

PROTOCOLS & APPLICATIONS GUIDE

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I. Introduction

The polymerase chain reaction (PCR) is a relatively simple technique that amplifies a DNA template to produce specific DNA fragments *in vitro*. Traditional methods of cloning a DNA sequence into a vector and replicating it in a living cell often require days or weeks of work, but amplification of DNA sequences by PCR requires only hours. While most biochemical analyses, including nucleic acid detection with radioisotopes, require the input of significant amounts of biological material, the PCR process requires very little. Thus, PCR can achieve more sensitive detection and higher levels of amplification of specific sequences in less time than previously used methods. These features make the technique extremely useful, not only in basic research, but also in commercial uses, including genetic identity testing, forensics, industrial quality control and *in vitro* diagnostics. Basic PCR is commonplace in many molecular biology labs where it is used to amplify DNA fragments and detect DNA or RNA sequences within a cell or environment. However, PCR has evolved far beyond simple amplification and detection, and many extensions of the original PCR method have been described. This chapter provides an overview of different types of PCR methods, applications and optimization. A detailed treatment of these methods is beyond the scope of this publication. However, an extensive bibliography is provided in the References section for researchers who require more comprehensive information.

A. Basic PCR

The PCR process was originally developed to amplify short segments of a longer DNA molecule (Saiki *et al.* 1985). A typical amplification reaction includes target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. Once assembled, the reaction is placed in a thermal cycler, an instrument that subjects the reaction to a series of different temperatures for set amounts of time. This series of temperature and time adjustments is referred to as one cycle of amplification. Each PCR cycle theoretically doubles the amount of targeted sequence (amplicon) in the reaction. Ten cycles theoretically multiply the amplicon by a factor of about one thousand; 20 cycles, by a factor of more than a million in a matter of hours.

Each cycle of PCR includes steps for template denaturation, primer annealing and primer extension (Figure 1.1). The initial step denatures the target DNA by heating it to 94°C or higher for 15 seconds to 2 minutes. In the denaturation process, the two intertwined strands of DNA separate from one another, producing the necessary single-stranded DNA template for replication by the thermostable DNA polymerase. In the next step of a cycle, the temperature is reduced to approximately 40–60°C. At this temperature, the oligonucleotide primers can form stable associations (anneal) with the denatured target DNA and serve as primers for the DNA polymerase. This step lasts approximately 15–60 seconds. Finally, the synthesis of new DNA begins as the reaction temperature is raised to the

optimum for the DNA polymerase. For most thermostable DNA polymerases, this temperature is in the range of 70–74°C. The extension step lasts approximately 1–2 minutes. The next cycle begins with a return to 94°C for denaturation.

Each step of the cycle should be optimized for each template and primer pair combination. If the temperature during the annealing and extension steps are similar, these two steps can be combined into a single step in which both primer annealing and extension take place. After 20–40 cycles, the amplified product may be analyzed for size, quantity, sequence, etc., or used in further experimental procedures.

An animated presentation (www.promega.com/paguide/animation/selector.htm?coreName=pcr01) illustrating the PCR process is available.

Additional Resources for Basic PCR

Technical Bulletins and Manuals

TB254	<i>GoTaq® PCR Core Systems Technical Bulletin</i> (www.promega.com/tbs/tb254/tb254.html)
9PIM750	<i>PCR Master Mix Promega Product Information</i> (www.promega.com/tbs/9pim750/9pim750.html)
9PIM300	<i>GoTaq® DNA Polymerase Promega Product Information</i> (www.promega.com/tbs/9pim300/9pim300.html)
9PIM829	<i>GoTaq® Flexi DNA Polymerase Promega Product Information</i> (www.promega.com/tbs/9pim829/9pim829.html)

Promega Publications

eNotes	Ensuring successful PCR using online resources (www.promega.com/enotes/features/fe0032.htm)
PN091	GoTaq® Green Master Mix: From amplification to analysis (www.promega.com/pnotes/91/12972_13/12972_13.html)
PN083	Introducing GoTaq® DNA Polymerase: Improved amplification with a choice of buffers (www.promega.com/pnotes/83/10492_21/10492_21.html)
PN078	Performance advantages designed into Promega's PCR Master Mix (www.promega.com/pnotes/78/9186_09/9186_09.html)

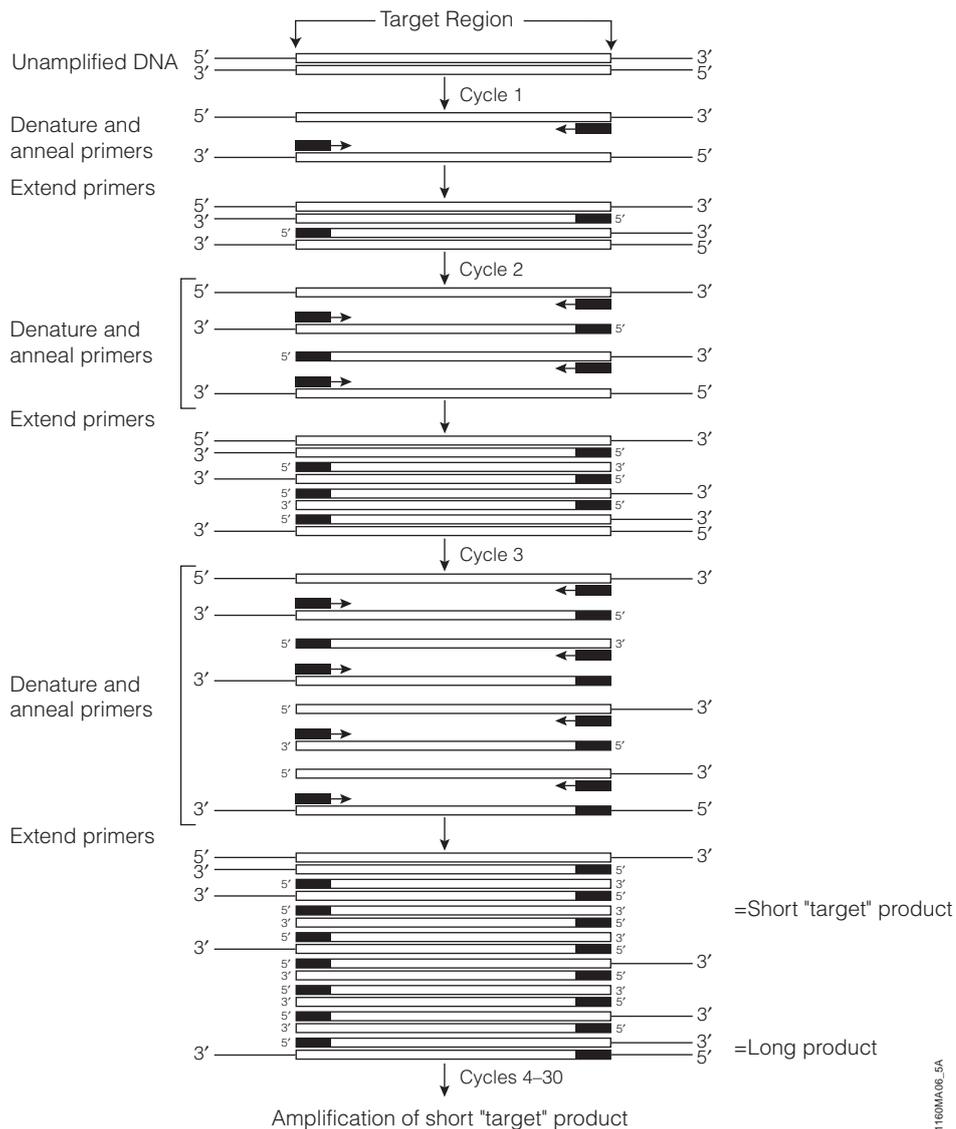


Figure 1.1. Schematic diagram of the PCR process.

More publications (www.promega.com/pnotes/apps/pcr/)

Online Tools

Amplification Product Selector (www.promega.com/selectors/amplification/)

Citations

Bermejo-Alvarez, P. *et al.* (2008) Can bovine in vitro-matured oocytes selectively process X- or Y-sorted sperm differentially? *Biol. Reprod.* **79**, 594–7.

To determine whether oocytes are able to select X-bearing or Y-bearing spermatozoa, the authors performed in vitro fertilization of bovine oocytes with X-sorted semen, Y-sorted semen, a mixture of X- and Y-sorted semen, and unsorted semen. The gender of the resulting embryos was determined by amplifying two DNA targets: a Y chromosome-specific target for gender assignment and a bovine-specific satellite sequence as a control. PCRs were

performed using GoTaq® Flexi DNA Polymerase (1 unit per 25µl reaction), and amplified products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining.

PubMed Number: 18579751

Staniszewska, I. *et al.* (2008) Integrin alpha9 beta1 is a receptor for nerve growth factor and other neurotrophins. *J. Cell Sci* **121**, 504–13.

The authors investigated the ability of α9β1 integrin to act as a neurotrophin receptor and affect cell signaling pathways. As part of the study, RT-PCR was used to detect the presence of other neurotrophin receptors in their model cell line SW480. Reverse transcription was performed using the Reverse Transcription System and 1µg of total RNA isolated using the SV Total RNA Isolation System. The resulting cDNA (5µg) was amplified for 35 cycles (β-actin

as a control) or 40 cycles (TrkA and p75NTR) using GoTaq® Green Master Mix. RT-PCR results were confirmed by Western blot analysis.

PubMed Number: 18230652

B. RT-PCR

Thermostable DNA polymerases used for basic PCR require a DNA template, and as such, the technique is limited to the analysis of DNA samples. Yet numerous instances exist in which amplification of RNA would be preferred. To apply PCR to the study of RNA, the RNA sample must first be reverse transcribed to cDNA to provide the necessary DNA template for the thermostable polymerase (Figure 1.2). This process is called reverse transcription (RT), hence the name RT-PCR.

Avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (M-MLV or MuLV) reverse transcriptases are generally used to produce a DNA copy of the RNA template using either random primers, an oligo(dT) primer or sequence-specific primers. Promega also offers GoScript™ Reverse Transcriptase (Cat.# A5000) and ImProm-II™ Reverse Transcriptase (Cat.# A3801). GoScript™ Reverse Transcriptase is qualified for use in qPCR, including GoTaq® qPCR and Plexor® qPCR Systems for performing RT-qPCR. Alternatively, some thermostable DNA polymerases (e.g., *Tth* DNA polymerase) possess a reverse transcriptase activity, which can be activated by using manganese instead of magnesium as a cofactor (Myers and Gelfand, 1991). After this initial reverse transcription step to produce the cDNA template, basic PCR is carried out to amplify the target sequence.

The quality and purity of the RNA template is crucial to the success of RT-PCR. Total RNA or poly(A)+ RNA can be used as the starting template, but both must be intact and free of contaminating genomic DNA. Specific capture of poly(A)+ RNA will enrich a targeted message so that less of the reverse transcription reaction is needed for subsequent amplification. The efficiency of the first-strand synthesis reaction, which can be related to the RNA quality, also will significantly affect amplification results.

First Strand Synthesis:

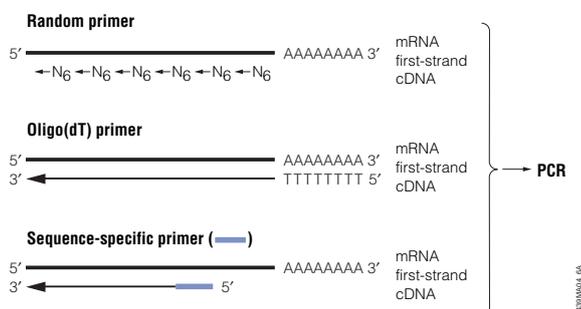


Figure 1.2. Schematic diagram of RT-PCR.

Additional Resources for RT-PCR

Technical Bulletins and Manuals

TB220	<i>Access RT-PCR System Technical Bulletin</i> (www.promega.com/tbs/tb220/tb220.html)
TM316	<i>GoScript™ Reverse Transcriptase Technical Manual</i> (www.promega.com/tbs/tm316/tm316.html)
TM236	<i>ImProm-II™ Reverse Transcription System Technical Manual</i> (www.promega.com/tbs/tm236/tm236.html)
TB099	<i>Reverse Transcription System Technical Bulletin</i> (www.promega.com/tbs/tb099/tb099.html)
9PIA170	<i>AccessQuick™ RT-PCR System Promega Product Information</i> (www.promega.com/tbs/9pia170/9pia170.html)

Promega Publications

PN079	<i>AccessQuick™ RT-PCR System: Simple, stable and sensitive</i> (www.promega.com/pnotes/79/9492_12/9492_12.html)
PN079	<i>Using ImProm-II™ Reverse Transcription System for coupled RT-PCR</i> (www.promega.com/pnotes/79/9492_15/9492_15.html)
PN078	<i>Technically speaking: Promega RT-PCR systems explained</i> (www.promega.com/pnotes/78/9186_21/9186_21.html)
PN073	<i>Using the Access RT-PCR System: Reaction parameters that affect efficient amplification</i> (www.promega.com/pnotes/73/8235_14/8235_14.html)
More publications	(www.promega.com/pnotes/apps/rt_cdna/)

Citations

Nanashima, N. *et al.* (2008) The hairless phenotype of the Hirosaki hairless rat is due to the deletion of an 80-kb genomic DNA containing five basic keratin genes. *J. Biol. Chem.* **283**, 16868–75.

The mutation responsible for the hairless phenotype was linked to a 80kb deletion of chromosome 7q36. Because many basic keratin genes are located at 7q36, the authors examined keratin gene expression in the Hirosaki rat using RT-PCR. Expression of kb21, kb23 and kb26 was not detected, whereas other basic keratin genes were expressed. RT-PCR was performed using the AccessQuick™ RT-PCR System and 0.5µg of total RNA isolated from rat skin for 21–30 cycles.

PubMed Number: 18420582

1 Nucleic Acid Amplification

Capozzo, A.V. *et al.* (2003) Development of DNA vaccines against hemolytic-uremic syndrome in a murine model. *Infect. Immun.* **71**, 3971–8.

Researchers used the pGEM[®]-T Vector System to clone the entire 1.4kb Shiga toxin type 2 gene (Stx2) from *E. coli* O157-H7 C600 (933W). The resultant construct, named pGEMTStx2, was used as a template in PCR to amplify each region of the gene corresponding to Shiga toxin type 2 subunits A and B. Each PCR product was digested with BamHI and EcoRI, then ligated into pCDNA 3.1+ to create pStx2ΔA and pStx2B. Mice then were immunized with either one or both of these constructs and another construct expressing murine granulocyte-macrophage colony-stimulating factor. Expression of each subunit in mouse tissue was verified by RT-PCR using specific primers and the AccessQuick[™] RT-PCR System.

PubMed Number: 12819084

C. Hot-Start PCR

Hot-start PCR is a common technique to reduce nonspecific amplification due to assembly of amplification reactions at room temperature. At these lower temperatures, PCR primers can anneal to template sequences that are not perfectly complementary. Since thermostable DNA polymerases have activity at these low temperatures (although in most cases the activity is less than 25%) the polymerase can extend misannealed primers. This newly synthesized region then acts as a template for primer extension and synthesis of undesired amplification products. However, if the reaction is heated to temperatures >60°C before polymerization begins, the stringency of primer annealing is increased, and synthesis of undesired PCR products is avoided or reduced.

Hot-start PCR also can reduce the amount of primer-dimer synthesized by increasing the stringency of primer annealing. At lower temperatures, PCR primers can anneal to each other via regions of complementarity, and the DNA polymerase can extend the annealed primers to produce primer dimer, which often appears as a diffuse band of approximately 50–100bp on an ethidium bromide-stained gel. The formation of nonspecific products and primer-dimer can compete for reagent availability with amplification of the desired product. Thus, hot-start PCR can improve the yield of specific PCR products.

To perform manual hot-start PCR, reactions are assembled on ice or at room temperature, but one critical component is omitted until the reaction is heated to 60–65°C, at which point the missing reagent is added. This omission prevents the polymerase from extending primers until the critical component is added at the higher temperature where primer annealing is more stringent. However, this method is tedious and increases the risk of contamination. A second, less labor-intensive approach involves the reversible inactivation or physical separation of one or more critical components in the reaction. For example, the magnesium or DNA polymerase can be sequestered in a wax bead,

which melts as the reaction is heated to 94°C during the denaturation step, releasing the component only at higher temperatures (Carothers *et al.* 1989; Krishnan *et al.* 1991; Clark, 1988). The DNA polymerase also can be kept in an inactive state by binding to an oligonucleotide, also known as an aptamer (Lin and Jayasena, 1997; Dang and Jayasena, 1996) or an antibody (Scalice *et al.* 1994; Sharkey *et al.* 1994). This bond is disrupted at the higher temperatures, releasing the functional DNA polymerase. Finally, the DNA polymerase can be maintained in an inactive state through chemical modification (Moretti, T. *et al.* 1998).

Additional Resources for Hot-Start PCR

Technical Bulletins and Manuals

9PIM500 *GoTaq[®] Hot Start Polymerase Promega Product Information*
(www.promega.com/tbs/9pim500/9pim500.html)

9PIM512 *GoTaq[®] Hot Start Green Master Mix Promega Product Information*
(www.promega.com/tbs/9pim512/9pim512.html)

9PIM513 *GoTaq[®] Hot Start Colorless Master Mix Promega Product Information*
(www.promega.com/tbs/9pim513/9pim513.html)

Promega Publications

PN099 Get the convenience of hot-start PCR with the new GoTaq[®] Hot Start Polymerase
(www.promega.com/pnotes/99/15674_08/15674_08.html)

D. Long PCR

Amplification of long DNA fragments is desirable for numerous applications such as physical mapping applications (Rose, 1991) and direct cloning from genomes. While basic PCR works well when smaller fragments are amplified, amplification efficiency (and therefore the yield of amplified fragments) decreases significantly as the amplicon size increases over 5kb. This decrease in yield can be attributed to the accumulation of truncated products, which are not suitable substrates for additional cycles of amplification. These products appear as smeared, as opposed to discrete, bands on a gel.

In 1994, Wayne Barnes (Barnes, 1994) and other researchers (Cheng *et al.* 1994) examined factors affecting polymerization across larger regions of DNA by thermostable DNA polymerases and identified key variables affecting the yield of longer PCR products. They devised an approach using a mixture of two thermostable polymerases to synthesize longer PCR products. The first polymerase lacks a 3'→5' exonuclease (proofreading) activity; the second enzyme, present at a reduced concentration, contains a potent proofreading activity. Presumably, when the nonproofreading DNA polymerase (e.g., *Taq* DNA polymerase) misincorporates a dNTP, subsequent extension of the newly synthesized DNA either

proceeds very slowly or stops completely. The proofreading polymerase (e.g., *Pfu* DNA polymerase or *Tli* DNA polymerase) serves to remove the misincorporated nucleotide, allowing the DNA polymerases to continue extension of the new strand.

Although the use of two thermostable DNA polymerases can significantly increase yield, other conditions can have a significant impact on the yield of longer PCR products (Cheng *et al.* 1995). Logically, longer extension times can increase the yield of longer PCR products because fewer partial products are synthesized. Extension times depend on the length of the target; times of 10–20 minutes are common. In addition, template quality is crucial. Depurination of the template, which is promoted at elevated temperatures and lower pH, will result in more partial products and decreased overall yield. In long PCR, denaturation time is reduced to 2–10 seconds to decrease depurination of the template. Additives, such as glycerol and dimethyl sulfoxide (DMSO), also help lower the strand-separation and primer-annealing temperatures, alleviating some of the depurination effects of high temperatures. Cheng *et al.* also found that reducing potassium concentrations by 10–40% increased the amplification efficiency of longer products (Cheng *et al.* 1995).

E. Quantitative Endpoint PCR

PCR and RT-PCR are generally used in a qualitative format to evaluate biological samples. However, a wide variety of applications, such as determining viral load, measuring responses to therapeutic agents and characterizing gene expression, would be improved by quantitative determination of target abundance. Theoretically, this should be easy to achieve, given the exponential nature of PCR, because a linear relationship exists between the number of amplification cycles and the logarithm of the number of molecules. In practice, however, amplification efficiency is decreased because of contaminants (inhibitors), competitive reactions, substrate exhaustion, polymerase inactivation and target reannealing. As the number of cycles increases, the amplification efficiency decreases, eventually resulting in a plateau effect.

Normally, quantitative PCR requires that measurements be taken before the plateau phase so that the relationship between the number of cycles and molecules is relatively linear. This point must be determined empirically for different reactions because of the numerous factors that can affect amplification efficiency. Because the measurement is taken prior to the reaction plateau, quantitative PCR uses fewer amplification cycles than basic PCR. This can cause problems in detecting the final product because there is less product to detect.

To monitor amplification efficiency, many applications are designed to include an internal standard in the PCR. One such approach includes a second primer pair that is specific for a “housekeeping” gene (i.e., a gene that has constant expression levels among the samples compared) in the reaction (Gaudette and Crain, 1991; Murphy *et al.* 1990).

Amplification of housekeeping genes verifies that the target nucleic acid and reaction components were of acceptable quality but does not account for differences in amplification efficiencies due to differences in product size or primer annealing efficiency between the internal standard and target being quantified.

The concept of competitive PCR—a variation of quantitative PCR—is a response to this limitation. In competitive PCR, a known amount of a control template is added to the reaction. This template is amplified using the same primer pair as the experimental target molecule but yields a distinguishable product (e.g., different size, restriction digest pattern, etc.). The amounts of control and test product are compared after amplification. While these approaches control for the quality of the target nucleic acid, buffer components and primer annealing efficiencies, they have their own limitations (Siebert and Larrick, 1993; McCulloch *et al.* 1995), including the fact that many depend on final analysis by electrophoresis.

Numerous fluorescent and solid-phase assays exist to measure the amount of amplification product generated in each reaction, but they often fail to discriminate amplified DNA of interest from nonspecific amplification products. Some of these analyses rely on blotting techniques, which introduce another variable due to nucleic acid transfer efficiencies, while other assays were developed to eliminate the need for gel electrophoresis yet provide the requisite specificity. Real-time PCR, which provides the ability to view the results of each amplification cycle, is a popular way of overcoming the need for analysis by electrophoresis.

Additional Resources for Quantitative Endpoint PCR

Promega Publications

PN068	Quantitative RT-PCR: Rapid construction of templates for competitive amplification (www.promega.com/pnotes/68/7381_16/7381_16.html)
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F. Quantitative Real-Time PCR

The use of fluorescently labeled oligonucleotide probes or primers or fluorescent DNA-binding dyes to detect and quantitate a PCR product allows quantitative PCR to be performed in real time. Specially designed instruments perform both thermal cycling to amplify the target and fluorescence detection to monitor PCR product accumulation. DNA-binding dyes are easy to use but do not differentiate between specific and nonspecific PCR products and are not conducive to multiplex reactions. Fluorescently labeled nucleic acid probes have the advantage that they react with only specific PCR products, but they can be expensive and difficult to design. Some qPCR technologies employ fluorescently labeled PCR primers instead of probes. One example, which will be discussed in more detail below, is the Plexor® technology, which requires only a single fluorescently labeled primer,

is compatible with multiplex PCR and allows specific and nonspecific amplification products to be differentiated (Sherrill *et al.* 2004; Frackman *et al.* 2006).

The use of fluorescent DNA-binding dyes is one of the easiest qPCR approaches. The dye is simply added to the reaction, and fluorescence is measured at each PCR cycle. Because fluorescence of these dyes increases dramatically in the presence of double-stranded DNA, DNA synthesis can be monitored as an increase in fluorescent signal. However, preliminary work often must be done to ensure that the PCR conditions yield only specific product. In subsequent reactions, specific amplification can be verified by a melt curve analysis. Thermal melt curves are generated by allowing all product to form double-stranded DNA at a lower temperature (approximately 60°C) and slowly ramping the temperature to denaturing levels (approximately 95°C). The product length and sequence affect melting temperature (T_m), so the melt curve is used to characterize amplicon homogeneity. Nonspecific amplification can be identified by broad peaks in the melt curve or peaks with unexpected T_m values. By distinguishing specific and nonspecific amplification products, the melt curve adds a quality control aspect during routine use. The generation of melt curves is not possible with assays that rely on the 5'→3' exonuclease activity of *Taq* DNA polymerase, such as the probe-based TaqMan® technology.

The GoTaq® qPCR Master Mix (Cat.# A6001) is a qPCR reagent system that contains a proprietary fluorescent DNA-binding dye that often exhibits greater fluorescence enhancement upon binding to double-stranded DNA and less PCR inhibition than the commonly used SYBR® Green I dye. The dye in the GoTaq® qPCR Master Mix enables efficient amplification, resulting in earlier quantification cycle (C_q , formerly known as cycle threshold [C_t]) values and an expanded linear range using the same filters and settings as SYBR® Green I. The GoTaq® qPCR Master Mix is provided as a simple-to-use, stabilized 2X formulation that includes all components for qPCR except sample DNA, primers and water. For more information, view the GoTaq® qPCR Master Mix video (www.promega.com/multimedia/VPA6001/VPA6001.html).

Real-time PCR using labeled oligonucleotide primers or probes employs two different fluorescent reporters and relies on energy transfer from one reporter (the energy donor) to a second reporter (the energy acceptor) when the reporters are in close proximity. The second reporter can be a quencher or a fluor. If the second reporter is a quencher, the energy from the first reporter is absorbed but re-emitted as heat rather than light, leading to a decrease in fluorescent signal. Alternatively, if the second reporter is a fluor, the energy can be absorbed and re-emitted at another wavelength through fluorescent resonance energy transfer (FRET, reviewed in Didenko, 2001), and the progress of the reaction can be monitored by the decrease in fluorescence of the energy donor or the

increase in fluorescence of the energy acceptor. During the exponential phase of PCR, the change in fluorescence is proportional to accumulation of PCR product.

Examples of a primer-based approach are the Plexor® qPCR and qRT-PCR Systems, which require two PCR primers, only one of which is fluorescently labeled. These systems take advantage of the specific interaction between two modified nucleotides (Sherrill *et al.* 2004; Johnson *et al.* 2004; Moser and Prudent, 2003). The two novel bases, isoguanine (iso-dG) and 5'-methylisocytosine (iso-dC), form a unique base pair in double-stranded DNA (Johnson *et al.* 2004). To perform fluorescent quantitative PCR using this new technology, one primer is synthesized with an iso-dC residue as the 5'-terminal nucleotide and a fluorescent label at the 5'-end; the second primer is unlabeled. During PCR, this labeled primer is annealed and extended, becoming part of the template used during subsequent rounds of amplification. The complementary iso-dGTP, which is available in the nucleotide mix as dabcyI-iso-dGTP, pairs specifically with iso-dC. When the dabcyI-iso-dGTP is incorporated, the close proximity of the dabcyI quencher and the fluorescent label on the opposite strand effectively quenches the fluorescent signal. This process is illustrated in Figure 1.3. The initial fluorescence level of the labeled primers is high in Plexor® System reactions. As amplification product accumulates, signal decreases.

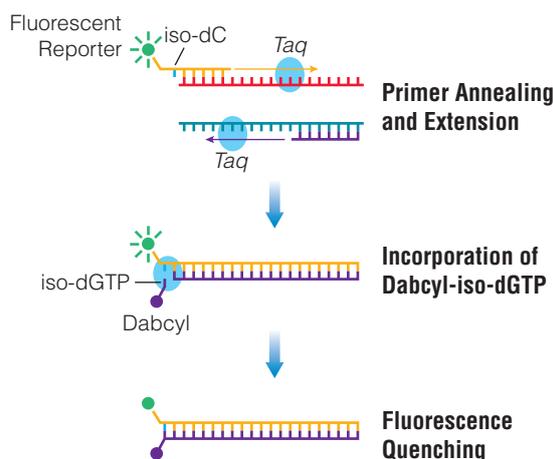


Figure 1.3. Quenching of the fluorescent signal by dabcyI during product accumulation.

Quenching of the fluorescent label by dabcyI is a reversible process. Fluorescence is quenched when the product is double-stranded. Denaturing the product separates the label and quencher, resulting in an increased fluorescent signal. Consequently, thermal melt curves can be used to characterize amplicon homogeneity.

A benefit of the Plexor® technology over detection using simple DNA-binding dyes is the capacity for multiplexing. The labeled primer can be tagged with one of many common fluorescent labels, allowing two- to four-color multiplexing, depending on the instrument used. The simplicity of primer design for the Plexor® technology is a

distinct advantage over probe-based quantitative PCR approaches. Also, the Plexor® technology does not rely on enzymatic cleavage to generate signal and does not have the complex hybridization kinetics that can be typical of other approaches to real-time PCR. The Plexor® technology also can be used for quantitative RT-PCR by incorporating a reverse transcription step.

Some qPCR strategies employ complementary nucleic acid probes to quantify the DNA target. These probes also can be used to detect single nucleotide polymorphisms (Lee *et al.* 1993; Bernard *et al.* 1998). There are several general categories of real-time PCR probes, including hydrolysis, hairpin and simple hybridization probes. These probes contain a complementary sequence that allows the probe to anneal to the accumulating PCR product, but probes can differ in the number and location of the fluorescent reporters.

Hydrolysis probes are labeled with a fluor at the 5'-end and a quencher at the 3'-end, and because the two reporters are in close proximity, the fluorescent signal is quenched. During the annealing step, the probe hybridizes to the PCR product generated in previous amplification cycles. The resulting probe:target hybrid is a substrate for the 5'→3' exonuclease activity of the DNA polymerase, which degrades the annealed probe and liberates the fluor (Holland *et al.* 1991). The fluor is freed from the effects of the energy-absorbing quencher, and the progress of the reaction and accumulation of PCR product is monitored by the resulting increase in fluorescence. With this approach, preliminary experiments must be performed prior to the quantitation experiments to show that the signal generated is proportional to the amount of the desired PCR product and that nonspecific amplification does not occur.

Hairpin probes, also known as molecular beacons, contain inverted repeats separated by a sequence complementary to the target DNA. The repeats anneal to form a hairpin structure, where the fluor at the 5'-end and a quencher at the 3'-end are in close proximity, resulting in little fluorescent signal. The hairpin probe is designed so that the probe binds preferentially to the target DNA rather than retains the hairpin structure. As the reaction progresses, increasing amounts of the probe anneal to the accumulating PCR product, and as a result, the fluor and quencher become physically separated. The fluor is no longer quenched, and the level of fluorescence increases. One advantage of this technique is that hairpin probes are less likely to mismatch than hydrolysis probes (Tyagi *et al.* 1998). However, preliminary experiments must be performed to show that the signal is specific for the desired PCR product and that nonspecific amplification does not occur.

The use of simple hybridization probes involves two labeled probes or, alternatively, one labeled probe and a labeled PCR primer. In the first approach, the energy emitted by the fluor on one probe is absorbed by a fluor on the second probe, which hybridizes nearby. In the second approach, the emitted energy is absorbed by a second fluor that is

incorporated into the PCR product as part of the primer. Both of these approaches result in increased fluorescence of the energy acceptor and decreased fluorescence of the energy donor. The use of hybridization probes can be simplified even further so that only one labeled probe is required. In this approach, quenching of the fluor by deoxyguanosine is used to bring about a change in fluorescence (Crockett and Wittwer, 2001; Kurata *et al.* 2001). The labeled probe anneals so that the fluor is in close proximity to G residues within the target sequence, and as probe annealing increases, fluorescence decreases due to deoxyguanosine quenching. With this approach, the location of probe is limited because the probe must hybridize so that the fluorescent dye is very near a G residue. The advantage of simple hybridization probes is their ability to be multiplexed more easily than hydrolysis and hairpin probes through the use of differently colored fluors and probes with different melting temperatures (reviewed in Wittwer *et al.* 2001).

Additional Resources for Real-Time PCR

Technical Bulletins and Manuals

TM318	GoTaq® qPCR Master Mix Technical Manual (www.promega.com/tbs/tm318/tm318.html)
TM262	Plexor® qPCR System Technical Manual (www.promega.com/tbs/tm262/tm262.html)
TM263	Plexor® One-Step qRT-PCR System Technical Manual (www.promega.com/tbs/tm263/tm263.html)
TM264	Plexor® Two-Step qRT-PCR System Technical Manual (www.promega.com/tbs/tm264/tm264.html)

Promega Publications

tpub_014	Introducing GoTaq® qPCR Master Mix: The bright choice for dye-based qPCR (www.promega.com/pubs/tpub_014.htm)
PN092	The Plexor™ Systems provide accurate quantitation in multiplex qPCR and qRT-PCR (www.promega.com/pnotes/92/13408_10/13408_10.html)
PN090	Plexor™ technology: A new chemistry for real-time PCR (www.promega.com/pnotes/90/12727_02/12727_02.html)

G. Rapid Amplified Polymorphic DNA Analysis

Genetic analysis of organisms at the molecular level is an important and widely practiced scientific tool. Several techniques developed over more than a decade offer the opportunity to identify each individual or type of individual in a species unambiguously.

One important PCR-based genetic analysis is random amplified polymorphic DNA analysis (RAPD; reviewed in McClelland and Welsh, 1994; Power, 1996; Black, 1993). RAPD uses small, nonspecific primers to amplify seemingly random regions of genomic DNA. Successful primer pairs produce different banding profiles of PCR products between individuals, strains, cultivars or species when analyzed by gel electrophoresis.

Slight modifications to the basic PCR method are made for RAPD. First, the primers are approximately 10 bases in length compared to the 17- to 23-base primer length of normal PCR. Because primers are shorter, the annealing temperature is reduced to less than 40°C.

As with most PCR techniques, RAPD requires very little material for analysis and is relatively insensitive to template integrity. No blotting techniques are required, thus eliminating the use of ³²P, bypassing probe generation and decreasing the amount of time required to obtain results.

H. Rapid Amplification of cDNA Ends (RACE)

Rapid amplification of cDNA ends (RACE) is a variation of RT-PCR that amplifies unknown cDNA sequences corresponding to the 3'- or 5'-end of the RNA. Numerous variations of the original protocols have been published (Troutt *et al.* 1992; Edwards *et al.* 1991; Edwards *et al.* 1993; Liu and Gorovskiy, 1993; Fromont-Racine *et al.* 1993; reviewed in Schaefer, 1995) but will not be discussed in detail here.

Two general RACE strategies exist: one amplifies 5' cDNA ends (5' RACE) and the other captures 3' cDNA end sequences (3' RACE). In either strategy, the first step involves the conversion of RNA to single-stranded cDNA using a reverse transcriptase. For subsequent amplification, two PCR primers are designed to flank the unknown sequence. One PCR primer is complementary to known sequences within the gene, and a second primer is complementary to an "anchor" site (anchor primer). The anchor site may be present naturally, such as the poly(A) tail of most mRNAs, or can be added in vitro after completion of the reverse transcription step. The anchor primer also can carry adaptor sequences, such as restriction enzyme recognition sites, to facilitate cloning of the amplified product. Amplification using these two PCR primers results in a product that spans the unknown 5' or 3' cDNA sequence, and sequencing this product will reveal the unknown sequence. The information obtained from partial cDNA sequences then can be used to assemble the full-length cDNA sequence (Frohman *et al.* 1988; Loh *et al.* 1989; Ohara *et al.* 1989).

In 5' RACE (Figure 1.4), the first-strand cDNA synthesis reaction is primed using an oligonucleotide complementary to a known sequence in the gene. After removing the RNA template, an anchor site at the 3'-end of the single-stranded cDNA is created using terminal deoxynucleotidyl transferase, which adds a nucleotide tail. A typical

amplification reaction follows using an anchor primer complementary to the newly added tail and another primer complementary to a known sequence within the gene.

The 3'-RACE procedure uses a modified oligo(dT) primer/adaptor as the reverse transcription primer. This oligo(dT) primer/adaptor is comprised of an oligo(dT) sequence, which anneals to the poly(A)⁺ tail of the mRNA, and an adaptor sequence at the 5' end. A single G, C or A residue at the 3' end ensures that cDNA synthesis is initiated only when the primer/adaptor anneals immediately adjacent to the junction between the poly(A)⁺ tail and 3' end of the mRNA. This oligo(dT) primer/adaptor is used as the anchor primer in the subsequent amplifications along with a primer complementary to known sequences within the gene. See Figure 1.5.

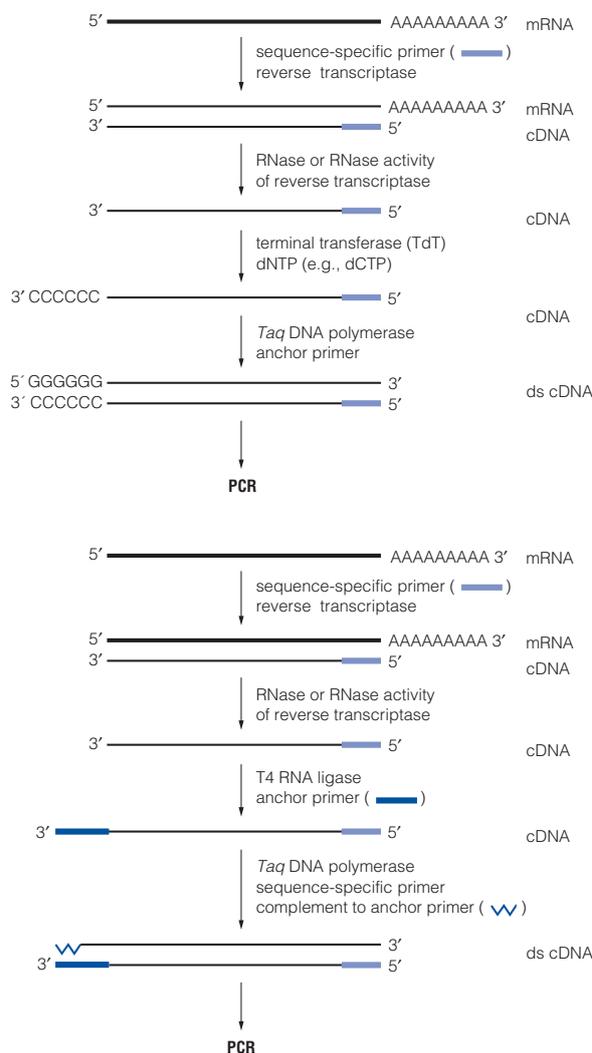


Figure 1.4. Schematic diagram of two 5' RACE methods.

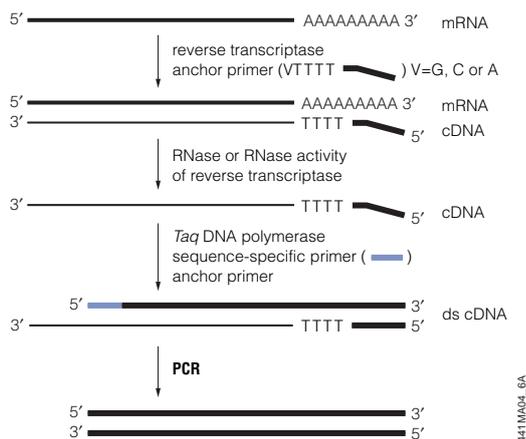


Figure 1.5. Schematic diagram of a typical 3'-RACE protocol.

I. Differential Display PCR

Differential display PCR is another variation of RT-PCR and is used to identify differences in mRNA expression patterns between two cell lines or populations. In one example of this procedure, cDNA synthesis is primed using a set of modified oligo(dT) primers, which anneal to the poly(A)+ tail of mRNA (Liang and Pardee, 1992). Each of the oligo(dT) primers carries an additional two nucleotides at the 3'-end. This ensures that extension only occurs if the primer anneals immediately adjacent to the junction between the poly(A)+ tail and 3' end of the mRNA. Because the two additional nucleotides will only anneal to a subset of the mRNA molecules, this also reduces the complexity of the RNA population that is reverse transcribed. The RNA is first reverse transcribed with one of the modified oligo(dT) primers to synthesize first-strand cDNA, which is then amplified by PCR using two random 10mer primers. After amplification, the reaction products are visualized by gel electrophoresis, and banding patterns for the two cell populations are compared to identify differentially expressed cDNAs.

Another form of analyzing differences between complex genomes is representational difference analysis (RDA). This method combines "subtractive" library techniques (Lisitsyn *et al.* 1993) with PCR amplification to find differences in complex genomes. A variation of this is cDNA RDA, where total RNA from the cell populations is first converted into cDNA, subtractive techniques are performed and the products are amplified by PCR (Hubank and Schatz, 1994). By using cDNA, the complexity is significantly reduced, providing another method to analyze differences in expression between cell types or in response to various treatments.

Additional Resources for Differential Display PCR

Promega Publications

- NN015 Targeted display: Identifying differentially expressed mRNAs
www.promega.com/nnotes/nn502/502_13.htm

J. In situ PCR

In situ PCR, first described in 1990, combines the sensitivity of PCR or RT-PCR amplification with the cellular or histological localization associated with in situ hybridization techniques (Haase *et al.* 1990). These features make in situ PCR a powerful tool to detect proviral DNA, oncogenesis and localization of rare messages.

The technique is amenable to analysis of fixed cells or tissue cross-sections. Detection of amplified products can be accomplished indirectly by subsequent hybridization using either radiolabeled, fluorescently labeled or biotin-labeled nucleic acid probes. PCR products also can be detected directly by incorporating a labeled nucleotide, although this method is subject to higher background levels.

The use of in situ PCR requires altering some of the reaction parameters typical of basic PCR (Nuovo *et al.* 1993; Thaker, 1999). For example, increased Mg^{2+} concentrations (approximately 4.5mM versus the normal 1.5–2.5mM) are used for in situ PCR. An increased amount of DNA polymerase is also required unless BSA is added to the reaction, presumably because the polymerase binds to the glass plate and coverslip.

Tissue preparation also plays a significant role in the success of in situ PCR. A strong relationship exists between the time of fixation and protease digestion and the intensity of PCR signal. Tissue preparation also affects the level of side reactions, resulting in primer-independent signals, which are not normally present in basic PCR. These primer-independent signals often arise from *Taq* DNA polymerase-mediated repair of single-stranded gaps in the genomic DNA.

As the use of the technique has spread, the process has been further optimized. Numerous publications (reviewed in Nuovo, 1995; Staskus *et al.* 1995) describe process improvements that increase sensitivity and decrease nonspecific amplification products.

K. High-Fidelity PCR

For some applications, such as gene expression, mutagenesis or cloning, the number of mutations introduced during PCR needs to be minimized. For these applications, we recommend using a proofreading polymerase. Proofreading DNA polymerases, such as *Pfu* and *Tli* DNA polymerases, have a 3'→5' exonuclease activity, which can remove any misincorporated nucleotides so that the error rate is relatively low. The accuracy of *Pfu* DNA polymerase is approximately twofold higher than that of *Tli* DNA polymerase and sixfold higher than that of *Taq* DNA polymerase (Cline *et al.*, 1996).

The most commonly used DNA polymerase for PCR is *Taq* DNA polymerase, which has an error rate of approximately 1×10^{-5} errors per base. This error rate is relatively high due to the enzyme's lack of 3'→5' exonuclease (proofreading) activity. The error rate of *Tfi* DNA polymerase, another nonproofreading polymerase, is similar to that of *Taq* DNA polymerase.

Reaction conditions can affect DNA polymerase fidelity, and DNA polymerases may be affected in different ways or to different degrees. In general, excess magnesium or the presence of manganese will reduce the fidelity of DNA polymerases (Eckert and Kunkel, 1990). Unequal nucleotide concentrations also can affect fidelity; nucleotides that are present at higher concentrations will be misincorporated at a higher frequency (Eckert and Kunkel, 1990). Reaction pH also can have a big effect on fidelity (Eckert and Kunkel, 1990; Eckert and Kunkel, 1991). For example, the fidelity of *Taq* DNA polymerase increases as pH decreases, with the lowest error rate occurring in the range of pH 5–6 (Eckert and Kunkel, 1990), but the opposite is true for *Pfu* DNA polymerase. *Pfu* DNA polymerase has higher fidelity at higher pH (Cline *et al.*, 1996). Finally, exposing the DNA template to high temperatures (i.e., 94°C) for extended periods of time can lead to DNA damage, specifically the release of bases from the phosphodiester backbone. The resulting abasic sites can cause some DNA polymerases to stall but also can result in a higher rate of mutations, most frequently transversions, as the DNA polymerase adds a random nucleotide at an abasic site (Eckert and Kunkel, 1991).

Additional Resources for High-Fidelity PCR

Promega Publications

- PN068 *Pfu* DNA Polymerase: A high fidelity enzyme for nucleic acid amplification (www.promega.com/pnotes/68/7381_07/7381_07.html)

L. PCR and DNA Sequencing: Cycle Sequencing

The PCR process also has been applied to DNA sequencing in a technique called cycle sequencing (Murray, 1989; Saluz and Jost, 1989; Carothers *et al.*, 1989; Krishnan *et al.*, 1991). Cycle sequencing reactions differ from typical PCR amplification reactions in that they use only a single primer, resulting in a linear (as opposed to theoretically exponential) amplification of the target molecule. Other reaction components are comparable, and either radioactive or fluorescent labels are incorporated for detection.

M. Cloning PCR Products

Amplification with a DNA polymerase lacking 3'→5' (proofreading) exonuclease activity (e.g., *Taq* DNA polymerase) yields products that contain a single 3'-terminal nucleotide overhang, typically an A residue (Clark, 1988; Hu, 1993). These PCR products can be conveniently cloned into T-vectors, which contain a single T overhang (reviewed in Mezei and Storts, 1994; Guo and Bi, 2002).

DNA polymerases that possess proofreading activity (e.g., *Tli* DNA polymerase or *Pfu* DNA polymerase) generate blunt-ended PCR products. These products are compatible with standard blunt-end cloning strategies. Conversely, blunt-end PCR products can be tailed with *Taq* DNA polymerase and dATP prior to cloning into a T-vector (Zhou *et al.*, 1995).

Additional Resources for Cloning PCR Products

Technical Bulletins and Manuals

- TM042 *pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual* (www.promega.com/tbs/tm042/tm042.html)
- TM044 *pTARGET™ Mammalian Expression Vector System Technical Manual* (www.promega.com/tbs/tm044/tm044.html)

Promega Publications

- PN082 Technically speaking: T-vector cloning (www.promega.com/pnotes/82/10203_24/10203_24.html)
- PN071 Rapid ligation for the pGEM®-T and pGEM®-T Easy Vector Systems (www.promega.com/pnotes/71/7807_08/7807_08.html)
- PN071 Cloning blunt-end *Pfu* DNA polymerase-generated PCR fragments into pGEM®-T Vector Systems (www.promega.com/pnotes/71/7807_10/7807_10.html)
- PN068 Technically speaking: Optimized cloning with T vectors (www.promega.com/pnotes/68/7381_31/7381_31.html)
- PN060 Digestion of PCR and RT-PCR products with restriction endonucleases without prior purification or precipitation (www.promega.com/pnotes/60/6079_23/promega.html)
- More publications (www.promega.com/pnotes/apps/cloning/)

Vector Maps

pGEM®-T and pGEM®-T Easy Vectors (www.promega.com/vectors/t_vectors.htm#b01)

pTARGET™ Mammalian Expression Vector (www.promega.com/vectors/t_vectors.htm#b02)

Citations

Kurth, E.G. *et al.* (2008) Involvement of BmoR and BmoG in n-alkane metabolism in *Pseudomonas butanovora*. *Microbiology* **154**, 139–47.

The authors characterized five open-reading frames flanking the alcohol-inducible alkane monooxygenase (BMO) structural gene of *Pseudomonas butanovora*. Strains with mutated *bmoR*, encoding a putative transcriptional regulator, or *bmoG*, encoding a putative chaperonin, were created by gene inactivation. The *bmoR* gene was amplified and cloned into the pGEM®-T Vector for disruption with a kanamycin cassette. The two termini of the *bmoG* gene were amplified separately, ligated to the kanamycin cassette and cloned into the pGEM®-T Easy Vector. Plasmids

encoding the disrupted genes were transformed into *Pseudomonas butanovorana* by electroporation.

PubMed Number: 18174133

Bröker, D. *et al.* (2008) The genomes of the non-clearing-zone-forming and natural-rubber-degrading species *Gordonia polyisoprenivorans* and *Gordonia westfalica* harbor genes expressing Lcp activity in *Streptomyces* strains. *Appl. Environ. Microbiol.* **74**, 2288–97.

Natural rubber-degrading bacteria fall into two categories: those forming clearing zones on latex overlay plates and those that do not. To investigate this degradation process, the authors amplified latex-clearing protein (*lcp*) homologs from non-clearing-zone-forming bacteria using degenerate PCR primers based on *lcp* sequences from clearing-zone forming species. The 3' region of the *lcp* gene in *G. westfalica* was amplified by nested PCR using biotinylated primers, and the amplified products were cloned in the pGEM®-T Easy Vector and sequenced using universal M13 forward and reverse primers.

PubMed Number: 18296529

II. General Considerations for PCR Optimization

This discussion focuses on the use of *Taq* DNA polymerase in PCR, since this is the enzyme most commonly used in PCR. Many of these suggestions also apply when using other DNA polymerases.

A. Magnesium Concentration

Magnesium is a required cofactor for thermostable DNA polymerases, and magnesium concentration is a crucial factor that can affect amplification success. Template DNA concentration, chelating agents present in the sample (e.g., EDTA or citrate), dNTP concentration and the presence of proteins all can affect the amount of free magnesium in the reaction. In the absence of adequate free magnesium, *Taq* DNA polymerase is inactive (Figure 1.6). Excess free magnesium reduces enzyme fidelity (Eckert and Kunkel, 1990) and may increase the level of nonspecific amplification (Williams, 1989; Ellsworth *et al.* 1993). For these reasons, researchers should empirically determine the optimal magnesium concentration for each target. To do so, set up a series of reactions containing 1.0–4.0mM Mg²⁺ in 0.5–1mM increments and visualize the results to determine which magnesium concentration produced the highest yield of product and the minimal amount of nonspecific product. The effect of magnesium concentration and the optimal concentration range can vary with the particular DNA polymerase. For example, the performance of *Pfu* DNA polymerase seems depend less on magnesium concentration, but when optimization is required, the optimal concentration is usually in the range of 2–6mM.

Many DNA polymerases are supplied with a magnesium-free reaction buffer and a tube of 25mM MgCl₂ so that you can adjust the Mg²⁺ concentration to the optimal level for each reaction. Before assembling the reactions, be sure to thaw the magnesium solution completely prior to

use and vortex the magnesium solution for several seconds before pipetting. Magnesium chloride solutions can form concentration gradients as a result of multiple freeze-thaw cycles, and vortex mixing is required to obtain a uniform solution. These two steps, though seemingly simple, eliminate the cause of many failed experiments.

Some scientists prefer to use reaction buffers that already contain MgCl₂ at a final concentration of 1.5mM. It should be noted, however, that Hu *et al.* reported performance variability of reaction buffer solutions containing magnesium (Hu *et al.* 1992). The free magnesium changes of 0.6mM observed in their experiments dramatically affected amplification yields in an allele-specific manner. The authors found that heating the buffer at 90°C for 10 minutes restored the homogeneity of the solution. They postulated that magnesium chloride precipitates as a result of multiple freeze-thaw cycles.

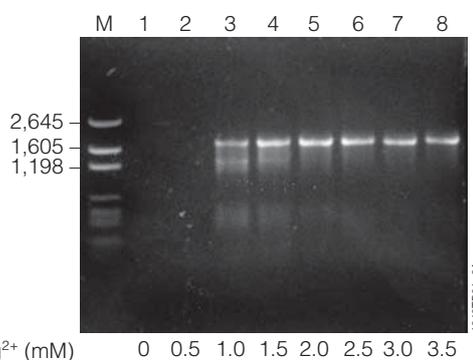


Figure 1.6. Effects of magnesium concentration on amplification. Amplifications were performed using various Mg²⁺ concentrations to demonstrate the effect on the amplification of a 1.8kb target luciferase gene. The reaction products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining. Lane M, Promega pGEM® DNA Markers (Cat.# G1741); lane 1, 0mM Mg²⁺; lane 2, 0.5mM Mg²⁺; lane 3, 1mM Mg²⁺; lane 4, 1.5mM Mg²⁺; lane 5, 2mM Mg²⁺; lane 6, 2.5mM Mg²⁺; lane 7, 3mM Mg²⁺ and lane 8, 3.5mM Mg²⁺.

B. Buffer Considerations

Most reaction buffers consist of a buffering agent, most often a Tris-based buffer, and salt, commonly KCl. The buffer regulates the pH of the reaction, which affects DNA polymerase activity and fidelity. Modest concentrations of KCl will increase DNA polymerase activity by 50–60% over activities in the absence of KCl; 50mM KCl is considered optimal (Gelfand, 1989).

GoTaq® DNA Polymerase contains native *Taq* DNA polymerase in a proprietary formulation. It is supplied with 5X Green GoTaq® Reaction Buffer and 5X Colorless GoTaq® Reaction Buffer. The 5X Green GoTaq® Reaction Buffer contains two dyes (blue and yellow) that separate during electrophoresis to monitor migration progress. The buffer also contains a compound that increases the density of the sample so that it will sink into the well of the agarose gel, allowing reactions to be directly loaded onto an agarose gel without the need for loading dye. The blue dye

comigrates at the same rate as a 3–5kb DNA fragment in a 1% agarose gel. The yellow dye migrates at a rate faster than primers (<50bp) in a 1% agarose gel. The 5X Colorless GoTaq® Reaction Buffer and 5X Green GoTaq® Reaction Buffer have the same formulation, except for the dyes. The 5X Colorless GoTaq® Reaction Buffer is recommended for any applications where absorbance or fluorescence measurements of the PCR amplifier will be taken without prior cleanup. Both buffers are supplied at pH 8.5 and contain MgCl₂ at a concentration of 7.5mM for a final concentration of 1.5mM.

GoTaq® Flexi DNA Polymerase is supplied with 5X Green GoTaq® Flexi Reaction Buffer and 5X Colorless GoTaq® Flexi Reaction Buffer. The compositions are identical to the 5X Green GoTaq® Reaction Buffer and 5X Colorless GoTaq® Reaction Buffer, except that the GoTaq® Flexi reaction buffers do not contain MgCl₂. Instead, the GoTaq® Flexi DNA Polymerase is supplied with a tube of 25mM MgCl₂ so that reactions can be supplemented with varying concentrations of magnesium.

C. Enzyme Concentration

We recommend using 1–1.25 units of *Taq* DNA polymerase in a 50µl amplification reaction. In most cases, this is an excess of enzyme, and adding more enzyme will not significantly increase product yield. In fact, increased amounts of enzyme increase the likelihood of generating artifacts associated with the intrinsic 5'→3' exonuclease activity of *Taq* DNA polymerase, resulting in smeared bands in an agarose gel (Longley *et al.* 1990; Bell and DeMarini, 1991).

Pipetting errors are a frequent cause of excessive enzyme levels. Accurate dispensing of small volumes of enzyme solutions in 50% glycerol is difficult, so we strongly recommend preparing a reaction master mix, which requires a larger volume of each reagent, to reduce pipetting errors.

D. PCR Primer Design

PCR primers define the target region to be amplified and generally range in length from 15–30 bases. Ideally primers will have a GC-content of 40–60%. Avoid three G or C residues in a row near the 3'-end of the primer to minimize nonspecific primer annealing. Also, avoid primers with intra- or intermolecular complementary sequences to minimize the production of primer-dimer. Intramolecular regions of secondary structure can interfere with primer annealing to the template and should be avoided.

Ideally, the melting temperature (T_m), the temperature at which 50% of the primer molecules are annealed to the complementary sequence, of the two primers will be within 5°C so that the primers anneal efficiently at the same temperature. Primers can be designed to include sequences that are useful for downstream applications. For example, restriction enzyme sites can be placed at the 5'-ends of PCR primers to facilitate subsequent cloning of the PCR product,

or a T7 RNA polymerase promoter can be added to allow *in vitro* transcription without the need to subclone the PCR product into a vector.

E. Template Quality

Successful amplification depends on DNA template quantity and quality. Reagents commonly used to purify nucleic acids (salts, guanidine, proteases, organic solvents and SDS) are potent inactivators of DNA polymerases. For example, 0.01% SDS will inhibit *Taq* DNA polymerase by 90%, while 0.1% SDS will inhibit *Taq* DNA polymerase by 99.9% (Konat *et al.* 1994). A few other examples of PCR inhibitors are phenol (Katcher and Schwartz, 1994), heparin (Beutler *et al.* 1990; Holodniy *et al.* 1991), xylene cyanol, bromophenol blue (Hoppe *et al.* 1992), plant polysaccharides (Demeke and Adams, 1992), and the polyamines spermine and spermidine (Ahokas and Erkkila, 1993). In some cases, the inhibitor is not introduced into the reaction with the nucleic acid template. A good example of this is an inhibitory substance that can be released from polystyrene or polypropylene upon exposure to ultraviolet light (Pao *et al.* 1993; Linquist *et al.* 1998).

If an amplification reaction fails and you suspect the DNA template is contaminated with an inhibitor, add the suspect DNA preparation to a control reaction with a DNA template and primer pair that has amplified well in the past. Failure to amplify the control DNA usually indicates the presence of an inhibitor. Additional steps to clean up the DNA preparation, such as phenol:chloroform extraction or ethanol precipitation, may be necessary.

F. Template Quantity

The amount of template required for successful amplification depends upon the complexity of the DNA sample. For example, of a 4kb plasmid containing a 1kb target sequence, 25% of the input DNA is the target of interest. Conversely, a 1kb target sequence in the human genome (3.3×10^9 bp) represents approximately 0.00003% of the input DNA. Thus, approximately 1,000,000-fold more human genomic DNA is required to maintain the same number of target copies per reaction. Common mistakes include using too much plasmid DNA, too much PCR product or too little genomic DNA as the template. Reactions with too little DNA template will have low yields, while reactions with too much DNA template can be plagued by nonspecific amplification. If possible, start with $>10^4$ copies of the target sequence to obtain a signal in 25–30 cycles, but try to keep the final DNA concentration of the reaction ≤ 10 ng/µl. When reamplifying a PCR product, the concentration of the specific PCR product is often not known. We recommend diluting the previous amplification reaction 1:10 to 1:10,000 before reamplification.

1µg of 1kb RNA = 1.77×10^{12} molecules

1µg of 1kb dsDNA = 9.12×10^{11} molecules

1µg of pGEM® Vector DNA = 2.85×10^{11} molecules

1µg of lambda DNA = 1.9×10^{10} molecules

1 μg of *E. coli* genomic DNA = 2×10^8 molecules

1 μg of human genomic DNA = 3.04×10^5 molecules

G. Cycling Parameters

The two most commonly altered cycling parameters are annealing temperature and extension time. The lengths and temperatures for the other steps of a PCR cycle do not usually vary significantly. However in some cases, the denaturation cycle can be shortened or a lower denaturation temperature used to reduce the number of depurination events, which can lead to mutations in the PCR products.

Primer sequence is a major factor that determines the optimal annealing temperature, which is often within 5°C of the melting temperature of the primers. Using an annealing temperature slightly higher than the primer T_m will increase annealing stringency and can minimize nonspecific primer annealing and decrease the amount of undesired products synthesized. Using an annealing temperature lower than the primer T_m can result in higher yields, as the primers anneal more efficiently at the lower temperature. We recommend testing several annealing temperatures, starting approximately 5°C below the T_m , to determine the best annealing conditions. In many cases, nonspecific amplification and primer-dimer formation can be reduced through optimization of annealing temperature, but if undesirable PCR products remain a problem, consider incorporating one of the many hot-start PCR methods.

Oligonucleotide synthesis facilities will often provide an estimate of a primer's T_m . The T_m also can be calculated using the Biomath Calculators (www.promega.com/biomath/). Numerous formulas exist to determine the theoretical T_m of nucleic acids (Baldino, Jr. *et al.* 1989; Rychlik *et al.* 1990). The formula below can be used to estimate the melting temperature for oligonucleotides:

$$T_m = 81.5 + 16.6 \times (\log_{10}[\text{Na}^+]) + 0.41 \times (\%G+C) - 675/n$$

where $[\text{Na}^+]$ is the molar salt concentration and n = number of bases in the oligonucleotide

Example:

To calculate the melting temperature of a 22mer oligonucleotide with 60% G+C in 50mM KCl:

$$T_m = 81.5 + 16.6 \times (\log_{10}[0.05]) + 0.41 \times (60) - 675/22$$

$$= 81.5 + 16.6 \times (-1.30) + 24.60 - 30.68 = 54^\circ\text{C}$$

The length of the extension cycle, which also may need to be optimized, depends on PCR product size and the DNA polymerase being used. In general, allow approximately 1 minute for every 1kb of amplicon (minimum extension time = 1 minute) for nonproofreading DNA polymerases and 2 minutes for every 1kb of amplicon for proofreading DNA polymerases. Avoid excessively long extension times, as they can increase the likelihood of generating artifacts associated with the intrinsic 5'→3' exonuclease activity of *Taq* DNA polymerase (Longley *et al.* 1990; Bell and DeMarini, 1991).

PCR typically involves 25–35 cycles of amplification. The risk of undesirable PCR products appearing in the reaction increases as the cycle number increases, so we recommend performing only enough cycles to synthesize the desired amount of product. If nonspecific amplification products accumulate before sufficient amounts of PCR product can be synthesized, consider diluting the products of the first reaction and performing a second amplification with the same primers or primers that anneal to sequences within the desired PCR product (nested primers).

H. PCR Enhancers and Additives

Addition of PCR-enhancing agents can increase yield of the desired PCR product or decrease production of undesired products. There are many PCR enhancers, which can act through a number of different mechanisms. These reagents will not enhance all PCRs; the beneficial effects are often template- and primer-specific and will need to be determined empirically. Some of the more common enhancing agents are discussed below.

Addition of betaine, DMSO and formamide can be helpful when amplifying GC-rich templates and templates that form strong secondary structures, which can cause DNA polymerases to stall. GC-rich templates can be problematic due to inefficient separation of the two DNA strands or the tendency for the complementary, GC-rich primers to form intermolecular secondary structures, which will compete with primer annealing to the template. Betaine reduces the amount of energy required to separate DNA strands (Rees *et al.* 1993). DMSO and formamide are thought to aid amplification in a similar manner by interfering with hydrogen bond formation between two DNA strands (Geiduschek and Herskovits, 1961).

Some reactions that amplify poorly in the absence of enhancers will give a higher yield of PCR product when betaine (1M), DMSO (1–10%) or formamide (1–10%) are added. Concentrations of DMSO greater than 10% and formamide greater than 5% can inhibit *Taq* DNA polymerase and presumably other DNA polymerases as well (Varadaraj and Skinner, 1994). Specific examples of the effects of DMSO and betaine on GoTaq® DNA Polymerase are published in *Neural Notes* (www.promega.com/nnotes/nn021/21_02.htm) (Knoche, K., 2002).

In some cases, general stabilizing agents such as BSA (0.1mg/ml), gelatin (0.1–1.0%) and nonionic detergents (0–0.5%) can overcome amplification failure. These additives can increase DNA polymerase stability and reduce the loss of reagents through adsorption to tube walls. BSA also has been shown to overcome the inhibitory effects of melanin on RT-PCR (Giambernardi *et al.* 1998). Nonionic detergents, such as Tween®-20, NP-40 and Triton® X-100, have the added benefit of overcoming inhibitory effects of trace amounts of strong ionic detergents, such as 0.01% SDS (Gelfand and White, 1990). Ammonium ions can make an amplification reaction more tolerant of nonoptimal conditions. For this reason, some PCR reagents

include 10–20mM (NH₄)₂SO₄. Other PCR enhancers include glycerol (5–20%), polyethylene glycol (5–15%) and tetramethyl ammonium chloride (60mM).

I. Nucleic Acid Cross-Contamination

It is important to minimize cross-contamination between samples and prevent carryover of RNA and DNA from one experiment to the next. Use separate work areas and pipettors for pre- and postamplification steps. Use positive displacement pipettes or aerosol-resistant tips to reduce cross-contamination during pipetting. Wear gloves, and change them often.

There are a number of techniques that can be used to prevent amplification of contaminating DNA. PCR reagents can be treated with isopropyl alcohol, a photo-activated, cross-linking reagent that intercalates into double-stranded DNA molecules and forms covalent, interstrand crosslinks, to prevent DNA denaturation and replication. These interstrand crosslinks effectively render contaminating DNA unamplifiable.

Treatment of PCR reagents with uracil-N-glycosylase (UNG), a DNA repair enzyme that hydrolyzes the base-ribose bond at uracil residues, eliminates one of the most common sources of DNA contamination: previously amplified PCR products. UNG treatment prevents replication of uracil-containing DNA by causing the DNA polymerase to stall at the resulting abasic sites. For UNG to be an effective safeguard against contamination, the products of previous amplifications must be synthesized in the presence of dUTP. This is easily accomplished by substituting dUTP for some or all of the dTTP in the reaction. Nonproofreading polymerases will readily incorporate dUTP into a PCR product, but proofreading polymerases incorporate dUTP much less efficiently (Slupphaug *et al.* 1993; Greagg *et al.* 1999; Lasken *et al.* 1996). Since dUTP incorporation has no noticeable effect on the intensity of ethidium bromide staining or electrophoretic mobility of the PCR product, reactions can be analyzed by standard agarose gel electrophoresis. While both methods are effective (Rys and Persing, 1993), UNG treatment has the advantage that both single-stranded and double-stranded DNA templates will be rendered unamplifiable (Longo *et al.* 1990).

III. General Considerations for RT-PCR

Please also read General Considerations for PCR Optimization. Many of the important parameters discussed there also apply to RT-PCR. For a discussion of reverse transcriptases commonly used for RT-PCR, see the Reverse Transcription section.

A. Overview of the Access and AccessQuick™ RT-PCR Systems

The Access RT-PCR System and AccessQuick™ RT-PCR System are designed for the reverse transcription and amplification of a specific target RNA from either total RNA or mRNA (Miller and Storts, 1995; Knoche and Denhart, 2001). These one-tube, two-enzyme systems provide sensitive, quick and reproducible analysis of even

rare RNAs (Miller and Storts, 1996). The systems use AMV Reverse Transcriptase for first-strand cDNA synthesis and the thermostable *Tfi* DNA Polymerase from *Thermus flavus* (Kaledin *et al.* 1981) for second-strand cDNA synthesis and DNA amplification. The systems include an optimized single-buffer system that permits sensitive detection of RNA transcripts without the need for buffer additions between reverse transcription and PCR amplification steps. This simplifies the procedure and reduces the potential for contamination. The elevated reaction temperature (45°C) possible with AMV reverse transcriptase minimizes problems encountered with RNA secondary structures (Brooks *et al.* 1995).

B. Template Considerations

For RT-PCR, successful reverse transcription depends on RNA integrity and purity. Procedures for creating and maintaining a ribonuclease-free (RNase-free) environment to minimize RNA degradation are described in Blumberg, 1987. The use of an RNase inhibitor (e.g., Recombinant RNasin® Ribonuclease Inhibitor) is strongly recommended. For optimal results, the RNA template, whether a total RNA preparation, an mRNA population or a synthesized RNA transcript, should be DNA-free to avoid amplification of contaminating DNA. The most commonly used DNA polymerases for PCR have no reverse transcriptase activity under standard reaction conditions, and thus, amplification products will be generated only if the template contains trace amounts of DNA with similar sequences.

Successful RT-PCR also depends on RNA quantity, which may need to be varied to determine the optimal amount. Excellent amplification results can be obtained with the Access and AccessQuick™ RT-PCR Systems using total RNA template levels in the range of 1pg–1µg per reaction (Figure 1.7) or poly(A)+ RNA template levels in the range of 1pg–100ng.

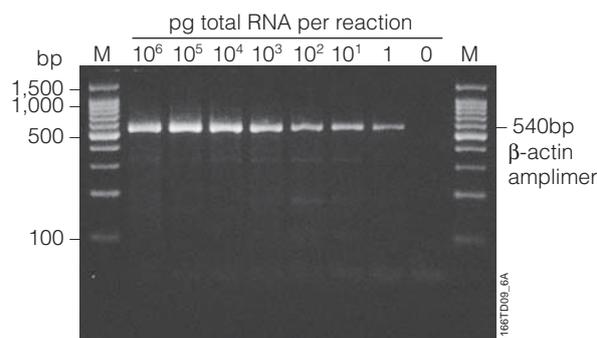


Figure 1.7. Amplification of a specific message in total RNA. RT-PCR amplifications containing the indicated amounts of mouse liver total RNA were performed using the Access RT-PCR System as described in the Access RT-PCR protocol using oligonucleotide primers specific to the mouse β -actin transcript. The specific 540bp amplicon is indicated. Equivalent aliquots of each amplification reaction were separated on a 3% NuSieve®/ 1% agarose gel in 1X TAE buffer containing 0.5µg/ml ethidium bromide. Lanes M, 100bp DNA Ladder (Cat.# G2101).

C. Reverse Transcription Primer Design

Selection of an appropriate primer for reverse transcription depends on target mRNA size and the presence of secondary structure. For example, a primer that anneals specifically to the 3'-end of the transcript (a sequence-specific primer or oligo(dT) primer) may be problematic when reverse transcribing the 5'-ends of long mRNAs or molecules that have significant secondary structure, which can cause the reverse transcriptase to stall during cDNA synthesis. Random hexamers prime reverse transcription at multiple points along the transcript. For this reason, they are useful for either long mRNAs or transcripts with significant secondary structure.

Whenever possible, we recommend using a primer that anneals only to defined sequences in particular RNAs (sequence-specific primers) rather than to entire RNA populations in the sample (e.g., random hexamers or oligo(dT) primer). To differentiate between amplification of cDNA and amplification of contaminating genomic DNA, design primers to anneal to sequences in exons on opposite sides of an intron so that any amplification product derived from genomic DNA will be much larger than the product amplified from the target cDNA. This size difference not only makes it possible to differentiate the two products by gel electrophoresis but also favors the synthesis of the smaller cDNA-derived product (amplification of smaller fragments is often more efficient than that of long fragments).

Regardless of primer choice, the final primer concentration in the reaction is usually within the range of 0.1–1.0 μM , but this may need to be optimized. We recommend using a final concentration of 1 μM primer (50 pmol in a 50 μl reaction) as a starting point for optimization. More information on PCR primer design is provided in the PCR Primer Design section.

D. Cycle Parameters

Efficient first-strand cDNA synthesis can be accomplished in a 20- to 60-minute incubation at 37–45°C using AMV reverse transcriptase or at 37–42°C for M-MLV reverse transcriptase. When using AMV RT we recommend using a sequence-specific primer and performing reverse transcription at 45°C for 45 minutes as a starting point. The higher reaction temperature will minimize the effects of RNA secondary structure and encourage full-length cDNA synthesis. First-strand cDNA synthesis with random hexamers and oligo(dT) primer should be conducted at room temperature (20–25°C) and 37°C, respectively.

The Access and AccessQuick™ RT-PCR Systems do not require RNA denaturation prior to initiation of the reverse transcription reaction. If desired, however, a denaturation step may be incorporated by incubating a separate tube containing the primers and RNA template at 94°C for 2 minutes. Do not incubate AMV reverse transcriptase at 94°C; it will be inactivated. The template/primer mixture then can be cooled to 45°C and added to the RT-PCR mix for the standard reverse transcription incubation at 45°C.

Following the reverse transcription, we recommend a 2-minute incubation at 94°C to denature the RNA/cDNA hybrid, inactivate AMV reverse transcriptase and dissociate AMV RT from the cDNA. It has been reported that AMV reverse transcriptase must be inactivated to obtain high yields of amplification product (Sellner *et al.* 1992; Chumakov, 1994).

Most RNA samples can be detected using 30–40 cycles of amplification. If the target RNA is rare or if only a small amount of starting material is available, it may be necessary to increase the number of cycles to 45 or 50 or dilute the products of the first reaction and reamplify.

IV. Thermostable DNA Polymerases

Prior to the use of thermostable DNA polymerases in PCR, researchers had to laboriously replenish the reaction with fresh enzyme (such as Klenow or T4 DNA polymerase) after each denaturation cycle. Thermostable DNA polymerases revolutionized and popularized PCR because of their ability to withstand the high denaturation temperatures. The use of thermostable DNA polymerases also allowed higher annealing temperatures, which improved the stringency of primer annealing.

Thermostable DNA polymerases can be used for either one-enzyme or two-enzyme RT-PCR (Myers and Gelfand, 1991; Chiochia and Smith, 1997). For example, *Tth* DNA polymerase can act as a reverse transcriptase in the presence of Mn^{2+} for one-enzyme RT-PCR (Myers and Gelfand, 1991). All of the DNA polymerases mentioned below can be used to amplify first-strand cDNA produced by a reverse transcriptase, such as AMV RT, in two-enzyme RT-PCR.

Thermostable DNA polymerases can be divided into two groups: those with a 3'→5' exonuclease (proofreading) activity, such as *Pfu* DNA polymerase, and those without the proofreading function, such as *Taq* DNA polymerase. These two groups have some important differences. Proofreading DNA polymerases are more accurate than nonproofreading polymerases due to the 3'→5' exonuclease activity, which can remove a misincorporated nucleotide from a growing DNA chain. When the amplified product is to be cloned, expressed or used in mutation analysis, *Pfu* DNA polymerase is a better choice due to its high fidelity. However, for routine PCR, where simple detection of an amplification product is the goal, *Taq* DNA polymerase is the most commonly used enzyme because yields tend to be higher with a nonproofreading DNA polymerase.

Amplification with nonproofreading DNA polymerases results in the template-independent addition of a single nucleotide to the 3'-end of the PCR product, whereas the use of proofreading DNA polymerases results in blunt-ended PCR products (Clark, 1988; Hu, 1993). The single-nucleotide overhang can simplify the cloning of PCR products.

Proofreading DNA polymerases also are used in blends with nonproofreading DNA polymerases, or amino-terminally truncated versions of *Taq* DNA

polymerase, to amplify longer stretches of DNA with greater accuracy than the nonproofreading DNA polymerase alone (Barnes, 1994; Cline *et al.* 1996). See Long PCR.

A. Taq DNA Polymerase

Taq DNA polymerase is isolated from *Thermus aquaticus* and catalyzes the primer-dependent incorporation of nucleotides into duplex DNA in the 5'→3' direction in the presence of Mg²⁺. The enzyme does not possess 3'→5' exonuclease activity but has 5'→3' exonuclease activity.

Taq DNA polymerase is suitable for most PCR applications that do not require a high-fidelity enzyme, such as detecting specific DNA or RNA sequences. The error rate of *Taq* DNA polymerase is approximately 1×10^{-5} errors/base, although the fidelity does depend somewhat on the reaction conditions. The fidelity is slightly higher at lower pH, lower magnesium concentration and relatively low dNTP concentration (Eckert and Kunkel, 1990; Eckert and Kunkel, 1991). See High-Fidelity PCR.

Taq DNA polymerase is commonly used to amplify PCR products of 5kb or less. PCR products in the range of 5–10kb can be amplified with *Taq* DNA polymerase but often require more optimization than smaller PCR products. For products larger than approximately 10kb, we recommend an enzyme or enzyme mix and reaction conditions that are designed for long PCR.

Taq DNA polymerase is a processive enzyme with an extension rate of >60 nucleotides/second at 70°C (Innis *et al.* 1988), so an extension step of 1 minute per 1kb to be amplified should be sufficient to generate full-length PCR products. The enzyme has a half-life of 40 minutes at 95°C (Lawyer *et al.* 1993). Because *Taq* DNA polymerase is a nonproofreading polymerase, PCR products generated with *Taq* DNA polymerase will contain a single-nucleotide 3' overhang, usually a 3' A overhang.

Additional Resources for *Taq* DNA Polymerase

Technical Bulletins and Manuals

9PIM300 *GoTaq® DNA Polymerase Promega Product Information*
(www.promega.com/tbs/9pim300/9pim300.html)

9PIM829 *GoTaq® Flexi DNA Polymerase Promega Product Information*
(www.promega.com/tbs/9pim829/9pim829.html)

Promega Publications

eNotes *GoTaq® Green Master Mix for quick and easy two-step RT-PCR*
(www.promega.com/enotes/applications/ap0069.htm)

B. Tfi DNA Polymerase

Tfi DNA polymerase catalyzes the primer-dependent polymerization of nucleotides into duplex DNA in the presence of Mg²⁺. In the presence of Mn²⁺, *Tfi* DNA polymerase can use RNA as a template. *Tfi* DNA polymerase exhibits a 5'→3' exonuclease activity but lacks a 3'→5' exonuclease activity. This enzyme is commonly used in PCR (Gaensslen *et al.* 1992), where its activity is similar to that of *Taq* DNA polymerase. *Tfi* DNA polymerase is used in the Access and AccessQuick™ RT-PCR Systems.

C. Tth DNA Polymerase

Tth DNA polymerase catalyzes polymerization of nucleotides into duplex DNA in the 5'→3' direction in the presence of MgCl₂. The enzyme can use an RNA template in the presence of MnCl₂ (Myers and Gelfand, 1991;

Ruttimann *et al.* 1985). *Tth* DNA polymerase exhibits a 5'→3' exonuclease activity but lacks detectable 3'→5' exonuclease activity. The error rate of *Tth* DNA polymerase has been measured at 7.7×10^{-5} errors/base (Arakawa *et al.* 1996). *Tth* DNA polymerase can amplify target DNA in the presence of phenol-saturated buffer (Katcher and Schwartz, 1994) and has been reported to be more resistant to inhibition by blood components than other thermostable polymerases (Ehrlich *et al.* 1991; Bej and Mahbubani, 1992).

Tth DNA polymerase is commonly used for PCR (Myers and Gelfand, 1991; Carballeira *et al.* 1990) and RT-PCR (Myers and Gelfand, 1991; Chiocchia and Smith, 1997). For primer extension, RT-PCR and cDNA synthesis using RNA templates with complex secondary structure, the high reaction temperature of *Tth* DNA polymerase may be an advantage over more commonly used reverse transcriptases, such as AMV and M-MLV reverse transcriptases. Recombinant *Tth* DNA polymerase has been shown to exhibit RNase H-like activity (Auer *et al.* 1995).

Additional Resources for *Tth* DNA Polymerase

Technical Bulletins and Manuals

9PIM210 *Tth DNA Polymerase Promega Product Information*
(www.promega.com/tbs/9pim210/9pim210.html)

D. Tli DNA Polymerase

Tli DNA polymerase replicates DNA through the polymerization of nucleotides into duplex DNA in the 5'→3' direction in the presence of Mg²⁺. This enzyme also contains a 3'→5' exonuclease activity, which results in increased fidelity of nucleotide incorporation. There is no detectable reverse transcriptase activity or 5'→3' exonuclease activity. *Tli* DNA polymerase will promote strand displacement at 72°C but will not displace DNA at 55°C (Kong *et al.* 1993). Greater than 95% of the amplified products will be blunt-ended.

Tli DNA polymerase is commonly used for PCR and RT-PCR, where its proofreading activity makes it useful for high-fidelity and long PCR (Keohavong *et al.* 1993). Due to the 3'→5' exonuclease activity of *Tli* DNA polymerase, the enzyme can degrade the oligonucleotide primers used to initiate DNA synthesis. This exonucleolytic attack can be effectively prevented by using hot-start PCR or introducing a single phosphorothioate bond at the 3' termini of the primers (Byrappa *et al.* 1995). *Tli* DNA polymerase also can be used for primer extension, where the high optimal temperature of the enzyme may be an advantage for templates with complex secondary structure.

E. Pfu DNA Polymerase

Pfu DNA polymerase has one of the lowest error rates of all known thermophilic DNA polymerases used for amplification due to the high 3'→5' exonuclease activity (Cline *et al.* 1996; Andre *et al.* 1997). For cloning and expressing DNA after PCR, *Pfu* DNA polymerase is often the enzyme of choice. *Pfu* DNA polymerase can be used alone to amplify DNA fragments up to 5kb by increasing the extension time to 2 minutes per kilobase. It is also used in blends with DNA polymerases lacking the proofreading function, such as *Taq* DNA polymerase, to achieve longer amplification products than with *Pfu* DNA polymerase alone (Barnes, 1994). However, the proofreading activity can shorten PCR primers, leading to decreased yield and increased nonspecific amplification. This exonucleolytic attack can be effectively prevented by using hot-start PCR or introducing a single phosphorothioate bond at the 3'-termini of the primers (Byrappa *et al.* 1995).

Additional Resources for *Pfu* DNA Polymerase

Technical Bulletins and Manuals

9PIM774 *Pfu* DNA Polymerase Promega Product Information
(www.promega.com/tbs/9pim774/9pim774.html)

Promega Publications

PN068 *Pfu* DNA Polymerase: A high fidelity enzyme for nucleic acid amplification
(www.promega.com/pnotes/68/7381_07/7381_07.html)

V. Reverse Transcriptases

The discovery of reverse transcriptases, or RNA-dependent DNA polymerases, and their role in retrovirus infection (Baltimore, 1970; Temin and Mizutani, 1970) altered molecular biology's central dogma of

DNA→RNA→protein. Reverse transcriptases use an RNA template to synthesize DNA and require a primer for synthesis, like other DNA polymerases. For *in vitro* applications, the primer can be either oligo(dT), which hybridizes to the poly(A)⁺ tails of eukaryotic mRNAs, random hexamers, which prime synthesis throughout the length of the RNA template, or a sequence-specific primer, which hybridizes to a known sequence within the RNA

template. Polymerization from a primer then proceeds as for DNA-dependent DNA polymerases. The commonly used reverse transcriptases, AMV reverse transcriptase, M-MLV reverse transcriptase and M-MLV reverse transcriptase, RNase H minus, perform the same reaction but at different optimum temperatures (AMV, 42°C; M-MLV, 37°C; and M-MLV RT, RNase H-, 42°C).

Some reverse transcriptases also possess intrinsic 3'- or 5'-exoribonuclease (RNase) activity, which is generally used to degrade the RNA template after first strand cDNA synthesis. Absence of the 5'-exoribonuclease (RNase H) activity may aid production of longer cDNAs (Berger *et al.* 1983).

Some DNA-dependent DNA polymerases also possess a reverse transcriptase activity, which can be favored under certain conditions. For example, the thermostable, DNA-dependent *Tth* DNA polymerase exhibits reverse transcriptase activity when Mn²⁺ is substituted for Mg²⁺ in a reaction.

A. AMV Reverse Transcriptase

AMV RT catalyzes DNA polymerization using template DNA, RNA or RNA:DNA hybrids (Houts *et al.* 1979). AMV reverse transcriptase is the preferred reverse transcriptase for templates with high secondary structure due to its higher reaction temperature (up to 58°C). AMV RT is used in a wide variety of applications including cDNA synthesis (Houts *et al.* 1979; Gubler and Hoffman, 1983), RT-PCR and rapid amplification of cDNA ends (RACE; Skinner *et al.* 1994). Although the high optimal temperature (42°C) makes it the enzyme of choice for cDNA synthesis using templates with complex secondary structure, its relatively high RNase H activity limits its usefulness for generation of long cDNAs (>5kb). For these templates, M-MLV RT or M-MLV RT, RNase H minus, may be a better choice.

Additional Resources for AMV Reverse Transcriptase

Technical Bulletins and Manuals

9PIM510 *AMV Reverse Transcriptase Promega Product Information*
(www.promega.com/tbs/9pim510/9pim510.html)

B. M-MLV Reverse Transcriptase

M-MLV RT is a single-polypeptide, RNA-dependent DNA polymerase. The enzyme also has DNA-dependent DNA polymerase activity at high enzyme levels (Roth *et al.* 1985). M-MLV RT is used in a variety of applications, including cDNA synthesis, RT-PCR and RACE (Gerard, 1983). Its relatively low RNase H activity compared to AMV RT makes M-MLV RT the enzyme of choice for generating long cDNAs (>5kb) (Sambrook and Russell, 2001). However, for short templates with complex secondary structure, AMV RT or M-MLV RT, RNase H minus, may be a better choice due to their higher optimal temperatures. M-MLV RT is less processive than AMV RT, so more units of M-MLV RT may be required to generate the same amount of cDNA (Schaefer, 1995).

Additional Resources for M-MLV Reverse Transcriptase

Technical Bulletins and Manuals

9PIM170 *M-MLV Reverse Transcriptase Promega Product Information*
www.promega.com/tbs/9pim170/9pim170.html

C. M-MLV Reverse Transcriptase, RNase H Minus

M-MLV reverse transcriptase, RNase H minus, is an RNA-dependent, 5'→3' DNA polymerase that has been genetically altered to remove the associated ribonuclease H activity, which causes degradation of the RNA strand of an RNA:DNA hybrid (Tanese and Goff, 1988). The absence of RNase H activity makes M-MLV, RNase H minus, the enzyme of choice for generating long cDNAs (>5kb). However, for shorter templates with complex secondary structure, AMV reverse transcriptase may be a better choice because it can be used at higher reaction temperatures.

There are two forms of M-MLV, RNase H minus: the deletion mutant and the point mutant. As the names suggest, the deletion mutant had a specific sequence in the RNase H domain deleted, and the point mutant has a point mutation introduced in the RNase H domain. While the native M-MLV RT has a recommended reaction temperature of 37°C, the deletion and point mutants are more stable at higher temperatures and can be used at reaction temperatures of up to 50°C and 55°C, respectively, depending upon the reverse transcription primers used. The point mutant is often preferred over the deletion mutant because the point mutant has DNA polymerase activity comparable to that of the wildtype M-MLV enzyme, whereas the deletion mutant has a slightly reduced DNA polymerase activity compared to that of the wildtype enzyme (Figure 1.8).

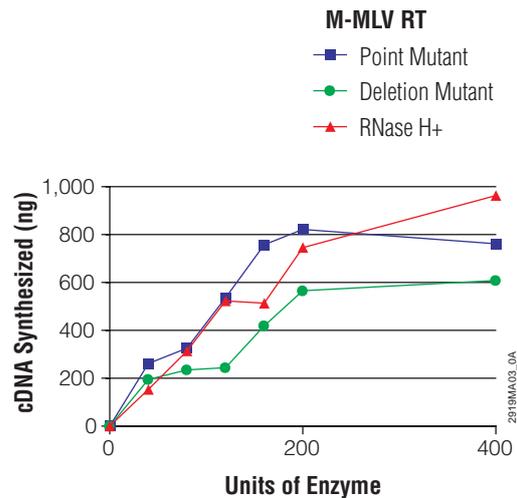


Figure 1.8. Comparison of the mass amount of total cDNA synthesized from 2µg of a 7.5kb RNA template by increasing amounts of three Promega M-MLV reverse transcriptases. Each first-strand cDNA reaction was performed using 2µg of a 7.5kb RNA template (1µl), 0.5µg of oligo(dT)₁₅ primer (1µl) and 14µl water. The RNA and oligo(dT)₁₅ primer were heated at 70°C for 5 minutes and cooled on ice for 5 minutes. Five microliters of M-MLV RT 5X Buffer, 1.25µl of 10µM dNTPs, 0.5µl of α-³²P dCTP (10µCi/µl, 400Ci/mmol) and either 25, 50, 100, 150, 200 or 400 units of M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant; M-MLV Reverse Transcriptase, RNase H Minus, Deletion Mutant; or native M-MLV Reverse Transcriptase (RNase H+) was used in a final volume of 25µl. Reactions were incubated at 42°C for 60 minutes. TCA precipitations were performed, and first-strand cDNA yields were calculated.

Additional Resources for M-MLV Reverse Transcriptase, RNase H Minus

Technical Bulletins and Manuals

9PIM530 *M-MLV Reverse Transcriptase, RNase H Minus, Promega Product Information*
www.promega.com/tbs/9pim530/9pim530.html

9PIM368 *M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant, Promega Product Information*
www.promega.com/tbs/9pim368/9pim368.html

VI. Example of a PCR Protocol

Materials Required:

(see Composition of Solutions section)

- template DNA
- downstream primer
- upstream primer
- GoTaq® DNA Polymerase (Cat.# M8291)
- MgCl₂, 25mM
- Nuclease-Free Water (Cat.# P1193)

- nuclease-free light mineral oil (e.g., Sigma Cat.# M5904) if using a thermal cycler without a heated lid; do not autoclave
- dNTP mix, 10mM of each dNTP

Note: To facilitate optimization, troubleshooting and validation, we strongly recommend including both positive and negative control reactions.

1. Combine the first five reaction components in the order listed below in a thin-walled 0.5ml reaction tube. Gently vortex the tube for 10 seconds, and briefly centrifuge in a microcentrifuge. Initiate the reaction by adding the template and primers.

Component	Volume	Final Concentration
Nuclease-Free Water (to a final volume of 50µl)	Xµl	
5X Green or Colorless GoTaq® Flexi Buffer	10µl	1X
dNTP mix, 10mM each dNTP	1µl	0.2mM each
GoTaq® DNA Polymerase (5u/µl)	0.25µl	0.025u/µl
25mM MgCl ₂	3µl	1.5mM
downstream primer	50pmol ¹	1µM
upstream primer	50pmol	1µM
template ²	Yµl	

¹A general formula for calculating the number of nanograms of primer equivalent to 50pmol is: 50pmol = 16.3ng × b; where b is the number of bases in the primer.

²If possible, start with >10⁴ copies of the target sequence to obtain a signal in 25–30 cycles, but keep the final DNA concentration of the reaction at ≤10ng/µl. Less than 10 copies of a target can be amplified (Saiki, 1988), but more cycles may be required to detect a signal by gel electrophoresis. Additional cycles may increase nonspecific amplification, evidenced by smeared bands upon gel electrophoresis.

2. Overlay the reaction with 1–2 drops (20–40µl) of nuclease-free mineral oil to prevent condensation and evaporation. Mineral oil addition is not necessary if you are using a thermal cycler with a heated lid.
3. Place tube in a thermal cycler, and proceed with the thermal cycling profile chosen for your reactions.
4. Analyze 5µl of the PCR products by agarose gel electrophoresis. The products should be readily visible by UV transillumination of the ethidium bromide-stained gel.
5. Store reaction products at –20°C until needed.

VII. Example of an RT-PCR Protocol

A. Access RT-PCR Protocol

These conditions work well to detect the 323bp PCR product generated from the Positive Control RNA using the Upstream and Downstream Control Primers provided with the Access RT-PCR System. We recommend optimizing the parameters for each target RNA.

Materials Required:

(see Composition of Solutions section)

- template RNA
- downstream oligonucleotide primer
- upstream oligonucleotide primer
- Access RT-PCR System (Cat.# A1250)
- Nuclease-Free Water (Cat.# P1193)
- nuclease-free light mineral oil (e.g., Sigma Cat.# M5904) if using a thermal cycler without a heated lid

1. Prepare the reaction mix by combining the indicated volumes of Nuclease-Free Water, AMV/*Tfl* 5X Reaction Buffer, dNTP Mix, 25mM MgSO₄ and the specific upstream and downstream primers in a thin-walled 0.5ml reaction tube on ice. Mix the components by pipetting. Add AMV Reverse Transcriptase and *Tfl* DNA Polymerase to the reaction. Gently vortex the tube for 10 seconds to mix.

Component	Volume	Final Concentration
Nuclease-Free Water (to a final volume of 50µl)	Xµl	
AMV/ <i>Tfl</i> 5X Reaction Buffer	10µl	1X
dNTP Mix, 10mM each dNTP	1µl	0.2mM each
downstream primer	50pmol ³	1µM
upstream primer	50pmol	1µM
25mM MgSO ₄	2µl	1mM
AMV Reverse Transcriptase (5u/µl)	1µl	0.1u/µl
<i>Tfl</i> DNA Polymerase (5u/µl)	1µl	0.1u/µl
RNA sample ⁴	Yµl	

³A general formula for calculating the number of nanograms of primer equivalent to 50pmol is: 50pmol = 16.3ng × b; where b is the number of bases in the primer. For the positive control reaction, use 3.3µl of both the Downstream and Upstream Control Primers (50pmol).

⁴Use 10³–10⁶ copies of a specific target template or 1pg–1µg total RNA. Use 2µl of the Positive Control RNA with Carrier (2.5 attomoles or 1 × 10⁶ copies).

2. Overlay the reaction with one or two drops (20–40µl) of nuclease-free mineral oil to prevent condensation and evaporation. Mineral oil addition is not necessary if you are using a thermal cycler with a heated lid.

- Place tube in a thermal cycler equilibrated at 45°C, and incubate for 45 minutes.
- Proceed directly to thermal cycling for second-strand cDNA synthesis and amplification (refer to Tables 1.1 and 1.2).

Table 1.1. First-Strand cDNA Synthesis.

1 cycle	45°C for >45 minutes	reverse transcription
1 cycle	94°C for >2 minutes	AMV RT inactivation and RNA/cDNA/primer denaturation

Table 1.2. Second-Strand cDNA Synthesis and PCR.

40 cycles	94°C for 30 seconds	denaturation
	60°C for 1 minute	annealing
	68°C for 2 minutes	extension
1 cycle	68°C for 7 minutes	final extension
1 cycle	4°C	soak

B. ImProm-II™ Reverse Transcription System Protocol

- Place sterile, thin-walled dilution tubes and reaction tubes on ice. Thaw the experimental RNA or 1.2kb Kanamycin Positive Control RNA on ice, and return any unused portion to the freezer as soon as aliquots are taken.
- On ice, combine RNA (up to 1µg) and primer in Nuclease-Free Water for a final volume of 5µl per reaction.

Experimental Reactions

Component	Volume
Experimental RNA (up to 1µg/reaction) ⁵	Yµl
Oligo(dT) ₁₅ Primer or Random Primers (0.5µg/reaction) or gene-specific primer (10–20pmol/reaction) ⁶	Xµl
Nuclease-Free Water to a final volume of	5µl

⁵Use 10²–10¹⁰ copies of a specific target RNA template or 1pg–1µg total RNA or poly(A)+ mRNA.

⁶10–20pmol of primer in a 20µl reaction is equal to 0.5–1µM. A general formula for calculating nanograms of primer equivalent to 10pmol is 3.3 × b, where b is the number of bases in the primer.

Positive Control Reaction

Component	Volume
1.2kb Kanamycin Positive Control RNA, 0.5µg/µl	2µl
Oligo(dT) ₁₅ Primer, 0.5µg/µl	1µl
Nuclease-Free Water	2µl
Final Volume	5µl

Negative (No Template) Control Reaction

Component	Volume
Oligo(dT) ₁₅ Primer or Random Primers (0.5µg/reaction) or gene-specific primer (10–20pmol/reaction)	Xµl
Nuclease-Free Water to a final volume of	5µl

- Close each tube of RNA tightly. Place tubes into a preheated 70°C heat block for 5 minutes. Immediately chill in ice-water for at least 5 minutes. Centrifuge each tube for 10 seconds in a microcentrifuge to collect the condensate and maintain the original volume. Keep the tubes closed and on ice until the reverse transcription reaction mix is added.
- Prepare the reverse transcription reaction mix by combining the following components of the ImProm-II™ Reverse Transcription System in the order listed in a sterile 1.5ml microcentrifuge tube on ice. Determine the volume of each component needed for the desired number of reaction, and combine the components in the order listed. Vortex gently to mix, and keep on ice prior to dispensing into the reaction tubes.

Experimental Reactions

Component	Volume
Nuclease-Free Water (to a final volume of 15µl)	Xµl
ImProm-II™ 5X Reaction Buffer	4.0µl
MgCl ₂ , 25mM (1.5–8.0mM final conc.) ⁷	12–64µl
dNTP Mix, 10mM each dNTP (0.5mM final conc.) ⁸	1.0µl
RNasin® Ribonuclease Inhibitor (optional)	20u
ImProm-II™ Reverse Transcriptase	1.0µl
Final Volume	15.0µl

⁷The final Mg²⁺ concentration should be optimized in the range of 1.5–8.0mM.

⁸If isotopic or nonisotopic incorporation is desired to monitor first-strand cDNA synthesis, α[³²P]-dCTP or other modified nucleotides may be added to the dNTP mixture.

Positive Control Reaction

Component	Volume
Nuclease-Free Water (to a final volume of 15µl)	Xµl
ImProm-II™ 5X Buffer	4.0µl
MgCl ₂ , 25mM (6mM final conc.)	4.8µl
dNTP Mix, 10mM each dNTP (0.5mM final conc.)	1.0µl
RNasin® Ribonuclease Inhibitor (optional)	20u
ImProm-II™ Reverse Transcriptase	1.0µl
Final Volume	15.0µl

Negative (No Reverse Transcriptase) Control Reaction

Component	Amount
Nuclease-Free Water (to a final volume of 15µl)	Xµl
ImProm-II™ 5X Reaction Buffer	4.0µl
MgCl ₂ , 25mM (1.5–8.0mM final conc.)	1.2–6.4µl
dNTP Mix, 10mM each dNTP (0.5mM final conc.)	1.0µl
RNasin® Ribonuclease Inhibitor (optional)	20u
Final Volume	15.0µl

- Dispense 15µl of reverse transcription reaction mix to each reaction tube on ice. Be careful to prevent cross-contamination. Add 5µl of RNA and primer mix to each reaction for a final reaction volume of 20µl per tube. If there is a concern about evaporation in subsequent steps, overlay the reaction with a drop of nuclease-free mineral oil.
- Anneal: Place tubes in a controlled-temperature heat block equilibrated at 25°C, and incubate for 5 minutes.
- Extend: Incubate tubes in a controlled-temperature heat block at 42°C for up to one hour. The extension temperature may be optimized between 37–55°C.
- Inactivate reverse transcriptase: If the experimental goal is to proceed with PCR, the reverse transcriptase must be thermally inactivated prior to amplification. Incubate tubes in a controlled-temperature heat block at 70°C for 15 minutes.
- Prepare the PCR mix by dispensing the appropriate volume of each component into a sterile, 1.5ml microcentrifuge tube on ice. Combine the components in the order listed, vortex gently to mix and keep on ice prior to dispensing to the reaction tubes. An aliquot

of the first-strand cDNA (1µl or 20µl) from the reverse transcription reaction is added last to the PCR mix. See Notes 1–3.

Component	Volume per 100µl reaction (1µl RT reaction)	Volume per 100µl reaction (20µl RT reaction)
Nuclease-Free Water	55.2µl	45.6µl
5X Green or Colorless GoTaq® Flexi Buffer	19.8µl	16.0µl
MgCl ₂ , 25mM (2mM final conc.) ⁹	7.8µl	3.2µl
PCR Nucleotide Mix, 10mM (0.2mM final conc.)	2.0µl	1.0µl
Upstream Control Primer (1µM final conc.)	6.6µl	6.6µl
Downstream Control Primer (1µM final conc.)	6.6µl	6.6µl
GoTaq® DNA Polymerase (5.0 units)	1.0µl	1.0µl
PCR mix per reaction	99µl	80µl
RT reaction per reaction	1.0µl	20.0µl
Total PCR Volume	100.0µl	100.0µl

⁹For experimental reactions, the final Mg²⁺ concentration should be optimized in the range of 1.5–2.5mM.

- Overlay the reaction with two drops of nuclease-free mineral oil to prevent evaporation and condensation.

Place the reactions in a thermal cycler that has been prewarmed to 94°C. An optimized program for amplification using the Upstream and Downstream Control Primers provided with the Access RT-PCR system is given in Table 1.3.

Table 1.3. Amplification Conditions for the Positive Control Reaction.

Denaturation: 94°C for 2 minutes
25 cycles:
Denaturation: 94°C for 1 minute
Annealing: 60°C for 1 minute
Extension: 72°C for 2 minutes
Final extension: 72°C for 5 minutes
Hold 4°C

- After the cycle is complete, analyze products or store amplifications at –20°C.
- Analyze PCR products by agarose gel electrophoresis of 10% of the total reaction. The products will be readily visible by UV transillumination of an ethidium bromide-stained gel. The amplification product obtained using the Positive Control RNA with the Upstream and Downstream Control Primers is 323bp long.

1 Nucleic Acid Amplification

13. Store reaction products at -20°C until needed.

Notes

1. In this example, the final volume of PCR mix should be sufficient for 100 μl reactions once the cDNA volume is added. The volume of each component may be scaled for reactions of less than 100 μl . Scale up volumes to accommodate the total number of PCR amplifications being performed.
2. The amount of reverse transcription reaction used in the PCR may be modified after experimental optimization.
3. The amounts of magnesium and dNTPs and volume of reaction buffer added to the PCR vary, depending

on how much RT reaction is used as template. For example, for a 100 μl PCR that contains 20 μl of RT product, 8 μl of 10X thermophilic polymerase reaction buffer is added to support the 80 μl PCR mix addition. If 5 μl of RT reaction were added to 95 μl of PCR mix, 9.5 μl of 10X thermophilic polymerase reaction buffer would be needed. Similar considerations must be given to the magnesium and dNTP additions. This example details the amplification conditions recommended to amplify either 1 μl or 20 μl of the cDNA synthesized in the positive control reverse transcriptase reactions containing the 1.2kb Kanamycin Positive Control RNA template in a 100 μl PCR.

VIII. Troubleshooting PCR and RT-PCR

Symptoms	Solutions
Low yield or no amplification product (PCR or RT-PCR)	<p>Template was degraded. Verify template integrity by electrophoresis. Repurify the DNA or RNA template if the nucleic acid appears degraded.</p> <p>Too much or too little template was used. Verify template concentration by comparing the staining intensity of the DNA template after agarose gel electrophoresis and ethidium bromide staining with that of DNA standards with known concentrations.</p> <p>Inhibitor was present in sample. Reduce template volume in the reaction. Perform an ethanol precipitation to remove inhibitors. Some common inhibitors are listed in the Template Quality section.</p> <p>Poor primer design. Make sure primers are not self-complementary or complementary to each other.</p> <p>Verify that primers are complementary to the appropriate strands.</p> <p>Insufficient number of cycles. Return reactions to thermal cycler for 5 more cycles.</p> <p>Primer concentration was too low. Verify primer concentration in the reaction. Increase primer concentration if necessary.</p> <p>Suboptimal reaction conditions. Optimize Mg²⁺ concentration, annealing temperature and extension time. Verify that primers are present at equal concentrations. Refer to General Considerations for PCR Optimization for more information about optimizing reaction conditions.</p> <p>Nucleotides were degraded. Keep nucleotides frozen in aliquots, thaw quickly and keep on ice once thawed. Avoid multiple freeze-thaw cycles.</p> <p>Target sequence was not present in target DNA or RNA. Redesign experiment, or try other sources of target DNA or RNA</p> <p>Reaction component was missing. Always perform a positive control reaction with a template/primer combination that has amplified well in the past to determine when a component was omitted. Check the reaction components, and repeat the reaction.</p> <p>Poor-quality mineral oil. The reaction must be overlaid with high-quality, nuclease-free light mineral oil when using a thermal cycler without a heated lid. Do not use autoclaved mineral oil.</p> <p>Thermal cycler was programmed incorrectly. Verify that times and temperatures are correct. Use step cycles, not hold segments.</p> <p>Thermal cycler did not reach the proper temperature. Calibrate the thermal cycler to be sure reactions are heated to the programmed temperatures. Depending on the primers and template, small changes in cycling conditions can affect yield.</p> <p>Temperature was too low in some positions of thermal cycler. Perform a set of control reactions to determine if certain positions in the thermal cycler give low yields.</p>
Nonspecific amplification products (PCR or RT-PCR)	<p>Reaction conditions were suboptimal. Optimize Mg²⁺ concentration, annealing temperature, primer size, extension time and cycle number to minimize nonspecific priming. Refer to General Considerations for PCR Optimization for more information about optimizing reaction conditions.</p> <p>Perform hot-start PCR to minimize nonspecific amplification. If you are not using a DNA polymerase designed for hot-start PCR, such as GoTaq® Hot Start Polymerase, assemble reactions on ice and preheat the thermal cycler to 95°C before adding reaction tubes.</p> <p>Poor primer design. Make sure primers are not self-complementary or complementary to each other, especially near the 3'-ends. Avoid using three G or C nucleotides in a row at the 3'-end of a primer. Try a longer primer.</p> <p>Primer concentration was too high. Verify primer concentration in the reaction. Try a lower concentration.</p>

Symptoms	Solutions
	<p>Reaction was contaminated by another RNA or DNA. Use positive displacement pipettes or aerosol-resistant tips to reduce cross-contamination during pipetting. Use a separate work area and pipettor for pre- and postamplification. Wear gloves, and change them often. Use UNG or another technique to prevent carryover of DNA produced in a previous amplification into subsequent reactions. See the Nucleic Acid Cross-Contamination section.</p> <p>Multiple target sequences exist. Design new primers with higher specificity to target sequence in template DNA or cDNA.</p>
Low yield or no first-strand product (RT-PCR)	<p>RNA was degraded. Verify RNA integrity by denaturing agarose gel electrophoresis. Ensure that reagents, tips and tubes are RNase-free. Isolate RNA in the presence of a ribonuclease inhibitor (e.g., Promega RNasin® Ribonuclease Inhibitor). Repurify the DNA or RNA template if the nucleic acid appears degraded.</p> <p>AMV reverse transcriptase was thermally inactivated. If an initial denaturation/annealing step is introduced into the protocol, be certain to add the enzyme mix containing AMV reverse transcriptase after denaturation and subsequent 45°C equilibration.</p> <p>Poor primer specificity. Verify that the reverse transcription primer is complementary to the downstream RNA sequence.</p> <p>Poor primer annealing. If oligo(dT) primers or random hexamers were used as the reverse transcription primer, verify that the annealing step was carried out at an appropriate temperature prior to reverse transcription.</p> <p>RNA template was impure. Carryover of reagents (e.g., SDS, NaCl, heparin, guanidine thiocyanate) from some RNA purification methods can interfere with RT-PCR. Reduce volume of target RNA, perform additional purification steps or change purification method.</p> <p>Target RNA was not present in the sample or was present at low levels. Use poly(A)+ RNA, rather than total RNA, as a template to increase mRNA target abundance. Alternatively, isolate RNA from different starting material with a higher abundance of the desired target RNA.</p>
Amplification product with a higher-than-expected molecular weight (RT-PCR)	Genomic DNA sequences related to the RNA template contaminated the RNA preparation. Treat the RNA sample with RQ1 RNase-Free DNase to degrade contaminating DNA.

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- RT-PCR reactions at temperatures above 45°C are covered by U.S. Pat. Nos. 5,817,465 and 5,654,143 and European Pat. No. 0 568 272.
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I. RNA Interference

A. Introduction

In this chapter, we provide a brief overview of the RNA interference (RNAi) process and discuss technologies and products that can be used for RNAi experiments. A protocol is provided for the enzymatic synthesis of double-stranded RNA *in vitro* that provides an inexpensive alternative to chemical synthesis of RNAs. We also discuss design and selection of short interfering RNA (siRNA) sequences and describe a vector system, the psiCHECK™ Vectors, that can be used to screen potential siRNA target sequences for effectiveness during RNAi optimization. In addition, the GeneClip™ U1 Hairpin Cloning Systems, a DNA-directed RNAi (ddRNAi) system specifically designed for expression of small hairpin RNAs in mammalian cells, provide a cloning-based approach to allow fast, easy ligation and expression of hairpin oligonucleotides. Various strategies for delivery of siRNA to target cells are discussed, and example protocols for transient and stable transfection of mammalian cells are provided. Finally, methods for quantitating target gene suppression are briefly summarized.

B. Overview and Mechanism of RNAi

RNA interference (RNAi) is a phenomenon in which double-stranded RNA (dsRNA) suppresses expression of a target protein by stimulating the specific degradation of the target mRNA (for reviews, see Hannon, 2003; Caplen, 2004; Fuchs *et al.* 2004; Betz, 2003a). RNAi has been used to study loss of function for a variety of genes in several organisms including various plants, *Caenorhabditis elegans* and *Drosophila*, and permits loss-of-function genetic screens and rapid tests for genetic interactions in mammalian cells (Hannon, 2002; Williams *et al.* 2003).

RNAi involves a multistep process (Figure 2.1). dsRNA is recognized by an RNase III family member (e.g., Dicer in *Drosophila*) and cleaved into siRNAs of 21–23 nucleotides (Agrawal *et al.* 2003; Elbashir *et al.* 2001b; Bernstein *et al.* 2001; Hammond *et al.* 2000). These siRNAs are incorporated into an RNAi targeting complex known as RISC (RNA-induced silencing complex), which destroys mRNAs homologous to the integral siRNA (Hammond *et al.* 2000; Bernstein *et al.* 2001). The target mRNA is cleaved in the center of the region complementary to the siRNA (Elbashir *et al.* 2001c), with the net result being rapid degradation of the target mRNA and decreased protein expression.

RNAi has revolutionized the study of gene function, and is being explored as a therapeutic tool (for reviews, see Dorsett and Tuschl, 2004; Hannon and Rossi, 2004). For example, RNAi has been used to identify gene products essential for cell growth (Harborth *et al.* 2001), to cause subtype and species-specific knockdown of various protein kinase C (PKC) isoforms in both human and rat cells (Irie *et al.* 2002), and to specifically target degradation of an oncogene product (Wilda *et al.* 2002). RNAi has also been used to specifically target and prevent viral infections by HIV-1 and HCV in cell culture (Park *et al.* 2002) and intact

animals (McCaffrey *et al.* 2002). These observations open the field for further studies toward novel gene therapy approaches for anti-cancer or anti-viral treatments using siRNAs or shRNAs.

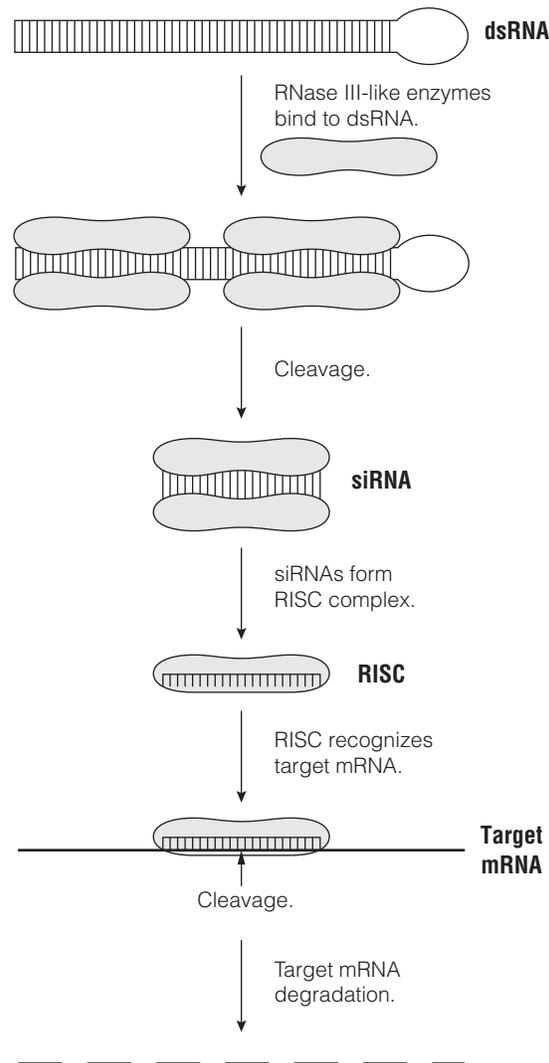


Figure 2.1. Simplified schematic diagram of the proposed RNA interference mechanism. dsRNA processing proteins (RNase III-like enzymes) bind to and cleave dsRNA into siRNA. The siRNA forms a multicomponent nuclease complex, the RNA-induced silencing complex (RISC). The target mRNA recognized by RISC is cleaved in the center of the region complementary to the siRNA and quickly degraded. An animated version (www.promega.com/paguide/animation/selector.htm?coreName=rnai01) of this illustration is also available.

An animated presentation (<http://www.nature.com/focus/rnai/animations/index.html>) illustrating the entire RNAi process is available on the *Nature* web site.

C. RNAi as a Tool for Targeted Inhibition of Gene Expression

The use of long dsRNAs (>400bp) has been successful in generating RNA interference effects in many organisms including *Drosophila* (Misquitta and Paterson, 1999),

zebrafish (Wargelius *et al.* 1999), *Planaria* (Sanchez-Alvarado *et al.* 1999) and numerous plants (Jorgensen, 1990, Fukusaki *et al.* 2004, Jensen *et al.* 2004). In mammalian systems, siRNA molecules of 21–22 nucleotides or short hairpin RNAs (shRNAs) are used to avoid endogenous nonspecific antiviral responses that target longer dsRNAs (Caplen *et al.* 2001, Elbashir *et al.* 2001a). Yu *et al.* (2002) and others (Brummelkamp *et al.* 2002b; McManus *et al.* 2002; Sui *et al.* 2002; Xia *et al.* 2002; Barton and Medzhitov, 2002) demonstrated that shRNAs bearing a fold-back, stem-loop structure of approximately 19 perfectly matched nucleotides connected by various spacer regions and ending in a 2-nucleotide 3'-overhang can be as efficient as siRNAs at inducing RNA interference. si/shRNAs can induce specific gene silencing in a wide range of mammalian cell lines without leading to global inhibition of mRNA translation (Caplen *et al.* 2001; Elbashir *et al.* 2001a; Paddison *et al.* 2002).

Generation of Short Interfering RNAs

siRNAs are the main effectors of the RNAi process. These molecules can be synthesized chemically or enzymatically *in vitro* (Micura, 2002; Betz, 2003b; Paddison *et al.* 2002) or endogenously expressed inside the cells in the form of shRNAs (Yu *et al.* 2002; McManus *et al.* 2002). Plasmid-based expression systems using RNA polymerase III U6 or H1, or RNA polymerase II U1, small nuclear RNA promoters, have been used for endogenous expression of shRNAs (Brummelkamp *et al.* 2002b; Sui *et al.* 2002, Novarino *et al.* 2004).

Rational Design of Effective siRNA Probes

Design of the siRNA sequence is crucial for effective gene silencing. Rational design strategies for effective siRNAs are being developed based on an understanding of RNAi biochemistry and of naturally occurring microRNA (miRNA) function. Several groups have proposed basic empirical guidelines for designing effective siRNAs that can be applied to the selection of potential target sequences (Chiu and Rana, 2002; Khvorova *et al.* 2003; Schwarz *et al.* 2003; Hsieh *et al.* 2004; Reynolds *et al.* 2004; Ui-Tei *et al.* 2004). In addition, strategies for experimentally screening effective siRNAs from pools of potential siRNAs are being developed (Kumar *et al.* 2003; Vidugiriene *et al.* 2004) and will remain a useful tool until potent siRNAs can be predicted accurately for each target gene.

Delivery of siRNA

The efficient delivery of siRNAs is a vital step in RNAi-based gene silencing experiments. Synthetic siRNAs can be delivered by electroporation or by using lipophilic agents (McManus *et al.* 2002; Kishida *et al.* 2004). siRNAs have been used successfully to silence target genes; however, these approaches are limited by the transient nature of the response. The use of plasmid systems to express small hairpin RNAs helps overcome this limitation by allowing stable suppression of target genes (Dykxhoorn *et al.* 2003). Various viral delivery systems have also been developed to deliver shRNA-expressing cassettes into cells that are difficult to transfect, creating new possibilities for RNAi usage (Brummelkamp *et al.* 2002a; Rubinson *et al.*

2003). Successful delivery of siRNAs in live animals has also been reported (Hasuwa *et al.* 2002; Carmell *et al.* 2003; Kobayashi *et al.* 2004).

II. siRNA Design and Optimization

A. Design of Target Sequences

Identifying an optimal target sequence is critical to the success of RNA interference experiments. Since it is not possible to predict the optimal siRNA sequence for a given target, multiple siRNAs will usually need to be evaluated. Recommendations for the design of siRNAs are constantly being improved upon as knowledge of the RNAi process continues to expand. At the time of writing this chapter, the recommendations are as follows: siRNA target sequences should be specific to the gene of interest and have ~20–50% GC content (Henshel *et al.* 2004). Ui-Tei *et al.* (2004) report that siRNAs satisfying the following conditions are capable of effective gene silencing in mammalian cells: 1) G/C at the 5' end of the sense strand; 2) A/U at the 5' end of the antisense strand; 3) at least 5 A/U residues in the first 7 bases of the 5' terminal of the antisense strand; 4) no runs of more than 9 G/C residues.

Additionally, primer design rules specific to the RNA polymerase used will apply. For example, for RNA polymerase III, the polymerase that transcribes from the U6 promoter, the preferred target sequence is 5'-GN₁₈-3'. Runs of 4 or more Ts (or As on the other strand) serve as terminator sequences for RNA polymerase III and should be avoided. In addition, regions with a run of any single base should be avoided (Czauderna *et al.* 2003). It is generally recommended that the mRNA target site be at least 50–200 bases downstream of the start codon (Sui *et al.* 2002; Elbashir *et al.* 2002, Duxbury and Whang, 2004) to avoid regions in which regulatory proteins might bind.

Several online design tools are available to assist in identifying potential siRNA targets. The siRNA Designer provides such a tool for identifying target sequences for use with our RNA interference systems. This tool searches for sequences satisfying siRNA design recommendations and also incorporates a BLAST search capability to ensure that selected sequences are specific to the gene of interest, an important requirement to ensure specificity and minimize off-target effects. The siRNA Designer program designs oligonucleotides for use with the GeneClip™ U1 Hairpin Cloning Systems (Cat.# C8750, C8760, C7910, C8770) and the T7 RiboMAX™ Express RNAi System (Cat.# P1700). The siRNA Designer analyzes input DNA or RNA sequences for regions that fit siRNA design requirements and displays siRNAs that could target these regions, along with the sequences of the oligonucleotides needed to produce these siRNAs with the chosen system.

Additional Resources for siRNA Design

Online Tools

siRNA Designer (www.promega.com/siRNA Designer/)

B. Use of Reporter Genes for RNAi Optimization

Not all siRNAs directed against a target gene are equally effective in suppressing expression of that target in mammalian cells. Therefore, it is important to identify siRNA sequences that are effective inhibitors of target gene expression. Although rational designs for selection of potential target sequences have been encouraging in generating effective siRNAs, accurate prediction of the most effective siRNAs still remains to be achieved. Current screening technologies are based on semiquantitative, time-consuming methods and are not easily modified to perform rapid, simultaneous screening of multiple siRNA/shRNA sequences. However, as the field of RNAi advances, and more high-throughput applications are adopted, there is a growing need for rapid, quantitative screening to confirm siRNA effectiveness (Kumar *et al.* 2003; Mousset *et al.* 2003).

Recently, several quantifiable procedures that use reporter genes to help rapidly identify effective siRNAs have been developed. In these approaches, the change in expression of a reporter gene fused to a target gene is used as an indicator of the effectiveness of an RNAi methodology. Here, we describe the psiCHECK™ Vector system, which is based on use of the bioluminescent *Renilla* luciferase reporter gene. The psiCHECK™ Vectors offer several advantages compared to other fusion approaches such as green fluorescent protein (GFP)- or Flag-tag-based methods. Measurement of net fluorescence from GFP in cell culture can be difficult and, in most cases, a flow cytometer is required for quantitation. Flag-tag quantitation requires Western blot analysis, which can be time-consuming. The high sensitivity of bioluminescence detection can readily tolerate lower expression levels, and introduction of a second reporter gene, firefly luciferase, allows normalization of changes in *Renilla* luciferase expression, making the psiCHECK™ Vector approach more robust and giving greater reproducibility of results.

The psiCHECK™-1 and -2 Vectors allow quantitative selection of optimal siRNA target sites and can be adapted for use in high-throughput applications. Figure 2.2 provides a basic illustration of how the psiCHECK™ Vectors are used. Both vectors contain a synthetic version of the *Renilla* luciferase (*hRluc*) reporter gene for monitoring RNAi activity. Several restriction sites are included 3' of the luciferase translational stop codon, allowing creation of transcriptional fusions between the gene of interest and the *Renilla* luciferase reporter gene. Because of the presence of a stop codon in-frame with the *Renilla* luciferase open reading frame, no fusion protein is produced. Consequently, there is no need to maintain frames when inserting the target gene. Also, toxic genes or gene fragments can be analyzed using this design without the danger of these genes killing the transfected cells.

The psiCHECK™-1 Vector (Cat.# C8011) is recommended for monitoring RNAi effects in live cells. Changes in *Renilla* luciferase activity can be measured with the EnduRen™

Live Cell Substrate (Cat.# E6481). This approach permits continuous monitoring of intracellular luminescence. *Renilla* luciferase expression can be monitored continuously for 2 days without interfering with normal cell physiology.

The psiCHECK™-2 Vector (Cat.# C8021) contains an additional reporter gene, a synthetic firefly luciferase gene (*hluc*), and is designed for endpoint lytic assays. Inclusion of the firefly luciferase gene permits normalization of changes in *Renilla* luciferase expression to firefly luciferase expression. *Renilla* and firefly luciferase activities can be measured using either the Dual-Luciferase® Reporter Assay System (Cat.# E1910) or the Dual-Glo® Luciferase Assay System (Cat.# E2920).

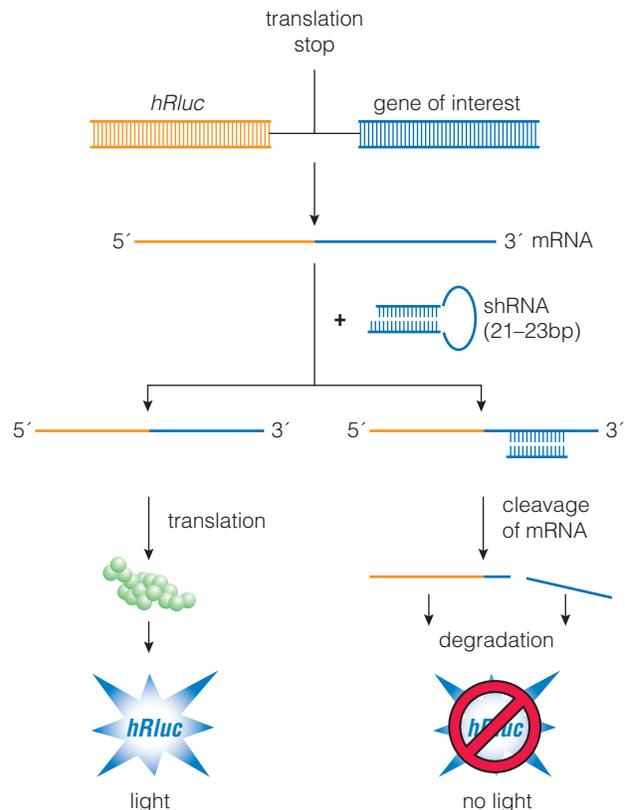


Figure 2.2. Mechanism of action of the psiCHECK™ Vectors.

To use the psiCHECK™ Vectors for screening siRNA targets, the gene of interest is cloned into the multiple cloning region located 3' to the synthetic *Renilla* luciferase gene and its translational stop codon. After cloning, the vector is transfected into a mammalian cell line, and a fusion of the *Renilla* gene and the target gene is transcribed. Functional *Renilla* luciferase is translated from the intact transcript. Depending on your experimental design, vectors expressing shRNA or synthetic siRNA can be either cotransfected simultaneously or sequentially. If a specific shRNA/siRNA effectively initiates the RNAi process on the target RNA, the fused *Renilla* target gene mRNA sequence will be degraded, resulting in reduced *Renilla* luciferase activity.

Additional Resources for psiCHECK™ Vectors**Technical Bulletins and Manuals**

TB329 *psiCHECK™ Vectors Technical Bulletin*
(www.promega.com/tbs/tb329/tb329.html)

Vector Maps

psiCHECK™ Vectors (www.promega.com/vectors/rna_interfer_vectors.htm#b01)

Promega Publications

PN087 The use of bioluminescent reporter genes for RNAi optimization
(www.promega.com/pnotes/87/11527_02/11527_02.html)

III. Enzymatic Synthesis of RNA in Vitro

siRNA synthesis in vitro provides a useful alternative to the potentially expensive chemical synthesis of RNA (Figure 2.3). The method relies on T7 phage RNA polymerase to produce individual sense and antisense strands that are annealed in vitro prior to delivery into the cells of choice (Fire *et al.* 1998; Donze and Picard, 2002; Yu *et al.* 2002, Shim *et al.* 2002).

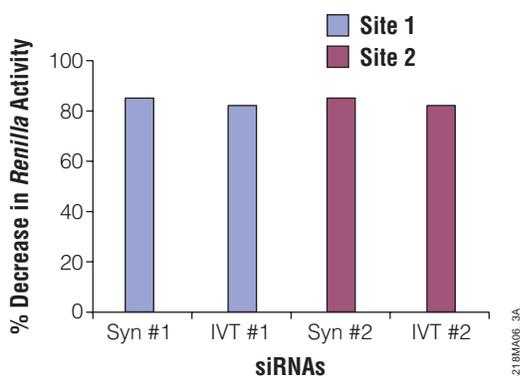


Figure 2.3. Comparison of RNA interference induced by siRNAs synthesized chemically or by in vitro transcription. Two different *Renilla* luciferase siRNA target sequences were synthesized chemically (Syn) or using the T7 RiboMAX™ Express RNAi System (IVT). The target sequences were then evaluated by RNA interference in CHO cells stably expressing *Renilla* luciferase.

The T7 RiboMAX™ Express RNAi System (Cat.# P1700) is an in vitro transcription system designed for rapid production of milligram amounts of double-stranded RNA (dsRNA). The system can be used to synthesize siRNAs for use in mammalian systems (Figure 2.4; Betz, 2003b, Hwang *et al.* 2004) or longer interfering RNAs for nonmammalian systems (Betz and Worzella, 2003; Betz, 2003c). The DNA templates for in vitro transcription of siRNAs are a pair of short, duplex oligonucleotides that contain T7 RNA polymerase promoters upstream of the sense and antisense RNA sequences. Each oligonucleotide of the duplex is a separate template for the synthesis of one strand of the siRNA. The separate short RNA strands that are synthesized are then annealed to form siRNA.

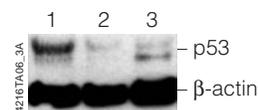


Figure 2.4. Suppression of endogenous p53 protein using siRNA prepared using the T7 RiboMAX™ Express RNAi System.

Twenty-four hours after plating in a 12-well plate, 293T cells were transfected with 200ng scrambled siRNA (lane 1), 200ng in vitro synthesized p53 siRNA (lane 2), or 200ng chemically synthesized p53 siRNA (lane 3). Twenty-four hours after transfection, cells were lysed using 1X Reporter Lysis Buffer (Cat.# E3971) containing protease inhibitors, and the protein was quantitated using the BCA Protein Assay (Pierce). Equal amounts of each lysate (10µg) were separated on a 4–12% polyacrylamide Bis-Tris gel (Invitrogen) and transferred to Hybond®-C membrane (Amersham). The blot was probed with both a p53 antibody (Calbiochem) and a β-actin antibody (Abcam). Detection was performed using Goat Anti-Mouse HRP Conjugate (Cat.# W4021) and the Transcend™ Chemiluminescent Non-Radioactive Translation Detection System (Cat.# L5080). The blot was exposed to Kodak X-OMAT® film for approximately 4 minutes. The simultaneous detection of the β-actin protein controlled for loading and transfer. The p53 and β-actin bands are indicated and are of the expected sizes.

A. In Vitro Synthesis of dsRNA for Use in Nonmammalian Systems

RNAi experiments in nonmammalian systems are typically performed with dsRNA of 400bp or larger (Elbashir *et al.* 2001b; Yang *et al.* 2000, Hammond *et al.* 2000). The minimum size of dsRNA recommended for RNAi in these systems is ~200bp. In general, templates for transcription of dsRNA for use in RNAi experiments correspond to most or all of the target message sequence. Data suggests that longer dsRNA molecules are more effective on a molar basis at silencing protein expression, but higher concentrations of smaller dsRNA molecules may have similar silencing effects. Data generated at Promega suggests that smaller dsRNAs can be as effective and efficient at inducing RNAi in nonmammalian systems (Betz, 2003c).

In the T7 RiboMAX™ Express RNAi System, dsRNA production requires a T7 RNA polymerase promoter at the 5'-ends of both DNA target sequence strands. To achieve this, separate DNA templates, each containing the target sequence in a different orientation relative to the T7 promoter, are transcribed in two separate reactions. The resulting transcripts are mixed and annealed post-transcriptionally. DNA templates can be created by PCR or by using two linearized plasmid templates, each containing the T7 polymerase promoter at a different end of the target sequence.

See the *T7 RiboMAX™ Express RNAi System Technical Bulletin #TB316* for a protocol for in vitro synthesis of dsRNA for RNAi in nonmammalian systems.

B. In Vitro Synthesis of siRNA for Use in Mammalian Systems

Figure 2.5 outlines the protocol for synthesis of siRNA using the T7 RiboMAX™ Express RNAi System. The initial step is generating the DNA template, which consists of two DNA oligonucleotides annealed to form a duplex. Generally

20pmol of duplex oligonucleotides are required per 20µl in vitro transcription reaction. Using the RiboMAX™ Express T7 Buffer and Enzyme Mix allows efficient synthesis of RNA in as little as 30 minutes. The annealed DNA oligonucleotide template is removed by a DNase digestion step, and the separate small RNA strands (sense and antisense) are annealed to form siRNA. The siRNA is precipitated using sodium acetate and isopropanol, and the resuspended product can be analyzed on polyacrylamide gels for size and integrity. Quantitation of the siRNA can be accomplished by either gel analysis or RiboGreen® analysis (Molecular Probes).

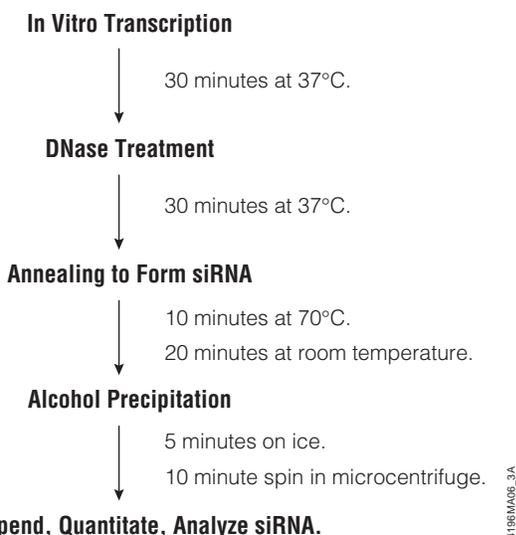


Figure 2.5. The T7 RiboMAX™ Express RNAi System protocol.

Materials Required:

- T7 RiboMAX™ Express RNAi System (Cat.# P1700)
- 2X oligo annealing buffer (20mM Tris-HCl [pH 7.5], 100mM NaCl)
- nuclease-free water
- gene-specific oligonucleotides
- isopropanol
- 70% ethanol

Designing DNA Oligonucleotides

The target mRNA sequence selected must be screened for the sequence 5'-GN₁₇C-3'. The generation of 3–5 different siRNA sequences for a particular target is recommended to allow screening for the optimal target site. The oligonucleotides consist of the target sequence plus the T7 RNA polymerase promoter sequence and 6 extra nucleotides upstream of the minimal promoter sequence to allow for efficient T7 RNA polymerase binding. Details on design of oligonucleotides for use with this system are found in the *T7 RiboMAX™ Express RNAi System Technical Bulletin* #TB316.

For further assistance, an siRNA finder and oligo design tool that specifically selects siRNA targets for use with the RiboMAX™ System is available in the siRNA Designer. Simply select the "T7 RiboMAX™ Express RNAi System: siRNA" option.

Additional Resources for siRNA Design

Online Tools

siRNA Designer (www.promega.com/siRNADesigner/)

Annealing DNA Oligonucleotides

1. Resuspend DNA oligonucleotides in nuclease-free water to a final concentration of 100pmol/µl.
2. Combine each pair of DNA oligonucleotides to generate either the sense strand RNA or antisense strand RNA templates as follows:

oligonucleotide 1 (100pmol/µl)	10µl
oligonucleotide 2 (100pmol/µl)	10µl
2X oligo annealing buffer	50µl
nuclease-free water	30µl
Final Volume	100µl

3. Heat at 90–95°C for 3–5 minutes, then allow the mixture to cool slowly to room temperature. The final concentration of annealed oligonucleotide is 10pmol/µl. Store annealed oligonucleotide DNA template at either 4°C or –20°C.

Synthesizing Large Quantities of siRNA

1. Set up the reaction at room temperature. The 20µl reaction may be scaled as necessary (up to 500µl total volume; use multiple tubes for reaction volumes >500µl). Add the components in the order shown below. For each siRNA, two separate reactions must be assembled as each RNA strand is synthesized separately, and then mixed following transcription.

T7 Reaction Components	Sample Reaction	Control Reaction
RiboMAX™ Express 2X Buffer	10µl	10µl
annealed oligonucleotide template DNA (10pmol/µl)	2.0µl	
pGEM® Express Positive Control Template	–	1.0µl
nuclease-free water	6.0µl	7.0µl
Enzyme Mix, T7 Express	2.0µl	2.0µl
Final Volume	20µl	20µl

2. Incubate for 30 minutes at 37°C.

Removing the DNA Template and Annealing siRNA

The DNA template can be removed by digestion with DNase following the transcription reaction. RQ1 RNase-Free DNase (Cat.# M6101) has been tested for its ability to degrade DNA while maintaining the integrity of RNA. If accurate RNA concentration determination is desired, the RNA should be DNase-treated and purified to remove potentially inhibitory or interfering components.

1. To each 20µl transcription reaction, add 1µl of RQ1 RNase-Free DNase and incubate for 30 minutes at 37°C.

- Combine separate sense and antisense reactions and incubate for 10 minutes at 70°C, then allow the tubes to cool to room temperature (approximately 20 minutes). This step anneals the separate short sense and antisense RNA strands, generating siRNA.

Purifying siRNA

- Add 0.1 volume of 3M Sodium Acetate (pH 5.2) and 1 volume of isopropanol. Mix and place on ice for 5 minutes. The reaction will appear cloudy. Spin at top speed in a microcentrifuge for 10 minutes.
- Carefully aspirate the supernatant, and wash the pellet with 0.5ml of cold 70% ethanol, removing all ethanol following the wash. Air-dry the pellet for 15 minutes at room temperature, and resuspend the RNA sample in nuclease-free water in a volume 2–5 times the original reaction volume (at least 2 volumes are required for adequate resuspension). Store at –20°C or –70°C.

Additional Resources for T7 RiboMAX™ Express RNAi System

Technical Bulletins and Manuals

TB316 *T7 RiboMAX™ Express RNAi System Technical Bulletin*
(www.promega.com/tbs/tb316/tb316.html)

Promega Publications

eNotes RNAi in *Drosophila* S2 cells: Effect of dsRNA size, concentration, and exposure time
(www.promega.com/enotes/applications/ap0050_tabs.htm)

PN084 The T7 RiboMAX™ Express RNAi System: Efficient synthesis of dsRNA for RNA interference
(www.promega.com/pnotes/84/10705_07/10705_07.html)

PN085 Produce functional siRNAs and hairpin siRNAs using the T7 RiboMAX™ Express RNAi System
(www.promega.com/pnotes/85/10904_15/10904_15.html)

Citations

Hwang, C.K. *et al.* (2004) Transcriptional regulation of mouse μ opioid receptor gene by PU.1. *J. Biol. Chem.* **279**, 19764–74.

In this article, siRNA was used to reduce the level of the PU.1 transcription factor. The siRNA was generated using the T7 RiboMAX™ Express RNAi System with design assistance from the siRNA Designer. Annealed siRNA was purified by isopropanol precipitation. Forty-eight hours after transfecting 2.5 μ g of siRNA into RAW264.7 cells, RNA and protein were isolated from the cells, and the siRNA effect was analyzed by RT-PCR and Western blot.

PubMed Number: 14998994

Kim, C.S. *et al.* (2004) Neuron-restrictive silencer factor (NRSF) functions as a repressor in neuronal cells to regulate μ opioid receptor gene. *J. Biol. Chem.* **279**, 46464–73.

In this article, siRNA was used to silence endogenous mouse and human NRSF expression in NS20Y and HeLa cells. siRNAs were generated using the T7 RiboMAX™ Express RNAi System. The siRNA Designer was used to select targets and to design primers.

PubMed Number: 15322094

IV. DNA-Directed RNAi

A. Overview of ddRNAi

DNA-directed RNA interference (ddRNAi) involves the use of DNA templates to synthesize si/shRNA in vivo. ddRNAi relies on U6 or H1 [RNA polymerase III], or U1 [RNA polymerase II] promoters for the expression of siRNA target sequences that have been transfected into mammalian cells (Miyagishi and Taira, 2002; Brummelkamp *et al.* 2002b; Novarino *et al.* 2004). si/shRNA target sequences can be generated by PCR, creating “expression cassettes” that can be transfected directly into cells (Csiszar *et al.* 2004; Castanotto *et al.* 2002) or cloned into expression vectors (Sui *et al.* 2002; Paul *et al.* 2002; Gou *et al.* 2003; Yu *et al.* 2002). PCR generation is recommended when rapid screening of numerous siRNAs is desired. Cloning-based approaches that allow direct ligation of hairpin oligonucleotides into a ddRNAi vector provide another method for quickly and easily screening various targets (Yeager *et al.* 2005). Screening can also be performed using synthetic RNAs, but this can become expensive for numerous targets.

Vector-based approaches also offer the potential of stable, long-term inhibition of gene expression by providing siRNAs on plasmids that allow selection of transfected cells. Vectors with markers, such as puromycin, neomycin or hygromycin, can be used for suppression of target genes for several weeks or longer. Transfection with synthetic siRNAs allows for only a transient measurement (usually 48–72 hours) of the RNAi effect.

The success of ddRNAi depends on several parameters including generation of vectors containing full-length sh/siRNA sequences, delivery of those vectors into cells, and expression of the si/shRNA constructs. Most strategies for cloning siRNA target sequences into expression vectors utilize the design of a hairpin structure. This design consists of two inverted repeats separated by a short spacer sequence (loop sequence). After transcription by RNA polymerase, the inverted repeats anneal and form a hairpin, which is then cleaved by Dicer to form an siRNA.

The GeneClip™ U1 Hairpin Cloning Systems facilitate easy expression of shRNAs in vivo by ddRNAi-based methods. This system provides a simple, cloning-based approach, allowing ligation of potential shRNA target sequences into vectors that allow transient or stable expression in mammalian cells.

B. GeneClip™ U1 Hairpin Cloning Systems

The GeneClip™ U1 Hairpin Cloning Systems (Cat.# C8750, C8760, C8770, C8780, C8790) are designed for rapid and efficient cloning of hairpin sequences for expression of shRNAs *in vivo*. The U1 promoter provides the benefit of production of shRNA with a defined termination site, mimicking siRNA produced *in vivo* when the Dicer complex cleaves the loop off the hairpin. Using the GeneClip™ Systems, complementary oligonucleotides supplied by the user are annealed and cloned into the predigested vector downstream of the U1 promoter. The linearized vectors contain overhangs for increased cloning efficiency. The GeneClip™ Systems are provided in five formats. The GeneClip™ U1 Hairpin Cloning System—Basic is designed for transient suppression of the gene of interest. The GeneClip™ U1 Hairpin Cloning System—hMGFP contains the green fluorescent protein gene from *Montastrea cavernosa* and can therefore be used to determine transfection efficiency; it also allows separation of transfected cells by fluorescent-activated cell sorting (FACS®). The three remaining GeneClip™ U1 Hairpin Cloning Systems provide the ability to stably select transfected cells using either neomycin, hygromycin or puromycin, so that experimental results do not depend on transfection efficiency.

An overview of the GeneClip™ U1 Hairpin Cloning System protocol is given in Figure 2.6.

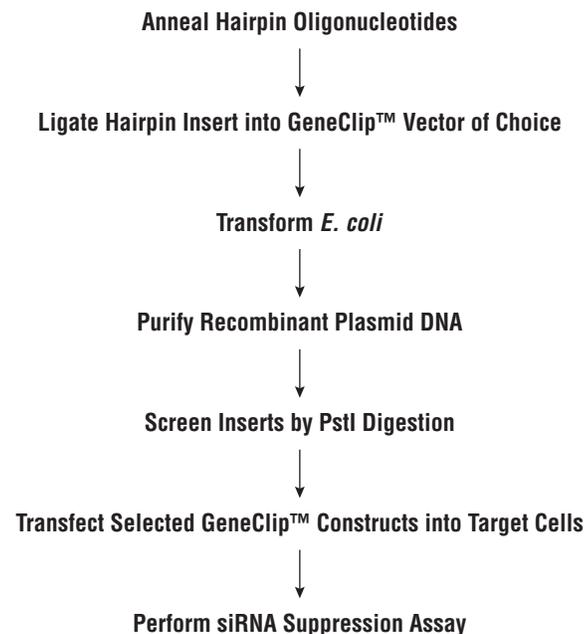


Figure 2.6. Overview of the GeneClip™ U1 Hairpin Cloning System protocol.

Hairpin Cloning

Once target sites have been selected, the hairpin oligonucleotides are annealed and ligated into the pGeneClip™ Vector, which is then screened for the proper insert (Figure 2.7). These two oligonucleotides form a DNA insert that contains a hairpin siRNA target sequence.

Standard desalting of the oligonucleotides is necessary, but gel purification and 5' phosphorylation are not required. Because the oligonucleotides inserted into the vector are short (~60nt), detection by standard agarose gel electrophoresis can be difficult. To make detection of inserts more convenient, a PstI site is engineered into the vector backbone (Figure 2.7). Upon successful ligation of the annealed oligonucleotides into the vector, a second PstI site is created. Digestion with PstI therefore results in two easily separated bands of approximately 3kb and 1kb. If no insert is present, PstI digestion will result in a single band. In our experiments, ligation of the insert typically results in over 50-fold more colonies than control ligations containing vector alone. In addition, the PstI digestion showed over 90% of the colonies contained insert.

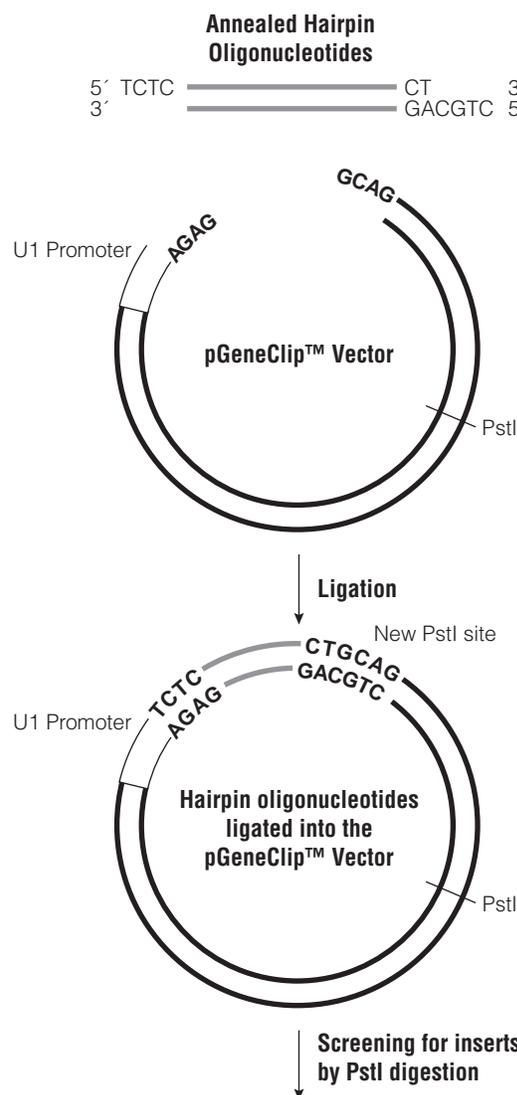


Figure 2.7. Cloning of a hairpin insert into a pGeneClip™ Vector. The simple cloning procedure involves ligation of the hairpin insert into the pGeneClip™ Vector, which is provided linearized and ready for ligation.

Target Site Selection

The introduction of long strands of dsRNA into mammalian cells induces a strong interferon response that can lead to an overall shutdown of protein synthesis and cell death. Short dsRNA molecules (<30nt) can bypass the interferon response and still function in the RNAi pathway (Elbashir *et al.* 2001a). Since the entire mRNA sequence cannot be used in mammalian cells, various target sites must be selected for use in RNAi experiments. Unfortunately not all target sequences in an mRNA will work equally well to suppress target gene expression. The siRNA Designer (www.promega.com/siRNA Designer/) is an online tool developed by Promega that is designed to assist in selecting the most likely target sites for RNAi (Hwang *et al.* 2004; Kim *et al.* 2004). The user enters the sequence of interest, and the program generates a list of target sites that will most likely be effective. The siRNA Designer uses a number of parameters, including examining the free energy of the 5' end of the antisense sequence, to evaluate siRNA candidates. Although programs such as the siRNA Designer allow selection of potentially effective siRNA sequences, they cannot guarantee that the chosen sequences will be highly effective. Experimental testing of the optimal sequences is required to confirm a high level of inhibition.

Transient and Stable In Vivo Suppression

The pGeneClip™ Basic and pGeneClip™ hMGFP Vectors are recommended for use in transient transfection assays. For many target sequences, transient transfection can quickly yield cells that can be assessed for the effects of gene suppression, including changes in phenotype, protein expression levels or other effects.

One consideration in evaluating transient transfection experiments is transfection efficiency. For example, if only 30% of the cells are transfected, it may be difficult to detect inhibition of expression, since 70% of the cells were not successfully transfected and still express the target mRNA. The GeneClip™ U1 Hairpin Cloning System—hMGFP (Cat.# C8790) provides an shRNA expression vector that contains an internal fluorescent marker for monitoring delivery efficiency of shRNA-expressing constructs. In addition to allowing transcription of hairpin target sequences and generation of siRNAs in vivo, the vector contains an improved, synthetic version of the green fluorescent protein (hMGFP) gene. The hMGFP gene encodes a 26kDa protein that gives improved fluorescence and reduced cytotoxicity compared with other GFP proteins. The presence of GFP allows easy determination of transfection efficiency and allows selection of transfected cells by FACS® (Cormack *et al.* 1996; Sorensen *et al.* 1999; Galbraith *et al.* 1999). Importantly, the expression of hMGFP does not affect gene silencing by shRNA molecules expressed from the same vector.

The pGeneClip™ Puromycin, pGeneClip™ Hygromycin and pGeneClip™ Neomycin Vectors allow selection of stably transfected cells. Thus, the results are no longer dependent on transfection efficiency. If necessary, clonal lines of transfected cells can be generated if the population

of selected cells does not show the expected inhibition levels. Because integration of the vector into different positions in the genome can affect expression of the RNAi hairpin, a population of cells may not show suppression. A clonal cell line that sufficiently expresses the RNAi hairpin may be required to demonstrate suppression with the pGeneClip™ Vectors.

To test the effectiveness of the GeneClip™ U1 promoter, we monitored reduction in p53 protein levels after targeting p53 mRNA. p53 expression was targeted using shRNAs cloned into a pGeneClip™ Basic Vector. More than 90% inhibition was observed for the protein compared to a nonspecific control (data not shown). To test p53 reduction in vivo, 293T cells, which contain elevated levels of p53, were transfected with a pGeneClip™ Basic Vector expressing p53 shRNA. Compared to nontransfected cells, the cells containing the pGeneClip™ Vector expressing p53 shRNA exhibited greater than 85% reduction in p53 protein levels (Figure 2.8, Panel A).

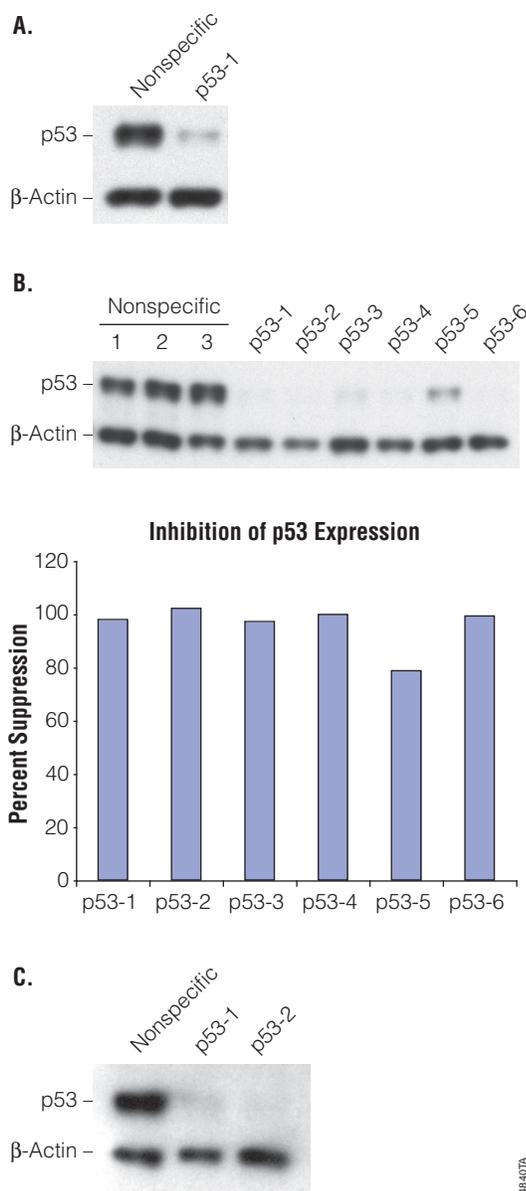


Figure 2.8. Inhibition of p53 expression in transient transfections and in stable clones. 293T cells were transfected with pGeneClip™ Basic Vectors containing either a hairpin target sequence directed against p53 or a nonspecific target sequence. After 48 hours, cells were collected, lysed, and protein levels quantitated by BCA assay (Pierce). For stable transfections, 293T cells were transfected with a pGeneClip™ Puromycin Vector containing either a nonspecific or a p53-specific target sequence. Cells were selected with puromycin for 7 days, and clones were assayed after passage 4 and passage 11. For Western analysis, 2µg protein per lane was loaded on an 8% Tris-Glycine gel and then transferred to nitrocellulose. Blots were probed with monoclonal antibodies to p53 (Oncogene Research Products, Ab-2, 1:1,000 dilution) and β-actin (Abcam, AC-12, 1:5,000 dilution). Detection was performed using ECL™ Plus (Amersham). Protein levels were quantitated by densitometry after exposure to film. **Panel A.** shRNA suppression of p53 expression in transiently transfected cells. **Panel B.** Stable reduction of p53 at passage 4. **Panel C.** Stable reduction of p53 at passage 11.

Additional Resources for the GeneClip™ U1 Hairpin Cloning Systems

Technical Bulletins and Manuals

TM256 *GeneClip™ U1 Hairpin Cloning Systems Technical Manual*
(www.promega.com/tbs/tm256/tm256.html)

Vector Maps

pGeneClip™ Vectors (www.promega.com/vectors/rna_interfer_vectors.htm)

Promega Publications

PN089 *GeneClip™ U1 Hairpin Cloning Systems for expression of short hairpin RNAs in vivo*
(www.promega.com/pnotes/89/12416_21/12416_21.html)

Online Tools

siRNA Designer (www.promega.com/siRNADesigner/)

V. RNA Delivery Strategies

Successful RNAi experiments are dependent on both siRNA design and effective delivery of siRNA duplexes into cells. RNAi delivery strategies vary depending on the target cells or organism. For example, *C. elegans* may be injected (Fire *et al.* 1998; Grishok *et al.* 2000), soaked in (Tabara *et al.* 1998), or fed (Timmons and Fire, 1998; Kamath *et al.* 2001; Fraser *et al.* 2000) dsRNA. Successful delivery of interfering RNA has also been achieved by microinjection of RNA into *Drosophila* embryos (Kennerdell and Carthew, 1998) and mouse oocytes (Wianny and Zernicka-Goetz, 2000). Delivery to *Drosophila* S2 cells in culture can be achieved by incubating the cells with the chosen RNA (Clemens *et al.* 2000; Betz and Worzella, 2003). Use of DNA-based approaches like ddRNAi vectors allows use of standard transfection reagents/methods; for example, cationic lipids, calcium phosphate, DEAE-dextran, polybrene-DMSO or electroporation (Caplen *et al.* 2001; Elbashir *et al.* 2001a).

A. Transfection of ddRNAi Vector Constructs

Once annealed, hairpin oligonucleotides are ligated to the appropriate pGeneClip™ Vector, the resulting constructs can be used for transient or stable transfection. The GeneClip™ U1 Hairpin Cloning Systems provide a choice of vectors containing various selectable markers (neomycin, hygromycin or puromycin) that can be used for stable expression of a pool of cells or individual clones. Transfection of the plasmid DNA into human cells may be mediated by cationic lipids, calcium phosphate, DEAE-Dextran, polybrene-DMSO or electroporation. Transfection conditions will need to be optimized for your particular system. Guidelines for transfection of the pGeneClip™ Vectors are provided in Technical Manual #TM256. General considerations for transient and stable transfection are given below.

Transient Transfection

High transfection efficiency is essential for achieving substantial suppression levels using a transient transfection approach. Prior to testing for suppression of the target protein, optimize the transfection conditions for maximum efficiency in the system to be tested. The optimal conditions will vary with cell type, transfection method used and the amount of DNA. When using the pGeneClip™ Basic, Puromycin, Hygromycin or Neomycin Vectors, optimization can be performed using a GFP reporter such as the Monster Green® Fluorescent Protein pHMGFP Vector (Cat.# E6421). The pGeneClip™ hMGFP Vector already contains the GFP reporter. The GFP reporter can also be used to determine transfection efficiency for the assay. To test the effectiveness of the pGeneClip™ Vector constructs (screening various sequences for levels of inhibition), the use of a reporter, such as GFP, is highly recommended. This control can be performed as a separate transfection to determine the percentage of the cell population transfected or as a cotransfection where flow cytometry can be used to sort GFP-positive cells. The level of target RNA suppression in transfected cells can then be determined by taking the transfection efficiency into account.

Obtaining maximum suppression requires optimizing specific assay conditions. We have observed variations in suppression efficiency as a result of the cell line, cell culture conditions, target sequence and transfection conditions. Varying the amount of transfection reagent, amount of DNA and cell density can influence transfection efficiency. Obtaining the highest transfection efficiency with low toxicity is essential for maximizing the siRNA interference (suppression) effect in a transient assay. Additionally, maintaining healthy cell cultures is essential for this application. The key considerations are discussed more fully below.

Cell Density (Confluence) at Transfection: The recommended cell density for most cell types at transfection is approximately 30–50%; this level is lower than standard transfection experiments where cells are plated at 50–70% confluency. The optimal cell density should be determined for each cell type. Continued proliferation and the need to passage cells should be considered when determining the number of cells to plate.

Cell Proliferation: The successful suppression of gene expression requires actively proliferating and dividing cells, so it is essential to maintain healthy cell cultures. It also is important to minimize the decrease in cell growth associated with nonspecific transfection effects and to maintain cell culture under subconfluent conditions to

assure rapid cell division. We recommend using the CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570) to monitor cell viability and growth.

Time: The optimal time after transfection for analyzing interference effects must be determined empirically by testing a range of incubation times. Typically little inhibition is seen after 24 hours, but the maximal suppression time can vary from 48–96 hours, depending on the cells used and the experimental targets tested.

Stable Transfection

For stable expression, antibiotic selection must be applied following transfection. Cell lines vary in the level of resistance to antibiotics, so the level of resistance of a particular cell line must be tested before attempting stable selection of the cells. A "kill curve" will determine the minimum concentration of the antibiotic needed to kill nontransfected cells. The antibiotic concentration for selection will vary depending on the cell type and the growth rate. In addition, cells that are confluent are more resistant to antibiotics, so it is important to keep the cells subconfluent. The typical effective ranges and lengths of time needed for selection are given in Table 2.1.

For example, to generate a kill curve for G-418 selection, test G-418 concentrations of 0, 100, 200, 400, 600, 800 and 1,000 µg/ml to determine the concentration that is toxic to nontransfected cells. Once the effective concentration of antibiotic has been determined, transfected cells can be selected for resistance.

Once the effective concentration of antibiotic has been determined, transfected cells can be selected for resistance, as outlined in the protocol below.

1. Following transfection, seed cells at a low cell density.
2. Apply antibiotic to the medium at the effective concentration determined from the kill curve.
3. Prepare a control plate for all selection experiments by treating nontransfected cells with antibiotic in medium under the experimental conditions. This control plate will confirm whether the conditions of antibiotic selection were sufficiently stringent to eliminate cells not expressing the resistance gene.
4. Change the medium every 2–3 days until drug-resistant clones appear.
5. Once clones (or pools of cells) are selected, grow the cells in media containing the antibiotic at a reduced antibiotic concentration, typically 25–50% of the level used during selection.

Table 2.1. Typical Conditions for Selection of Stable Transfectants.

Vector	Antibiotic	Effective Concentration	Time Needed for Selection
pGeneClip™ Puromycin Vector	Puromycin	1–10 µg/ml	2–7 days
pGeneClip™ Hygromycin Vector	Hygromycin	100–1,000 µg/ml	3–10 days
pGeneClip™ Neomycin Vector	G-418	100–1,000 µg/ml	3–14 days

See Figure 2.8, Panels B and C for stably transfected cells that suppressed expression of p53.

B. Delivery of dsRNA to *Drosophila* S2 Cells in Culture

The protocol outlined below was used to successfully deliver PCR products of various sizes (180bp or 505bp) generated either from the 778bp ERK-A target or from a control plasmid containing the *Renilla* luciferase gene (phRL-null Vector; 500bp or 1,000bp) to *Drosophila* S2 cells in culture (Figure 2.9; Betz and Worzella, 2003). Purified, in vitro-synthesized ERK-A dsRNA was introduced into *Drosophila* S2 cells using the method described by Clemens *et al.* (2000) following the protocol described below.

1. Incubate 1×10^6 S2 cells in 1ml of *Drosophila* expression system (DES) serum-free medium (Invitrogen) in triplicate wells of a six-well culture dish in the presence or absence of various amounts (0, 9.5, 38, or 190nM) of the test (ERK-A) dsRNA or a nonspecific (*Renilla* luciferase) dsRNA.
2. Incubate the S2 cells at room temperature with the dsRNA for 1 hour, then add 2ml of complete growth medium.
3. Incubate the cells at room temperature for an additional 3 days to allow for turnover of the target protein.

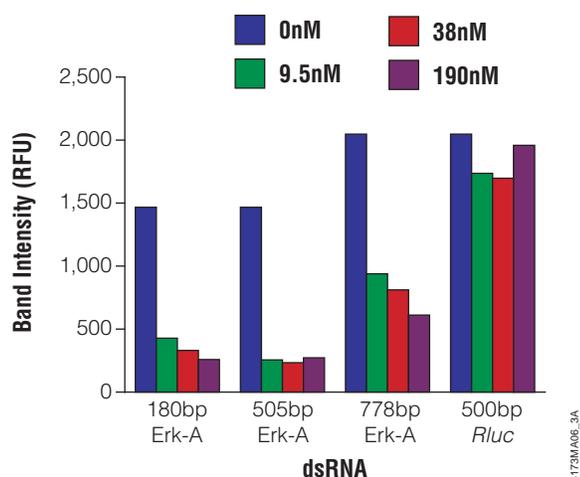


Figure 2.9. Effect of Erk-A dsRNA length and concentration of Erk-A protein levels in S2 cells. Erk-A dsRNAs and a nonspecific control dsRNA (*Renilla* luciferase; *Rluc*) were synthesized, purified, and quantitated using the T7 RiboMAX™ Express RNAi System. The Erk-A dsRNAs were 180bp, 505bp, or 778bp. The *Rluc* negative-control dsRNA was 500bp. *Drosophila* S2 cells were treated with increasing concentrations of each dsRNA (0, 9.5, 38 or 190nM) in triplicate for 3 days. The dsRNA concentration refers to the initial 1ml treatment. Replicate wells were pooled and a cell lysate prepared. The cell lysates were then subjected to Western blot analysis for Erk-A protein levels (Betz and Worzella, 2003). The quantity of Erk-A protein in each sample was quantitated using enhanced chemifluorescent detection reagents (Amersham) and a STORM® fluorescent scanner (blue mode). The basal level of Erk-A in the 180bp and 505bp Erk-A samples is different than in the other two samples because these samples were processed on different blots.

VI. Quantitating siRNA Target Gene Expression

Reduction of the targeted gene expression can be measured by 1) monitoring phenotypic changes of the cell, 2) measuring changes in mRNA levels (e.g., using RT-PCR), or 3) detecting changes in protein levels by immunocytochemistry or Western blot analysis (Huang *et al.* 2003; Kullmann *et al.* 2002; Lang *et al.* 2003). The suppression effect will vary depending on the target, cell line and experimental conditions.

Controlling for nonspecific effects on other targets is very important. As a negative control, cells can be transfected with either a nonspecific or scrambled target sequence. This will show that suppression of gene expression is specific to the expression of the hairpin siRNA target sequences. When suppression is determined by Western analysis, positive controls for other genes (e.g., tubulin or actin) should be included (Huang *et al.* 2003). Additional controls may also be desirable (Editorial (2003) *Nat. Cell Biol.* 5, 489–90).

A. Confirming the RNAi Effect

Several summary articles are available that suggest various options for controls that should be incorporated into RNAi experimental design to ensure accuracy and correct identification of an RNAi effect (Editorial (2003) *Nat. Cell Biol.* 5, 489–90; Duxbury and Whang, 2004). The preferred control is to show restoration of functionality of a gene through artificial overexpression of the target gene in a form that is not affected by RNAi. For example, the target gene can be engineered to contain silent mutations that render the mRNA invulnerable to the RNAi effect and introduced into the cell on a plasmid vector. If such constructs “rescue” the original function of the gene, this is a good indication that the observed suppression is mediated by RNAi. Use of siRNAs targeting several different areas of the same gene to suppress expression may also be used to provide evidence that an effect is mediated by RNAi. The observation of the same suppression effect using more than one target RNA can confirm that the observed effect is indeed RNAi.

For experiments using in vitro-synthesized siRNAs, the minimum concentration of RNAi showing an effect should be used to avoid nonspecific effects due to the introduction of large quantities of RNA into the cell. Ideally, any observed suppression should be confirmed at both the mRNA and protein levels. Northern blotting and quantitative, real-time RT-PCR can be used to demonstrate reduction of expression at the RNA level. Quantitative Western blotting, phenotypic and functional assays are some of the options available to show protein knockdown.

B. Negative and Positive Controls

Scrambled siRNAs and siRNAs containing a single mismatch can be used as negative controls. However, the latter are regarded as more informative. Positive controls with RNAs known to exhibit an RNAi effect may also be useful.

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I. Introduction

A. Cell Death as Cell Fate: Historical Context

Although apoptosis is often described as a "hot topic" or a "new and exploding" area of biological research, the concept of cell death as a normal cell fate was articulated only three years after Schleiden and Schwann introduced the Cell Theory when, in 1874, Vogt described natural cell death as an integral part of toad development (Cotter and Curtin, 2003). Since these early observations, natural cell death has been described "anew" several times. In 1885 Flemming provided the first morphological description of a natural cell death process, which we now label "apoptosis", a term coined by Kerr and colleagues to describe the unique morphology associated with a cell death that differs from necrosis (Kerr *et al.* 1972). The revolution that has occurred in apoptosis research is a direct result of a better understanding of the genetic program and biochemical mechanisms of apoptosis.

In the 1970s and 1980s, studies revealed that apoptosis not only had specific morphological characteristics but that it was also a tightly regulated process with specific biochemical characteristics. Studies of cell lineage in the nematode, *Caenorhabditis elegans*, showed that apoptosis was a normal feature of the nematode's invariant developmental program. Of the 1,090 somatic cells of the *C. elegans* adult hermaphrodite, 131 die during normal development (Hengartner, 1997). By documenting every cell division from the zygote to the adult, researchers discovered that the lineage and the timing of apoptosis for each of these 131 cells were constant, demonstrating that apoptosis was a tightly regulated process, presumably genetically programmed (i.e., programmed cell death). At the biochemical level, Wyllie showed that DNA degradation by a specific endonuclease during apoptosis resulted in a DNA ladder composed of mono- and oligonucleosomal-sized fragments (Wyllie, 1980).

B. Morphology and Overview of Apoptosis

Morphologically, apoptosis is first characterized by a change in the refractive index of the cell (Hengartner, 1997) followed by cytoplasmic shrinkage and nuclear condensation. The cell membrane begins to show blebs or spikes (protrusions of the cell membrane), depending on cell type (Figure 3.1), and eventually these blebs and spikes separate from the dying cell and form "apoptotic bodies". Apoptotic cells also cease to maintain phospholipid asymmetry in the cell membrane, and phosphatidylserine (PS) appears on the outer leaflet (Williamson, 2000). The mitochondrial outer membrane (MOM) also undergoes changes that include loss of its electrochemical gradient, possibly by the formation of pores in the MOM, and substances such as cytochrome c leak from the MOM into the cytoplasm. Finally, adjacent cells or macrophages phagocytose apoptotic bodies and the dying cell. The apoptotic cell does not provoke an inflammatory response, and only individual cells are affected by apoptosis in vivo.

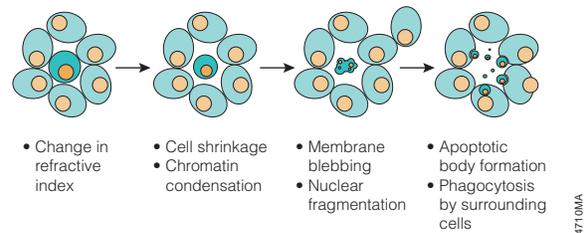


Figure 3.1. Morphology changes during apoptosis. The cell membrane begins to show blebs or spikes, depending on cell type. Eventually these separate from the dying cell and form "apoptotic bodies" that are phagocytosed by neighboring cells.

The events of apoptosis stand in contrast to necrosis, which is first marked by a loss of cell membrane integrity. The cytoplasm and mitochondria of the necrotic cell swell, and ultimately the cell and many of its internal organelles lyse. There is no vesicle or apoptotic body formation, and often necrosis affects groups of adjacent cells. The necrotic cell remnants are phagocytosed by macrophages, and inflammatory responses are provoked in vivo.

Apoptosis and necrosis represent two extremes of a continuum of cell death. This continuum includes many variations. "Apoptosis-like programmed cell death" refers to a cell death process that has some of the hallmarks of apoptosis such as chromatin condensation and the appearance of PS on the outer leaflet of the cell membrane but does not necessarily require caspase activity (Leist and Jäätelä, 2001). "Necrosis-like programmed cell death" describes programmed cell death that does not include chromatin condensation and has varying degrees of other apoptotic features. Caspase-1 and caspase-8 have been implicated in some cases of this type of programmed cell death (Leist and Jäätelä, 2001). "Paraptosis" describes a cell death that requires gene expression but morphologically does not resemble either apoptosis or necrosis (Sperandio *et al.* 2000).

In addition, apoptotic cells cultured in vitro will eventually undergo "secondary necrosis". After extended incubation, apoptotic cells ultimately shut down metabolism, lose membrane integrity and release their cytoplasmic contents into the culture medium (Riss and Moravec, 2004). Therefore, cells that have initiated apoptosis may exhibit some of the morphological phenotypes associated with necrosis. Because programmed cell death takes many forms, both morphologically and biochemically, researchers need to examine multiple biochemical markers at carefully selected time points to determine the mechanism of cell death in their experimental system.

C. Molecular Players in Apoptosis

Caspases

Large-scale mutagenesis experiments in the nematode *C. elegans* identified mutations that disrupted the programmed cell death fates during development, the *cell death abnormal (ced)* genes (Hedgecock *et al.* 1983; Ellis and Horvitz, 1986). The gene *ced-3* was cloned and found to encode a protease that contained a cysteine residue at the active site and

cleaved its substrates after the amino acid *aspartate* (*caspase*; Yuan, J. *et al.* 1993). Genetic analysis showed that *ced-3* was absolutely required for apoptosis in *C. elegans*.

Caspases constitute a large protein family that is highly conserved among multicellular organisms. The family can be divided into two major subfamilies: caspases that are involved primarily in inflammation and have homology to caspase-1 (Interleukin-1 β -Converting Enzyme), and those caspases that are related to CED-3 and are primarily involved in apoptosis. Caspases are constitutively expressed in most cell types as inactive zymogens that are proteolytically processed before they gain full activity. The caspase zymogens contain several domains including an N-terminal prodomain, a large subunit and a small subunit. Caspase activation involves cleaving the zymogen at a specific aspartic acid in the region between the large and small subunits and removing the prodomain. The active site is formed by a heterodimer containing one large and one small subunit, and the fully active caspase protein is a tetramer composed of two heterodimers (Figure 3.2). Because caspases exist as zymogens, their activity is thought to be regulated primarily post-translationally. However recent studies indicate that expression of the caspase-9 gene is regulated transcriptionally as well (Csiszar, 2003), and endoplasmic reticulum (ER) stress can induce expression of mouse caspase-12 in transfected cells (Rao *et al.* 2001).

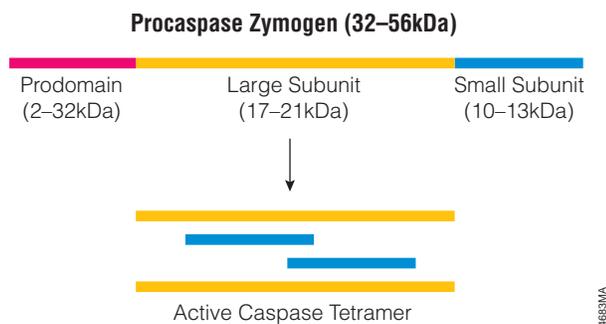


Figure 3.2. The active caspase enzyme. Caspase zymogens are cleaved between the large and small subunits, and the prodomains are removed. The active site is formed by a heterodimer that contains one large and one small subunit. Two heterodimers associate to form the fully active tetramer.

Human caspase-8 and caspase-9 are involved in initiating apoptosis through two different signaling mechanisms and are known as "initiator caspases." They can activate the effector caspases, including caspase-3, by proteolytic processing. In turn, caspase-3 cleaves downstream targets and irreversibly commits the cell to the apoptotic fate.

Bcl-2 Family Proteins

The gene *ced-9* protects against apoptosis in *C. elegans*, and genetic loss of function of *ced-9* leads to increased apoptosis (Hengartner *et al.* 1992). The *ced-9*-encoded protein is homologous with the *bcl-2* gene, a proto-oncogene involved in human lymphoma (Tsujimoto and Croce, 1986). The conservation of function of *ced-9* and *bcl-2* is demonstrated by transgenic experiments in which the human *bcl-2* gene rescues the *ced-9* loss-of-function phenotype in *C. elegans*

mutants (Hengartner and Horvitz, 1994). The Bcl-2 protein family includes a large number of proteins that share common Bcl-2 homology (BH) domains. Structurally, the Bcl-2 proteins can be divided into three groups. Group I proteins include Bcl-2, and these proteins are anti-apoptotic. Group II and III family members are pro-apoptotic. The group II family members contain all three of the BH domains; the group III family members contain only the BH-3 domain. The pro-apoptotic members of the Bcl-2 family are implicated in permeabilizing the MOM and allowing leakage of mitochondrial proteins such as cytochrome c. The anti-apoptotic members of the protein family, such as Bcl-2, appear to protect cells from apoptosis by sequestering pro-apoptotic proteins or interfering with their activity (Daniel *et al.* 2003).

D. Activating Apoptosis

Apoptosis can be induced in response to many external stimuli (extrinsic pathway) including activation of cell surface receptors such as Fas, TNFR1 (tumor necrosis factor receptor 1), TRAIL-R1 (TNF-related apoptosis-inducing ligand receptor 1), TRAIL-R2, p75-NGFR (p75-nerve growth factor receptor) and others (Wajant *et al.* 2003). These "death receptors" have two distinct signaling motifs: death domains (DD) and death effector domains (DED) that allow them to interact with other proteins involved in the apoptosis cascade. Typically the extrinsic pathway involves activating the initiator caspase, caspase-8, which in turn either activates caspase-3 or cleaves the Bcl-2 family member, Bid, leading to the formation of the apoptosome and activation of caspase-9 (Hengartner, 2000). The alternative mitochondrial pathway (intrinsic pathway) can be activated by events such as DNA damage (Rich *et al.* 1999). The mitochondrial pathway involves members of the Bcl-2 family that regulate cytochrome c release from the mitochondria.

Other studies have suggested that a third pathway for activating apoptosis may involve the endoplasmic reticulum. In mice, caspase-12 has been implicated in an ER stress pathway that induces apoptosis (Nakagawa *et al.* 2000). Caspase-12 in the mouse localizes to the ER and is cleaved in response to ER stress such as the accumulation of unfolded proteins in the ER (Nakagawa *et al.* 2000). Mouse caspase-12 activation appears to be mediated by calpain, and Ca²⁺ homeostasis may be an important indicator of cell health (Rao *et al.* 2001). Caspase-12-deficient mice show less sensitivity to amyloid beta (A β)-induced cell death (Nakagawa, 2000), suggesting that the ER may be involved in A β -induced cell death pathways. The amyloid plaques characteristic of Alzheimer's disease contain A β fragments. These A β fragments are neurotoxic and are implicated in many neurodegenerative diseases (Yuan and Yankner, 2000). Enzymatic activity has not been demonstrated for human caspase-12, but neuroblastoma cells expressing mouse caspase-12 are more sensitive to ER stress (Hitomi *et al.* 2003). Screens for human genes closely related to caspase-12 have identified human caspase-4 as a potential candidate for activating apoptosis through an

ER stress pathway (Hitomi *et al.* 2004). Caspase-4 is cleaved when cells are treated with ER stress-inducing agents, and caspase-4 localizes to the ER and mitochondria in SK-N-SH neuroblastoma and HeLa cells (Hitomi *et al.* 2004). Treating cells with caspase-4 siRNA increases their resistance to ER stress-induced apoptosis and also increases the resistance of SK-N-SH cells to A β -induced cell death (Hitomi *et al.* 2004).

E. Fas: An Example of the Death Receptor Pathway

Extrinsic signaling at the cell surface can be initiated by aggregation of Fas receptors when they bind to the multivalent Fas Ligand (FasL). This aggregation brings the cytoplasmic domains of the membrane receptors into close proximity and induces a conformational change that allows the assembly of a signaling complex, the death inducing signaling complex (DISC; Figure 3.3), at the cytoplasmic tail of the receptors. Some studies have suggested that the death receptors may be pre-aggregated in the membrane through interaction of pre-ligand-binding assembly domains (PLAD; Chan *et al.* 2000; Siegel, *et al.* 2000). The DISC comprises the receptors and ligand as well as an "adaptor" protein, Fas associated death domain protein (FADD), that binds through its C-terminal DD to the ligand-bound receptor and recruits procaspase-8.

Procaspase-8 in turn binds to the DED of FADD via its own N-terminal DED domains. As a consequence of DISC formation at ligand-bound receptors, several molecules of procaspase-8 are brought into close proximity, resulting in high local concentration of procaspase-8. One hypothesis suggests that the low intrinsic activity of procaspase-8 allows the procaspase-8 zymogens to cleave and activate each other (induced proximity activation; Hengartner, 2000). Induced proximity activation has also been proposed for human caspase-2 and nematode CED-3 (Hengartner, 2000). However, other studies have suggested that the activation of caspase-8 requires dimerization (Boatright *et al.* 2003). Active caspase-8 heterotetramers are released from DISC and are free to cleave and activate the effector caspase, caspase-3. An animated presentation

(www.promea.com

[/paguide/animation/selector.htm?coreName=apop01](http://paguide/animation/selector.htm?coreName=apop01))

illustrating the death receptor pathway is available. In some cells caspase-8 leads to an amplification loop that involves caspase-8 cleavage of the Bcl-2 protein family member, Bid. When Bid is cleaved it can induce Bax-mediated release of cytochrome c from the mitochondria, further committing the cell to the apoptosis fate.

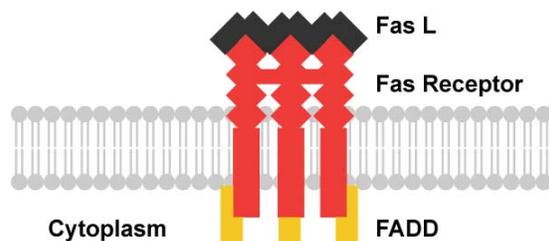


Figure 3.3. DISC Formation. Aggregation of activated death receptors brings the cytoplasmic domains into close proximity and induces a conformational change that allows the assembly of the death inducing signaling complex (DISC) at the cytoplasmic tail of the receptors.

F. The Mitochondrial Pathway (Intrinsic)

The mitochondrial pathway involves members of the Bcl-2 family of proteins and can be activated by the death receptor pathway (Section I.E) or by other stimuli that are independent of death receptors including DNA damage, topoisomerase inhibition or withdrawal of trophic factors (Parone *et al.* 2003). Many of the Group II and Group III Bcl-2 family members, such as Bax, Bad and Bid, shuttle between the mitochondria and the other parts of the cell. Their activity is regulated by a variety of mechanisms including proteolytic processing, phosphorylation and sequestration by inhibitory proteins.

Pro-apoptotic signals direct the Group II and III Bcl-2 family proteins to the mitochondria where the pro-apoptotic members interact with anti-apoptotic Bcl-2 family members including Bcl-2 and Bcl-XL to determine whether or not apoptosis will be initiated. If the pro-apoptotic proteins "win," cytochrome c and other molecules are released from the MOM. Once cytochrome c is released from the mitochondria, it can interact with Apaf-1 (a mammalian homolog of *C. elegans* CED-4; Zou *et al.* 1997), dATP and procaspase-9 in a protein complex called the apoptosome. Caspase-9 is processed and activated when it is part of the apoptosome, where it can cleave and activate caspase-3. An animated presentation (www.promea.com [/paguide/animation/selector.htm?coreName=apop02](http://paguide/animation/selector.htm?coreName=apop02)) illustrating the mitochondrial pathway is available.

G. Clinical Applications of Apoptosis Research

Many diseases—cancers, autoimmune diseases and neurodegenerative diseases, including Alzheimer's Huntington's, and ALS—demonstrate either a failure of apoptosis to eliminate harmful cells or the inappropriate activation of apoptosis leading to loss of essential cells. The complexity of apoptosis regulation and the large numbers of molecular players in the apoptotic signaling pathways provide ample opportunity for developing therapeutics to modulate the pathway. Potential therapeutic strategies include small molecules that inhibit or activate specific proteins involved in the pathway, antisense oligos directed

against specific genes involved in apoptosis, and antibodies that can oligomerize cell membrane receptors to modulate the pathway, among others (Murphy *et al.* 2003).

One obvious target for modulating apoptosis is the caspase family of proteins. The natural delay in activation of the caspases after injury allows time for treatment, and molecules that target the caspases have shown therapeutic potential in preclinical animal models (Reed, 2002; Nicholson, 2000). In mouse models of ischemic injury, active site inhibitors of caspases have been used and result in decreased apoptosis and increased survival and organ function (Nicholson, 2000; Hayakawa *et al.* 2003). Caspase inhibitors have also been used to treat sepsis in mouse models. In these models, caspase inhibition decreased lymphocyte apoptosis and improved survival rates. One pharmaceutical company, Vertex, has a caspase inhibitor in preclinical trials for treating sepsis (Murphy *et al.* 2003).

Molecules called "inhibitors of apoptosis" or IAPs are also potential therapeutic targets. These proteins, which function to suppress apoptosis, are evolutionarily conserved. Some cancers overexpress IAPs, and IAP expression is associated with resistance to apoptosis (Reed, 2002). Survivin is an IAP that has been associated with many human cancers, including lung cancer and malignant melanoma (Nicholson, 2000). Eliminating survivin activity has the potential of rendering cancer cells more sensitive to drugs that initiate apoptosis. IAPs are also being investigated in gene therapy strategies as a way of preventing excessive cell loss after stroke (Reed, 2002).

Both the death receptor and mitochondrial pathways present potential therapeutic targets as well. Normal and cancer cells show different sensitivities to TRAIL-mediated apoptosis, with approximately 80 percent of human cancer cell lines being sensitive to TRAIL-mediated apoptosis (Nicholson, 2000). In studies where TRAIL (Apo-2L) was administered with cisplatin or etoposide, cancer cells showed increased apoptosis (Nicholson, 2000). In experiments with SCID mice, recombinant TRAIL was able to slow the growth of tumors after transplantation or decrease the size of established tumors. Recombinant TRAIL also showed lower liver toxicity than CD95 ligand or TNF- α (Nicholson, 2002).

The Bcl-2 family members that play essential roles in the mitochondrial pathway are also being targeted by drug companies. Bcl-2 protein is upregulated in many cancer cells. An antisense Bcl-2 oligo has shown promise in preclinical trials in SCID mice and in Phase III clinical trials (Nicholson, 2000; Reed, 2002). Bad is a pro-apoptotic Bcl-2 family member that is implicated in neuronal apoptosis. It is a substrate of calcineurin/calmodulin-dependent phosphatase, and dephosphorylation of Bad allows Bad to bind and neutralize the anti-apoptotic protein Bcl-XL. Current therapeutics that target this part of the apoptotic pathway include active site inhibitors of calcineurin and compounds like the NMDA receptor antagonist,

memantine, that prevent calcium influx. Memantine is in clinical trials for treatment of Alzheimer's disease and multi-infarct dementia (Reed 2002).

Many other regulators and players in the apoptotic signaling pathways are also being targeted for developing therapeutics. There are many signaling cascades in cells that influence the decision of a cell to undergo apoptosis. Modifying these signaling inputs is another way to influence cell fate. MAPK family members, JUN kinases, and AKT kinase pathways all provide ways for potentially modulating inputs into apoptosis pathways of target cells (Reed, 2002; Murphy *et al.* 2003; Nicholson, 2000).

Much remains to be understood about the precise regulation of natural cell death. Understanding these cell death pathways will provide opportunity to influence and modulate cell death signaling so that inappropriate cell death can be prevented or inappropriately dividing cells can be killed using the cell's own molecular machinery.

H. Methods and Technologies for Detecting Apoptosis

Apoptosis occurs via a complex signaling cascade that is tightly regulated at multiple points, providing many opportunities to evaluate the activity of the proteins involved. The initiator and effector caspases are particularly good targets for detecting apoptosis in cells. These ubiquitous enzymes exist as inactive zymogens in cells and are cleaved before forming active heterotetramers that drive apoptotic events. Luminescent and fluorescent substrates for specific caspases have allowed the development of homogeneous assays to detect their activity. Additionally, specific antibodies that recognize the active form of the caspases or the products of caspase cleavage can be used to detect apoptosis within cells. Fluorescently conjugated caspase inhibitors can also be used to label active caspases within cells.

In addition to monitoring caspase activity, many reagents exist for monitoring molecules in the mitochondria that are indicators of apoptosis, such as cytochrome c. Some of the biochemical features of apoptosis such as loss of membrane phospholipid asymmetry and DNA fragmentation can also be used to identify apoptosis. Cell viability assays can be combined with apoptosis assays to provide more information about mechanisms of cell death through multiplexing assays on a single sample. The remainder of this chapter will describe technologies, protocols and tools to allow you to detect apoptosis in a variety of experimental systems.

II. Detecting Caspase Activity and Activation

A. Luminescent Assays for Measuring Caspase Activity

The caspase family of cysteine proteases are the central mediators of the proteolytic cascade leading to cell death and elimination of compromised cells. As such, the caspases are tightly regulated both transcriptionally and by endogenous anti-apoptotic polypeptides, which block productive activation (Earnshaw *et al.* 1999). Furthermore, the enzymes involved in this process dictate distinct

pathways and demonstrate specialized functions consistent with their primary biological roles (Stennicke *et al.* 1999). Assays that directly measure caspase activity can provide valuable information for the researcher about the mechanism of death in dying cells.

The Caspase-Glo® Assays use the luminogenic caspase-8 tetrapeptide substrate (Z-LETD-aminoluciferin), the caspase-9 tetrapeptide substrate (Z-LEHD-aminoluciferin) or the caspase-3/7 substrate (Z-DEVD-aminoluciferin) and a stable luciferase in proprietary buffers. The buffers are optimized for the specific caspase activity, cell lysis and luciferase activity. In the absence of active caspase, the caspase substrates do not act as substrates for luciferase and thus produce no light. Upon cleavage of the substrates by the respective caspase, aminoluciferin is liberated and can contribute to the generation of light in a luminescence reaction (Figure 3.4). The resulting luminescent signal is directly proportional to the amount of caspase activity present in the sample.

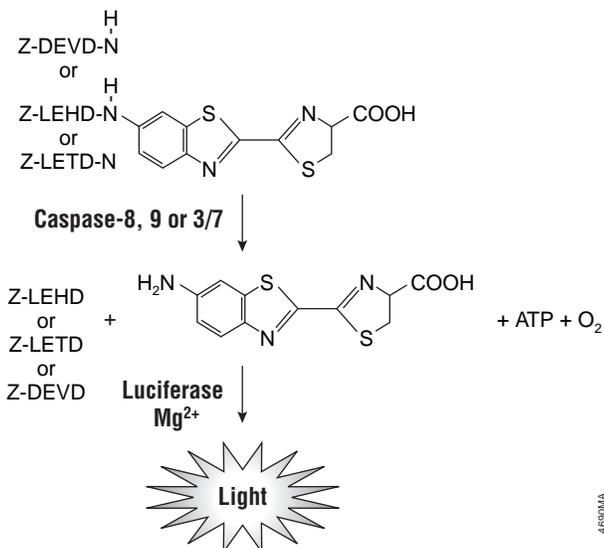


Figure 3.4. Caspase-8, -9, -3/7 cleavage of the pro-luminogenic substrates containing LETD, LEHD or DEVD, respectively. Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the production of light.

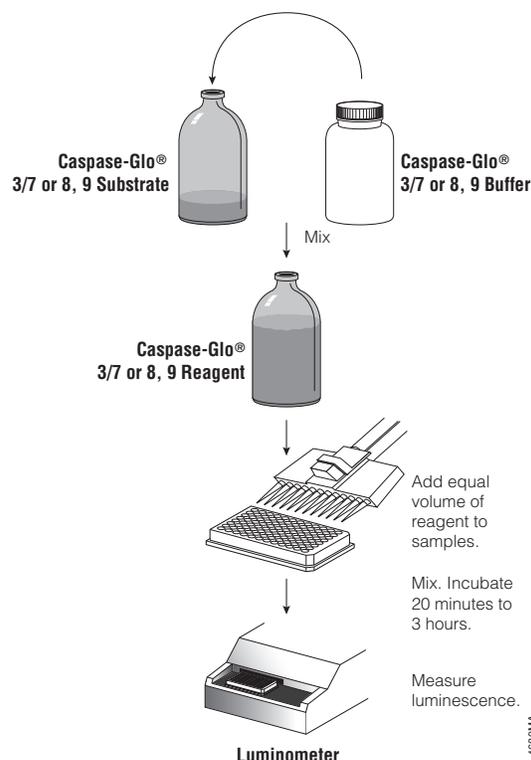


Figure 3.5. Schematic diagram of the Caspase-Glo® Assay protocols.

The Caspase-Glo® 8, 9 and 3/7 Assays are configured for ease of use and are the most sensitive caspase assays available. The reagents are prepared by adding buffer directly to the lyophilized substrate. These homogeneous reagents can then be added to the sample in a convenient 1:1 ratio (Figure 3.5) without a separate lysis step. Because the luminescent signal “glows” rather than “flashes,” reagent injectors are not required, and the assay is suitable for high-throughput applications. Figure 3.6 illustrates the linearity of the Caspase-Glo® 3/7 Assay. For a detailed protocol and more background information on the Caspase-Glo® Assays, please see Technical Bulletins #TB332, #TB333, or #TB323.

Materials Required:

- Caspase-Glo® 8 Assay and protocol (Cat.# G8200, G8201, G8202), Caspase-Glo® 9 Assay and protocol (Cat.# G8210, G8211, G8212) or Caspase-Glo® 3/7 Assay and protocol (Cat.# G8090, G8091, G8092)
- White-walled multiwell luminometer plates adequate for cell culture
- Multichannel pipettor or automated pipetting station
- Plate shaker, for mixing multiwell plates
- Luminometer capable of reading multiwell plates
- Purified caspase enzyme (e.g., BIOMOL Cat.# SE-172)
- 10mM HEPES buffer (pH 7.4) with 0.1% Prionex® stabilizer to dilute purified enzyme
- Caspase inhibitor, if performing assays to examine caspase inhibition

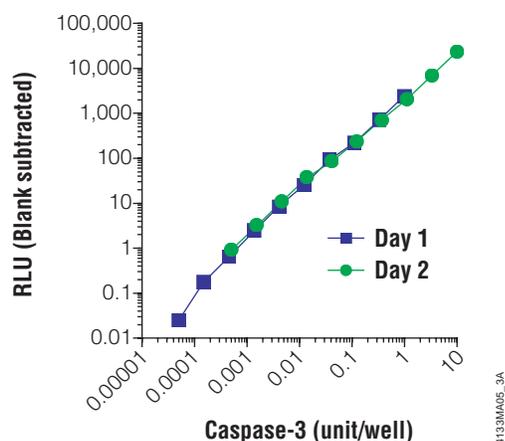


Figure 3.6. The Caspase-Glo® 3/7 Assay is linear over four orders of magnitude of caspase concentration. Purified caspase-3 was titrated and assayed in 96-well plates using the Caspase-Glo® 3/7 Assay on two different days. Luminescence was measured 1 hour after adding the Caspase-Glo® Reagent to the cells. The graph shows that the assay is linear over 4 orders of magnitude of caspase concentration. One unit of caspase = 0.07ng protein = 1pmol of substrate (Ac-DEVD-pNA) hydrolyzed/minute per the manufacturer's unit definition. Each point represents the average of 4 wells. The no-caspase control was subtracted from each point.

Additional Resources for Caspase-Glo® Assays

Technical Bulletins and Manuals

- TB332 *Caspase-Glo® 8 Assay Technical Bulletin*
(www.promega.com/tbs/tb332/tb332.html)
- TB333 *Caspase-Glo® 9 Assay Technical Bulletin*
(www.promega.com/tbs/tb333/tb333.html)
- TB323 *Caspase-Glo® 3/7 Technical Bulletin*
(www.promega.com/tbs/tb323/tb323.html)

Promega Publications

- PN087 Correlation of caspase activity and chemo-response in epithelial ovarian cancer cell lines
(www.promega.com/pnotes/87/11527_15/11527_15.html)
- CN008 Detect caspase-8 and -9 activities using the Caspase-Glo® Assays
(www.promega.com/cnotes/cn008/cn008_09.htm)
- CN009 Characterizing responses to treatments using homogeneous caspase activity and cell viability assays
(www.promega.com/cnotes/cn009/cn009_11.htm)
- CN006 Choosing the right cell-based assay for your research
(www.promega.com/cnotes/cn006/cn006_06.htm)

- CN006 Caspase-Glo® 3/7 Assay: Use fewer cells and spend less time with this homogeneous assay.
(www.promega.com/cnotes/cn006/cn006_13.htm)
- CN010 Multiplexing homogeneous cell-based assays
(www.promega.com/cnotes/cn010/cn010_15.htm)
- CN010 Miniaturizing and automating cell viability and reporter assays for high-throughput and ultrahigh-throughput screening
(www.promega.com/cnotes/cn010/cn010_10.htm)

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)

B. Fluorescent Assays for Measuring Caspase Activity

Apo-ONE® Homogeneous Caspase-3/7 Assay

The Apo-ONE® Homogeneous Caspase-3/7 Assay detects caspase-3/7 activity based on the cleavage of a profluorescent DEVD peptide-rhodamine 110 substrate [(Z-DEVD)2-R110]. The Apo-ONE® Reagent is prepared by combining buffer and substrate. The reagent is added directly to culture wells using a 1:1 ratio of reagent to culture medium. The contents are mixed and incubated for 1–2 or more hours, and the fluorescent signal is measured. The reagent permeabilizes the cells to release the caspase, delivers the profluorescent substrate and provides optimized conditions to stabilize caspase activity. Because the fluorescent R110 product continues to accumulate in the presence of active caspase-3 and -7, extending the incubation period up to 18 hours increases the signal-to-background ratio, providing greater sensitivity. The assay is easily scalable to meet miniaturization needs of HTS screening as long as the 1:1 ratio is maintained. Figure 3.7 provides an overview of the assay protocol. For a detailed protocol and background information about this system, please see Technical Bulletin #TB295 (www.promega.com/tbs/tb295/tb295.html).

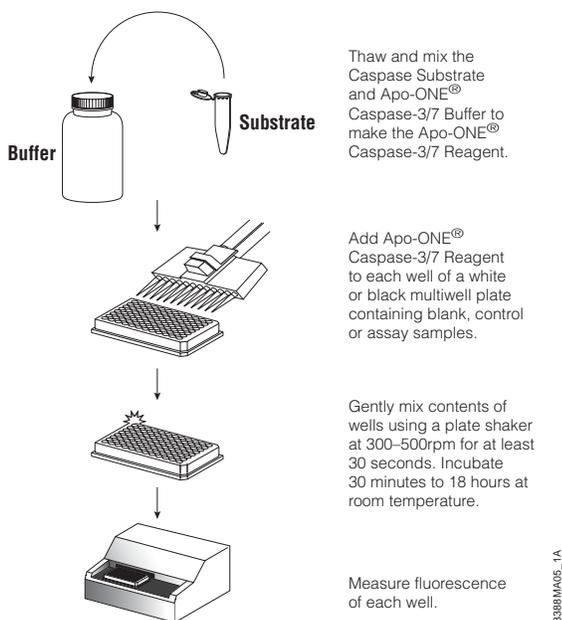


Figure 3.7. Schematic of Apo-ONE® Assay protocol.

Materials Required:

- Apo-ONE® Homogeneous Caspase-3/7 Assay and protocol (Cat.# G7790, G7791, G7792)
- 96- or 384-well opaque white or black plate suitable for cell culture (Nalge Nunc International has FluoroNunc™ Products for such applications)
- fluorescent plate reader (e.g., LabSystems Cat.# 9502887 or equivalent)
- single and multichannel pipettors
- plate shaker

Additional Resources for the Apo-ONE® Homogeneous Caspase-3/7 Assay

Technical Bulletins and Manuals

TB295 *Apo-ONE® Homogeneous Caspase-3/7 Assay* (www.promega.com/tbs/tb295/tb295.html)

Promega Publications

CN002 The Apo-ONE® Homogeneous Caspase-3/7 Assay: A simplified "solution" for apoptosis detection (www.promega.com/cnotes/cn002/cn002_02.htm)

CN010 Multiplexing homogeneous cell-based assays (www.promega.com/cnotes/cn010/cn010_15.htm)

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)

Citations

Wagner, K-D. *et al.* (2003) Oxygen regulated expression of the Wilms' tumor suppressor Wt1 involves hypoxia-inducible factor-1 (HIF-1). *FASEB J.* **17**, 1364–6.

The authors of this study used the Apo-ONE® Assay to assess apoptosis in kidney homogenates.

PubMed Number: 12738801

CaspACE™ Assay System, Fluorometric

The CaspACE™ Assay System, Fluorometric, provides reagents for measuring the activity of the caspase-1, or the Interleukin-1β-Converting Enzyme (ICE/CED-3), and caspase-3, or CPP32, families of cysteine aspartic acid-specific proteases. The CaspACE™ Assay System provides fluorogenic substrates and inhibitors that allow quantitative measurement of both ICE (caspase-1) and CPP32 (caspase-3/DEVDase) protease activities (Figure 3.8). The use of the two selective substrates and inhibitors provided allows discrimination between ICE and CPP32 activities. This assay system may be used with purified enzyme preparations or cell extracts. For a detailed protocol and background information on this system, please see Technical Bulletin #TB248 (www.promega.com/tbs/tb248/tb248.html).

Materials Required:

- CaspACE™ Assay System, Fluorometric (Cat.# G3540)
- 30°C incubator (or 37°C incubator, see Technical Bulletin #TB248)
- spectrofluorometer
- 96-well plate and fluorescence plate reader (if using 96-well plate format)
- dimethyl sulfoxide (DMSO)
- DTT, 100mM in deionized water
- deionized water
- Parafilm® laboratory film or plate sealer

Standard Assay

1. Prepare duplicate microcentrifuge tubes for each of the three assay conditions as shown.

Reagent	Blank	Assay	Negative Control
Caspase Assay Buffer	160µl	160µl	160µl
DMSO	10µl	10µl	–
DTT, 100mM	50µl	50µl	50µl
cell extract	–	Xµl	Xµl
2.5mM appropriate inhibitor	–	–	10µl
deionized water to final volume	490µl	490µl	490µl

2. Mix the contents of the tubes by vortexing gently. Incubate at 30°C for 30 minutes.
3. Add 10µl of the appropriate 2.5mM substrate to each tube.
4. Incubate at 30°C for 60 minutes. Measure the fluorescence of each reaction at an excitation wavelength of 360nm and an emission wavelength of 460nm. Fluorescence measurements must be completed within 2 hours of the addition of substrate.

96-Well Assay

1. Use a flat-bottom, white or black polystyrene 96-well plate for the assay. Prepare duplicate wells containing blank, assay and negative control reaction mixtures as follows:

Reagent	Blank	Assay	Negative Control
Caspase Assay Buffer	32 μ l	32 μ l	32 μ l
DMSO	2 μ l	2 μ l	–
DTT, 100mM	10 μ l	10 μ l	10 μ l
cell extract	–	X μ l	X μ l
2.5mM appropriate inhibitor	–	–	2 μ l
deionized water to final volume	98 μ l	98 μ l	98 μ l

2. Cover the plate with Parafilm® laboratory film and incubate at 30°C for 30 minutes.
3. Add 2 μ l of the appropriate 2.5mM substrate to all wells.
4. Cover the plate with Parafilm® laboratory film and incubate at 30°C for 60 minutes. Measure the fluorescence of the reactions at an excitation wavelength of 360nm and an emission wavelength of 460nm. Fluorescence measurements must be completed within 2 hours of the addition of substrate.

Calculation of Results

1. Determine the mean fluorescence values, DFU1 (change in fluorescence in the absence of inhibitor) and DFU2 (change in fluorescence in the presence of inhibitor) as follows: DFU1 = (mean assay FU) – (mean blank FU)
DFU2 = (mean negative control FU) – (mean blank FU)
2. Calculate the value for (DFU1 – DFU2)/time to determine the change in fluorescence units per unit time at 30°C due to ICE/ CPP32 enzyme activity.
3. Calculate the enzyme specific activity as described in Technical Bulletin #TB248 (www.promega.com/tbs/tb248/tb248.html).

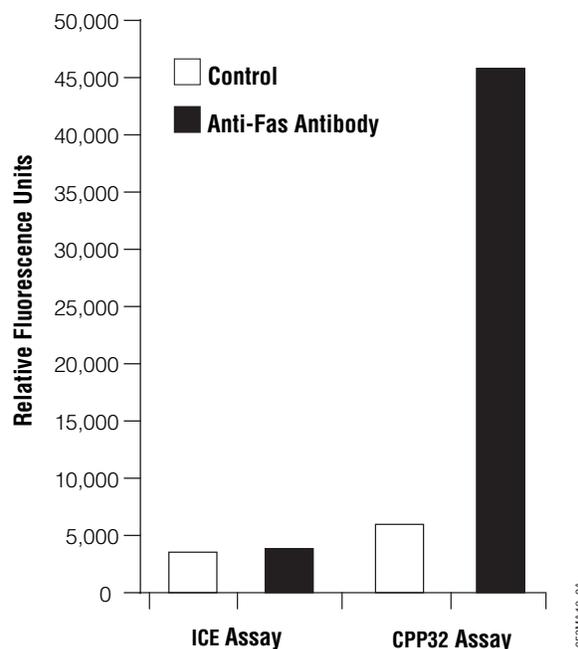


Figure 3.8. Measurement of ICE and CPP32 protease activities in anti-Fas antibody-treated human Jurkat T-cells. Jurkat T-cells (5×10^5 cells/ml) were treated with 100ng/ml of anti-Fas mAb or PBS (control) for 4 hours at 37°C. Cell lysates were tested for ICE and CPP32 activity according to the assay conditions described in Technical Bulletin #TB248.

Additional Resources for the CaspACE™ Assay System, Fluorometric

Technical Bulletins and Manuals

- TB248 *CaspACE™ Assay System, Fluorometric, Technical Bulletin*
(www.promega.com/tbs/tb248/tb248.html)

Promega Publications

- eNotes Sensitivity of the fluorometric and colorimetric CaspACE™ Assay Systems and purification of fragmented DNA from apoptotic cells
(www.promega.com/enotes/applications/ap0003_tabs.htm)
- PN066 CaspACE™ Assay System, Fluorometric
(www.promega.com/pnotes/66/7014_07/7104_07.html)

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)

Citations

McGinty, A. *et al.* (2000) Cyclooxygenase-2 expression inhibits trophic withdrawal apoptosis in nerve growth factor-differentiated PC12 cells. *J. Biol. Chem.* **275**, 12095–101.

C12 cells were transfected to express cyclooxygenase-2 (Cox-2). Six hours after withdrawal of NGF, the Cox-2-producing cells contain near control levels of caspase-3 activity as judged by the CaspACE™ Assay

System, Fluorometric. The proliferation was measured with the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS/PMS).

PubMed Number: 10766843

C. Colorimetric Assay for Detecting Caspase Activity

The CaspACE™ Assay System, Colorimetric (Cat.# G7220, G7351), provides a colorimetric substrate and a cell-permeable inhibitor that allow quantitative measurement of caspase-3 (DEVDase) protease activity, which is an early regulatory event in the apoptotic cell death process.

The colorimetric substrate (Ac-DEVD-pNA) is labeled with the chromophore *p*-nitroaniline (pNA). pNA is released from the substrate upon cleavage by DEVDase. Free pNA produces a yellow color that is monitored by a spectrophotometer at 405nm. The amount of yellow color produced upon cleavage is proportional to the amount of DEVDase activity in the sample.

The potent, irreversible and cell-permeable pan-caspase inhibitor Z-VAD-FMK is provided in the CaspACE™ Assay System, Colorimetric. The addition of the Z-VAD-FMK Inhibitor before inducing apoptosis in cell culture inhibits the activation of the caspase cascade, including caspase-3. This compound inhibits the activation of caspases in several models of apoptosis. In some systems, inhibition occurs through blocking the cleavage sites of caspases.

Materials Required:

- CaspACE™ Assay System, Colorimetric (Cat.# G7220)
- 37°C incubator
- 96-well plate (flat-bottom, clear polystyrene)
- 96-well plate reader
- dimethyl sulfoxide (DMSO)
- DTT, 100mM in deionized water
- deionized water
- Parafilm® laboratory film or plate sealer

CaspACE™ Assay System, Colorimetric Protocol

1. Thaw the Substrate stock solution and the Caspase Assay Buffer. Warm to room temperature and mix thoroughly before use.
2. Prepare replicate wells containing blank (no cell extract), negative control (extract from untreated cells), induced apoptosis (extract from induced cells) and inhibited apoptosis (extract from induced, inhibitor-treated cells) samples.

Reagent	Blank	Negative Control	Induced	Inhibited
Caspase Assay Buffer	32µl	32µl	32µl	32µl
DMSO	2µl	2µl	2µl	2µl
DTT, 100mM	10µl	10µl	10µl	10µl
Neg. control extract	–	Xµl	–	–
induced extract	–	–	Xµl	–
inhibited extract	–	–	–	Xµl
deionized H ₂ O to:	98µl	98µl	98µl	98µl

3. Add 2µl of the DEVD-pNA Substrate (10mM stock) to all wells.
4. Cover the plate with Parafilm® laboratory film or a plate sealer and incubate at 37°C for 4 hours.
Note: The assay may be incubated overnight at 22–25°C or at 37°C. Sample absorbance should not change with overnight incubation; however, background absorbance may increase.
5. Measure the absorbance in the wells at 405nm. Calculate caspase-specific activity as described in Technical Bulletin #TB270.

Additional Resources for the CaspACE™ Assay System, Colorimetric

Technical Bulletins and Manuals

- TB270 *CaspACE™ Assay System, Colorimetric Technical Bulletin*
(www.promega.com/tbs/tb270/tb270.html)

Promega Publications

- eNotes Sensitivity of the fluorometric and colorimetric CaspACE™ Assay Systems and purification of fragmented DNA from apoptotic cells
(www.promega.com/enotes/applications/ap0003_tabs.htm)

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)

Citations

Leite, F. *et al.* (2002) Inflammatory cytokines enhance the interaction of *Mannheimia haemolytica* leukotoxin with bovine peripheral blood neutrophils in vitro. *Infect. Immun.* **70**, 4336–43.

Recombinant Human Tumor Necrosis Factor- α (rhTNF- α) was added to bovine peripheral blood neutrophils (PMN) to induce expression of lymphocyte function-associated antigen 1 (LFA-1). Researchers incubated 1×10^6 cultured bovine PMNs with 50ng of rhTNF- α for 15 or 60 minutes. LFA-1 was detected by flow cytometry. The authors also

used the Colorimetric CaspACE™ Assay System to assess apoptosis in bovine PMNs. For these studies, protein-normalized cell lysates were used to compare caspase-3 activity to LFA-1 expression.

PubMed Number: 12117943

D. In Situ Marker for Caspase-3: FITC-VAD-FMK

CaspACE™ FITC-VAD-FMK In Situ Marker is a fluorescent analog of the pan caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone). The fluorescein isothiocyanate (FITC) group has been substituted for the carbobenzoxy (Z) N-terminal blocking group to create the fluorescent apoptosis marker. This structure allows delivery of the inhibitor into the cell where it irreversibly binds to activated caspases. The FITC label allows for a single-reagent addition to assay for caspase activity in situ. The FITC-VAD-FMK Marker is supplied as a 5mM solution in DMSO and is intended for in situ monitoring of caspase activity by fluorescence detection. The suggested concentration for use in anti-Fas-treated Jurkat cell culture is 10µM.

Method for Detecting Apoptosis in Jurkat Cells

Materials Required:

- CaspACE™ FITC-VAD-FMK In Situ Marker (Cat.# G7461, G7462)
 - poly-L-lysine coated slides
 - anti-Fas mAb (Clone CH-11 MBL International Cat.# SY-100)
 - PBS
 - formalin
 - mounting medium
 - fluorescence microscope
1. Seed Jurkat cells at 1×10^5 cells/ml and grow in RPMI-1640 + 10% FBS in a 37°C, 5% CO₂ incubator for 2–3 days, until they reach a density of 5×10^5 cells/ml.
 2. Prepare poly-L-lysine-coated slides. Coat each chamber of multi-chamber slides with 0.01% poly-L-lysine solution. When partially dry, rinse the slides in NANOpure® water and then air-dry. Poly-L-lysine-coated slides can be prepared in advance and stored at 4°C for up to 7 days before use.
 3. Add anti-Fas mAb (Clone CH-11, MBL International Cat.# SY-100) to a final concentration of 0.1µg/ml. Do not add to controls. Incubate for 3–4 hours at 37°C.
 4. Add CaspACE™ FITC-VAD-FMK In Situ Marker to the Jurkat cells at a final concentration of 10µM. Protect cells from light and incubate for 20 minutes in the incubator. Keep cells protected from light for the remaining steps.
 5. Centrifuge at $300 \times g$ for 5 minutes.
 6. Wash cells with PBS, then centrifuge at $300 \times g$ for 5 minutes.

7. Suspend cells in PBS to 1.5×10^6 cells/ml.
8. Add cells to poly-L-lysine-coated slides and incubate at room temperature for 5 minutes to allow the cells to adhere to the poly-L-lysine.
9. Fix in 10% buffered formalin for 30 minutes at room temperature (protected from light).
10. Rinse 3 times for 5 minutes each time in PBS.
11. Add mounting medium and coverslips to the slides. Analyze under a fluorescence microscope.

Additional Resources for the CaspACE™ FITC-VAD-FMK In Situ Marker

Technical Bulletins and Manuals

- 9PIG746 *CaspACE™ FITC-VAD-FMK In Situ Marker Product Information*
www.promega.com/tbs/9pig746/9pig746.html

Promega Publications

- eNotes *CaspACE™ FITC-VAD-FMK In Situ Marker as a probe for flow cytometry detection of apoptotic cells*
www.promega.com/enotes/applications/ap0020_tabs.htm
- PN075 *CaspACE™ FITC-VAD-FMK In Situ Marker for Apoptosis: Applications for flow cytometry*
www.promega.com/pnotes/75/8554_20/8554_20.html
- NN016 *Live/Dead Assay: In situ labeling of apoptotic neurons with CaspACE™ FITC-VAD-FMK Marker*
www.promega.com/nnotes/nn503/503_14.htm

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)

Citations

Allombert-Blaise, C. *et al.* (2003) Terminal differentiation of human epidermal keratinocytes involves mitochondria- and caspase-dependent cell death pathway. *Cell Death Differ.* **10**, 850–2.

The CaspACE™ FITC-VAD-FMK In Situ Marker was used at a concentration of 5µM in primary human epidermal keratinocyte culture to visualize active caspases during cell differentiation induced with calcium. In this experiment, the authors cultured primary human epidermal keratinocytes for 48 hours in 1.2mM calcium with or without 100mM z-VAD-FMK to demonstrate specific caspase activation and cell differentiation in calcium-induced keratinocytes upon labeling with the CaspACE™ FITC-VAD-FMK In Situ Marker.

PubMed Number: 12815468

Qi, H. *et al.* (2003) Inactivation of Cdc13p triggers MEC-1-dependent apoptotic signals in yeast. *J. Biol. Chem.* **278**, 15136–41.

Apoptosis in yeast cells was detected using the CaspACE™ FITC-VAD-FMK In Situ Marker. Yeast cells were stained with the marker at room temperature, washed and resuspended. FACS® analysis of cells was performed with excitation at 488nm and emission of 520–550nm.

PubMed Number: 12569108

Elbaz, M., Avni, A. and Weil, M. (2002) Constitutive caspase-like machinery executes programmed cell death in plant cells. *Cell Death Differ.* **9**, 726–33.

In this article, the CaspACE™ FITC-VAD-FMK In Situ Marker was used to stain tobacco plant cells induced to undergo apoptosis.

PubMed Number: 12058273

E. Detecting Active Caspase-3 Using an Antibody

Anti-ACTIVE® Caspase-3 pAb is intended for use as a marker of apoptosis; it specifically stains apoptotic human cells without staining nonapoptotic cells. All known caspases are synthesized as pro-enzymes activated by proteolytic cleavage. Anti-ACTIVE® Caspase-3 pAb is an affinity-purified rabbit polyclonal antibody directed against a peptide from the p18 fragment of human caspase-3. The antibody is affinity purified using a peptide corresponding to the cleaved region of caspase-3.

General Immunochemical Staining Protocol

Materials Required:

- Anti-ACTIVE® Caspase-3 pAb (Cat.# G7481)
 - prepared, fixed samples on slides
 - Triton® X-100
 - PBS
 - blocking buffer (PBS/0.1% Tween® 20 + 5% horse serum)
 - donkey anti-rabbit Cy®3 conjugate secondary antibody (Jackson Laboratories Cat.# 711-165-152)
 - mounting medium
 - humidified chamber
1. Permeabilize the fixed cells by incubating in PBS/0.2% Triton® X-100 for 5 minutes at room temperature. Wash three times in PBS, in Coplin jars, for 5 minutes at room temperature.
 2. Drain the slide and add 200µl of blocking buffer (PBS/0.1% Tween® 20 + 5% horse serum). Use of serum from the host species of the conjugate antibody (or closely related species) is suggested. Lay the slides flat in a humidified chamber and incubate for 2 hours at room temperature. Rinse once in PBS.
 3. Add 100µl of the Anti-ACTIVE® Caspase-3 pAb diluted 1:250 in blocking buffer. Prepare a slide with no Anti-ACTIVE® Caspase-3 pAb as a negative control. Incubate slides in a humidified chamber overnight at 4°C.

4. The following day, wash the slides twice for 10 minutes in PBS, twice for 10 minutes in PBS/0.1% Tween® 20 and twice for 10 minutes in PBS at room temperature.
5. Drain slides and add 100µl of donkey anti-rabbit Cy®3 conjugate diluted 1:500 in PBS. (We recommend Jackson Laboratories Cat.# 711-165-152.) Lay the slides flat in a humidified chamber, protected from light, and incubate for 2 hours at room temperature. Wash twice in PBS for 5 minutes, once in PBS/0.1% Tween® 20 for 5 minutes and once in PBS for 5 minutes, protected from light.
6. Drain the liquid, mount the slides in a permanent or aqueous mounting medium and observe with a fluorescence microscope.

Additional Resources for the Anti-ACTIVE® Caspase-3 pAb

Technical Bulletins and Manuals

- 9PIG748 *Anti-ACTIVE® Caspase-3 pAb Product Information*
(www.promega.com/tbs/9PIG748/9pig748.html)

Promega Publications

- CN001 Immunohistochemical staining using promega Anti-ACTIVE® and apoptosis antibodies
(www.promega.com/cnotes/cn001/cn001_4.htm)
- PN075 Anti-ACTIVE® Caspase-3 pAb for the detection of apoptosis
(www.promega.com/pnotes/75/8554_17/8554_17.html)

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)
Antibody Assistant (www.promega.com/techserv/tools/abasst/)

Citations

Kommers, G.D. *et al.* (2004) Pathogenesis of six pigeon-origin isolates of Newcastle disease virus for domestic chickens. *Vet. Pathol.* **39**, 353–62.

The Anti-ACTIVE® Caspase-3 polyclonal antibody was used to immunohistochemically stain Newcastle Disease Virus (NDV)-infected chicken spleens. Sections were deparaffinized, peroxidase-treated and microwaved for 10 minutes to retrieve antigens. The Anti-ACTIVE® Caspase-3 polyclonal antibody was utilized and detected with a biotinylated anti-rabbit antibody, streptavidin-phosphatase and DAB.

PubMed Number: 12014499

F. Using an Antibody Against a Cleaved Caspase-3 Substrate (Anti-PARP p85 Fragment pAb)

Poly (ADP-ribose) polymerase (PARP), a nuclear enzyme involved in DNA repair, is a well-known substrate for caspase-3 cleavage during apoptosis. Anti-PARP p85 Fragment pAb is a rabbit polyclonal antibody specific for the p85 fragment of PARP that results from caspase cleavage of the 116kDa intact molecule and thus provides an in situ marker for apoptosis. Each batch of antibody is tested for use in immunostaining applications and contains sufficient antibody for 50 immunocytochemical reactions at a working dilution of 1:100.

General Immunocytochemistry Protocol**Materials Required:**

- Anti-PARP p85 Fragment pAb (Cat.# G7341)
 - cells fixed on slides
 - PBS
 - blocking buffer (PBS/0.1% Tween® 20 + 5% horse serum)
 - donkey anti-rabbit biotin conjugate (Jackson Cat.# 711-065-152) or donkey anti-rabbit Cy®3 conjugate (Jackson Cat.# 711-165-152)
 - H₂O₂ (if using biotin conjugate)
 - DAB solution (if using biotin conjugate)
 - ultrapure water
 - humidified chamber
 - peroxidase-labeled streptavidin (eg., KPL Cat.# 14-300-00, diluted 1µg/ml in PBS)
1. Permeabilize cells fixed on slides in 0.2% Triton® X-100/PBS for 5 minutes at room temperature.
 2. Wash in 1X PBS in coplin jars for 5 minutes at room temperature. Repeat twice for a total of 3 washes.
 3. Drain the slides and add blocking buffer (PBS/0.1% Tween® 20 + 5% normal serum). Cover cells with blocking buffer (200µl per slide). Lay the slides flat in a humidified chamber and incubate for 2 hours at room temperature.
 4. Rinse once in PBS.
 5. Add 100µl of the Anti-PARP p85 Fragment pAb diluted in blocking buffer. We recommend a starting dilution of 1:100. Include a slide with no Anti-PARP p85 Fragment pAb as a negative control. Incubate slides in a humidified chamber overnight at 4°C.
 6. The following day, wash the slides twice for 10 minutes in 1X PBS, twice for 10 minutes in PBS/0.1% Tween® 20, and twice for 10 minutes in 1X PBS at room temperature.
 7. If the secondary antibody is a horseradish peroxidase (HRP) conjugate, block endogenous peroxidases by incubating with 0.3% hydrogen peroxide for 4–5 minutes at room temperature. If you are using a different method of detection with a secondary antibody, proceed to Step 9.

8. Wash in 1X PBS in coplin jars for 5 minutes. Repeat twice for a total of 3 washes.
9. Drain slides and add 100–200µl of diluted secondary antibody to each slide. We recommend donkey anti-rabbit biotin conjugate (Jackson Cat.# 711-065-152) or donkey anti-rabbit Cy®3 conjugate (Jackson Cat.# 711-165-152) diluted 1:500 in PBS/0.1% Tween® 20. Lay the slides flat in a humidified chamber and incubate for 2 hours at room temperature.
10. Wash several times in 1X PBS.
11. For the biotin conjugate, drain the slides and add 100–200µl of Streptavidin-HRP solution to each slide. Lay the slides flat in a humidified chamber and incubate for 45 minutes at room temperature. For HRP-conjugated secondary antibodies, proceed to Step 13. For other secondary antibodies, proceed to Step 15.
12. Wash in 1X PBS in coplin jars for 5 minutes. Repeat twice for a total of 3 washes.
13. Add 100–200µl of freshly made diaminobenzidine (DAB) solution to each slide. Lay the slides flat and incubate for ~10 minutes at room temperature.
14. Rinse the slides in NANOpure® water. Bleach is frequently used to inactivate the DAB before disposal; however, local requirements for hazardous waste should be followed.
15. Drain the liquid and mount the slides in a permanent or aqueous mounting medium (slides mounted in 70% glycerol can be stored for several weeks at 4°C or –20°C).

Method for Staining Postnatal Day 0 Mouse Brain, Paraffin-Embedded Sections. (All steps are performed at room temperature.)**Materials Required:**

- Anti-PARP p85 Fragment, pAb (Cat.# G7341)
 - paraffin-embedded, fixed sample
 - HemoDe® (Fisher Scientific) or xylene
 - ethanol (100, 95 and 70%)
 - PBS
 - Triton® X-100
 - H₂O₂
 - biotin-conjugated donkey anti-rabbit pAb
 - RTU ABC reagent (Vector Laboratories)
 - DAB substrate kit (Vector Laboratories)
 - VECTASHIELD® DAPI anti-fade Reagent (Vector Laboratories)
1. Embed tissue in paraffin after fixation in 4% paraformaldehyde. Six micron sections are used for this protocol.

Note: Best results will be obtained if the animal is perfused with fix and postfixed after dissection.

2. Deparaffinize by washing tissue 3 times for 5 minutes each in Hemo De® (Fisher Scientific) or xylene. Rinse tissue sections for 2 minutes in 100% ethanol. Transfer sections to 95% ethanol for 2 minutes, then transfer them to 70% ethanol for 2 minutes. Finally, rinse tissue sections 2 times for 2 minutes each in PBS.
3. Permeabilize for 10 minutes in PBS + 0.1% Triton® X-100.
4. Wash sections 2 times for 5 minutes each in PBS.
5. Block endogenous peroxidase activity by incubating sections in 0.3% H₂O₂ in PBS for 30 minutes.
6. Wash sections 2 times for 5 minutes each in PBS.
7. Block for 45 minutes in PBS + 5% donkey serum
8. Incubate with Anti-PARP p85 Fragment pAb diluted 1:50 in PBS + 1.0 % donkey serum for 60 minutes.
9. Wash sections 3 times for 5 minutes each in PBS.
10. Incubate with biotin-conjugated donkey anti-rabbit pAb (Jackson Laboratories) diluted 1:500 in PBS for 60 minutes.
11. Wash sections 3 times for 5 minutes each in PBS.
12. Incubate in RTU (Ready To Use) ABC reagent (Vector Laboratories) for 60 minutes.
13. Wash sections 3 times for 5 minutes each in PBS.
14. Develop with DAB substrate kit (Vector Laboratories) for 10 minutes.
15. Wash 3 times for 5 minutes each in water.
16. Mount in VECTASHIELD® + DAPI anti-fade reagent (Vector Laboratories).
17. Analyze samples immediately using a fluorescence microscope.

Additional Resources for the Anti-PARP p85 Fragment pAb

Technical Bulletins and Manuals

- TB273 *Anti-PARP p85 Fragment pAb Technical Bulletin*
(www.promega.com/tbs/tb273/tb273.html)

Promega Publications

- PN072 Cleaved PARP as a marker for apoptosis in tissue sections
(www.promega.com/notes/72/8094_07/8094_07.html)

- CN001 Immunohistochemical staining using Promega Anti-ACTIVE® and apoptosis antibodies
(www.promega.com/notes/cn001/cn001_4.htm)

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)
Antibody Assistant (www.promega.com/techserv/tools/abasst/)

Citations

Davidson, B. *et al.* (2003) Expression and activation of the nerve growth factor receptor TrkA in serous ovarian carcinoma. *Clin. Cancer Res.* **9**, 2248–59.

Anti-PARP p85 Fragment pAb was used to stain human peritoneal and pleural effusions.

PubMed Number: 12796393

O'Brien, M.A., Moravec, R.A. and Riss, T. (2001) Poly (ADP-ribose)polymerase cleavage monitored in situ in apoptotic cells. *Biotechniques.* **30**, 886–91.

The authors demonstrate specificity of an affinity-purified polyclonal antibody to the p85 fragment of PARP with Western blots that show that the antibody recognizes the 85kDa (p85) fragment of PARP but not full-length PARP.

PubMed Number: 11314271

III. Detecting Cell Death Using Mitochondrial Markers

A. Detecting Apoptosis Using Anti-Cytochrome c mAb

Anti-Cytochrome c mAb (Cat.# G7421) is a monoclonal IgG antibody (clone 6H2.B4) against cytochrome c, an electron carrier protein identified as essential to the mitochondrial respiratory process. This protein is an important molecule in the apoptosis pathway. Cytochrome c is translocated from the mitochondria to the cytoplasm, where it associates with Apaf-1 and caspase-9 in the apoptosome to activate apoptosis through caspase-9 activity.

This antibody is suited for immunocytochemistry (1:1,000 dilution) and immunohistochemistry (1:1,000 dilution). This antibody is not recommended for Western blotting.

Generally, a double staining procedure is performed using a mitochondrial-specific dye such as CMX-rosamine. In nonapoptotic cells, the cytochrome c labeling should give a punctate staining that mirrors that of CMX-rosamine. In apoptotic cells, cytochrome c is released, and this colocalization of staining disappears. In most cases it may not be possible to see any staining at all, as cytochrome c becomes unstable once it is released into the cytoplasm. Therefore, it is important to have a nonapoptotic control to ensure that the staining conditions used are able to detect any available cytochrome c.

Additional Resources for the Cytochrome c mAb

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)

Antibody Assistant (www.promega.com/techserv/tools/abasst/)

Citations

Paroni, G. *et al.* (2001) Caspase-2-induced apoptosis is dependent on caspase-9, but its processing during UV- or tumor necrosis factor-dependent cell death requires caspase-3. *J. Biol. Chem.* **276**, 21907–15.

Caspase-2 processing in human cell lines with mutations in caspase-3 and caspase-9 was examined. Procaspase-2 is the preferred caspase cleaved by caspase-3, while caspase-7 cleaves procaspase-2 with reduced efficiency. Cytochrome c release during apoptosis induction was monitored by immunocytochemistry. IMR90-E1A cells were fixed with 3% paraformaldehyde in PBS for 1 hour at room temperature. Fixed cells were washed with PBS and 0.1M glycine (pH 7.5) and permeabilized with 0.1% Triton® X-100 in PBS for 5 minutes. Cells were stained with Anti-Cytochrome c mAb diluted in PBS for 1 hour in a moist chamber at 37°C. Cells were washed with PBS twice and incubated with a tetramethylrhodamine isothiocyanate-conjugated anti-mouse for 30 minutes at 37°C. Nuclei were evidenced by Hoechst staining.

PubMed Number: 11399776

B. Detecting Cell Death with Mitochondrial Dyes

Although early stages of apoptosis do not result in immediate changes in mitochondrial metabolic activity, during apoptosis the electrochemical gradient across the mitochondrial outer membrane (MOM) collapses. One theory suggests that the change in the electrochemical gradient results from the formation of pores in the MOM by the activation and assembly of Bcl-2 family proteins in the mitochondria. One common method for observing the change in MOM properties involves a fluorescent cationic dye. In healthy nonapoptotic cells, the lipophilic dye accumulates in the mitochondria. Once the molecules reach a critical concentration inside the mitochondria, they form aggregates that emit a specific fluorescence (bright red for the cationic dye, JC-1). But, in apoptotic cells, the MOM does not maintain the electrochemical gradient, and the cationic dye diffuses into the cytoplasm, where the monomeric form emits a specific fluorescence that is different from the fluorescence of the aggregated form (green for the cationic dye, JC-1; Zamzami *et al.* 2000).

Other mitochondrial dyes can be used to measure the redox potential or metabolic activity of the mitochondria in the cells. Late in cell death processes, mitochondria lose their ability to metabolize such dyes. Although mitochondrial dyes can provide information about the overall “health” of the cells, they cannot specifically address the mechanism of cell death (apoptosis or necrosis) and are usually used in conjunction with other apoptosis detection methods (such as a caspase assay) to determine the mechanism of cell death (Zamzami *et al.* 2000; Waterhouse *et al.* 2003).

IV. Detecting Apoptosis by Measuring Changes in the Cell Membrane

Normally, eukaryotic cells maintain a specific asymmetry of phospholipids in the inner and outer leaflets of the cell membrane. During cell death phosphatidylserine (PS) becomes abundant on the outer leaflet. Detecting this change in phospholipid asymmetry is one way to detect cell death. Annexin V is a phospholipid binding protein that has a high affinity for PS. Normally, Annexin V does not bind to intact cells; however, if a cell is dying, Annexin V will bind to the PS in the outer leaflet of the cell membrane. If Annexin V is conjugated to a dye or fluorescent molecule, it can be used to label apoptotic cells (van Genderen *et al.* 2003; Bossy-Wetzel and Green, 2000).

V. Using DNA Fragmentation to Detect Apoptosis

Many of the assays used to detect apoptosis analyze the characteristic DNA fragmentation that occurs during apoptosis. In apoptotic cells the genomic DNA is cleaved to multimers of 180–200bp (based on the nucleosomal repeat length). This cleaved DNA is easily observed as a “ladder” upon analysis by gel electrophoresis. To detect this DNA fragmentation at the single-cell level, assays rely on labeling the ends of the nucleosomal fragments followed by either colorimetric or fluorescent detection. The DeadEnd™ Assays use this approach, commonly called the TUNEL (TdT-mediated dUTP Nick End Labeling) assay. With this system cells are treated so that the membrane is permeable to the reagents and enzymes necessary to label the DNA fragments. After cellular uptake of the reagents, the 3' OH ends of the multimers are “tailed” with labeled fluorescein-12-dUTP (DeadEnd™ Fluorometric TUNEL System) or with biotinylated nucleotides (DeadEnd™ Colorimetric TUNEL System). For the fluorometric assay, the fragments produced are fluorescently labeled. For the colorimetric assay, the biotinylated DNA fragments are detected using streptavidin-conjugated horseradish peroxidase.

A. DeadEnd™ Fluorometric TUNEL System

Materials Required:

- DeadEnd™ Fluorometric TUNEL System (Cat.# G3250)
- PBS
- propidium iodide (Sigma Cat.# P4170)
- optional: *SlowFade*® Light Anti-Fade Kit (Molecular Probes Cat.# S7461) or VECTASHIELD® (Vector Labs Cat.# H-1000)
- optional: VECTASHIELD® + DAPI (Vector Labs Cat.# H-1200)

For Cultured Cells

Materials Required:

- 1% methanol-free formaldehyde (Polysciences Cat.# 18814) in PBS
- 4% methanol-free formaldehyde (Polysciences Cat.# 18814) in PBS
- 70% ethanol
- 0.2% Triton® X-100 solution in PBS

- 0.1% Triton® X-100 solution in PBS containing 5mg/ml BSA
- DNase I (e.g., RQ1 RNase-Free DNase, Cat.# M6101)
- 20mM EDTA (pH 8.0)
- DNase buffer
- DNase-free RNase A

For Paraffin-Embedded Tissue Sections

Materials Required:

- 4% methanol-free formaldehyde (Polysciences Cat.# 18814) in PBS
- xylene
- ethanol (100%, 95%, 85%, 70% and 50% diluted in deionized water)
- 0.85% NaCl solution
- proteinase K buffer
- DNase I
- DNase I buffer

Equipment for Cultured Adherent Cells and Tissue Sections

Materials Required:

- poly-L-lysine-coated or silanized microscope slides
- cell scraper
- Coplin jars (separate jar needed for optional DNase I positive control)
- forceps
- humidified chambers for microscope slides
- 37°C incubator
- micropipettors
- glass coverslips
- rubber cement or clear nail polish
- fluorescence microscope

Equipment for Cell Suspensions

Materials Required:

- tabletop centrifuge
- 37°C incubator or a 37°C covered water bath
- poly-L-lysine-coated or silanized microscope slides
- Coplin jars (separate jar needed for optional DNase I positive control)
- forceps
- glass coverslips
- humidified chambers for microscope slides
- micropipettors
- flow cytometer or fluorescence microscope

Apoptosis Detection by Fluorescence Microscopy (protocol)

1. Attach cells to slides and fix in methanol-free formaldehyde solution.
2. Wash slides in PBS then permeabilize with Triton® X-100.
3. Rinse slides in PBS and tap dry. Pre-equilibrate slides with Equilibration Buffer (5–10 minutes at room temperature).
4. Thaw nucleotide mix and prepare the rTdT incubation buffer for reactions and controls as described in Technical Bulletin #TB235.

5. Label DNA strand breaks with fluorescein-12-dUTP for 60 minutes at 37°C in a humidified chamber protected from light.
6. Stop reactions by immersing slides in 2X SSC (15 minutes at room temperature).
7. Wash the slides 3 times for 5 minutes each in PBS to remove unincorporated fluorescein-12-dUTP.
8. Stain the samples in a Coplin jar by immersing the slides in 40ml of propidium iodide solution freshly diluted to 1µg/µl in PBS for 15 minutes at room temperature in the dark.
9. Wash the slides 3 times for 5 minutes each in PBS.
10. Analyze samples immediately using a fluorescence microscope. Alternatively, add 1 drop of Anti-Fade solution (Molecular Probes Cat.# S7461) to the area containing the treated cells and mount slides using glass coverslips. Seal the edges with rubber cement or clear nail polish and let dry for 5–10 minutes.

Analysis of Suspension Cells By Flow Cytometry (protocol overview)

1. Wash 3–5 × 10⁶ cells with PBS and centrifuge at 300 × g at 4°C. Repeat this wash and resuspend in 0.5ml of PBS.
2. Fix the cells by adding 5ml of 1% methanol-free formaldehyde for 20 minutes or overnight on ice.
3. Centrifuge the cells at 300 × g for 10 minutes at 4°C, remove the supernatant and resuspend cells in 5ml of PBS. Repeat wash once and resuspend cells in 0.5ml of PBS.
4. Add the cell suspension to 5ml of 70% ice-cold ethanol and keep at –20°C for at least 4 hours.
5. Centrifuge the cells at 300 × g for 10 minutes and resuspend in 5ml of PBS. Repeat centrifugation and resuspend the cells in 1ml of PBS.
6. Transfer 2 × 10⁶ cells into a 1.5ml microcentrifuge tube.
7. Centrifuge at 300 × g for 10 minutes, remove supernatant and resuspend the pellet in 80µl of Equilibration Buffer. Incubate at room temperature for 5 minutes.
8. While the cells are equilibrating, thaw the Nucleotide Mix on ice and prepare sufficient rTdT incubation buffer for all reactions according to Technical Bulletin #TB235. To determine the total volume of rTdT incubation buffer needed, multiply the number of reactions times 50µl, the volume of a standard reaction using 2 × 10⁶ cells. For negative controls, prepare a control incubation buffer without rTdT Enzyme, substituting deionized water for the enzyme.

9. Centrifuge cells at $300 \times g$ for 10 minutes. Remove supernatant and resuspend the pellet in 50 μ l rTdT incubation buffer. Incubate in a water bath for 60 minutes at 37°C, protecting from direct light exposure. Resuspend the cells by pipetting at 15-minute intervals.
10. Terminate the reaction by adding 1ml of 20mM EDTA. Vortex gently.
11. Centrifuge cells at $300 \times g$ for 10 minutes. Remove supernatant and resuspend the pellet in 1ml of 0.1% Triton® X-100 solution in PBS containing 5mg/ml BSA. Repeat once for a total of 2 rinses.
12. Centrifuge cells at $300 \times g$ for 10 minutes. Remove supernatant and resuspend the cell pellet in 0.5ml propidium iodide solution (freshly diluted to 5 μ g/ml in PBS) containing 250 μ g of DNase-free RNase A.
13. Incubate the cells at room temperature for 30 minutes in the dark.
14. Analyze cells by flow cytometry. Measure green fluorescence of fluorescein-12-dUTP at 520 \pm 20nm and red fluorescence of propidium iodide at >620nm.

Additional Resources for the DeadEnd™ Fluorometric TUNEL System

Technical Bulletins and Manuals

- TB235 *DeadEnd™ Fluorometric TUNEL System Technical Bulletin*
(www.promega.com/tbs/tb235/tb235.html)

Promega Publications

- PN059 Analysis of DNA fragmentation in epidermal keratinocytes using the Apoptosis Detection System, Fluorescein
(www.promega.com/pnotes/59/5644e/5644e.html)
- PN057 Detection of apoptotic cells using the Apoptosis Detection System, Fluorescein
(www.promega.com/pnotes/57/5573b/5573b.html)

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)

Citations

DeCoster, M.A. (2003) Group III secreted phospholipase A2 causes apoptosis in rat primary cortical neuronal cultures. *Brain Res.* **988**, 20–8.

The DeadEnd™ Fluorometric TUNEL System was used to demonstrate the apoptotic effect of secreted phospholipase A2 (sPLA2) on primary rat cortical neurons in culture. Dual staining with the DeadEnd™ Fluorometric TUNEL System and propidium iodide allowed quantification of the TUNEL staining area by analysis of digitized images.

PubMed Number: 14519523

Davis, D.W. *et al.* (2003) Automated quantification of apoptosis after neoadjuvant chemotherapy for breast cancer: Early assessment predicts clinical response. *Clin. Cancer Res.* **9**, 955–60.

The authors developed an automated, laser scanning, cytometer-based method to quantify the percentage of tumor cells containing DNA fragmentation characteristic of apoptosis. They used the DeadEnd™ Fluorometric TUNEL System to analyze sections from breast tumor biopsies.

PubMed Number: 12631592

B. DeadEnd™ Colorimetric TUNEL System

Materials Required:

- DeadEnd™ Colorimetric TUNEL System (Cat.# G7360, G7130)
- phosphate-buffered saline (PBS)
- 0.3% hydrogen peroxide for blocking endogenous peroxidases
- fixative (e.g., 10% buffered formalin, 4% paraformaldehyde, 4% methanol-free formaldehyde)
- mounting medium

For Cultured Cells

Materials Required:

- poly-L-lysine
- 0.2% Triton® X-100 solution in PBS
- DNase I (e.g., RQ1 RNase-Free DNase, Cat.# M6101)
- DNase buffer

For Paraffin-Embedded Tissue Sections

Materials Required:

- xylene or xylene substitute [e.g., Hemo-De® Clearing Agent (Fisher Cat.# 15-182-507A)]
- ethanol (100%, 95%, 85%, 70% and 50%) diluted in deionized water
- 0.85% NaCl solution
- proteinase K buffer
- DNase I
- DNase I buffer

Equipment for Tissue Sections and Cultured Cells

Materials Required:

- poly-L-lysine-coated or silanized microscope slides
- forceps
- Coplin jars (separate jar needed for optional DNase I positive control)
- humidified chambers for microscope slides
- 37°C incubator
- micropipettors
- glass coverslips
- clear nail polish or rubber cement
- microscope

Apoptosis Detection (protocol)

1. Prepare samples by attaching sections or cells to a microscope slide, fixing the sample, washing and permeabilizing the cells with 0.2% Triton® X-100 in PBS.

2. Pre-equilibrate the slides with Equilibration Buffer.
3. Label DNA strand breaks with Biotinylated Nucleotide Mix (60 minutes at 37°C).
4. Stop the reaction by immersing slides in 2X SSC (15 minutes at room temperature).
5. Wash the slides 3 times for 5 minutes each in PBS.
6. Block with hydrogen peroxide (3–5 minutes at room temperature).
7. Wash the slides 3 times for 5 minutes each in PBS.
8. Add Streptavidin HRP diluted in PBS (30 minutes at room temperature).
9. Wash the slides 3 times for 5 minutes each in PBS.
10. Add DAB and develop (approximately 10 minutes).
11. Rinse slides several times in deionized water and analyze sample with a light microscope.

Additional Resources for the DeadEnd™ Colorimetric TUNEL System

Technical Bulletins and Manuals

- TB199 *DeadEnd™ Colorimetric TUNEL System Technical Bulletin*
(www.promega.com/tbs/tb199/tb199.html)

Promega Publications

- NN016 *DeadEnd™ Colorimetric Apoptosis Detection System for the analysis of retinal apoptosis*
(www.promega.com/nnotes/nn503/503_13.htm)
- PN069 *DeadEnd™ Colorimetric Apoptosis Detection System: Applications in pathology*
(www.promega.com/pnotes/69/7542_02/7542_02.html)

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)

Citations

Teder, P. *et al.* (2002) Resolution of Lung Inflammation by CD44. *Science*. **250**, 155–8.

Apoptotic cells were detected in paraffin-embedded sections of mouse lung tissue with the DeadEnd™ Colorimetric TUNEL System

PubMed Number: 11935029

Bezzi, P. *et al.* (2001) CXCR4-activated astrocyte glutamate release via TNF α : amplification by microglia triggers neurotoxicity. *Nat. Neurosci.* **4**, 702–10.

The apoptotic nature of neuron cell death via a chemokine-activated cell-cell communication system involving microglia was characterized. Hippocampal pyramidal neurons were obtained from embryonic day 17

rat brain and exposed to gp120IIIB and stained for neuronal death by apoptosis using the DeadEnd™ Colorimetric TUNEL System. Neuronal death was also detected by immunocytochemistry using the Anti-ACTIVE® Caspase-3 pAb (1:250 dilution).

PubMed Number: 11426226

VI. Using Two or More Detection Methods to Confirm Apoptosis

Typically, more than one method is necessary to confirm that cell death is occurring via apoptosis. Cultured cells undergoing apoptosis in vitro eventually undergo secondary necrosis. After extended incubation, apoptotic cells ultimately shut down metabolism, lose membrane integrity and release their cytoplasmic contents into the culture medium. Markers of apoptosis such as caspase activity may be expressed only transiently. Therefore, to determine if apoptosis is the primary mechanism of cell death, understanding the kinetics of the cell death process in your model system is critical. If detailed information on the mechanism of cell death is desired, the duration of exposure to the toxin, the concentration of the test compound and the choice of assay endpoint become critical.

VII. Multiplexing Assays

The ability to gather more than one set of data from the same sample (i.e., multiplexing) is becoming increasingly important. Multiplexing more than one assay from the same culture well can provide internal controls and eliminate the need to repeat work. Figure 3.9 shows data obtained from multiplexing a luminescent caspase-8 and a fluorescent caspase-3/7 assay. Figure 3.10 shows an example of multiplexing a cell viability assay (CellTiter-Blue® Assay) and a caspase assay (Apo-ONE® Assay) sequentially in the same well. Below we provide several sample protocols for multiplexing Promega cell-based viability, cytotoxicity and apoptosis assays. These protocols are intended as starting points. As with any homogeneous assay, multiplexing assays will require optimization for each experimental system. We strongly recommend running appropriate controls, including performing each assay individually on the samples. Additional background, optimization and recommended controls for each assay are provided in the technical literature that accompanies each individual assay. We strongly advise reading this information before attempting a multiplexing experiment.

A. Distinguishing Caspase-3/7 and Caspase-8 or -9 Activity (Sample Protocol)

Materials Required:

- Caspase-Glo® 8 or 9 Reagent (Cat.# G8200, G8201, G8202 or Cat.# G8210, G8211, G8212)
- Apo-ONE® Homogeneous Caspase-3/7 Assay (Cat.# G7790, G7791, G7792)
- plate-reading luminometer
- fluorescent plate reader

1. Culture and treat cells with drug of interest in 100µl of medium in a 96-well plate.

Note: It is often beneficial to identify and include control compounds that induce specific caspase response profiles (e.g., TNF-superfamily ligands or agonists for extrinsic pathway or small molecule inducers or insults for intrinsic pathway). In addition, a vehicle control should always be included to matched wells at the same time as any test compound.

2. During the cell exposure to the compounds, prepare either the Caspase-Glo® 8 or 9 reagents by adding the Caspase-Glo® Buffer to the lyophilized Substrate.
3. Thaw the Apo-ONE® substrate and add it to either the Caspase-Glo® 8 or 9 reagent at a dilution of 1:200 (50µl/10ml of Caspase-Glo® 8 or 9 reagent). The Apo-ONE® buffer will not be used in this multiplexed assay. Shield the multiplexing reagent from ambient light and allow it to equilibrate to room temperature.
4. Remove plated cells from the incubator (37°C) and add an equal volume of the multiplexing reagent (e.g., 100µl to 100µl).

5. Mix briefly at 500–700rpm on an orbital shaker and shield them from ambient light.

Note: Mixing by pipetting is discouraged, because it may create excess bubbles.

6. Incubate for 30 minutes to 1 hour at room temperature to achieve steady-state signal associated with the Caspase-Glo® 8 or 9 Assays. Measure luminescence.
7. Read fluorescence signal at 485_{Ex}/525_{Em}.

Note: Fluorescence intensity of the caspase-3/7 assay will increase as a function of time. Therefore, the fluorescence signal will likely be greater after a 2– to 3-hour incubation. Although the luminescent Caspase-Glo® 8 or 9 Assays have stable luminescence profiles with a half-life approaching 5 hours, measurements should be taken within 3 hours.

Other Considerations: Caspases-8 and -9 are initiator enzymes which activate the effector caspases-3 or -7. To this end, the kinetics of the useable induction windows of the multiplexed assay differ somewhat. Although, maximal caspase-8 or -9 activities mirror those of caspase-3 or -7, the activity half-lives differ in a manner consistent with their biological function. In other words, caspase-3 or -7 may be measurable much longer than the more transient caspase-8 or -9 activities. The optimal response should be determined by time course studies.

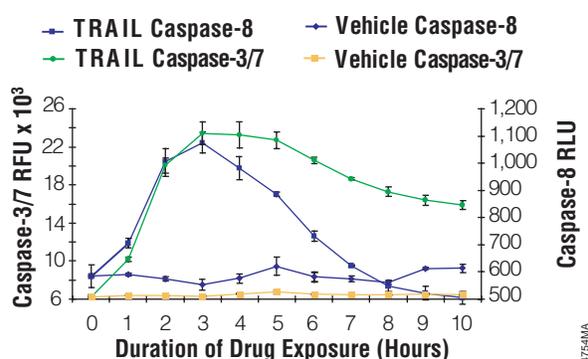


Figure 3.9. Multiplexing luminescent caspase-8 and fluorescent caspase-3/7 assays. Jurkat cells were seeded at 25,000 cells/well. Fifty microliters of rTRAIL (Chemicon, 100ng/ml final) or a vehicle control (RPMI 1640 with 10% FBS) was added to replicate wells every hour for 10 hours. Caspase-Glo® 8 reagent was prepared by combining the assay buffer with the substrate. The fluorescent Apo-ONE® Assay caspase-3/7 substrate was mixed into the Caspase-Glo® 8 reagent at a final concentration of 50µM. The combined reagent/substrate was added in 100µl volumes, incubated 60 minutes, and then luminescence and fluorescence were measured.

B. Multiplexing a Fluorescent Caspase-3/7 Assay with a Cell Viability Assay (Sample Protocol)

Materials Required:

- CellTiter-Blue® Cell Viability Assay (Cat.# G8080, G8081, G8082)
 - Apo-ONE® Homogeneous Caspase-3/7 Assay (Cat.# G7790, G7791, G7792)
 - fluorescent plate reader
1. Culture and treat cells with drug of interest in 100µl of medium in a 96-well plate. During the final 1–2 hours of treatment, add 20µl/well of CellTiter-Blue® Reagent directly to the culture wells.
 2. Return plate to incubator for duration of the treatment period.
 3. Record CellTiter-Blue® fluorescence (viability) at 560nm/590nm.
 4. Add an equal volume of Apo-ONE® Reagent (120µl/well).
 5. Record Apo-ONE® fluorescence (caspase) at 485_{Ex}/527_{Em}.

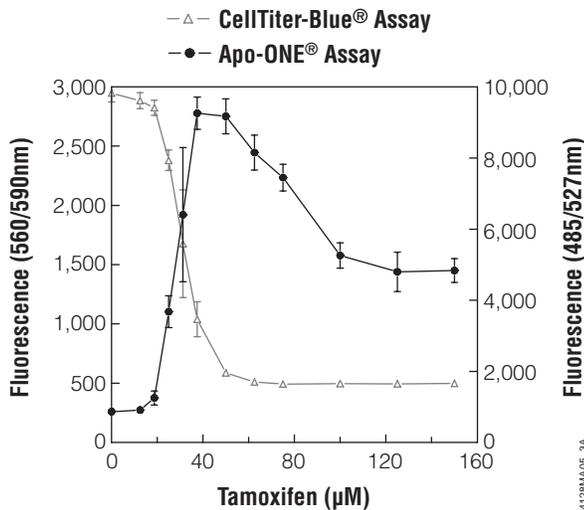


Figure 3.10. Multiplexing cell viability assays. HepG2 cells (10,000 cells/100µl cultured overnight) were treated with various concentrations of tamoxifen for 5 hours. Viability was determined by adding CellTiter-Blue® Reagent (20µl/well) to each well after 3.5 hours of drug treatment and incubating for 1 hour before recording fluorescence (560_{Ex}/590_{Em}). Caspase activity was then determined by adding 120µl/well of Apo-ONE® Reagent and incubating for 0.5 hour before recording fluorescence (485_{Ex}/527_{Em}).

C. Multiplexing a Luminescent Caspase Assay with a Fluorescent Cell Viability Assay (Sample Protocol)

Materials Required:

- CellTiter-Blue® Cell Viability Assay (Cat.# G8080, G8081, G8082)
- Caspase-Glo® 3/7 Assay (Cat.# G8090, G8091, G8092)
- fluorescent plate reader
- plate-reading luminometer

1. Culture and treat cells with drug of interest in 100µl of medium in a 96-well plate.
2. During the final 1–2 hours of treatment, add 20µl/well CellTiter-Blue® Reagent using diluted 1:4 with Dulbecco's PBS.
3. Return the plate to the incubator for the duration of the treatment period.
4. Record the CellTiter-Blue® fluorescence (viability) at 560_{Ex}/590_{Em}.
5. Add an equal volume of Caspase-Glo® 3/7 Reagent (120µl/well). The wells will slowly turn bright pink.
6. Incubate one hour at room temperature and record luminescence (caspase activity).

D. Determine the Mechanism of Cytotoxicity (Sample Protocol)

Materials Required:

- CytoTox-ONE™ Homogeneous Membrane Integrity Assay (2X concentration; Cat.# G7890, G7891)
- Caspase-Glo® 3/7 Assay (Cat.# G8090, G8091, G8092)

- fluorescent plate reader
- plate-reading luminometer

1. Culture and treat cells with the drug of interest in 100µl of medium in a 96-well plate.
2. Reconstitute CytoTox-ONE™ Substrate at 2X concentration and add 25µl/well.
3. Shake while incubating for 10 minutes at room temperature. Record fluorescence (560_{Ex}/590_{Em}) as described in the CytoTox-ONE™ System Technical Bulletin #TB306.
4. Add an equal volume (125µl) of Caspase-Glo® 3/7 Reagent to each well.
5. Incubate for 1 hour at room temperature to achieve luminescence steady state. Record luminescence as described in the Caspase-Glo® 3/7 Assay Technical Bulletin #TB323.

E. Assessing Gene Regulation and Apoptosis Involvement (Sample Protocol)

Materials Required:

- EnduRenµ Live Cell Substrate (Cat.# E6481, E6482, E6485)
- Apo-ONE® Homogeneous Caspase-3/7 Assay Reagent (Cat.# G7790, G7791, G7792)
- cells transfected with appropriate Renilla luciferase reporter
- plate-reading luminometer
- fluorescent plate reader

1. Culture and treat cells with drug of interest in 90µl of medium in a 96-well plate.
2. Add EnduRen™ Substrate (60µM final 10µl/well) to a portion of the wells containing drug treated cells and incubate for an additional 2 hours 37°C, 5% CO₂. You may add the Substrate before or after experimental treatment, depending on cell tolerance to the EnduRen™ Substrate.
3. Record luminescence.
4. Add an equal volume of Apo-ONE® Reagent (100µl/well) and incubate for 1 hour at room temperature.
5. Record fluorescence (485_{Ex}/527_{Em}).as described in Technical Bulletin TB295.

Note: We strongly recommend the following controls: Drug-treated cells with Apo-ONE® Reagent added alone and drug-treated cells with EnduRen™ Substrate added alone.

VIII. General Protocols for Inducing Apoptosis in Cells

Apoptosis may be induced in experimental systems through a variety of methods, including:

Treating cells with the protein synthesis inhibitor, anisomycin, or the DNA topoisomerase I inhibitor, camptothecin, induces apoptosis in the human promyelocytic cell line HL-60 (Del Bino *et al.* 1991; Li *et al.* 1995; Gorczyca *et al.* 1993; Darzynkiewicz *et al.* 1992).

Withdrawal of growth factors induces apoptosis of growth factor-dependent cell lines. For example, NGF-deprivation of PC12 cells or sympathetic neurons in culture induces apoptosis (Batistatou and Greene, 1991).

In vitro treatment with the glucocorticoid, dexamethasone, induces apoptosis in mouse thymus lymphocytes (Gavrieli *et al.* 1992; Cohen and Duke 1984).

Activation of either Fas or TNF-receptors by the respective ligands or by cross-linking with agonist antibody induces apoptosis of Fas- or TNF receptor-bearing cells (Tewari and Dixit 1995).

A. Anti-Fas mAb Induction of Apoptosis in Jurkat Cells

1. Grow Jurkat cells in RPMI-1640 medium containing 10% fetal bovine serum in a humidified, 5% CO₂ incubator at 37°C.
2. Suspend the cells in fresh medium at a concentration of 1×10^5 cells/ml. After two to three days of incubation in a 37°C, 5% CO₂ incubator, harvest the cells by centrifugation at $300\text{--}350 \times g$ for 5 minutes.
3. Resuspend cells in fresh medium to 5×10^5 cells/ml and add anti-Fas mAb to a final concentration of 0.05–0.1 µg/ml. Incubate for 3–6 hours in a 37°C incubator. As a negative control, incubate untreated cells (no anti-Fas mAb) under the same conditions. (Stop here for homogeneous assay, or plate the cells in a 96-well plate.)
4. Harvest the cells by centrifugation at $300\text{--}350 \times g$ for 5 minutes.
5. Remove all medium and resuspend cells in PBS.
6. Repeat centrifugation and resuspend the cell pellet in PBS to 1.5×10^6 cells/ml.

B. Anisomycin-Induced Apoptosis in HL-60 Cells

Treatment with the protein synthesis inhibitor, anisomycin induces apoptosis in the human promyelocytic cell line HL-60.

1. Grow HL-60 cells in RPMI-1640 medium containing 10% fetal bovine serum in a humidified 5% CO₂ incubator at 37°C.
2. Adjust the cell density to 5×10^5 cells/ml and treat with anisomycin at a final concentration of 2 µg/ml (dissolved in DMSO). Incubate for 2 hours in a humidified 5% CO₂

incubator at 37°C. Treat negative control cells with an equal volume of DMSO, and incubate under the same conditions.

3. Harvest the cells and resuspend in PBS to 1.5×10^6 /ml.

C. Staurosporine-Induced Apoptosis in SH-SY5Y Neuroblastoma Cells

1. Culture cells in a 1:1 mixture of Ham's F12 nutrients and minimal essential medium supplemented with 10% fetal bovine serum (FBS), 100IU/ml penicillin and 100mg/ml streptomycin in an atmosphere of 95% air and 5% CO₂ at 37°C.
2. Allow cells to reach 70% confluence. Trypsinize to release cells from the flask, and plate in a 96-well plate in 45% MEM, 45% F12K and 10% FBS.
3. After 24 hours, treat cells with 100 µl of 3.125 µM staurosporine in DMSO.
4. Incubate with staurosporine for 24 hours before performing cell-based assay.

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I. Introduction

Choosing a cell viability or cytotoxicity assay from among the many different options available can be a challenging task. Picking the best assay format to suit particular needs requires an understanding of what each assay is measuring as an endpoint, of how the measurement correlates with cell viability, and of what the limitations of the assay chemistries are. Here we provide recommendations for characterizing a model assay system and some of the factors to consider when choosing cell-based assays for manual or automated systems.

A. Establishing an In Vitro Model System

The species of origin and cell types used in cytotoxicity studies are often dictated by specific project goals or the drug target that is being investigated. Regardless of the model system chosen, establishing a consistent and reproducible procedure for setting up assay plates is important. The number of cells per well and the equilibration period prior to the assay may affect cellular physiology. Maintenance and handling of stock cultures at each step of the manufacturing process should be standardized and validated for consistency. Assay responsiveness to test compounds can be influenced by many subtle factors including culture medium surface-to-volume ratio, gas exchange, evaporation of liquids and edge effects. These factors are especially important considerations when attempting to scale up assay throughput.

B. Choosing an Endpoint to Measure

One of the first things to decide before choosing an assay is exactly what information you want to measure at the end of a treatment period. Assays are available to measure a variety of different markers that indicate the number of dead cells (cytotoxicity assay), the number of live cells (viability assay), the total number of cells or the mechanism of cell death (e.g., apoptosis). Table 4.1 compares Promega homogeneous cell-based assays and lists the measured parameters, sensitivity of detection, incubation time and detection method for each assay.

A basic understanding of the changes that occur during different mechanisms of cell death will help you decide which endpoint to choose for a cytotoxicity assay (Riss and Moravec, 2004). Figure 4.1 shows a simplified example illustrating chronological changes occurring during apoptosis and necrosis and the results that would be expected from using the assays listed in Table 4.1 to measure different markers.

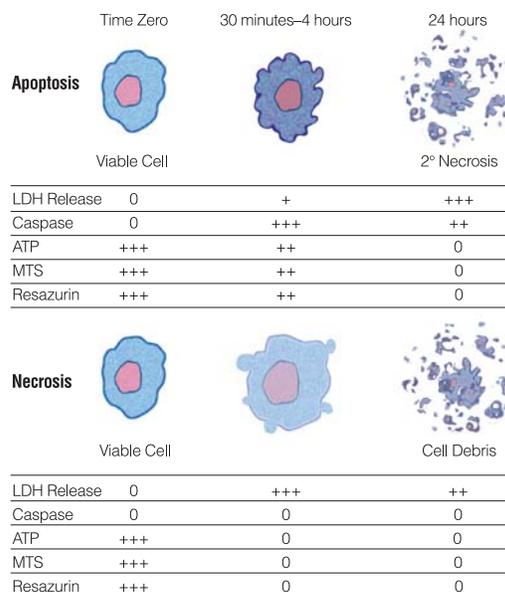


Figure 4.1. Mechanisms of cell death can be determined by measuring different markers of cell viability and apoptosis in vitro.

Cultured cells that are undergoing apoptosis in vitro eventually undergo secondary necrosis. After extended incubation, apoptotic cells ultimately shut down metabolism, lose membrane integrity and release their cytoplasmic contents into the culture medium. Markers of apoptosis such as caspase activity may be present only transiently. Therefore, to determine if apoptosis is the primary mechanism of cell death, understanding the kinetics of the cell death process in your model system is critical.

Cells undergoing necrosis typically undergo rapid swelling, lose membrane integrity, shut down metabolism and release their cytoplasmic contents into the surrounding culture medium. Cells undergoing rapid necrosis in vitro do not have sufficient time or energy to activate apoptotic machinery and will not express apoptotic markers. [For additional information about mechanisms of cell death, please visit the Apoptosis Chapter (www.promega.com/paguide/chap3.htm) of this *Protocols and Applications Guide*.]

If the information sought is simply whether there is a difference between “no treatment” negative controls and “toxin treatment” of experimental wells, the choice between measuring the number of viable cells or the number of dead cells may be irrelevant. However, if more detailed information on the mechanism of cell death is being sought, the duration of exposure to toxin, the concentration of the test compound, and the choice of the assay endpoint become critical (Riss and Moravec, 2004).

C. Characterizing Assay Responsiveness

Protocols used to measure cytotoxicity in vitro differ widely. Often assay plates are set up containing cells and allowed to equilibrate for a predetermined period before adding test compounds. Alternatively, cells may be added

Table 4.1. Comparison of Promega Cell Viability, Cytotoxicity and Apoptosis Assays

Characteristic	CellTiter-Glo® Luminescent Cell Viability Assay	BacTiter-Glo® Microbial Cell Viability Assay	CellTiter-Blue® Cell Viability Assay	CellTiter 96® Aqueous One Solution Cell Proliferation Assay
Incubation	10 minutes	5 minutes	1–4 hours	1–4 hours
Parameter measured	ATP	ATP	resazurin reduction	MTS reduction
Sensitivity: 96-well/384-well	50 cells/15 cells (also 1536-well format)	~40 cells/N.D.	390 cells/50 cells	800 cells/200 cells
Sample Type	suspension or adherent cells	bacteria, yeast	suspension or adherent cells	suspension or adherent cells
Detection	luminescent	luminescent	fluorometric or colorimetric	colorimetric
Characteristic	Apo-ONE® Homogeneous Caspase-3/7 Assay	Caspase-Glo® 3/7 Assay	Caspase-Glo® 8 or 9 Assays	
Incubation	1–18 hours	30 minutes–2 hours	30 minutes–2 hours	
Parameter measured	effector caspase activity	effector caspase activity	initiator caspase activity	
Sensitivity: 96-well/384-well	Several hundred cells in a population	20 cells/20 cells	20 cells/20 cells	
Sample Type	culture cells or purified enzyme	culture cells or purified enzyme	culture cells or purified enzyme	
Detection	fluorometric	luminescent	luminescent	
Characteristic	CytoTox-ONE™ Membrane Integrity Assay	MultiTox-Fluor Multiplex Cytotoxicity Assay	CytoTox-Fluor™ Cytotoxicity Assay	CytoTox 96® Non-Radioactive Cytotoxicity Assay
Incubation	10 minutes	30 minutes	30 minutes	30 minutes
Parameter measured	LDH release	live- and dead-cell protease activity	dead-cell protease activity	LDH Release
Sensitivity: 96-well/384-well	800 cells/200 cells	several hundred cells or cell equivalents (also in 1536-well format)	several hundred cell equivalents	several hundred cells or cell equivalents
Sample Type	suspension or adherent cells	suspension or adherent cells	suspension or adherent cells	suspension or adherent cells
Detection	fluorometric	fluorometric	fluorometric	colorimetric

directly to plates that already contain test compounds. The duration of exposure to the toxin may vary from less than an hour to several days, depending on specific project goals. Brief periods of exposure may be used to determine if test compounds cause an immediate necrotic insult to cells, whereas exposure for several days is commonly used to determine if test compounds inhibit cell proliferation. Cell viability or cytotoxicity measurements usually are determined at the end of the exposure period. Assays that require only a few minutes to generate a measurable signal (e.g., ATP quantitation or LDH-release assays) provide information representing a snapshot in time and have an advantage over assays that may require several hours of incubation to develop a signal (e.g., MTS or resazurin). In addition to being more convenient, rapid assays reduce the chance of artifacts caused by interaction of the test compound with assay chemistry.



Figure 4.2. Generalized scheme representing an in vitro cytotoxicity assay protocol.

In vitro cultured cells exist as a heterogeneous population. When populations of cells are exposed to test compounds, they do not all respond simultaneously. Cells exposed to toxin may respond over the course of several hours or days, depending on many factors, including the mechanism of cell death, the concentration of the toxin and the duration of exposure. As a result of culture heterogeneity, the data from most plate-based assay formats represent an average of the signal from the population of cells.

D. Determining Dose and Duration of Exposure

Characterizing assay responsiveness for each in vitro model system is important, especially when trying to distinguish between different mechanisms of cell death (Riss and Moravec, 2004). Initial characterization experiments should include a determination of the appropriate assay window using an established positive control.

Figures 4.3 and 4.4 show the results of two experiments to determine the kinetics of cell death caused by different concentrations of tamoxifen in HepG2 cells. The two experiments measured different endpoints: ATP as an indicator of viable cells and caspase activity as a marker for apoptotic cells.

The ATP data in Figure 4.3 indicate that high concentrations of tamoxifen are toxic after a 30-minute exposure. The longer the duration of tamoxifen exposure the lower the IC_{50} value or dose required to “kill” half of the cells, suggesting the occurrence of a cumulative cytotoxic effect. Both the concentration of toxin and the duration of exposure contribute to the cytotoxic effect. To illustrate the importance of taking measurements after an appropriate duration of exposure to test compound, notice that the ATP

assay indicates that 30 μ M tamoxifen is not toxic at short incubation times but is 100% toxic after 24 hours of exposure. Choosing the appropriate incubation period will affect results.

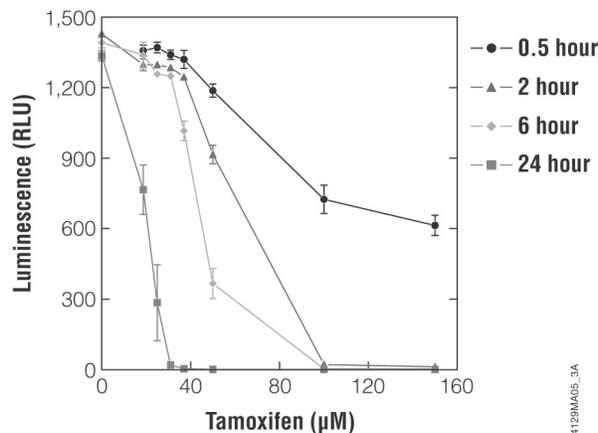


Figure 4.3. Characterization of the toxic effects of tamoxifen on HepG2 cells using the CellTiter-Glo[®] Luminescent Cell Viability Assay to measure ATP as an indication of cell viability.

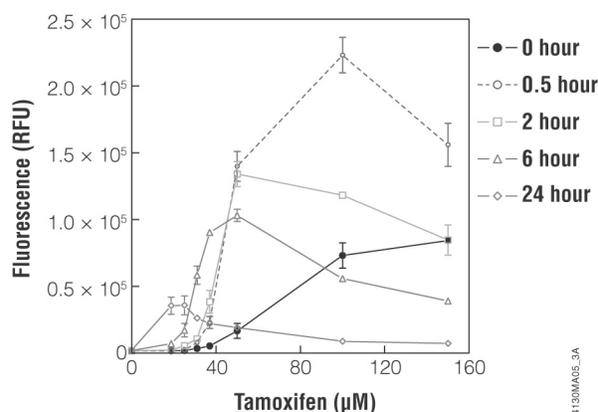


Figure 4.4. Characterization of the effects of tamoxifen on HepG2 cells using the Apo-ONE[®] Homogeneous Caspase-3/7 Assay to measure caspase-3/7 activity as a marker of apoptosis.

The appearance of some apoptosis markers is transient and may only be detectable within a limited window of time. The data from the caspase assay in Figure 4.4 illustrate the transient nature of caspase activity in cells undergoing apoptosis. The total amount of caspase activity measured after a 24-hour exposure to tamoxifen is only a fraction of earlier time points. There is a similar trend of shifting to lower IC_{50} values after increased exposure time. The combined ATP and caspase data may suggest that, at early time points with intermediate concentrations of tamoxifen, the cells are undergoing apoptosis; but after a 24-hour exposure most of the population of cells are in a state of secondary necrosis.

E. Homogeneous Assays for Multiwell Formats and Automated Screening

Promega produces a complete portfolio of homogeneous assays (assays that can be performed in your cell culture plates) that are designed to meet a variety of experimental requirements. The general protocol for these "homogeneous" assays is "add, mix and measure." Some of these homogeneous assay systems require combining components to create the "reagent," and some protocols require incubation or agitation steps, but none require removing buffer or medium from assay wells. The available homogeneous assay systems include assays designed to measure cell viability, cytotoxicity and apoptosis. Promega also offers some non-homogeneous cell viability assays.

F. Additional Factors to Consider When Choosing a Cell Viability Assay

Among the many factors to consider when choosing a cell-based assay, the primary concern for many researchers is the **ease of use**. Homogeneous assays do not require removal of culture medium, cell washes or centrifugation steps. When choosing an assay, the time required for reagent preparation and the total length of time necessary to develop a signal from the assay chemistry should be considered. The stability of the absorbance, fluorescence or luminescence signal is another important factor that provides convenience and flexibility in recording data and minimizes differences when processing large batches of plates.

Another factor to consider when selecting an assay is **sensitivity of detection**. Detection sensitivity will vary with cell type if you choose to measure a metabolic marker, such as ATP level or MTS tetrazolium reduction. The signal-to-background ratios of some assays may be improved by increasing incubation time. The sensitivity not only depends upon the parameter being measured but also on other parameters of the model system such as the plate format and number of cells used per well. Cytotoxicity assays that are designed to detect a change in viability in a population of 10,000 cells may not require the most sensitive assay technology. For example, a tetrazolium assay should easily detect the difference between 10,000 and 8,000 viable cells. On the other hand, assay model systems that use low cell numbers in a high-density multiwell plate format may require maximum sensitivity of detection such as that achieved with the luminescent ATP assay technology.

For researchers using automated screening systems, the **reagent stability** and compatibility with robotic components is often a concern. The assay reagents must be stable at ambient temperature for an adequate period of time to complete dispensing into several plates. In addition, the signal generated by the assay should also be stable for extended periods of time to allow flexibility for recording data. For example, the luminescent signal from the ATP assay has a half-life of about 5 hours, providing adequate

flexibility. With other formats such as the MTS tetrazolium assay or the LDH release assay, the signal can be stabilized by the addition of a detergent-containing stop solution.

In some cases the choice of assay may be dictated by the **availability of instrumentation** to detect absorbance, fluorescence or luminescence. The Promega portfolio of products contains an optional detection format for each of the three major classes of cell-based assays (viability, cytotoxicity or apoptosis). In addition, results from some assays such as the ATP assay can be recorded with more than one type of instrument (luminometer, fluorometer or CCD camera).

Cost is an important consideration for every researcher; however, many factors that influence the **total cost of running an assay** are often overlooked. All of the assays described above are homogeneous and as such are more efficient than multistep assays. For example, even though the reagent cost of an ATP assay may be higher than other assays, the speed (time savings), sensitivity (cell sample savings) and accuracy may outweigh the initial cost. Assays with good detection sensitivity that are easier to scale down to 384- or 1536-well formats may result in savings of cell culture reagents and enable testing of very small quantities of expensive or rare test compounds.

The ability to gather more than one set of data from the same sample (i.e., multiplexing) also may contribute to saving time and effort. **Multiplexing** more than one assay in the same culture well can provide internal controls and eliminate the need to repeat work. For instance, the LDH-release assay is an example of an assay that can be multiplexed. The LDH-release assay offers the opportunity to gather cytotoxicity data from small aliquots of culture supernatant that can be removed to a separate assay plate, thus leaving the original assay plate available for any other assay such as gene reporter analysis, image analysis, etc. Several of our homogeneous apoptosis and viability assays can be multiplexed without transferring media, allowing researchers to assay multiple parameters in the same sample well.

Reproducibility of data is an important consideration when choosing a commercial assay. However, for most cell-based assays, the variation among replicate samples is more likely to be caused by the cells rather than the assay chemistry. Variations during plating of cells can be magnified by using cells lines that tend to form clumps rather than a suspension of individual cells. Extended incubation periods and edge effects in plates may also lead to decreased reproducibility among replicates and less desirable Z'-factor values.

Promega Publications

CN016 Timing Your Apoptosis Assays
(www.promega.com/cnotes/cn016/cn016_18.htm)

II. Cell Viability Assays that Measure ATP

A. CellTiter-Glo® Luminescent Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture. Detection is based on using the luciferase reaction to measure the amount of ATP from viable cells. The amount of ATP in cells correlates with cell viability. Within minutes after a loss of membrane integrity, cells lose the ability to synthesize ATP, and endogenous ATPases destroy any remaining ATP; thus the levels of ATP fall precipitously. The CellTiter-Glo® Reagent does three things upon addition to cells. It lyses cell membranes to release ATP; it inhibits endogenous ATPases, and it provides luciferin, luciferase and other reagents necessary to measure ATP using a bioluminescent reaction.

The unique properties of a proprietary stable luciferase mutant enabled a robust, single-addition reagent. The "glow-type" signal can be recorded with a luminometer, CCD camera or modified fluorometer and generally has a half-life of five hours, providing a consistent signal across large batches of plates. The CellTiter-Glo® Assay is extremely sensitive and can detect as few as 10 cells. The luminescent signal can be detected as soon as 10 minutes after adding reagent, or several hours later, providing flexibility for batch processing of plates.

Materials Required:

- CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570, G7571, G7572, G7573) and protocol #TB288 (www.promega.com/tbs/tb288/tb288.html)
- opaque-walled multiwell plates adequate for cell culture
- multichannel pipette or automated pipetting station
- plate shaker, for mixing multiwell plates
- luminometer (e.g., GloMax™ 96 Microplate Luminometer (Cat.# E6501) or CCD imager capable of reading multiwell plates
- ATP (for use in generating a standard curve)

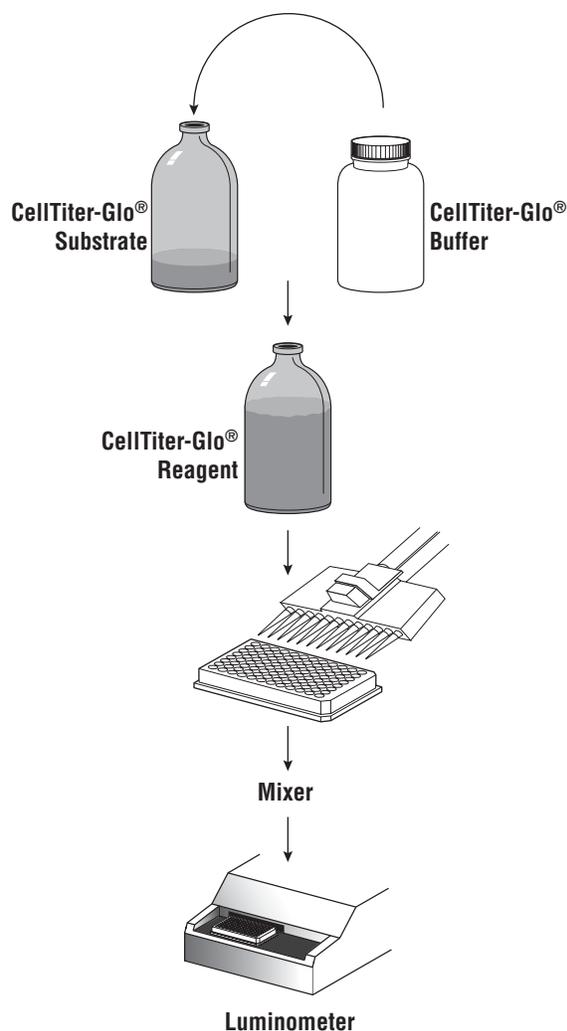


Figure 4.5. Schematic diagram of CellTiter-Glo® Luminescent Cell Viability Assay protocol. For a detailed protocol and considerations for performing this assay, see Technical Bulletin #TB288 (www.promega.com/tbs/tb288/tb288.html).

Additional Considerations for Performing the CellTiter-Glo® Luminescent Cell Viability Assay

Temperature: The intensity and rate of decay of the luminescent signal from the CellTiter-Glo® Assay depends on the rate of the luciferase reaction. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates to a constant temperature before performing the assay. Transferring eukaryotic cells from 37°C to room temperature has little effect on the ATP content (Lundin *et al.* 1986). We have demonstrated that removing cultured cells from a 37°C incubator and allowing them to equilibrate to 22°C for 1–2 hours has little effect on the ATP content.

For batch-mode processing of multiple assay plates, take precautions to ensure complete temperature equilibration. Plates removed from a 37°C incubator and placed in tall stacks at room temperature will require longer equilibration than plates arranged in a single layer. Insufficient

equilibration may result in a temperature gradient effect between the wells in the center and on the edge of the plates. The temperature gradient pattern may depend on the position of the plate in the stack.

Chemicals: Differences in luminescence intensity have been observed using different types of culture media and sera. The presence of phenol red in culture medium should have little impact on luminescence output. Assay of 0.1 μM ATP in RPMI medium without phenol red showed ~5% increase in relative light units (RLU) compared to RPMI containing the standard concentration of phenol red, whereas RPMI medium containing 2X the normal concentration of phenol red showed a ~2% decrease in RLU. Solvents used for the various test compounds may interfere with the luciferase reaction and thus affect the light output from the assay. Interference with the luciferase reaction can be determined by assaying a parallel set of control wells containing medium without cells. Dimethylsulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations up to 2% in the assay and only minimally affects light output.

Plate Recommendations: We recommend using opaque-walled multiwell plates suitable for luminescence measurements. Opaque-walled plates with clear bottoms to allow microscopic visualization of cells also may be used; however, these plates will have diminished signal intensity and greater cross-talk between wells. Opaque white tape can be used to decrease luminescence loss and cross-talk.

Cellular ATP Content: Values reported for the ATP level in cells vary considerably (Lundin *et al.* 1986; Kangas *et al.* 1984; Stanley, 1986; Beckers *et al.* 1986; Andreotti *et al.* 1995). Factors that affect the ATP content of cells may affect the relationship between cell number and luminescence. Anchorage-dependent cells that undergo contact inhibition at high densities may show a change in ATP content per cell at high densities, resulting in a nonlinear relationship between cell number and luminescence. Factors that affect the cytoplasmic volume or physiology of cells also can affect ATP content. For example, depletion of oxygen is one factor known to cause a rapid decrease in ATP (Crouch *et al.* 1993).

Mixing: Optimum assay performance is achieved when the CellTiter-Glo® Reagent is completely mixed with the sample of cultured cells. Suspension cell lines (e.g., Jurkat cells) generally require less mixing to achieve lysis and extraction of ATP than adherent cells (e.g., L929 cells). Several additional parameters related to reagent mixing include: the force of delivery of CellTiter-Glo® Reagent, the sample volume and the dimensions of the well. All of these factors may affect assay performance. The degree of mixing required may be affected by the method used for adding the CellTiter-Glo® Reagent to the assay plates. Automated pipetting devices using a greater or lesser force of fluid delivery may affect the degree of subsequent mixing required. Complete reagent mixing in 96-well plates should be achieved using orbital plate shaking devices, which are built into many luminometers, and shaking for the

recommended 2 minutes. Special electromagnetic shaking devices using a radius smaller than the diameter of the well may be required when using 384-well plates. The depth of the medium and the geometry of the multiwell plates may also affect mixing efficiency.

Additional Resources for CellTiter-Glo® Luminescent Cell Viability Assay

Technical Bulletins and Manuals

- TB288 *CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin*
(www.promega.com/tbs/tb288/tb288.html)
- EP014 *Automated CellTiter-Glo® Luminescent Cell Viability Assay Protocol*
(www.promega.com/tbs/ep014/ep014.html)

Promega Publications

- CN013 Selecting cell-based assays for drug-discovery screening
(www.promega.com/cnotes/cn013/cn013_16.htm)
- CN010 Multiplexing homogeneous cell-based assays
(www.promega.com/cnotes/cn010/cn010_15.htm)
- CN006 Choosing the right cell-based assay for your research
(www.promega.com/cnotes/cn006/cn006_06.htm)
- CN005 CellTiter-Glo® Luminescent Cell Viability Assay for cytotoxicity and cell proliferation studies
(www.promega.com/cnotes/cn005/cn005_15.htm)

Online Tools

Cell Viability Assistant (www.promega.com/techserv/tools/cellviaasst/)

Citations

Nguyen, D.G. *et al.* (2006) "UnPAKing" Human Immunodeficiency Virus (HIV) replication: Using small interfering RNA screening to identify novel cofactors and elucidate the role of Group I PAKs in HIV infection. *J. Virol.* **80**, 130–7.

The CellTiter-Glo® Luminescent Cell Viability Assay was used to assess viability of HeLaCD4βgal or U373-Magi-CCR5E cells transfected with siRNAs that targeted potential proviral host factors for HIV infection.

PubMed Number: 16352537

Boutros, M. *et al.* (2004) Genome-wide RNAi analysis of growth and viability in *Drosophila* cells. *Science* **303**, 832–5. This paper describes use of RNA interference (RNAi) to screen the genome of *Drosophila melanogaster* for genes affecting cell growth and viability. The CellTiter-Glo® Luminescent Cell Viability Assay and a Molecular Dynamics Analyst HT were used. The authors report

finding 438 target genes that affected cell growth or viability.

PubMed Number: 14764878

B. BacTiter-Glo™ Microbial Cell Viability Assay

The BacTiter-Glo™ Microbial Cell Viability Assay is based on the same assay principles and chemistries as the CellTiter-Glo® Assay. However, the buffer supports bacterial cell lysis of Gram+ and Gram- bacteria and yeast. Figure 4.6 provides a basic outline of the BacTiter-Glo™ Assay procedure. The formulation of the reagent supports bacterial cell lysis and generation of a luminescent signal in an “add, mix and measure” format. This assay can measure ATP from as few as ten bacterial cells from some species and is a powerful tool for determining growth curves of slow-growing microorganisms (Figure 4.7), screening for antimicrobial compounds (Figure 4.8) and evaluating antimicrobial compounds (Figure 4.9).

Materials Required:

- BacTiter-Glo™ Luminescent Cell Viability Assay (Cat.# G8230, G8231, G8232, G8233) and protocol #TB337 (www.promega.com/tbs/tb337/tb337.html)
- opaque-walled multiwell plates
- multichannel pipette or automated pipetting station
- plate shaker, for mixing multiwell plates
- luminometer (e.g., GloMax™ 96 Microplate Luminometer (Cat.# E6501) or CCD imager capable of reading multiwell plates
- ATP (for generating a standard curve; Cat.# P1132)

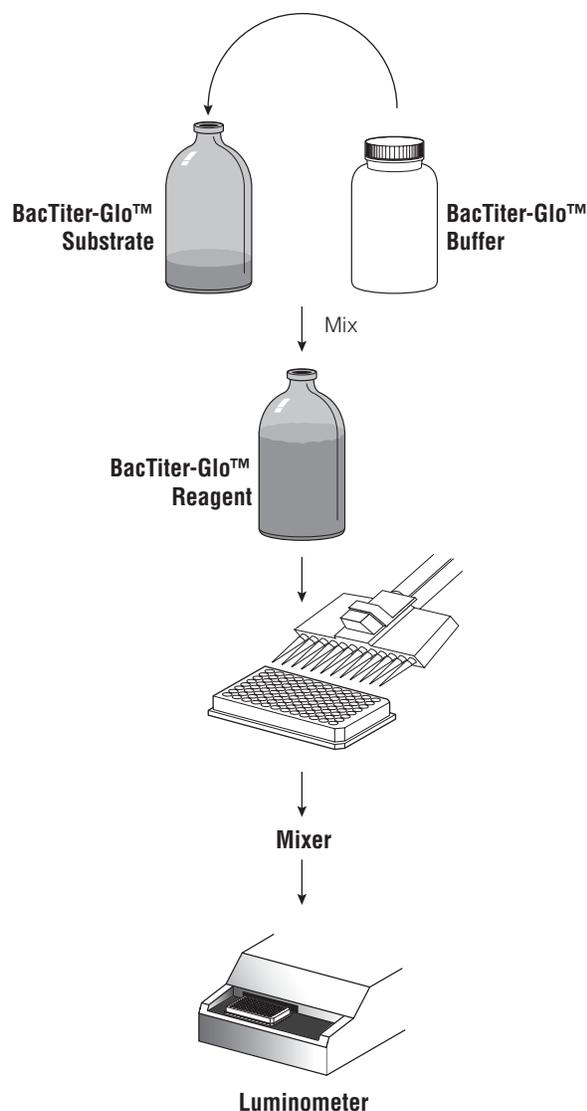


Figure 4.6. Schematic diagram of BacTiter-Glo™ Assay protocol. For a detailed protocol and considerations for performing this assay, see Technical Bulletin #TB337 (www.promega.com/tbs/tb337/tb337.html). The assay is suitable for single-tube or multiwell-plate formats.

Additional Considerations for Performing the BacTiter-Glo™ Microbial Cell Viability Assay

Temperature: The intensity and rate of decay of the luminescent signal from the BacTiter-Glo™ Assay depend on the rate of the luciferase reaction. Environmental factors that affect the rate of the luciferase reaction will result in a change in the intensity of light output and the stability of the luminescent signal. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates to room temperature before performing the assay. Insufficient equilibration may create a temperature gradient effect between the wells in the center and on the edge of the plates.

Microbial Growth Medium: Growth medium is another factor that can contribute to the background luminescence and affect the luciferase reaction in terms of signal level and signal stability. We have used MH II Broth (cation-adjusted Mueller Hinton Broth; Becton, Dickinson and Company Cat.# 297963) for all our experiments unless otherwise mentioned. It supports growth for most commonly encountered aerobic and facultative anaerobic bacteria and is selected for use in food testing and antimicrobial susceptibility testing by Food and Drug Administration and National Committee for Clinical Laboratory Standards (NCCLS) (Association of Official Analytical Chemists, 1995; NCCLS, 2000). MH II Broth has low luminescence background and good batch-to-batch reproducibility.

Chemicals: The chemical environment of the luciferase reaction will affect the enzymatic rate and thus luminescence intensity. Solvents used for the various compounds tested for their antimicrobial activities may interfere with the luciferase reaction and thus the light output from the assay. Interference with the luciferase reaction can be detected by assaying a parallel set of control wells containing medium without compound. Dimethylsulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations up to 2% in the assay and has less than 5% loss of light output.

Plate and Tube Recommendations: The BacTiter-Glo™ Assay is suitable for multiwell-plate or single-tube formats. We recommend standard opaque-walled multiwell plates suitable for luminescence measurements. Opaque-walled plates with clear bottoms to allow microscopic visualization of cells also may be used; however, these plates will have diminished signal intensity and greater cross-talk between wells. Opaque white tape can be used to reduce luminescence loss and cross-talk. For single-tube assays, the standard tube accompanying the luminometer should be suitable.

Cellular ATP Content: Different bacteria have different amounts of ATP per cell, and values reported for the ATP level in cells vary considerably (Stanley, 1986; Hattori *et al.* 2003). Factors that affect the ATP content of cells such as growth phase, medium, and presence of metabolic inhibitors, may affect the relationship between cell number and luminescence (Stanley, 1986).

Mixing: Optimum assay performance is achieved when the BacTiter-Glo™ Reagent is completely mixed with the sample of cultured cells. For all of the bacteria we tested, maximum luminescent signals were observed after efficiently mixing and incubating for 1–5 minutes. However, complete extraction of ATP from certain bacteria, yeast or fungi may take longer. Automated pipetting devices using a greater or lesser force of fluid delivery may affect the degree of subsequent mixing required. Ensure complete reagent mixing in 96-well plates by using orbital plate shaking devices built into many luminometers. We

recommend considering these factors when performing the assay and determining whether a mixing step and/or longer incubation is necessary.

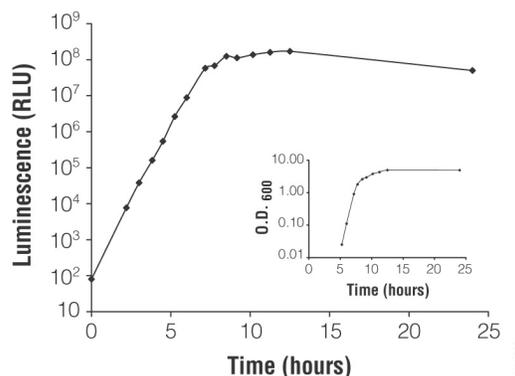


Figure 4.7. Evaluating bacterial growth using the BacTiter-Glo™ Assay. *E. coli* ATCC 25922 strain was grown in Mueller Hinton II (MH II) broth (B.D. Cat.# 297963) at 37°C overnight. The overnight culture was diluted 1:10⁶ in 50ml of fresh MH II broth and incubated at 37°C with shaking at 250rpm. Samples were taken at various time points, and the BacTiter-Glo™ Assay was performed according to the protocol described in Technical Bulletin #TB337. Luminescence was recorded on a GloMax™ 96 Microplate Luminometer (Cat.# E6501). Optical density was measured at 600nm (O.D.₆₀₀) using a Beckman DU650 spectrophotometer. Diluted samples were used when readings of relative light units (RLU) and O.D. exceeded 10⁸ and 1, respectively.

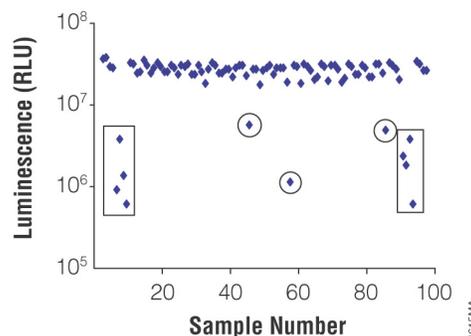


Figure 4.8. Screening for antimicrobial compounds using the BacTiter-Glo™ Assay. *S. aureus* ATCC 25923 strain was grown in Mueller Hinton II (MH II) Broth (BD Cat.# 297963) at 37°C overnight. The overnight culture was diluted 100-fold in fresh MH II Broth and used as inoculum for the antimicrobial screen. Working stocks (50X) of LOPAC compounds and standard antibiotics were prepared in DMSO. Each well of the 96-well plate contained 245µl of the inoculum and 5µl of the 50X working stock. The multiwell plate was incubated at 37°C for 5 hours. One hundred microliters of the culture was taken from each well, and the BacTiter-Glo™ Assay was performed according to the protocol described in Technical Bulletin #TB337. Luminescence was measured using a GloMax™ 96 Microplate Luminometer (Cat.# E6501). The samples and concentrations are: wells 1–4 and 93–96, negative control of 2% DMSO; wells 5–8 and 89–92, positive controls of 32µg/ml standard antibiotics tetracycline, ampicillin, gentamicin, chloramphenicol, oxacillin, kanamycin, piperacillin and erythromycin; wells 9–88, LOPAC compounds at 10µM.

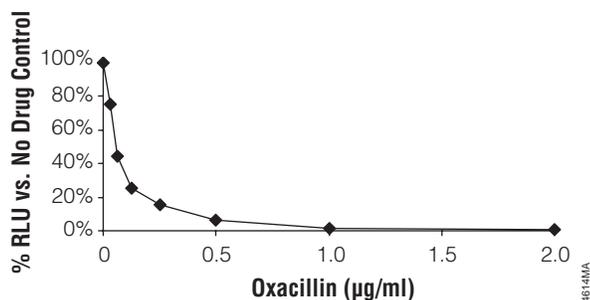


Figure 4.9. Evaluating antimicrobial compounds using the BacTiter-Glo™ Assay. *S. aureus* ATCC 25923 strain and oxacillin were prepared as described in Figure 4.8 and incubated at 37°C; the assay was performed after 19 hours of incubation as recommended for MIC determination by NCCLS. The percentage of relative light units (RLU) compared to the no-oxacillin control is shown. Luminescence was recorded on a GloMax™ 96 Microplate Luminometer (Cat.# E6501).

Additional Resources for BacTiter-Glo™ Microbial Cell Viability Assay

Technical Bulletins and Manuals

TB337 *BacTiter-Glo™ Microbial Cell Viability Assay Technical Bulletin*
(www.promega.com/tbs/tb337/tb337.html)

Promega Publications

CN010 Determining microbial viability using a homogeneous luminescent assay
(www.promega.com/cnotes/cn010/cn010_02.htm)

PN088 Quantitate microbial cells using a rapid and sensitive ATP-based luminescent assay
(www.promega.com/pnotes/88/12162_02/12162_02.html)

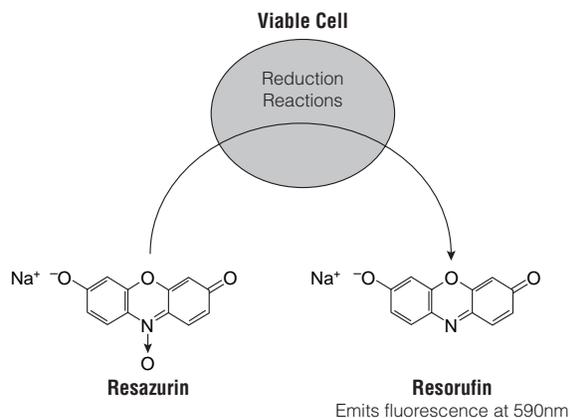


Figure 4.10. Conversion of resazurin to resorufin by viable cells results in a fluorescent product. The fluorescence produced is proportional to the number of viable cells.

The CellTiter-Blue® Assay is a simple and inexpensive procedure that is amenable to multiplexing applications with other assays to collect a variety of data (Figures 4.11 and 4.12). The incubation period is flexible, and the data can be collected using either fluorescence or absorbance, though fluorescence is preferred because of superior sensitivity. The assay provides good Z'-factor values in high-throughput screening situations and is amenable to automation.

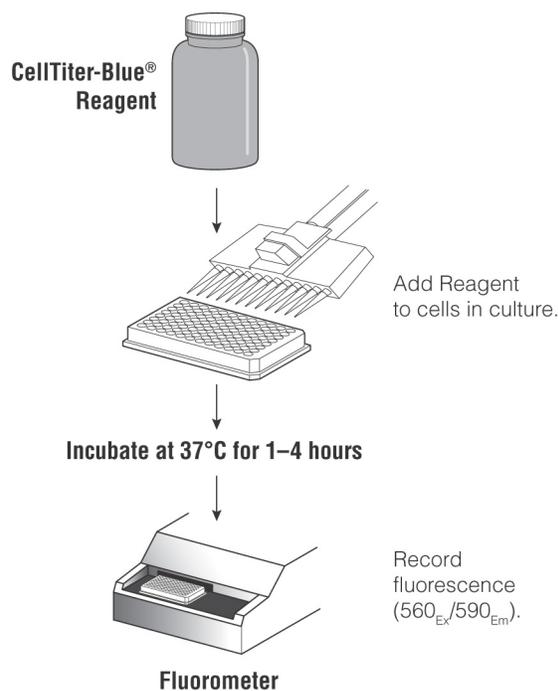


Figure 4.11. Schematic outlining the CellTiter-Blue® Assay protocol. Multiwell plates that are compatible with fluorescent plate readers are prepared with cells and compounds to be tested. CellTiter-Blue® Reagent is added to each well and incubated at 37°C to allow cells to convert resazurin to resorufin. The fluorescent signal is read using a fluorescence plate reader.

III. Cell Viability Assays that Measure Metabolic Capacity

A. CellTiter-Blue® Cell Viability Assay (resazurin)

The CellTiter-Blue® Cell Viability Assay uses an optimized reagent containing resazurin. The homogeneous procedure involves adding the reagent directly to cells in culture at a recommended ratio of 20µl of reagent to 100µl of culture medium. The assay plates are incubated at 37°C for 1–4 hours to allow viable cells to convert resazurin to the fluorescent resorufin product. The conversion of resazurin to fluorescent resorufin is proportional to the number of metabolically active, viable cells present in a population (Figure 4.10). The signal is recorded using a standard multiwell fluorometer. Because different cell types have different abilities to reduce resazurin, optimizing the length of incubation with the CellTiter-Blue® Reagent can improve assay sensitivity for a given model system. The detection sensitivity is intermediate between the ATP assay and the MTS reduction assay.

Materials Required:

- CellTiter-Blue® Cell Viability Assay (Cat.# G8080, G8081, G8082) and protocol #TB317 (www.promega.com/tbs/tb317/tb317.html)
- multichannel pipettor
- fluorescence reader with excitation 530–570nm and emission 580–620nm filter pair
- absorbance reader with 570nm and 600nm filters (optional)
- 96-well plates compatible with a fluorescence plate reader

General Considerations for the CellTiter-Blue® Cell Viability Assay

Incubation Time: The ability of different cell types to reduce resazurin to resorufin varies depending on the metabolic capacity of the cell line and the length of incubation with the CellTiter-Blue® Reagent. For most applications a 1- to 4-hour incubation is adequate. For optimizing screening assays, the number of cells/well and the length of the incubation period should be empirically determined. A more detailed discussion of incubation time is available in Technical Bulletin #TB317

(www.promega.com/tbs/tb317/tb317.html).

Volume of Reagent Used: The recommended volume of CellTiter-Blue® Reagent is 20µl of reagent to each 100µl of medium in a 96-well format or 5µl of reagent to each 25µl of culture medium in a 384-well format. This ratio may be adjusted for optimal performance, depending on the cell type, incubation time and linear range desired.

Site of Resazurin Reduction: Resazurin is reduced to resorufin inside living cells (O'Brien *et al.* 2000). Resazurin can penetrate cells, where it becomes reduced to the fluorescent product, resorufin, probably as the result of the action of several different redox enzymes. The fluorescent resorufin dye can diffuse from cells and back into the surrounding medium. Culture medium harvested from rapidly growing cells does not reduce resazurin (O'Brien *et al.* 2000). An analysis of the ability of various hepatic subcellular fractions suggests that resazurin can be reduced by mitochondrial, cytosolic and microsomal enzymes (Gonzalez and Tarloff, 2001).

Optical Properties of Resazurin and Resorufin: Both the light absorbance and fluorescence properties of the CellTiter-Blue® Reagent are changed by cellular reduction of resazurin to resorufin; thus either absorbance or fluorescence measurements can be used to monitor results. We recommend measuring fluorescence because it is more sensitive than absorbance and requires fewer calculations to account for the overlapping absorbance spectra of resazurin and resorufin. More details about making fluorescence and absorbance measurements are provided in Technical Bulletin #TB317 (www.promega.com/tbs/tb317/tb317.html).

Background Fluorescence and Light Sensitivity of

Resazurin: The resazurin dye (blue) in the CellTiter-Blue® Reagent and the resorufin product produced in the assay

(pink) are light-sensitive. Prolonged exposure of the CellTiter-Blue® Reagent to light will result in increased background fluorescence and decreased sensitivity. Background fluorescence can be corrected by including control wells on each plate to measure the fluorescence from serum-supplemented culture medium in the absence of cells. There may be an increase in background fluorescence in wells without cells after several hours of incubation.

Multiplexing with Other Assays: Because CellTiter-Blue® Reagent is relatively non-destructive to cells during short-term exposure, it is possible to use the same culture wells to do more than one type of assay. An example showing the measurement of caspase activity using the Apo-ONE® Homogeneous Caspase-3/7 Assay (Cat.# G7792) is shown in Figure 4.12. A protocol for multiplexing the CellTiter-Blue® Assay and the Apo-ONE® Caspase-3/7 Assay is provided in chapter 3 (www.promega.com/paguide/chap3.htm) of this *Protocols and Applications Guide*.

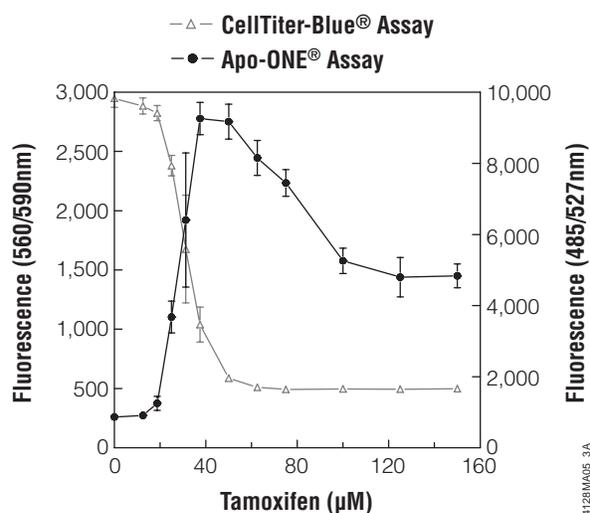


Figure 4.12. Multiplexing the CellTiter-Blue® Assay with the Apo-ONE® Homogeneous Caspase-3/7 Assay. HepG2 cells (10,000cells/100µl cultured overnight) were treated with various concentrations of tamoxifen for 5 hours. Viability was determined by adding CellTiter-Blue® Reagent (20µl/well) to each well after 3.5 hours of drug treatment and incubating for 1 hour before recording fluorescence (560_{Ex}/590_{Em}). Caspase activity was then determined by adding 120µl/well of Apo-ONE® Homogeneous Caspase-3/7 Reagent and incubating for 0.5 hour before recording fluorescence (485_{Ex}/527_{Em}).

Stopping the Reaction: The fluorescence generated in the CellTiter-Blue® Assay can be stopped and stabilized by adding SDS. We recommend adding 50µl of 3% SDS per 100µl of original culture volume. The plate can then be stored at ambient temperature for up to 24 hours before recording data, provided that the contents are protected from light and covered to prevent evaporation.

Additional Resources for the CellTiter-Blue® Cell Viability Assay

Technical Bulletins and Manuals

- TB317 *CellTiter-Blue® Cell Viability Assay Technical Bulletin*
(www.promega.com/tbs/tb317/tb317.html)
- EP015 *Automated CellTiter-Blue® Cell Viability Assay Protocol*
(www.promega.com/tbs/ep015/ep015.html)

Promega Publications

- CN013 Selecting cell-based assays for drug discovery screening
(www.promega.com/cnotes/cn013/cn013_16.htm)
- CN010 Multiplexing homogeneous cell-based assays
(www.promega.com/cnotes/cn010/cn010_15.htm)
- CN006 Choosing the right cell-based assay for your research
(www.promega.com/cnotes/cn006/cn006_06.htm)
- PN083 Introducing the CellTiter-Blue® Cell Viability Assay
(www.promega.com/pnotes/83/10492_10/10492_10.html)

Online Tools

Cell Viability Assistant (www.promega.com/techserv/tools/cellviasst/)

Citations

Bruno, I.G., Jin, W. and Cote, C.J. (2004) Correction of aberrant FGFR1 alternative RNA splicing through targeting of intronic regulatory elements. *Hum. Mol. Genet.* **13**, 2409–20.

Human U251 glioblastoma cell lines treated with antisense morpholino oligonucleotides were assessed for viability and apoptosis by multiplexing the CellTiter-Blue® Cell Viability Assay and Apo-ONE® Homogeneous Caspase-3/7 Assay on single-cell cultures.

PubMed Number: 15333583

B. Tetrazolium-Based Assays

Metabolism in viable cells produces "reducing equivalents" such as NADH or NADPH. These reducing compounds pass their electrons to an intermediate electron transfer reagent that can reduce the tetrazolium product, MTS, into an aqueous, soluble formazan product. At death, cells rapidly lose the ability to reduce tetrazolium products. The production of the colored formazan product, therefore, is proportional to the number of viable cells in culture.

The CellTiter 96® AQueous products are MTS assays for determining the number of viable cells in culture. The MTS tetrazolium is similar to the widely used MTT tetrazolium,

with the advantage that the formazan product of MTS reduction is soluble in cell culture medium and does not require use of a Solubilization Solution.

The CellTiter 96® AQueous One Solution Cell Proliferation Assay is an MTS-based assay that involves adding a single reagent directly to the assay wells at a recommended ratio of 20µl reagent to 100µl of culture medium. Cells are incubated 1–4 hours at 37°C and then absorbance is measured at 490nm. This assay chemistry has been widely accepted and is cited in hundreds of published articles.

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation assay is also an MTS-based assay. The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Reagent is prepared by combining two solutions, MTS and an electron coupling reagent, phenazine methosulfate (PMS). The reagent is then added to cells. During the assay, MTS is converted to a soluble formazan product. Samples are read after a 1- to 4-hour incubation at 490nm.

CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)

Materials Required:

- CellTiter 96® AQueous One Solution Cell Proliferation Assay (Cat.# G3582, G3580, G3581) and protocol #TB245 (www.promega.com/tbs/tb245/tb245.html)
- 96-well plates suitable for tissue culture
- repeating, digital or multichannel pipettors
- 96-well spectrophotometer

General Protocol

1. Thaw the CellTiter 96® AQueous One Solution Reagent. It should take approximately 90 minutes at room temperature on the bench top, or 10 minutes in a water bath at 37°C, to completely thaw the 20ml size.
2. Pipet 20µl of CellTiter 96® AQueous One Solution Reagent into each well of the 96-well assay plate containing the samples in 100µl of culture medium.
3. Incubate the plate for 1–4 hours at 37°C in a humidified, 5% CO₂ atmosphere.

Note: To measure the amount of soluble formazan produced by cellular reduction of the MTS, proceed immediately to Step 4. Alternatively, to measure the absorbance later, add 25µl of 10% SDS to each well to stop the reaction. Store SDS-treated plates protected from light in a humidified chamber at room temperature for up to 18 hours. Proceed to Step 4.

4. Record the absorbance at 490nm using a 96-well spectrophotometer.

Additional Resources for the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay

Technical Bulletins and Manuals

TB245 *CellTiter 96[®] AQueous One Solution Cell Proliferation Assay Technical Bulletin* (www.promega.com/tbs/tb245/tb245.html)

Promega Publications

CN013 Selecting cell-based assays for drug discovery screening (www.promega.com/cnotes/cn013/cn013_16.htm)

CN006 Choosing the right cell-based assay for your research (www.promega.com/cnotes/cn006/cn006_06.htm)

eNotes Compatibility of the CellTiter 96[®] AQueous One Solution Assay with the Luciferase Assay System (www.promega.com/enotes/applications/ap0008_tabs.htm)

Online Tools

Cell Viability Assistant (www.promega.com/techserv/tools/cellviaasst/)

Citations

Gauduchon, J. *et al.* (2005) 4-Hydroxytamoxifen inhibits proliferation of multiple myeloma cells in vitro through down-regulation of c-Myc, up-regulation of p27Kip1, and modulation of Bcl-2 family members. *Clin. Cancer Res.* **11**, 2345–54.

The CellTiter 96[®] AQueous One Solution Cell Proliferation Assay was used to evaluate cell viability of six different multiple myeloma cell lines.

PubMed Number: 15788686

Berglund, P. *et al.* (2005) Cyclin E overexpression obstructs infiltrative behavior in breast cancer: A novel role reflected in the growth pattern of medullary breast cancers. *Cancer Res.* **65**, 9727–34.

Attachment assays were performed with MDA-MB-468 cell lines stably transfected with a cyclin-E GFP fusion construct. Cells were allowed to adhere to 96-well plates, washed then incubated with the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay.

PubMed Number: 16266993

CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay

Materials Required:

- CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay (Cat.# G5440) and protocol #TB169 (www.promega.com/tbs/tb169/tb169.html)
- 96-well plate
- 37°C incubator
- 10% SDS

General Protocol for One 96-Well Plate Containing Cells Cultured in 100µl Volume

1. Thaw the MTS Solution and the PMS Solution.
2. Remove 2.0ml of the MTS Solution using aseptic technique and transfer to a test tube.
3. Add 100µl of PMS Solution to the 2.0ml of MTS Solution immediately before use.
4. Gently swirl the tube to completely mix the combined MTS/PMS solution.
5. Pipet 20µl of the combined MTS/PMS solution into each well of the 96-well assay plate.
6. Incubate the plate for 1–4 hours at 37°C in a humidified, 5% CO₂ chamber.
7. Record the absorbance at 490nm using a plate reader.

Additional Resources for the CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay

Technical Bulletins and Manuals

TB169 *CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay Technical Bulletin* (www.promega.com/tbs/tb169/tb169.html)

Promega Publications

PN044 CellTiter 96[®] and CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assays (www.promega.com/pnotes/44/44p46/44p46.html)

PN081 Technically speaking: Cell viability assays (www.promega.com/pnotes/81/9939_32/9939_32.html)

Online Tools

Cell Viability Assistant (www.promega.com/techserv/tools/cellviaasst/)

Citations

Zhang, L. *et al.* (2004) A transforming growth factor beta-induced Smad3/Smad4 complex directly activates protein kinase A. *Mol. Cell. Biol.* **24**, 2169–80.

The cell proliferation of fetal mink lung cells was measured using the CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay.

PubMed Number: 14966294

Tamasloukht, M. *et al.* (2003) Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. *Plant Physiol.* **131**, 1468–78.

The CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay was used to measure metabolic activity of germinating fungal spores.

PubMed Number: 12644696

CellTiter 96® Non-Radioactive Cell Proliferation Assay

The CellTiter 96® Non-Radioactive Cell Proliferation Assay (Cat.# G4000, G4100) is a colorimetric assay system that measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by viable cells. After incubation of the cells with the Dye Solution for approximately 1–4 hours, a Solubilization Solution is added to lyse the cells and solubilize the colored product. These samples can be read using an absorbance plate reader at a wavelength of 570nm. The amount of color produced is directly proportional to the number of viable cells.

**Additional Resources for the CellTiter 96®
Non-Radioactive Cell Proliferation Assay**
Technical Bulletins and Manuals

TB112 *CellTiter 96® Non-Radioactive Cell Proliferation Assay*
(www.promega.com/tbs/tb112/tb112.html)

Promega Publications

PN044 *CellTiter 96® and CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assays*
(www.promega.com/pnotes/44/44p46/44p46.html)

PN081 *Technically speaking: Cell viability assays*
(www.promega.com/pnotes/81/9939_32/9939_32.html)

Online Tools

Cell Viability Assistant (www.promega.com/techserv/tools/cellviasast)

Other Cell Viability Assays

The MultiTox-Fluor Multiplex Cytotoxicity Assay (Cat.# G9200, G9201, G9202) is a single-reagent-addition fluorescent assay that simultaneously measures the relative number of live and dead cells in cell populations. The MultiTox-Fluor Multiplex Cytotoxicity Assay gives ratiometric, inversely correlated measures of cell viability and cytotoxicity. The ratio of viable cells to dead cells is independent of cell number and therefore can be used to normalize data. Having complementary cell viability and cytotoxicity measures reduces errors associated with pipetting and cell clumping. Assays are often subject to chemical interference by test compounds, media components and can give false-positive or false-negative results. Independent cell viability and cytotoxicity assay chemistries serve as internal controls and allow identification of errors resulting from chemical interference from test compounds or media components. More information about the MultiTox-Fluor Assay can be found in Section IV "Cytotoxicity Assays" of this chapter.

IV. Cytotoxicity Assays**A. Determining the Number of Live and Dead Cells in a Cell Population: MultiTox-Fluor Multiplex Cytotoxicity Assay**

Cell-based assays are important tools for contemporary biology and drug discovery because of their predictive potential for in vivo applications. However, the same

cellular complexity that allows the study of regulatory elements, signaling cascades or test compound bio-kinetic profiles also can complicate data interpretation by inherent biological variation. Therefore, researchers often need to normalize assay responses to cell viability after experimental manipulation.

Although assays for determining cell viability and cytotoxicity that are based on ATP, reduction potential and LDH release are useful and cost-effective methods, they have limits in the types of multiplexed assays that can be performed along with them. The MultiTox-Fluor Multiplex Cytotoxicity Assay (Cat.# G9200, G9201, G9202) is a homogeneous, single-reagent-addition format (Figure 4.13) that allows the measurement of the relative number of live and dead cells in a cell population. This assay gives ratiometric, inversely proportional values of viability and cytotoxicity (Figure 4.15) that are useful for normalizing data to cell number. Also, this reagent is compatible with additional fluorescent and luminescent chemistries.

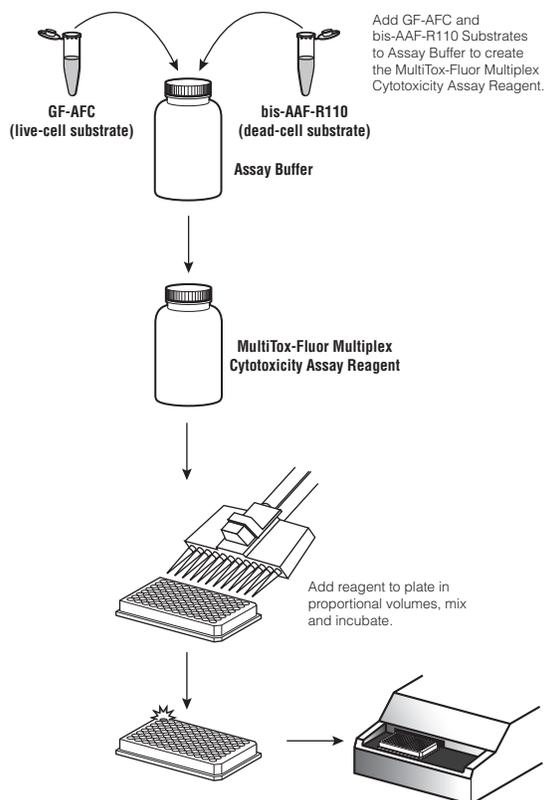


Figure 4.13. Schematic diagram of the MultiTox-Fluor Multiplex Cytotoxicity Assay. The assay uses a homogeneous, single-reagent-addition format to determine live- and dead-cell numbers in a cell population.

The MultiTox-Fluor Multiplex Cytotoxicity Assay simultaneously measures two protease activities; one is a marker of cell viability, and the other is a marker of cytotoxicity. The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant peptide substrate (glycyl-phenylalanyl-amino-fluorocoumarin; GF-AFC). The

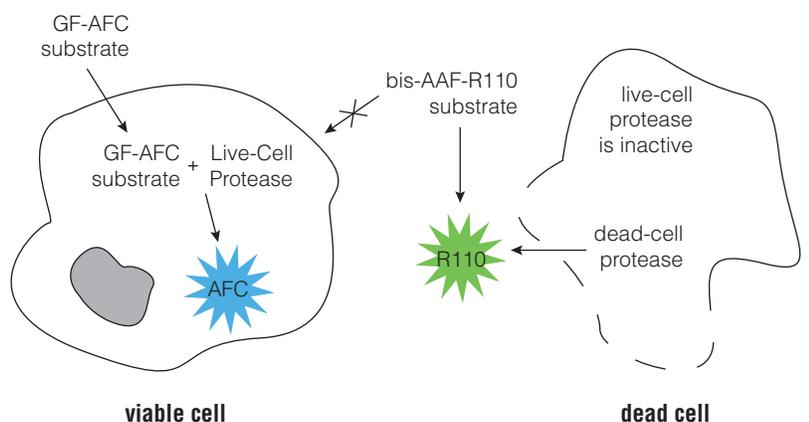


Figure 4.14. Biology of the MultiTox-Fluor Multiplex Cytotoxicity Assay. The GF-AFC Substrate can enter live cells where it is cleaved by the live-cell protease to release AFC. The bis-AAF-R110 Substrate cannot enter live cells, but instead can be cleaved by the dead-cell protease activity to release R110.

substrate enters intact cells where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells (Figure 4.14). This live-cell protease becomes inactive upon loss of membrane integrity and leakage into the surrounding culture medium. A second, fluorogenic, cell-impermeant peptide substrate (bis-alanyl-alanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity (Figure 4.14). Because bis-AAF-R110 is not cell-permeant, essentially no signal from this substrate is generated by intact, viable cells. The live- and dead-cell proteases produce different products, AFC and R110, which have different excitation and emission spectra, allowing them to be detected simultaneously.

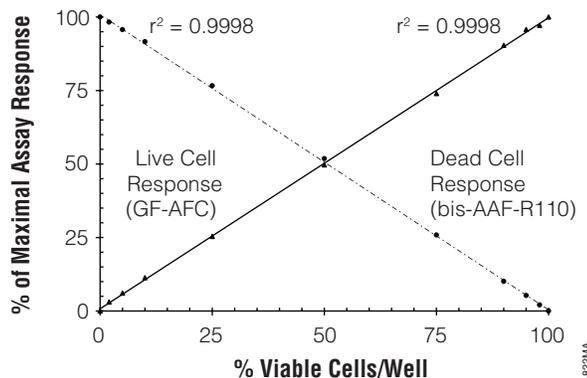


Figure 4.15. Viability and cytotoxicity measurements are inversely correlated and ratiometric. When viability is high, the live-cell signal is highest, and the dead-cell signal is lowest. When viability is low, the live-cell signal is lowest, and the dead-cell signal is highest.

MultiTox-Fluor Multiplex Cytotoxicity Assay Materials Required:

- MultiTox-Fluor Multiplex Cytotoxicity Assay (Cat.# G9200, G9201, G9202) and protocol #TB348 (www.promege.com/tbs/tb348/tb348.html)

- 96- or 384-well opaque-walled tissue culture plates compatible with fluorometer (clear or solid bottom)
- multichannel pipettor
- reagent reservoirs
- fluorescence plate reader with filter sets: 400nm_{Ex}/505nm_{Em} and 485nm_{Ex}/520nm_{Em}
- orbital plate shaker
- positive control cytotoxic reagent or lytic detergent

Example Cytotoxicity Assay Protocol

- If you have not performed this assay on your cell line previously, we recommend determining assay sensitivity using your cells. Protocols to determine assay sensitivity are available in the *MultiTox-Fluor Multiplex Cytotoxicity Assay Technical Bulletin #TB348* (www.promege.com/tbs/tb348/tb348.html).
- Set up 96-well or 384-well assay plates containing cells in culture medium at the desired density.
- Add test compounds and vehicle controls to appropriate wells so that the final volume is 100µl in each well (25µl for 384-well plates).
- Culture cells for the desired test exposure period.
- Add MultiTox-Fluor Multiplex Cytotoxicity Assay Reagent in an equal volume to all wells, mix briefly on an orbital shaker, then incubate for 30 minutes at 37°C.
- Measure the resulting fluorescence: live cells, 400nm_{Ex}/505nm_{Em}, and dead cells, 485nm_{Ex}/520nm_{Em}.

General Considerations for the MultiTox-Fluor Multiplex Cytotoxicity Assay

Background Fluorescence and Inherent Serum Activity: Tissue culture medium that is supplemented with animal serum may contain detectable levels of the protease marker used for dead-cell measurement. The quantity of this protease activity may vary among different lots of serum.

To correct for variability, background fluorescence should be determined using samples containing medium plus serum without cells.

Temperature: The generation of fluorescent product is proportional to the protease activity of the markers associated with cell viability and cytotoxicity. The activity of these proteases is influenced by temperature. For best results, we recommend incubating at a constant controlled temperature to ensure uniformity across the plate.

Assay Controls: In addition to a no-cell control to establish background fluorescence, we recommend including an untreated cells (maximum viability) and positive (maximum cytotoxicity) control in the experimental design. The maximum viability control is established by the addition of vehicle only (used to deliver the test compound to test wells). In most cases, this consists of a buffer system or medium and the equivalent amount of solvent added with the test reagent. The maximum cytotoxicity control can be determined using a compound that causes cytotoxicity or a lytic reagent added to compromise viability (non-ionic or Zwitterionic detergents).

Cytotoxicity Marker Half-Life: The activity of the protease marker released from dead cells has a half-life estimated to be greater than 10 hours. In situations where cytotoxicity occurs very rapidly (necrosis) and the incubation time is greater than 24 hours, the degree of cytotoxicity may be underestimated. The addition of a lytic detergent may be useful to determine the total cytotoxicity marker activity remaining (from remaining live cells) in these extended incubations.

Light Sensitivity: The MultiTox-Fluor Multiplex Cytotoxicity Assay uses two fluorogenic peptide substrates. Although the substrates demonstrate good general photostability, the liberated fluors (after contact with protease) can degrade with prolonged exposure to ambient light sources. We recommend shielding the plates from ambient light at all times.

Cell Culture Medium: The GF-AFC and bis-AAF-R110 Substrates are introduced into the test well using an optimized buffer system that mitigates differences in pH from treatment. In addition, the buffer system supports protease activity in a host of different culture media with varying osmolarity. With the exception of media formulations with either very high serum content or phenol red indicator, no substantial performance differences will be observed among media.

Additional Resources for the MultiTox-Fluor Multiplex Cytotoxicity Assay

Technical Bulletins and Manuals

- TB348 *MultiTox-Fluor Multiplex Cytotoxicity Assay Technical Bulletin*
(www.promega.com/tbs/tb348/tb348.html)

Promega Publications

- CN016 Multiplexed viability, cytotoxicity and apoptosis assays for cell-based screening
(www.promega.com/cnotes/cn016/cn016_12.htm)
- CN015 MultiTox-Fluor Multiplex Cytotoxicity Assay technology
(www.promega.com/cnotes/cn015/cn015_11.htm)

Online Tools

Cell Viability Assistant (www.promega.com/techserv/tools/cellviaasst)

B. Measuring the Relative Number of Dead Cells in a Population: CytoTox-Fluor™ Cytotoxicity Assay

The CytoTox-Fluor™ Cytotoxicity Assay is a single-reagent-addition, homogeneous fluorescent assay that measures the relative number of dead cells in cell populations (Figure 4.16). The CytoTox-Fluor™ Assay measures a distinct protease activity associated with cytotoxicity. The assay uses a fluorogenic peptide substrate (bis-alanyl-alanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) to measure "dead-cell protease" activity, which has been released from cells that have lost membrane integrity. The bis-AAF-R110 Substrate cannot cross the intact membrane of live cells and therefore gives no signal from live cells.

The CytoTox-Fluor™ Assay is designed to accommodate downstream multiplexing with most Promega luminescent assays or spectrally distinct fluorescent assay methods, such as assays measuring caspase activation, reporter expression or orthogonal measures of viability (Figure 4.17).

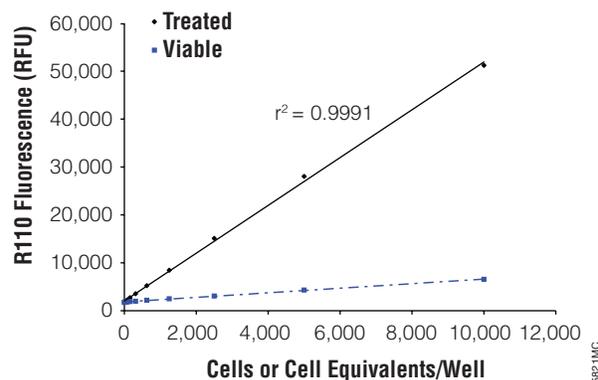


Figure 4.16. The CytoTox-Fluor™ Cytotoxicity Assay signals derived from viable cells (untreated) or lysed cells (treated) are proportional to cell number.

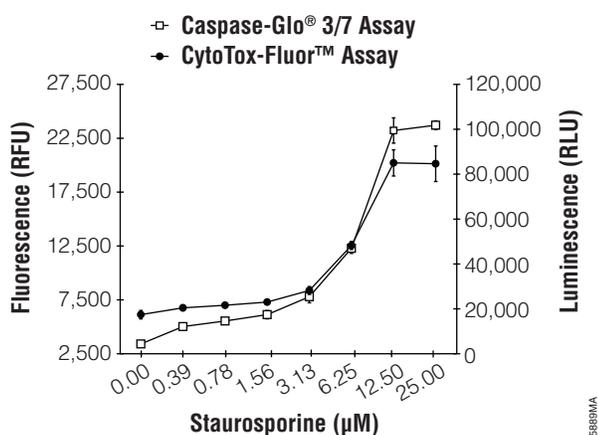


Figure 4.17. CytoTox-Fluor™ Assay multiplexed with Caspase-Glo® 3/7 Assay. The CytoTox-Fluor™ Assay Reagent is added to wells and cytotoxicity measured after incubation for 30 minutes at 37°C. Caspase-Glo® 3/7 Reagent is added and luminescence measured after a 30-minute incubation.

CytoTox-Fluor™ Cytotoxicity Assay

Materials Required:

- CytoTox-Fluor™ Cytotoxicity Assay (Cat.# G9206, G9207, G9208) and protocol #TB350 (www.promega.com/tbs/tb350/tb350.html)
- 96-, 384- or 1536-well opaque-walled tissue culture plates compatible with fluorometer (clear or solid bottom)
- multichannel pipettor
- reagent reservoirs
- fluorescence plate reader with filter sets: 485nm_{Ex}/520nm_{Em}
- orbital plate shaker
- positive control cytotoxic reagent or lytic detergent

Example Cytotoxicity Assay Protocol

1. If you have not performed this assay on your cell line previously, we recommend determining assay sensitivity using your cells. Protocols to determine assay sensitivity are available in the *CytoTox-Fluor™ Cytotoxicity Assay Technical Bulletin #TB350* (www.promega.com/tbs/tb350/tb350.html).
2. Set up 96-well or 384-well assay plates containing cells in culture medium at desired density.
3. Add test compounds and vehicle controls to appropriate wells so that the final volume is 100μl in each well (25μl for 384-well plates).
4. Culture cells for the desired test exposure period.
5. Add CytoTox-Fluor™ Cytotoxicity Assay Reagent in an equal volume to all wells, mix briefly on an orbital shaker, then incubate for 30 minutes at 37°C.
6. Measure the resulting fluorescence: 485nm_{Ex}/520nm_{Em}.

Example Multiplex Protocol (with luminescent caspase assay)

1. Set up 96-well assay plates containing cells in culture medium at desired density.
2. Add test compounds and vehicle controls to appropriate wells so that the final volume is 100μl in each well (25μl for 384-well plates).
3. Culture cells for the desired test exposure period.
4. Add CytoTox-Fluor™ Cytotoxicity Assay Reagent in an equal volume to all wells, mix briefly on an orbital shaker, then incubate for 30 minutes at 37°C.
5. Measure the resulting fluorescence: 485nm_{Ex}/520nm_{Em}.
6. Add an equal volume of Caspase-Glo® 3/7 Reagent to the wells, incubate for 30 minutes and measure luminescence.

General Considerations for the CytoTox-Fluor™ Cytotoxicity Assay

Background Fluorescence and Inherent Serum Activity:

Tissue culture medium that is supplemented with animal serum may contain detectable levels of the protease marker used for dead-cell measurement. The quantity of this protease activity may vary among different lots of serum. To correct for variability, background fluorescence should be determined using samples containing medium plus serum without cells.

Temperature: The generation of fluorescent product is proportional to the protease activity of the marker associated with cytotoxicity. The activity of this protease is influenced by temperature. For best results, we recommend incubating at a constant controlled temperature to ensure uniformity across the plate.

Assay Controls: In addition to a no-cell control to establish background fluorescence, we recommend including an untreated cells (maximum viability) and positive (maximum cytotoxicity) control in the experimental design. The maximum viability control is established by the addition of vehicle only (used to deliver the test compound to test wells). In most cases, this consists of a buffer system or medium and the equivalent amount of solvent added with the test compound. The maximum cytotoxicity control can be determined using a compound that causes cytotoxicity or a lytic compound added to compromise viability (non-ionic or Zwitterionic detergents).

Cytotoxicity Marker Half-Life: The activity of the protease marker released from dead cells has a half-life estimated to be greater than 10 hours. In situations where cytotoxicity occurs very rapidly (necrosis) and the incubation time is greater than 24 hours, the degree of cytotoxicity may be underestimated. The addition of a lytic detergent may be useful to determine the total cytotoxicity marker activity remaining (from any live cells) in these extended incubations.

Light Sensitivity: Although the bis-AAF-R110 Substrate demonstrates good general photostability, the liberated fluorors (after contact with protease) can degrade with prolonged exposure to ambient light sources. We recommend shielding the plates from ambient light at all times.

Cell Culture Medium: The bis-AAF-R110 Substrate is introduced into the test well using an optimized buffer system that mitigates differences in pH from treatment. In addition, the buffer system supports protease activity in a host of different culture media with varying osmolarity. With the exception of media formulations with either very high serum content or phenol red indicator, no substantial performance differences will be observed among media.

Additional Resources for the CytoTox-Fluor™ Cytotoxicity Assay

Technical Bulletins and Manuals

TB350 *CytoTox-Fluor™ Cytotoxicity Assay Technical Bulletin*
(www.promega.com/tbs/tb350/tb350.html)

Online Tools

Cell Viability Assistant (www.promega.com/techserv/tools/cellviasast)

C. Cytotoxicity Assays Measuring LDH Release

Cells that have lost membrane integrity release lactate dehydrogenase (LDH) into the surrounding medium. The CytoTox-ONE™ Homogeneous Membrane Integrity Assay is a fluorescent method that uses coupled enzymatic reactions to measure the release of LDH from damaged cells as an indicator of cytotoxicity. The assay is designed to estimate the number of nonviable cells present in a mixed population of living and dead cells. Alternatively, if a cell lysis reagent is used, the same assay chemistry can be used to determine the total number of cells in a population.

LDH catalyzes the conversion of lactate to pyruvate with the concomitant production of NADH. The CytoTox-ONE™ Reagent contains excess substrates (lactate and NAD⁺) to drive the LDH reaction and produce NADH. This NADH, in the presence of diaphorase and resazurin, is used to drive the diaphorase-catalyzed production of the fluorescent resorufin product. Because reaction conditions proceed at physiological pH and salt conditions, the CytoTox-ONE™ Reagent does not damage living cells, and the assay can be performed directly in cell culture using a homogeneous method. The CytoTox-ONE™ Assay is fast, typically requiring only a 10-minute incubation period. Under these assay conditions, there is no significant reduction of resazurin by the population of viable cells.

The CytoTox-ONE™ Assay is compatible with 96- and 384-well formats. The detection sensitivity is a few hundred cells but can be limited by the LDH activity present in serum used to supplement culture medium. When automated on the Biomek® 2000 workstation, the CytoTox-ONE™ Assay gives excellent Z'-factor values (Figure 4.14). Because the CytoTox-ONE™ Assay is

relatively nondestructive, it can be multiplexed with other assays to allow researchers to measure more than one parameter from the same sample. For multiplexing protocols using the CytoTox-ONE™ Assay see *Cell Notes* Issue 10 (www.promega.com/cnotes/cnotedex_00.htm#10) or Chapter 3 "Apoptosis" (www.promega.com/paguide/chap3.htm#title7) of this Protocols and Applications Guide.

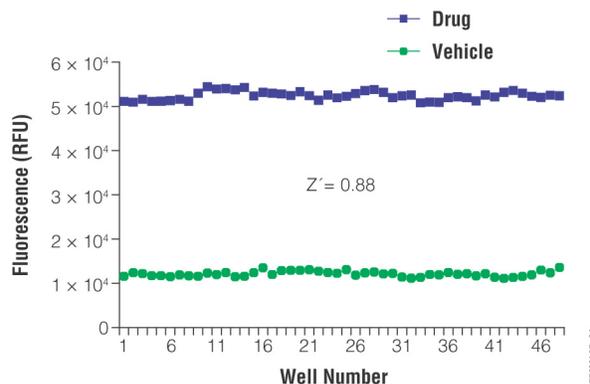


Figure 4.18. Representation of Z'-factor equal to 0.88 using the CytoTox-ONE™ Assay for one of the plates processed by the Biomek® 2000 Workstation. HepG2 cells were plated in 96-well tissue culture, treated white plates with clear bottoms at a density of 40,000 cells/well. Cells were allowed to grow to confluence and were treated with 3.125µM staurosporine on one half of the plate or DMSO vehicle control on the other half. A two-plate protocol was written for the Biomek® 2000 workstation, and plates were read on a fluorescent plate reader.

CytoTox-ONE™ Homogeneous Membrane Integrity Assay

Materials Required:

- CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Cat.# G7890, G7891, G7892) and protocol #TB306 (www.promega.com/tbs/tb306/tb306.html)
- 96- or 384-well opaque-walled tissue culture plates compatible with fluorometer (clear or solid bottom)
- multichannel pipettor
- reservoirs to hold CytoTox-ONE™ Reagent and Stop Solution
- fluorescence plate reader with excitation 530–570nm and emission 580–620nm
- plate shaker

Example Cytotoxicity Assay Protocol

1. Set up 96-well assay plates containing cells in culture medium.
2. Add test compounds and vehicle controls to appropriate wells such that the final volume is 100µl in each well (25µl for a 384-well plate).
3. Culture cells for desired test exposure period.
4. Remove assay plates from the 37°C incubator and equilibrate to 22°C (approximately 20–30 minutes).

- Optional:** If the Lysis Solution is used to generate a Maximum LDH Release Control, add 2µl of Lysis Solution (per 100µl original volume) to the positive control wells. If a larger pipetting volume is desired, use 10µl of a 1:5 dilution of Lysis Solution.
- Add a volume of CytoTox-ONE™ Reagent equal to the volume of cell culture medium present in each well, and mix or shake for 30 seconds (e.g., add 100µl of CytoTox-ONE™ Reagent to 100µl of medium containing cells for the 96-well plate format or add 25µl of CytoTox-ONE™ Reagent to 25µl of medium containing cells for the 384-well format).
- Incubate at 22°C for 10 minutes.
- Add 50µl of Stop Solution (per 100µl of CytoTox-ONE™ Reagent added) to each well. For the 384-well format (where 25µl of CytoTox-ONE™ Reagent was added), add 12.5µl of Stop Solution. This step is optional but recommended for consistency.
- Shake plate for 10 seconds and record fluorescence with an excitation wavelength of 560nm and an emission wavelength of 590nm.
- Remove assay plates from the 37°C incubator and equilibrate to 22°C (approximately 20–30 minutes).
- Add a volume of CytoTox-ONE™ Reagent equal to the volume of cell culture medium present in each well, and mix or shake for 30 seconds (e.g., add 100µl of CytoTox-ONE™ Reagent to 100µl of medium containing cells for the 96-well plate format or add 25µl of CytoTox-ONE™ Reagent to 25µl of medium containing cells for the 384-well format).
- Incubate at 22°C for 10 minutes.
- Add 50µl of Stop Solution (per 100µl of CytoTox-ONE™ Reagent added) to each well in the 96-well format. For the 384-well format (where 25µl of CytoTox-ONE™ Reagent was added), add 12.5µl Stop Solution. This step is optional but recommended for consistency.
- Shake plate for 10 seconds and record fluorescence at 560_{Ex}/590_{Em}.

Calculation of Results

- Subtract the average fluorescence values of the Culture Medium Background from all fluorescence values of experimental wells.
- Use the average fluorescence values from Experimental, Maximum LDH Release, and Culture Medium Background to calculate the percent cytotoxicity for a given experimental treatment.
Percent cytotoxicity = $100 \times (\text{Experimental} - \text{Culture Medium Background}) / (\text{Maximum LDH Release} - \text{Culture Medium Background})$

Example Total Cell Number Assay Protocol

The CytoTox-ONE™ Assay can be used to estimate the total number of cells in assay wells at the end of a proliferation assay. The procedure involves lysing all the cells to release LDH followed by adding the CytoTox-ONE™ Reagent. The total number of cells present will be directly proportional to the background-subtracted fluorescence values, which represent LDH activity.

- Set up 96-well assay plates containing cells in culture medium.
- Add test compounds and vehicle controls to appropriate wells so the final volume is 100µl in each well (25µl per well for 384-well plates).
- Culture cells for desired test exposure period.
- Add 2µl of Lysis Solution (per 100µl of original volume) to all wells. If a larger pipetting volume is desired, use 10µl of a 1:5 dilution of Lysis Solution.

- Remove assay plates from the 37°C incubator and equilibrate to 22°C (approximately 20–30 minutes).
- Add a volume of CytoTox-ONE™ Reagent equal to the volume of cell culture medium present in each well, and mix or shake for 30 seconds (e.g., add 100µl of CytoTox-ONE™ Reagent to 100µl of medium containing cells for the 96-well plate format or add 25µl of CytoTox-ONE™ Reagent to 25µl of medium containing cells for the 384-well format).
- Incubate at 22°C for 10 minutes.
- Add 50µl of Stop Solution (per 100µl of CytoTox-ONE™ Reagent added) to each well in the 96-well format. For the 384-well format (where 25µl of CytoTox-ONE™ Reagent was added), add 12.5µl Stop Solution. This step is optional but recommended for consistency.
- Shake plate for 10 seconds and record fluorescence at 560_{Ex}/590_{Em}.

General Considerations for Performing the CytoTox-ONE™ Assay

Background Fluorescence/Serum LDH: Animal serum used to supplement tissue culture medium may contain significant amounts of LDH that can lead to background fluorescence. The quantity of LDH in animal sera will vary depending on several factors, including the species and the health or treatment of the animal prior to collecting serum. Background fluorescence can be corrected by including a control to measure the fluorescence from serum-supplemented culture medium in the absence of cells. Using reduced serum concentrations or serum-free medium can reduce or eliminate background fluorescence resulting from LDH in serum and improve assay sensitivity.

Temperature: The generation of fluorescent product in the CytoTox-ONE™ Assay is proportional to the quantity of LDH. The enzymatic activity of LDH is influenced by temperature. We recommend equilibrating the temperature of the assay plate and the CytoTox-ONE™ Reagent to 22°C (20–30 minutes) before adding the CytoTox-ONE™ Reagent to initiate the reaction. The recommended incubation period for the CytoTox-ONE™ Reagent is 10 minutes when reagents and samples are at 22°C. At longer incubation times or higher temperatures, assay linearity may decrease due to substrate depletion. In some situations, the time required for manual or robotic addition of CytoTox-ONE™ Reagent to the assay plate may be a significant portion of the 10-minute incubation period. To minimize the difference in incubation interval among wells within a plate, we recommend adding Stop Solution using the same sequence used for adding the CytoTox-ONE™ Reagent.

Assay Controls: In a standard cytotoxicity assay, a 100% cell lysis control may be performed to determine the maximum amount of LDH present. Individual laboratories may prefer to use a positive control that is known to be toxic for their specific cell type, culture conditions, and assay model system. For convenience, we include the Lysis

Solution, which is a 9% (weight/volume) solution of Triton® X-100 in water. Use of Lysis Solution at the recommended dilution will result in almost immediate lysis of most cell types and subsequent release of cytoplasmic LDH into the surrounding culture medium. Use of Lysis Solution at the recommended dilution is compatible with the CytoTox-ONE™ Assay chemistry.

Considerations for the **Maximum LDH Release Control experimental design** will influence the values for the Maximum LDH Release Control. The mechanism of cytotoxicity, and thus the kinetics of release of LDH, may vary widely for different experimental compounds being tested. The method by which the Maximum LDH Release Control is prepared as well as the timing of the addition of Lysis Solution (i.e., beginning, middle or end of experimental/drug treatment period) may both affect the value obtained for 100% LDH release. For example, if the indicator cells are growing throughout the duration of exposure to test compounds, untreated control wells may have more cells and thus may have more LDH present at the end of the exposure period. Adding Lysis Solution after cultured cells are exposed to test compounds may give a different Maximum LDH Release Control value than adding Lysis Solution before the exposure period. The half-life of LDH that has been released from cells into the surrounding medium is approximately 9 hours. If Lysis Solution is added at the beginning of an experimental exposure period, the quantity of active LDH remaining in the culture medium at the end of the experiment may underestimate the quantity of LDH present in untreated cells. The recommended dilution of Lysis Solution is compatible with the enzymatic reactions and fluorescence of the assay. Using higher concentrations of Lysis Solution may increase the rate of enzymatic reactions and inflate maximum cell lysis values.

Light Sensitivity of Resazurin: The resazurin dye in the CytoTox-ONE™ Reagent and the resorufin product formed during the assay are light-sensitive. Prolonged exposure of the CytoTox-ONE™ Assay Buffer or reconstituted CytoTox-ONE™ Reagent to light will result in increased background fluorescence in the assay and decreased sensitivity.

Use of Stop Solution to Stop Development of Fluorescent Signal: The Stop Solution provided is designed to rapidly stop the continued generation of fluorescent product and allow the plate to be read at a later time. There may be situations where the researcher will want to take multiple kinetic readings of the same plate and not stop the assay. After adding the Stop Solution, provided that there is some serum (5–10%) present in the samples, the resulting fluorescence is generally stable for up to two days if the assay plate has been protected from light exposure and the wells have been sealed with a plate sealer to prevent evaporation. If no serum is present, the resulting fluorescence is stable for 1–2 hours.

Cell Culture Media: Pyruvate-supplemented medium is recommended for some cell lines. Common examples of culture media that contain pyruvate include: Ham's F12, Iscove's and some formulations of DMEM. Culture media containing pyruvate may cause a reduction in the fluorescent signal due to product inhibition of the LDH reaction catalyzing conversion of lactate to pyruvate. For most situations, the recommended assay conditions of 10 minutes at 22°C will provide adequate signal. However, assay conditions can be empirically optimized. To increase the fluorescent signal, we recommend omitting pyruvate during the assay period, if the cell line does not require it. Alternatively, conditions known to increase the fluorescent signal include increasing the time of incubation with the CytoTox-ONE™ Reagent prior to adding Stop Solution or incubating the assay at temperatures above the recommended 22°C (up to 37°C). In all cases, all samples within a single assay should be measured using the same conditions.

Use of Resazurin as an Indicator in both Cytotoxicity and Cell Viability Assays: Resazurin reduction is a common reporter for both cytotoxicity and cell viability assays. Using the reaction conditions recommended for the CytoTox-ONE™ Assay (i.e., reduced temperature and short incubation time), only a negligible amount of resazurin is reduced by the viable cell population. In the CytoTox-ONE™ Assay, the rate of the LDH reaction is increased by providing excess substrates (pyruvate, NAD⁺, and diaphorase) so that the reaction proceeds relatively quickly (10 minutes at ambient temperature). By contrast, the CellTiter-Blue® Cell Viability Assay requires longer incubation times (1–4 hours) and a higher incubation temperature (37°C). Additionally, the concentration of resazurin is different between the two assays.

Additional Resources for the CytoTox-ONE™ Homogeneous Membrane Integrity Assay Technical Bulletin

Technical Bulletins and Manuals

- | | |
|-------|---|
| TB306 | <i>CytoTox-ONE™ Homogeneous Membrane Integrity Assay Technical Bulletin</i>
(www.promega.com/tbs/tb306/tb306.html) |
| EP016 | <i>Automated CytoTox-ONE™ Homogeneous Membrane Integrity Assay Protocol</i>
(www.promega.com/tbs/ep016/ep016.html) |

Promega Publications

- | | |
|-------|---|
| PN085 | Automating Promega cell-based assays in multiwell formats
(www.promega.com/pnotes/85/10904_25/10904_25.html) |
| CN006 | Frequently asked questions: CytoTox-ONE™ Homogeneous Membrane Integrity Assay
(www.promega.com/cnotes/cn006/cn006_19.htm) |

Online Tools

Cell Viability Assistant (www.promega.com/techserv/tools/cellviasst/)

Citations

Chen, J. *et al.* (2003) Effect of bromodichloromethane on chorionic gonadotrophin secretion by human placental trophoblast cultures. *Toxicol. Sci.* **76**, 75–82.

The CytoTox-ONE™ Homogeneous Membrane Integrity Assay was used to assess LDH release in trophoblasts exposed to BDCM for 25 hours.

PubMed Number: 12970577

CytoTox 96® Non-Radioactive Cytotoxicity Assay

The CytoTox 96® Non-Radioactive Cytotoxicity Assay is a colorimetric method for measuring lactate dehydrogenase (LDH), a stable cytosolic enzyme released upon cell lysis, in much the same way as [⁵¹Cr] is released in radioactive assays. Released LDH in culture supernatants is measured with a 30-minute coupled enzymatic assay that results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color formed is proportional to the number of lysed cells. Visible wavelength absorbance data are collected using a standard 96-well plate reader. The assay can be used to measure membrane integrity for cell-mediated cytotoxicity assays in which a target cell is lysed by an effector cell, or to measure lysis of target cells by bacteria, viruses, proteins, chemicals, etc. This assay can be used to determine general cytotoxicity or total cell number.

Two factors in tissue culture medium can contribute to background in the CytoTox 96® Assay: phenol red and LDH from animal sera. The absorbance value of a culture medium control is used to normalize the values obtained from other samples. Background absorbance from phenol red also can be eliminated by using a phenol red-free medium. The quantity of LDH in animal sera will vary depending on several parameters, including the species and the health or treatment of the animal prior to collecting serum. Human AB serum is relatively low in LDH activity, while calf serum is relatively high. The concentration of serum can be decreased to reduce the amount of LDH contribution to background absorbance. In general decreasing the serum concentration to 5% will significantly reduce background without affecting cell viability. Certain detergents (e.g., SDS and Cetrimide) can inhibit LDH activity. The Lysis Solution included with the CytoTox 96® Assay does not affect LDH activity and does not interfere with the assay. Technical Bulletin #TB163 provides a detailed protocol for performing this assay.

Additional Resources for the CytoTox 96®**Non-Radioactive Cytotoxicity Assay Technical Bulletin Technical Bulletins and Manuals**

TB163 *CytoTox 96® Non-Radioactive Cytotoxicity Assay Technical Bulletin*
(www.promega.com/tbs/tb163/tb163.html)

Promega Publications

CN004 *In vitro* toxicology and cellular fate determination using Promega's cell-based assays
(www.promega.com/cnotes/cn004/cn004_02.htm)

Online Tools

Cell Viability Assistant (www.promega.com/techserv/tools/cellviasst/)

Citations

Hernandez, J.M. *et al.* (2003) Novel kidney cancer immunotherapy based on the granulocyte-macrophage colony-stimulating factor and carbonic anhydrase IX fusion gene. *Clin. Cancer Res.* **9**, 1906–16.

The CytoTox 96® Non-Radioactive Cytotoxicity Assay was used to determine specific cytotoxicity of human dendritic cells that were transduced with recombinant adenoviruses containing the gene encoding a fusion protein of granulocyte-macrophage colony stimulating factor and carbonic anhydrase IX.

PubMed Number: 12738749

V. Assays to Detect Apoptosis

A variety of methods are available for detecting apoptosis to determine the mechanism of cell death. The Caspase-Glo® Assays are highly sensitive, luminescent assays with a simple “add, mix, measure” protocol that can be used to detect caspase-8 (Cat.# G8200), caspase-9 (Cat.# G8210) and caspase-3/7 (Cat.# G8090) activities. If you prefer a fluorescent assay, the Apo-ONE® Homogeneous Caspase-3/7 Assay (Cat.# G7792) is useful and, like the Caspase-Glo® Assays, can be multiplexed with other assays. A later marker of apoptosis is TUNEL analysis to identify the presence of oligonucleosomal DNA fragments in cells. The DeadEnd™ Fluorometric (Cat.# G3250) and the DeadEnd™ Colorimetric (Cat.# G7360) TUNEL Assays allow users to end-label the DNA fragments to detect apoptosis. A detailed discussion of apoptosis and methods and technologies for detecting apoptosis can be found in Chapter 3 of this Protocols & Applications Guide: Apoptosis (www.promega.com/paguide/chap3.htm).

VI. Multiplexing Cell Viability Assays

The latest generation of Promega cell-based assays includes luminescent and fluorescent chemistries to measure markers of cell viability, cytotoxicity and apoptosis, as well as to perform reporter analysis. Using these tools researchers can investigate how cells respond to growth factors, cytokines, hormones, mitogens, radiation, effectors, compound libraries and other signaling molecules. However, researchers often need more than one type of data from a sample, so the ability to multiplex, or analyze more than one parameter from a single sample, is desirable. Chapter three of this *Protocols & Applications Guide* (www.promega.com/paguide/chap3.htm#title7) presents basic protocols for multiplexing experiments using Promega

homogeneous apoptosis assays. Here we present protocols for multiplexing cell viability with cytotoxicity assays or reporter assays. For protocols describing multiplex experiments using cell viability and apoptosis assays, please see Chapter 3 (www.promega.com/paguide/chap3.htm#title7) of this guide.

The protocols provided are guidelines for multiplexing cell-based assays and are intended as starting points. As with any homogeneous assay, multiplexing assays will require researchers to optimize their assays for specific experimental systems. We strongly recommend running appropriate controls, including performing each assay individually on the samples. Additional background, optimization and control information for each assay is provided in its accompanying technical literature.

The CellTiter-Glo[®] Luminescent Cell Viability Assay (Cat.# G7571; Technical Bulletin #TB288 (www.promega.com/tbs/tb288/tb288.html)) is a homogeneous assay that measures ATP. This viability assay can be multiplexed with a live-cell luciferase reporter assay using the EnduRen[™] Live Cell Substrate (Cat.# E6482; Technical Manual #TM244 (www.promega.com/tbs/tm244/tm244.html)) or with the CytoTox-ONE[™] Homogeneous Membrane Integrity Assay (Cat.# G7891; Technical Bulletin #TB306 (www.promega.com/tbs/tb306/tb306.html)), which assesses cytotoxicity by measuring LDH release. Figure 4.14 illustrates data obtained from a multiplexing experiment using a *Renilla* reporter assay using the EnduRen[™] Live-Cell Substrate and the CellTiter-Glo[®] Assay.

The MultiTox-Fluor and CytoTox-Fluor[™] assays can be multiplexed with luminescent assays measuring caspase activities to obtain information about apoptosis while controlling for cytotoxic or proliferative events. Example protocols for multiplex experiments using the MultiTox-Fluor or CytoTox-Fluor[™] Assays with luminescent caspase assays are provided below. Additionally, the CytoTox-ONE[™] Assay, which measures LDH release can be multiplexed with the Apo-ONE[®] Homogeneous Caspase-3/7 Assay to give information on cytotoxicity and mechanism of cell death.

A. Normalizing Reporter Gene Signal with Cell Viability

1. Culture and treat cells with the drug of interest in 90 μ l of medium in a 96-well plate.
2. Dilute the EnduRen[™] Live Cell Substrate (Cat.# E6482) as directed in Technical Manual #TM244 (www.promega.com/tbs/tm244/tm244.html). Add 10 μ l/well of EnduRen[™] Substrate (60 μ M) and incubate for an additional 2 hours at 37°C, 5% CO₂. You may add the EnduRen[™] Substrate before or after experimental treatment, depending on cell tolerance.
3. Record luminescence to indicate reporter activity.

4. Add an equal volume of CellTiter-Glo[®] Reagent (100 μ l/well), mix for 2 minutes on an orbital shaker to induce cell lysis, and incubate an additional 10 minutes at room temperature to stabilize luminescent signal.
5. Record luminescence as described in Technical Bulletin #TB288 (www.promega.com/tbs/tb288/tb288.html) to indicate cell viability.

Note: We suggest these controls: 1) Drug-treated cells with the CellTiter-Glo[®] Reagent alone, 2) Drug-treated cells with the EnduRen[™] Substrate alone.

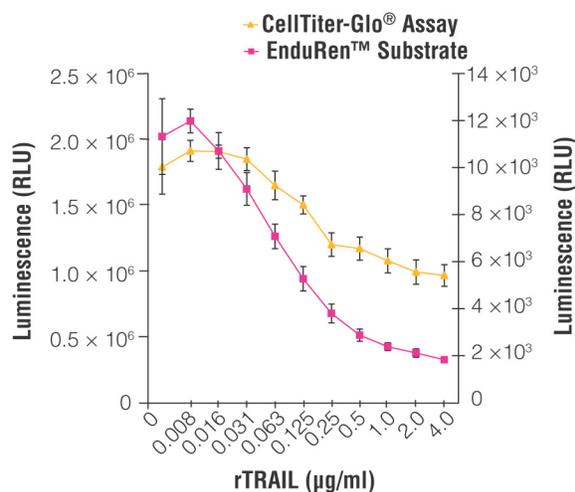


Figure 4.19. Multiplex of a *Renilla* reporter assay with a luminescent cell viability assay. HeLa cells stably expressing humanized *Renilla* luciferase were plated at 10,000 cells/well in a 96-well plate. EnduRen[™] Live Cell Substrate (Cat.# E6482) was added to all of the wells at a 1:1,000 dilution in DMEM + 10% FBS. The TRAIL protein (CalBiochem Cat.# 616375) was added starting at 4 μ g/ml with subsequent twofold serial dilutions. Cells were incubated for 16 hours and assayed. *Renilla* expression was measured using a GloMax[™] 96 Microplate Luminometer (Cat.# E6521) immediately after incubation. CellTiter-Glo[®] Reagent was then added at a volume of 1:1 to each well and luminescence read on the GloMax[™] 96 Microplate Luminometer. RLU=Relative Light Units.

B. Determining Cytotoxicity and Cell Viability

1. Culture and treat cells with drug of interest in 75 μ l of medium in a 96-well plate (black or white).
2. Reconstitute CytoTox-ONE[™] Substrate at 1X concentration, and add 50 μ l/well.
3. Shake gently and incubate for 10 minutes at room temperature. Record fluorescence (560_{Ex}/590_{Em}) as described in Technical Bulletin #TB306.
4. Reconstitute the CellTiter-Glo[®] Substrate. Add 124 μ l of CellTiter-Glo[™] Substrate plus 1 μ l 20mM DTT to each well such that the final concentration of DTT in the well is 0.8mM.

- Shake gently and incubate for 1 hour at room temperature. Record luminescence as described in Technical Bulletin #TB288.

Note: Ensure that all of the wells change to an even pink color after incubating with CellTiter-Glo® Reagent. If all of the wells contain the same pink color when luminescence is recorded, the light is quenched evenly throughout the sample, regardless of the initial CytoTox-ONE™ Substrate activity.

C. Multiplexing CytoTox-Fluor™ Cytotoxicity Assay with a Luminescent Caspase Assay

- Set up 96-well assay plates containing cells in culture medium at desired density.
- Add test compounds and vehicle controls to appropriate wells so the final volume in the well is 100µl in each well (25µl for a 384-well plate).
- Culture cells for the desired test exposure period.
- Add 10µl CytoTox-Fluor™ Cytotoxicity Assay Reagent (prepared as 10µl substrate in 1ml Assay Buffer) to all wells, and mix briefly by orbital shaking. Incubate for at least 30 minutes at 37°C. **Note:** Longer incubations may improve assay sensitivity and dynamic range. However, do not incubate longer than 3 hours.
- Measure resulting fluorescence using fluorometer (485nm_{Ex}/520nm_{Em}). **Note:** Adjustment of instrument gains (applied photomultiplier tube energy) may be necessary.
- Add an equal volume of Caspase-Glo® 3/7 Reagent to wells (100–110µl per well), incubate for 30 minutes, then measure luminescence using a luminometer.

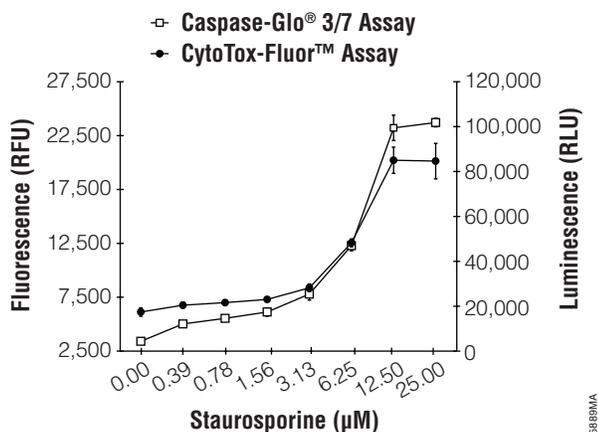


Figure 4.20. CytoTox-Fluor™ Assay multiplexed with Caspase-Glo® 3/7 Assay. The CytoTox-Fluor™ Assay Reagent is added to wells and cytotoxicity measured after incubation for 30 minutes at 37°C. Caspase-Glo® 3/7 Reagent is added and luminescence measured after a 30-minute incubation.

D. Multiplexing the MultiTox-Fluor Multiplex Cytotoxicity Assay with a Luminescent Caspase Assay

- Set up 96-well assay plates containing cells in medium at the desired density.
- Add test compounds and vehicle controls to appropriate wells so that the final volume in the well is 100µl (25µl for a 384-well plate).
- Culture cells for the desired test exposure period.
- Add 10µl of MultiTox-Fluor Reagent (prepared as 10µl Substrate in 1ml Assay Buffer) to all wells, and mix briefly by orbital shaking. Incubate for at least 30 minutes at 37°C. **Note:** Longer incubations may improve assay sensitivity and dynamic range. However, do not incubate more than 3 hours.
- Measure resulting fluorescence using a fluorometer (live-cell fluorescence 400_{Ex}/505_{Em}; dead-cell fluorescence 485_{Ex}/520_{Em}).
- Add an equal volume of Caspase-Glo® 3/7 Reagent to wells (100–110µl per well), incubate for 30 minutes, then measure luminescence using a luminometer.

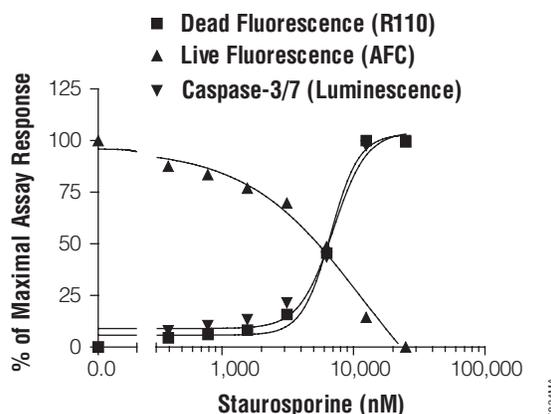


Figure 4.21. The MultiTox-Fluor Assay technology can be multiplexed with other assays. LN-18 cells were plated at a density of 10,000 cells per well in 50µl volumes of MEM + 10% fetal bovine serum and allowed to attach overnight. Staurosporine was twofold serially diluted and added to wells in 50µl volumes. The plate was incubated at 37°C in 5% CO₂ for 6 hours. MultiTox-Fluor Reagent was prepared as 10µl Substrate in 1ml Assay Buffer, and 10µl was used. The plate was mixed and incubated for 30 minutes at 37°C. Fluorescence was read on a BMG PolarStar plate reader. Caspase-Glo® 3/7 Reagent was then added in an additional 100µl volume, and luminescence measured after a 10-minute incubation. The resulting signals were normalized to a percentage of maximal response and plotted using GraphPad Prism® software.

E. Multiplexing the CytoTox-ONE™ Homogeneous Membrane Integrity Assay with the Apo-ONE® Homogeneous Caspase-3/7 Assay

1. Plate cells at the desired density (e.g., 10,000 cells/well) in a white, clear-bottom 96-well plate.
2. Add treatment compound at desired concentration (e.g., tamoxifen).
3. Culture cells for the desired test exposure period.
4. To assay LDH activity transfer 50 μ l of the culture supernatants to a 96-well plate and add 50 μ l of CytoTox™ Reagent and incubate the plate for 10 minutes at 22°C. **Note:** Pyruvate in the cell-culture medium can inhibit the LDH reaction. Cells in medium supplemented with pyruvate may require a longer incubation.
5. Stop the reaction by adding 25 μ l of Stop solution to each well.
6. Measure fluorescence at 560_{Em}/590_{Ex}.
7. To determine the activity of caspase-3/7, add 50 μ l of Apo-ONE® Reagent to the original culture plate containing cells, and incubate at room temperature for 45 minutes.
8. Measure fluorescence at 485_{Ex}/527_{Em}.

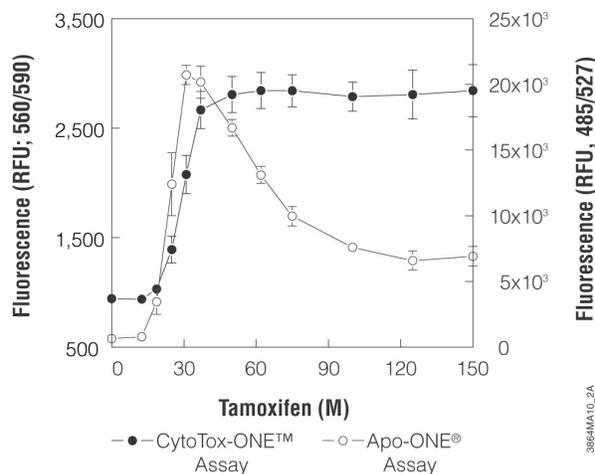


Figure 4.22. Example data showing multiplexed caspase-3/7 and LDH release assays using different plates. HepG2 cells were plated at 10,000 cells/well in a white, clear-bottom 96-well plate and cultured overnight. Various concentrations of tamoxifen were added to the wells and incubated for 4 hours at 37°C. To assay LDH activity, 50 μ l/well culture supernatants were transferred to a 96-well plate to which 50 μ l/well of CytoTox-ONE™ Reagent was added. LDH samples were incubated at ambient temperature for 30 minutes prior to stopping with 25 μ l/well of Stop Solution. Fluorescence 560/590nm was determined. For caspase-3/7 determination, 50 μ l/well of Apo-ONE® Reagent was added to the original culture plate containing cells, and incubated at ambient temperature for 45 minutes prior to determining fluorescence 485/527nm. (**Note:** The 30-minute incubation period for the assay was longer than the 10 minutes recommended in the technical bulletin because the medium for these cells was supplemented with pyruvate (which inhibits the LDH reaction) and the assay was performed at ambient temperature, which was a little colder than the 22°C recommended in the technical bulletin.

Promega Publications

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| CN012 | Perform multiplexed cell-based assays on automated platforms
(www.promega.com/cnotes/cn012/cn012_13.htm) |
| CN010 | Multiplexing homogeneous cell-based assays
(www.promega.com/cnotes/cn010/cn010_15.htm) |

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I. Introduction

Cell-free systems for *in vitro* gene expression and protein synthesis have been described for many different prokaryotic (Zubay, 1973) and eukaryotic (Pelham and Jackson, 1976; Anderson *et al.* 1983) systems. Eukaryotic cell-free systems, such as rabbit reticulocyte lysate and wheat germ extract, are prepared from crude extract containing all the components required for translation of *in vitro*-transcribed RNA templates. Eukaryotic cell-free systems use isolated RNA synthesized *in vivo* or *in vitro* as a template for the translation reaction (e.g., Rabbit Reticulocyte Lysate Systems [Cat.# L4960, L4540] or Wheat Germ Extract Systems [Cat.# L4380]). Coupled eukaryotic cell-free systems combine a prokaryotic phage RNA polymerase with eukaryotic extracts and utilize an exogenous DNA or PCR-generated templates with a phage promoter for *in vitro* protein synthesis (Figure 5.1) (TNT® Coupled Reticulocyte Lysate [Cat.# L4600, L4610, L4950, L5010, L5020], TNT® Quick Coupled Transcription/Translation Systems [Cat.# L1170, L2080], TNT® T7 Quick for PCR DNA [Cat.# L5540] and TNT® Wheat Germ Extract Systems [Cat.# L4120, L4130, L4140, L5030, L5040]).

Proteins translated using the TNT® Coupled Systems can be used in many types of functional studies. TNT® Coupled Transcription/Translation reactions have traditionally been used to confirm open reading frames, study protein mutations and make proteins *in vitro* for protein:DNA binding studies, protein activity assays, or protein:protein interaction studies. Recently, proteins expressed using the TNT® Coupled Systems have also been used in assays to confirm yeast two-hybrid interactions, perform *in vitro* expression cloning (IVEC) and make protein substrates for enzyme activity or protein modification assays. For a listing of recent citations using the TNT® Coupled Systems in various applications, please visit:

www.promega.com/citations/ (www.promega.com/citations)

Transcription and translation are typically coupled in prokaryotic systems; that is, they contain an endogenous or phage RNA polymerase, which transcribes mRNA from an exogenous DNA template. This RNA is then used as a template for translation. The DNA template may be either a gene cloned into a plasmid vector (cDNA) or a PCR generated template. A ribosome binding site (RBS) is required for templates translated in prokaryotic systems. During transcription, the 5'-end of the mRNA becomes available for ribosome binding and translation initiation, allowing transcription and translation to occur simultaneously. Prokaryotic systems are available that use DNA templates containing either prokaryotic promoters (such as *lac* or *tac*; *E. coli* S30 Extract System for Circular and Linear DNA [Cat.# L1020 and L1030] or a phage RNA polymerase promoter; *E. coli* T7 S30 Extract System for Circular DNA [Cat.# L1130]).

Most *in vitro* systems produce picomole (or nanogram) amounts of proteins per 50µl reaction. This yield is usually sufficient for most types of radioactive, fluorescent and antibody analyses, such as polyacrylamide gel separation, Western blotting, immunoprecipitation and/or, depending on the protein of interest, enzymatic or biological activity assays. For radioactive detection, a radioactive amino acid is added to the translation reaction and, after incorporation, the gene product is identified by autoradiography following SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Alternatively, non-radioactive labeling methods such as fluorescent, chemiluminescent or colorimetric detection may be used (i.e., Transcend™ and FluoroTect™ Systems; Sections VIII and IX, respectively). If antibodies to the protein are available, then techniques such as immunoblotting or immunoprecipitation can be used. The functional activity of *in vitro*-translated products can often be detected directly in the reaction mixture. If protein purification is necessary, fusion of the protein to a purification “tag” allows the protein to be isolated from the *in vitro* translation reaction and subsequently studied.

Since protein synthesis reactions can be driven by RNA templates (translation; Section I.A) or DNA templates (coupled transcription/translation; Section I.B), the type of template is generally the first consideration when choosing an appropriate system. Promega translation and coupled transcription/translation systems are summarized in Tables 5.1 and 5.2, respectively. All systems provide reliable, convenient and efficient methods to initiate translation and produce full-size protein products. For assistance in choosing a cell-free protein expression system, the Cell-Free Protein Expression Product Selector (www.promega.com/selectors/tnt/) is available.

Cell-free protein synthesis systems have become standard tools for the *in vitro* expression of proteins from cloned genes. Applications for *in vitro* expression systems include analysis of protein:protein ([/multimedia/tnt01.htm](http://multimedia/tnt01.htm)) and protein:nucleic acid interactions ([/multimedia/tnt02.htm](http://multimedia/tnt02.htm)) (www.promega.com/multimedia/tnt01.htm) and protein:nucleic acid interactions ([/multimedia/tnt02.htm](http://multimedia/tnt02.htm)) (www.promega.com/multimedia/tnt02.htm). In addition, these systems can be used for mutational analysis, epitope mapping, *in vitro* evolutionary studies, protein truncation test (PTT) (Powell *et al.* 1993; Roest *et al.* 1993), clone verification, functional analysis, mutagenesis and domain mapping, ribosome display (Mattheakis *et al.* 1994; Hanes and Pluckthun, 1997) and *in vitro* expression cloning (IVEC) (Lustig *et al.* 1997; King *et al.* 1997), molecular diagnostics and high-throughput screening (Novac *et al.* 2004). *In vitro* expression systems also offer significant time savings over *in vivo* systems. The primary advantage of *in vitro* translation over *in vivo* protein expression is that *in vitro* systems allow the use of a defined template to direct protein synthesis. *In vitro* systems also have the ability to express toxic, proteolytically sensitive, or unstable gene products, and allow the specific labeling of gene products so that individual proteins can be monitored in complex reaction mixtures.

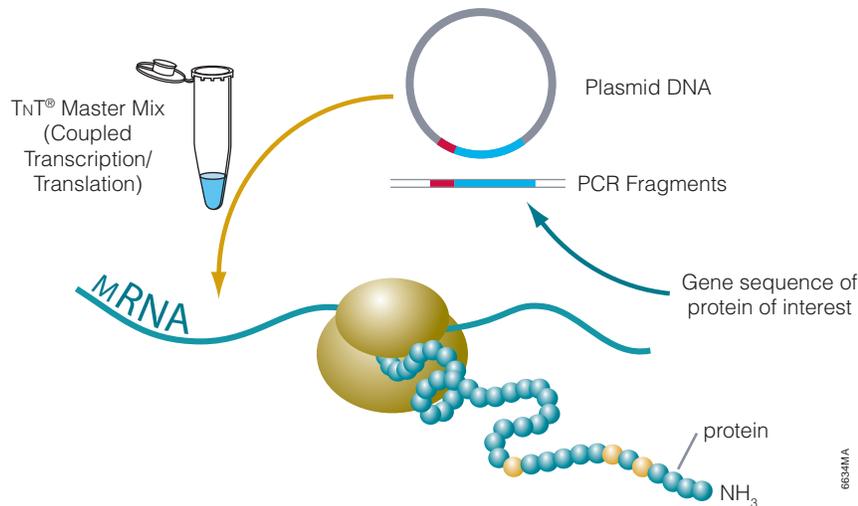


Figure 5.1. Cell-Free expression using the TNT® systems.

A. mRNA-Driven Translation

The Rabbit Reticulocyte Lysate Translation Systems (Nuclease-treated and Untreated), Flexi® Rabbit Reticulocyte Lysate System and Wheat Germ Extract System are all used for translation of mRNA. Table 5.1 summarizes these systems.

The Rabbit Reticulocyte Lysate, Nuclease-Treated, and the Flexi® Rabbit Reticulocyte Lysate have been optimized for mRNA translation by adding several supplements. These include hemin, which prevents activation of the heme-regulated eIF-2a kinase (HRI); an energy-generating system consisting of pretested phosphocreatine kinase and phosphocreatine; and calf liver tRNAs, to balance the accepting tRNA populations, thus optimizing codon usage and expanding the range of mRNAs that can be translated efficiently. In addition both lysates are treated with micrococcal nuclease to eliminate endogenous mRNA, thus reducing background translation. The Flexi® Rabbit Reticulocyte Lysate System provides greater flexibility of reaction conditions than the Rabbit Reticulocyte Lysate, Nuclease-Treated, by allowing translation reactions to be optimized for a wide range of parameters, including Mg²⁺ and K⁺ concentrations, and offers the choice of adding DTT. In contrast, the Rabbit Reticulocyte Lysate, Untreated, contains the cellular components necessary for protein

synthesis (tRNA, ribosomes, amino acids, initiation, elongation and termination factors) but has not been treated with micrococcal nuclease. Untreated Rabbit Reticulocyte Lysate is used primarily for the isolation of translation components, as an abundant source of endogenous globin mRNA and to study protein synthesis of the endogenous globin mRNA. Untreated Rabbit Reticulocyte Lysate is not recommended for in vitro translation of specific mRNAs.

Wheat Germ Extract contains the cellular components necessary for protein synthesis (tRNA, ribosomes, initiation, elongation and termination factors). The extract is optimized further by the addition of an energy-generating system consisting of phosphocreatine and phosphocreatine kinase, spermidine to stimulate the efficiency of chain elongation and thus overcome premature termination, and magnesium acetate at a concentration recommended for the translation of most mRNA species. Only the addition of exogenous amino acids (including an appropriately labeled amino acid) and mRNA are necessary to stimulate translation. Finally, Potassium Acetate is supplied as a separate component so that the translational system may be optimized for a wide range of mRNAs.

Table 5.1. Summary of mRNA-Driven Translation Systems.

System	Micrococcal Nuclease-Treated	Untreated	Salt Optimization Necessary	Protein Yield in 50µl Reaction
Rabbit Reticulocyte Lysate, Nuclease-Treated (Cat.# L4960)	X	-	-	50-200ng
Rabbit Reticulocyte Lysate, Untreated (Cat.# L4151)	-	X	-	endogenous mRNA synthesized
Flexi® Rabbit Reticulocyte Lysate (Cat.# L4540)	X	-	X	50-200ng
Wheat Germ Extract (Cat.# L4380)	X	-	-	30-150ng

B. DNA-Driven Cell-Free Expression

Both eukaryotic and prokaryotic coupled transcription/translation systems are available from Promega. Table 5.2 summarizes these systems.

The TNT® Coupled Reticulocyte Lysate Transcription/Translation Systems and the TNT® Quick Coupled Transcription/Translation Systems transcribe and translate proteins from plasmid templates using a single-tube format. The TNT® Coupled Systems provide all the reaction components separately, including three separate amino acid mixtures: minus methionine, cysteine, or leucine. The TNT® Quick Coupled System provides a master mix containing all the reaction components (including a minus methionine amino acid mix), thus saving time by requiring fewer pipetting steps. TNT® T7 Quick for PCR DNA is specially formulated for transcription/translation of linear, PCR-generated templates, which often require higher potassium and magnesium concentrations than plasmid DNA. For transcription/translation of linear or PCR-generated templates with the TNT® Quick Coupled System, a T7 TNT® PCR Enhancer is provided and must be added to the reactions. Only linear templates containing the T7 promoter are recommended for the TNT® Coupled Reticulocyte Lysate Transcription/Translation Systems.

The TNT® Coupled Wheat Germ Extract System offers an alternative to the rabbit reticulocyte systems for eukaryotic coupled transcription/translation in a single-tube format. Unlike standard wheat germ extract translations, which commonly use RNA synthesized *in vitro* from SP6, T3 or T7 RNA polymerase promoters, the TNT® Coupled Wheat Germ Extracts incorporate transcription directly in the translation mix.

The *E. coli* S30 Extract Systems simplify transcription/translation of DNA sequences cloned in plasmid or lambda vectors. The S30 extracts are prepared from *E. coli* B strains deficient in ompT endoproteinase and Ion protease activity. All three S30 systems contain an S30 Premix that includes all the components required for coupled transcription/translation except for amino acids. Separate amino acid mixtures minus methionine, cysteine or leucine are provided to facilitate radiolabeling of translation products. The *E. coli* S30 Extract Systems for Circular DNA and Linear DNA require that the gene of interest be under the control of a good *E. coli* promoter such as lambda PR, lambda PL, tac, trc or lacUV5. The *E. coli* T7 S30 Extract System for Circular DNA contains T7 RNA Polymerase as well as the components required for translation, thus simplifying transcription/translation of DNA sequences cloned into plasmid or lambda vectors containing a T7 promoter.

II. Eukaryotic Cell-Free Translation Systems

This section provides information on specific parameters you need to be aware of when using eukaryotic *in vitro* translation systems. We recommend reviewing the

considerations that apply to the particular system being used before proceeding to the translation protocols detailed in Sections III–VII.

A. DNA Template Considerations

DNA purified using the PureYield™ Plasmid Midiprep System (Cat.# A2492, A2495) is sufficiently pure for use in TNT® Rabbit Reticulocyte Lysate or Wheat Germ Extract reactions. A standard (50µl) TNT® translation reaction requires 1µg of plasmid DNA as a template. However, 0.2–2.0µg of DNA template can provide satisfactory levels of translation, and adding more than 2µg of plasmid does not necessarily increase the amount of protein produced. For simultaneous expression from two or more DNA templates, we recommend adding approximately 0.5–1.0µg of each template, keeping the total amount of DNA added to 2µg or less.

Two template elements that are very helpful for increasing the efficiency of *in vitro* translation are an optimal Kozak sequence and a synthetic poly(A) tail of at least 30 nucleotides. Neither of these elements is required for translation using the TNT® Systems, but each can help improve translation efficiency. The Kozak sequence (Kozak, 1986) serves to position the ribosome at the initiating AUG codon of the translated RNA. Poly(A)⁺ sequences have been reported to affect the stability and, therefore, the level of protein synthesized in Rabbit Reticulocyte Lysate (Jackson and Standart, 1990). We have noticed a two- to fivefold increase in luciferase production when the luciferase gene is cloned into the pSP64 Poly(A) Vector (Cat.# P1241). Another important consideration is the length of untranslated sequence between the transcription start site and the translation start site—a long 5′ untranslated region can form secondary structures, which may inhibit translation. In addition, there may be additional AUG sequences present in the untranslated region that could be recognized as a translation start site, resulting in fusion proteins or incorrect products. We recommend limiting the length of 5′ untranslated regions to less than 100bp.

B. Protein Labeling

Most researchers label *in vitro* translation products with [³⁵S]methionine. If the protein of interest contains only a few methionine residues, however, it may be necessary to label with an alternative radioactive amino acid or with a non-radioactive labeling system (Table 5.3). If there are sufficient cysteine or leucine residues in the protein, or if both methionine and cysteine or leucine will be used together to label the protein, then the appropriate amino acid mixture can be included in the TNT® Coupled Reticulocyte Lysate Systems reaction. The TNT® Coupled Systems contain amino acid mixtures lacking either methionine, cysteine or leucine. Amino Acid Mixture Minus Methionine and Cysteine is available separately (Cat.# L5511). Conversely, we don't recommend using alternative radioactively labeled amino acids in the TNT® Quick

Table 5.2. Summary of Coupled Transcription/Translation Systems.

System	Template			PCR-Generated	RBS Required	Kozak Preferred	Labeling Options	Expected Yield ¹
	Linear DNA	Circular DNA						
TNT [®] Coupled Reticulocyte Lysate Transcription/Translation (T7, T3 or SP6 RNA Polymerase) (Cat.# L4610, L4950, L4600)	X ²	X	Not Recommended		-	X	Met, Cys, Leu, FluoroTect [™] , Transcend [™]	150-300ng
TNT [®] Quick Coupled Transcription/Translation (T7/SP6 RNA Polymerase) (Cat.# L1170, L2080)	X ³	X	X ³		-	X	Met, FluoroTect [™] , Transcend [™]	200-350ng
TNT [®] T7 Quick for PCR DNA (Cat.# L5540)	X	Not Recommended	X		-	X	Met, FluoroTect [™] , Transcend [™]	150-300ng
TNT [®] Insect Cell Extract Protein Expression System (Cat.# L1101)	Not Recommended	X	Not Recommended		-	X	Met, Cys, Leu, Transcend [™] , FluoroTect [™]	1-4µg
TNT [®] SP6 High-Yield Wheat Germ Protein Expression System (Cat.# L3260)	X	X	X		-	X	Met, Cys, Leu, Transcend [™] , FluoroTect [™]	1-5µg
TNT [®] Coupled Wheat Germ (T7, T3 and SP6 RNA Polymerase)(Cat.# L4120, L4130, L4140)	X ⁴	X ⁵	X		-	X	Met, Cys, Leu, FluoroTect [™]	150-300ng
<i>E. coli</i> T7 S30 Extract for Circular DNA (Cat.# L1130)	Not Recommended	X	Not Recommended		X	-	Met, Cys, Leu, Transcend [™] , FluoroTect [™]	~300ng
<i>E. coli</i> S30 for Circular DNA (Cat.# L1020)	Not Recommended	X	Not Recommended		X	-	Met, Cys, Leu, Transcend [™] , FluoroTect [™]	150-200ng
<i>E. coli</i> S30 for Linear DNA (Cat.# L1030)		Not Recommended	X		X	-	Met, Cys, Leu, Transcend [™] , FluoroTect [™]	100-250ng
S30 T7 High-Yield Protein Expression System (Cat.# L1110)	Not Recommended	X	Not Recommended		X	-	Met, Cys, Leu, Transcend [™] , FluoroTect [™]	10-25µg

¹Expected yield for 50µl reaction volumes.

²Only T7 linear templates.

³T7 promoter only, must use provided T7 TNT[®] PCR Enhancer.

⁴Must be linearized for T7.

Coupled Systems, since the master mix contains all amino acids except methionine, and the labeling efficiency with other amino acids will be significantly reduced.

Table 5.3. Recommended Concentrations of Alternative Radiolabeled Amino Acids.

Amino Acid	Final Concentration in Reaction	Volume Required
[³ H]leucine (100–200Ci/mmol)	0.5mCi/ml	5μl
[¹⁴ C]leucine (300mCi/mmol)	5μCi/ml	5μl
[³⁵ S]cysteine (1,200Ci/mmol)	0.3mCi/ml	5μl

[³⁵S] Methionine

We recommend using a “translational grade” [³⁵S]methionine such as PerkinElmer EasyTag™ L-[³⁵S]methionine (PerkinElmer Cat.# NEG709A). We have obtained acceptable results using 1–4μl of [³⁵S]methionine (1,200Ci/mmol at 10mCi/ml). Depending upon the translational efficiency of the experimental RNA/DNA and number of methionines present in the protein, the amount of [³⁵S]methionine can be adjusted to balance exposure time against label cost. When using and storing [³⁵S]methionine, follow the manufacturer’s recommendations.

Non-Radioactive Protein Labeling

The Transcend™ Non-Radioactive Translation Detection Systems (Cat.# L5070 and L5080) and the FluoroTect™ Green_{Lys} in vitro Translation Labeling System (Cat.# L5001) can be used with any of the TNT® Coupled or Quick Coupled Systems. These systems use a precharged lysine tRNA, which is incorporated into the translated protein. The Transcend™ System incorporates a biotinylated lysine, which can be detected by blotting and probing with streptavidin/AP or streptavidin HRP. The FluoroTect™ Reagent incorporates a fluorescently labeled lysine (BODIPY®), which can be detected directly in the gel.

III. Eukaryotic Cell-Free Expression Systems

The TNT® Coupled Reticulocyte Lysate Systems offer researchers an alternative for eukaryotic in vitro transcription and translation: a one-tube, coupled transcription/translation system. Standard Rabbit Reticulocyte Lysate or Wheat Germ Extract translations (Pelham and Jackson, 1976) commonly use RNA synthesized in vitro (Krieg and Melton, 1984) from SP6, T3 or T7 RNA polymerase promoters. The RNA is then used as a template for translation. The TNT® Systems bypass many of these steps by incorporating transcription directly in the translation mix.

In most cases, the TNT® System reactions produce significantly more protein (two- to sixfold) in a 1- to 2-hour reaction than standard in vitro Rabbit Reticulocyte Lysate

or Wheat Germ Extract translations using RNA templates. In addition, TNT® Lysates also can be used with microsomal membranes to study processing events (see Section VII).

A. TNT® SP6 High-Yield Protein Expression System

The TNT® SP6 High-Yield Protein Expression System uses a high-activity wheat germ extract supplemented with SP6 RNA polymerase and other components to express 10–100μg/ml of soluble protein in a one-tube coupled transcription/translation format. The system produces substantially more protein than conventional wheat germ systems while maintaining the ease of use that cell-free and coupled transcription and translation conditions afford. Following a two-hour incubation, the expressed protein can be used directly in downstream applications; no protein purification steps are necessary. For a detailed protocol and background information on this system, please see Technical Manual #TM282 (www.promega.com/tbs/tm282/tm282.html).

B. TNT® T7 Insect Cell Extract Protein Expression System

The TNT® T7 Insect Cell Extract Protein Expression System is a single-tube, coupled transcription/translation system for the cell-free expression of proteins. The extract is made from the commonly used *Spodoptera frugiperda* Sf21 cell line (Ezure *et al.* 2006) However, the extract contains no endogenous glycosylation machinery. All necessary components are present in the TNT® T7 ICE Master Mix (ICE = insect cell extract). Protein is expressed from genes cloned downstream of the T7 polymerase promoter. The best expression will be achieved using vectors that contain the baculovirus polyhedrin 5’ and 3’ untranslated region (Suzuki *et al.* 2006). Two vectors, the pF25A and pF25K ICE T7 Flexi® Vectors (Cat.# L1061 and L1081), have been designed specifically to produce optimal protein yields in this system. For a detailed protocol and background information on this system, please see Technical Manual #TM305 (www.promega.com/tbs/tm305/tm305.html).

The pF25A and pF25K ICE T7 Flexi® Vectors are compatible with the Flexi® Cloning System (Cat.# C8640), which allows easy transfer of sequences to and from additional Flexi® Vectors. Additional Flexi® Vectors are available for use with other protein expression systems. For a detailed protocol and background information on this system, please see Technical Manual #TM254 (www.promega.com/tbs/tm254/tm254.html).

C. TNT® Coupled Wheat Germ Extract Systems—Coupled Transcription/Translation

The TNT® Wheat Germ Extract Systems are available in five configurations for transcription and translation of genes cloned downstream from the SP6, T3 or T7 RNA polymerase promoter. With these systems, a 50μl reaction is programmed with 0.2–2μg of template and incubated for 1.5 hours at 30°C. For a detailed protocol and background information about this system, please see Technical Bulletin #TB165 (www.promega.com/tbs/tb165/tb165.html).

D. TNT® Quick Coupled Transcription/Translation Systems

The TNT® Quick Coupled Transcription/Translation Systems simplify the transcription/translation process by including all of the reaction components (RNA Polymerase, Nucleotides, salt and RNasin® Ribonuclease Inhibitor) together with the reticulocyte lysate solution in a single TNT® Quick Master Mix. The components of this Master Mix have been carefully adjusted to maximize both expression and fidelity for most gene constructs. When necessary, Magnesium Acetate and Potassium Chloride can be used to optimize *in vitro* translation reactions with the TNT® Quick Systems. The inclusion of RNasin® Ribonuclease Inhibitor directly in the Master Mix protects against potential disaster from the introduction of RNases carried over in the DNA solutions prepared using some miniprep protocols. The TNT® Quick System is available in two configurations for transcription and translation of genes cloned downstream from either the T7 or SP6 RNA polymerase promoters. For a detailed protocol and background information on this system, please see Technical Manual #TM045 (www.promega.com/tbs/tm045/tm045.html).

Protocol

- appropriate TNT® Quick Coupled Transcription/Translation System (Cat.# L1170, L1171, L2080, or L2081)
- Nuclease-Free Water (Cat.# P1193)
- radiolabeled amino acid (for radioactive detection) or Transcend™ tRNA (Cat.# L5061) or Transcend™ Colorimetric (Cat.# L5070) or Chemiluminescent (Cat.# L5080) Translation Detection System (for non-radioactive detection) or FluoroTect™ Green_{Lys} *in vitro* Translation Labeling System (for fluorescent detection; Cat.# L5001)

To use these systems, 0.2–2.0 µg of circular plasmid DNA containing a T7 or SP6 promoter, or a linearized plasmid or PCR-generated fragment containing a T7 promoter, is added to the TNT® Quick Master Mix and incubated for 60–90 minutes at 30°C. The synthesized proteins are then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detected (Figure 5.2).

The following is a general guideline for setting up transcription/translation reactions using plasmid or PCR-generated DNA as template. Examples of standard reaction setup using [³⁵S]methionine, Transcend™ Non-Radioactive Detection System or FluoroTect™ Green_{Lys} Systems are provided.

Plasmid DNA

Assemble the reaction components in a 0.5ml or 1.5ml microcentrifuge tube. After addition of all the components, gently mix by pipetting. If necessary, centrifuge briefly to return the reaction to the bottom of the tube. For the control reaction, use 1 µl of the Luciferase Control DNA supplied.

Note: We recommend also including a negative control reaction containing no added template to allow measurement of background incorporation of labeled amino acids.

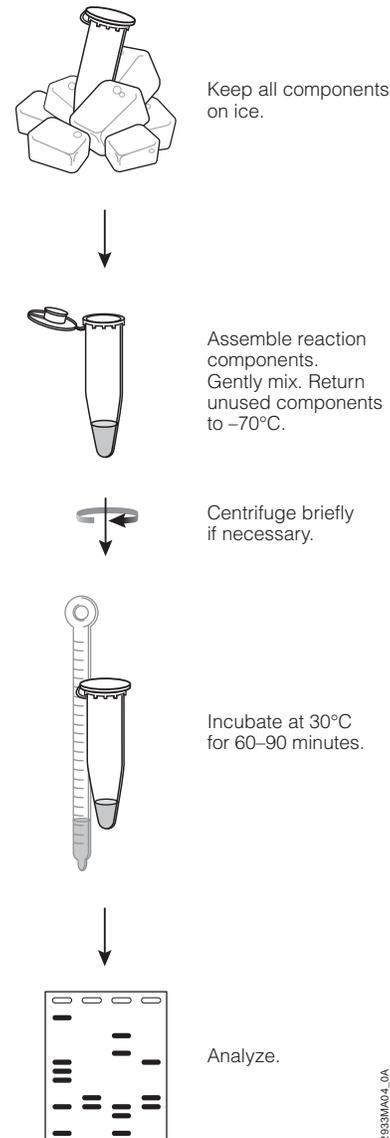


Figure 5.2. Flow chart illustrating the TNT® systems protocol.

Standard Reaction Conditions Using [³⁵S] Methionine, Transcend™ tRNA or FluoroTect™ Green_{Lys} tRNA.

Components	[³⁵ S] methionine	Transcend™ tRNA	FluoroTect™ Green _{Lys} tRNA
TNT® Quick Master Mix	40µl	40µl	40µl
Methionine, 1mM (mix gently prior to use)	–	1µl	1µl
[³⁵ S]methionine 1,000Ci/mmol at 10mCi/ml)	2µl	–	–
plasmid DNA template (0.5µg/µl)	2µl	2µl	2µl
Transcend™ Biotin-Lysyl-tRNA	–	1–2µl	–
FluoroTect™ Green _{Lys} tRNA	–	–	1–2µl
Nuclease-Free Water to a final volume of	50µl	50µl	50µl

PCR-Generated DNA Templates

For PCR-generated templates, the T7 TNT® PCR Enhancer should be included in the transcription/translation reaction.

Assemble the reaction components (below) in a 0.5ml or 1.5ml microcentrifuge tube. After addition of all the components, gently mix by pipetting. If necessary,

centrifuge briefly to return the reaction to the bottom of the tube. For the control reaction, use 1µl of the Luciferase Control DNA supplied.

Note: We recommend also including a negative control reaction containing no added template to allow measurement of background incorporation of labeled amino acids.

Standard Reaction Conditions Using PCR-Generated Templates with [³⁵S]Methionine, Transcend™ tRNA or FluoroTect™ Green_{Lys} tRNA.

Components	[³⁵ S] methionine	Transcend™ tRNA	FluoroTect™ Green _{Lys} tRNA
TNT® Quick Master Mix	40µl	40µl	40µl
Methionine, 1mM (mix gently prior to use)	–	1µl	1µl
[³⁵ S]methionine 1,000Ci/mmol at 10mCi/ml)	2µl	–	–
PCR-generated DNA template	2.5–5µl	2.5–5µl	2.5–5µl
T7 TNT® PCR Enhancer	1µl	1µl	1µl
Transcend™ Biotin-Lysyl-tRNA	–	1–2µl	–
FluoroTect™ Green _{Lys} tRNA	–	–	1–2µl
Nuclease-Free Water to a final volume of	50µl	50µl	50µl

Additional Resources for Eukaryotic Cell-Free Expression Systems

Technical Bulletins and Manuals

TM282	<i>TNT® SP6 High-Yield Protein Expression System Technical Manual</i> (www.promega.com/tbs/tm282/tm282.html)
TM305	<i>TNT® T7 Insect Cell Extract Protein Expression System Technical Manual</i> (www.promega.com/tbs/tm305/tm305.html)

TB165	<i>TNT® Coupled Wheat Germ Extract Systems Technical Bulletin</i> (www.promega.com/tbs/tb165/tb165.html)
TM045	<i>TNT® Quick Coupled Transcription/Translation Systems Technical Manual</i> (www.promega.com/tbs/tm045/tm045.html)

Promega Publications

PN058	<i>The TNT® T7 Quick Coupled Transcription/Translation System</i> (www.promega.com/pnotes/58/5189b/5189b.html)
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- PN060 Applications of the TNT® T7 Quick Coupled Transcription/Translation System (www.promega.com/pnotes/60/6079_07/promega.html)
- PN066 Application of the TNT® T7 Quick System to Selection and Evolution of Antibody Combining Sites (www.promega.com/pnotes/66/7014_14/7014_14.html)
- PN067 In vitro Expression Cloning Using the TNT® Coupled Reticulocyte Lysate System (www.promega.com/pnotes/67/7201_02/7201_02.html)
- PN070 Applications of Promega's In Vitro Expression Systems (www.promega.com/pnotes/70/7618_02/7618_02.html)
- PN081 Express More Functional Protein: TNT® Quick Coupled Transcription/Translation Systems (www.promega.com/pnotes/81/9939_16/9939_16.html)
- PN088 Technically Speaking: TNT® Rabbit Reticulocyte Lysate Systems—Easy Protein Expression (www.promega.com/pnotes/88/12162_24/12162_24.html)
- PN093 TNT® SP6 High-Yield Protein Expression System: More Protein from a Coupled Transcription/Translation System (www.promega.com/pnotes/93/14023_15/14023_15.html)
- PN100 Cell-Free Protein Expression with the TNT® T7 Insect Cell Extract Protein Expression System (www.promega.com/pnotes/100/16620_11/16620_11.html)

Citations

Nagase, T. *et al.* (2008) Exploration of human ORFeome: High-throughput preparation of ORF clones and efficient characterization of their protein products. *DNA Research* 15, 137–149.

The authors created HaloTag® fusion proteins and examined expression of these proteins in vitro and in COS7 and HEK293 cells. They also performed comparisons between the Flexi® System and Gateway® cloning system, specifically examining the effects of flanking sequences on protein expression in in vitro translation systems including the SP6 High-Yield Protein Expression System. They confirmed that the cellular localization of the HaloTag® fusion proteins was consistent with results obtained using GFP-fusions.

PubMed Number: 18316326

Citations

Muik, M. *et al.* (2008) Dynamic coupling of the putative coiled-coil domain of ORAI1 with STIM1 mediates ORAI1 channel activation. *J. Biol. Chem.* 283, 8014–8022.

The authors performed protein pull-down assays to characterize the interaction of ORAI1 and STIM1, two protein components of the calcium-release calcium current. His6-STIM1 C terminus and ORAI1 were synthesized using the TNT® Coupled Reticulocyte Lysate System in the presence of 35S, and His6-STIM1 C terminus was immobilized using MagZ™ Binding Particles. An aliquot of the TNT® reaction expressing ORAI1 was added to the particles, and proteins were washed, eluted using increasing concentrations of imidazole (10–40mM) and analyzed by SDS-PAGE. In a second set of pull-down assays, His6-STIM1 C terminus was used to pull down ORAI1 N- and C-terminal fragments expressed as GST fusion proteins. The His6-STIM1 C terminus protein was purified from transiently transfected HEK293 cells using the MagneHis™ Protein Purification System.

PubMed Number: 18187424

IV. Rabbit Reticulocyte Lysate Translation Systems**A. Standard Rabbit Reticulocyte Lysate Translation**

The Rabbit Reticulocyte Lysate Translation System plays an important role in characterization of mRNA translation products, investigation of transcriptional and translational control, and co-translational processing of secreted proteins by the addition of microsomal membranes to the translation reaction. Rabbit Reticulocyte Lysate is prepared from New Zealand white rabbits injected with phenylhydrazine using a standard protocol to increase reticulocyte production (Pelham and Jackson, 1976). The reticulocytes are harvested, and any contaminating cells that could otherwise alter the translational properties of the final extract are removed. After lysis of the reticulocytes, the extract is treated with micrococcal nuclease to digest endogenous mRNA and thus reduce background translation to a minimum. The lysate contains the cellular components necessary for protein synthesis: tRNA, ribosomes, amino acids, and initiation, elongation and termination factors. Reticulocyte Lysate is further optimized for mRNA translation by adding several supplements as described in Section I.A.

Rabbit reticulocyte lysate has been reported to contain a variety of post-translational processing activities, including acetylation, isoprenylation, proteolysis and some phosphorylation activity (Glass and Pollard, 1990). Processing events such as signal peptide cleavage and core glycosylation can be examined by adding canine microsomal membranes to a translation reaction (Andrews, 1987; Walter and Blobel, 1983; Thompson and Beckler, 1992)

B. Flexi® Rabbit Reticulocyte System—In Vitro Translation

The Flexi® Rabbit Reticulocyte Lysate System allows greater flexibility of reaction conditions than the standard Rabbit Reticulocyte Lysate System, Nuclease Treated. Different

mRNAs commonly exhibit different optimum salt concentrations for translation. Furthermore, small variations in salt concentration can lead to dramatic differences in translation efficiency. The Flexi® Rabbit Reticulocyte Lysate System allows optimization of a wide range of parameters, including Mg²⁺ and K⁺ concentrations, and offers the choice of adding DTT. To help optimize Mg²⁺ for a specific message, the endogenous Mg²⁺ concentration of each lysate batch is stated on the product insert. The Flexi® System also offers the choice of three amino acid mixtures and includes a control RNA encoding the firefly luciferase gene. For a detailed protocol and background information about this system, please see Technical Bulletin #TB127 (www.promega.com/tbs/tb127/tb127.html).

Protocol

Materials Required:

- Flexi® Rabbit Reticulocyte Lysate System (Cat.# L4540)
- RNasin® Ribonuclease Inhibitor or RNasin® Plus RNase Inhibitor (Cat.# N2111 or N2611)
- Nuclease-Free Water (Cat.# P1193)
- radiolabeled amino acid (for radioactive detection) or Transcend™ tRNA (Cat.# L5061) or Transcend™ Colorimetric (Cat.# L5070) or Chemiluminescent (Cat.# L5080) Translation Detection System (for non-radioactive detection) or FluoroTect™ Green_{Lys} in vitro Translation Labeling System (for fluorescent detection; Cat.# L5001)

The following is a general guideline for setting up a Flexi® Lysate translation reaction. Also provided is an example of a standard reaction. The reaction uses [³⁵S]methionine as the radiolabel; other isotopes may also be used (see Table 5.3). For the positive control reaction, use 1–2 µl of the Luciferase Control RNA supplied.

1. Assemble the following reaction components in a 0.5ml or 1.5ml tube.

Note: We recommend also including a negative control reaction containing no added template to allow measurement of background incorporation of labeled amino acids.

Component	Volume
Flexi® Rabbit Reticulocyte Lysate	33 µl
Amino Acid Mixture Minus Methionine, 1mM	1 µl
[³⁵ S]methionine (1,200Ci/mmol at 10mCi/ml)	2 µl
Magnesium Acetate, 25mM	0–4 µl
Potassium Chloride, 2.5M	1.4 µl
DTT, 100mM	0–1 µl
RNasin® Ribonuclease Inhibitor (40u/ml)	1 µl
RNA substrate	1–12 µl
Nuclease-Free Water to final volume	50 µl

2. Incubate the translation reaction at 30°C for 60–90 minutes.
3. Analyze the results of translation.

C. RNA Template Considerations

Use a final concentration of 5–80 µg/ml of in vitro transcripts produced with the RiboMAX™ Large Scale RNA Production Systems (Cat.# P1280 and P1300) for the translation. RNA from other standard transcription procedures may contain components at concentrations that inhibit translation. Therefore, a lower concentration, 5–20 µg/ml of in vitro transcript, should be used with these systems. The optimal RNA concentration should be determined before performing experiments. Average preparations of mRNA stimulate translation about 10- to 20-fold over background (i.e., no exogenous RNA template). To determine the optimal concentration, serially dilute your RNA template first and then add the same volume of RNA to each reaction. This ensures that other variables are kept constant. In addition, the presence of certain nucleic acid sequence elements can have profound effects on initiation fidelity and translation efficiency; 3'-poly(A)⁺ sequences, 5'-caps, 5'-untranslated regions and the sequence context around the AUG start, or secondary AUGs in the sequence (Kozak, 1990).

The presence of inhibitors can significantly reduce translation efficiency. Oxidized thiols, low concentrations of double-stranded RNA and polysaccharides are typical inhibitors of translation in rabbit reticulocyte lysate (Jackson and Hunt, 1983). To determine if inhibitors are present in your mRNA preparation, mix your RNA with Luciferase Control RNA and determine if translation of luciferase RNA is inhibited relative to a control translation containing only the luciferase RNA. Residual ethanol should also be removed from mRNA preparations and labeled amino acids before they are added to the translation reaction.

You may need to optimize the potassium and magnesium concentrations in your translation reactions. Addition of 0.5–2.5mM Mg²⁺ is generally sufficient for the majority of mRNAs. See Tables 5.4 and 5.5 for the concentrations of key components present in the lysate.

Table 5.4. Final Concentrations of Rabbit Reticulocyte Lysate Components in a 50 µl Translation Reaction.

Components	Final Concentration
Creatine phosphate	10mM
Creatine phosphokinase	50 µg/ml
DTT	2mM
Calf liver tRNA	50 µg/ml
Potassium acetate	79mM
Magnesium acetate	0.5mM
Hemin	0.02mM

Table 5.5. Approximate Endogenous Amino Acid Pools.

Amino Acid	Concentration (μM) ¹	Amino Acid	Concentration (μM) ¹
Ala	157	Leu	5
Asn	51	Lys	51
Asp	1093	Met	5
Arg	41	Phe	4
Cys	2	Pro	87
Gln	200	Ser	93
Glu	260	Thr	59
Gly	1050	Trp	1
His	14	Tyr	3
Ile	9	Val	30

¹These amino acid concentrations should be used only as estimates. These values are not determined for individual lots of Rabbit Reticulocyte Lysate.

For many years, there have been discussions in the literature concerning which potassium salt is preferable in rabbit reticulocyte translation reactions. Potassium acetate (KOAc) often was used, rather than potassium chloride (KCl), because the chloride ion was shown to be inhibitory to translation, while higher levels of the acetate salt were not (Weber *et al.* 1977). However, several advantages of adding potassium chloride to rabbit reticulocyte translation reactions have been identified. Adding KCl, in addition to KOAc, can improve the fidelity of initiation from capped messages (Kozak, 1990). Uncapped *in vitro*-generated RNAs are reported to be translated with greater initiation fidelity using KCl instead of KOAc (Jackson, 1991). It also has been reported that high (120mM) levels of added KCl greatly enhance translational efficiency of EMCV (encephalomyocarditis virus) RNA (Jackson, 1991; Beckler, 1992). Although each RNA transcript will have its own optimal KCl concentration for translation, Table 5.6 can be used as a rough guideline (the volumes recommended are for a 50μl final reaction volume). If further optimization of salt concentrations is required, we recommend using the Flexi® Rabbit Reticulocyte Lysate.

Table 5.6. KCl Concentration for Translation.

Type of RNA	Suggested KCl Concentration	Volume of 2.5M KCl Required
"Run-Off" transcript	40–70mM	0.8–1.4ml
Poly(A)-containing transcript (e.g., pSP64 poly(A) Vector)	60–90mM	1.2–1.8ml
EMCV UTR-containing transcript	100–120mM	2.0–2.4ml
Capped RNAs	70–100mM	1.4–2.0ml

D. Optimization

The 66% concentration of lysate in the standard reaction is optimal for most applications. If desired, the lysate can be diluted 50 to 60% without a substantial reduction in

translational efficiency. If optimal expression is desired in a reduced lysate concentration reaction, then the levels of Mg²⁺ and K⁺ must be adjusted to account for the reduction in Mg²⁺ and K⁺ from the rabbit reticulocyte lysate. The endogenous Mg²⁺ concentration of each lysate batch is listed on the product insert. Because the endogenous K⁺ concentration of each lysate batch is not determined, the optimal amount of K⁺ will have to be determined empirically.

Mg²⁺ is absolutely required and is the most critical component affecting translation. The range of Mg²⁺ for optimal translation is very narrow, and therefore, small changes in Mg²⁺ concentration can dramatically affect the efficiency of translation. Furthermore, each RNA transcript will exhibit an individual optimal Mg²⁺ concentration. To provide information useful for optimizing translation, the endogenous Mg²⁺ concentration of each lysate batch is stated on the product insert. For many RNA transcripts, this endogenous level should be very close to the optimal concentration. To determine if additional Mg²⁺ stimulates translation for a specific transcript, add 0–4μl of the provided Magnesium Acetate to a 0–2mM final added concentration in the standard 50μl reaction. High Mg²⁺ concentrations, though, can reduce the fidelity of translation and should be avoided (Snyder and Edwards, 1991).

No DTT is added to the Flexi® Rabbit Reticulocyte Lysate during production. DTT can prevent the formation of disulfide bridges in proteins. If the alteration in structure affects the active site of the protein, the protein may be inactive. To study protein activity, we recommend that DTT not be added to the translation reaction. We have compared lysates prepared with or without added DTT. In those lysates prepared without DTT, we have added back DTT after thawing the stored lysate. We found no differences in translational efficiency or fidelity from these lysate combinations. If desired, 1μl of the provided 100mM DTT can be added to a 50μl (66%) lysate reaction to provide an identical concentration of DTT to that found in a standard Rabbit Reticulocyte Lysate reaction (2mM).

Additional Resources for Rabbit Reticulocyte Lysate Translation Systems

Technical Bulletins and Manuals

TB127	<i>Flexi® Rabbit Reticulocyte Lysate System Technical Bulletin</i> (www.promega.com/tbs/tb127/tb127.html)
TM232	<i>Rabbit Reticulocyte Lysate System Technical Manual</i> (www.promega.com/tbs/tm232/tm232.html)

Citations

Tanaka, M., Chock, P.B. and Stadtman, E.R. (2007) Oxidized messenger RNA induces translation errors. *Proc. Natl. Acad. Sci. USA* **104**, 66-71..

Oxidative damage has been associated with a range of age-related neurological conditions. In this study, the effect of mRNA oxidation was investigated. A direct correlation was observed between the extent of oxidation and the frequency of translation errors. The effects of oxidation of mRNA were investigated in in vitro translation experiments using mRNA treated with an iron-ascorbate mixture and hydrogen peroxide. Translation in vitro was performed using rabbit reticulocyte lysate supplemented with protease inhibitors. The translation products were detected using anti-FLAG and anti-c-Myc antibodies.

PubMed Number: 17190801

V. Wheat Germ Extract—Cell-Free Translation

A. Description

Wheat Germ Extract is prepared by grinding wheat germ in an extraction buffer, followed by centrifugation to remove cell debris. The supernatant is then separated by chromatography from endogenous amino acids and plant pigments that inhibit translation. The extract is treated with micrococcal nuclease to destroy endogenous mRNA and thus reduce background translation to a minimum. The extract contains the cellular components necessary for protein synthesis: tRNA, rRNA, and initiation, elongation and termination factors, and optimized further by the addition of several supplements as described in Section I.A.

Only the addition of exogenous amino acids (including an appropriate labeled amino acid) and mRNA are necessary to stimulate translation. Potassium acetate is supplied as an individual component so that the translational system may be additionally enhanced for a wide range of mRNAs.

The reaction conditions provided below are optimized for the BMV Control supplied with the system and should be considered a starting point for experiments. However, many factors affect translation efficiency of specific RNAs in Wheat Germ Extracts and should be considered when designing in vitro translation experiments (see Section II). For a detailed protocol and background information about this system, please see Technical Manual #TM230 (www.promega.com/tbs/tm230/tm230.html).

The optimal RNA concentration for translation should be determined before performing definitive experiments. To do this, serially dilute your RNA template first and then add the same volume of RNA to each reaction to ensure that other variables are kept constant.

Optimum potassium concentration varies from 50–200mM, depending on the mRNA. The optimal potassium concentration for translation of BMV RNA is 130mM. If this concentration results in poor translation of your sample mRNA, potassium levels should be adjusted to an optimum concentration. Certain mRNAs may also require altered magnesium concentration; optimum magnesium

concentration for the majority of mRNAs is expected to fall in the range of 2–5mM. See Table 5.7 for the concentrations of key exogenous components of Wheat Germ Extract.

Table 5.7. Final Concentrations of Supplements added to the Wheat Germ Extract in a 50µl Translation Reaction.

Components	Final Concentration
Creatine phosphate	10mM
Creatine phosphokinase	50µg/ml
DTT	5mM
Calf liver tRNA	50µg/ml
Magnesium acetate	2.1mM
Potassium acetate	53mM ¹
Spermidine	0.5mM
ATP	1.2mM
GTP	0.1mM

¹Additional potassium acetate may need to be added to optimize translation for each sample RNA.

B. Protocol

Materials Required:

- Wheat Germ Extract System (Cat.# L4380)
- RNasin® Ribonuclease Inhibitor (Cat.# N2111 or N2511)
- radiolabeled amino acid
- Nuclease-Free Water (Cat.# P1193)

The reaction below uses [³⁵S]methionine; other isotopes may also be used (see Table 5.3). For the control reaction, use 2µl of BMV Control RNA.

1. Set up the following reaction in a 0.5 or 1.5 ml tube.

Note: We recommend also including a negative control reaction containing no added template to allow measurement of background incorporation of labeled amino acids.

Component	Volume
Wheat Germ Extract	25µl
1mM Amino Acid Mixture (Minus Methionine)	4µl
RNA substrate in water	2µl
Potassium Acetate, 1M	0–7µl
RNasin® Ribonuclease Inhibitor (at 40u/µl)	1µl
[³⁵ S]methionine (1,200Ci/mmol) at 10mCi/ml ¹	2.5µl
Nuclease-Free Water to final volume	50µl

¹Final [³⁵S]methionine concentration = 0.5mCi/ml.

2. Incubate at 25°C for 60–120 minutes.
3. Analyze results.

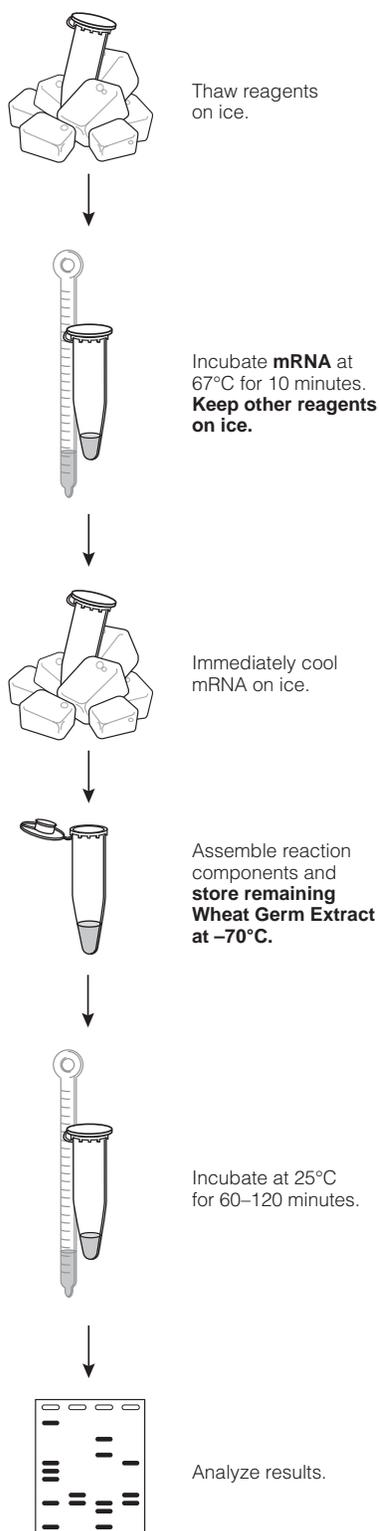


Figure 5.3. Schematic of translation using the Wheat Germ Extract.

Additional Resources for the Wheat Germ Extract

Technical Bulletins and Manuals

TM230 *Wheat Germ Extract Technical Manual*
www.promega.com/tbs/tm230/tm230.html

VI. Co-Translational Processing Using Canine Pancreatic Microsomal Membranes

A. Description

Microsomal vesicles are used to study co-translational and initial post-translational processing of proteins (Rando, 1996; Han and Martinage, 1992; Chow *et al.* 1992). Processing events such as signal peptide cleavage, (MacDonald *et al.* 1988), membrane insertion (Ray *et al.* 1995), translocation and core glycosylation (Bocco *et al.* 1988) can be examined by translation of the appropriate mRNA in vitro in the presence of microsomal membranes. Processing and glycosylation events may also be studied by transcription/translation of the appropriate DNA in TNT® Rabbit Reticulocyte Lysate Systems when used with microsomal membranes. To assure consistent performance with minimal translational inhibition and background, Promega microsome preparations have been isolated free from contaminating membrane fractions and stripped of endogenous membrane-bound ribosomes and mRNA. Membrane preparations are assayed for both signal peptidase and core glycosylation activities using two different control mRNAs. The two control mRNAs supplied with the Canine Microsomal Membranes are the precursor for β -lactamase (or ampicillin resistance gene product) from *E. coli* and the precursor for α -mating factor (or α -factor gene product) from *S. cerevisiae*.

For a detailed protocol and background information about this system, please see Technical Manual #TM231 (www.promega.com/tbs/tm231/tm231.html).

B. General Protocols for Translation with Microsomal Membranes

While the reaction conditions described here are suitable for most applications, the efficiency of processing using other membranes may vary. Thus, reaction parameters may have to be altered to suit individual requirements. In general, increasing the amount of membranes in the reaction increases the proportion of polypeptides translocated into vesicles but reduces the total amount of polypeptide synthesized. The amount of protein produced in lysates using Microsomal Membranes will be less than the amount produced in lysate alone. Depending on the DNA or RNA used, translation efficiency can be expected to drop between 10–50% in the presence of Microsomal Membranes.

Materials Required:

- Canine Pancreatic Microsomal Membranes (Cat.# Y4041)
- appropriate Reticulocyte Lysate System
- Recombinant RNasin® Ribonuclease Inhibitor (Cat.# N2511)

- radiolabeled amino acid
 - Nuclease-Free Water (Cat.# P1193)
1. Mix the following components on ice, in the order given, in a sterile 1.5ml microcentrifuge tube.

Translation Reaction with TNT® Rabbit Reticulocyte

Components	Volume
TNT® Lysate	12.5µl
TNT® Reaction Buffer	0.5µl
Amino Acid Mixture Minus Methionine, 1mM	0.5µl
TNT® RNA Polymerase (SP6, T3 or T7)	0.5µl
[³⁵ S]methionine (1,200Ci/mmol, at 10mCi/ml)	2.0µl
Nuclease-Free Water	5.5µl
Plasmid DNA, 0.5mg	0.5µl
Canine Microsomal Membranes	2.5µl
final volume	25.0µl

Translation Reaction with Rabbit Reticulocyte Lysate System, Nuclease-Treated

Components	Volume
Rabbit Reticulocyte Lysate, Nuclease-Treated	17.5µl
1mM Amino Acid Mixture (Minus Methionine)	0.5µl
[³⁵ S]methionine (1,200Ci/mmol, at 10mCi/ml)	2.0µl
Nuclease-Free Water	2.2µl
Canine Microsomal Membranes	1.8µl
RNA substrate in water ¹	1.0µl
final volume	25.0µl

¹For the control reactions, use pre β -lactamase and α -factor mRNA at 0.1µg/ml.

2. Incubate for 90 minutes at 30°C.
3. Analyze results.

C. Analysis of Results

When using 1.8µl of Microsomal Membranes per 25µl of translation mix, 90% of pre- β -lactamase will be processed to β -lactamase. The same amount of membranes will process 75–90% of α -factor to core glycosylated forms of α -factor. Upon SDS-PAGE, the precursor for β -lactamase migrates at 31.5kDa and the processed β -lactamase at 28.9kDa. The precursor for the α -factor migrates at 18.6kDa, and the core-glycosylated α -factor has a molecular weight of 32.0kDa but will migrate faster than the β -lactamase precursor (Figure 5.4).

In some cases, it is difficult to determine if efficient processing or glycosylation has occurred by gel analysis alone. These alternative assays for detecting co-translational processing events may be useful. A general assay for co-translational processing uses the protection afforded the translocated protein domain by the lipid bilayer of the

microsomal membrane. In this assay, protein domains are judged to be translocated if they are observed to be protected from exogenously added protease. To confirm that protection is due to the lipid bilayer, addition of 0.1% non-ionic detergent (such as Triton® X-100 or Nikkol) solubilizes the membrane and restores susceptibility to protease. Many proteases have proven useful for monitoring translocation in this fashion including protease K and trypsin (final concentration 0.1mg/ml; Gross *et al.* 1988).

An alternative procedure uses endoglycosidase H to determine the extent of glycosylation of translation products (Andrews, 1987). In cell-free systems, N-linked glycosylation occurs only within intact microsomes. Endoglycosidase H cleaves the internal N-acetylglucosamine residues of high mannose carbohydrates, resulting in a shift in apparent molecular weight on SDS-polyacrylamide gels to a position very close to that of the nonglycosylated species. The reaction conditions (0.1% SDS, 0.1M sodium citrate [pH 5.5] incubation at 37°C for 12 hours) are not compatible with those required to maintain membrane integrity. For this reason, translocated polypeptides are not “protected” from digestion with endoglycosidase H.

Additional Resources for Canine Microsomal Membranes**Technical Bulletins and Manuals**

TM231 *Canine Pancreatic Microsomal Membranes Technical Manual*
(www.promega.com/tbs/tm231/tm231.html)

Promega Publications

PN038 Post-translational processing: Use of the TNT® Lysate Systems with Canine Microsomal Membranes
(www.promega.com/pnotes/38/38_15/38_15.htm)

PN070 Applications of Promega's In Vitro Expression Systems
(www.promega.com/pnotes/70/7618_02/7618_02.html)

Citations

He, W. *et al.* (2007) The membrane topology of RTN3 and its effect on binding of RTN3 to BACE1. *J. Biol. Chem.* **282**, 29144–29151.

The authors of this study determined the membrane topology of reticulon 3 (RTN3), an integral membrane protein that is expressed at high levels in neurons and has been shown to negatively regulate the activity of BACE1 (Beta site APP-Cleaving Enzyme). Disruption of RTN3 is associated with incidence of dystrophic neurites in AD brain. RTN3 was translated using the TNT® Quick Coupled Transcription/Translation System in the presence of Canine Microsomal Membranes and labeled using the Transcend™ Non-Radioactive Translation Detection System.

PubMed Number: 17699523

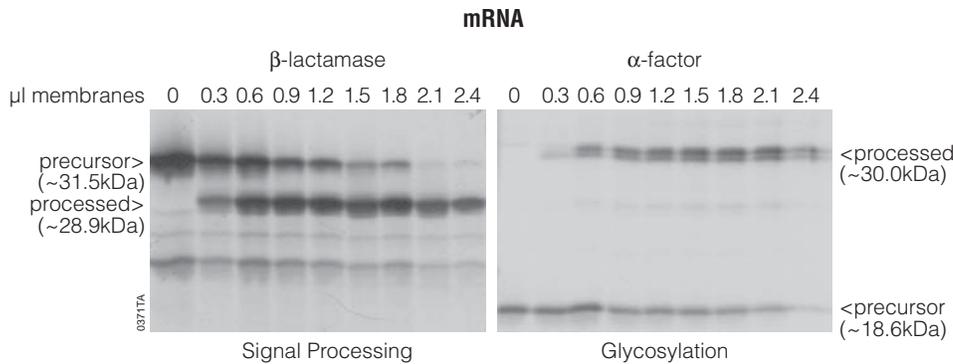


Figure 5.4. Processing and glycosylation activity of Canine Microsomal Membranes. The positive control mRNAs (0.1µg of *E. coli* β-lactamase and 0.1µg of *S. cerevisiae* α-factor) were translated using Rabbit Reticulocyte Lysate in a 25µl reaction for 60 minutes in the presence of the indicated amounts of Microsomal Membranes. Aliquots (1.67µl) were then analyzed by SDS-PAGE and autoradiography of the ³⁵S-labeled proteins.

VII. Prokaryotic Cell-Free Translation Systems

This section provides information on specific parameters that require optimization for prokaryotic in vitro translation reactions. We recommend that you review the considerations that apply to the particular system being used before proceeding to the translation protocols (Section VII.D).

A. Description

The S30 extracts in the *E. coli* S30 Extract Systems are prepared by modifications of the methods described by Zubay (Zubay, 1973; Zubay, 1980; Lesley *et al.* 1991). The *E. coli* S30 Extract System for Linear Templates (Cat.# L1030) is prepared from *E. coli* B strains deficient in ompT endoproteinase and lon protease activity. This results in greater stability of expressed proteins, which would otherwise be degraded by proteases if expressed in vivo (Pratt, 1984; Studier and Moffatt, 1986). The *E. coli* B strain used to produce the S30 Extract for Linear Templates is also deficient in exonuclease V (the recBCD enzyme). The recD mutation of this strain produces a more active S30 Extract System for Linear DNA than previously described nuclease-deficient recBC-derived S30 extracts (Lesley *et al.*, 1991; Pratt, 1984; Chen and Zubay, 1983). However, the S30 Extract for Linear Templates is less active than the S30 Extract System for Circular DNA and T7 S30 Extract. The *E. coli* S30 Extract System for Circular DNA also allows higher expression levels of proteins that are normally expressed at low levels in vivo due to the action of host-encoded repressors (Collins, 1979). For a detailed protocol and background information about the *E. coli* S30 Extract Systems, please see Technical Bulletin #TB092 (www.promega.com/tbs/tb092/tb092.html), Technical Bulletin #TB102 (www.promega.com/tbs/tb102/tb102.html) and Technical Bulletin #TB219 (www.promega.com/tbs/tb219/tb219.html).

The *E. coli* T7 S30 Extract System for Circular DNA simplifies transcription/translation of DNA sequences cloned in plasmid or lambda vectors containing a T7 promoter by providing an extract that contains T7 RNA Polymerase for transcription and all necessary components

for translation. The researcher only supplies the cloned DNA containing a T7 promoter and a ribosome binding site.

The *E. coli* S30 Systems contain an S30 Premix Without Amino Acids that is optimized for each lot of S30 Extract and contains all other required components, including rNTPs, tRNAs, an ATP-regenerating system, IPTG and appropriate salts. Amino acid mixtures lacking cysteine, methionine or leucine are provided to facilitate radiolabeling of translation products.

The most common application of *E. coli* S30 Extract Systems is the synthesis of small amounts of radiolabeled protein. The synthesis of a protein of the correct size is a useful way to verify gene products. Proteins expressed in the *E. coli* S30 Extract Systems may also be used for a variety of functional transcription and translation studies. Additional applications of the *E. coli* S30 Extract Systems include synthesis of small amounts of radiolabeled protein for use as a tracer in protein purification (Promega Notes 26, 1990) and incorporation of unnatural amino acids into proteins for structural studies (Noren *et al.* 1989).

B. Template Considerations

Use only highly purified DNA templates (e.g., CsCl- or gel-purified) and avoid adding high concentrations of salts or glycerol with the DNA template. The activity of the *E. coli* S30 Systems may be inhibited by NaCl (≥50mM), glycerol (≥1%), and by small amounts of Mg²⁺ (1–2mM) or potassium salts (50mM). The DNA template should be ethanol-precipitated with sodium acetate rather than ammonium acetate. Protein yields from the *E. coli* S30 Extract Systems vary with the template and the conditions used. Typical protein yields range from 50–250ng per 50µl reaction.

S30 Extract and T7 S30 Extract for Circular DNA

Expression of cloned DNA fragments in the *E. coli* S30 Extract System for Circular DNA requires that the gene be under the control of a good *E. coli* promoter. Examples of such promoters include lambda PR, lambda PL, tac, trc and lacUV5. Expression of cloned DNA fragments in the T7 S30

Circular System requires that the gene be under the control of either a T7 or a good *E. coli* promoter. Expression levels from T7 promoters are typically higher than that from *E. coli* promoters in this extract. Expression from *E. coli* promoters can be inhibited by the addition of rifampicin to the extract; transcription by T7 RNA Polymerase is resistant to rifampicin.

S30 Extract for Linear DNA or RNA

Expression of gene products from linear DNA containing supercoiling-sensitive promoters can be reduced in the S30 System by up to 100-fold (Chen and Zubay, 1983). Examples of good promoters that are supercoiling-insensitive include lacUV5, tac, λ and λ PR. DNA from other prokaryotic species may not contain promoters that direct transcription in the *E. coli* S30 Extract System for Linear Templates. RNA generated in vitro from cloned genes lacking an *E. coli* promoter is also suitable. Larger templates, such as bacteriophage lambda DNA, may also be used.

PCR-Generated Templates

PCR technology has introduced many methods for site-specific in vitro mutagenesis. Combining PCR with phage λ exonuclease treatments has produced mutated fragments larger than 2.5kb (Shyamala and Ames, 1991). PCR products can be added to the *E. coli* S30 Extract System for Linear Templates for rapid confirmation of expected protein size or activity.

Avoid contaminating the S30 Extract reaction with the wrong PCR product or primer dimers. If agarose gel analysis indicates that your PCR reaction produced a unique band, primer dimers can be removed by ethanol precipitation with sodium acetate. Otherwise, PCR-amplified DNA should be gel purified before use.

Restriction Enzyme-Digested Templates

For restriction enzyme-digested DNA, 10–20 μ g of DNA should be digested in a 100–200 μ l reaction volume. Ethanol precipitate and resuspend the DNA at a concentration of 1 μ g/ μ l in TE buffer or water. Add 2–4 μ g of this DNA directly to the S30 reaction. However, if the desired results are not obtained, the DNA should be further purified by phenol extraction followed by ethanol precipitation.

RNA Templates

The amount of in vitro RNA added to the extract can vary from 10–100 μ g. For synthesizing milligram quantities of highly pure, “translatable” RNA, we recommend using one of the RiboMAX™ Large Scale RNA Production Systems (Cat.# P1280 and P1300; RiboMAX™ Large Scale RNA Production Systems—SP6 and T7 Technical Bulletin, #TB166 (www.promega.com/tbs/tb166/tb166.html)).

C. S30 T7 High-Yield Protein Expression System

The S30 T7 High-Yield Protein Expression System is an *E. coli* extract-based cell-free protein synthesis system. It simplifies the transcription and translation of DNA sequences cloned in plasmid or lambda vectors containing a T7 promoter by providing an extract that contains T7 RNA polymerase for transcription and all necessary components for translation. This system can produce high

levels of recombinant proteins (up to hundreds of micrograms of recombinant protein per milliliter of reaction) within an hour using a vector containing the sequence of interest, a T7 promoter and a ribosome-binding site (RBS).

The S30 T7 High-Yield Protein Expression System contains the T7 S30 Extract, Circular, which is prepared by modifications of the method described by Zubay (Zubay, 1973 and Zubay, 1980) from an *E. coli strain* B deficient in OmpT endoproteinase and lon protease activity. This results in greater stability for translated proteins that would otherwise be degraded by proteases if expressed in vivo (Studier and Moffatt, 1986; Pratt, 1984). An optimized S30 Premix Plus provides all other required components, including amino acids, rNTPs, tRNAs, an ATP-regenerating system, IPTG and appropriate salts to express high levels of recombinant proteins.

Using this system, protein can be expressed in volumes as small as 5 μ l for high-throughput screening in a 96-well plate format. The amount of protein synthesized increases proportionally with reaction volume (up to 250 μ l). Because it is a cell-free system, the S30 T7 High-Yield Protein Expression System can be used to express proteins that are toxic to *E. coli* cells. Finally, small amounts of target protein can be purified using affinity tags (e.g., metal-affinity tag) for downstream analysis. For a detailed protocol and background information about the S30 T7 High-Yield Protein Expression System, please see Technical Manual #TM306 (www.promega.com/tbs/tm306/tm306.html).

D. *E. coli* S30 Extract Systems Protocol

Materials Required:

- appropriate *E. coli* S30 Extract System (Cat.# L1020, L1030 or L1130)
- radiolabeled amino acid
- Nuclease-Free Water (Cat.# P1193)

The following protocol is designed for use with the *E. coli* S30 Extract Systems for Circular (including the T7 System) or Linear DNA Templates and [³⁵S]methionine as the radiolabel. Other radioisotopes may also be used (see Table 5.3). For positive control reactions, use 4 μ l of the Control DNA provided. For multiple reactions, create a master mix by combining the appropriate volumes of Amino Acid Mixture Minus Methionine (or Cysteine or Leucine), S30 Premix Without Amino Acids, radiolabeled amino acid (optional), S30 Extract and Nuclease-Free Water. Aliquot the master mix into 1.5ml microcentrifuge tubes and initiate the reactions by adding the DNA template to the tubes.

1. Set up the following reaction in a 1.5ml tube.

Component	Volume
DNA template	≤4μg
Amino Acid Mixture Minus Methionine (mix gently prior to use)	5μl
S30 Premix Without Amino Acids (mix gently prior to use)	20μl
[³⁵ S]methionine (1,200Ci/mmol, at 10mCi/ml)	1μl
S30 Extract, Circular/Linear/T7 (mix gently prior to use)	15μl
Nuclease-Free Water to final volume	50μl

2. Vortex gently, then centrifuge in a microcentrifuge for 5 seconds to collect the reaction mixture at the bottom of the tube.
3. Incubate the reaction at 37°C for 1–2 hours.
4. Stop the reaction by placing the tubes in an ice bath for 5 minutes.
5. Analyze the results of the reaction.

Optimization

The amount of DNA added should be optimized. In general, reactions should not contain more than 4μg of DNA. An increased amount of DNA can result in higher incorporation of label but can also increase the number of internal translational starts or prematurely arrested translation products detected. For the positive control reactions, use the PinPoint™ Control Vector DNA for T7 systems and pBEST_{luc}™ DNA for non-T7 systems. Template DNA/RNA and water purity are extremely important. If efficiencies are low, examine the quality of the template DNA and water. See Section VII.B for general template considerations.

For the T7 S30 Extract, transcription by the endogenous *E. coli* RNA polymerase can be inhibited by the addition of the antibiotic rifampicin, while transcription by the phage T7 RNA Polymerase is unaffected. To inhibit the endogenous RNA polymerase, add 1μl of a 50μg/ml solution of rifampicin in water prior to the addition of the DNA template to the reaction. Addition of excess rifampicin is unnecessary and may decrease protein synthesis. The T7 S30 Extract contains nuclease activity, which prevents the use of linear DNA templates such as PCR products in the reaction. PCR products containing a T7 promoter and a ribosome binding site can be used by adding a small amount (1μl) of the T7 S30 Extract to the *E. coli* S30 Extract for Linear DNA.

Controls

The S30 Extracts for Linear and Circular DNA use pBEST_{luc}™ DNA (Linear or Circular, respectively) as a control to synthesize luciferase. Luciferase protein migrates at 61kDa in denaturing gels. An apparent internal translation start results in a second major gene product of 48kDa. The control plasmid also contains the gene for ampicillin resistance (β-lactamase). β-lactamase may appear

as a faint band migrating at 31.5kDa. Unlabeled luciferase is used in a luminescence assay to monitor the efficiency of the S30 reaction. To generate unlabeled luciferase, use a complete Amino Acid Mixture, rather than a Minus Amino Acid Mixture, and omit the radiolabeled amino acid.

The T7 S30 System for Circular DNA uses the PinPoint™ Control Vector DNA template as a positive control. Translation of this vector will result in the synthesis of the proteins shown in Figure 5.5. The largest molecular weight band corresponds to the PinPoint™-CAT fusion protein (39kDa). A prominent band corresponding to β-lactamase (28kDa) migrates below the PinPoint™-CAT fusion. Expression of β-lactamase is significantly higher in the T7 S30 Extract. This is the result of transcription from the T7 promoter upstream of the fusion protein, which reads through the ampicillin resistance gene. Some full-length CAT protein is also observed, which is probably due to an internal translation initiation site. For a negative control, omit the DNA from the reaction. Use the negative control to determine background radiolabel incorporation.

Reaction Temperature

The reaction may be incubated between 24–37°C. The fastest linear rate occurs at 37°C for approximately 2 hours, although the reaction will continue for several hours at a slower rate. Lower temperatures produce a slower rate of translation but often extend the time of the linear rate to several hours. Enhanced expression at lower temperatures for longer times appears to be gene/protein-specific and may be tried if the standard reaction at 37°C for 1 hour does not produce the desired results.

Additional Resources for *E. coli* S30 Extract Systems

Technical Bulletins and Manuals

TB306	<i>S30 T7 High-Yield Protein Expression Systems Technical Manual</i> (www.promega.com/tbs/tm306/tm306.html)
TB092	<i>E. coli S30 Extract System for Circular DNA Technical Bulletin</i> (www.promega.com/tbs/tb092/tb092.html)
TB102	<i>E. coli S30 Extract System for Linear Templates Technical Bulletin</i> (www.promega.com/tbs/tb102/tb102.html)
TB219	<i>E. coli T7 S30 Extract for Circular DNA Technical Bulletin</i> (www.promega.com/tbs/tb219/tb219.html)

Promega Publications

PN080	Optimized Gene Expression with the T7 Sample System (www.promega.com/pnotes/80/9748_10/9748_10.html)
PN100	The S30 T7 High-Yield Protein Expression System (www.promega.com/pnotes/100/16620_09/16620_09.html)

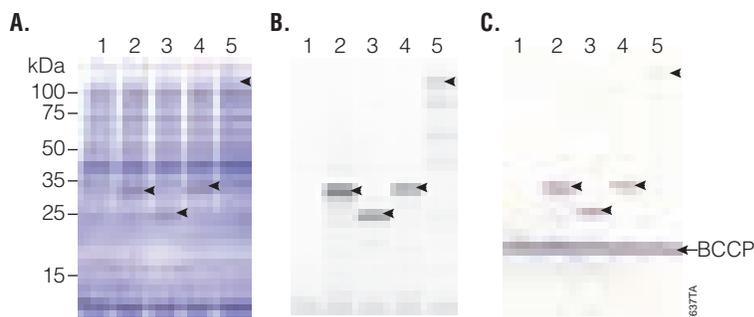


Figure 5.5. Coupled in vitro transcription/translation of circular DNA templates using the S30 T7 High-Yield Protein Expression System. The protein-coding sequences cloned into pFN6A (HQ) Flexi® Vector were expressed as described in the S30 T7 High-Yield Protein Expression System Technical Manual #TM306, resolved by SDS-polyacrylamide gel electrophoresis (PAGE; 4–20% Tris-glycine) and visualized by Coomassie® blue staining (Panel A), fluorescent scanning (Panel B), or transferred to PVDF membrane, treated with Streptavidin Alkaline Phosphatase and stained with Western Blue® Stabilized Substrate for Alkaline Phosphatase (Panel C). For each gel: lane 1, no DNA; lane 2, *Renilla* luciferase; lane 3, Monster Green® Fluorescent Protein; lane 4, HaloTag® protein; lane 5, β-galactosidase. (BCCP = *E. coli* biotin carboxyl carrier protein.)

PN101 A Guide to Optimizing Protein Synthesis in the S30 T7 High-Yield Protein Expression System (www.promega.com/pnotes/101/17252_10/17252_10.html)

Citations

Boddeker, N. *et al.* (2002) Characterization of a novel antibacterial agent that inhibits bacterial translation. *RNA* 8(9), 1120–8.

The effect of the novel antibiotic GS7128 on translation was determined in cell-free systems. The effective concentration for inhibition of translation was determined for both prokaryotes and eukaryotes by translating β-galactosidase from a pGEM® vector in *E. coli* S30 extracts, and luciferase control RNA in rabbit reticulocyte lysates. GS7128 partially inhibited initiation and fully inhibited elongation of peptides by blocking the peptidyl transferase reaction. GS7128 was shown to bind ribosomes differently than any other characterized antibiotic.

PubMed Number: 12358431

colorimetric or chemiluminescent detection. Typically, 0.5–5ng of protein can be detected within 3–4 hours after gel electrophoresis. This sensitivity is equivalent to that achieved with [³⁵S]methionine incorporation and autoradiographic detection 6–12 hours after gel electrophoresis. For a detailed protocol and background information, please see Technical Bulletin #TB182 (www.promega.com/tbs/tb182/tb182.html)

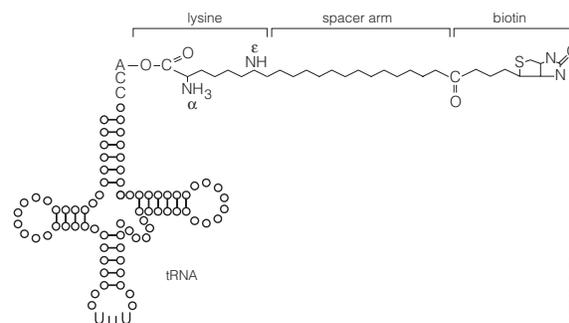


Figure 5.6. Schematic representation of Transcend™ tRNA structure.

VIII. Transcend™ Non-Radioactive Translation Detection System

A. Description

The Transcend™ Non-Radioactive Translation Detection Systems enable non-radioactive detection of proteins synthesized in vitro. Using this system, biotinylated lysine residues are incorporated into nascent proteins during translation, eliminating the need for labeling with [³⁵S]methionine or other radioactive amino acids. Biotinylated lysine is added to the translation reaction as a pre-charged ε-labeled biotinylated lysine-tRNA complex (Transcend™ tRNA) rather than a free amino acid. After SDS-PAGE and electroblotting, the biotinylated proteins can be visualized by binding either Streptavidin-Alkaline Phosphatase (Streptavidin-AP) or Streptavidin-Horseradish Peroxidase (Streptavidin-HRP), followed either by

- The use of Transcend™ tRNA offers several advantages
- No radioisotope handling, storage or disposal is needed.
 - The biotin tag allows detection (0.5–5ng sensitivity).
 - The biotin tag is stable for 12 months, both as the Transcend™ tRNA Reagent and within the labeled proteins. It is not necessary to periodically resynthesize biotin-labeled proteins, unlike ³⁵S-labeled proteins, whose label decays over time.
 - Labeled proteins are detected as sharp gel bands, regardless of the intensity of labeling or amount loaded on the gel, thus allowing the detection of poorly expressed gene products.
 - Results can be visualized quickly, using either colorimetric or chemiluminescent detection.

The precharged *E. coli* lysine tRNAs provided in this system have been chemically biotinylated at the ε-amino group using a modification of the methodology developed by

Johnson *et al.* (1976). The biotin moiety is linked to lysine by a spacer arm, which greatly facilitates detection by avidin/streptavidin reagents (Figure 5.6). The resulting biotinylated lysine tRNA molecule (Transcend™ tRNA) can be used in either eukaryotic or prokaryotic in vitro translation systems such as the TNT® Coupled Transcription/Translation Systems, Rabbit Reticulocyte Lysate, Wheat Germ Extract or *E. coli* S30 Extract (Kurzchalia *et al.* 1988). Lysine is one of the more frequently used amino acids. On average, lysine constitutes 6.6% of a protein's amino acids, whereas methionine constitutes only 1.7% (Dayhoff, 1978).

B. Effects of Biotinylated Lysine Incorporation on Expression Levels and Enzyme Activity

Lysine residues are common in most proteins and usually are exposed at the aqueous-facing exterior. The presence of biotinylated lysines may or may not affect the function of the modified protein. In gel shift experiments, c-Jun synthesized in TNT® Reticulocyte Lysate reactions and labeled with Transcend™ tRNA performed identically to unlabeled c-Jun (Crowley *et al.* 1993).

C. Estimating Incorporation Levels of Biotinylated Lysine

Incorporation of radioactively labeled amino acids into proteins typically is quantitated as percent incorporation of the label added. This value can include incorporation of radioactivity into spurious gene products such as truncated polypeptides. Thus, percent incorporation values provide only a rough estimate of the amount of full-length protein synthesized and do not provide any information on translation fidelity. With Transcend™ tRNA reactions, it is difficult to directly determine the percent incorporation of biotinyl-lysines into a translated protein. An alternative means of estimating translation efficiency and fidelity in Transcend™ tRNA reactions is to determine the minimum amount of products detectable after SDS-PAGE. In all cases tested, we detected translation products in 1µl of a 50µl translation reaction using as little as 0.5µl of Transcend™ tRNA (Figure 5.7). The amount of biotin incorporated increases linearly with the amount of Transcend™ tRNA added to the reaction, up to a maximum at approximately 2µl.

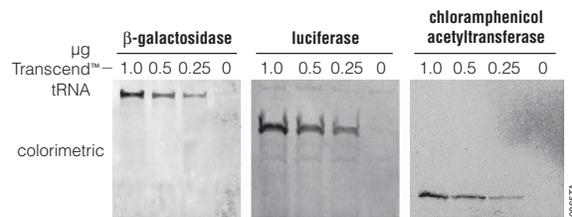


Figure 5.7. Effects of Transcend™ tRNA concentration on detection of proteins synthesized in vitro. Coupled transcription/translation reactions were performed as described in Section III. The indicated amounts of Transcend™ tRNA (equivalent to 2.0, 1.0, 0.5 or 0µl) were added to the translation reactions prior to incubation at 30°C for 1 hour. One microliter of the reaction was used for SDS-PAGE. The separated proteins were transferred to PVDF membrane (100V for 1 hour). The membrane was blocked in TBS + 0.5% Tween® 20 for 15 minutes, probed with Streptavidin-AP (45 minutes), washed twice with TBS + 0.5% Tween® 20 and twice with TBS, and incubated with Western Blue® Substrate for 2 minutes.

D. Capture of Biotinylated Proteins

Biotinylated proteins can be removed from the translation reaction using biotin-binding resins such as SoftLink™ Soft Release Monomeric Avidin Resin. Nascent proteins containing multiple biotins bind strongly to SoftLink™ Resin and cannot be eluted using “soft-release” nondenaturing conditions. SoftLink™ Resin is useful, however, as a substitute for immunoprecipitation.

E. Non-Radioactive Translation and Detection Protocol

Materials Required:

- Transcend™ Non-Radioactive Translation Detection System (Cat.# L5070, L5080)
- RNasin® Ribonuclease Inhibitor (Cat.# N2111)
- Nuclease-Free Water (Cat.# P1193)
- translation extract
- salts, DTT and other components as needed to optimize translation reaction
- complete amino acid mix or a combination of two minus amino acid mixes
- PVDF or nitrocellulose membrane
- Tris-buffered saline (TBS)
- TBS + 0.5% Tween® 20 (TBST)
- Optional: Ponceau S stain (Sigma Cat.# P7170)

Use the following protocol as a guideline for setting up translation reactions using Transcend™ tRNA. In general, Transcend™ tRNA may be used in any in vitro translation protocol at a concentration of 1µl Transcend™ tRNA per 50µl reaction. An example reaction using Rabbit Reticulocyte Lysate is provided.

The number of lysines in the translated polypeptide and the efficiency of translation are the two most important factors affecting band intensity of the translation product. To increase the intensity of weak bands, add up to double the standard amount of Transcend™ tRNA to the reaction (see Figure 5.7).

To reduce the chance of RNase contamination, wear gloves and use microcentrifuge tubes and pipette tips that have been handled only with gloves. Addition of RNasin® Ribonuclease Inhibitor to the translation reaction is recommended but not required.

If the amount of translation product must be estimated, add radioactive amino acid(s) (in addition to Transcend™ tRNA) to either a control translation reaction or all translation reactions. Percent incorporation of the radioactive amino acid can be used in combination with knowledge of the protein's amino acid composition to estimate the amount of translation product produced.

1. Thaw the Transcend™ tRNA on ice. Thaw the translation lysate by hand-warming and immediately place on ice. Thaw all other components at 37°C, and then store on ice.
2. Set up reactions on ice, adding all components except Transcend™ tRNA. Gently mix and briefly centrifuge if necessary. Add Transcend™ tRNA.

Components	Volume
Rabbit Reticulocyte Lysate	35µl
Nuclease-Free Water	10µl
RNasin® Ribonuclease Inhibitor (40u/µl)	1µl
1mM complete amino acid mixture (or mixture of two minus amino acid mixtures)	1µl
RNA template in Nuclease-Free Water	2.0µl
Transcend™ tRNA	1–2µl
final volume	50µl

3. Immediately incubate the translation reaction at 30°C for 60 minutes.
4. Place the tube on ice to terminate the reaction.

Endogenous Biotinylated Proteins

Commonly used translation extracts contain endogenous biotinylated proteins, which may be detected when translation products are analyzed by SDS-PAGE, electroblotting and Streptavidin-AP detection. Rabbit Reticulocyte Lysate contains one biotinylated protein which migrates as a faint band at 100kDa and, in some lots, an additional very faint band at 47kDa. *E. coli* S30 Extract contains one endogenous protein, migrating at 22.5kDa. Wheat Germ Extract contains five major endogenous biotinylated proteins, migrating at 200kDa, 80kDa, 32kDa and a doublet at 17kDa. Comparison to a negative control reaction (without template) will distinguish endogenous biotinylated protein(s) from newly synthesized biotinylated translation product.

Optimization

Biotin labeling of poorly expressed proteins or proteins containing few lysines can be increased by doubling the amount of Transcend™ tRNA added per 50µl translation

reaction (see Figure 5.7). For maximal expression, optimize the amount of template added to the reaction and use highly purified RNA or DNA.

Prokaryotic Coupled Transcription/Translation

In *E. coli* S30 reactions, increased band intensities often can be obtained by using 2–3 times the recommended DNA concentration.

F. Colorimetric and Chemiluminescent Detection of Translation Products

Biotin-containing translation product can be analyzed in either of two ways. The product can be resolved directly by SDS-PAGE, transferred to an appropriate membrane and detected by either a colorimetric or chemiluminescent reaction (Figure 5.8). Alternatively, biotinylated protein can be captured from the translation mix using a biotin-binding resin such as SoftLink™ Resin. This approach is useful as a replacement for immunoprecipitation of protein complexes.

Additional Resources for Transcend™ Non-Radioactive Translation Detection Systems

Technical Bulletins and Manuals

TB182	<i>Transcend™ Non-Radioactive Translation Detection Systems Technical Bulletin</i> (www.promega.com/tbs/tb182/tb182.html)
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Promega Publications

PN067	A General Method for Isolating Targets of RNA and DNA Binding Proteins (www.promega.com/pnotes/67/7201_05/7201_05.html)
PN070	Applications of Promega's In Vitro Expression Systems (www.promega.com/pnotes/70/7618_02/7618_02.html)

IX. FluoroTect™ Green_{Lys} in vitro Translation Labeling System

A. Description

The FluoroTect™ Green_{Lys} in vitro Translation Labeling System uses a charged lysine tRNA molecule labeled with the fluorophore BODIPY®-FL at the epsilon (ε) amino acid position of lysine (Figure 5.9). For the FluoroTect™ System, lysine was chosen as the labeled amino acid because it is one of the more frequently used amino acids, comprising, on average, 6.6% of a protein's amino acids. Detection of the labeled proteins is accomplished in 2–5 minutes directly “in-gel” by use of a laser-based fluorescent gel scanner. This eliminates any requirement for protein gel manipulation such as fixing/drying or any safety, regulatory or waste disposal issues such as those associated with the use of radioactively labeled amino acids. The convenience of non-isotopic “in-gel” detection also avoids the time-consuming electroblotting and detection steps of conventional non-isotopic systems. For a detailed protocol

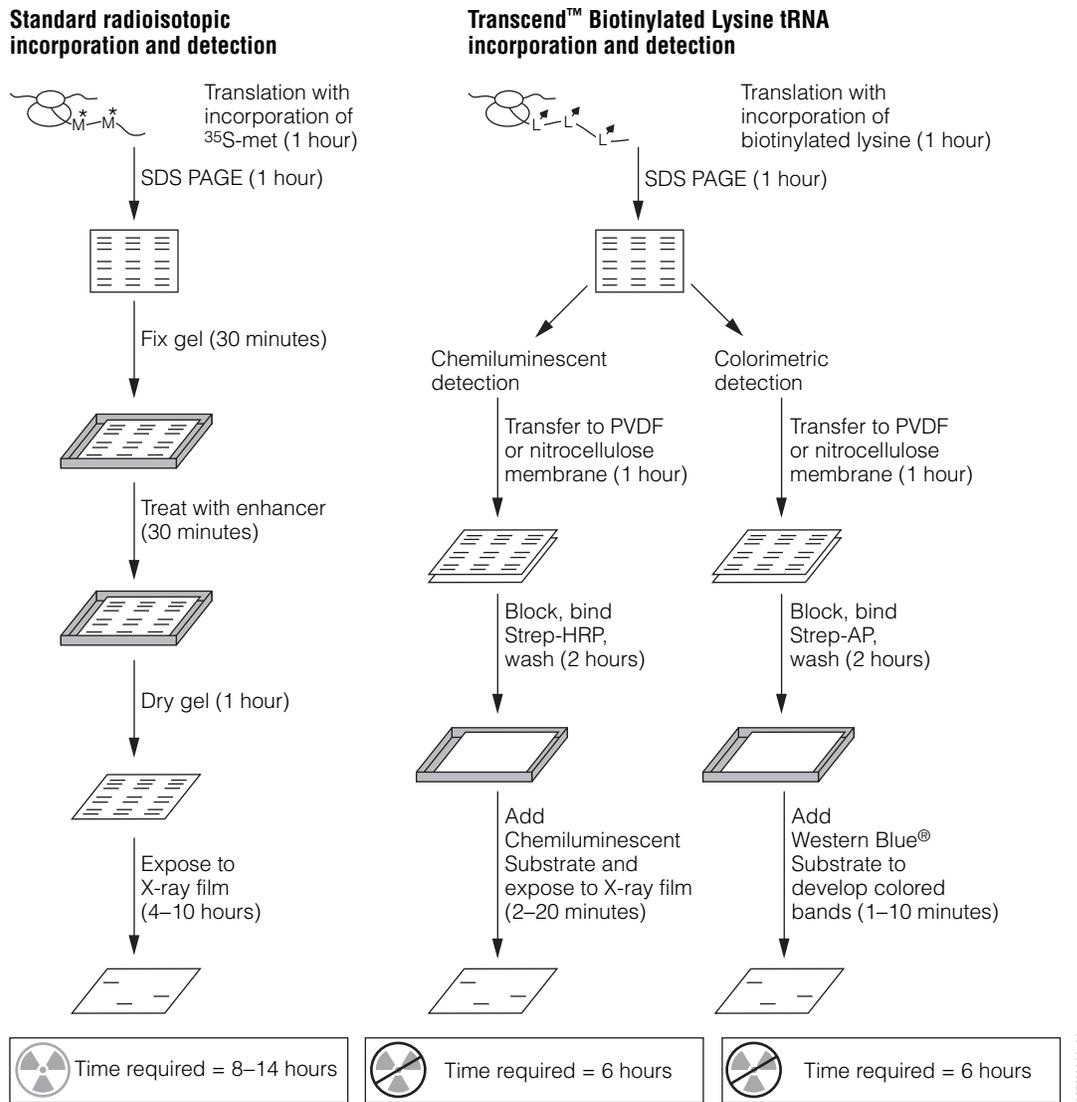


Figure 5.8. Schematic of colorimetric and chemiluminescent detection of translation products.

and background information about this system, please see Technical Bulletin #TB285 (www.promega.com/tbs/tb285/tb285.html).

Dynamics], with a 532nm excitation, or the FMBIO® II [Hitachi], with a 505 channel)

B. Translation Protocol

- FluoroTect™ Green_{Lys} in vitro Translation Labeling System (Cat.# L5001)
- Nuclease-Free Water (Cat.# P1193)
- translation system (e.g., TNT® Coupled Transcription/Translation System, Rabbit Reticulocyte Lysate, Wheat Germ Extract or *E. coli* S30 Extract)
- complete amino acid mix or a combination of two minus amino acid mixes
- salts, DTT and other components as needed to optimize the translation reaction
- fluorescent imaging instrument (i.e., FluorImager® SI or FluorImager® 595 [Molecular Dynamics], both with a 488 argon laser; the Typhoon® 8600 [Molecular

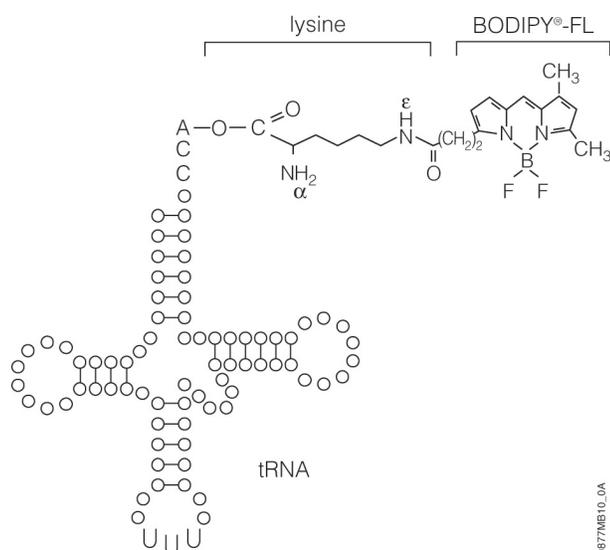


Figure 5.9. Structure of FluoroTect™ GreenLys tRNA.

Use the following protocol as a guideline for setting up translation reactions using FluoroTect™ GreenLys tRNA. In general, FluoroTect™ GreenLys tRNA may be used in an in vitro translation protocol at a concentration of 1μl of FluoroTect™ GreenLys tRNA per 50μl reaction. Examples of standard reactions using TNT® T7 Quick for PCR DNA and Rabbit Reticulocyte Treated Lysate are provided.

1. Assemble the following reactions on ice. Add all components except the FluoroTect™ GreenLys tRNA, and gently mix by pipetting the reaction while stirring the reaction with the pipette tip. If necessary, spin briefly in a microcentrifuge to return the sample to the bottom of the tube. Add the FluoroTect™ GreenLys tRNA.

Example Using TNT® T7 Quick for PCR DNA with FluoroTect™ GreenLys tRNA

Component	Volume
TNT® T7 PCR Quick Master Mix	40μl
1mM methionine	1μl
PCR-generated DNA template	2.5–5μl
FluoroTect™ GreenLys tRNA	1–2μl
Nuclease-Free Water to a final volume of	50μl

Example Using Rabbit Reticulocyte Lysate with FluoroTect™ GreenLys tRNA

Component	Volume
Rabbit Reticulocyte Lysate, Nuclease Treated	35μl
RNasin® Ribonuclease Inhibitor	1μl
Amino Acid Mixture, Complete	1μl
FluoroTect™ GreenLys tRNA	1–2μl
Luciferase Control RNA	1μl
Nuclease-Free Water to a final volume of	50μl

2. Incubate at 30°C for 60–90 minutes (conditions will vary depending on the translation system used).
3. Terminate the reaction by placing on ice. If necessary, the translation reaction can be stored for several months at –20°C or –70°C.

Fluorescence Detection

The fluorescent translation product should be resolved by running a sample on an SDS-PAGE and then visualized by placing the gel on a laser-based fluorescence scanning device.

Additional Resources for FluoroTect™ GreenLys in vitro Translation Labeling System

Technical Bulletins and Manuals

TB285 *FluoroTect™ GreenLys in vitro Translation Labeling System Technical Bulletin*
www.promega.com/tbs/tb285/tb285.html

Promega Publications

PN077 *FluoroTect™ GreenLys in vitro Translation Labeling System*
www.promega.com/pnotes/77/9028_23/9028_23.html

Citations

Shibuya, N. and Nakashima, N. (2008) Characterization of the 5′ internal ribosome entry site of *Plautia stali* intestine virus *J. Gen. Virol.* **87**, 3679–3686 .

The *Plautia stali* virus contains two open reading frames and includes a 5′ internal ribosome entry site (IRES) and an intergenic IRES region. These authors showed that the 5′ IRES was functional and initiated translation in insect cell lysate but not in rabbit reticulocyte lysate or wheat germ extract. The efficiency of translation mediated by the 5′ IRES region was tested with and without cap analog using various firefly and *Renilla* luciferase reporter constructs. They also used deletion mutants to identify the specific regions required for translation initiation.

PubMed Number: 17098985

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I. Introduction

In recent years, the complete genomes of many organisms have been sequenced, allowing genes to be identified by searching the sequence for protein-coding regions, transcription start sites and other features of a typical gene. However, this process tells us little about the gene's role in biological processes. In some cases, we can obtain clues about a gene product's function by identifying regions of sequence homology with known genes or motifs encoding well characterized protein domains. For many genes, sequence analysis alone provides insufficient insight. In these cases, knowing when and where genes are expressed often can help us gain more understanding of the gene's role in a cell. Examining the temporal and spatial patterns of gene expression can help assign function to genes involved in physiological changes (e.g., aging or fruit ripening), tumorigenesis, pathogenicity, cellular responses to stimuli and a wide variety of other cellular events.

II. Techniques to Monitor Gene Expression Levels

Many techniques have been developed to examine absolute and relative levels of gene expression (reviewed in Roth, 2002; Bartlett, 2002). Historically gene expression was monitored by Northern analysis or RNase protection assays (RPA). More recently, microarrays and PCR-based techniques, such as quantitative PCR and differential display PCR, have become popular.

A. Northern Blot Analysis

Once a gene is identified, it is useful to determine the size of the mRNA and determine if alternative splice variants of different sizes are present. This information can be used to estimate the size of the putative protein and confirm DNA sequencing data. The method used to analyze RNA in this way is Northern blot analysis, in which total or poly(A)+ mRNA is run on a denaturing agarose gel, and a specific RNA target is detected by hybridization to a labeled probe in the dried gel itself (Wang *et al.* 1991) or on a membrane (Ausubel *et al.* 2003; Sambrook and Russell, 2001). The resulting signal is proportional to the amount of target RNA in the RNA population. An example is shown in Figure 6.1, and a protocol for Northern blot analysis is provided in Section III.A.

Comparing signals from two or more cell populations or tissues reveals relative differences in gene expression levels. Absolute quantitation can be performed by comparing the signal to a standard curve generated using known amounts of an *in vitro* transcript corresponding to the target RNA. Analysis of housekeeping genes, genes whose expression levels are expected to remain fairly constant regardless of conditions, is often used to normalize the results, eliminating any apparent differences caused by unequal transfer of RNA to the membrane or unequal loading of RNA on the gel. Recent studies have shown that some genes thought to be suitable housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase and β -actin, in fact may have varying levels of expression under certain

circumstances (Goidin *et al.* 2001; Warrington, 2000), so take care when choosing an appropriate housekeeping gene.

The first step in Northern blot analysis is isolating pure, intact RNA from the cells or tissue of interest. Because Northern blots distinguish RNAs by size, sample integrity influences the degree to which a signal is localized in a single band (Lee and Costlow, 1987). Partially degraded RNA samples will result in the signal being smeared or distributed over several bands with an overall loss in sensitivity and possibly erroneous data interpretation. Ribonucleases (RNases) are ubiquitous enzymes that are difficult to completely and irreversibly inactivate. When isolating RNA, use precautions to avoid inadvertently introducing RNases during or after RNA isolation. Guidelines for creating an RNase-free environment and protocols for RNA isolation can be found in Section IV.A.

In Northern blot analysis, DNA, RNA and oligonucleotide probes can be used (Section IV.B), and these probes can be radiolabeled or non-radioactively labeled. The size of the target RNA, not the probe, will determine the size of the detected band, so methods that generate probes of variable lengths, such as random-primed labeling, are suitable for probe synthesis. The specific activity of the probe will determine the level of sensitivity, so we recommend using probes with high specific activities ($>10^9$ cpm/ μ g), particularly for low-abundance RNAs. Labeling methods that produce probes with lower specific activities can be used to detect more abundant RNAs.

B. RNase Protection Assay

In an RNase protection assay (RPA, reviewed in Prediger, 2001), the RNA target and an RNA probe of a defined length are hybridized in solution. Following hybridization, the RNA is digested with RNases specific for single-stranded nucleic acids to remove any unhybridized, single-stranded target RNA and probe. RNases are inactivated, and the RNA is separated by denaturing polyacrylamide gel electrophoresis. The amount of intact RNA probe is proportional to the amount of target RNA in the RNA population. RPA can be used for relative and absolute quantitation of gene expression and also for mapping RNA structure, such as intron/exon boundaries and transcription start sites. An RNase protection assay protocol is given in Section III.B.

The RNase protection assay is superior to Northern blots to detect and quantify low-abundance RNAs. In Northern blots, some RNA fails to transfer or bind to the membrane, and some molecules may not be accessible for hybridization. However, this is not a concern in an RNase protection assay when hybridization takes place in solution. Thus RPA often has lower limits of detection. Also RPA can be used to distinguish and quantify RNAs with high sequence homology. When using RNase I, single-nucleotide mismatches between the probe and target RNA are sufficient for cleavage by RNases, so only perfectly matched probes will yield a protected fragment of the expected size.

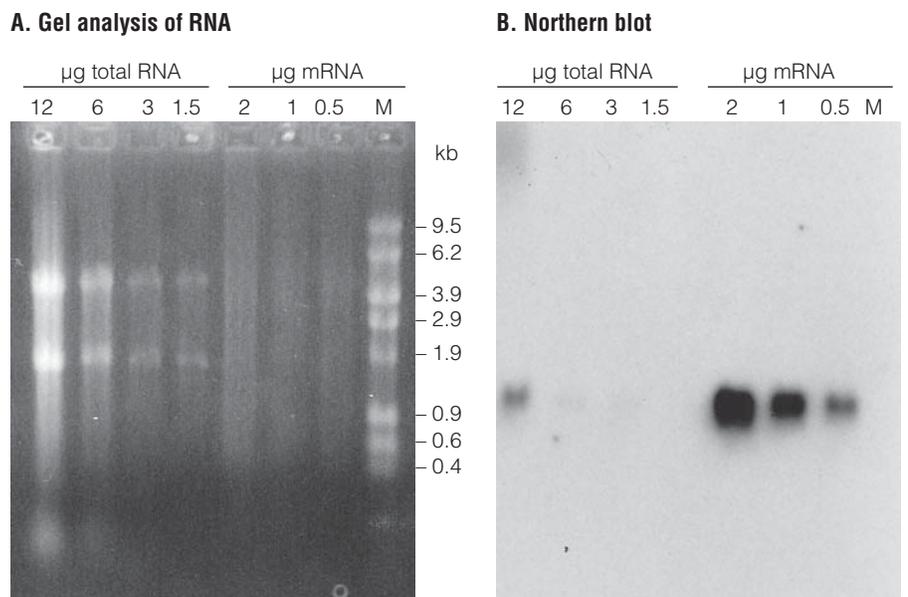


Figure 6.1. Detection of a α -1-proteinase inhibitor by Northern blot analysis. **Panel A.** Photograph of ethidium bromide-stained gel containing samples of total RNA isolated from mouse liver and poly(A)⁺ RNA (mRNA) isolated using the PolyATtract[®] System 1000 (Cat.# Z5400). Amounts of RNA loaded are given at the top of the lanes. Lane M contains RNA Markers (Cat.# G3191), the sizes of which are given along the side of the gel. **Panel B.** Northern blot of the gel in Panel A. The probe used was an alkaline phosphatase-oligonucleotide conjugate specific to the α -1-proteinase inhibitor mRNA, which is an abundant message in liver. The probe was prepared and analyzed by a chemiluminescent detection method. The film was exposed to X-ray film for 2 hours at 37°C.

The antisense RNA probes used in RPA are generated by *in vitro* transcription of a DNA template with a defined endpoint and are typically in the range of 50–600 nucleotides. The use of RNA probes that include additional sequences not homologous to the target RNA allows the protected fragment to be distinguished from the full-length probe. RNA probes typically are used instead of DNA probes due to the ease of generating single-stranded RNA probes and the reproducibility and reliability of RNA:RNA duplex digestion with RNases (Ausubel *et al.* 2003). For the most sensitive detection of low-abundance RNAs, we recommend using probes with high specific activities. A protocol for generating single-stranded, high-specific-activity RNA probes that are suitable for RNase protection assays can be found in the *Riboprobe[®] in vitro Transcription Systems Technical Manual #TM016* (www.promega.com/tbs/tm016/tm016.html).

Additional Resources for RNase Protection Assays

Promega Publications

PN038 RNase ONE[™]: Advantages for nuclease protection assays
www.promega.com/notes/38/38_01/38_01.htm

C. Microarrays

One of the biggest limitations of Northern blot analysis and RNase protection assays is the inability to analyze more than a few genes at a time. The use of microarrays overcomes this limitation and enables researchers to

examine thousands of genes in one experiment, giving scientists a more comprehensive view of the genes involved in a specific cellular event.

The microarray process can be divided into two main parts. First is the printing of known gene sequences onto glass slides or other solid support followed by hybridization of fluorescently labeled cDNA (containing the unknown sequences to be interrogated) to the known genes immobilized on the glass slide. After hybridization, arrays are scanned using a fluorescent microarray scanner. Analyzing the relative fluorescent intensity of different genes provides a measure of the differences in gene expression.

One method of creating arrays is by immobilizing PCR products onto activated glass surfaces. Typically, these probes are generated by PCR or RT-PCR and cloned into a plasmid vector to create a library of 10,000 or more clones. This plasmid library is stored in *E. coli*. Whenever the researcher needs to make a new array, the *E. coli* clones are grown, plasmids are isolated and the cloned genes are amplified with primers common to the plasmid backbone. These amplified products tend to be longer sequences, typically in the range of 100–1,000 bases. A robot is then used to print thousands of the amplified clones in an array of 50–200 µm spots on a specially prepared glass slide or other suitable support.

DNA arrays also can be generated by immobilizing presynthesized oligonucleotides onto prepared glass slides. In this case, representative gene sequences are manufactured and prepared using standard oligonucleotide

synthesis and purification methods. These synthesized gene sequences are complementary to the genes of interest and tend to be shorter sequences in the range of 25–70 nucleotides. Alternatively, immobilized oligos can be chemically synthesized in situ on the surface of the slide. In situ oligonucleotide synthesis involves the consecutive addition of the appropriate nucleotides to the spots on the microarray; spots not receiving a nucleotide are protected during each stage of the process using physical or virtual masks.

In expression profiling microarray experiments, the RNA templates used are representative of the transcription profile of the cells or tissues under study. First RNA is isolated from the cell populations or tissues to be compared. Each RNA sample then is used as a template to generate fluorescently labeled cDNA via reverse transcription. Fluorescent labeling of the cDNA can be accomplished by either direct-labeling or indirect-labeling methods. During direct labeling, fluorescently modified nucleotides (e.g., Cy³- or Cy⁵-dCTP) are incorporated directly into the cDNA during the reverse transcription. Alternatively, indirect labeling can be achieved by incorporating aminoallyl-modified nucleotides during cDNA synthesis, then conjugating an N-hydroxysuccinimide (NHS)-ester dye to the aminoallyl-modified cDNA after the reverse transcription reaction is complete.

To perform differential gene expression analysis, cDNA pools generated from different RNA samples are labeled in different colors. For example, when comparing RNA from tumor and normal tissue samples, the cDNA generated from the tumor RNA can be labeled with Cy³, while the cDNA generated from the normal RNA sample can be labeled with Cy⁵. The resulting labeled cDNA is purified to remove unincorporated nucleotides, free dye and residual RNA. Following purification, the labeled cDNA samples are combined, then hybridized to the microarray. The microarray is scanned post-hybridization using a fluorescent microarray scanner (Figure 6.2). The fluorescent intensity of each spot indicates the level of expression for that gene; bright spots correspond to strongly expressed genes, while dim spots indicate weak expression. Multiple targets labeled in different dye colors can be analyzed simultaneously to determine which genes are differentially expressed.

Once the images are obtained, the raw data must be analyzed (reviewed in Dharmadi and Gonzalez, 2004; Roth, 2002). First, the background fluorescence must be subtracted from the fluorescence of each spot. The data then are normalized to a control sequence, such as an exogenously added RNA (Schena *et al.* 1995) or a housekeeping gene to account for any nonspecific hybridization, array imperfections or variability in the array setup, cDNA labeling, hybridization or washing. Care must be taken when choosing an appropriate housekeeping gene; recent studies have shown that some genes thought to be suitable housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase and β -actin, may in fact have varying levels

of expression under certain circumstances (Goidin *et al.* 2001; Warrington, 2000). Data normalization allows the results of multiple arrays to be compared.

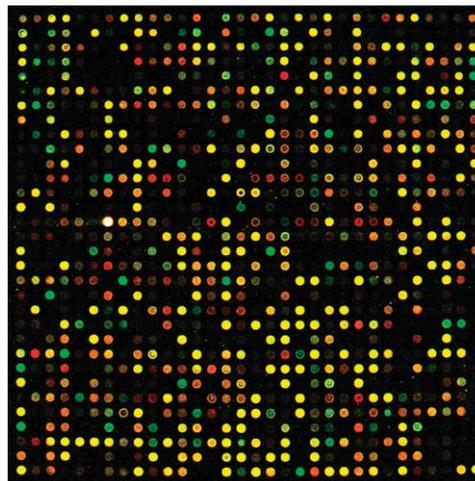


Figure 6.2. Cy³- and Cy⁵-labeled cDNA hybridized to a DNA microarray. Cy³-labeled cDNA from 293T mRNA (green) and Cy⁵-labeled cDNA from HeLa mRNA (red) were hybridized to custom 4K arrays provided by Corning Incorporated. Data were acquired using a Genepix[®] 4000B scanner (Axon Instruments, Inc.). A representative subgrid is shown. The scanner generates separate data images for green (532nm) and red (635nm) wavelengths. By overlaying the red and green data images, you can view the differential expression of various genes. Green spots indicate expression in the 293T sample, while red spots indicate expression in the HeLa sample. Yellow spots indicate genes that were expressed in both samples. The fluorescence intensity of each color at each spot indicates the level of expression of that gene in each sample.

Table 6.1. Expression Analysis Techniques.

Technique	Nucleic Acid Labeled	Advantages
Northern blot analysis	RNA or DNA probe	<ul style="list-style-type: none"> provides information about the quantity and size of the RNA target can be used to determine whether splice variants of different sizes exist
RNase Protection Assay	RNA probe	<ul style="list-style-type: none"> offers superior quantitation of RNA, allowing more sensitive analysis of low-abundance RNAs can be used to map RNA structure can distinguish and quantify RNAs with high sequence homology
Microarrays	cDNA	<ul style="list-style-type: none"> allows analysis of thousands of genes in a single experiment can simultaneously analyze multiple targets labeled with different dyes

Additional Resources for Microarrays

Promega Publications

PN085	High-throughput DNA fragment purification using the MagneSil® automated 384-well clean-up systems (www.promega.com/pnotes/85/10904_03/10904_03.html)
PN082	Introducing the Wizard® SV 96 PCR Clean-Up System (www.promega.com/pnotes/82/10203_06/10203_06.html)
PN081	Indirect fluorescent labeling of microarray targets using ImProm-II™ Reverse Transcriptase (www.promega.com/pnotes/81/9939_14/9939_14.html)
PN080	MagneSil® Paramagnetic Particles: A high-throughput PCR purification system for microarrays (www.promega.com/pnotes/80/9748_14/9748_14.html)
PN080	Technically speaking: Hit the spot with microarrays (www.promega.com/pnotes/80/9748_24/9748_24.html)
BR120	RNA Analysis Notebook (www.promega.com/guides/ma_guide/microarrays.pdf)

eNotes Microarray applications using Promega products
(www.promega.com/enotes/applications/ap0037_tabs.htm)

D. Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) is a common method to quantify target mRNA levels in a sample. To perform qRT-PCR, RNA samples are reverse transcribed to produce cDNA, then the cDNA is quantified by quantitative PCR. qPCR can be performed as an end-point assay or, more commonly, in real time using fluorescently labeled oligonucleotide probes or primers or DNA-binding fluorescent dyes such as SYBR® Green. For more information about qPCR, see the Nucleic Acid Amplification chapter. (www.promega.com/paguide/chap1.htm)

III. Expression Analysis Protocols

Tips to create an RNase-free environment and choose an RNA isolation protocol can be found in Section IV.A. A discussion of RNA and DNA labeling techniques can be found in Section IV.B.

A. Northern Blot Analysis Protocol

Agarose/Formaldehyde Gel Electrophoresis of RNA

Materials Required:

(see Composition of Solutions section)

- agarose
- MOPS 5X and 1X buffer
- 37% formaldehyde
- DEPC-treated water
- RNA markers (optional)
- RNA sample buffer
- RNA loading buffer

1. Prepare a 1% gel by combining the following components (sufficient for a 280ml gel):

MOPS 5X buffer	56.0ml
DEPC-treated water	174.0ml
agarose	2.79g

Mix well, and boil to dissolve the agarose.

2. Cool to 55°C, and add 50ml of 37% formaldehyde. Mix thoroughly and pour a 0.5–1.0cm thick gel. Allow to solidify.

Note: Do not add ethidium bromide to the gel or running buffer because it will decrease the efficiency of RNA transfer to the membrane.

3. Prepare the RNA samples by mixing 1 part RNA with 2 parts RNA sample buffer up to a total volume of 10–30µl, depending upon the thickness of the gel. Heat samples at 65°C for 5 minutes, cool to room temperature, then add 2µl of RNA loading buffer.

Note: We recommend loading 0.2–10 μ g of total RNA for a high-abundance message. The amount of RNA required depends upon transcript abundance. Up to 30 μ g of total RNA per well can be used. For rare messages, we recommend isolating poly(A)⁺ RNA and loading at least 3 μ g per well.

- Prerun the gel for 10 minutes in MOPS 1X buffer prior to loading the samples. Load samples, then run the gel at 4–5V/cm. Continue electrophoresis until the bromophenol blue has migrated at least 10cm from the wells.

Note: RNA markers should be run along with the RNA samples of interest if size determination is an important factor in the experiment. If desired, load duplicate sets of samples and excise one set after electrophoresis for ethidium bromide staining. The integrity of the samples can be confirmed by the presence of the 28S and 18S ribosomal RNA bands.

Transfer of RNA to Membranes

Many different setups to transfer nucleic acids to membranes exist. The following procedure is a general guideline for upward capillary transfer of RNA to a membrane and may not be optimal for all types and brands of hybridization membranes. Follow the manufacturer's recommendations for optimal results. An alternative protocol for downward capillary transfer can be found in Sambrook and Russell, 2001.

Materials Required:

(see Composition of Solutions section)

- SSC: 20X, 5X
- NaOH, 0.05N (optional)
- DEPC-treated water
- nylon hybridization membrane
- Whatman[®] 3MM paper or equivalent
- 12 blotting pads per Northern transfer procedure
- Stratalinker[®] UV Crosslinker or equivalent, or vacuum oven

- Soak the gel several times in DEPC-treated water at room temperature to remove the formaldehyde.

Note: Use care in handling the gel because it will be more fragile than standard agarose gels.

- Optional:** Soak the gel in 0.05N NaOH for 20 minutes at room temperature to partially hydrolyze the RNA. Rinse the gel in DEPC-treated water.

Note: This step is beneficial if the RNA to be transferred is >2.5kb in length, the gel is >1% agarose or the gel is >5mm thick. The partial hydrolysis of the RNA will improve the transfer efficiency of high-molecular-weight RNA from the gel.

- Soak the gel in 20X SSC for 45 minutes at room temperature.

- While the gel is soaking, cut 2 pieces of Whatman[®] 3MM filter paper and 1 piece of hybridization membrane to the exact size of the gel.

Note: Wear gloves, and handle the hybridization membrane by the edges using forceps to avoid background artifacts. Prewet the hybridization membrane in distilled water, and soak it in 20X SSC until needed.

- Stack 4 blotting pads, and completely saturate them in 20X SSC.

Note: We have noted that sponges can be a source of background in biotin-streptavidin systems. We highly recommend using only blotting pads for the transfer process. These work exceptionally well in providing even and efficient transfers without background.

- Place the presoaked (completely saturated) blotting pads in a clean glass or plastic container. Wet 2 pieces of Whatman[®] 3MM paper in 20X SSC. Place one of the moistened pieces of filter paper on top of the blotting pads.

- Place the gel on top of the filter paper with the bottom side of the gel up. Surround the gel with cut pieces of Parafilm[®] to prevent short-circuit wicking of the transfer solution.

- Using gloved hands, place the membrane on top of the gel. Do not move the membrane once it has been placed on the gel. Remove any air bubbles by gently rubbing a gloved hand over the back of the membrane or by gently rolling a clean 25ml pipette over the surface of the membrane.

- Place the second piece of Whatman[®] 3MM filter paper (prewet with 20X SSC) on top of the hybridization membrane.

Note: Keep the membrane out of direct contact with the blotting pads to reduce background problems.

- Place the remaining 8 (dry) precut blotting pads on top of the Whatman[®] 3MM filter paper.

- Place a glass plate over the top of the blotting pads along with a full 250ml bottle as a weight. Allow the transfer to proceed for 1–16 hours at room temperature.

Note: Transfer times should be about 10 minutes per millimeter of gel thickness. Transfer under these conditions ensures complete denaturation of RNA during transfer without significant RNA degradation. The rapid-transfer process ensures minimum gel compression and high transfer efficiency.

- After the transfer is complete, carefully disassemble the blotting apparatus, leaving the hybridization membrane in contact with the gel. It is usually

convenient at this time to mark the back of the membrane with a pencil to indicate which is the RNA side.

Note: The transferred gel can be stained with 0.02% methylene blue in 0.3M sodium acetate (pH 5.2) and destained with water to determine if the transfer was complete. Alternatively, the gel can be stained with ethidium bromide and visualized under ultraviolet light.

- Carefully remove the membrane, and wash the blot once in 5X SSC for 5 minutes at room temperature. Remove any pieces of agarose that may be stuck to the membrane with a gloved hand.
- Allow the membrane to dry for 5 minutes. For nylon membranes, UV-crosslink the RNA to the membrane (RNA side up). For a Stratilinker® UV Crosslinker, we routinely irradiate at the recommended 120 millijoules (using the Auto-Crosslink setting). For nitrocellulose membranes, bake the membrane for 2 hours at 80°C in a vacuum oven between two pieces of Whatman® 3MM filter paper. In place of UV irradiation, nylon membranes also can be baked for 2 hours in a vacuum oven; however, the hybridization signal is decreased.
- If it is not convenient to perform the prehybridization at this time, the crosslinked membrane may be placed between two sheets of Whatman® 3MM paper, wrapped in aluminum foil and stored at room temperature.

Hybridization of Probe

Materials Required:

(see Composition of Solutions section)

- radiolabeled DNA or RNA probe (see Section IV.B)
 - heat-sealable bags or containers
 - boiling water bath
 - water bath, preheated to 42°C
 - water bath, preheated to 68°C
 - prehybridization/hybridization solution, prewarmed to 42°C
 - SSC: 5X, 2X
 - stringency wash solution I
 - stringency wash solution II, preheated to 68°C
- Wet the membrane completely in 5X SSC for 2 minutes.
 - Place the membrane in a heat-sealable bag or a sealable container.

Note: Ensure that the container will not leak if a radioactively labeled probe is to be used.
 - Add 0.2ml of prehybridization/hybridization solution (preheated to 42°C) per square centimeter of the membrane. Seal the container. Incubate at 42°C for 1–2 hours.

Note: If a sealable bag is used, leave room between the membrane and the seal to allow a corner of the bag to be removed when the probe is added in Step 6.

- Denature the probe in a boiling water bath for 5 minutes, and quick-chill on crushed ice.
- Prepare the hybridization solution by adding an appropriate amount of denatured, labeled probe to fresh prehybridization/hybridization solution (0.2ml per square centimeter of the membrane, preheated to 42°C), and mix.

Note: For a radioactively labeled probe with a specific activity of 10⁸cpm/μg, add the probe to a concentration of 10ng/ml. For a probe with a specific activity of >10⁹cpm/μg, add the probe to a concentration of 2ng/ml. For a non-radioactively labeled probe, add probe to a final concentration of 10–100ng/ml.
- Open the container or resealable bag (by cutting off a corner), and decant the prehybridization/hybridization solution. Immediately add the hybridization solution, and reseal the container or bag. Incubate at 42°C overnight.

Note: It is possible to add the denatured probe directly to the prehybridization/hybridization solution in the container or bag, reseal and mix.
- After hybridization, transfer the blots immediately to 300ml of stringency wash solution I. Wash with gentle shaking for 5 minutes at room temperature. Repeat for a second wash.

Note: For oligonucleotide probes, reduce the wash times to 1–2 minutes.
- Pour off the stringency wash solution I, and immediately add 300ml of stringency wash solution II prewarmed to 68°C. Wash with gentle shaking for 15 minutes at 68°C. Repeat for a second wash.

Note: This is a high-stringency wash. For a moderate-stringency wash, reduce the temperature of the wash to 42°C. For a low-stringency wash, increase the SSC concentration to 0.2X and wash twice for 5 minutes at room temperature. Conditions that may require lower stringency washes include the use of probes with low melting temperatures (e.g., short probes or AU- or AT-rich probes) and probes that do not have high homology with the RNA target (e.g., degenerate probes or probes derived from related gene sequences).
- Wash the blots with 300ml of 2X SSC for 10 minutes at room temperature with gentle shaking to remove excess SDS.
- Wrap the blot in plastic wrap, and perform autoradiography if the probe is radioactively labeled. For a non-radioactive probe, follow the appropriate detection protocol. Do not allow the blot to dry out, especially if it is to be probed again.

Note: Nylon blots can be stripped of probe and rehybridized. To strip a nylon membrane, incubate the blot for 1–2 hours in 10mM Tris-HCl (pH 7.4), 0.2% SDS preheated to 70–75°C or in 50% deionized formamide, 0.1X SSC, 0.1% SDS preheated to 68°C (Sambrook and Russell, 2001).

B. RNase Protection Assay Protocol

The following protocol has been optimized for use with RNase ONE™ Ribonuclease. Further details on ribonuclease protection assays are provided in Ausubel *et al.* 2003; Sambrook and Russell, 2001; Melton *et al.* 1984.

Many factors, including Na⁺ concentration, probe sequences, reaction temperature, annealing temperature and RNase ONE™ Ribonuclease concentration, affect the detection of complementary RNAs by this method. We recommend performing titration experiments to optimize conditions specific for your sequences. A good discussion of these experimental variables can be found elsewhere (Lee and Costlow, 1987). When mapping AU-rich regions (>75% A + U), such as the regions found at the 3'-end of oncogenes, we recommend the alternative procedures found in Brewer and Ross, 1990, and Brewer *et al.* 1992. In addition, the use of an RNase that does not cut at A or U residues (e.g., RNase T1) can reduce the background due to cleavage in AU-rich regions, as these duplex regions "breathe" (spontaneously and temporarily denature to form single-stranded regions).

Experimental Considerations

RNA Probe

Prepare a labeled RNA probe by *in vitro* transcription as described in Section IV.B. Remove the template DNA by RQ1 RNase-Free DNase digestion, phenol:chloroform extraction and ethanol precipitation to prevent background hybridization. Gel purification is not a substitute for DNase treatment, as residual DNA fragments can copurify with the probe and compete for hybridization with the RNAs to be analyzed.

Longer probes (>300bp) may require gel purification after DNase treatment because the probe may contain shorter RNA species due to sequence-specific pausing or premature termination of the bacteriophage polymerase before completion of the transcript. Short probes (150–300bp) can often be used without gel purification. Purify the probe from an acrylamide gel by incubating the gel slice in 0.5M ammonium acetate, 1mM EDTA, 0.2% SDS at 37°C for 1–2 hours or overnight (overnight incubation will produce higher yields).

It is important to use a molar excess of the probe. When using a radioactively labeled probe, use only ³²P-labeled probes with a minimum specific activity of 1–3 × 10⁸cpm/μg. We recommend 1–5 × 10⁵cpm of probe per reaction. The probe should be stored at –70°C and used within 3 days of preparation to minimize background.

RNA Sample

The RNA should have a minimum A_{260/280} ratio of 1.9. If the A_{260/280} ratio of your sample is less than 1.9, repeat the extraction and ethanol precipitation. Use 5–10μg of total RNA to detect more abundant sequences. Use 30–40μg of total RNA or 500ng–1μg of poly(A)⁺ RNA to detect rare sequences.

The target RNA must be intact. Check the integrity of total RNA by separating the RNA on a denaturing agarose gel and staining with ethidium bromide. For mammalian RNA, the staining intensity of the 28S ribosomal RNA band should be approximately twice that of the 18S ribosomal RNA band if the RNA is undegraded.

Amount of RNase

The RNases commonly used in an RPA are RNase I, RNase T1 or a combination of RNase T1 and RNase A. RNase I cleaves after all four ribonucleotides, RNase T1 cleaves after G residues and RNase A cleaves after A and U residues. For most efficient cleavage of the single-stranded regions immediately adjacent to the double-stranded RNA, we recommend RNase I (RNase ONE™ Ribonuclease, Cat.# M4261). To map sequences, use 1–10u RNase ONE™ Ribonuclease per 10μg of total RNA or 0.1–1u of RNase ONE™ Ribonuclease per 1μg of poly(A)⁺ RNA. To analyze sequences containing a single-base mismatch, it may be necessary to add 40 times more RNase ONE™ Ribonuclease to the reaction.

Annealing and Digestion Temperatures

Most samples anneal efficiently at 37–45°C in hybridization buffer. RNase ONE™ Ribonuclease digestion works most efficiently at 20–37°C (Brewer *et al.* 1992). Annealing at lower temperatures may be required to maintain hybrids of AU-rich sequences during RNase ONE™ Ribonuclease digestion.

Protocol

Materials Required:

(see Composition of Solutions section)

- RPA hybridization buffer
 - RNase digestion buffer
 - ³²P-labeled RNA probe with a minimum specific activity of 1–3 × 10⁸cpm/μg
 - purified total RNA or poly(A)⁺ RNA from the tissue or cells of interest
 - RNase-ONE™ Ribonuclease (Cat.# M4261)
 - ice-cold 100% ethanol
 - 3.0M ammonium acetate (pH 5.2)
 - ice-cold 70% ethanol
 - 20% w/v SDS
 - 20mg/ml proteinase K
 - phenol:chloroform:isoamyl alcohol
 - carrier transfer RNA (tRNA), 10mg/ml
 - RPA loading dye
1. Combine sample RNA and RNA probe in an RNase-free 1.5ml microcentrifuge tube. Include a sample containing 10μg of tRNA and the probe as a control for background hybridization and complete digestion.

Note: Use 5–10 µg of total RNA to detect more abundant sequences. Use 30–40 µg of total RNA or 500 ng–1 µg of poly(A)⁺ RNA to detect rare sequences.

- Ethanol precipitate the samples by adding 0.1 volume of 3.0M ammonium acetate (pH 5.2) and 2.5 volumes of ice-cold 100% ethanol. Mix and incubate at –20°C for 30 minutes. Centrifuge at maximum speed in a microcentrifuge for 15 minutes at 4°C.

Note: Coprecipitation of the probe and sample ensures that the subsequent annealing is reproducible. The probe and sample may be added without ethanol precipitation to the hybridization buffer, but the additional volume will alter the final hybridization conditions. Consider how possible changes in stringency will affect the results. Furthermore, the omission of the ethanol precipitation step makes this direct approach more susceptible to inhibitors and contaminants in the RNA sample or probe. However, some RNA may be lost during the precipitation step.

- Remove the supernatant. Wash the pellet with 1 ml of ice-cold 70% ethanol, centrifuge briefly in a microcentrifuge and remove all ethanol. Dry the sample at room temperature for 5 minutes. The tubes may be monitored with a Geiger counter to avoid pellet loss.
- Resuspend the pellets completely in 30 µl of RPA hybridization buffer.
- Incubate the samples at 85°C for 5 minutes to denature the RNAs, then incubate each sample for 2–16 hours at an appropriate annealing temperature. See Annealing and Digestion Temperature in Experimental Considerations.
- Add 300 µl of RNase digestion buffer and the appropriate amount of RNase ONE™ Ribonuclease (see Amount of RNase in Experimental Considerations). Incubate the samples for 30–60 minutes at 20–37°C.
- Stop the reaction as follows: Add 10 µl of 20% w/v SDS and 2.5 µl of 20 mg/ml proteinase K. Incubate for 15 minutes at 37°C. Extract once with phenol:chloroform:isoamyl alcohol, and remove the aqueous phase to a clean microcentrifuge tube containing 1 µl of 10 mg/ml carrier tRNA .
Note: Alternatively, stop the reaction by adding 3.3 µl of 10% SDS and 20 µg of carrier tRNA and mixing.
- Add 825 µl of ice-cold 100% ethanol, and chill at –20°C for 30 minutes, then centrifuge at maximum speed in a microcentrifuge for 15 minutes at 4°C to pellet the RNA. Carefully remove the supernatant. Wash the pellet using 300 µl of 70% ethanol and dry.
- Resuspend the pellet by vortexing in 10 µl of RPA loading dye.

- Incubate at 85°C for 5 minutes to denature the RNA, and place on ice. Vortex and centrifuge briefly in a microcentrifuge.
- Resolve the samples on a 5–8% polyacrylamide/7M urea gel, and detect the fragments by autoradiography.

C. Microarray Protocol

Promega offers two systems for labeling cDNA for microarray analysis. The ChipShot™ Direct Labeling and Clean-Up System (Cat.# Z4100) provides an efficient, reproducible method to generate fluorescent cDNA by direct incorporation of Cy®-labeled nucleotides in a reverse transcription reaction. The ChipShot™ Direct Labeling System protocol is optimized to account for differences in the efficiency of incorporating Cy®3- vs Cy®5-labeled dCTP, resulting in robust synthesis of labeled cDNA with both Cy®-labeled nucleotides.

The ChipShot™ Indirect Labeling and Clean-Up System (Cat.# Z4000) provides reagents and protocols to generate fluorescent cDNA without the use of Cy®-labeled nucleotides. Indirect labeling is achieved by incorporating aminoallyl-modified nucleotides during cDNA synthesis, followed by conjugation of a CyDye® NHS-ester dye to the aminoallyl-modified cDNA after the reverse transcription reaction is complete.

Both the ChipShot™ Direct Labeling and ChipShot™ Indirect Labeling Systems are optimized for use of total RNA or poly(A)⁺ mRNA as templates for cDNA synthesis. When total RNA is used, only 5 µg of RNA template is required to generate sufficient labeled cDNA for hybridization to a minimum of two or three full 22 × 50 mm arrays. Compared to many other commercially available systems that require 10–25 µg of total RNA template, the ChipShot™ Labeling Systems allow users to conserve limited RNA template and increase the number of replicates performed. When poly(A)⁺ mRNA is used as the template for cDNA synthesis, only 1.5 µg is required. The PolyATtract® mRNA Isolation System provides an efficient method for isolating mRNA for use in cDNA-labeling experiments.

IV. Related Protocols

A. RNA Isolation

Successful analysis of gene expression by RPA, Northern analysis, RT-PCR or microarray analysis requires pure, intact RNA. The RNA must be free of DNA and potential inhibitors that can interfere with labeling or hybridization. Successful isolation of intact RNA requires four essential steps: i) effective disruption of cells or tissue; ii) denaturation of nucleoprotein complexes; iii) inactivation of endogenous RNase activity; and iv) removal of contaminating DNA and proteins. Most important is the immediate inactivation of endogenous RNase activity, which is released from membrane-bound organelles upon cell disruption, to minimize RNA degradation. RNA is notoriously susceptible to degradation, and special care is

required for its isolation. All methods of RNA isolation use strong denaturants to inhibit endogenous RNases. RNases, in contrast to deoxyribonucleases (DNases), are difficult to inactivate because they do not require cofactors and are heat-stable, refolding following heat denaturation. Some tissues such as pancreas and spleen are naturally rich in RNases, while other tissues such as liver are low in RNases.

About RNA

RNA is found in the nucleus, cytoplasm and mitochondria of eukaryotic cells. Total cytoplasmic RNA consists of ribosomal RNA (rRNA), transfer RNA (tRNA), messenger RNA (mRNA) and other small species of RNA.

Heteronuclear RNA (hnRNA), the precursor of mRNA, is present in the nucleus. Only 1–2% of the total RNA in eukaryotic cells is mRNA; the majority of total RNA consists of rRNA (Ausubel *et al.* 2003). The amount of mRNA in mammalian cells has been estimated at approximately 500,000 mRNA molecules per cell (Ausubel *et al.* 2003).

With rare exceptions, all species of eukaryotic mRNAs are polyadenylated. Some viral RNAs also are polyadenylated and reside in the cytoplasm or mitochondria. In contrast, bacterial mRNAs are generally not polyadenylated, although some bacterial RNA is polyadenylated (Gopalakrishna *et al.* 1981).

Polyadenylic acid is added in the nucleus to the free 3'-OH of hnRNA following cleavage and is required for mRNA transport into the cytoplasm (Huang and Carmichael, 1996). The typical length of poly(A) addition is 200 bases in mammalian cells (Huang and Carmichael, 1996), while mRNA isolated from plant chloroplasts contains poly(A)+ tails of only approximately 20 bases (Murillo *et al.* 1995). The length of the poly(A)+ tail can vary during the life of the message and decreases with age for a given message (Lewin, 1980). In higher eukaryotic cells, changes in polyadenylation function to control translation in the cytoplasm and to stabilize the message during early development (Winkles and Grainger, 1985; Pfarr *et al.* 1986; Salles *et al.* 1992).

The steady state level of mRNA in the cytoplasm is a combination of three factors: the rate of production, the rate of degradation and the rate of transport from the nucleus. The half-life of mRNAs in mammalian cells ranges from hours to days (Ausubel *et al.* 2003), while in yeast the half-life is 4–45 minutes (Herrick *et al.* 1990). In bacteria, the half-life is much shorter, typically a few minutes (Selinger *et al.* 2003).

Creating a Ribonuclease-Free Environment

Ribonucleases are extremely difficult to inactivate. Great care should be taken to avoid inadvertently introducing RNases into the RNA preparation during or after isolation. This is especially important if the starting material has been difficult to obtain or is irreplaceable. The following notes

may be helpful in preventing the accidental contamination of the sample with RNases, allowing the isolation of full-length RNA.

- Two of the most common sources of RNase contamination are the researcher's hands and bacteria or molds, which may be present on airborne dust particles or laboratory glassware. To prevent contamination from these sources, sterile technique should be employed when handling any reagents used for RNA isolation or analysis. Gloves should be worn at all times.
- Whenever possible, use sterile, disposable plasticware when handling RNA. These materials are generally RNase-free and do not require pretreatment to inactivate RNases.
- Nondisposable glassware and plasticware should be treated before use to ensure that it is RNase-free. Glassware should be baked at 200°C overnight. Plasticware should be thoroughly rinsed before use with 0.1N NaOH/1mM EDTA, then with diethyl pyrocarbonate (DEPC)-treated water. Equipment that cannot be conveniently treated with DEPC can be treated with an RNase decontamination solution, such as RNaseZap® (Ambion).

Note: COREX® tubes should be rendered RNase-free by treatment with DEPC and not by baking; baking will increase the failure rate of this type of tube during centrifugation. COREX® tubes should be treated with 0.05% DEPC overnight at room temperature, then autoclaved for 30 minutes to remove any trace of DEPC.

- Autoclaving alone is not sufficient to inactivate RNases. Solutions supplied by the researcher should be treated with 0.05% DEPC overnight at room temperature, then autoclaved for 30 minutes to remove any trace of DEPC. Alternatively, RNases in a reaction can be inactivated by adding RNasin® Ribonuclease Inhibitor, which inhibits a broad spectrum of RNases, including RNase A, RNase B, RNase C and human placental RNase, and is active over a broad pH range (pH 5.5–9).

- **Note:** Tris buffers and any chemicals containing primary amine groups cannot be treated with DEPC. Use caution when weighing out Tris to avoid RNase contamination, and use DEPC-treated water and glassware when preparing Tris buffers.
- While most sources of fresh deionized water are free of contaminating RNase activity, deionized water is a potential source of RNase activity. If degradation of the target or probe RNA occurs, it may be necessary to test the laboratory's water source for RNase activity.
- We recommend that chemicals for use in RNA isolation and analysis be reserved separately from those for other uses. Wear gloves when handling labware and reagents, and use only baked spatulas and untouched weigh boats or weigh paper.

Choosing an RNA Isolation Protocol

One of the first considerations when deciding on an RNA purification protocol is whether you will be using total RNA or poly(A)+ RNA for your application. The source of RNA, type of RNA to be purified, relative abundance of the RNA, sample size and convenience of the isolation procedure are all factors that also must be considered. For valuable tissue samples, we suggest that a portion of each sample be reserved at -70°C in the event that loss of a sample occurs during RNA purification. Promega offers both total RNA isolation systems (SV Total RNA Isolation System, PureYield™ RNA Midiprep System and MagneSil® Total RNA mini-Isolation System) and poly(A)+ RNA isolation systems (PolyAtract® System 1000) that yield clean, intact RNA from a variety of cell and tissue types.

Additional Resources for RNA Isolation**Technical Bulletins and Manuals**

TM048	<i>SV Total RNA Isolation System Technical Manual</i> (www.promega.com/tbs/tm048/tm048.html)
TB294	<i>SV 96 Total RNA Isolation System Technical Bulletin</i> (www.promega.com/tbs/tb294/tb294.html)
TM021	<i>PolyAtract® mRNA Isolation Systems Technical Manual</i> (www.promega.com/tbs/tm021/tm021.html)
TM228	<i>PolyAtract® System 1000 Technical Manual</i> (www.promega.com/tbs/tm228/tm228.html)

Promega Publications

PN099	RNA purification kit comparison: Yield, quality and real-time RT-PCR performance (www.promega.com/pnotes/99/15674_12/15674_12.html)
PN094	Cleanup of TRIzol® reagent-purified total RNA using the PureYield™ RNA Midiprep System (www.promega.com/pnotes/94/14410_07/14410_07.html)
PN092	PureYield™ RNA Midiprep System: Isolating pure total RNA without DNase (www.promega.com/pnotes/92/13408_14/13408_14.html)
PN086	MagneSil® Total RNA mini-Isolation System (www.promega.com/pnotes/86/11217_18/11217_18.html)
PN084	Quantitative, real-time RT-PCR expression using the SV 96 Total RNA Isolation System (www.promega.com/pnotes/84/10705_23/10705_23.html)

PN079	High-throughput purification using the SV 96 Total RNA Isolation System (www.promega.com/pnotes/79/9492_29/9492_29.html)
PN068	Isolation of RNA from plant, yeast and bacteria (www.promega.com/pnotes/68/7381_28/7381_28.html)
PN064	RNA purification: A rapid and versatile protocol for the isolation of total RNA (www.promega.com/pnotes/64/9331_07/promega.html)
PN063	Technically speaking: Tips for working with RNA and troubleshooting downstream applications (www.promega.com/pnotes/63/8581_17/promega.html)
PN055	A rapid protocol for the isolation of total RNA suitable for RT-PCR (www.promega.com/pnotes/55/5265e/5265e.html)
PN044	Isolation of mRNA from plant tissues using the PolyAtract® System 1000 (www.promega.com/pnotes/44/neille/neille.html)
PN039	30-Minute poly(A)+ RNA isolation directly from tissue (www.promega.com/pnotes/39/39_07/39_07.htm)
BR120	RNA Analysis Notebook (www.promega.com/guides/rna_guide/purifyingrna.pdf)
eNotes	Measuring gene expression from mammalian brain tissue (www.promega.com/enotes/applications/ap0007_tabs.htm)

Total RNA Isolation Using the SV Total RNA Isolation System

The SV Total RNA Isolation System provides a fast and simple technique to purify intact total RNA from tissues, cultured cells and white blood cells in as little as one hour, depending on the number of samples to be processed. Up to 60mg of tissue can be processed per purification, depending on the type and RNA expression levels of the tissue. The system also incorporates a DNase treatment step to substantially reduce genomic DNA contamination, which can interfere with amplification-based methods. For best results from this system, use fresh samples when processing tissue. Older samples may yield less total RNA. If necessary freeze the samples immediately after collection in liquid nitrogen, and store at -70°C for future use. Samples homogenized in SV RNA Lysis Buffer also may be stored at -70°C .

For a protocol for RNA isolation from cultured cells, tissue samples, white blood cells, plant tissue, yeast and bacterial cells, see the *SV Total RNA Isolation System Technical Manual* #TM048 (www.promega.com/tbs/tm048/tm048.html).

Materials Required:

(see Composition of Solutions section)

- SV Total RNA Isolation System (Cat.# Z3100, Z3101 or Z3105) and protocol
- small tissue homogenizer (for RNA isolation from tissue)
- ethanol, 95%, RNase-free
- microcentrifuge
- 10X phosphate-buffered saline (PBS), sterile (for RNA isolation from cultured cells)
- sterile hypodermic syringe fitted with a sterile 20-gauge needle (for RNA isolation from cultured cells)
- water bath or heating block, preheated to 70°C
- Laboratory Vacuum Manifold (e.g., Vac-Man®, Cat.# A7231, or Vac-Man® Jr. Laboratory Vacuum Manifold, Cat.# A7660) and Vacuum Adapters (Cat.# A1331) (required for RNA purification by vacuum)

Poly(A)+ RNA Isolation Using the PolyAtract® System 1000

The PolyAtract® System 1000 isolates poly(A)+ RNA directly from crude cell or tissue lysates using the Promega MagneSphere® technology, eliminating the need for oligo(dT) cellulose columns. The system uses a biotinylated oligo(dT) primer to hybridize in solution to the 3' poly(A) region present in most mature eukaryotic mRNA species. The hybrids are captured using streptavidin coupled to paramagnetic particles and a magnetic separation stand, then washed at high stringency. The mRNA is eluted from the solid phase by the simple addition of RNase-free deionized water. This procedure yields an essentially pure fraction of mature mRNA after only a single round of magnetic separation.

To isolate poly(A)+ RNA directly from tissue samples or cultured cells, see the *PolyAtract® System 1000 Technical Manual* #TM228 (www.promega.com/tbs/tm228/tm228.html). This technical manual also describes precipitation and concentration of mRNA and determination of mRNA concentration.

Materials Required:

(see Composition of Solutions section)

- PolyAtract® System 1000 (Cat.# Z5400 or Z5420) and protocol
- small tissue homogenizer (for RNA isolation from tissue)
- 50ml sterile screw-cap conical tubes
- 15ml sterile COREX® or other glass centrifuge tubes
- 70°C water bath
- Beckman Model J2-21 centrifuge or equivalent
- 1X PBS (for RNA isolation from cell cultures)
- scale or balance (to weigh tissue samples)
- MagneSphere® Magnetic Separation Stand (see Table 1 of the *PolyAtract® System 1000 Technical Manual*)

#TM228 (www.promega.com/tbs/tm228/tm228.html) to determine the appropriate magnetic stand)

B. DNA and RNA Labeling

A number of methods exist to attach a label to a nucleic acid molecule. These consist of techniques to incorporate the label into the substrate or attach the label to the ends of a nucleic acid fragment. The choice of method is determined largely by the nature of the substrate to be labeled. Some other factors to consider include: the amount of substrate available for labeling, its size in base pairs, the type of nucleic acid (DNA or RNA), the desired specific activity and whether it is double-stranded or single-stranded. Promega provides several nucleic acid-labeling systems, which are described briefly in this section.

Random-Primed Labeling

Random-primed labeling (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984) uses a mixture of random hexadeoxyribonucleotides to prime DNA synthesis in vitro from any linear double-stranded DNA template. With this method, it is possible to generate probes of high specific activity ($>1 \times 10^6$ cpm/ μ g), even using DNA fragments cut from agarose gels (Feinberg and Vogelstein, 1984). Since the input DNA serves as a template and remains intact during the reaction, minimal amounts of DNA (25ng) can be labeled to a high specific activity. Using the Prime-a-Gene® Labeling System, 40–80% of the labeled deoxyribonucleotide can typically be incorporated into the DNA template, depending on the template and reaction conditions used. Using a template greater than 500bp, probes generated by random-primed labeling generally are 250–300bp in length and are suitable for a variety of applications, including Northern analysis.

5' End Labeling

5' end labeling uses T4 polynucleotide kinase, which catalyzes the transfer of the γ -phosphate group from ATP to the 5'-hydroxyl terminus of double-stranded or single-stranded DNA or RNA molecules (the forward reaction). Suitable substrates include synthetic oligonucleotides, most of which lack a 5'-phosphate group, and DNA fragments that have been dephosphorylated with alkaline phosphatase to remove the 5'-phosphate groups. Under certain conditions, the reaction with T4 polynucleotide kinase can be made reversible, permitting exchange of the γ -phosphate of ATP with the 5' terminal phosphate of a polynucleotide (the exchange reaction, see the *T4 Polynucleotide Kinase Technical Bulletin* #TB519 (www.promega.com/tbs/tb519/tb519.html)), thus circumventing the need to dephosphorylate the substrate (Donis-Keller *et al.* 1977). The specific activity of a probe generated using the forward reaction is typically 2×10^6 cpm/pmol, while the specific activity of a probe generated using the exchange reaction is approximately 6×10^5 cpm/pmol (Berger and Kimmel, 1987). The Promega 5' End-Labeling System includes both T4 Polynucleotide Kinase and Calf Intestinal Alkaline Phosphatase and their optimal reaction buffers to perform the dephosphorylation

and labeling reactions (see the *DNA 5' End-Labeling System Technical Bulletin* #TB096 (www.promega.com/tbs/tb096/tb096.html)).

3' End Labeling

Terminal deoxynucleotidyl transferase (TdT) is an enzyme that catalyzes the repetitive addition of mononucleotides from dNTPs to the terminal 3'-OH of a DNA initiator accompanied by the release of inorganic phosphate (Kato *et al.* 1967). The enzyme, which is available from Promega as Terminal Deoxynucleotidyl Transferase, Recombinant (Cat.# M1871), provides several methods to label the 3' termini of DNA. The first involves adding an [α - 32 P] dNTP "tail" to the 3' termini of single-stranded DNA fragments. The number of nucleotides that will be added to the DNA template depends on the ratio of nucleotides to 3'-OH termini (Grosse and Manns, 1993). Alternatively, incorporation can be limited to a single nucleotide by using [α - 32 P] cordycepin-5'-triphosphate, which lacks a free 3' hydroxyl group, preventing incorporation of additional nucleotides (Tu and Cohen, 1980). The specific activity of probes generated by 3' end labeling are typically 5×10^6 cpm/ μ g (Brown, 1998).

Nick Translation

To label DNA by nick translation, free 3'-hydroxyl ends (nicks) are created within the unlabeled DNA by DNase I. DNA polymerase I then catalyzes the addition of a nucleotide residue to the 3'-hydroxyl terminus of the nick.

At the same time, the 5'→3' exonuclease activity of this enzyme removes the nucleotide from the 5'-phosphoryl terminus of the nick. The new nucleotide is incorporated at the position where the original nucleotide was excised, and the nick is thus shifted along one nucleotide at a time in a 3' direction. This 3' shift of the nick results in the sequential addition of labeled nucleotides to the DNA, while the pre-existing nucleotides are removed (Sambrook and Russell, 2001). DNA probes prepared by nick translation can be used for a wide variety of hybridization techniques, such as gel blots and colony plaque lifts. Typically greater than 65% of the labeled deoxyribonucleotide is incorporated, generating high-specific-activity probes (routinely 10^8 dpm/ μ g) approximately 400–750 nucleotides in length (Sambrook and Russell, 2001).

in vitro Transcription

RNA probes can be synthesized by in vitro transcription (Melton *et al.* 1984) in the presence of a radioactive or non-radioactive label. Suitable radioactive labels include 32 P-, 33 P-, 35 S- or 3 H-labeled ribonucleotide. These probes have a defined length and are useful for Northern and Southern blots, in situ hybridization and RNase protection assays (Melton *et al.* 1984; Sambrook and Russell, 2001; Uhlig *et al.* 1991). Using an [α - 32 P]rCTP label and the conditions described in the *Riboprobe® in vitro Transcription System Technical Manual* #TM016 (www.promega.com/tbs/tm016/tm016.html), RNA transcribed in vitro will typically have a specific activity of 2 – 2.5×10^8 cpm/ μ g.

Additional Resources for DNA and RNA Labeling

Technical Bulletins and Manuals

TB049	<i>Prime-a-Gene® Labeling System Technical Bulletin</i> (www.promega.com/tbs/tb049/tb049.html)
9PIM187	<i>Terminal Deoxynucleotidyl Transferase, Recombinant, Promega Product Information</i> (www.promega.com/tbs/9pim187/9pim187.html)
TB096	<i>DNA 5' End-Labeling System Technical Bulletin</i> (www.promega.com/tbs/tb096/tb096.html)
TB519	<i>T4 Polynucleotide Kinase Technical Bulletin</i> (www.promega.com/tbs/tb519/tb519.html)
TM016	<i>Riboprobe® in vitro Transcription Systems Technical Manual</i> (www.promega.com/tbs/tm016/tm016.html)

V. Composition of Solutions

Denhardt's Reagent, 50X (500ml)

- 5g Ficoll® (Type 400)
- 5g polyvinylpyrrolidone
- 5g bovine serum albumin (Fraction V)

Dissolve in DEPC-treated water, and adjust the volume to 500ml. Sterilize by filtration (0.45mm), and store at -20° C.

DEPC-treated water

Add diethyl pyrocarbonate (DEPC) to deionized water at a final concentration of 0.1%. Incubate overnight at room temperature in a fume hood. Autoclave for 20 minutes.

Caution: DEPC is a suspected carcinogen. Work in a fume hood, and follow standard laboratory safety procedures.

MOPS 5X buffer (2L)

- 0.2M 3-[N-morpholino]-2-hydroxypropanesulfonic acid (MOPS) (pH 7.0)
- 0.05M sodium acetate
- 0.005M EDTA (pH 8.0)

To prepare 2 liters of buffer, add 83.72g of MOPS (free acid) and 8.23g of sodium acetate to 1.6 liters of DEPC-treated water, and stir until completely dissolved. Add 20ml of DEPC-treated 0.5M EDTA, and adjust the pH to 7.0 with 10N NaOH. Bring the final volume to 2 liters with DEPC-treated water. Dispense into 200ml aliquots, and autoclave. The solution will turn yellow, but this will not affect the quality of the buffer.

PBS (1L)

0.2g KCl
8.0g NaCl
0.2g KH₂PO₄
1.15g Na₂HPO₄

Add components one at a time to 900ml of room-temperature deionized water, and stir until completely dissolved. Adjust the pH to 7.4 using 1N HCl or 1N NaOH if necessary. Bring the final volume to 1 liter. If stored for long periods filter the solution through a 0.45mm filter, and store in a tightly capped sterile bottle.

Prehybridization/hybridization solution

50% deionized formamide
5X SSPE
2X Denhardt's Reagent
0.1% SDS

RNase digestion buffer

10mM Tris-HCl (pH 7.5)
5mM EDTA
200mM sodium acetate

RNA loading buffer

50% glycerol
1mM EDTA
0.4% bromophenol blue

Prepare in nuclease-free water. Use very high-grade glycerol to avoid ribonuclease activity. Dispense into 500µl aliquots, and store at -20°C.

RNA sample buffer

10.0ml deionized formamide
3.5ml 37% formaldehyde
2.0ml MOPS 5X Buffer

Mix and dispense into 500µl aliquots. Store at -20°C in tightly sealed screw-cap tubes. These can be stored for up to 6 months. **Caution:** Formamide is a teratogen, and formaldehyde is a toxic carcinogen. Work in a fume hood, and follow standard laboratory safety procedures.

RPA hybridization buffer

80% deionized formamide
40mM PIPES (pH 6.4)
0.4M sodium acetate
1mM EDTA

RPA loading dye

80% deionized formamide
10mM EDTA
0.1% bromophenol blue
0.1% xylene cyanol
0.1% SDS

SSC, 20X (500ml)

87.7g NaCl
44.1g sodium citrate

Dissolve in 400ml of DEPC-treated water. Adjust the pH to 7.2 with 10N NaOH, and bring the volume to 500ml. Dispense into aliquots. Sterilize by autoclaving.

SSPE, 20X (1L)

175.3g NaCl
27.6g NaH₂PO₄ • H₂O
7.4g EDTA, disodium salt

Dissolve in 800ml of DEPC-treated water. Adjust the pH to 7.4 with 10N NaOH, and bring the volume to 1 liter. Autoclave.

Stringency wash solution I

2X SSC
0.1% SDS

Stringency wash solution II

0.1X SSC
0.1% SDS

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I. Introduction

Signal transduction is one of the most widely studied areas in biology. Extracellular information perceived at the surface of a cell must be translated into an intracellular response that involves a complex network of interwoven signaling cascades. These signaling events ultimately regulate such cellular responses as proliferation, differentiation, secretion and apoptosis. Signal transduction cascades are generally triggered by the binding of extracellular ligands, such as growth factors, cytokines, neurotransmitters or hormones, to a cell-surface receptor. These receptors transmit the stimulus to the interior of the cell, where the signal is amplified and directed a targeted signaling pathway.

The propagation and amplification of the primary signal involves a wide array of enzymes with specialized functions. Many of these signaling enzymes propagate the signal by post-translationally modifying other cellular proteins that are involved in the signaling cascade. Protein phosphorylation, one of the most common post-translational modifications, plays a dominant role in almost all signaling events and involves the transfer of a phosphate group from adenosine triphosphate (ATP) to the target protein (van der Geer *et al.* 1994). In general, phosphorylation either activates or inactivates a given protein to perform a certain function. Protein kinases and phosphatases are the enzymes responsible for determining the phosphorylation state of cellular proteins and, thus, whether a signal gets transduced within a cell. Changes in the level, subcellular localization and activity of kinases and phosphatases have consequences for normal cell function and maintenance of cellular homeostasis (De Meyts, 1995; Denton and Tavaré, 1995).

A. The MAPK Pathways

The Mitogen-Activated Protein Kinase (MAPK) signaling pathways play an important role in signal transduction in eukaryotic cells, where they modulate many cellular events including: mitogen-induced cell cycle progression through the G1 phase, regulation of embryonic development, cell movement and apoptosis, and cell differentiation (Murray, 1998; Schaeffer and Weber, 1999). These evolutionarily conserved pathways are organized in three-kinase modules consisting of a MAP kinase, an activator of MAP kinase (MAP Kinase Kinase or MEK) and a MAP Kinase Kinase Kinase (MEK Kinase, MEKK, or MAPK Kinase Kinase). There are at least three distinct MAP kinase signal transduction pathways in mammalian cells, each named after the particular MAPK associated with it (Figure 7.1). These include the extracellular signal-regulated kinases, ERK 1/2 (also known as MAPKs), the c-JUN N-terminal kinases/stress-activated protein kinase (JNK/SAPK) and the p38 kinases. An animated presentation (www.promega.com/paguide/animation/selector.htm?coreName=mapk01) highlighting some of the events during MAPK signaling is available.

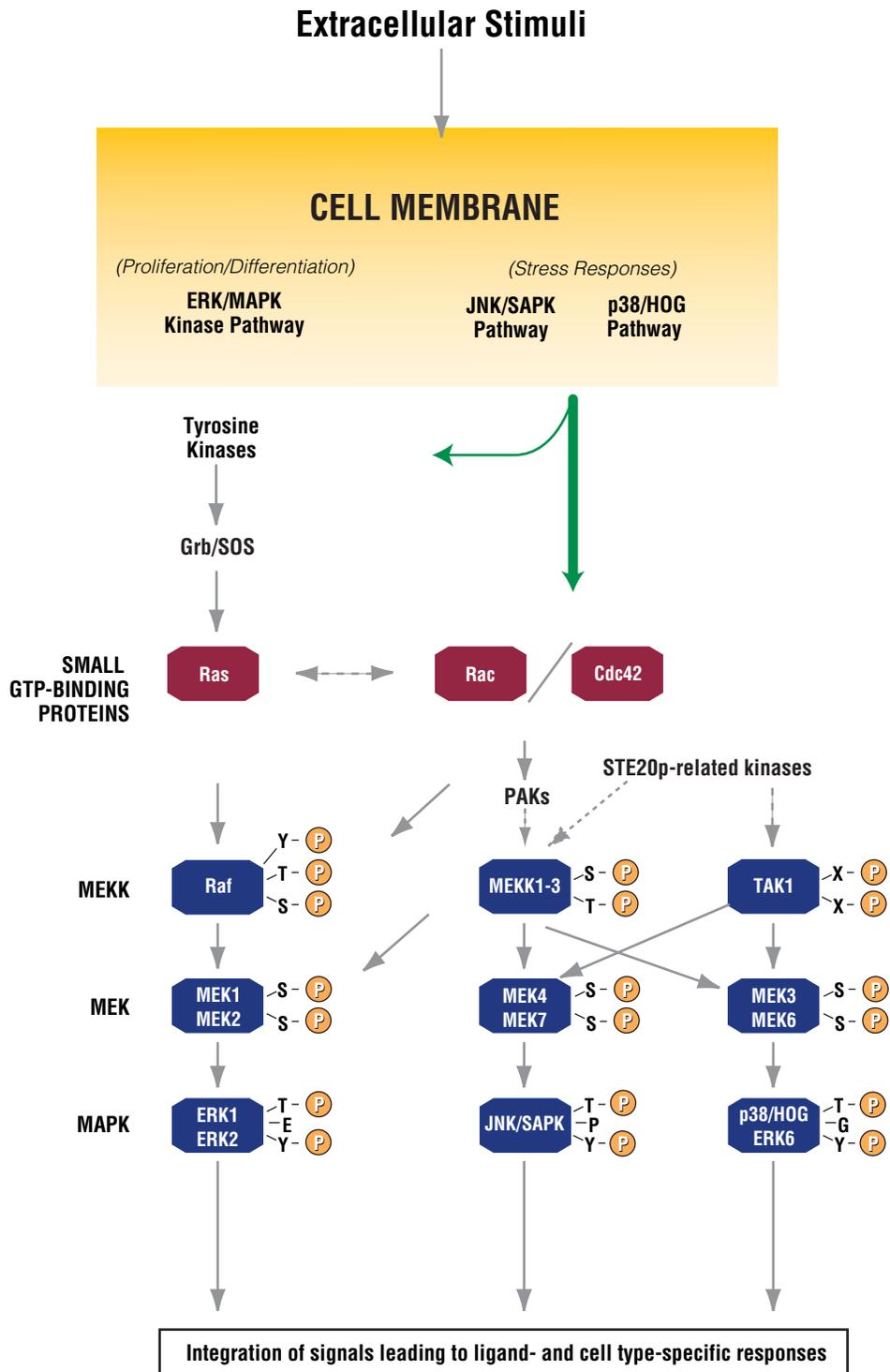
Signal transduction cascades involving ERK/MAPK enzymes are also regulated by the activities of a variety of protein phosphatases. Several dual-specificity protein phosphatases have been identified that can differentially dephosphorylate MAPK, JNK or p38 enzymes (Neel and Tonks, 1997; Ellinger-Ziegelbauer *et al.* 1997). In addition, individual Ser/Thr (e.g., PP2A) or Tyr (e.g., PTP1) phosphatases also appear to regulate the activity of the ERK/MAPK enzymes by dephosphorylating either core residue (Hunter, 1995; Keyse, 1995; Alessi, 1995; Doza, 1995). Thus, the cell can tightly regulate the activity of the ERK/MAPK enzymes by judicious use of different combination of MEKS, mono- and dual-specificity protein phosphatases and the subcellular localization of each enzyme to elicit the appropriate physiological response (Payne, 1991; Zhang, 2001).

B. The Phosphoinositol 3-Kinases (PI3-Ks)

Phosphoinositol 3-Kinases (PI3-Ks) catalyze the transfer of the gamma phosphate group from ATP to the 3-OH of three different substrates: phosphatidylinositol (PI), phosphatidyl inositol 4 phosphate (PI4P), and phosphatidyl inositol 4,5 phosphate (PI(4,5)P₂; Vanhaesebroeck *et al.* 2001). PI3-Ks modulate the levels of PIs in cells to influence many cellular functions including cell growth, gluconeogenesis and glycolysis, motility, and cell development and differentiation. These enzymes are divided into three classes, largely based on substrate preferences (Rameh and Cantley, 1999; Okkenhaug and Vanhaesebroeck, 2003). This review will focus on the class I PI3-Ks. Class I PI3-Ks comprise a 110kDa catalytic subunit (p110) and a regulatory/adaptor subunit. The class I PI3-Ks can be divided into subclasses A and B based on their upstream signaling partners. Class IA PI3-Ks signal downstream of tyrosine kinases; class IB PI3-Ks signal downstream of G protein-coupled receptors (GPCRs; Vanhaesebroeck *et al.* 2001).

The adaptor/regulatory subunit of Class IA PI3-K contains two Src homology 2 (SH2) domains through which it can bind to activated receptor tyrosine kinases (RTKs) or to cytosolic tyrosine kinases such as Src family kinases or JAK kinases. Binding to phosphotyrosine in the RTKs is thought to bring the cytosolic PI3-Ks to the membrane where the PI substrates reside (Cooray, 2004; Vanhaesebroeck *et al.* 2001). All mammalian cells investigated to date express at least one Class IA PI3-K, and stimuli that result in tyrosine kinase activity generally lead to class IA PI3-K activation (Vanhaesebroeck *et al.* 2001).

After the PI3-K is activated, it can phosphorylate its phosphoinositide substrates. PI can be phosphorylated to produce PI3P, which appears to bind selectively proteins that contain an FYVE domain, a Zn²⁺ finger domain that has been found in a diverse group of proteins, many of which are involved in membrane trafficking (Vanhaesebroeck *et al.* 2001). PI(4,5)P₂ can be phosphorylated by activated PI3-K to produce phosphatidyl inositol 3,4,5 triphosphate (PIP₃). PIP₃ in turn interacts with



Integration of signals leading to ligand- and cell type-specific responses

Figure 7.1. Activation of different MAPK signaling cascades by different extracellular stimuli. The ERK, JNK and p38 cascades all contain the same series of three kinases. A MEK Kinase (MEKK) phosphorylates and activates a MAP Kinase Kinase (MEK), then MEK phosphorylates and activates a MAP Kinase (MAPK).

a variety of molecules, such as Akt Kinase (also called PKB) via the pleckstrin homology (PH) domains of these downstream targets (Cooray, 2004). Interaction of PIP₃ with Akt allows phosphorylation of Akt by PDK 1 and

subsequent activation of Akt (Rameh and Cantley, 1999). Akt is a serine/threonine kinase that phosphorylates many different target proteins. Many pro-apoptotic proteins are substrates of Akt that are inactivated by Akt

phosphorylation, including Bad, caspase-9 and GSK-3 (Cooray, 2004). Akt also regulates transcription of many genes including forkhead transcription factors and NF- κ B (Cooray, 2004; Sliva, 2004). An animated presentation (www.promega.com/paguide/animation/selector.htm?coreName=pi3k01) that shows some events associated with the PI3-K pathway is available.

In *Drosophila*, PI3-K is implicated in regulating cell growth without affecting cell division rates. Studies of wing imaginal discs show that overexpression of the p110 subunit of PI3-K results in increased growth. Reducing activity reduces the size of the wing imaginal disc, producing adult flies with small wings (Vanhaesebroeck *et al.* 2001). This size effect does not appear to be tied to differences in cell division rates. Similar results have been observed in studies of PI3-K signaling in mouse heart, where cell growth is affected but cell division rates are not.

PI3-K signaling is also implicated in progression to S phase and DNA synthesis in cells. PI3-K activity is tied to the accumulation of cyclin D in cells and may act at a variety of levels, transcription, post transcription, and post translation, to affect cyclin D accumulation. PI3-Ks may also play roles in relieving inhibition of the cell cycle (Vanhaesebroeck *et al.* 2001).

PI3-Ks and Cancer

PI3-Ks are implicated in breast, colon, endometrial, head and neck, kidney, liver, lymphoma, melanoma, sarcoma and stomach cancers (Sliva, 2004), making them an important therapeutic target for human cancer therapy. In fact, PI3-K mutations found in human cancers have oncogenic activity (Kang *et al.* 2005), and PI3-K might mediate its activity through mTOR (Aoki *et al.* 2001). Cell motility is one of many cell functions influenced by PI3-K signaling. Invasive breast cancer MDA-MB-231 cells, have higher than normal PI3-K activity. Inhibition of PI3-K by dominant negative mutations of the PI3-K regulatory/adaptor subunits or treatment with LY 294002 or wortmannin (PI3-K-specific inhibitors) suppresses motility of these cells (Sliva, 2004). Studies indicate that PI3-K may play a role in actin cytoskeleton rearrangements, perhaps through guanosine nucleotide exchange factors and GTPase-activating proteins (Vanhaesebroeck *et al.* 2001).

PI3-Ks can also activate NF- κ B through a variety of mechanisms in different cells. In HepG2 cells, IL-1 stimulates the phosphorylation and activation of NF- κ B through a PI3-K-dependent pathway (Sliva, 2004). Expression of a dominant negative regulatory subunit of PI3-K or treatment with PI3-K inhibitors suppressed NF- κ B activation as well as motility in the MDA-MB-231 cell line (Sliva, 2004).

A viral oncogene that encodes a variant PI3-K was isolated from a chicken retrovirus. Expression of this oncogene increases cellular PI, activates Akt and transforms chicken embryo fibroblasts (Rameh and Cantley, 1999). These

oncogenic effects may be mediated through the pathways by which PI3-Ks normally influence cell growth, cell cycle progression and transcription.

PI3-K signaling is balanced by the activities of inositol lipid phosphatases. The most well studied PI phosphatase is PTEN, which was first described as a tumor suppressor that is deleted or mutated in several human cancers (Rameh and Cantley, 1999). Furthermore, physical interaction of PTEN with the MSP58 oncogene inhibits cellular transformation, thus validating the role of PTEN as a tumor suppressor (Okamura *et al.* 2005).

C. Investigating Phosphatases and Kinases as Potential Therapeutic Targets

The human genome is reported to contain 518 protein kinases that are involved in phosphorylation of 30% all cellular proteins (Manning *et al.* 2002). Taken together, genes for protein kinases and phosphatases represent five percent of the human genome (Cohen, 2001). Many other phosphotransferases play equally important roles in cellular reactions that use ATP as substrate but are not classified as protein kinases. These include PI3-kinases (Shears, 2004), lipid kinases such as sphingosine kinases (French *et al.* 2003) and sugar kinases such as glucokinase (Grimsby *et al.* 2003). Changes in the level, activity or localization of these kinases, phosphotransferases and phosphatases greatly influence the regulation of key cellular processes. Because of the role that these enzymes play in cellular functions and in various pathologies, they represent important drug targets (Cohen, 2002). By 2002, more than twenty-six small molecule inhibitors of protein kinases alone were either approved for clinical use or in phase I, II or III clinical trials (Cohen, 2002; Pearson and Fabbro, 2004).

This chapter describes the tools available for investigating the activities of kinases and phosphatases that are involved in signaling cascades. We describe a variety of technologies including luminescent and fluorescent assays for kinase and phosphatases. The phosphorylation state of the substrates of kinases can also be informative when studying cell signaling. We describe a variety of antibodies for detecting the phosphorylated forms of some kinase substrates as well as kinase substrates and inhibitors that can be used as tools to analyze kinase activities in samples.

II. Kinase Activity Assays

A. Luminescent Kinase Assays

Kinases are enzymes that catalyze the transfer of a phosphate group from ATP to a substrate. The depletion of ATP as a result of kinase activity can be monitored in a highly sensitive manner through the use of Kinase-Glo[®] or Kinase-Glo[®] Plus Reagent, which uses luciferin, oxygen and ATP as substrates in a reaction that produces oxyluciferin and light (Figure 7.2).

The Kinase-Glo[®] and Kinase-Glo[®] Plus Reagents rely on the properties of a proprietary thermostable luciferase (Ultra-Glo[™] Recombinant Luciferase) that is formulated to generate a stable “glow-type” luminescent signal. The

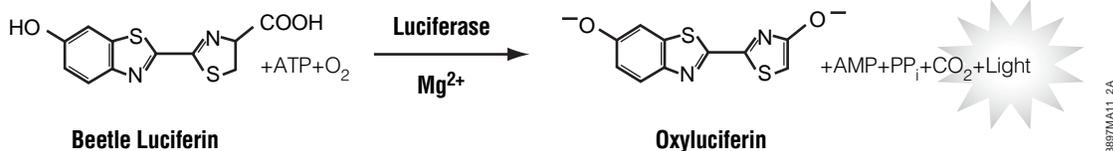


Figure 7.2. The luciferase reaction. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg^{2+} , ATP and molecular oxygen and produces one photon of light per turnover.

reagents are prepared by combining the Kinase-Glo[®] or Kinase-Glo[®] Plus Buffer with the lyophilized substrate provided with each system.

The protocol for both systems involves a single addition of an equal volume of Reagent to a completed kinase reaction that contains ATP, purified kinase and substrate. The plate is mixed and luminescence read. The luminescence is directly proportional to the ATP present in the kinase reaction, and kinase activity is inversely correlated with luminescent output.

The Kinase-Glo[®] Luminescent Kinase Assay (Cat.# V6711) and the Kinase-Glo[®] Plus Luminescent Kinase Assay (Cat.# V3771) can be used with virtually any kinase and substrate combination. The Kinase-Glo[®] Assay is extremely sensitive and is linear from 0 to 10 μ M ATP. It routinely provides Z'-factor values greater than 0.8 in both 96-well and 384-well formats (Figure 7.3). Z'-factor is a statistical measure of assay dynamic range and variability; a Z'-factor greater than 0.5 is indicative of a robust assay (Zhang *et al.* 1999).

We have demonstrated the utility of the Kinase-Glo[®] Assay for high-throughput screening (Somberg *et al.* 2003; Goueli *et al.* 2004a). We tested the Kinase-Glo[®] Assay using a commercially available Library of Pharmacologically Active Compounds (LOPAC) to determine if the assay could score true kinase hits in that library. When we screened the LOPAC collection for inhibitors of PKA using the manual protocol, we found six wells in which we could detect kinase inhibition (Somberg *et al.* 2003). The same six wells also showed detectable kinase inhibition when we tested the Kinase-Glo[®] Assay in low-volume 384 and 1536-well formats (Goueli *et al.* 2004b; Figure 7.4). The Kinase-Glo[®] Assay can also be used to determine IC₅₀ values for kinase inhibitors. The IC₅₀ values for one of the six hits from the LOPAC library were determined using the Kinase-Glo[®] Assay. The Kinase-Glo[®] Assay gave values similar to values reported in the literature, further establishing the utility of the Kinase-Glo[®] Assay for high-throughput screening (Goueli *et al.* 2004b).

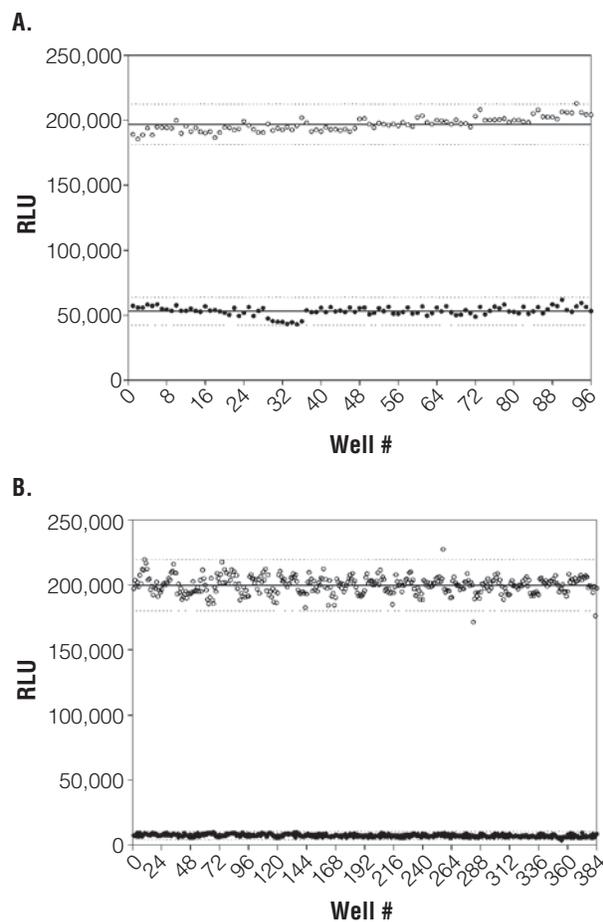


Figure 7.3. Determining Z'-factor for the Kinase-Glo[®] Assay. **Panel A.** The reaction was performed using 0.25 units/well PKA (solid circles) or no PKA (open circles) in 100 μ l volume. PKA was diluted in 50 μ l kinase reaction buffer (40mM Tris [pH 7.5], 20mM $MgCl_2$ and 0.1mg/ml BSA), containing 5 μ M Kemptide Substrate (Cat.# V5161) and 1 μ M ATP. The kinase reaction was run for 20 minutes at room temperature. **Panel B.** The 384-well plate assay was performed using 0.05 units/well (solid circles) or no PKA (open circles) in 20 μ l volume. Solid lines indicate mean, and dotted lines indicate \pm 3 S.D. Z'-factor values were ~0.8 in both formats.

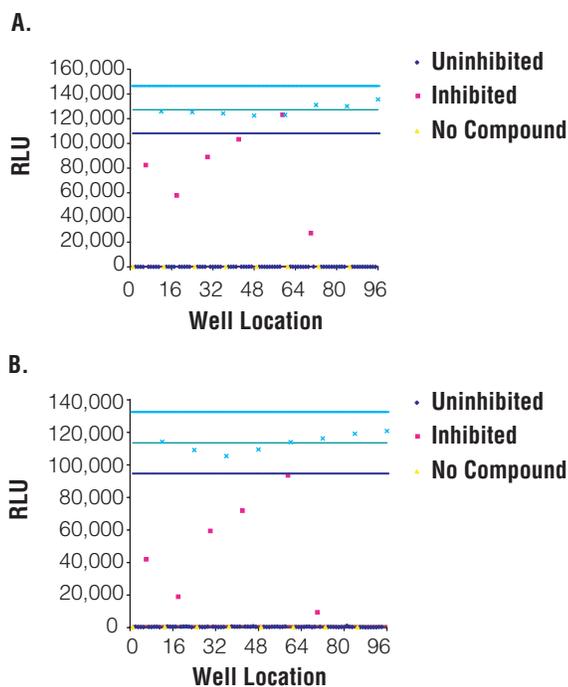


Figure 7.4. Compound screen using Plate 6 of the LOPAC (Sigma-RBI) performed in LV384- (Panel A) and 1536-well (Panel B) formats. Compounds were screened at 10 μ M. See Goueli *et al.* 2004a (www.promega.com/cnotes/cn010/cn010_20.htm) for percent inhibition of compounds that inhibited kinase activity.

The Kinase-Glo[®] Plus Assay not only allows users to detect kinase inhibitors, but also to distinguish between ATP competitive and noncompetitive inhibitors. Because the concentration of ATP in cells is fairly high, inhibitors of protein kinases that are not ATP-competitive are more desirable as therapeutic agents than ATP-competitive kinase inhibitors. Because the catalytic domains and active sites of protein kinases have been evolutionarily conserved, inhibitors that are not only ATP non-competitive, but also selective toward the target kinase are most desirable. The Kinase-Glo[®] Plus Assay is optimized to work at ATP concentrations that more closely reflect cellular ATP concentrations and is linear up to 100 μ M ATP.

Materials Required:

- Kinase-Glo[®] Assay System (Cat.# V6711, V6712, V6713, V6714) or Kinase-Glo[®] Plus Assay System (Cat.# V3771, V3772, V3773, V3774) and Protocol (Technical Bulletin #TB318 (www.promega.com/tbs/tb318/tb318.html) or #TB343 (www.promega.com/tbs/tb343/tb343.html)).
- solid white multiwell plates
- multichannel pipet or automated pipetting station
- plate shaker
- luminometer capable of reading multiwell plates
- ATP
- appropriate kinase substrate
- appropriate kinase reaction buffer

Figure 7.5 provides an overview of the Kinase-Glo[®] Assay Protocol. The Kinase-Glo[®] Plus Assay follows the same format.

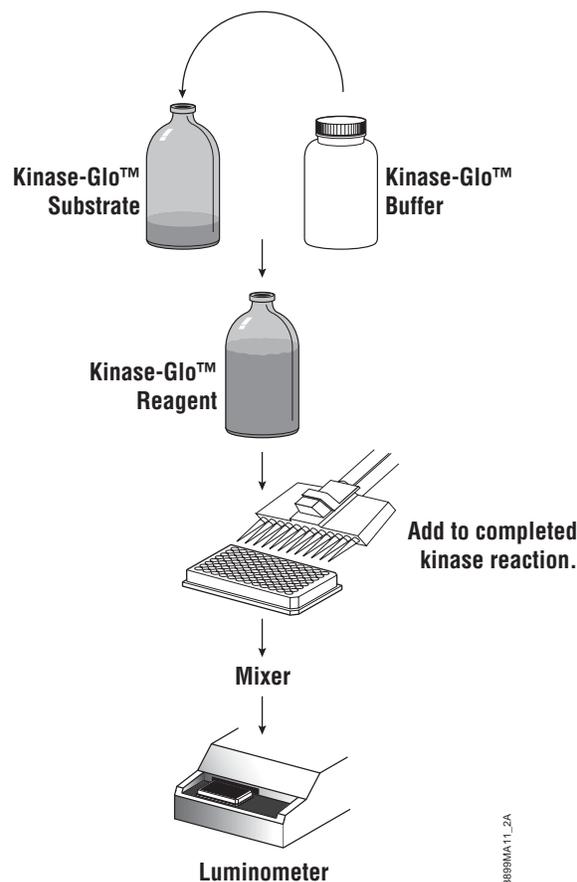


Figure 7.5. Schematic diagram of the Kinase Glo[®] Assay protocol.

Additional Resources for Kinase-Glo[®] and Kinase-Glo[®] Plus Luminescent Kinase Assays

Technical Bulletins and Manuals

- | | |
|-------|---|
| TB318 | <i>Kinase-Glo[®] Luminescent Kinase Assay</i>
(www.promega.com/tbs/tb318/tb318.html) |
| TB343 | <i>Kinase-Glo[®] Plus Luminescent Kinase Assay</i>
(www.promega.com/tbs/tb343/tb343.html) |

Promega Publications

- | | |
|-------|--|
| CN011 | Citation Note: Measuring LPS-induced PKC activity in U937 cells
(www.promega.com/cnotes/cn011/cn011_17.htm) |
| CN010 | High-throughput screening using a universal luminescent kinase assay
(www.promega.com/cnotes/cn010/cn010_20.htm) |
| CN005 | Kinase-Glo [®] Assay: Detect virtually any kinase
(www.promega.com/cnotes/cn005/cn005_05.htm) |

PN083 Introducing the Kinase-Glo® Luminescent Kinase Assay
 (www.promega.com/notes/83/10492_14/10492_14.html)

Citations

Koresawa, M. and Okabe, T. (2004) High-throughput screening with quantitation of ATP consumption: A universal non-radioisotope, homogeneous assay for protein kinase. *Assay Drug Dev. Technol.* **2**, 153–60.

The authors describe the advantages of the Kinase-Glo® Assay for high-throughput screening. Cyclin-dependent kinase 4 (Cdk4) was used as a model kinase to draw comparisons between the Kinase-Glo® Assay and a "gold standard" radioactive filter assay in terms of reproducibility and use screening for true hits of kinase inhibitors in chemical libraries.

PubMed Number: 15165511

B. Fluorescent Kinase Assays

The ProFluor® Kinase Assays measure PKA (Cat.# V1240, V1241) or PTK (Cat.# V1270, V1271) activity using purified kinase in a multiwell plate format and involve "add, mix, read" steps only. The user performs a standard kinase reaction with the provided bisamide rhodamine 110 substrate. The provided substrate is nonfluorescent. After the kinase reaction is complete, the user adds a Termination Buffer containing a Protease Reagent. This simultaneously stops the reaction and removes amino acids specifically from the nonphosphorylated R110 Substrate, producing highly fluorescent rhodamine 110. Phosphorylated substrate is resistant to protease digestion and remains nonfluorescent. Thus, fluorescence is inversely correlated with kinase activity (Figure 7.6).

We tested the ability of several tyrosine kinases to phosphorylate the peptide substrate provided in the ProFluor® Src-Family Kinase Assay using protease cleavage and fluorescence output as an indicator of enzyme activity.

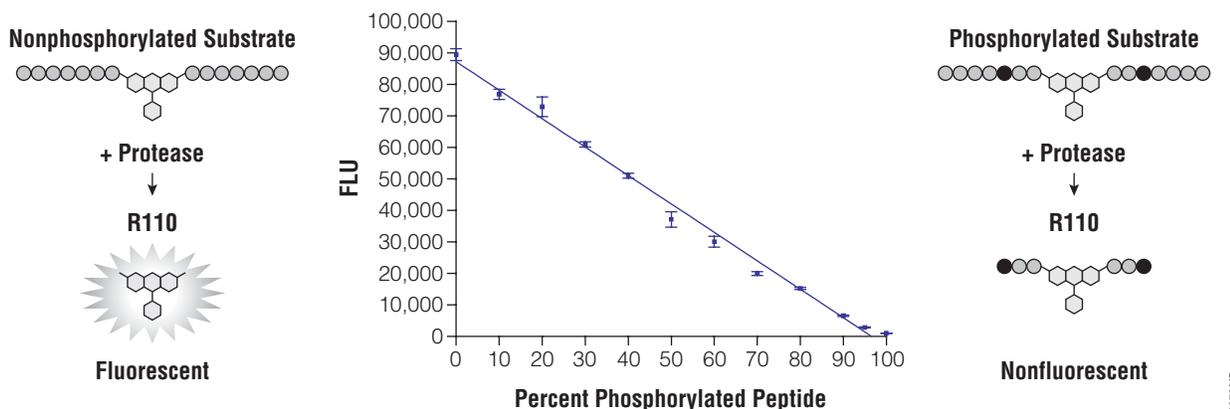


Figure 7.6. Schematic graph demonstrating that the presence of a phosphorylated amino acid (black circles) blocks the removal of amino acids by the protease. The graph shows the average FLU (n = 6) obtained after a 30-minute Protease Reagent digestion using mixtures of nonphosphorylated PKA R110 Substrate and phosphorylated PKA R110 Substrate. (FLU = Fluorescence Light Unit, excitation wavelength 485nm, emission wavelength, 530nm, $r^2 = 0.992$). As the concentration of the phosphopeptide increases in the reaction, FLU decreases.

The PTK peptide substrate served as an excellent substrate for all of the Src-family PTKs such as Src, Lck, Fyn, Lyn, Jak and Hck and the recombinant epidermal growth factor receptor (EGFR) and insulin receptor (IR). The fluorescence decreases with increasing concentrations for four Src family enzymes tested (Goueli *et al.* 2004a). The amount of enzyme required to phosphorylate 50% of the peptide (EC_{50}) was quite low (EC_{50} for Src, Lck, Fyn, Lyn A and Hck were 14.0, 1.38, 4.0, 4.13 and 1.43ng, respectively). As low as a few nanograms of Lck could be detected using this system.

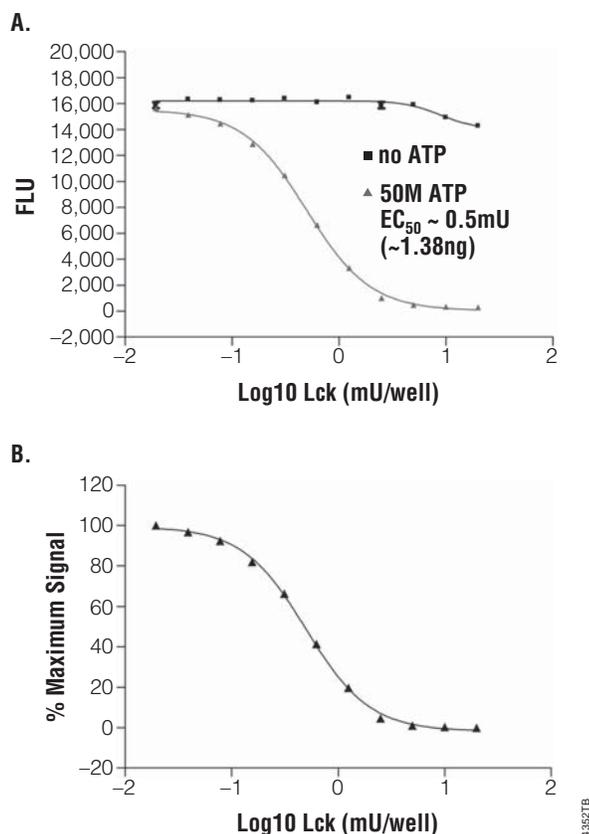


Figure 7.7. Kinase activity is inversely correlated with R110 fluorescence. Results of titration curves performed according to the protocol in Technical Bulletin #TB331 (www.promega.com/tbs/tb331/tb331.html) in solid black, flat-bottom 96-well plates. Panels A and B show the results of a Lck titration (Upstate Biotech Cat.# 14-442). **Panel A** shows the data collected (actual R110 FLU) with or without ATP. Data points are the average of 4 determinations. Curve fitting was performed using GraphPad Prism® 4.0 sigmoidal dose response (variable slope) software. The R² value is 0.99, EC₅₀ is 0.5mU/well, and the maximum dynamic range in the assay is ~50- to ~60-fold. Normalizing the data allows quick determination of the amount of kinase required for the percent conversion desired (**Panel B**).

Generalized Protocol for the ProFluor® Kinase Assays

Materials Required:

- ProFluor® PKA Assay (Cat.# V1240, V1241) or ProFluor® Src-Family Kinase Assay (Cat.# V1270, V1271) and protocol (Technical Bulletin #TB315 (www.promega.com/tbs/tb315/tb315.html) or #TB331 (www.promega.com/tbs/tb331/tb331.html), respectively)
- black-walled multiwell plates (e.g., Microfluor 2, black 96-well plate; ThermoElectron Cat.# 7805)
- multichannel pipet or automated pipetting station
- plate shaker (e.g., DYNEX MICRO-SHAKER® II)
- plate-reading fluorometer with filters capable of reading R110 and AMC fluorescence
- protein kinase

General Assay Protocol and Format for ProFluor® Kinase Assays

1. Dilute kinase and R110 Substrate in 1X Reaction Buffer.
2. Dilute ATP in 1X Reaction Buffer.
3. Mix contents in wells of plate and incubate at room temperature (20 minutes for PKA Assay, 60 minutes for Src-Family Kinase Assay).
4. Dilute Protease Reagent in 1X Termination Buffer A.
5. Mix plate and incubate at room temperature (30 minutes for PKA Assay, 60 minutes for Src-Family Kinase Assay).
6. Dilute Stabilizer Reagent in 1X Termination Buffer A.
7. Mix plate and R110 and AMC fluorescence.

We highly recommend performing a kinase titration to determine the optimal amount of kinase to use for screening and to determine whether or not the enzyme preparation contains components that negatively affect the performance of the assay. Please see Technical Bulletins #TB315 (www.promega.com/tbs/tb315/tb315.html) or #TB331 (www.promega.com/tbs/tb331/tb331.html) for additional information.

Additional Resources for ProFluor® Kinase Assays

Technical Bulletins and Manuals

- | | |
|-------|---|
| TB315 | <i>ProFluor® PKA Assay Technical Bulletin</i>
(www.promega.com/tbs/tb315/tb315.html) |
| TB331 | <i>ProFluor® Src-Family Kinase Assay Technical Bulletin</i>
(www.promega.com/tbs/tb331/tb331.html) |

Promega Publications

- | | |
|-------|---|
| CN005 | ProFluor® PKA Assay: Excellent Z'-factor values mean reliable results
(www.promega.com/cnotes/cn005/cn005_02.htm) |
| CN008 | Assay protein tyrosine kinase and protein tyrosine phosphatase activity in a homogeneous, non-radioactive, high-throughput format
(www.promega.com/cnotes/cn008/cn008_15.htm) |

Citations

Kupcho, K. *et al.* (2003) A homogeneous, nonradioactive high-throughput fluorogenic protein kinase assay. *Anal. Biochem.* **317**, 210–7.

This paper presents the ProFluor® Assays to measure enzyme activity of low concentration protein kinases.

PubMed Number: 12758259

III. Radioactive Kinase Assays

A. SAM²® Biotin Capture Membrane and Biotin Capture Plate

The SAM²® Biotin Capture Membrane (Cat.# V2861, Cat.# V7861; Figure 7.8) is a proprietary technology that relies on the high-affinity streptavidin:biotin interaction for the capture and detection of biotinylated molecules regardless of their sequence. The unique features of the SAM²® Membrane compared to other membranes or substrates (e.g., P81 phosphocellulose or streptavidin-coated plates), is the high density of covalently linked streptavidin per square centimeter and the selective mode of capture. This high-density streptavidin matrix efficiently captures biotinylated molecules or substrates, providing high signal-to-noise ratios even in assays using low enzyme concentrations or crude cell extracts. The SAM²® Biotin Capture Membrane offers superior assay performance by providing high binding capacity, low nonspecific binding, sequence-independent capture and the flexibility of multiple format configurations. The SAM²® Membrane is available as a sheet containing 96 numbered and partially cut squares. This format is used in the SignaTECT® Kinase Assay Systems. The SAM²® Membrane is also available as a 7.6 × 10.9cm solid sheet, which can be used for high-throughput applications. The membrane can be analyzed by autoradiography, PhosphorImager® analysis, or scintillation counting.

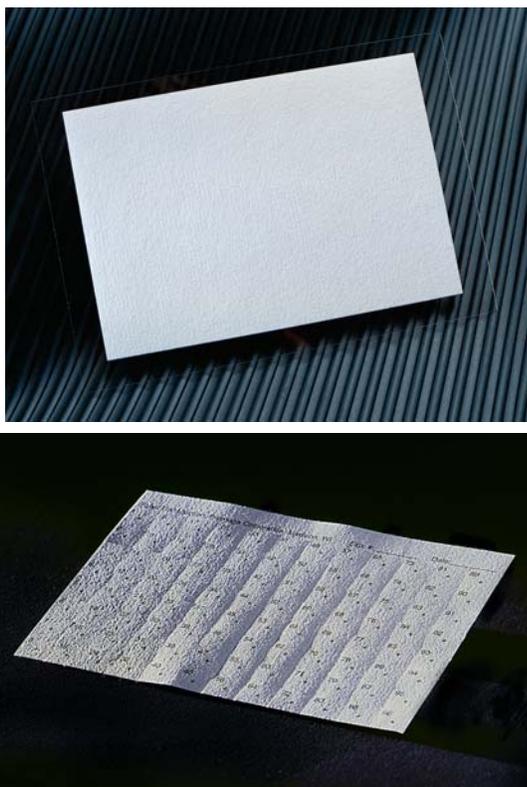


Figure 7.8. SAM²® Biotin Capture Membrane shown as a 7.6 × 10.9cm sheet (top) and in a 96-square format (bottom).

The SAM²® 96 Biotin Capture Plate (Cat.# V7541, V7542) contains the SAM²® Biotin Capture Membrane in the wells of a microfiltration plate. The 96-well plate configuration allows users to perform washes with a vacuum manifold or a plate washer. The plate is supplied with a transparent top seal and opaque bottom seal for adding scintillation fluid to perform quantitation using a microplate liquid scintillation counter.



Figure 7.9. The SAM²® 96 Biotin Capture Plate.

Additional Resources for SAM²® Membranes

Technical Bulletins and Manuals

- TB547 SAM²® Biotin Capture Membrane Technical Bulletin
(www.promega.com/tbs/tb547/tb547.html)
- TB249 SAM²® 96 Biotin Capture Plate Technical Bulletin
(www.promega.com/tbs/tb249/tb249.html)

Promega Publications

- CN005 From one to 9,000 samples: Using high-density streptavidin-coated membranes for kinase detection
(www.promega.com/cnotes/cn005/cn005_09.htm)
- PN064 Advances in SAM²® Membrane technology: High-throughput biotin capture systems for use in rapid screening
(www.promega.com/pnotes/64/9331_02/promega.html)
- PN075 Protein kinases as drug targets in high-throughput systems
(www.promega.com/pnotes/75/8554_24/8554_24.html)
- BR095 Signal Transduction Resource
(www.promega.com/guides/sigtrans_guide/default.htm)

Citations

Xuei, X. *et al.* (2003) Use of SAM²® Biotin Capture Membrane in microarrayed compound screening (μ ARCS) format for nucleic acid polymerization assays *J. Biol. Mol. Screening* **8**, 273–82.

This study evaluated the feasibility of using the μ ARCS technology for nucleic acid polymerization assays. To ensure the efficient capture of the nucleic acid polymerization reaction and to minimize the nonspecific binding, the authors used a SAM2® Biotin Capture Membrane in the assay. In both studies, the nucleic acid substrate was biotinylated on one end and was bound to the SAM2® Membrane.

PubMed Number: 12857381

Huynk, Q.K. *et al.* (2000) Characterization of the recombinant IKK1/IKK2 heterodimer. Mechanisms regulating kinase activity. *J. Biol. Chem.* **275**, 25883–91. Kinase activity of IKK1/IKK2 was measured using a biotinylated IKB α peptide. The reaction was run and added to a SAM2® 96-well Biotin Capture plate. The plate was washed, dried, and γ^{33} ATP was measured to indicate kinase activity.

PubMed Number: 10823818

B. SignaTECT® Protein Kinase Assay Systems

The SignaTECT® Protein Kinase Assay Systems use biotinylated peptide substrates in conjunction with the streptavidin-coated SAM2® Biotin Capture Membrane. The binding of biotin to the streptavidin is rapid and strong, and the association is unaffected by rigorous washing procedures, denaturing agents, wide extremes in pH, temperature and salt concentration. High signal-to-noise ratios are generated even with complex samples, while the high substrate capacity allows optimum reaction kinetics. The systems can be used to measure protein kinase activities using low femtomole levels of purified enzyme or crude cellular extracts. SignaTECT® Assays are available to measure protein tyrosine kinases (Cat.# V6480), cdc2 kinase (Cat.# V6430), cAMP-dependent protein kinase (Cat.# V7480), protein kinase C (Cat.# V7470), DNA-dependent protein kinase (Cat.# V7870) and calmodulin-dependent protein kinase (Cat.# V8161).

As outlined in Figure 7.10, the assay steps and analysis of results are straightforward and require only common laboratory equipment. Following phosphorylation and binding of the biotinylated substrate to the numbered and partially cut squares of SAM2® Biotin Capture Membrane, unincorporated $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ is removed by a simple washing procedure. This procedure also removes nonbiotinylated proteins that have been phosphorylated by other kinases in the sample. The bound, labeled substrate is then quantitated by scintillation counting or PhosphorImager® analysis. Typical results generated using the SignaTECT® Assays are presented in Figure 7.11.

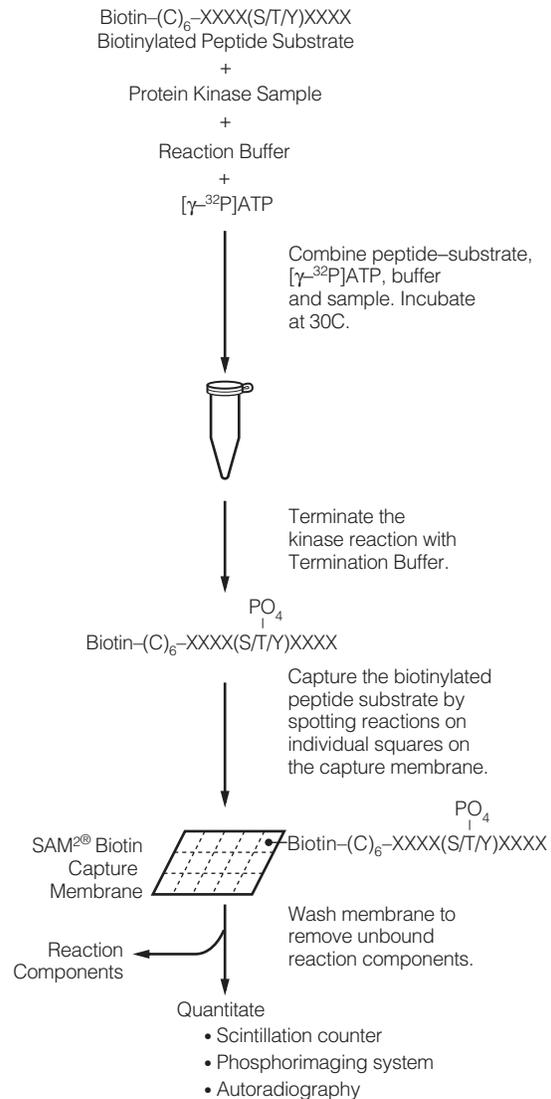


Figure 7.10. The SignaTECT® Protein Kinase Assay protocol.

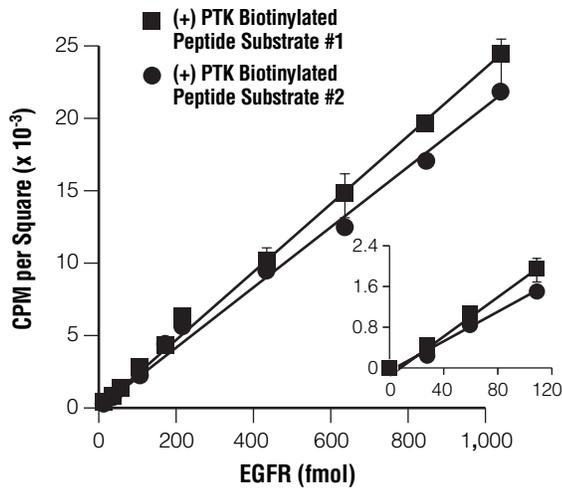


Figure 7.11. Linear detection of EGFR kinase activity with the SignaTECT® PTK Assay System. EGFR (Cat.# V5551) activity was measured in the presence of PTK Biotinylated Peptide Substrate 1 or PTK Biotinylated Peptide Substrate 2, provided with the SignaTECT® PTK System (Cat.# V6480). Inset: enlargement of the data using 120fmol of EGFR.

Additional Resources for the SignaTECT® Kinase Assay Systems

Technical Bulletins and Manuals

- TB211 *SignaTECT® Protein Tyrosine Kinase (PTK) Assay System Technical Bulletin*
(www.promega.com/tbs/tb211/tb211.html)
- TB227 *SignaTECT® cdc2 Protein Kinase Assay System Technical Bulletin*
(www.promega.com/tbs/tb227/tb227.html)
- TB241 *SignaTECT® cAMP-Dependent Protein Kinase Assay System Technical Bulletin*
(www.promega.com/tbs/tb241/tb241.html)
- TB242 *SignaTECT® Protein Kinase C (PKC) Assay System Technical Bulletin*
(www.promega.com/tbs/tb242/tb242.html)
- TB250 *SignaTECT® DNA-Dependent Protein Kinase Assay System Technical Bulletin*
(www.promega.com/tbs/tb250/tb250.html)
- TB279 *SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaM KII) Assay System Technical Bulletin*
(www.promega.com/tbs/tb279/tb279.html)

Promega Publications

- CN001 Store operated calcium entry activates at the GVBD stage of *Xenopus* meiosis
(www.promega.com/cnotes/cn001/cn001_12.htm)

- PN058 SAM2® Biotin Capture Membrane and SignaTECT® Protein Kinase Assay Systems
(www.promega.com/pnotes/58/5189d/5189d.html)
- PN059 Detection and quantitation of protein tyrosine kinases
(www.promega.com/pnotes/59/5644a/5644a.html)
- PN063 SignaTECT® DNA-Dependent Protein Kinase Assay System
(www.promega.com/pnotes/63/8581_07/promega.html)
- PN076 Tools to study the activation of CaM KII in neuronal functions
(www.promega.com/pnotes/76/8840_06/8840_06.html)
- BR095 Signal Transduction Resource
(www.promega.com/guides/sigtrans_guide/default.htm)

Citations

Zhang, L. *et al.* (2004) A transforming growth factor beta-induced Smad3/Smad4 complex directly activates protein kinase A. *Mol. Cel Biol.* **24**, 2169–80.

These authors investigated the possible interaction between TGFβ and PKA signaling pathways using the SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System.

PubMed Number: 14966294

C. Other Kinase Assay Formats (non-radioactive)

The PepTag® Protein Kinase Assays are fast and quantitative non-radioactive alternatives to [γ -³²P]ATP-based assays for measuring protein kinase C (Cat.# V5330) and cAMP-dependent protein kinase (Cat.# V5340) activity. The assays use fluorescently-tagged peptide substrates with a net positive charge. Phosphorylation changes the charge of the peptide to a net negative, which influences the migration of the peptide in an agarose gel. This is the basis for detecting changes in phosphorylation via a rapid, 15-minute agarose gel separation (Figure 7.12).

General PepTag® Assay Protocol

Materials Required:

- PepTag® Non-Radioactive PKC Assay (Cat.# V5330) or PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay (Cat.# V5340) and protocol (#TB132 (www.promega.com/tbs/tb132/tb132.html))
- PKA or PKC dilution buffer
- horizontal agarose gel apparatus
- glycerol, 80%
- Tris-HCl, 50mM (pH 8.0)
- agarose, 0.8% in 50mM Tris-HCl (pH 8.0)
- probe sonicator

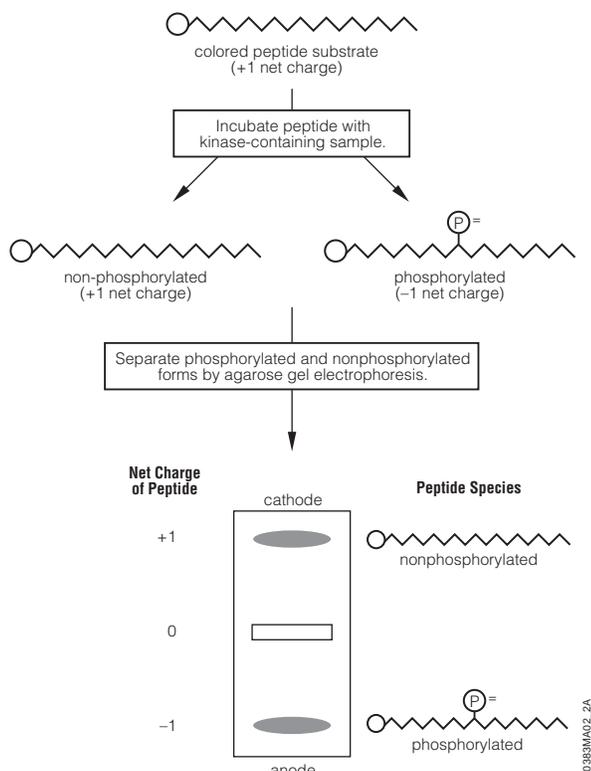


Figure 7.12. Schematic diagram of the PepTag® Non-Radioactive Protein Kinase Assay procedure.

Additional Resources for the PepTag® Non-Radioactive Protein Kinase Assay

Technical Bulletins and Manuals

- TB132 *PepTag® Assay for Non-Radioactive Detection of Protein Kinase C or cAMP-Dependent Protein Kinase Technical Bulletin* (www.promega.com/tbs/tb132/tb132.html)

Promega Publications

- BR095 *Signal Transduction Resource* (www.promega.com/guides/sigtrans_guide/default.htm)

Citations

DiPasquale, G. and Chiorini, J.A. (2004) PKA/PrKX activity is a modulator of AAV/adenovirus interaction. *EMBO J.* **22**, 1716–24.

The PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay was used to analyze PKA activity in HeLa cells infected with a human parvovirus. Lysates were prepared by sonication of cells in PBS. Data is presented as either a percent of control or fold increase over control.

PubMed Number: 12660177

IV. Phosphorylation-Specific Antibodies

The Anti-ACTIVE® phosphorylation-specific antibodies were developed to provide an accurate measure of enzyme activation. These antibodies specifically recognize the active, phosphorylated form of a given kinase. The Anti-ACTIVE® Antibodies are raised against phosphorylated peptide sequences present in the activating loop of a number of protein kinases. Whether used in Western analysis, immunocytochemistry or immunohistochemical staining, the Anti-ACTIVE® MAPK, JNK, p38 and CaM KII Antibodies will recognize only the active form of the enzyme.

A. Phosphorylation-Specific Antibodies in MAPK Signaling Pathways

Anti-ACTIVE® MAPK, pAb, Rabbit, (pTEpY)

This antibody is an affinity purified polyclonal antibody that specifically recognizes the dually phosphorylated, active form of MAPK. The antibody is raised against a dually phosphorylated peptide sequence representing the catalytic core of the active ERK enzyme and recognizes the active forms of ERK1, ERK2 and ERK7.

Anti-ACTIVE® JNK pAb, Rabbit, (pTPpY)

Anti-ACTIVE® JNK pAb is an affinity purified polyclonal antibody that recognizes the dually phosphorylated, active form of cJun N-terminal protein Kinase (JNK).

Anti-ACTIVE® JNK pAb is raised against a dually phosphorylated peptide sequence representing the catalytic core of the active JNK enzyme. The antibody recognizes the active forms of JNK1, JNK2, and JNK3 isoforms.

Anti-ACTIVE® p38 pAb, Rabbit, (pTGpY)

Anti-ACTIVE® p38 Ab, Rabbit, is an affinity purified polyclonal antibody that recognizes the active form of p38 kinase. The Anti-ACTIVE® p38 pAb is raised against the dually phosphorylated peptide sequence representing the catalytic core of the active p38 enzyme. The Anti-ACTIVE® p38 pAb recognizes the active forms of p38 α , γ , and δ isoforms.

Western Blot Analysis with Anti-ACTIVE® MAPK, JNK and p38 pAbs

Materials Required:

- Anti-ACTIVE® MAPK (Cat.# V8031), JNK (Cat.# V7931), or p38 (Cat.# V1211) pAb
- Anti-ACTIVE® Qualified Donkey Anti-Rabbit IgG (H+L), HRP (Cat.# V7951) or Donkey Anti-Rabbit IgG (H+L) AP (Cat.# V7971) Secondary Antibodies
- protein sample transferred to nitrocellulose or PVDF membrane
- bovine serum albumin, 1%
- TBS buffer
- TBST or PVDF buffer
- shaking platform

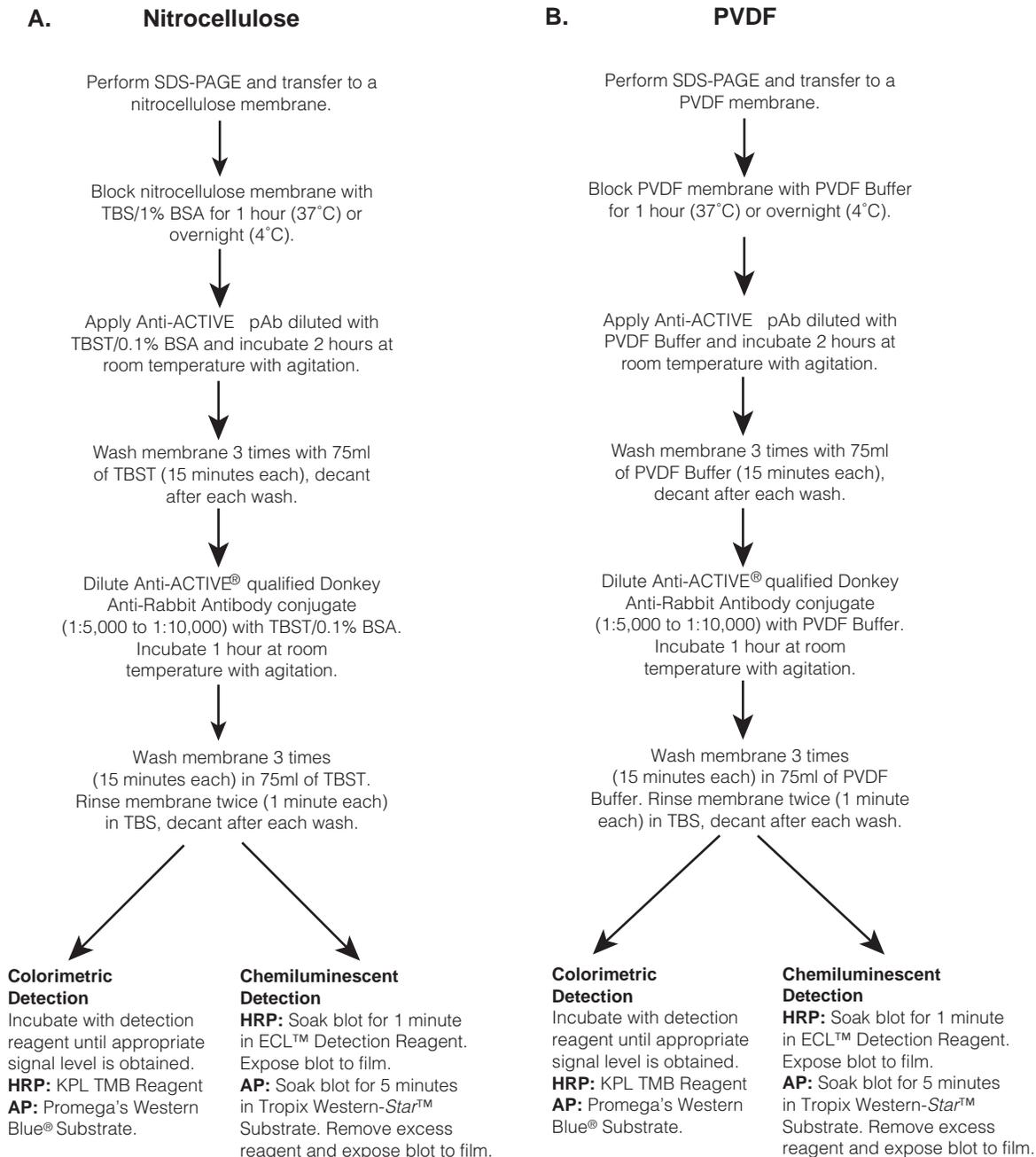


Figure 7.13. Schematic diagram illustrating the use of nitrocellulose and PVDF membranes in Western blot analysis with Anti-ACTIVE® pAbs. Protocols for use with nitrocellulose (Panel A) and PVDF (Panel B) membranes. Recommended dilutions of the Anti-ACTIVE® pAbs are 1:5,000 for Anti-ACTIVE® MAPK pAb, 1:2,000 for Anti-ACTIVE® p38 pAb, 1:5,000 for Anti-ACTIVE® JNK pAb and 1:5,000 to 1:10,000 for the Anti-ACTIVE® Donkey Anti-Rabbit IgG (H+L) secondary antibodies (both HRP- and AP-conjugated). KPL is an abbreviation for Kirkegaard and Pery Laboratories. See Technical Bulletin #TB262 (www.promega.com/tbs/tb262/tb262.html) for more information about this protocol. You may need to determine the optimal dilutions of primary and secondary antibodies for your system. Use of secondary antibodies other than those available from Promega may require additional optimization.

Immunocytochemistry with Anti-ACTIVE® MAPK, JNK and p38 pAbs

The following method is for preparing and immunostaining PC12 cells stimulated by either nerve growth factor to activate MAP kinases or soribitol to activate JNK and p38 kinases. For additional information see Technical Bulletin

#TB262 (www.promega.com/tbs/tb262/tb262.html)

Materials Required:

- Anti-ACTIVE® Qualified Donkey Anti-Rabbit IgG (H+L), HRP (Cat.# V7951) or Donkey Anti-Rabbit IgG (H+L), AP (Cat.# V7971) Secondary Antibodies

- LabTek® 4-chambered slides (Fisher Cat.# 12-565-21)
- rat-tail collagen (Collaborative BioScience Products)
- RPMI 1640 with 25mM HEPES, 300mg/l L-glutamine, 10% horse serum, 5% fetal bovine serum and 0.5mM EGTA
- NGF (Cat.# G5141) or sorbitol
- PBS
- 10% paraformaldehyde
- methanol, -20°C
- blocking buffer
- donkey anti-rabbit Cy®3 conjugate (Jackson ImmunoResearch Cat.# 741-165-152)

Preparation and Activation of PC12 Cells

1. Coat 4-chambered slides with rat tail collagen (6µg/cm² in sterile PBS) for one hour.
2. Grow PC12 cells in chambers at 37° in 5% CO₂ in medium containing RPMI 1640 with 25mM HEPES, 300mg/L L-glutamine, 10% horse serum, 5% fetal bovine serum and 0.5mM EGTA. The medium should be changed every other day until the cells reach 80% confluence.
3. Activate the cells in 2 chambers as described below. Use the cells in the remaining 2 chambers as untreated controls.

NGF: The day before immunocytochemistry, add fresh medium with serum. The next day add 200ng/ml NGF in RPMI. Incubate for 5 minutes at 37°C.

Sorbitol: The day before immunocytochemistry, add fresh medium without serum. The next day add sorbitol to a final concentration of 1M. Incubate for 30 minutes at 37°C.
4. Proceed with staining as outlined in Figure 7.14.

Additional Resources for the Anti-ACTIVE® Antibodies

Technical Bulletins and Manuals

- TB262 *Anti-ACTIVE® MAPK, JNK and p38 Polyclonal Antibodies and Anti-ACTIVE® Qualified Secondary Antibody Conjugates* (www.promega.com/tbs/tb262/tb262.html)

Promega Publications

- PN069 New Anti-ACTIVE® MAPK and 'pan ERK 1/2' antibodies for Western analysis (www.promega.com/pnotes/69/7542_09/7542_09.html)
- PN076 Technically speaking: Anti-ACTIVE® Antibodies and MAPK signaling pathways (www.promega.com/pnotes/76/8840_23/8840_23.html)

- PN080 Demonstration of immunohistochemical staining using Promega Anti-ACTIVE® and apoptosis Antibodies (www.promega.com/pnotes/80/9748_20/9748_20.html)

Online Tools

Antibody Assistant (www.promega.com/techserv/tools/abasst/)

Citations

Hsu, C.Y. *et al.* (2004) Characterization of active mitogen-activated protein kinase in ovarian serous carcinomas *Clin. Can. Res.* **10**, 6432–6.

The Anti-ACTIVE® MAPK polyclonal antibody was used to immunohistochemically stain and type patient ovarian serous carcinomas using paraffin-fixed tissue sections on tissue microarrays. Western blots were also performed on tissue lysates using a 1:3,000 dilution of the antibody.

PubMed Number: 15475429

Le'Negrate, G. *et al.* (2003) Downregulation of caspases and Fas ligand expression, and increased lifespan of neutrophils after transmigration across intestinal epithelium *Cell Death Differ.* **10**, 153–62.

Anti-ACTIVE® JNK pAb was used in immunoblot analysis of human polymorphonuclear leukocyte protein lysates.

PubMed Number: 12700643

Aballay, A. *et al.* (2003) *Caenorhabditis elegans* innate immune response triggered by *Salmonella enterica* requires intact LPS and is mediated by a MAPK signaling pathway *Curr. Biol.* **13**, 47–52.

Activation of the p38 homolog in the worm was monitored by Western analysis using the Anti-ACTIVE® p38 pAb.

PubMed Number: 12526744

B. Phosphorylation-Specific CaM KII Antibody

This antibody recognizes calcium/calmodulin-dependent protein kinase CaM KII that is phosphorylated on threonine 286. The Anti-ACTIVE® CaM KII pAb (Cat.# V1111) was raised against the phosphothreonine-containing peptide derived from this region.

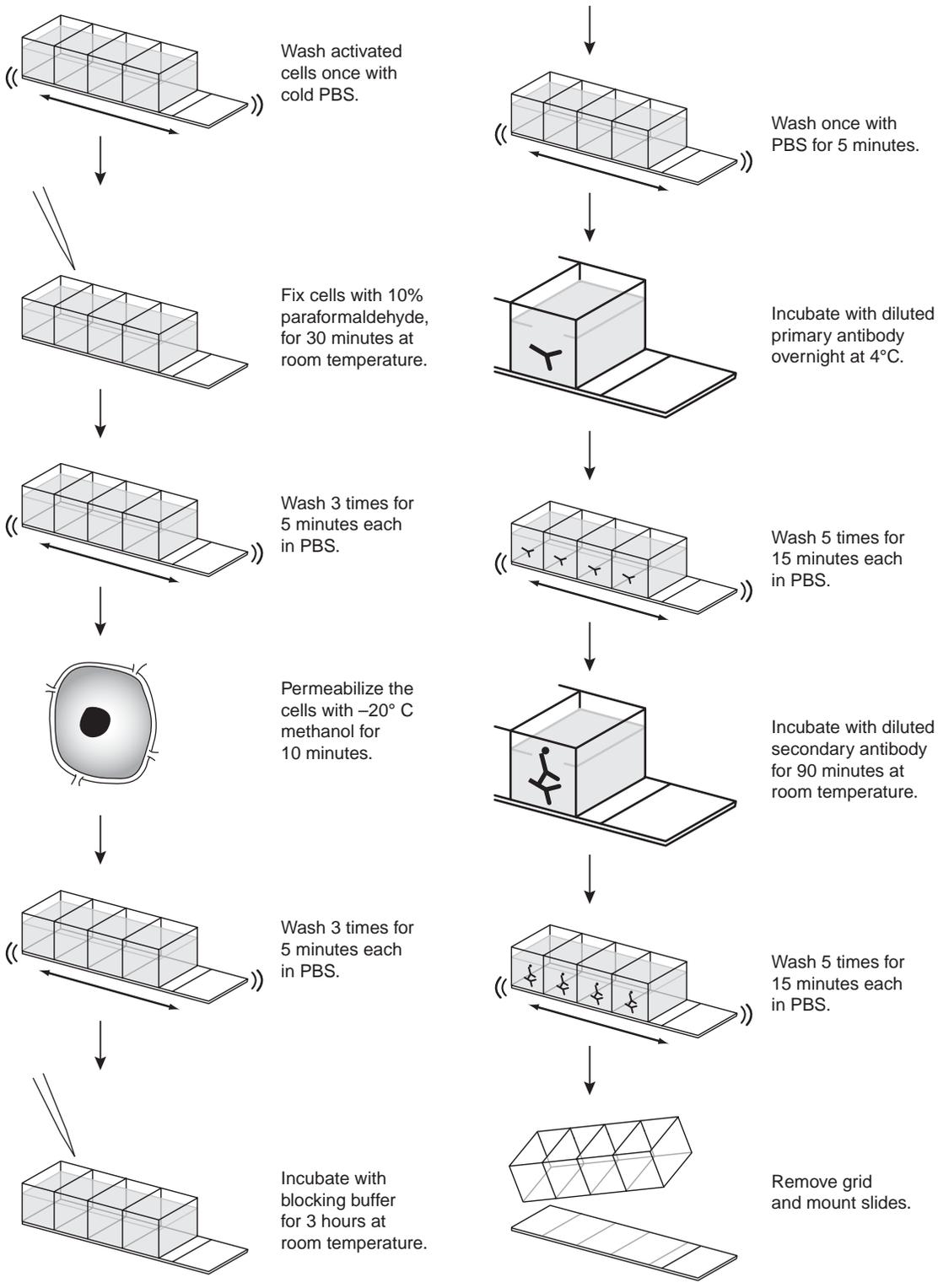
Additional Information for the Anti-ACTIVE® CaM KII pAb

Technical Bulletins and Manuals

- TB264 *Anti-ACTIVE® CaM KII pAb, (pT²⁸⁶) and Anti-ACTIVE® Qualified Secondary Antibody Conjugates Technical Bulletin* (www.promega.com/tbs/tb264/tb264.html)

Promega Publications

- PN067 Anti-ACTIVE® Antibody for specific detection of phosphorylated CaM KII protein kinase (www.promega.com/pnotes/67/7201_09/7201_09.html)



(continued in next column)

Figure 7.14. Immunostaining of activated PC12 cells. This protocol is for immunostaining of activated PC12 cells and may need to be optimized for your particular experimental system.

BR095 Signal Transduction Resource
(www.promega.com/guides/sigtrans_guide/default.htm)

Online Tools

Antibody Assistant (www.promega.com/techserv/tools/abasst/)

Citations

Matsumoto, Y. and Maller, J.L. (2002) Calcium, calmodulin and CaM KII requirement for initiation of centrosome duplication in *Xenopus* egg extracts *Science* **295**, 499–502. CaM KII(281-309) was added to metaphase-arrested extracts. After adding calcium, the extracts were incubated at room temperature. Anti-ACTIVE® CaM KII pAb and Anti-ACTIVE® Qualified HRP secondary antibodies were used to probe immunoblots for phospho-T²⁸⁶ CaM KII α .

PubMed Number: 11799245

BR095 Signal Transduction Resource
(www.promega.com/guides/sigtrans_guide/default.htm)

Citations

Cancedda, L. *et al.* (2003) Patterned vision causes CRE-mediate gene expression in the visual cortex through PKA and ERK. *J. Neurosci.* **23**, 7912–20.

MEK Inhibitor U0126 was used to demonstrate specific CRE-mediated gene expression through Erk activation in mouse brains.

PubMed Number: 12904462

V. Kinase Inhibitors

A. MEK Inhibitor U0126

MEK Inhibitor U0126 (Cat.# V1121) inhibits the activity of MAP Kinase Kinase (MEK 1/2) and thus prevents the activation of MAPK. U0126 inhibits MEK 1 with an IC₅₀ of 0.5 μ M in vitro (Favata *et al.* 1998). U0126 inhibits phosphorylation activated MEK 1 and MEK 2 as well as constitutively active MEK 1 and MEK 2 mutants (Favata *et al.* 1998; Goueli *et al.* 1998). U0126 is noncompetitive with respect to the MEK substrates ATP and ERK (Favata *et al.* 1998; Tolwinski *et al.* 1999).

Additional Resources for MEK Inhibitor U0126

Technical Bulletins and Manuals

9PIV112 MEK Inhibitor U0126 Promega Product Information
(www.promega.com/tbs/9piv112/9piv112.html)

Promega Publications

- CN001 Frequently asked questions: Kinase inhibitors and activators
(www.promega.com/cnotes/cn001/cn001_10.htm)
- NN021 Using MAPK antibodies and reagents to study cell signaling in neurons
(www.promega.com/nnotes/nn021/21_09.htm)
- PN069 U0126: A novel, selective and potent inhibitor of MAP Kinase Kinase (MEK)
(www.promega.com/pnotes/69/7542_06/7542_06.html)
- PN071 U0126: An inhibitor of MKK/ERK signal transduction in mammalian cells
(www.promega.com/pnotes/71/7807_04/7807_04.html)

B. PD 98059

PD 98059 (Cat.# V1191) inhibits MEK activation (Alessi *et al.* 1995; Dudley *et al.* 1995; Favata *et al.* 1998). PD 98059 inhibits MEK 1 but is an inefficient inhibitor of MEK 2. (Alessi *et al.* 1995; Dudley *et al.* 1995). It inhibits activation of MEK 1 by Raf with an IC₅₀ of 5 μ M and of the active MEK 1 mutant with an IC₅₀ of 10 μ M (Alessi *et al.* 1995; Dudley *et al.* 1995).

Additional Resources for PD 98059

Promega Publications

- CN001 Frequently asked questions: Kinase inhibitors and activators
(www.promega.com/cnotes/cn001/cn001_10.htm)
- NN021 Using MAPK antibodies and reagents to study cell signaling in neurons
(www.promega.com/nnotes/nn021/21_09.htm)
- BR095 Signal Transduction Resource
(www.promega.com/guides/sigtrans_guide/default.htm)

Citations

Schmidt, H. *et al.* (2000) Involvement of mitogen-activated protein kinase in agonist-induced phosphorylation of the mu-opioid receptor in HEK 293 cells *J. Neurochem.* **74**, 414–22.

HEK 293 cells stably expressing the mu-opioid receptor respond to agonists by MAPK phosphorylation. Activation of the MAPK was completely inhibited by the MEK Inhibitor U0126 at 100nM as judged by immunocytochemistry. The PD 98059 required 20 μ M for the same inhibitory effect.

PubMed Number: 10617147

C. SB 203580

SB 203580 (Cat.# V1161) is a specific, cell-permeant inhibitor of the stress and inflammatory cytokine-activate MAP kinase homologues p38 α , β and β 2. It acts as a competitive inhibitor of ATP binding to the kinase. Reported IC₅₀ values range from 21nM to 1 μ M. SB 203580 has no significant effect on the activities of ERKs, JNKs, p38 γ or p38 δ .

Promega Publications

- CN001 Frequently asked questions: Kinase inhibitors and activators
(www.promega.com/cnotes/cn001/cn001_10.htm)
- BR095 Signal Transduction Resource
(www.promega.com/guides/sigtrans_guide/default.htm)

Citations

Wytenbach, A. *et al.* (2002) Heat shock protein 27 prevents cellular polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin. *Hum. Mol. Genet.* **11**, 1137–51.

SB 203580 MAP kinase p38 α , p38 β and p38 β 2 inhibitor was used in both COS-7 and SK-N-SH huntingtin exon 1-transfected cell cultures. Decreased nuclear fragmentation was reported when 1 or 10 μ M SB 203580 inhibitor was added to the transfected cell cultures.

PubMed Number: 11978772

D. PI3 Kinase Inhibitor LY 294002

LY 294002 (Cat.# V1201) is a potent and specific cell-permeant inhibitor of phosphatidylinositol 3-kinases (PI3-K) with an IC₅₀ value in the 1–50 μ M range. LY 294002 competitively inhibits ATP binding to the catalytic subunit of PI3-Ks and does not inhibit PI4-Kinase, DAG-kinase, PKC, PKA, MAPK, S6 kinase, EGFR or c-src tyrosine kinases and rabbit kidney ATPase (Rameh and Cantley, 1999; Fruman *et al.* 1998). LY 294002 has improved stability and specificity compared to Wortmannin, which is an irreversible inhibitor that covalently interacts with PI3-Ks.

Additional Resources for LY 294002**Promega Publications**

- CN001 Frequently asked questions: Kinase inhibitors and activators
(www.promega.com/cnotes/cn001/cn001_10.htm)
- BR095 Signal Transduction Resource
(www.promega.com/guides/sigtrans_guide/default.htm)

Citations

Yamaguchi, K. *et al.* (2004) Identification of nonsteroidal anti-inflammatory drug-activated gene (NAG-1) as a novel, downstream target of phosphatidylinositol 3-kinase/Akt/GSK-3B pathway. *J. Biol. Chem.* **279**, 49617–23.

The authors used inhibition of PI3-Kinase by LY 294002 to determine that NAG-1 expression in human colorectal cancer cells is regulated by a PI3-kinase pathway.

PubMed Number: 15377673

E. cAMP-Dependent Protein Kinase (PKA) Peptide Inhibitor

The cAMP-Dependent Protein Kinase Inhibitor (Cat.# V5681), also known as PKI, TTYADFIASGRRNAIHD, inhibits phosphorylation of target proteins by binding to

the protein-substrate site of the catalytic subunit of PKA. It corresponds to the region 5–24 of the naturally occurring PKI.

Additional Resources for the PKA Peptide Inhibitor**Promega Publications**

- BR095 Signal Transduction Resource
(www.promega.com/guides/sigtrans_guide/default.htm)

Citations

Zhang, B. *et al.* (2004) Rac1 inhibits apoptosis in human lymphoma cells by stimulating Bad phosphorylation on Ser-75. *Mol. Cell. Biol.* **24**, 6205–14.

Researchers used the cAMP-Dependent Protein Kinase Peptide Inhibitor to demonstrate that BAD kinase is phosphorylated through a cAMP-Dependent Protein Kinase (PKA) dependent pathway in Burkitt's lymphoma BL-41 cells.

PubMed Number: 15226424

F. InCELLect® AKAP St-Ht31 Inhibitor Peptide

The InCELLect® AKAP St-Ht31 Inhibitor Peptide (Cat.# V8211) and the InCELLect® Control Peptide (Cat.# V8221) can be used for in vivo studies of PKA activation. The Inhibitor Peptide is a steared (St) form of the peptide Ht31 derived from the human thyroid AKAP (A-kinase anchoring protein). The presence of the hydrophobic steared moiety enhances the cellular uptake of the peptides through the lipophilic microenvironment of the plasma membrane.

Additional Resources for InCELLect® AKAP St-Ht31 Inhibitor Peptide**Promega Publications**

- PN075 InCELLect® cell-permeable, steared peptides to probe cAMP-dependent protein kinase-mediated cellular signaling reactions in vivo.
(www.promega.com/pnotes/75/8554_29/8554_29.html)

G. Myristoylated Protein Kinase C Peptide Inhibitor

Myristoylated Protein Kinase C Peptide Inhibitor (Cat.# V5691) specifically inhibits calcium- and phospholipid-dependent protein kinase C. It is based on the pseudosubstrate region of PKC- α and PKC- β (Eicholtz, 1993).

Additional Resources for Myristoylated Protein Kinase C Peptide Inhibitor**Promega Publications**

- BR095 Signal Transduction Resource
(www.promega.com/guides/sigtrans_guide/default.htm)

Citations

Spyridopoulos, I. *et al.* (2002) Divergence of angiogenic and vascular permeability signaling by VEGF inhibition of protein kinase C suppresses VEGF-induced angiogenesis but promotes VEGF-induced, NO-dependent vascular permeability. *Atheroscler. Thromb. Vasc. Biol.* **22**, 901–6.

The Myristoylated Protein Kinase C Peptide Inhibitor and cAMP-Dependent Protein Kinase Peptide Inhibitor were used in cell and animal studies to help specifically identify Protein Kinase A and C activities.

PubMed Number: 12067896

H. Olomoucine cdc2 Protein Kinase Inhibitor

Olomoucine is a chemically synthesized inhibitor that is specific for p34^{cdc2} and related protein kinases. Its molecular weight is 298, and its molecular formula is C₁₅H₁₈N₆O.

Additional Resources for Olomoucine cdc2 Protein Kinase Inhibitor**Promega Publications**

BR095 Signal Transduction Resource
(www.promega.com/guides/sigtrans_guide/default.htm)

Citations

Yan, X. *et al.* (2003) Human Nudel and NudE as regulators of cytoplasmic dynein in poleward protein transport along the mitotic spindle. *Mol. Cell. Biol.* **23**, 1239–50.

Mitotic extracts were prepared from HEK293T cells transfected with plasmids encoding FLAG/Nudel fusion protein. Kinase assays were performed on the immunoprecipitated mitotic extracts in the presence or absence of olomoucine.

PubMed Number: 12556484

VI. Phosphatase Assays

Protein phosphorylation plays a key role in signal transduction, and genes for protein kinases and phosphatases represent a large portion of the human genome (Goueli *et al.* 2004b; Cohen, 2001). They are the opposing partners to the kinases in the cell, catalyzing the dephosphorylation of molecules involved in cellular pathways. Protein phosphatases can be divided into three general categories: a) protein tyrosine phosphatases, which remove phosphate from phosphotyrosine-containing proteins, b) protein serine/threonine phosphatases, which remove phosphate from phosphoserine- or phosphothreonine-containing proteins, and c) dual-specificity phosphatases, which can remove phosphate from phosphotyrosine, phosphothreonine, and phosphoserine (Hunter, 1995).

A. Fluorescent Phosphatase Assays

We have developed the ProFluor® Phosphatase Assays to overcome safety issues associated with radioactive assays while maintaining sensitivity and specificity. The ProFluor® Phosphatase Assays use bisamide R110-linked phosphopeptides that serve as substrates for PTPases. Phosphorylation of the peptide substrate renders it resistant to cleavage by the Protease Reagent that is included with these assay systems, reducing the fluorescence generated. However, when the phosphoryl moiety is removed by a phosphatase, the peptides become cleavable by the protease, releasing the highly fluorescent, free R110 molecule (Figure 7.15).

The ProFluor® PPase Assays offer the simplicity, sensitivity and specificity required for screening chemical libraries for novel inhibitors of protein phosphatases. These assays are robust with Z' factor values routinely greater than 0.8 (Figure 16; Goueli *et al.* 2004b)

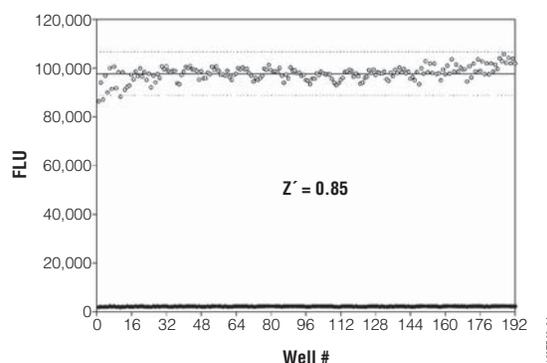


Figure 7.16. Z' factor values obtained in 384-well plates for the ProFluor® S/T PPase Assay. The assay was performed manually according to the protocol provided in Technical Bulletin #TB324 (www.promega.com/tbs/tb324/tb324.html) using solid black, flat-bottom plates with phosphatase (open circles) and without phosphatase (solid circles). Solid lines indicate the mean, and the dotted lines indicate \pm S.D. 6.25milliunits/well PP1 (Calbiochem Cat.# 539493) was used. Z' factor was 0.85).

Z' factor is a statistical description of the dynamic range and variability of an assay. Z' factor values >0.5 are indicative of a robust assay (Zhang *et al.* 1999). These fluorescent assays can be performed in single tubes, 96-well plates or 384-well plates, giving the user flexibility in format. The signal-to-noise ratio is very high, and the generated signal is stable for hours.

General Protocol for the ProFluor® Phosphatase Assays
Materials Required:

- ProFluor® Ser/Thr Phosphatase Assay (Cat.# V1260, V1261) or ProFluor® Tyrosine Phosphatase Assay (Cat.# V1280, V1281) and protocol (Technical Bulletin #TB324 (www.promega.com/tbs/tb324/tb324.html) or TB334 (www.promega.com/tbs/tb334/tb334.html), respectively)
- opaque-walled multiwell plates
- multichannel pipet or automated pipetting station
- plate shaker (DYNEX MICRO-SHAKER® or equivalent)

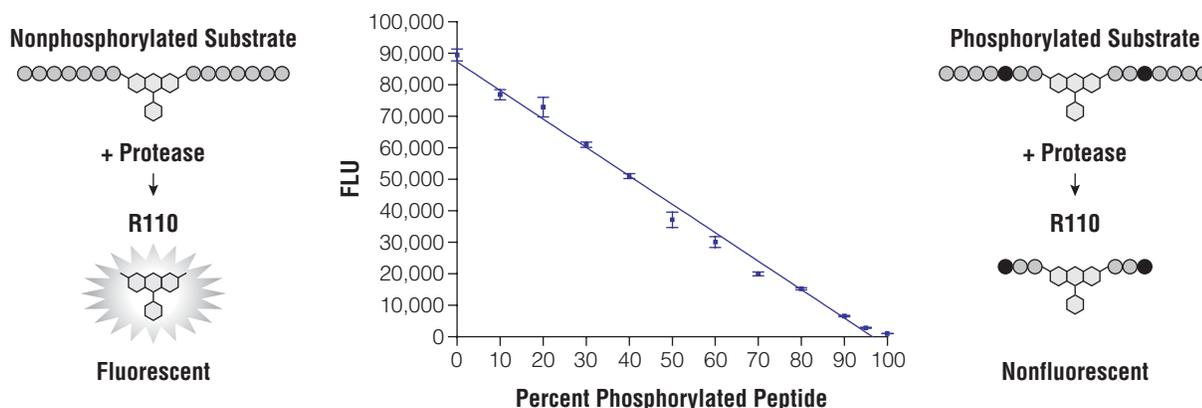


Figure 7.15. Schematic and graph demonstrating that Rhodamine 110 is essentially nonfluorescent in the bisamide form and that the presence of a phosphorylated amino acid (dark circle) blocks the removal of amino acids by the protease. The graph shows the average FLU obtained after a 30-minute protease reagent digestion using mixtures of nonphosphorylated R1110 PKA Substrate and phosphorylated R1110 PKA Substrate as indicated (n = 6).

- plate-reading fluorometer with filters for reading R110 and AMC fluorescence
 - protein tyrosine phosphatase or S/T protein phosphatase
 - okadaic acid (for PP1 and PP2A)
 - calmodulin (for PP2B)
1. Dilute the phosphatase in Reaction Buffer and add to wells.
 2. Dilute the PTPase R110 Substrate and the Control AMC Substrate in Reaction Buffer and add to wells.
 3. Mix the contents of the plate for 15 seconds and incubate at room temperature (10 minutes for PP1 and PP2A; 30 minutes for PP2B; 60 minutes for tyrosine PPase).
 4. Add Protease Solution.
 5. Mix the contents of the plate briefly and incubate at room temperature (90 minutes for PP2A, PP2B or PP1; 30 minutes for tyrosine PPase).
 6. Add Stabilizer Solution.
 7. Mix the contents of the plate and read fluorescence.

Additional Resources for ProFluor® Phosphatase Assays

Technical Bulletins and Manuals

- TB324 *ProFluor® Ser/Thr PPase Assay Technical Bulletin*
(www.promega.com/tbs/tb324/tb324.html)
- TB334 *ProFluor® Tyrosine Phosphatase Assay Technical Bulletin*
(www.promega.com/tbs/tb334/tb334.html)

Promega Publications

- CN007 Monitor purified phosphatase activity with a homogeneous non-radioactive high-throughput fluorogenic assay (www.promega.com/cnotes/cn007/cn007_05.htm)
- CN008 Assay protein tyrosine kinase and protein tyrosine phosphatase activity in a homogeneous, non-radioactive high-throughput format (www.promega.com/cnotes/cn008/cn008_15.htm)

Citations

Brisson, M. *et al.* (2004) Discovery and characterization of novel small molecule inhibitors of human Cdc25B dual specificity phosphatase. *Mol. Pharmacol.* **66**, 824–33.

The ProFluor® Ser/Thr PPase Assay was used to screen small molecule inhibitors Cdc25B on a panel of S/T PPases in order to characterize the specificity of these inhibitors for Cdc25B.

PubMed Number: 15231869

Kupcho, K. *et al.* (2004) A homogeneous, nonradioactive high-throughput fluorogenic protein phosphatase assay. *J. Biomol. Screen.* **9**, 223–31.

This article describes the use of the ProFluor® Phosphatase Assays to measure the activity of protein phosphatases at low concentrations.

PubMed Number: 15140384

B. Colorimetric Phosphatase Assays

Both the Tyrosine Phosphatase (Cat.# V2471) and the Serine/Threonine Phosphatase (Cat.# V2460) Assay Systems detect the release of phosphate from specific peptide substrates by measuring the appearance of a phosphate complex of molybdate:malachite green. For assays of crude extracts, endogenous phosphate and other inhibitory molecules are first removed by a simple 20-minute

procedure using Spin Columns that are supplied with each system. This step is unnecessary for assays using pure or partially purified enzyme preparations. Each system includes ready-to-use, specific substrates: the Tyrosine Phosphatase System provides two phosphotyrosine-containing peptides; the Serine/Threonine Phosphatase Assay System provides a phosphothreonine-containing peptide. Other phosphopeptides or phosphoproteins can be used as substrates to increase specificity or to use natural substrates. The simple assay procedure is outlined in Figure 7.17.

Materials Required:

- Serine/Threonine Phosphatase Assay System (Cat.# V2460) or Tyrosine Phosphatase Assay System (Cat.# V2471) and protocol (Technical Bulletin # TB218 or #TB212, respectively)
- 50ml disposable conical centrifuge tubes (e.g., Corning Cat.# 25330-50)
- appropriate storage buffer (see TB212 or TB218)
- Sephadex® G-25 storage buffer (for storing column)

Additional Resources for Serine/Threonine and Tyrosine Phosphatase Assay Systems

Technical Bulletins and Manuals

- | | |
|-------|---|
| TB218 | <i>Serine/Threonine Phosphatase Assay System Technical Bulletin</i>
(www.promega.com/tbs/tb218/tb218.html) |
| TB212 | <i>Tyrosine Phosphatase Assay System Technical Manual</i>
(www.promega.com/tbs/tb212/tb212.html) |

Promega Publications

- | | |
|-------|--|
| NN003 | Promega non-radioactive phosphatase systems
(www.promega.com/nnotes/nn103/103_12.htm) |
| BR095 | Signal Transduction Resource
(www.promega.com/guides/sigtrans_guide/default.htm) |

Citations

Bandyopadhyay, J. *et al.* (2002) Calcineurin, a calcium/calmodulin-dependent protein phosphatase, is involved in movement, fertility, egg laying, and growth in *Caenorhabditis elegans*. *Mol. Biol. Cell* **13**, 3281–93.

Purified fusion proteins created *C. elegans* that resemble insect and mammalian calcineurin were tested in the Serine/Threonine Phosphatase Assay System

PubMed Number: 12221132

Liu, T *et al.* (2004) Reactive oxygen species mediate virus-induced STAT activation: Role of tyrosine phosphatases. *J. Biol. Chem.* **279**, 2461–9.

The Tyrosine Phosphatase Assay System was used to assess the level of tyrosine phosphatase activity in human alveolar type II-like epithelial cells (the A549 cell line).

PubMed Number: 14578356

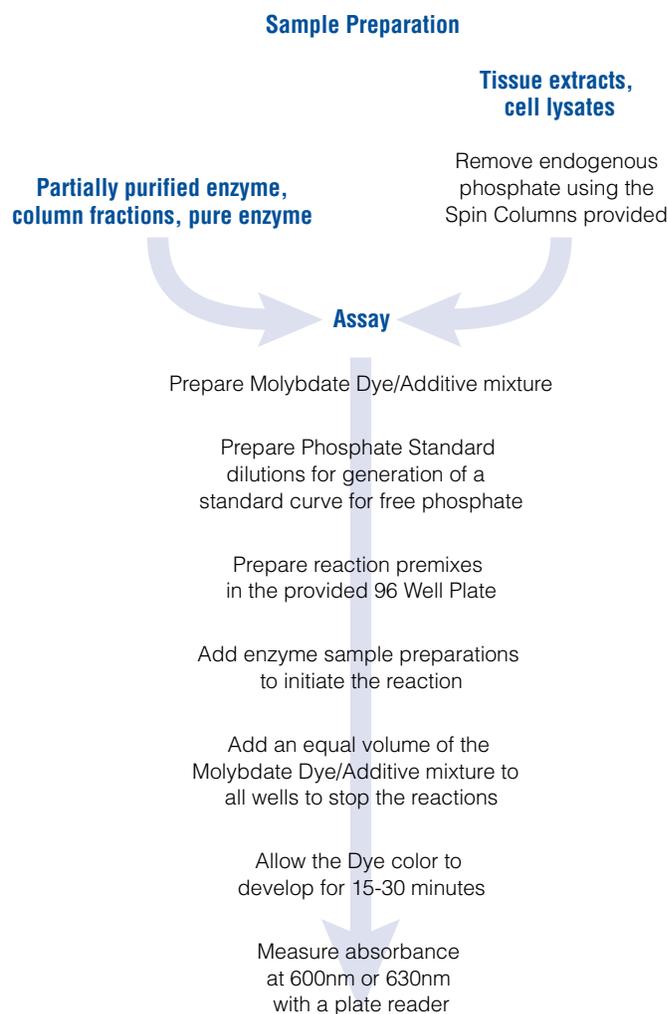


Figure 7.17. Steps required for measuring phosphatase activity using the Serine/Threonine or the Tyrosine Phosphatase Assay System. These systems can be used to measure phosphatase activity from partially purified enzyme preparations and tissue extracts or cell lysates.

VII. References

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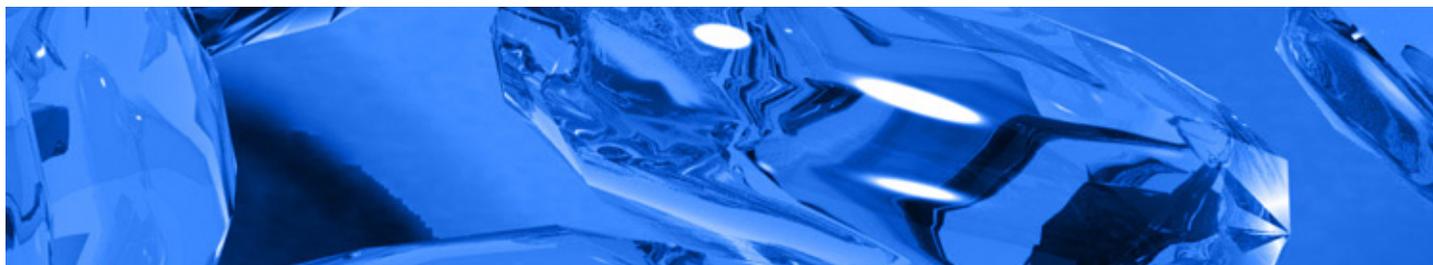
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I. Introduction

Genetic reporter systems have contributed greatly to the study of eukaryotic gene expression and regulation. Although reporter genes have played a significant role in numerous applications, both *in vitro* and *in vivo*, they are most frequently used as indicators of transcriptional activity in cells.

Typically, a reporter gene is joined to a promoter sequence in an expression vector that is transferred into cells. Following transfer, the cells are assayed for the presence of the reporter by directly measuring the reporter protein itself or the enzymatic activity of the reporter protein. An ideal reporter gene is one that is not endogenously expressed in the cell of interest and is amenable to assays that are sensitive, quantitative, rapid, easy, reproducible and safe.

Analysis of *cis*-acting transcriptional elements is a frequent application for reporter genes. Reporter vectors allow functional identification and characterization of promoter and enhancer elements because expression of the reporter correlates with transcriptional activity of the reporter gene. For these types of studies, promoter regions are cloned upstream or downstream of the gene. The promoter-gene fusion is introduced into cultured cells by standard transfection methods or into a germ cell to produce transgenic organisms. The use of reporter gene technology has allowed characterization of promoter and enhancer elements that regulate cell, tissue and development-defined gene expression.

Trans-acting factors can be assayed by co-transfer of the promoter-reporter gene fusion DNA with another cloned DNA expressing a trans-acting protein or RNA of interest. The protein could be a transcription factor that binds to the promoter region of interest cloned upstream of the reporter gene. For example, when tat protein is expressed from one vector in a transfected cell, the activity of different HIV-1 LTR sequences linked to a reporter gene increases and the activity increase is reflected in the increase of reporter gene protein activity.

Reporters can be assayed by detecting endogenous characteristics, such as enzymatic activity or spectrophotometric characteristics, or indirectly with antibody-based assays. In general, enzymatic assays are quite sensitive due to the small amount of reporter enzyme required to generate the products of the reaction. A potential limitation of enzymatic assays is high background if there is endogenous enzymatic activity in the cell (e.g., β -galactosidase). Antibody-based assays are generally less sensitive, but will detect the reporter protein whether it is enzymatically active or not.

Fundamentally, an assay is a means for translating a biomolecular effect into an observable parameter. While there are theoretically many strategies by which this can be achieved, in practice the reporter assays capable of delivering the speed, accuracy and sensitivity necessary for effective screening are based on photon production.

A. Luminescence versus Fluorescence

Photon production is realized primarily through fluorescence and chemiluminescence. Both processes yield photons as a consequence of energy transitions from excited-state molecular orbitals to lower energy orbitals. However, they differ in how the excited-state orbitals are created. In chemiluminescence, the excited states are the product of exothermic chemical reactions, whereas in fluorescence the excited states are created by absorption of light.

This distinction of how the excited states are created greatly affects the character of the photometric assay. For instance, fluorescence-based assays tend to be much brighter, since the photon used to create the excited states can be pumped into a sample at a very high rate. In chemiluminescence assays, the chemical reactions required to generate excited states usually proceed at a much lower rate, so yield a lower rate of photon emission. The greater brightness of fluorescence would appear to correlate with better assay sensitivity, but this is commonly not the case. Assay sensitivity is determined by a statistical analysis of signal relative to background or "noise", where the signal represents a sample measurement minus the background measurement. The limitation of fluorescence is that it tends to have much higher backgrounds, leading to lower relative signals.

The reason fluorescence assays have higher backgrounds is primarily because fluorometers must discriminate between the very high influx of photons into the sample and the much smaller emission of photons from the analytical fluorophores. This discrimination is accomplished largely by optical filtration, since emitted photons have longer wavelengths than excitation photons, and by geometry, since the emitted photons typically travel a different path than the excitation photons. But optical filters are not perfect in their ability to differentiate between wavelengths, and photons can also change directions through scattering. Chemiluminescence has the advantage that, since photons are not required to create the excited states, they do not constitute an inherent background when measuring photon efflux from a sample. The resulting low background permits precise measurement of very small changes in light.

Fluorescence assays can also be limited by the presence of interfering fluorophores within the samples. This is especially problematic in biological samples, which can be replete with a variety of heterocyclic compounds that fluoresce, typically in concentrations much above the analytical fluorophores of interest. The problem is minimized in simple samples, such as purified proteins. But in drug discovery, living cells are increasingly used for high-throughput screening. Unfortunately, cells are enormously complex in their chemical constitutions, which can exhibit substantial inherent fluorescence. Screening compound libraries is also inherently complex, since, although each assay sample may contain only one or a few compounds, the data set from which the drug leads are

sifted is cumulated from many thousands of compounds. These compounds may also present problems with fluorescence interference, since drug-like molecules typically have heterocyclic structures.

For image analysis of microscopic structure, fluorescence is almost universally preferred over chemiluminescence. Brightness counts because the optics required for imaging cellular structures are relatively inefficient at light gathering. Thus the low background inherent in chemiluminescence is of little advantage since it is usually far below the detection capabilities of imaging devices. Furthermore, imaging is largely a matter of edge detection, which has different signal-to-noise characteristics than simply detecting the presence of an analyte. Edge detection relies heavily on signal strength and suffers less from uniform background noise.

But in macroscopic measurements (such as in a plate well) requiring accurate quantification with high sensitivity, chemiluminescent assays often outperform analogous assays based on fluorescence. Macroscopic measurements are the foundation for most high-throughput screening, which relies heavily on the use of multiwell plates, typically with 96-, 384- or 1536-wells, to measure a single parameter in a large number of samples as quickly as possible. Assays based on fluorescence or chemiluminescence can yield high sample throughput. However, fluorescence is more likely to be hindered by light contamination (from the excitation beam) or the chemical compositions of the samples and compound libraries. The use of chemiluminescence in high-throughput screening has been limited largely by the lack of available assay methods. Due to its long history, fluorescence has been more commonly used. But new capabilities in chemiluminescence, particularly in bioluminescence, are now adding new bioluminescence techniques to high-throughput screening.

B. Bioluminescence Reporters

Bioluminescence is a form of chemiluminescence that has developed through natural selection. Although most people are aware of bioluminescence primarily through the nighttime displays of fireflies, there are many distinct classes of bioluminescence derived through separate evolutionary histories. These classes are widely divergent in their chemical properties, yet they all undergo similar chemical reactions, namely the formation and destruction of a dioxetane structure. The classes are all based on the interaction of the enzyme luciferase with a luminescent substrate luciferin (Figure 8.1). The luciferases that have been used most widely in high-throughput screening are beetle luciferases (including firefly luciferase), *Renilla* luciferase and aequorin. The beetle luciferases are the most versatile of this group, and the number of new applications is rapidly expanding. Click beetle luciferases, which also belong to the beetle group, are becoming better known and offer a range of new luminescence color options. *Renilla* luciferase has been used primarily for reporter gene

applications, although its use has also recently expanded. Aequorin has been used almost exclusively for monitoring intracellular calcium concentrations.

In nature, achieving efficient chemiluminescence is not a trivial matter, as evidenced by the lack of this phenomenon in daily life. The large energy transitions required for visible luminescence generally are disfavored over smaller ones that dissipate energy as heat, normally through interactions with surrounding molecules, especially water molecules in aqueous solutions. Because energy can be lost through these interactions, chemiluminescence depends strongly on environmental conditions. Thus chemiluminescence assays are often designed to incorporate hydrophobic compounds such as micelles to protect the excited state from water, or to rely on energy transfer to fluorophores that are less sensitive to solvent quenching. Another difficulty with chemiluminescence is efficient coupling of the reaction pathway to the creation of excited-state orbitals.

While chemiluminescence has relied on the ingenuity of chemists, bioluminescence has instead relied on the processes of natural evolution. As luminous organisms through the eons were selected by the brightness of their light, their luciferases have evolved both to maximize chemical couplings to generate the excited states and to protect the excited states from water. In firefly luciferase, the enzyme appears to exclude water by wrapping around the substrate, so that the excited-state reaction products are completely secluded. The enzyme structure shows two domains connected by a single polypeptide, which may act as a hinge. It is likely that the substrates bind between the domains, causing them to close like a lid onto a box. The enzyme would thus act as an insulator between the excited-state products and the environment around them. This strongly contrasts with synthetic forms of chemiluminescence, where the excited states are exposed to the solvent. In effect, a distinctive feature of bioluminescence is that the luciferase serves as a box that both generates and protects excited states.

Intracellular luciferase is typically quantified by adding a buffered solution containing detergent to lyse the cells and luciferase substrates to initiate the luminescent reaction.

The luminescence will slowly decay due to side reactions, causing irreversible inactivation of the enzyme. The nature of these side reactions is not well understood, but they are probably due to the formation of damaging free radicals. To maintain a steady luminescence over an extended period of time, ranging from minutes to hours, it is often necessary to inhibit the luminescent reaction to various degrees. This reduces the rate of luminescence decay to the point that it will not interfere over the time required to measure multiple samples. However, even under these conditions the luciferase may be quantified to as few as 10^{-20} moles per sample or less, which corresponds to roughly 10 molecules per cell. These assays are convenient for reporter gene applications because sample processing is not necessary prior to reagent addition. Simply add the reagent and read the resulting luminescence.

In a system where a second reporter is used, a "control" vector can be used to normalize for transfection efficiency or cell lysate recovery between treatments or transfection experiments. Typically, the control reporter gene is driven by a constitutive promoter and is cotransfected with "experimental" vectors. The experimental regulatory sequences are linked to a different reporter gene so that the relative activities of the two reporter gene products can be assayed individually. Control vectors can also be used to optimize transfection methods. Gene transfer efficiency is typically monitored by assaying reporter activity in cell lysates or by staining the cells *in situ* to estimate the percentage of cells expressing the transferred gene.

In general, bioluminescence reporters are preferred when experiments require high sensitivity, accurate quantitation or rapid analysis of multiple samples. Dual-bioluminescence assays can be particularly useful for efficiently extracting information.

II. Luciferase Genes and Vectors

A. Biology and Enzymology

Bioluminescence as a natural phenomenon is widely experienced with amazement at the prospect of living organisms creating their own light. Basic research into this phenomenon has also led to practical applications, particularly in molecular biology where bioluminescence enzymes have been widely used as genetic reporters. Moreover, the value of this application has grown considerably over the past decade as the traditional use of reporter genes has broadened to cover wide ranging aspects of cell physiology.

The conventional use of reporter genes has been largely to analyze and dissect the function of cis-acting genetic elements such as promoters and enhancers (so-called "promoter bashing"). In typical experiments, deletions or mutations are made in a promoter region, and their consequential effects on coupled expression of a reporter gene are then quantitated. However, the broader aspect of gene expression entails much more than transcription alone, and reporter genes can also be used to study these other cellular events.

Some examples of analytical methodologies that use luciferase include:

- Stable cell lines that integrate the reporter gene of interest into the chromosome can be selected and propagated when a selectable marker is included in a transfection vector. These types of engineered cell lines have been used for drug screening and to monitor the effect of exogenous agents and stimuli upon gene expression.
- Identification of interacting pairs of proteins *in vivo* using a system known as the two-hybrid system (Fields *et al.* 1989). The interacting proteins of interest are brought together as fusion partners—one is fused with a specific DNA binding domain, and the other protein is fused with a transcriptional activation domain. The physical interaction of the two fusion partners is

necessary for the functional activation of a reporter gene driven by a basal promoter and the DNA motif recognized by the DNA binding protein. This system was originally developed with yeast but has also been used in mammalian cells.

- Bioluminescence resonance energy transfer (BRET) for monitoring protein-protein interactions, where a fusion protein is made using the bioluminescent *Renilla* luciferase and another protein fused with a fluorescent molecule. Interaction of the two fusion proteins results in energy transfer from the bioluminescent molecule to the fluorescent molecule, with a concomitant change from blue light to green light (Angers *et al.* 2000).

Luciferase genes have been cloned from bacteria, beetles (e.g., firefly and click beetle), *Renilla*, *Aequorea*, *Vargula* and *Gonyaulax* (a dinoflagellate). Of these, only the luciferases from bacteria, beetles and *Renilla* have found general use as indicators of gene expression. Bacterial luciferase, although the first luciferase to be used as a reporter, is generally used to provide autonomous luminescence in bacterial systems through expression of the lux operon. Ordinarily it is not useful for analysis in mammalian cells.

Firefly Luciferase

Firefly luciferase is by far the most commonly used bioluminescent reporter. This monomeric enzyme of 61kDa catalyzes a two-step oxidation reaction to yield light, usually in the green to yellow region, typically 550–570nm (Figure 8.1). The first step is activation of the luciferyl carboxylate by ATP to yield a reactive mixed anhydride. In the second step, this activated intermediate reacts with oxygen to create a transient dioxetane that breaks down to the oxidized products, oxyluciferin and CO₂. Upon mixing with substrates, firefly luciferase produces an initial burst of light that decays over about 15 seconds to a low level of sustained luminescence. This kinetic profile reflects the slow release of the enzymatic product, thus limiting catalytic turnover after the initial reaction (Figure 8.1).

Various strategies to generate a stable luminescence signal have been tried to make the assay more convenient for routine laboratory use. The most successful of these incorporates coenzyme A to yield maximal luminescence intensity that slowly decays over several minutes. The mechanism of action for coenzyme A in the luminescent reaction is unclear, although it probably stems from the evolutionary ancestry of firefly luciferase. The amino acid sequence of firefly luciferase is related to a diverse family of acyl-CoA synthetases. By analogy to the catalytic mechanism of these related enzymes, formation of a thiol ester between CoA and luciferin seems likely. An optimized assay containing coenzyme A generates relatively stable luminescence in less than 0.3 seconds with linearity to enzyme concentration over a 100-millionfold range. The assay sensitivity allows quantitation to fewer than 10⁻²⁰ moles of enzyme.

The popularity of native firefly luciferase as a genetic reporter is due both to the sensitivity and convenience of the enzyme assay and to the tight coupling of protein

synthesis with enzyme activity. The gene encoding firefly luciferase, *luc*, is a monomer that does not require any post-translational modifications; it is available as a mature enzyme directly upon translation from its mRNA. It has been shown that catalytic competence is attained immediately after release from the ribosome. Hence, the luciferase assay provides a nearly instantaneous measure of total reporter expression in the cell.

Renilla Luciferase

Renilla luciferase is a 36kDa monomeric enzyme that catalyzes the oxidation of coelenterazine to yield coelenteramide and blue light of 480nm (Figure 8.1). The host organism, *Renilla reniformis* (sea pansy), is a coelenterate that creates bright green flashes upon tactile stimulation, apparently to ward off potential predators. The green light is created through association of the luciferase with a green fluorescent protein.

Although *Renilla* and *Aequorea* are both luminous coelenterates based on coelenterazine oxidation, and both have a green fluorescent protein, their respective luciferases are structurally unrelated. In particular, the *Renilla* luciferase does not require calcium in the luminescent reaction. The reporter gene for *Renilla* luciferase, *Rluc*, like that for firefly luciferase, is a cDNA. As a reporter molecule, *Renilla* luciferase provides many of the same benefits as firefly luciferase. Historically, the presence of nonenzymatic luminescence, termed autoluminescence, reduced assay sensitivity; however, improvements in assay chemistry have nearly eliminated this problem. In addition, the simplicity of the *Renilla* luciferase chemistry and, more recently, improvements to the luciferase substrate have enabled the quantitation of *Renilla* luciferase from living cells, in situ or in vivo.

Click Beetle Luciferase

Click beetle and firefly luciferase belong to the same beetle luciferase family. Hence, the size and enzymatic mechanism of click beetle luciferase are similar to those of firefly luciferase. What makes the click beetle unique is the variety of luminescence colors they emit. The Chroma-Luc™ genes were developed from naturally occurring luciferase genes of a luminous click beetle, *Pyrophorus plagiophthalmus*. Complementary DNAs cloned from the ventral light organ encode four luciferases capable of emitting luminescence ranging from green to orange (544–593nm). The Chroma-Luc™ luciferases were developed to generate luminescence colors as different as possible; a red luciferase (611nm) and two green luciferases (544nm each). These luciferase genes were codon optimized for mammalian cells and are nearly identical to one another, with a maximum of 8 amino acids difference between any two of these genes. The two green luciferases generate very similar luciferase proteins; however, one is maximally similar to (~98%) while the other is divergent from (~68%) the DNA sequence for the red luciferase. Experimental and control reporter genes and proteins within an experiment can

therefore be almost identical. Under circumstances where genetic recombination is a concern, the divergent luciferase pair may also be useful.

B. Gene Optimization

An ideal genetic reporter should: i) express uniformly and optimally in the host cells; ii) only generate responses to the effectors that the assay intends to monitor (avoid anomalous expression); and iii) have a low intrinsic stability to quickly reflect the transcriptional dynamics. Despite the biology and enzymology of the native luciferases, they are not necessarily optimal as genetic reporters. In the past decade, we have made significant improvements in expression, reducing the risk of anomalous expression and destabilizing these reporters. The key strategies to achieve these improvements are described here.

Peroxisomal Targeting Site Removal for the Beetle *luc* Gene

Normally, in the firefly light organ, luciferase is located in specialized peroxisomes of the photocytic cells. When expressed in foreign hosts, a conserved translocation signal within the enzyme structure causes it to accumulate in peroxisomes and glyoxysomes. In moderate to high levels of expression, the peroxisomes typically become saturated with luciferase and much of the reporter is found in the cytoplasm. Localization to the peroxisomes, however, might interfere with normal cellular physiology in two ways. First, large amounts of a foreign protein in the peroxisomes could impair their normal function. Second, many other peroxisomal proteins use the same translocation signals, so saturation with luciferase implies competition for the import of other peroxisomal proteins. Peroxisomal and glyoxysomal location of luciferase may also interfere with the performance of the genetic reporter. For instance, the luciferase accumulation in the cell might be differentially affected if it is distributed into two different subcellular compartments.

The stability of luciferase in peroxisomes is not known, but could well be different than its stability in the cytosol. If so, expression of luciferase could be affected by changes in the distribution of luciferase between peroxisomes and the cytosol. Measurements of in vivo luminescence might also be affected, since the availability of ATP, O₂, and luciferin within peroxisomes is not known.

The peroxisomal translocation signal in firefly and click beetle luciferases has been identified as the C-terminal tripeptide sequence, -Ser-Lys-Leu. Removal of this sequence abolishes import into peroxisomes. However, the relative specific activity of this modified luciferase has not been determined. To develop an optimal cytoplasmic form of the luciferase gene, we followed two strategies: i) we designed a new C-terminal tripeptide sequence based on the available data to minimize peroxisomal import, -Gly-Lys-Thr; and ii) we applied random mutagenesis to the C-terminal region and selected brightly luminescent colonies of *E. coli* transformed with the mutagenized luciferase genes. From sequence data of these selected mutants, we chose a clone with the sequence -Ile-Ala-Val.

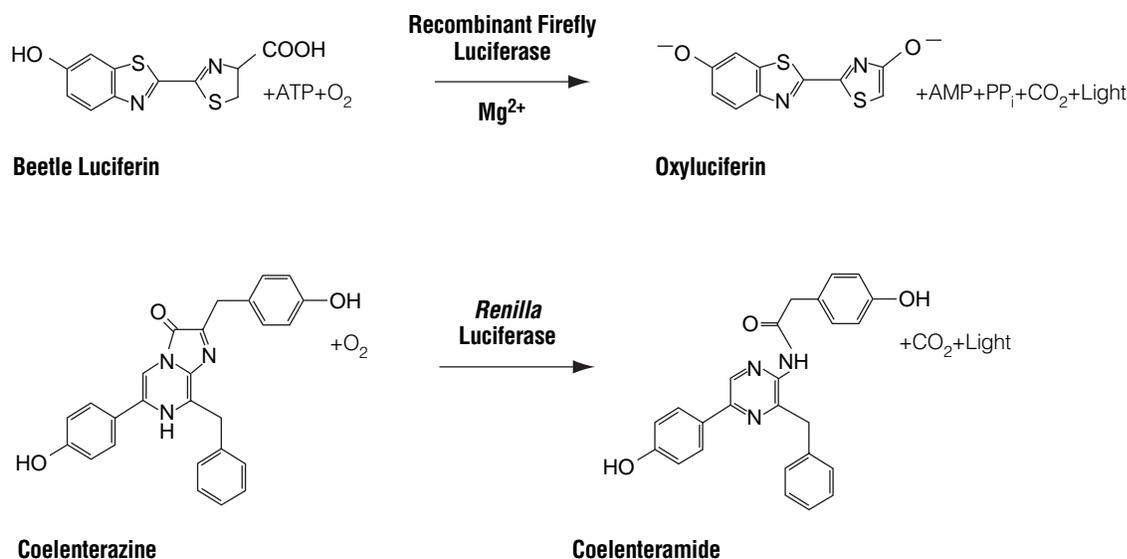


Figure 8.1. Diagram of firefly and *Renilla* luciferase reactions with their respective substrates, beetle luciferin and coelenterazine, to yield light.

Consistently, both modified luciferases yielded about 4- to 5-fold greater luminescence than the native enzyme when expressed in NIH/3T3 cells. We chose the luciferase containing an -Ile-Ala-Val sequence for the cytoplasmic form because it usually yielded slightly greater luminescence than the luciferase with -Gly-Lys-Thr. *Renilla* luciferase does not contain a targeting sequence and so is not affected by peroxisomal targeting.

Codon Optimization

Although redundancy in the genetic code allows amino acids to be encoded by multiple codons, different organisms favor some codons over others. The efficiency of protein translation in a non-native host cell can be substantially increased by adjusting the codon usage frequency but maintaining the same gene product. The native luciferase genes cloned from beetles (firefly or click beetle) or sea pansy (*Renilla reniformis*) use codons that are not optimal for expression in mammalian cells. Therefore, we systematically altered the codons to the preferred ones while removing inappropriate or unintended transcription regulatory sequences used in mammalian cells. As a result, a significant increase in luciferase expression levels was achieved, up to several hundredfold in some cases (Figures 8.2 and 8.3).

Cryptic Regulatory Sequence Removal

Anomalous expression is defined as departure from normal or expected levels of expression. The presence of cryptic regulatory sequences in the reporter gene may adversely affect transcription, resulting in anomalous expression of the reporter gene. Removal of these sequences has been shown to reduce the risk of anomalous expression. A cryptic regulatory sequence can be a transcription factor binding site and/or a promoter module (defined as two transcription factor binding sites separated by a spacer; Klingenhoff *et al.* 1999). Transcription factor binding sites located downstream from a promoter are believed to affect

promoter activity. Additionally, it is not uncommon for an enhancer element to exert activity and result in elevated levels of DNA transcription in the absence of a promoter sequence or in the presence of transcription regulatory sequences to increase the basal levels of gene expression in the absence of a promoter sequence. Promoter modules can exhibit synergistic or antagonistic functions (Klingenhoff *et al.* 1999).

We removed these cryptic regulatory sequences in the *luc* genes without changing the encoded amino acids. In addition, sequences resembling splice sites, poly(A) addition sequences, Kozak sequence (translation start for mammalian cells), *E. coli* promoters or *E. coli* ribosome binding sites were also removed wherever possible. This process has led to a greatly reduced number of cryptic regulatory sequences (Figure 8.4) in the *luc* genes and therefore a reduced risk of anomalous transcription.

Degradation Signal Addition

When performing reporter assays, measurements are made on the total accumulated reporter protein within cells. This accumulation occurs over the intracellular lifetime of the reporter, which is determined by both protein and mRNA stability. If transcription is changing during this lifetime, then the resulting accumulation of reporter will reflect a collection of different transcriptional rates. The longer the lifetime, the greater the collection of different transcriptional rates pooled into the reporter assay. This pooling process has a "dampening effect" on the representation of transcriptional dynamics, making changes in the transcriptional rate more difficult to detect. This can be remedied by reducing the lifetime of the reporter, thus reducing the pooling of different transcriptional rates into each reporter measurement. The resulting improvement in reporter dynamics is applicable to both upregulation and downregulation of gene expression.

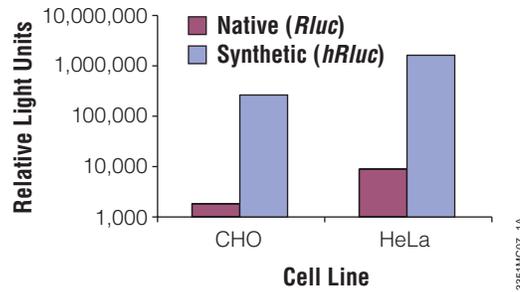


Figure 8.2. The synthetic *Renilla* luciferase gene supports higher expression than the native *Renilla* gene in mammalian cells. CHO and HeLa cells were transfected with pGL3-Control Vector (containing SV40 enhancer/promoter) harboring either the synthetic *hRluc* or native *Rluc* gene. Cells were harvested 24 hours after transfection and *Renilla* luciferase activity assayed using the Dual-Luciferase® Reporter Assay System.

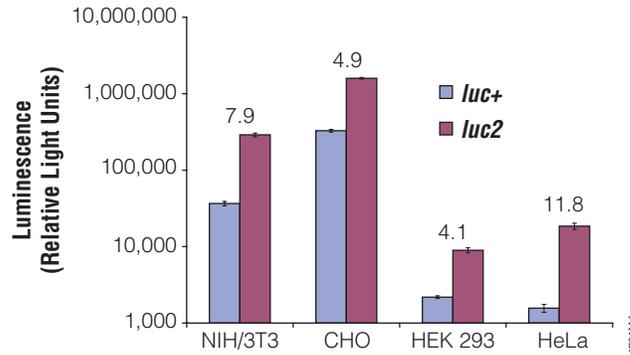


Figure 8.3. The firefly *luc2* gene displays higher expression than the *luc+* gene. The *luc2* gene was cloned into the pGL3-Control Vector (Cat.# E1741), replacing the *luc+* gene. Thus both firefly luciferase genes were in the same pGL3-Control Vector backbone. The two vectors containing either of the firefly luciferase genes were co-transfected into NIH/3T3, CHO, HEK 293 and HeLa cells using the pHRL-TK Vector for a transfection control. Twenty-four hours post-transfection the cells were lysed with Passive Lysis 5X Buffer (Cat.# E1941) and luminescence was measured using the Dual-Luciferase® Reporter Assay System (Cat.# E1910). Relative light units were normalized to the *Renilla* luciferase expression from the pHRL-TK Vector control. The fold increase in expression values is listed above each pair of bars. A repeat of this experiment yielded similar results.

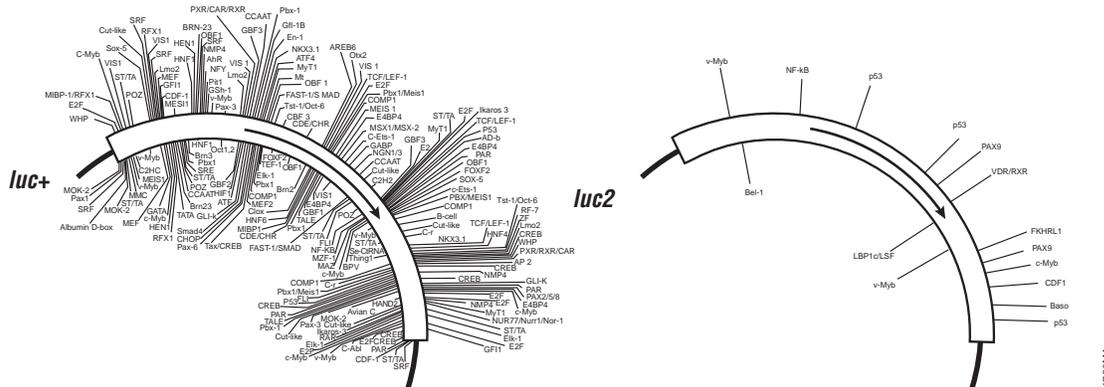


Figure 8.4. Reduced number of consensus transcription factor binding sites for the *luc2* gene. The number of consensus transcription factor binding sites identified in the *luc+* gene have been greatly reduced in the *luc2* gene.

Ideally, the reporter lifetime would be reduced to zero, completely eliminating the pooling of different transcriptional rates in each assay measurement. Only the transcription rate at the instant of the assay would be represented by the accumulation of reporter protein within the cells. Unfortunately, a zero lifetime would also yield zero accumulation, and thus no reporter could be measured. A compromise must be reached since, as lifetime reduces,

so does the amount of reporter available for detection in the assay. This is where the high sensitivity of luminescent assays is useful. Relative to other reporter technologies, the intracellular stability of luciferase reporters may be greatly reduced without losing measurable signals. Thus, the high sensitivity of luciferase assays permits greater dynamics in the luciferase reporters.

The speed by which a genetic reporter can respond to changes in the transcriptional rate is correlated to the stability of the reporter within cells. Highly stable reporters accumulate to greater levels in cells, but their concentrations change slowly with changes in transcription. Conversely, lower stability yields less accumulation but a much faster rate of response. To provide reporters designed to meet different experimental needs, families of luciferase genes have been developed yielding different intracellular stabilities. The genes conferring lower stabilities are referred to as the Rapid Response™ Reporters.

Beetle and *Renilla* luciferase reporters have an intrinsic protein half-life of ~3 hours. However, reporter response may still lag behind the underlying transcriptional events by several hours. To further improve reporter performance, we have developed destabilized luciferase reporters by genetically fusing a protein degradation sequence to the luciferase genes (Li *et al.* 1998). After evaluation of many degradation sequences for their effect on response rate and signal magnitude, two sequences were chosen, one composed of the PEST protein degradation sequence and a second composed of two protein (CL1 and PEST) degradation sequences. Due to their increased rate of degradation, these destabilized reporters respond faster and often display a greater magnitude of response to rapid transcriptional events and are therefore called the Rapid Response™ Reporters.

Vector Backbone Design

Vectors that are used to deliver the reporter gene to the host cells are also critical for the overall performance of the reporter assay. Cryptic regulatory sequences such as transcription factor binding sites and/or promoter modules found on the vector backbone could lead to high background and anomalous responses. This is a common issue for mammalian reporter vectors including our pGL3 Luciferase Reporter Vectors, which have recently been improved. We have extended our successful "cleaning" strategy for reporter genes to the entire pGL3 Vector backbone, removing cryptic regulatory sequences wherever possible, while maintaining functionality. Other modifications include a redesigned multiple cloning region to facilitate easy transfer of the DNA element of interest, removal of the f1 origin of replication and deletion of an intronic sequence. In addition, a synthetic poly(A) signal/transcriptional pause site was placed upstream of either the multiple cloning region (in promoterless vectors) or the HSV-TK, CMV or SV40 promoter (in promoter-containing vectors). This extensive effort resulted in the totally redesigned and unique vector backbone of the pGL4 Luciferase Reporter Vectors.

The pGL4 family of luciferase vectors incorporates a variety of features such as a choice of luciferases, Rapid Response™ versions, mammalian-selectable markers, basic vectors without promoters and promoter-containing control vectors (Figure 8.5).

By manipulating luciferase genes we've developed a series of optimized reporter genes featuring additional luminescence colors and improved codon usage, while deleting cryptic regulatory sequences such as transcription factor binding sites that could decrease protein expression in mammalian cells. The following section provides information about specific bioluminescence reporters and assays, including how to choose the correct reporter genes and vectors to suit your research needs.

Advantages of the pGL4 Luciferase Reporter Vectors

- Improved sensitivity and biological relevance due to:**
 - Increased reporter gene expression: Codon optimization of synthetic genes for mammalian expression
 - Reduced background and risk of expression artifacts: Removal of cryptic DNA regulatory elements and transcription factor binding sites
 - Improved temporal response: Rapid Response™ technology available using destabilized luciferase genes
- Additional advantages include:**
 - Flexible detection options: Choice of reporter genes
 - Easy transition from transient to stable cells: Choice of mammalian selectable markers
 - Easy transfer from one vector to another: Common multiple cloning site and a unique SfiI transfer scheme

III. Luciferase Reporter Assays and Protocols

The challenge for designing bioluminescence assays is harnessing this efficient light-emitting chemistry into analytical methodologies. Most commonly this is done by holding the reaction component concentrations constant, except for one component that is allowed to vary in relation to a biomolecular process of interest. When the reaction is configured properly, the resultant light is directly proportional to the variable component, thus coupling an observable parameter to the reaction outcome. In assays using luciferase, the variable component may be the luciferase itself or its substrates or cofactors. Because of very low backgrounds in bioluminescence, the linear range of this proportionality can be enormous, typically extending 10⁴- to 10⁸-fold over the concentration of the variable component.

Choosing the assay appropriate for your research needs is assisted by the following considerations and Tables 8.1 and 8.2, showing available luciferase genes, assays and reagents.

A. Single-Reporter Assays

Assays based on a single reporter provide the quickest and least expensive means for acquiring gene expression data from cells. However, because cells are inherently complex, the quantity of information gleaned from a single-reporter assay may be insufficient for achieving detailed and accurate results. Thus one of the first considerations in choosing a reporter methodology is deciding whether the speed and depth of information from a single reporter is

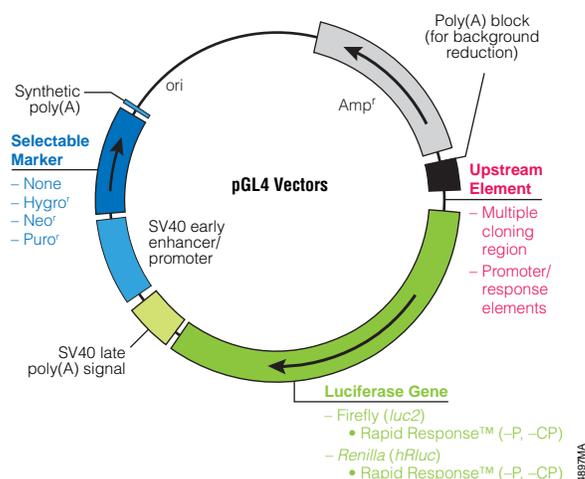


Figure 8.5. The family of pGL4 Luciferase Reporter Vectors incorporates a variety of additional features, such as a choice of luciferase genes, Rapid Response™ versions, a variety of mammalian selectable markers, and vectors with or without promoters.

sufficient or whether a greater density of information is desired. If a greater density of information is required, see the Dual-Reporter (and dual-color) Assays, Section III.B.

Using an assay reagent that produces stable luminescence is more convenient when performing assays in multiwell plates. Unfortunately, because bright reactions fade relatively quickly, a trade-off is necessary between luminescence intensity and duration. The Bright-Glo™ Reagent is designed for firefly luciferase to yield maximal luminescence intensity and sufficient duration for analysis in a multiwell plate. The Steady-Glo® Reagent provides even greater luminescence duration but with lower intensity. Both reagents are designed to work directly in culture medium for mammalian cells, so prior cell lysis is not necessary. This allows the user to grow cells in multiwell plates and then measure expression with a single step.

Additional Resources for Single-Reporter Assays

Technical Bulletins and Manuals

TB281	<i>Luciferase Assay System Technical Bulletin</i> (www.promega.com/tbs/tb281/tb281.html)
TM055	<i>Renilla Luciferase Assay System Technical Manual</i> (www.promega.com/tbs/tm055/tm055.html)
TM052	<i>Bright-Glo™ Luciferase Assay System Technical Manual</i> (www.promega.com/tbs/tm052/tm052.html)
TM051	<i>Steady-Glo® Luciferase Assay System Technical Manual</i> (www.promega.com/tbs/tm051/tm051.html)

TM259 *pGL4 Luciferase Reporter Vectors Technical Manual*
(www.promega.com/tbs/tm259/tm259.html)

Citations

Rifas, L. *et al.* (1997) Gestational exposure to ethanol suppresses msx2 expression in developing mouse embryos. *Proc. Natl. Acad. Sci. USA* **94**, 7549–54.

Luciferase studies were performed on transfected MC3T3-E1 cell lysates using the Luciferase Assay System. Constructs were prepared in the pGL2 Promoter Vector.

PubMed Number: 9207129

Ashfield, T. *et al.* (2004) Convergent evolution of disease resistance gene specificity in two flowering plant families. *Plant Cell* **16**, 309–18.

Leaves of *Glycine max* (soybean) were co-transfected by particle bombardment with various combinations of vectors encoding plant disease resistance genes and a luciferase reporter construct containing the constitutive 35S promoter of cauliflower mosaic virus. Leaf disks from the transfected areas were frozen in liquid nitrogen, ground and resuspended in 240µl of Luciferase Cell Culture Lysis Reagent. The lysates were then assayed for luciferase activity with the Luciferase Assay System. The luciferase values correlated to plant leaf cell survival of the various constructs.

PubMed Number: 14742871

Citations

de Haan, C.A.M. *et al.* (2004) Cleavage inhibition of the murine coronavirus spike protein by a furin-like enzyme affects cell-cell but not virus-cell fusion. *J. Virol.* **78**, 6048–54.

The *Renilla* Assay System was used to analyze mouse hepatitis coronavirus strain A59 (MHV-A59) entry into cells. A mouse hepatitis coronavirus construct expressing

Renilla luciferase was used to infect LR7 cells in the presence or absence of a furin protease inhibitor.

PubMed Number: 15141003

Lin, P.F. *et al.* (2003) A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. *Proc. Natl. Acad. Sci. USA* **100**, 11013–8.

To test the effect of BMS-378806, a new small molecule inhibitor of HIV-1, a cell fusion assay was developed. Target cells that stably expressed CD4, CXCR4 or CCR5 receptors and carried a responsive luciferase plasmid were prepared. Effector cells were transiently transfected with HIV coat protein gp160 from various strains of virus, and a plasmid to activate the responsive element promoting luciferase. Therefore, if the cells fused, luciferase was activated. To measure the activation, effector cells were plated with target cells 1:2 and seeded into 96-well plates at 1×10^4 cells/well, then incubated with various concentrations of BMS-378806 for 12–24 hours. Luciferase activity was determined using Steady-Glo[®] Reagent.

PubMed Number: 12930892

Promega Publications

PN075	Bright-Glo [™] and Steady-Glo [®] : Reagents for academic and industrial applications. (www.promega.com/pnotes/75/8554_03/8554_03.html)
PN089	pGL4 Vectors: A new generation of luciferase reporter vectors. (www.promega.com/pnotes/89/12416_07/12416_07.html)

B. Dual-Reporter Assays

The most commonly used dual-reporter assay is based on combining the chemistries for firefly and *Renilla* luciferases. These luciferases use different substrates and thus can be differentiated by their enzymatic specificities. The method comprises adding two reagents to each sample, with a measurement of luminescence following each addition. Addition of the first reagent activates the firefly luciferase reaction; addition of the second reagent extinguishes firefly luciferase and initiates the *Renilla* luciferase reaction. The Dual-Luciferase[®] Assay Reagent relies on cell lysis prior to performing the assay and thus requires the use of reagent injectors if used with multiwell plates. The Dual-Glo[™] Reagent is optimized for multiwell plates, providing longer luminescence duration (in other words, a longer luciferase half-life). As with other reagents designed for use in multiwell plates, the Dual-Glo[™] Assay works directly in the culture medium for mammalian cells without the prior cell lysis.

Generally the benefits of a dual assay can improve experimental efficiency by: i) reducing variability that can obscure meaningful correlations; ii) normalizing interfering phenomena that may be inherent in the experimental system; and iii) normalizing differences in transfection efficiencies between samples.

Reducing Variability

Because cells are complex micro-environments, significant variability may occur between samples within an experiment and between experiments performed at different times. Challenges include trying to maintain uniform cell density and viability between samples and accomplishing reproducible transfection of exogenous DNA. Multiwell plates introduce variables such as edge effects, brought about by differences in heat capacity and humidity across a plate. Dual assays can control for much of this variability, leading to more accurate and meaningful comparisons between samples (Hawkins *et al.* 2002; Hannah *et al.* 1998; Wood, 1998; Faridi *et al.* 2003).

Dual-Color Assays

In some cases, researchers may prefer to activate both luciferase assays simultaneously by adding a single reagent. This reduces total assay volume and liquid handling requirements. The light emission of the two luciferases can be differentiated by the color of the luminescence. We have developed click beetle luciferases, which are related to firefly luciferase, to yield red and green luminescence. The structures of these luciferases are nearly identical, containing only a few amino acid substitutions necessary to create the different colors. This structural similarity means that both the control reporter and the experimental reporter are likely to respond similarly to biochemical changes within the cell, resulting in even more accurate normalization to the control.

The genes encoding these reporters, the Chroma-Luc[™] genes, are codon optimized for mammalian cells. We developed two genes encoding the green-emitting reporter, one which is nearly identical to the gene encoding red luminescence, and one that is maximally divergent from it but that encodes the same protein. The divergent gene may be useful under circumstances where genetic recombination is a concern.

The Chroma-Glo[™] Assay reagent is designed for use in multiwell plates. Its formulation supports optimal reaction kinetics for both reporters simultaneously, and it works directly in culture medium. Because color differentiation is required for the Chroma-Glo[™] Assay, a luminometer capable of using colored optical filters is required. Since the light is transmitted through the optical filters, sensitivity relative to other assay methods is reduced. Both luciferases may be detectable using optical filters when the relative concentrations differ by approximately 100-fold. This is less than dual-luciferase assays using chemical differentiation, where the relative concentration may differ by over 1,000-fold.

Distinguishing among the Dual Assays

Dual-reporter and dual-color assays allow the user either to measure expression of two different reporter genes or one reporter gene and cell viability. In all cases, the assays allow both measurements to be made sequentially from each sample. Most dual assays are optimized for use in multiwell plates.

Additional Resources for Dual-Reporter and Dual-Color Assays

Technical Bulletins and Manuals

- TM040 *Dual-Luciferase® Reporter Assay System Technical Manual*
(www.promega.com/tbs/tm040/tm040.html)
- TM058 *Dual-Glo™ Luciferase Assay System Technical Manual*
(www.promega.com/tbs/tm058/tm058.html)
- TM062 *Chroma-Glo™ Luciferase Assay System Technical Manual*
(www.promega.com/tbs/tm062/tm062.html)
- TM259 *pGL4 Luciferase Reporter Vectors Technical Manual*
(www.promega.com/tbs/tm259/tm259.html)

Citations

Elbashir, S.M. *et al.* (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells *Nature* **411**, 494–8.

In this landmark paper describing RNA interference in mammalian cells, the firefly and *Renilla* luciferase genes were targeted for degradation. NIH/3T3, HEK293, HeLa S3, COS-7 and S2 cells were transfected with 1µg of pGL2-Control or pGL3-Control Vector, 0.1µg pRL-TK Vector and 0.21µg siRNA duplex targeting either firefly or *Renilla* luciferase. The Dual-Luciferase® Reporter Assay was used 20 hours post-transfection to monitor luciferase expression. It was found that transfection with 21bp dsRNA can cause the specific degradation of a targeted sequence. This was the first demonstration of the RNAi effect in mammalian cells.

PubMed Number: 11373684

Yamaguchi, K. *et al.* (2004) Identification of nonsteroidal anti-inflammatory drug-activated gene (NAG-1) as a novel downstream target of phosphatidylinositol 3-kinase/AKT/GSK-3 pathway. *J. Biol. Chem.* **279**, 49617–23.

The LY294002 phosphatidylinositol 3-kinase (PI3K) inhibitor was used to demonstrate nonsteroidal anti-inflammatory drug-activated gene (NAG-1) as a novel downstream target of the PI3K pathway during cell activation. For these experiments, HCT-116 cells were treated with 50µM LY294002, and NAG-1 protein expression was assessed by Western blotting. Gene upregulation during LY294002 treatment was measured with a luciferase reporter construct containing the NAG-1 promoter, the pRL-null Vector as a transfection control and the Dual-Luciferase® Assay System.

PubMed Number: 15377673

Verge, V. *et al.* (2004) Localization of a promoter in the putative internal ribosome entry site of *Saccharomyces cerevisiae* TIF4631 gene. *RNA* **10**, 277–86.

Researchers cloned the *Photinus* and *Renilla* luciferase ORFs into the pSP64 Poly(A) Vector to create a dual-reporter vector named SP6P. A similar vector, SP6R.4G(-508/-3).P, was created in which a 5' untranslated region from the *Saccharomyces* TIF4631 gene was cloned between the two reporter genes. These two vectors were used to transform yeast strains. The resultant transformants were lysed using Passive Lysis Buffer and a modified lysis procedure. Lysates were analyzed for luciferase activities with the Dual-Luciferase® Reporter Assay System and a TD20/20 luminometer. The researchers also cloned and sequenced the 5' untranslated region of TIF4631 using a RACE-PCR technique followed by cloning the PCR amplicons.

PubMed Number: 14730026

Citations

Chen, Q.Y. *et al.* (2004) Human CD1D gene has TATA boxless dual promoters: An SP1-binding element determines the function of the proximal promoter. *J. Immunol.* **172**, 5512–21.

The authors demonstrated that the human CD1D gene has distal and proximal TATA boxless promoter sequences. Distal and proximal promoters to CD1D were cloned into the pGL3-Basic Vector to create reporter constructs. One construct contained the entire 4,986 base pair region, including the distal and proximal CD1D promoter.

Transient transfections were performed using 5 x 10⁵ Jurkat cells in 24-well plates, 0.8µg of pGL3-Basic Vector with the insert of interest and 30ng of pRL-CMV Vector as a transfection normalization control. The Dual-Glo™ Luciferase Assay System was used to assay luciferase activities.

PubMed Number: 15100293

Promega Publications

- PN089 pGL4 Vectors: A new generation of luciferase reporter vectors.
(www.promega.com/pnotes/89/12416_07/12416_07.html)
- PN085 Increased *Renilla* luciferase sensitivity in the Dual-Luciferase® Reporter Assay System.
(www.promega.com/pnotes/85/10904_31/10904_31.html)
- PN085 Introducing Chroma-Luc™ Technology
(www.promega.com/pnotes/85/10904_11/10904_11.html)
- PN081 Dual-Glo™ Luciferase Assay System: Convenient dual reporter measurements in 96- and 384-well plates.
(www.promega.com/pnotes/81/9939_22/9939_22.html)

C. Live Cell Substrates

Researchers strive to monitor cellular activities with as little impact on the cell as possible. The endpoint of an experiment, however, sometimes requires complete disruption of the cells so that the environment surrounding the reporter enzyme can be carefully controlled. Recently, nondestructive live cell substrates were developed, which allow monitoring of *Renilla* luciferase without cell lysis. *Renilla* luciferase requires only oxygen and coelenterazine to generate luminescence, providing a simple luciferase system with which to measure luminescence from living cells. Unfortunately, coelenterazine is unstable in aqueous solutions and so it has been difficult and inconvenient to measure *Renilla* luciferase. EnduRen™ and ViviRen™ Live Cell Substrates have been designed to overcome this difficulty and to easily generate luminescence from live cells expressing *Renilla* luciferase. Because the *Renilla* luciferase luminescence is generated from living cells, these substrates are also ideal for multiplexing with assays that determine cell number.

Normalizing Interfering Phenomena

When making correlations between experimental conditions and the expression of a reporter gene, other events associated with cell physiology may affect reporter gene expression. Of particular concern is the effect of cytotoxicity, which can appear as genetic downregulation when using a single-reporter assay. Live cell assays that can be multiplexed with a cell viability assay allow independent monitoring of both reporter expression and cell viability to avoid misinterpretation of the data (Farfan *et al.* 2004). The use of multiplexed assays allow correlation of events within cells, such as the coupling of target suppression by RNAi to its consequences on cellular physiology (Hirose *et al.* 2002).

The CellTiter-Glo® Luminescent Cell Viability Assay provides a rapid and sensitive assay of cell viability based on luminescent detection of cellular ATP. Because the CellTiter-Glo® Assay uses a stabilized firefly luciferase in its formulation, it cannot be directly combined with a reporter assay for expression of firefly luciferase. However, it can be readily combined with nondestructive reporter assays of *Renilla* luciferase.

Expression of *Renilla* luciferase may be quantitated, or continuously monitored, by adding EnduRen™ Substrate to the culture medium. When reporter measurements are completed, CellTiter-Glo® Reagent is added to the sample to inactivate the *Renilla* luminescence and initiate the ATP-dependent luminescence. Because the CellTiter-Glo® Assay is an endpoint assay, further sample monitoring after measuring cell viability is not possible.

Additional Resources for Live Cell Substrates**Technical Bulletins and Manuals**

TM244	<i>EnduRen™ Live Cell Substrate Technical Manual</i> (www.promega.com/tbs/tm244/tm244.html)
TM064	<i>ViviRen™ Live Cell Substrate Technical Manual</i> (www.promega.com/tbs/tm064/tm064.html)
TM259	<i>pGL4 Luciferase Reporter Vectors Technical Manual</i> (www.promega.com/tbs/tm259/tm259.html)

Citations

Dinh, D.T. *et al.* (2005) Helix I of Beta-arrestin is involved in postendocytic trafficking but is not required for membrane translocation, receptor binding and internalization. *Mol. Pharmacol.* **67**, 375–82.

Type 1 angiotensin II receptor *Renilla* luciferase (AT1R-RLuc), and beta-arrestin1 and 2 GFP fusion constructs were transfected into COS-7 cells. The COS-7 cell cultures were then activated with 100µM angiotensin II in the presence of 60µM EnduRen™ Live Cell Substrate, and BRET fluorescence readings were taken at 475 and 515nm over a 1-hour period. The authors also describe analysis of helix I mutants of β-arrestin1 and β-arrestin2 in similar β-arrestin-GFP construct BRET studies. Data are displayed as a ratio of fluorescence readings with both constructs compared to fluorescence from the AT1R-RLuc construct alone.

PubMed Number: 15523053

Promega Publications

PN090	Measuring <i>Renilla</i> luminescence in living cells. (www.promega.com/pnotes/90/12727_10/12727_10.html)
PN089	pGL4 Vectors: A new generation of luciferase reporter vectors. (www.promega.com/pnotes/89/12416_07/12416_07.html)

D. Luciferase Reporter Cell Lines

Luciferase reporter assays have been widely used to investigate cellular signaling pathways and as high-throughput screening tools for drug discovery (Brasier *et al.* 1992, Zhuang *et al.* 2006) The GloResponse™ Cell Lines contain optimized, state-of-the-art luciferase reporter technology integrated into a cell line. The GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line and CRE-*luc2P* HEK293 Cell Line are designed for rapid and convenient analysis of cell signaling through the nuclear factor of activated T-cells (NFAT) pathway or cAMP response pathways via activation of a luciferase reporter gene. Activity of

non-native activators of these pathways (including GPCRs) can be studied after they have been introduced by transfection.

The GPCR assays configured using the GloResponse™ Cell Lines are amenable for high-throughput screening. These assays typically have greater response dynamics (fold of induction) than other assay formats and good quality as indicated by the high Z' values (Zhang *et al.* 1999).

GPCRs regulate a wide-range of biological functions and are one of the most important target classes for drug discovery (Klabunde *et al.* 2002). GPCR signaling pathways can be categorized into three classes based on the G protein α -subunit involved: Gs, Gi/o and Gq. The GloResponse™ CRE-*luc2P* HEK293 Cell Line can be used to study and configure screening assays for Gs- and Gi/o-coupled GPCRs, which signal through cAMP and CRE. For Gq-coupled GPCRs, which signal through calcium ion and NFAT-RE, the GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line should be used.

The GloResponse™ Cell Lines were generated by clonal selection of HEK293 cells stably transfected with pGL4-based vectors carrying specific response elements for the pathway of interest. These cell lines incorporate the improvements developed for the pGL4 family of reporter vectors for enhanced performance. The destabilized *luc2P* luciferase reporter is used for improved responsiveness to transcriptional dynamics. The *luc2P* gene is codon optimized for enhanced expression in mammalian cells, and the pGL4 plasmid backbone has been engineered to reduce background reporter expression. The result is a cell line with very high induction levels when the pathway of interest is activated.

Additional Resources for Live Cell Substrates

Technical Bulletins and Manuals

TB362	GloResponse™ CRE- <i>luc2P</i> HEK293 Cell Line Technical Bulletin (www.promega.com/tbs/tb362/tb362.html)
TB363	GloResponse™ NFAT-RE- <i>luc2P</i> HEK293 Cell Line Technical Bulletin (www.promega.com/tbs/tb363/tb363.html)

E. Selecting a Reporter Gene and Assay: Tables 8.1–8.3

The tables in this section are designed to show the various features of reporter vectors (Tables 8.1 and 8.2), including the reporter gene, whether the vector contains a multiple cloning region or not, what gene promoter and protein degradation sequence the vector has, and its mammalian selectable marker, as well as the features of our reporter assays (Table 8.3). In addition to the tables, the following tools will help in your choice of pGL4 Vector or a reporter assay.

For a step-by-step guide to help you choose the best pGL4 Vector for your studies use the pGL4 Vector Selector (<http://faqs.promega.com>). To go to the tool, click the link, then select the "Solution Finder" tab, and choose "pGL4 Vector Selector".

The Introduction to Reporter Gene Assays (www.promega.com/multimedia/reporter01.htm) animation demonstrates the basic design of a reporter gene assay using the Dual-Luciferase® Assay to study promoter structure, gene regulation and signaling pathways.

Table 8.1. pGL4 Luciferase Reporter Vectors.

Vector	Reporter Gene	Multiple Cloning Region	Protein Degradation Sequence	Gene Promoter	Mammalian Selectable Marker
pGL4.10	<i>luc2</i>	Yes	No	No	No
pGL4.11	<i>luc2P</i>	Yes	hPEST	No	No
pGL4.12	<i>luc2CP</i>	Yes	CL1-hPEST	No	No
pGL4.13	<i>luc2</i>	No	No	SV40	No
pGL4.14	<i>luc2</i>	Yes	No	No	Hygro
pGL4.15	<i>luc2P</i>	Yes	hPEST	No	Hygro
pGL4.16	<i>luc2CP</i>	Yes	CL1-hPEST	No	Hygro
pGL4.17	<i>luc2</i>	Yes	No	No	Neo
pGL4.18	<i>luc2P</i>	Yes	hPEST	No	Neo
pGL4.19	<i>luc2CP</i>	Yes	CL1-hPEST	No	Neo
pGL4.20	<i>luc2</i>	Yes	No	No	Puro
pGL4.21	<i>luc2P</i>	Yes	hPEST	No	Puro
pGL4.22	<i>luc2CP</i>	Yes	CL1-hPEST	No	Puro
pGL4.23	<i>luc2</i>	Yes	No	minP	No
pGL4.24	<i>luc2P</i>	Yes	hPEST	minP	No
pGL4.25	<i>luc2CP</i>	Yes	CL1-hPEST	minP	No
pGL4.26	<i>luc2</i>	Yes	No	minP	Hygro
pGL4.27	<i>luc2P</i>	Yes	hPEST	minP	Hygro
pGL4.28	<i>luc2CP</i>	Yes	CL1-hPEST	minP	Hygro
pGL4.29	<i>luc2P</i>	No	hPEST	CRE	Hygro
pGL4.30	<i>luc2P</i>	No	hPEST	NFAT-RE	Hygro
pGL4.31	<i>luc2P</i>	No	hPEST	GAL4UAS	Hygro
pGL4.32	<i>luc2P</i>	No	hPEST	NF-kB-RE	Hygro
pGL4.70	<i>hRluc</i>	Yes	No	No	No
pGL4.71	<i>hRlucP</i>	Yes	hPEST	No	No
pGL4.72	<i>hRlucCP</i>	Yes	CL1-hPEST	No	No
pGL4.73	<i>hRluc</i>	No	No	SV40	No
pGL4.74	<i>hRluc</i>	No	No	HSV-TK	No
pGL4.75	<i>hRluc</i>	No	No	CMV	No
pGL4.76	<i>hRluc</i>	Yes	No	No	Hygro
pGL4.77	<i>hRlucP</i>	Yes	hPEST	No	Hygro
pGL4.78	<i>hRlucCP</i>	Yes	No	No	Hygro
pGL4.79	<i>hRluc</i>	Yes	No	No	Neo
pGL4.80	<i>hRlucP</i>	Yes	hPEST	No	Neo
pGL4.81	<i>hRlucCP</i>	Yes	CL1-hPEST	No	Neo
pGL4.82	<i>hRluc</i>	Yes	No	No	Puro
pGL4.83	<i>hRlucP</i>	Yes	hPEST	No	Puro
pGL4.84	<i>hRlucCP</i>	Yes	CL1-hPEST	No	Puro

Table 8.2. Other Luciferase Reporter Vectors.

Vector	Reporter Gene	Multiple Cloning Region	Protein Degradation Sequence	Gene Promoter	Mammalian Selectable Marker
pGL3-Basic	<i>luc+</i>	Yes	No	No	No
pGL3-Control	<i>luc+</i>	Yes	No	SV40	No
pGL3-Enhancer	<i>luc+</i>	Yes	No	No	No
pGL3-Promoter	<i>luc+</i>	Yes	No	SV40	No
pCBR-Basic	<i>CBRLuc</i>	Yes	No	No	No
pCBR-Control	<i>CBRLuc</i>	No	No	No	No
pCBG68-Basic	<i>CBG68luc</i>	Yes	No	No	No
pCBG68-Control	<i>CBG68luc</i>	No	No	No	No
pCBG99-Basic	<i>CBG99luc</i>	Yes	No	No	No
pCBG99-Control	<i>CBG99luc</i>	No	No	No	No

Table 8.3. Luciferase Reporter Assays.

Assay System	Gene Assayed	Single Sample or Plate Assay	Signal Stability	Live Cell Assay
Single Reporter				
Luciferase Assay System	<i>luc, luc+, luc2</i>	Single or Plate ²	Short (<0.5h)	No
Steady-Glo [®] Luciferase Assay System	<i>luc, luc+, luc2</i>	Plate ¹	Long (>0.5h)	No
Bright-Glo [™] Luciferase Assay System	<i>luc, luc+, luc2</i>	Plate ¹	Long (>0.5h)	No
<i>Renilla</i> Luciferase Assay System	<i>Rluc, hRluc</i>	Single or Plate ²	Short (<0.5h)	No
Dual Reporter				
Dual-Glo [™] Luciferase Assay System	<i>luc+, luc2, Rluc, hRluc</i>	Plate ¹	Long (>0.5h)	No
Dual-Luciferase [®] Reporter Assay System	<i>luc+, luc2, Rluc, hRluc</i>	Single (Cat.# E1910)	Short (<0.5h)	No
		Plate ² (Cat.# E1980)	Short (<0.5h)	No
Chroma-Glo [™] Luciferase Assay System	<i>CBRLuc, CBG99luc, CBG68luc</i>	Plate ¹	Long (>0.5h)	No
Live Cell				
EnduRen [™] Live Cell Substrate	<i>Rluc, hRluc</i>	Plate ²	Long (>0.5h)	Yes
ViviRen [™] Live Cell Substrate	<i>Rluc, hRluc</i>	Plate	Short (<0.5h)	Yes

¹Do not use this product/reagent with automated reagent injectors, available on certain luminometers.

²Use with plate assays only when luminometer has a reagent injector.

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- Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.
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I. Introduction

In today's world of DNA analysis by multiplex and real-time PCR, the importance of high-quality, purified DNA cannot be underestimated. Finding a suitable DNA isolation system to satisfy your downstream application needs is vital for the successful completion of experiments. This DNA purification chapter addresses general information on the basics of DNA isolation, plasmid growth and DNA quantitation as well as how purification by silica can help increase your productivity so you spend less time purifying DNA and more time developing experiments and analyzing data. In addition, this chapter covers the wide variety of Promega products available for plasmid, genomic and fragment/PCR product purification and includes a sample protocol for each type of isolation system. Along with the discussion of Promega's DNA purification systems, we also consider the issues of scalability, downstream applications and yield to assist in finding the best system for your needs.

A. Basic Isolation Procedure

The basic steps of DNA isolation are disruption of the cellular structure to create a lysate, separation of the soluble DNA from cell debris and other insoluble material and purification of the DNA of interest from soluble proteins and other nucleic acids. Historically, this was done using organic extraction (e.g., phenol:chloroform) followed by ethanol precipitation. In the case of plasmid preparations, the multiple-day protocol typically involved cesium chloride banding followed by dialysis of the plasmid DNA. These methods were time consuming and used a variety of hazardous reagents.

For ease-of-use, Promega offers an array of conveniently packaged DNA purification products that can isolate DNA in less than an hour using much safer methods. Disruption of most cells is done by chaotropic salts, detergents or alkaline denaturation, and the resulting lysate is cleared by centrifugation, filtration or magnetic clearing. DNA is purified from the soluble portion of the lysate. When silica matrices are used, the DNA is eluted in an aqueous buffer such as TE or nuclease-free water. The purified, high-quality DNA is ready-to-use for a wide variety of demanding downstream applications such as multiplex PCR, coupled *in vitro* transcription/translation systems, transfection and sequencing reactions. Eluting and storing the DNA in TE buffer is helpful if the EDTA does not affect downstream applications. EDTA chelates or binds magnesium present in the purified DNA and can help inhibit possible contaminating nuclease activity.

DNA fragment purification from an amplification reaction or restriction enzyme digestion involves a direct treatment of the solution to remove the enzyme and reaction buffer and for PCR products, reduce the amount of nucleotides and primers present. Historically, this was done with phenol:chloroform extraction followed by precipitation. However, safety issues and the expense associated make organic extraction a less convenient DNA purification method. Promega's option is adding chaotropic salt to the

reaction volume and purifying the PCR products by silica chemistry. This method is quick and results in pure DNA ready for sequencing and cloning.

B. Basis for Purification by Silica

The majority of Promega's DNA isolation systems for genomic, plasmid and PCR product purification are based on purification by silica. Regardless of the method used to create a cleared lysate, the DNA of interest can be isolated by virtue of its ability to bind silica in the presence of high concentrations of chaotropic salts (Chen and Thomas, 1980; Marko *et al.* 1982; Boom *et al.* 1990). These salts are then removed with an alcohol-based wash and the DNA eluted in a low-ionic-strength solution such as TE buffer or water. The binding of DNA to silica seems to be driven by dehydration and hydrogen bond formation, which competes against weak electrostatic repulsion (Melzak *et al.* 1996). Hence, a high concentration of salt will help drive DNA adsorption onto silica, and a low concentration will release the DNA.

Promega has sold and supported silica-based DNA purification systems for nearly two decades. The first technology available was silica resin, exemplified by the Wizard® *Plus* Minipreps DNA Purification System. The protocol for purification by silica resin involves combining the cleared lysate with a resin slurry and using vacuum filtration to wash the bound DNA, followed by centrifugation to elute the purified DNA.

More recent purification systems consist of two different formats: silica membrane column (e.g., the PureYield™ Plasmid Midiprep System) and silica-coated MagneSil® Paramagnetic Particles (PMPs; e.g., Wizard® Magnetic 96 DNA Plant System). While both methods yield high-quality DNA, the silica membrane column is more convenient. For automated purification, either the 96-well silica membrane plates or the MagneSil® PMPs are easily adapted to a variety of robotic platforms. In order to process the DNA samples, the MagneSil® PMPs require a magnet for particle capture rather than centrifugation or vacuum filtration. The MagneSil® PMPs are considered a "mobile solid phase" with binding of nucleic acids occurring in solution. Particles can also be completely resuspended during the wash steps of a purification protocol, thus enhancing the removal of contaminants. See Figure 9.1 for images of a silica membrane column and the MagneSil® PMPs.



Figure 9.1. Images of two Promega silica purification matrices. **Panel A.** A PureYield™ Midiprep binding column. The membrane is present at the bottom of the column. **Panel B.** An electron micrograph of MagneSil® PMPs.

Additional Resources for Silica Purification

Promega Publications

PN100	Transfection-quality plasmid DNA in as little as ten minutes using the PureYield™ Plasmid Miniprep System (www.promega.com/pnotes/100/16620_06/16620_06.html)
PN082	Promega's SV membrane technology: The evolution of an indispensable laboratory tool (www.promega.com/pnotes/82/10203_10/10203_10.html)
PN069	MagneSil® Paramagnetic Particles: Novel magnetics for DNA purification (www.promega.com/pnotes/69/7542_12/7542_12.html)

C. Overview of Plasmid DNA Purification

The primary consideration for plasmid purification is separation of plasmid DNA from the chromosomal DNA and cellular RNA of the host bacteria. A number of methods have been developed to generate a cleared lysate that not only removes protein and lipids but also efficiently removes contaminating chromosomal DNA while leaving plasmid DNA free in solution. Methods for the preparation of cleared lysates that enrich for plasmid DNA include

SDS-alkaline denaturation (Birnboim and Doly, 1979; Birnboim, 1983), salt-SDS precipitation (Hirt, 1967) and rapid boiling (Holmes and Quigley, 1981).

The SDS-alkaline denaturation method, which is used in all Promega plasmid isolation systems, is a popular procedure for purifying plasmid DNA because of its overall versatility and consistency. This technique exploits the difference in denaturation and renaturation characteristics of covalently closed circular plasmid DNA and chromosomal DNA fragments. Under alkaline conditions (at pH 11), both plasmid and chromosomal DNA are efficiently denatured. Rapid neutralization with a high-salt buffer such as potassium acetate in the presence of SDS has two effects that contribute to the overall effectiveness of the method. First, rapid neutralization causes the chromosomal DNA to base-pair in an intrastrand manner, forming an insoluble aggregate that precipitates out of solution. The covalently closed nature of the circular plasmid DNA promotes interstrand rehybridization, allowing the plasmid to remain in solution. Second, the potassium salt of SDS is insoluble, so the protein and detergent precipitate and aggregate, which assists in the entrapment of the high-molecular-weight chromosomal DNA. Separation of soluble and insoluble material is accomplished by a clearing method (e.g., filtration, magnetic clearing or centrifugation). The soluble plasmid DNA is ready to be further purified.

There are several methods available to purify plasmid DNA from cleared lysate. These include:

- binding plasmid to silica in the presence of high concentrations of chaotropic salts (Chen and Thomas, 1980; Marko *et al.* 1982; Boom *et al.* 1990)
- differential precipitation of plasmid DNA from aqueous chaotropic salt/ethanol solutions (Hamaguchi and Geiduschek, 1962; Wilcockson, 1973; Wilcockson, 1975)
- ion exchange chromatography over DEAE-modified cellulose membranes (van Huynh *et al.* 1993)
- precipitation with polyethylene glycol (Lis, 1980; Paithankar and Prasad, 1991)
- organic extraction using phenol (Wang and Rossman, 1994)

Promega products like the Wizard® Plus SV Minipreps DNA Purification System and the PureYield™ Plasmid Systems combine the benefits of alkaline lysis with the rapid and easy purification by silica. This is done by using a silica-based membrane in a column format to bind the plasmid DNA contained in the cleared alkaline lysates. Purification is based on selective adsorption of DNA to the silica membrane in the presence of high concentrations of chaotropic salts, washes to efficiently remove contaminants, and elution of the DNA with low-salt solutions such as TE buffer or water. See *Promega Notes 82* (www.promega.com/pnotes/82/10203_10/10203_10.html) for additional discussion of the SV membrane.

Ideal for use with automated platforms, the silica-coated MagneSil® PMP systems are also easily scalable for larger volumes or multiwell format. For plasmid miniprep purification, the MagneSil® PMPs are used for both lysate clearing and DNA binding, eliminating the need for centrifugation or vacuum filtration, as the binding of nucleic acids occurs in solution. The particles are also completely resuspended during the wash steps of a purification protocol, enhancing the removal of impurities from the DNA. The Wizard® MagneSil® Plasmid DNA Purification System uses these PMPs for the purification of plasmid DNA in a 96-well format. This plasmid purification system can be used on automated workstations such as the Beckman Coulter Biomek® FX or the Tecan Genesis® RSP. See our web site for further information on compatibility of Promega DNA isolation products with various liquid-handling platforms at the Automated Methods (www.promega.com/automethods/) web page.

Purified plasmid DNA is used in many applications from preparing vectors for cloning to generating templates for transcription or coupled transcription/translation reactions. The silica-based purification systems from Promega minimize the amount of salts and other impurities carried over during isolation, which can negatively affect downstream applications, lower yield or prevent enzyme systems from synthesizing the product of interest.

Additional Resources for Plasmid DNA Purification

Promega Publications

BR129	DNA Analysis Notebook (www.promega.com/guides/dna_guide/default.htm)
BR152	Subcloning Notebook (www.promega.com/guides/subcloning_guide/)

D. Overview of Genomic DNA Isolation

Promega provides several systems designed to isolate genomic DNA from a variety of sources. One method, the solution-based Wizard® Genomic DNA Purification Kit, is the most versatile system available from Promega. This purification system relies on a series of precipitation steps to purify high-molecular-weight DNA from a prepared lysate. It is an excellent choice when a pure population of dsDNA molecules is required for downstream applications such as Southern blotting, real-time PCR and restriction digestion. Alternatively, Promega offers genomic DNA isolation systems based on sample lysis by detergents and purification by silica (see Basis for Purification by Silica and Overview of Plasmid DNA Purification for more details). These include both membrane-based systems (e.g., the single-column Wizard® SV Genomic DNA Purification Kit or the high-throughput, 96-well Wizard® SV 96 Genomic DNA Purification System) and the easily automated paramagnetic silica systems (e.g., MagneSil® Genomic, Large Volume System or the MagneSil® Blood Genomic,

Max Yield System). All of these systems purify genomic DNA that is amenable for use in many downstream applications.

Although techniques like Southern blotting, which require microgram amounts of DNA, are still performed in molecular biology laboratories, most assessment of chromosomal DNA is done by PCR technology including monoplex or multiplex PCR, SNP analysis and real-time PCR. These latter techniques use nanogram amounts of DNA per reaction. Regardless of the system chosen, Promega genomic DNA purification kits not only yield DNA suitable for a wide range of DNA quantity specifications but provide the required amount of high-quality DNA with minimal contaminants.

Additional Resources for Genomic DNA Purification

Promega Publications

BR129	DNA Analysis Notebook (www.promega.com/guides/dna_guide/default.htm)
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E. Overview of DNA Fragment Purification from Agarose Gels and PCR Amplifications

Applications such as cloning, labeling and sequencing DNA frequently require the purification of DNA fragments from agarose gels or amplification reactions. Promega provides multiple systems for DNA fragment purification, including two based on silica membrane technology (Wizard® SV Gel and PCR Clean-Up System and Wizard® SV 96 PCR Clean-Up System) and one based on MagneSil® PMPs (Wizard® MagneSil® Sequencing Reaction Clean-Up System).

The Wizard® SV Gel and PCR Clean-Up System provides a reliable method to purify double-stranded, PCR-amplified DNA either directly from the reaction or from agarose. The quick protocol is simple to perform, and the PCR products are purified from contaminants, including primer dimers, PCR additives and amplification primers. To purify PCR product from nonspecific amplification products, the reaction products can be separated in an agarose gel prior to purification. The agarose is dissolved by chaotropic buffer, freeing the DNA for binding to the silica SV membrane. After removal of contaminants by alcohol-based washes, the DNA bound to the SV column is eluted in water or TE buffer, free of salt or macromolecular contaminants. The Wizard® SV Gel and PCR Clean-Up System can also be used to purify DNA from enzymatic reactions such as restriction digestion and alkaline phosphatase treatment.

Additional Resources for DNA Fragment Purification from Agarose Gels and PCR Reactions

Promega Publications

BR129	DNA Analysis Notebook (www.promega.com/guides/dna_guide/default.htm)
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BR152 Subcloning Notebook
www.promega.com/guides/subcloning_guide/

F. Overview of Personal Automation™ Systems for Purification

Automation is increasingly used to improve productivity for research, diagnostics and applied testing. Traditionally, automation refers to the use of large, specialized and costly equipment that requires extensive training to operate and maintain. Promega has developed Personal Automation™ with the Maxwell® 16 System which provides a flexible, reliable, compact and easy-to-use alternative to traditional automated systems.

The Maxwell® 16 System combines instrumentation, automated methods, prefilled reagent cartridges, service and support, providing everything needed for purification from a single source. The Maxwell® 16 System is designed for low- to moderate-throughput automated purification of 1–16 small samples. Currently, there are predispensed reagent cartridges in kits for genomic DNA purification, total RNA purification and recombinant protein purification. These multiple cartridges make the Maxwell® 16 Instrument flexible for laboratories that may use one or all of these different systems. For genomic DNA purification, add blood, mouse tail, tissue (fresh or preprocessed formalin-fixed, paraffin-embedded [FFPE]), or bacteria samples directly to the prefilled reagent cartridge and press “Start”. You avoid the time and hands-on labor of Proteinase K or other preprocessing aside from FFPE, and the purified genomic DNA sample is ready in about 30 minutes. The eluted DNA can be used in PCR and other applications. RNA purification follows a similar process, involving preparation of a DNA-free lysate followed by RNA purification. The eluted RNA can then be used in qRT-PCR and other applications. Recombinant polyhistidine- or HQ-tagged proteins can be purified from multiple sample types, including bacteria, mammalian cells, insect cells and culture medium. Purified protein is compatible with many common downstream applications including polyacrylamide gel electrophoresis and detection, functionality studies, Western blot analysis and mass spectrometry.

There are two versions of the Maxwell® 16 Instrument and kits to accompany these choices. The Maxwell® 16 Instrument (Cat.# AS2000) plus the Maxwell® 16 SEV (standard-elution volume) Hardware Kit (Cat.# AS1200) elutes macromolecules (DNA, RNA and protein) in 300µl of elution buffer. Maxwell® 16 Instrument (Cat.# AS2000) with the Maxwell® 16 LEV (low-elution volume) Hardware Kit (Cat.# AS1250) can elute the purified product in 30–100µl of elution buffer. The lower elution volume is advantageous for some applications that benefit from concentrated DNA or RNA. If you have one version of the Maxwell® 16 Instrument (i.e., LEV), purchasing the other hardware kit (i.e., SEV) will allow you to convert your instrument to use the SEV purification kits.

The Maxwell® 16 Flexi Method Firmware makes the system even more versatile by allowing you to create custom methods when isolating DNA, RNA or recombinant protein with the Maxwell® 16 Instrument. You can optimize purification from challenging sample types or unique applications. The Maxwell® 16 Flexi Method Firmware allows you to set sample processing times for lysis, binding, drying and elution. In addition, the number of passes can be customized for particular samples. The firmware can be installed on a new instrument or can be purchased separately to install on an existing Maxwell® 16 Instrument (Cat.# AS1000 or AS2000).

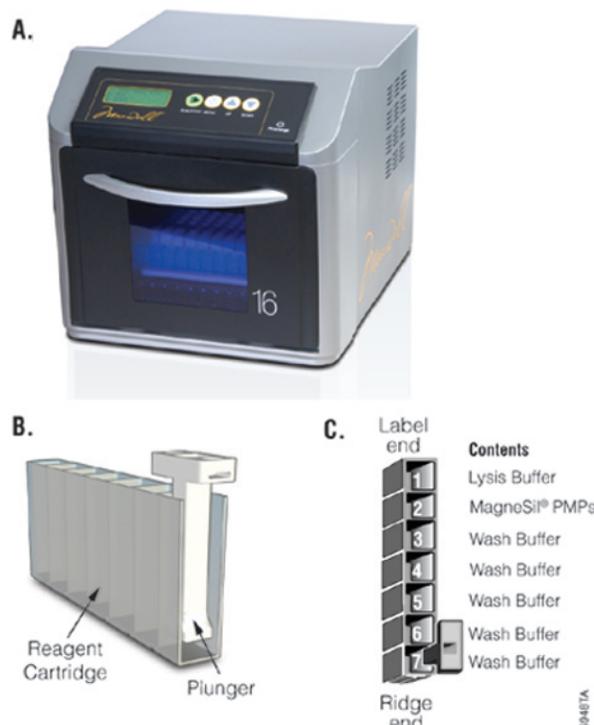


Figure 9.2. The Maxwell® 16 Instrument is used with optimized reagents predispensed into disposable cartridges. **Panel A.** The Maxwell® 16 Instrument. **Panel B.** A Maxwell® 16 reagent cartridge, cut away to show the plunger. The unique design of the cartridge allows direct processing of a variety of liquid and solid sample types with no need for preprocessing. **Panel C.** Top view of the Maxwell® 16 DNA Purification Cartridge.

G. Methods for Determining DNA Yield and Purity

DNA yield can be assessed using four different methods: absorbance (optical density), agarose gel electrophoresis, fluorescent DNA-binding dyes and a luciferase-pyrophosphorylation-coupled quantitation system. Each technique is described and includes information on necessary accessories (e.g., equipment). While all methods are useful, each has caveats to consider when choosing a quantitation system.

The most common technique to determine DNA yield and purity is also the easiest method—absorbance. All that is needed for measurement is a spectrophotometer equipped

with a UV lamp, UV-transparent cuvettes (depending on the instrument) and a solution of purified DNA. Absorbance readings are performed at 260nm (A_{260}) where DNA absorbs light most strongly, and the number generated allows one to estimate the concentration of the solution (see Estimation of DNA Concentration, Yield and Purity by Absorbance for more details). To ensure the numbers are useful, the A_{260} reading should be between 0.1–1.0.

However, DNA is not the only molecule that can absorb UV light at 260nm. Since RNA also has a great absorbance at 260nm, and the aromatic amino acids present in protein absorb at 280nm, both contaminants, if present in the DNA solution, will contribute to the total measurement at 260nm. Additionally, the presence of guanidine will lead to higher 260nm absorbance. This means that if the A_{260} number is used for calculation of yield, the DNA quantity may be overestimated (Adams, 2003).

To evaluate DNA purity by spectrophotometry, measure absorbance from 230nm to 320nm in order to detect other possible contaminants present in the DNA solution [detailed in the *MagneSil® Genomic, Large Volume System Technical Bulletin* (www.promega.com/tbs/tb549/tb549.html)]. The most common purity calculation is determining the ratio of the absorbance at 260nm divided by the reading at 280nm. Good-quality DNA will have an A_{260}/A_{280} ratio of 1.7–2.0. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate more contaminants are present. However, the best test of DNA quality is functionality in the application of interest (e.g., real-time PCR).

Strong absorbance around 230nm can indicate that organic compounds or chaotropic salts are present in the purified DNA. A ratio of 260nm to 230nm can help evaluate the level of salt carryover in the purified DNA. The lower the ratio, the greater the amount of thiocyanate salt is present, for example. As a guideline, the A_{260}/A_{230} is best if greater than 1.5. A reading at 320nm will indicate if there is turbidity in the solution, another indication of possible contamination. Therefore, taking a spectrum of readings from 230nm to 320nm is most informative.

Agarose gel electrophoresis of the purified DNA eliminates the issues associated with absorbance readings. To use this method, a horizontal gel electrophoresis tank with an external power supply, analytical-grade agarose, an appropriate running buffer (e.g., 1X TAE) and an intercalating DNA dye along with appropriately sized DNA standards are needed for quantitation. A sample of the isolated DNA is loaded into a well of the agarose gel and then exposed to an electric field. The negatively charged DNA backbone migrates toward the anode. Since small DNA fragments migrate faster, the DNA is separated by size. The percentage of agarose in the gel will determine what size range of DNA will be resolved with the greatest clarity (Sambrook *et al.* 1989). Any RNA, nucleotides and

protein in the sample migrate at different rates compared to the DNA so the band(s) containing the DNA will be distinct.

Concentration and yield can be determined after gel electrophoresis is completed by comparing the sample DNA intensity to that of a DNA quantitation standard. For example, if a 2 μ l sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100ng standard, then the solution concentration is 50ng/ μ l (100ng divided by 2 μ l). Standards used for quantitation should be labeled as such and be the same size as the sample DNA being analyzed. In order to visualize the DNA in the agarose gel, staining with an intercalating dye such as ethidium bromide or SYBR® Green is required. Because ethidium bromide is a known mutagen, precautions need to be taken for its proper use and disposal (Adams, 2003).

DNA-binding dyes compare the unknown sample to a standard curve of DNA, but genomic, fragment and plasmid DNA will each require their own standard curves and cannot be used interchangeably. If the DNA sample has been diluted, you will need to account for the dilution factor when calculating final concentration. Hoechst bisbenzimidazole dyes or PicoGreen® selectively bind double-stranded DNA (dsDNA). To use this method, a fluorometer to detect the dyes, dilution of the DNA solution and appropriate DNA standards are required. However, there are size qualifications: the DNA needs to be at least 1 kilobase in length for Hoechst and at least 200bp for PicoGreen® for successful quantitation. The range of measurement is 10–250ng/ml for Hoechst, 25pg/ml–1 μ g/ml for PicoGreen®, and the dyes are sensitive to GC content. In addition, the usual caveats for handling fluorescent compounds apply—photobleaching and quenching will affect the signal. While the dyes bind preferentially to dsDNA, RNA and nucleotides may contribute to the signal. [Adams, 2003; *The Handbook — A Guide to Fluorescent Probes*

(<http://probes.invitrogen.com/handbook/sections/0803.html>) and Selection Guide Quant-iT™ Nucleic Acid Quantitation Assays

(<http://probes.invitrogen.com/media/publications/511.pdf>) accessed October 16, 2008].

The fourth method is a Promega product called the DNA Quantitation System (Cat.# K4000). To measure DNA mass, a luminometer is required for light detection. When using the DNA Quantitation System, a light signal is produced in proportion to the amount of linear dsDNA present in a sample. The number of Relative Light Units (RLU) produced is compared to a standard curve each time a sample or samples are measured, giving the mass of DNA as the final calculation. The detection/quantitation of DNA using the DNA Quantitation System requires: 1) the activity of coupled enzymatic reactions to produce an amount of ATP proportional to the amount of DNA present followed by; 2) the generation of a light signal proportional to the amount of ATP using the ENLITEN® L/L Reagent. The first set of coupled reactions consists of a pyrophosphorylation

and transphosphorylation (pyro/transphosphorylation) reaction. The pyrophosphorylation reaction is the reverse of the DNA polymerization reaction (see Deutscher and Kornberg, 1969) and is catalyzed by T4 DNA Polymerase. In the presence of pyrophosphate and dsDNA, deoxynucleotide triphosphates (dNTPs) are released from the 3' termini of the DNA strands. The transphosphorylation reaction is catalyzed by the enzyme Nucleoside Diphosphate Kinase (NDPK). In this reaction, the terminal phosphate of the dNTP is transferred to ADP to form ATP. Thus, the ATP formed is proportional to the amount of dsDNA added to the reaction. In order to generate light, the ENLITEN® L/L Reagent requires ATP. Thus, the amount of ATP determines the brightness of the light signal, which in turn indicates how much DNA was present in the sample.

While the DNA Quantitation System method is sensitive (i.e., able to detect picogram amounts of DNA) and more accurate than spectrophotometry and agarose gel analyses, the DNA analyzed can only be linear and double-stranded with fragments in the range of 20–6,000bp. Therefore, chromosomal DNA will need to be digested by restriction enzymes prior to quantitation. The total amount of DNA used for analysis must be between 10–500pg/μl and no more than 2μl DNA sample used for detection. If the DNA concentration is greater, the sample must be diluted. The linear range of the DNA Quantitation System is 20pg to 1ng. Single-stranded DNA, if present, does not generate a signal unless a dimer or hairpin structure is formed. Since the assay is based on the amount of ATP present, care should be taken with the DNA samples. They should not contain dNTPs or NTPs, which can help form ATP, nor ATPase activity, which can decrease the light signal and cause a false low reading. Unlike absorbance methods, the DNA Quantitation System is not affected by protein contamination.

Choosing which quantitation method to use is based on many factors including access to equipment or reagents, reliability and consistency of the concentration calculations. Use caution when comparing yields between methods as the level of potential contaminants may cause variable determinations among the different methods.

H. Estimation of DNA Concentration, Yield and Purity by Absorbance

DNA concentration can be estimated by adjusting the A_{260} measurement for turbidity (measured by absorbance at A_{320}), multiplying by the dilution factor, and using the relationship that an A_{260} of 1.0 = 50μg/ml pure DNA.

Concentration (μg/ml) = (A_{260} reading – A_{320} reading) × dilution factor × 50μg/ml

Total yield is obtained by multiplying the DNA concentration by the final total purified sample volume.

DNA Yield (μg) = DNA Concentration × Total Sample Volume (ml)

A_{260}/A_{280} ratio can be used as an estimate of DNA purity [with a number of important limitations (Wilfinger, Mackey and Chanczynski, 1997; Glasel, 1997; Manchester, 1995)]. An A_{260}/A_{280} ratio between 1.7 and 2.0 is generally accepted as representative of a high-quality DNA sample. The ratio can be calculated after subtracting the non-nucleic acid absorbance at A_{320} .

DNA Purity (A_{260}/A_{280}) = (A_{260} reading – A_{320} reading) ÷ (A_{280} reading – A_{320} reading)

Note that the spectrophotometer is most accurate when measurements are between 0.1–1.0.

II. General Considerations for Plasmid DNA Purification

A. Bacterial Growth and Culture Conditions

Successful isolation of quality plasmid DNA begins with culture preparation. A number of factors can influence the growth of bacterial cells. Bacterial growth in liquid culture occurs in three phases: 1) a short lag phase in which the bacteria become acclimated to the media and begin to divide; 2) a log phase, characterized by exponential growth in which most strains of *E. coli* will divide every 20–30 minutes; and 3) a stationary phase in which growth slows and eventually stops in response to the lack of nutrients in the medium. No net increase in biomass will occur in the stationary phase, but plasmid replication will continue for several hours after reaching stationary phase. Most strains of *E. coli* will reach a concentration of $1.0\text{--}4.0 \times 10^9$ cells/ml of culture at this stage, depending on culture media and aeration conditions. Depending on inoculation size and the size of the culture, stationary phase will be reached in 6–8 hours.

Aeration and temperature are of critical importance. The culture volume should be less than or equal to 1/4 the volume of the container (e.g., 250ml medium in a 1 liter flask); using 1/10 the container volume (e.g., 100ml medium in a 1,000ml flask) produces optimal results. The culture tube or flask should be placed in an orbital shaker (approximately 250rpm) to ensure adequate aeration (Ausubel *et al.* 1989). Since most strains of *E. coli* grow best at 37°C, this incubation temperature is recommended unless the strain of interest requires different conditions for optimal growth.

Different culture media will also have a profound effect on the growth of different bacterial strains. Promega plasmid DNA purification systems are appropriate for bacterial cultures grown in 1X Luria-Bertani (LB) medium. However, use of LB-Miller medium containing more NaCl will produce significantly greater yields and is highly recommended. Richer media such as 2X YT, CIRCLEGROW® or Terrific Broth may be used to increase plasmid yields by increasing the biomass for a given volume of culture. Keep the biomass in a range acceptable for the plasmid isolation system used, as overloading may result in poor purity and yield of the plasmid DNA (see Biomass Processed for more information).

Culture incubation time affects both the yield and quality of plasmid DNA isolated. Bacterial cultures grown to insufficient density will yield relatively low amounts of DNA. Overgrown cultures may result in suboptimal yields and excessive chromosomal DNA contamination due to autolysis of bacterial cells after they have reached stationary phase. We do not recommend the use of cultures grown longer than 18–20 hours.

B. Antibiotic Selection

Most plasmids carry a marker gene for a specific antibiotic resistance. By supplementing the growth medium with the antibiotic of choice, only cells containing the plasmid of interest will propagate. Adding antibiotic to the required concentration will help to maximize plasmid yields. Note that adding too much antibiotic can inhibit growth and too little may cause a mixed population of bacteria to grow—both with and without the plasmid of interest. For more information on optimal antibiotic ranges to use in culture as well as the mechanisms of antibiotic action and resistance, see Table 9.1 and the review reference Davies and Smith, 1978.

C. Recommended Inoculation Procedures

1–100ml of Culture

Pick an isolated colony from a freshly streaked plate (less than 5 days old) and inoculate LB medium containing the required antibiotic(s). Incubation with shaking for 8–16 hours at 37°C before harvesting generally results in maximum yields of a high-copy-number plasmid. To achieve a highly reproducible yield, determine the cell density used in a typical experiment, and grow cultures to this density in each subsequent experiment. Typically, after overnight incubation, the absorbance of a tenfold dilution of the culture at a wavelength of 600nm (A_{600}) with a 1cm path length should range from 0.10–0.35.

100–1,000ml of Culture

Using a colony from a freshly streaked plate (less than 5 days old), inoculate 5–50ml of LB medium containing the required antibiotic(s). Grow this starter culture from 8 hours to overnight at 37°C. The following day, use this culture to inoculate the larger culture flask containing antibiotic-supplemented medium by diluting the starter culture between 100- to 500-fold (e.g., adding 10ml overnight culture to 1 liter medium). Incubate this

Table 9.1. Antibiotic Mode of Action and Mechanism of Resistance.

Antibiotic	Mode of Action	Mechanism of Resistance	Working Concentration	Stock Solution
Ampicillin (Amp)	A derivative of penicillin that kills growing cells by interfering with bacterial cell wall synthesis.	The resistance gene (<i>bla</i>) specifies a periplasmic enzyme, β -lactamase, which cleaves the β -lactam ring of the antibiotic.	50–125 μ g/ml	50mg/ml in water
Chloramphenicol (Cm)	A bacteriostatic agent that interferes with bacterial protein synthesis by binding to the 50S subunit of ribosomes and preventing peptide bond formation.	The resistance gene (<i>cat</i>) specifies an acetyltransferase that acetylates, and thereby inactivates, the antibiotic.	20–170 μ g/ml	34mg/ml in ethanol
Hygromycin (Hygro)	A protein synthesis inhibitor that interferes with 80S ribosome translocation and causes mistranslation.	The resistance gene (<i>hph</i>) specifies a phosphotransferase that catalyzes the phosphorylation of the 4-hydroxyl group on the cyclitol ring (hyosamine), thereby producing 7'-O-phosphoryl-hygromycin B, which lacks biological activity both in vivo and in vitro.	20–200 μ g/ml	100mg/ml in water
Kanamycin (Kan)	A bactericidal agent that binds to 70S ribosomes and causes misreading of messenger RNA.	The resistance gene (<i>kan</i>) specifies an enzyme (aminoglycoside phosphotransferase) that modifies the antibiotic and prevents its interaction with ribosomes.	30 μ g/ml	50mg/ml in water
Neomycin (Neo)	A bactericidal agent that blocks protein synthesis by binding to the prokaryotic 70S ribosomal subunit.	Expression of the bacterial APH (aminoglycoside phosphotransferase) gene (derived from Tn5).	50 μ g/ml	25mg/ml in water
Tetracycline (Tet)	A light-sensitive bacteriostatic agent that prevents bacterial protein synthesis by binding to the 30S subunit of ribosomes.	The resistance gene (<i>tet</i>) specifies a protein that modifies the bacterial membrane and prevents transport of the antibiotic into the cell.	10 μ g/ml in liquid culture; 12.5 μ g/ml in plates	12.5mg/ml in ethanol

secondary culture for 12–16 hours before harvesting cells. The A_{600} of a tenfold dilution of the culture should be 0.10–0.35. As with smaller cultures, to achieve a highly reproducible yield, determine the cell density used in a typical experiment and grow cultures to this density in each subsequent experiment.

Harvesting

When harvesting bacteria, follow the conditions outlined in either the Wizard® Plus SV Miniprep DNA Purification System (www.promega.com/tbs/tb225/tb225.html) or the PureYield™ Plasmid Midiprep System (www.promega.com/tbs/tm253/tm253.html) protocol. If the recommended centrifugation time or speed is exceeded, the pelleted cells may be more difficult to resuspend. Insufficient centrifugation time or speed may result in incomplete harvesting of cells and loss of starting material. Consult a centrifuge instruction manual for conversion of rpm to g-force. Once the bacteria are pelleted, this is a good stopping point in the purification process. Storing the pellet at –20°C results in little loss of plasmid DNA and may enhance lysis.

III. Factors That Affect Plasmid DNA Quality and Yield

A. Bacterial Strain Selection

The choice of host bacterial strain can have a significant impact on the quality and yield of DNA using any purification method. We recommend the use of host strains such as DH5α™, JM109 (Cat.# L2001, L1001) and XL1-Blue, which contain mutations in the *endA* gene. *E. coli* strains that are listed as *endA1* contain such mutations.

The *endA* gene encodes a 12kDa periplasmic protein called endonuclease I. This enzyme is a double-stranded DNase that can copurify with plasmid DNA, thus causing potential degradation. RNA acts as a competitive inhibitor and alters the endonuclease specificity from that of a double-stranded nucleolytic enzyme yielding seven-base oligonucleotides to a nickase that cleaves an average of one time per substrate (Lehman *et al.* 1962; Goebel and Helinski 1970). The function of endonuclease I is not fully understood, and strains bearing *endA1* mutations have no obvious phenotype other than improved stability and yield of plasmid obtained from them.

The expression of endonuclease I has been characterized and was found to be dependent on bacterial growth phase (Shortman and Lehman, 1964). In this study, endonuclease I levels were found to be more than 300 times higher during exponential phase compared to stationary phase. In addition, media compositions that encouraged rapid growth (e.g., high glucose levels and addition of amino acids) resulted in high endonuclease I levels.

Strains that contain the wildtype endonuclease A (*endA*) gene can yield high-quality, undegraded plasmid DNA if special precautions are used to reduce the probability of nuclease contamination and plasmid degradation (Shortman and Lehman, 1964). Promega has performed a thorough investigation of methods at different points in

the purification process to ensure the isolation of high-quality DNA from EndA+ (wildtype) bacterial strains. These include: 1) inclusion of an alkaline protease treatment step that degrades nucleases in the Wizard® Plus SV Minipreps DNA Purification System; 2) optimization of culture conditions to limit in vivo expression during bacterial growth; 3) heat inactivation during and after purification; 4) optimization of protocol conditions to limit binding of the nuclease to the resin and 5) post-purification methods to remove endonuclease. These methods and results are summarized in Schoenfeld *et al.* 1995 and the Wizard® Plus SV Plasmid DNA Purification System Technical Bulletin (www.promega.com/tbs/tb225/tb225.html). Information on genetic markers in bacterial strains can also be found in Ausubel *et al.* 1989 and Sambrook *et al.* 1989.

B. Plasmid Copy Number

One of the most critical factors affecting the yield of plasmid from a given system is the copy number of the plasmid. Copy number is determined primarily by the region of DNA surrounding and including the origin of replication in the plasmid. This area, known as the replicon, controls replication of plasmid DNA by bacterial enzyme complexes. Plasmids derived from pBR322 (Cat.# D1511) contain the ColE1 origin of replication from pMB1. This origin of replication is tightly controlled, resulting in approximately 25 copies of the plasmid per bacterial cell (low copy number). Plasmids derived from pUC contain a mutated version of the ColE1 origin of replication, which results in reduced replication control and approximately 200–700 plasmid copies per cell (high copy number).

Some plasmids contain the p15A origin of replication (e.g., pALTER®-Ex2 Vector), which is considered a low-copy-number origin. The presence of the p15A origin of replication allows for replication of that particular plasmid in conjunction with a plasmid containing the ColE1 origin of replication. A compatibility group is defined as a set of plasmids whose members are unable to coexist in the same bacterial cell. They are incompatible because they cannot be distinguished from one another by the bacterial cell at a stage that is essential for plasmid maintenance. The introduction of a new origin, in the form of a second plasmid of the same compatibility group, mimics the result of replication of the resident plasmid. Thus, any further replication is prevented until after the two plasmids have been segregated to different cells to create the correct prereplication copy number (Lewin, 2004).

Most plasmids provided by Promega, including the pGEM® Vectors, are considered to be high-copy-number. The only exceptions are the pALTER® series of mutagenesis vectors.

Some DNA sequences, when inserted into a particular vector, can lower the copy number of the plasmid. Furthermore, large DNA inserts can also reduce plasmid copy number. In many cases, the exact copy number of a particular construct will not be known. However, many of these plasmids are derived from a small number of commonly used parent constructs.

C. Appropriate Sample Size and Throughput

Depending on the volume of the bacterial culture, there are different isolation systems for your needs. For small-volume bacterial cultures of 0.6–3ml, use a system like the PureYield™ Plasmid Miniprep System, which gives a plasmid DNA yield of 1.5–7.5µg with an $A_{260}/A_{280} \geq 1.8$ from a 0.6ml overnight bacterial culture with a total biomass (O.D.₆₀₀ of culture × volume of culture in µl) of 1.3–8. For larger cultures with volumes ranging from 50–100ml, the PureYield™ Plasmid Midiprep System is a good choice. With this system, a 50ml culture of a high-copy-number plasmid with a total biomass of 100–200 O.D.₆₀₀ units will yield 100–200µg of plasmid. The PureYield™ Plasmid Maxiprep System can isolate plasmid from 100–250ml of culture with yields up to 1mg of plasmid DNA with an $A_{260}/A_{280} > 1.7$ from 250ml of overnight bacterial culture, transformed with a high-copy-number plasmid.

For high-throughput processing, systems based on a 96-well format can be performed manually with a vacuum manifold (e.g., Vac-Man® 96 Vacuum Manifold; Figure 9.3) using silica membrane technology such as the Wizard® SV 96 Plasmid DNA Purification System. Alternatively, an automated liquid-handling workstation can process multiwell plates with MagneSil® PMPs and a 96-well magnet (e.g., MagnaBot® 96 Magnetic Separation Device; Figure 9.4) using the Wizard® MagneSil® Plasmid Purification System. Yields for these systems using high-copy-number plasmid range from 3–5µg for the Wizard® SV 96 Plasmid DNA Purification System and up to 6µg for the Wizard® MagneSil® Plasmid Purification System. For more information on plasmid DNA automation, go to the Automated Methods (www.promega.com/automethods/) web site.



Figure 9.3. The Vac-Man® 96 Vacuum Manifold. This 96-well vacuum manifold is used for processing SV 96 plates for plasmid, genomic and PCR product purification.



Figure 9.4. The MagnaBot® 96 Magnetic Separation Device. This 96-well magnet is used for capturing MagneSil® PMPs for DNA purification.

Smaller plasmid amounts are helpful for assessing the success of a cloning experiment by PCR or restriction digestion or for use in a coupled transcription/translation system like the TNT® Coupled Reticulocyte Lysate Systems (Cat.# L1170, L2080).

D. Biomass Processed

Optical density (O.D.) is the measure of how much light is blocked by the biomass of the bacterial culture in a path length of 1cm. The density of the culture is measured at a wavelength of 600nm and can have a great effect on plasmid isolation success. For example, the Wizard® SV 96 Plasmid Purification System has a maximum biomass recommendation of 4.0 O.D.₆₀₀ to avoid clogging of the SV 96 Lysate Clearing Plate, so calculating the O.D. of the culture is necessary.

O.D./ml culture = 600nm absorbance reading × dilution factor

For O.D. measurement, a 1:10 dilution is typically used (e.g., 0.1ml culture in 0.9ml culture media) to keep the reading in the range of 0.1–1.0, where the spectrophotometer is most accurate. For the example above, if the 1:10 dilution reading is 0.15, meaning that each milliliter of culture is 1.5 O.D., no more than 2.67ml culture can be processed (4 O.D. divided by 1.5 O.D./ml = 2.67ml). Exceeding the recommendations of the plasmid purification system may cause clogging or contamination of the system.

E. Plasmid Purification Method and Transfection

Many plasmid isolation systems indicate they are transfection-quality (e.g., the PureYield™ Plasmid Systems or the Wizard MagneSil Tfx™ System). This may be important, as some cultured cells are sensitive to the amount of endotoxin and other contaminants present in the plasmid preparation. Endotoxin is a lipopolysaccharide cell wall component of the outer membrane of Gram-negative bacteria (i.e., all *E. coli* strains) that can copurify with the plasmid DNA regardless of the purification system used. The amount of this molecule varies by bacterial strain, growth conditions and isolation method. In the PureYield™ Plasmid Systems, there is an Endotoxin Removal Wash solution that reduces the amount of endotoxin, proteins and other contaminants eluted with the plasmid DNA. For many common cell lines like 293

and HeLa, the amount of endotoxin present for routine transfections has a minimal effect on the efficiency of transfection (Butash *et al.* 2000).

Many factors influence transfection efficiency and/or cellular death including the type and amount of transfection reagent, cell confluency, DNA amount and incubation time with the reagent:DNA complex. Each of these factors will need to be optimized for each cell line-plasmid combination transfected in order to minimize cell death and maximize transfection efficiency. In our experience, transfection experiments with HeLa and NIH/3T3 cells demonstrated that there was little DNA preparation difference with four different plasmid isolation systems used (based on silica membrane, anion exchange and silica resin) when comparing efficiencies using the same transfection reagent. However, the transfection reagent used for DNA uptake had a significant effect on transfection efficiency and cell death. For a starting place for optimization, visit the Transfection Calculator (www.promega.com/techserv/tools/transcalc/). To see if your cell line has been successfully transfected with Promega reagents, go to the Transfection Assistant (www.promega.com/transfectionasst/) for peer-reviewed citations and transfection information.

Additional Resources about Endotoxin

Online Tools

eNotes FAQspeak: What methods exist to remove endotoxin contamination of plasmid DNA? (www.promega.com/enotes/faqspeak/0004/fq0021.htm)

IV. Plasmid DNA Purification Systems

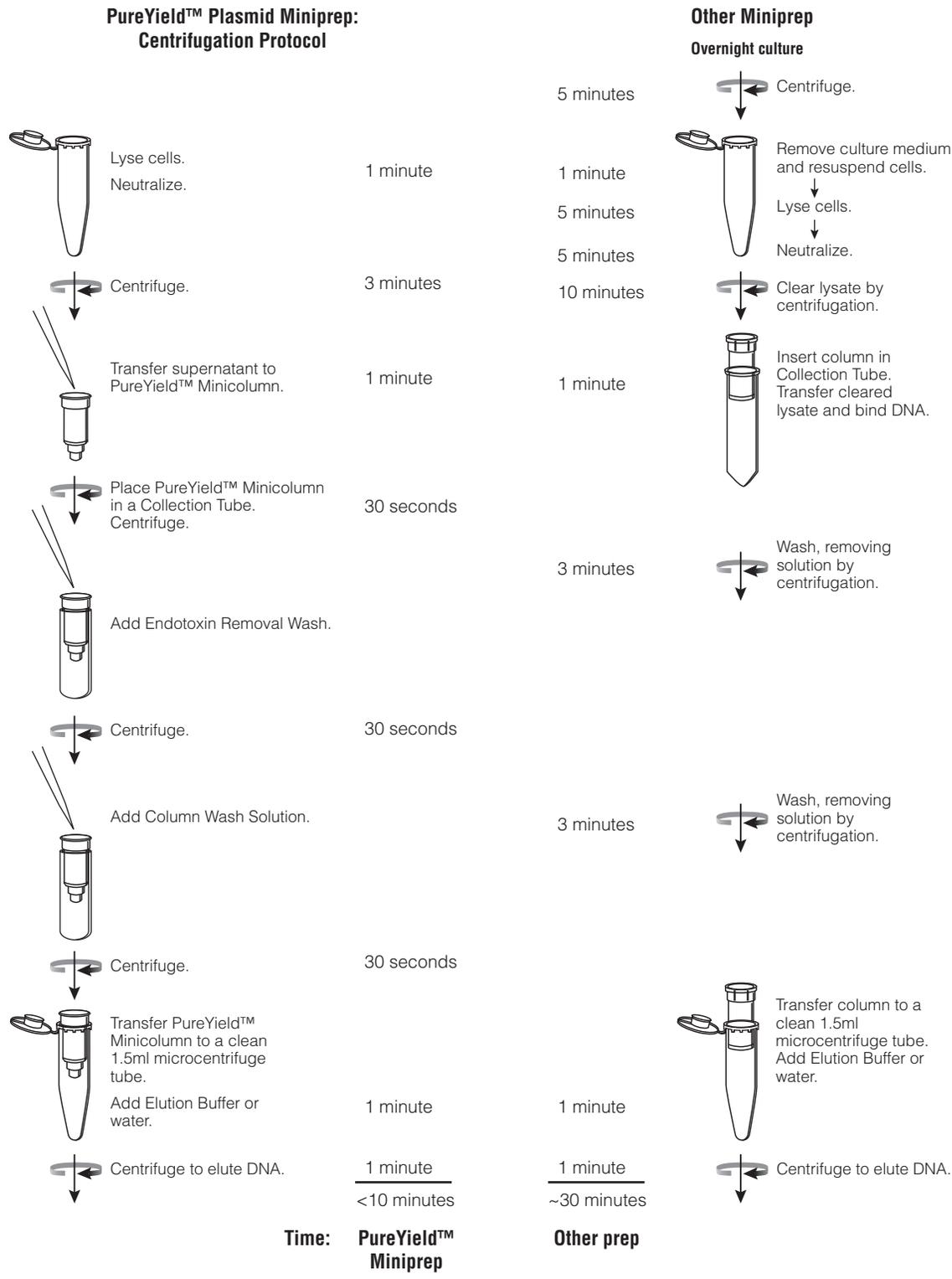
A. PureYield™ Plasmid Miniprep System

The PureYield™ Plasmid Miniprep System (Cat.# A1221, A1222) isolates high-quality plasmid DNA for use in eukaryotic transfection and in vitro expression experiments. The unique reagents, proprietary matrix and silica membrane-based minicolumn design of the PureYield™ Plasmid Miniprep System allow rapid DNA purification directly from bacterial culture in less than 10 minutes with elution volumes as low as 30µl, resulting in more concentrated plasmid DNA. The low elution volume is possible because the column design retains virtually no buffer. While the unique Endotoxin Removal Wash removes protein, RNA and endotoxin contaminants from the bound DNA, the Column Wash Solution followed by membrane drying eliminates salts and alcohols from the plasmid prep, allowing use of the purified plasmid for highly sensitive applications such as transfection, in vitro transcription and coupled in vitro transcription/translation. An additional benefit is that the same degree of purification can be obtained even with low-copy-number plasmids. Although the system works best for plasmids less than 10kb, plasmids as large as 18kb have been purified.

The unique combination of reagents in the PureYield™ Plasmid Miniprep System allows purification of plasmid either directly from bacterial culture or cell pellets of up to 3ml of cell culture (Figure 9.5). A typical overnight culture is grown in LB medium for 16–18 hours, and 0.6ml is used for the direct isolation method. If a larger volume is chosen, cells are harvested by centrifugation, then resuspended in 600µl of TE buffer or water. A transfection comparison of plasmid isolated using the PureYield™ Plasmid Miniprep in various cell lines can be found in Figure 9.6.



9 DNA Purification



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Figure 9.5. The PureYield™ Plasmid Miniprep System yields transfection-quality DNA in approximately 10 minutes.

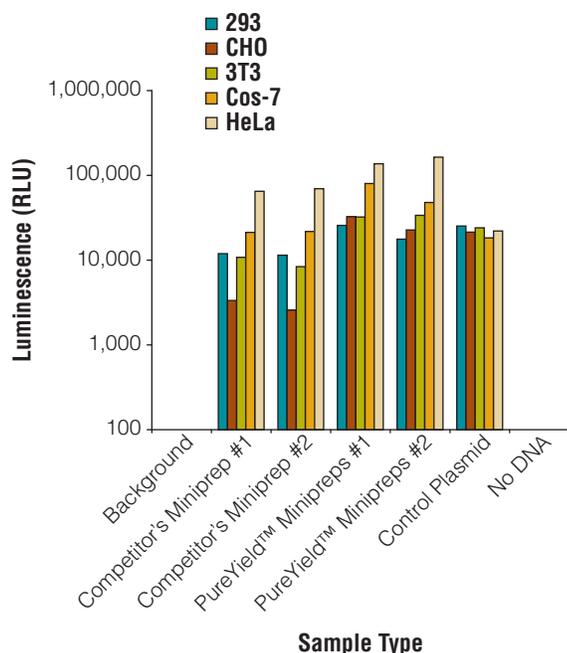


Figure 9.6. Plasmid DNA prepared using the PureYield™ Plasmid Miniprep System consistently works well in transfection experiments. The pGL4.13[*luc2*/SV40] Vector (Cat.# E6681) was prepared using a competing system or the PureYield™ Plasmid Miniprep System. Five different commonly used mammalian cell lines were transfected with the plasmid, and transfection efficiency was assessed by measuring the luciferase activity using the ONE-Glo™ Luciferase Assay System (Cat.# E6110; n = 6).

Additional Resources about the PureYield™ Plasmid Miniprep System

Technical Bulletins and Manuals

- TB374 *PureYield™ Plasmid Miniprep System Technical Bulletin*
(www.promega.com/tbs/tb374/tb374.html)

Promega Publications

- CN021 Transfection-quality plasmid DNA in as little as ten minutes using the PureYield™ Plasmid Miniprep System
(www.promega.com/cnotes/cn021/cn021_03.htm)

Online Tools

PureYield™ Plasmid Miniprep System Video Podcast Protocol
(<http://www.promega.com/multimedia/VPA1221/VPA1221.mov>)

B. PureYield™ Plasmid Midiprep System

As research moves from DNA sequencing to analysis of gene function, the need for rapid methods by which to isolate large quantities of high-quality plasmid DNA has increased. The PureYield™ Plasmid Midiprep System (Cat.# A2492, A2495) is designed to isolate high-quality plasmid DNA for use in eukaryotic transfection and in vitro expression experiments. This midiprep system uses a silica membrane column to purify plasmid DNA in as little as

30 minutes, greatly reducing the time spent on purification compared to silica resin or other membrane column methods.

The PureYield™ Plasmid Midiprep System also incorporates a unique Endotoxin Removal Wash, designed to remove substantial amounts of protein, RNA and endotoxin contaminants from purified plasmid DNA, improving the robustness of sensitive applications such as eukaryotic transfection, in vitro transcription and coupled in vitro transcription/translation. Purification is achieved without isopropanol precipitation of purified plasmid DNA or extensive centrifugation, providing rapid purification as well as a high concentration of pure plasmid DNA from a single method.

The PureYield™ Plasmid Midiprep System is designed to purify 100–200µg of plasmid DNA with an $A_{260}/A_{280} > 1.7$ from a 50ml overnight culture of bacteria, transformed with a high-copy-number plasmid, with a total optical density ($O.D._{600}$ of culture × volume of culture) of 100–200. Larger volumes up to 250ml can be processed, but require greater volumes of solutions than that supplied with the PureYield™ Plasmid Midiprep System.

The PureYield™ Plasmid Midiprep System is designed for purification by vacuum using a manifold such as the Vac-Man® Laboratory Vacuum Manifold (Cat.# A7231), but there are alternative protocols that use all centrifugation or vacuum and centrifugation together. All protocols generate high-quality purified plasmid DNA. A swinging-bucket tabletop centrifuge or the Eluator™ Vacuum Elution Device (Cat.# A1071) is required for the final elution step regardless of the protocol chosen.

Additional Resources for the PureYield™ Plasmid Midiprep System

Technical Bulletins and Manuals

- TM253 *PureYield™ Plasmid Midiprep System Technical Manual*
(www.promega.com/tbs/tm253/tm253.html)

Promega Publications

- PN088 Fast, reliable, high-quality midiprep plasmid purification using the PureYield™ Plasmid Midiprep System
(www.promega.com/pnotes/88/12162_05/12162_05.html)
- eNotes Remove the high-speed spin from PureYield™ Plasmid Preps
(www.promega.com/enotes/applications/ap0101.htm)

Citations

Abdel-Latif, M. *et al.* (2008) An epoxide hydrolase involved in the biosynthesis of an insect sex attractant and its use to localize the production site. *Proc. Natl. Acad. Sci. USA* **105**, 8914–9.

These authors amplified and characterized a putative epoxide hydrolase gene from the jewel wasp *Nasonia vitripennis*. PCR fragments were amplified from genomic DNA, purified from gels using the Wizard® SV Gel and PCR Clean-Up System and then subcloned into the pGEM®-T Easy Vector. The plasmid DNA was purified using the PureYield™ Midiprep System. Linearized plasmids were used for in vitro transcription of RNA for use in RNA interference experiments.

PubMed Number: 18579785

C. PureYield™ Plasmid Maxiprep System

As with the PureYield™ Plasmid Midiprep System, the PureYield™ Plasmid Maxiprep System (Cat.# A2392, A2393) is designed to isolate high-quality plasmid DNA for use in eukaryotic transfection and cell-free expression experiments. The system provides a rapid method for purification using a silica membrane column. Plasmid DNA can be purified in approximately 60 minutes, greatly reducing the time spent on purification compared to silica resin or other membrane column methods.

Like the other PureYield™ Plasmid Systems, the maxiprep system also incorporates a unique Endotoxin Removal Wash, designed to remove substantial amounts of protein, RNA and endotoxin contaminants from purified plasmid DNA, and improve the robustness of sensitive applications such as eukaryotic transfection, in vitro transcription and cell-free expression. Purification is achieved without isopropanol precipitation of purified plasmid DNA, providing rapid purification as well as a high concentration of pure plasmid DNA.

The PureYield™ Plasmid Maxiprep System purifies up to 1mg of plasmid DNA with an $A_{260}/A_{280} > 1.7$ from 250ml of overnight bacterial culture, transformed with a high-copy-number plasmid. The PureYield™ System requires a vacuum pump and manifold (e.g., the Vac-Man® Laboratory Vacuum Manifold, 20-sample [Cat.# A7231]), a centrifuge with a fixed-angle rotor for lysate clearing and either a tabletop centrifuge with a swinging bucket rotor or the Eluator™ Vacuum Elution Device (Cat.# A1071) for the final elution step.

Additional Resources for the PureYield™ Plasmid Maxiprep System

Technical Bulletins and Manuals

TM280 *PureYield™ Plasmid Maxiprep System Technical Manual*
(www.promega.com/tbs/tm280/tm280.html)

Promega Publications

eNotes Remove the high-speed spin from PureYield™ Plasmid Preps
(www.promega.com/enotes/applications/ap0101.htm)

D. Wizard® Plus SV Minipreps DNA Purification System

The Wizard® Plus SV Minipreps DNA Purification System (Cat.# A1330, A1340, A1460, A1470) provides a simple and reliable method for rapid isolation of plasmid DNA using a column-based silica membrane (see Figure 9.7 for overview of method). The entire miniprep procedure can be completed in 30 minutes or less, depending on the number of samples processed. The plasmid DNA can be purified by using either a vacuum manifold like the Vac-Man® Laboratory Vacuum Manifold (process up to 20 samples) or a microcentrifuge (number of samples processed depends on rotor size). This system can be used to isolate any plasmid hosted in *E. coli* but works most efficiently when the plasmid is less than 20,000bp in size. Purified plasmids can be used without further manipulation for automated fluorescent DNA sequencing as well as for other standard molecular biology techniques.

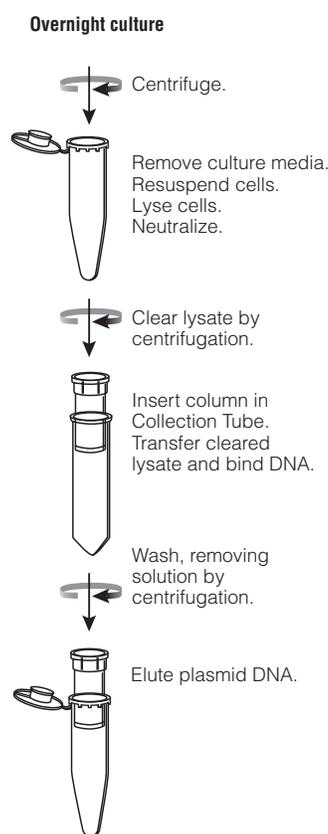


Figure 9.7. Overview of the Wizard® Plus SV Minipreps DNA Purification System centrifugation protocol.

The miniprep protocol in Technical Bulletin #TB225 is for the isolation of plasmid DNA from 1–10ml of overnight *E. coli* culture. The yield of plasmid will vary depending on a number of factors, including the volume of bacterial culture, plasmid copy number, type of culture medium and the bacterial strain used as discussed in Factors that Affect Plasmid DNA Quality and Yield. The DNA binding capacity of the SV membrane is up to 20µg of high-quality

plasmid DNA. An alkaline protease treatment step in the isolation procedure improves plasmid quality by digesting proteins like endonuclease I.

Additional Resources for the Wizard® Plus SV Minipreps DNA Purification System

Technical Bulletins and Manuals

- TB225 *Wizard® Plus SV Minipreps DNA Purification System Technical Bulletin*
(www.promega.com/tbs/tb225/tb225.html)

Promega Publications

- PN087 Yeast plasmid isolation using the Wizard® Plus SV Minipreps DNA Purification System
(www.promega.com/pnotes/87/11527_27/11527_27.html)
- PN059 Wizard® Plus SV Minipreps DNA Purification System: The next generation in miniprep purification
(www.promega.com/pnotes/59/5644b/5644b.html)
- eNotes Isolation of DNA from *Bacillus subtilis* using the Wizard® Plus SV Miniprep DNA Purification System
(www.promega.com/enotes/applications/ap0074.htm)
- eNotes A simple and reproducible method to isolate plasmid DNA from yeast after a two-hybrid screening
(www.promega.com/enotes/applications/ap0012_tabs.htm)

Citations

Cimerman, A. *et al.* (2006) Stolbur phytoplasma genome survey achieved using a suppression subtractive hybridization approach with high specificity. *Appl. Environ. Microbiol.* **72**, 3274–83.

To purify stolbur phytoplasma DNA from total DNA of infected periwinkle plants, two rounds of suppression subtractive hybridization (SSH) were carried out ending in amplification. The resultant PCR products (1µl) were ligated into 50ng of pGEM®-T Easy Vector using 3 units of T4 DNA Ligase. After transforming DH10B cells, ampicillin-resistant colonies were grown and the plasmids purified using the Wizard® Plus SV Miniprep DNA Purification System. The insert lengths were estimated with EcoRI digestion and agarose gel electrophoresis before amplification and labeling with digoxigenin. These probes were used for dot hybridization with denatured healthy or infected plant DNA (10µg) and the corresponding plasmid as a positive control (100ng).

PubMed Number: 16672467

E. Wizard® SV 96 and SV 9600 Plasmid DNA Purification Systems

To process more samples at once, consider the 96-well format of the Wizard® SV 96 (Cat.# A2250, A2255) and

SV 9600 (Cat.# A2258) Plasmid DNA Purification Systems. These high-throughput systems provide a simple and reliable method for the rapid isolation of plasmid DNA using a silica-membrane 96-well plate. A single plate can be processed in 60 minutes or less. The purified plasmid can be used directly for automated fluorescent DNA sequencing as well as for other standard molecular biology techniques, including restriction enzyme digestion and PCR. The Wizard® SV 96 and SV 9600 Systems are designed for use either in a manual format or with automated instruments. Methods to support automated plasmid DNA purification can be found online at: Automated Methods (www.promega.com/automethods/).

In order to use the Wizard® SV 96 and SV 9600 Systems, a vacuum manifold [e.g., Vac-Man® 96 Vacuum Manifold (Cat.# A2291)] and a vacuum pump capable of generating 15–20 inches of mercury or equivalent with a vacuum trap is needed for sample processing. Figure 9.3 shows the Vac-Man® 96 Manifold set up for purification.

Additional Resources for the Wizard® SV 96 and SV 9600 Plasmid DNA Purification Systems

Technical Bulletins and Manuals

- TB272 *Wizard® SV 96 Plasmid DNA Purification System Technical Bulletin*
(www.promega.com/tbs/tb272/tb272.html)
- TB292 *Wizard® SV 9600 Plasmid DNA Purification System Technical Bulletin*
(www.promega.com/tbs/tb292/tb292.html)

Promega Publications

- PN073 *Wizard® SV 96 Plasmid DNA Purification System: High quality plasmid DNA for use in fluorescent sequencing methods*
(www.promega.com/pnotes/73/8235_08/8235_08.html)

Citations

Scorpio, D.G. *et al.* (2008) Sequential analysis of *Anaplasma phagocytophilum msp2* transcription in murine and equine models of human granulocytic anaplasmosis. *Clin. Vaccine Immunol.* **15**, 418–24.

The authors examined the pattern of *Anaplasma phagocytophilum msp2* expression, a gene that modulates with little immune pressure and has decreased virulence with prolonged in vitro passage. C57BL/6J mice were inoculated with HL-60 cells infected with low-passage (passage 5) or high-passage (passage 26) *A. phagocytophilum*. Blood samples were taken 2–21 days post-inoculation and total RNA isolated. The purified RNA was subjected to RT-PCR, cloned into the pGEM®-T Easy Vector, transformed and plated. Plasmids were purified using the Wizard® SV 96 Plasmid DNA Purification System and the insert size analyzed using EcoRI digestion. The inserts were sequenced, aligned with *A. phagocytophilum* Webster strain

mSP2 using ClustalX and the resulting *mSP2* transcripts divided by origin from low- or high-passage bacteria.

PubMed Number: 18094110

F. Wizard® MagneSil® Plasmid Purification System

For automated, high-throughput plasmid purification, the Wizard® MagneSil® Plasmid DNA Purification System (Cat.# A1630, A1631, A1635) provides a simple and reliable method for the rapid isolation of plasmid DNA in a multiwell format. The purified plasmid can be used directly for automated fluorescent DNA sequencing, as well as for other standard molecular biology techniques including restriction enzyme digestion and PCR.

The purification procedure uses MagneSil® PMPs for lysate clearing as well as DNA capture, circumventing the need for centrifugation or vacuum filtration. The MagnaBot® 96 Magnetic Separation Device (Cat.# V8151; Figure 9.4) is needed for plasmid purification. The protocol also requires a multiwell plate shaker. This protocol has been optimized using the Micro Mix 5 shaker on the Beckman Coulter Biomek® 2000. To see workstations on which the Wizard® MagneSil® Plasmid Purification System has been automated, visit the Automated Methods (www.promega.com/automethods/) page on our web site.

Additional Resources for the Wizard® MagneSil® Plasmid Purification System

Technical Bulletins and Manuals

TB286 *Wizard® MagneSil® Plasmid Purification System Technical Bulletin*
(www.promega.com/tbs/tb286/tb286.html)

Promega Publications

PN079 Automated plasmid purification using MagneSil® Paramagnetic Particles
(www.promega.com/pnotes/79/9492_22/9492_22.html)

eNotes Isolation of genomic DNA from agricultural bacteria using the Wizard® MagneSil® Plasmid Purification System
(www.promega.com/enotes/applications/ap0038_tabs.htm)

Citations

Gualtieri, G. *et al.* (2006) A segment of the apospory-specific genomic region is highly microsyntenic not only between the apomicts *Pennisetum squamulatum* and buffelgrass, but also with a rice chromosome 11 centromeric-proximal genomic region. *Plant Physiol.* **140**, 963–71.

Eight *Pennisetum squamulatum* and seven buffelgrass BACs containing the apospory-specific genomic region (ASGR) marker *ugt197* were randomly sheared to 1.5–3.0kb in size. The fragments were blunt ended, size fractionated on a gel and the appropriate fraction excised and ligated into a plasmid. Random transformants were grown in deep-well plates and incubated for 18 hours. Plasmid DNA was

isolated using the Wizard® MagneSil® Plasmid DNA Purification System on a Biomek® 2000 workstation. The purified plasmid was sequenced with BigDye® reaction mix using 150–300ng DNA.

PubMed Number: 16415213

G. Wizard MagneSil Tfx™ System

For high-throughput processing, the Wizard MagneSil Tfx™ System (Cat.# A2380, A2381) provides a simple and reliable method for the rapid isolation of transfection-quality plasmid DNA in a multiwell format. The purified plasmid can be used directly for transfection, as well as for other standard molecular biology techniques.

Like the Wizard® MagneSil® Plasmid DNA Purification System, the Wizard MagneSil Tfx™ System uses MagneSil® PMPs for lysate clearing as well as DNA capture. In addition, a proprietary paramagnetic endotoxin removal resin reduces the level of endotoxin present in the purified plasmid DNA. By avoiding the need for centrifugation or vacuum filtration, DNA purification with the Wizard MagneSil Tfx™ System can be completely automated, requiring the MagnaBot® 96 Magnetic Separation Device (Cat.# V8151) and Heat Transfer Block (Cat.# Z3271) for the protocol.

DNA purified with the Wizard MagneSil Tfx™ System is greatly reduced in chemical contaminants as well as RNA, protein, and endotoxin, providing high-quality plasmid DNA suitable for transfection. The amount of DNA needed will vary depending on the transfection reagent and the cell line used and should be optimized whenever a new transfection reagent or cell line is examined.

An automated method has been developed for the Biomek® FX robotic workstation. The procedure requires no manual intervention and takes approximately 45 minutes to process a single 96-well plate. This automated protocol can be adapted to other robotic workstations. Visit our web site (www.promega.com/automethods/) for information on an automated protocol for your platform. An Automation Support Team member will contact you regarding a method for use with your particular system.

Additional Resources for the Wizard MagneSil Tfx™ System

Technical Bulletins and Manuals

TB314 *Wizard MagneSil Tfx™ System Technical Bulletin*
(www.promega.com/tbs/tb314/tb314.html)

Promega Publications

PN083 Wizard MagneSil Tfx™ System for the purification of transfection-grade DNA
(www.promega.com/pnotes/83/10492_18/10492_18.html)

V. Plasmid DNA Purification Protocol Featuring the PureYield™ Plasmid Midiprep System

Materials Required:

- PureYield™ Plasmid Midiprep System (Cat.# A2492; 25 preps)
- Eluator™ Vacuum Elution Device (Cat.# A1071) or swinging bucket rotor
- isopropanol
- ethanol, 95%
- tabletop centrifuge at room temperature (22–25°C)
- 50ml disposable plastic screw-cap tubes (e.g., Corning® or Falcon® brand)
- high-speed centrifuge capable of at least 15,000 × g and appropriate tubes
- vacuum pump, single- or double-stage, producing a pressure of approx. 650mm Hg
- vacuum manifold (e.g., Vac-Man® Laboratory Vacuum Manifold)

Endotoxin Removal Wash and Column Wash must be prepared as described below before lysing cells and purifying DNA (close cap tightly after additions):

Endotoxin Removal Wash

25 preps system: Add 57ml of isopropanol to the Endotoxin Removal Wash bottle.

Column Wash

25 preps system: Add 350ml of 95% ethanol to the Column Wash bottle.

Regardless of the purification method used, **keep these important protocol points in mind:**

- To differentiate the PureYield™ Clearing and PureYield™ Binding columns, note that the clearing columns are blue, while the binding columns are white.
- Perform all purification steps at room temperature (22–25°C).
- The concentration of the plasmid is dependent on copy number and elution volume. If a higher concentration is desired for subsequent applications, perform an ethanol precipitation after plasmid isolation. Add 1/10 volume 3M sodium acetate (pH 5.2), 2.5 volumes 95% ethanol. Place on ice for 15 minutes. Pellet the DNA by centrifugation at 14,000 × g for 10 minutes in a microcentrifuge. Wash pellet with 70% ethanol and centrifuge at 14,000 × g for 10 minutes. Resuspend DNA pellet in desired volume of nuclease-free water.

A. Standard DNA Purification Protocol

1. Grow 50–250ml of transformed *E. coli* bacterial cell culture overnight (16–21 hours) at optimal culture conditions.
Note: This protocol is optimized for 50–250ml of culture at an O.D.₆₀₀ = 2–4.
2. Pellet the cells using centrifugation at 5,000 × g for 10 minutes and discard supernatant. Drain tubes on a paper towel to remove excess media.
3. Resuspend pellet in Cell Resuspension Solution (see Table 9.2 for appropriate volumes).

Table 9.2. Solution Volumes Required to Generate Lysate.

Solution Volume	Bacterial Culture Volume	
	50–100ml	101–250ml
Cell Resuspension	3ml	6ml ¹
Cell Lysis Solution	3ml	6ml ¹
Neutralization Solution	5ml	10ml ¹

¹ Additional solutions will need to be purchased or made for processing 101–250ml culture volumes.

4. Add Cell Lysis Solution. Invert 3–5 times to mix. Incubate 3 minutes at room temperature (22–25°C).
5. Add Neutralization Solution. Invert 5–10 times to mix.
6. Centrifuge lysate at 15,000 × g for 15 minutes.
7. Assemble a column stack by placing a blue PureYield™ Clearing Column into the top of a white PureYield™ Binding Column. Place the assembled column stack onto a vacuum manifold as shown in Figure 9.8.



Figure 9.8. The PureYield™ Vacuum Purification Configuration. This image shows a blue PureYield™ Clearing Column nested on top of a white PureYield™ Binding Column. Both columns are sitting on the vacuum manifold port.

8. Pour the lysate into the clearing column. Apply maximum vacuum, continuing until all the liquid has passed through both the clearing and binding columns.
9. **Slowly release** the vacuum from the filtration device before proceeding. Remove the clearing column, leaving the binding column on the vacuum manifold.

Note: If the binding membrane has been dislodged from the bottom of the column, tap it back into place using a sterile pipet.

Wash

10. Add 5.0ml of Endotoxin Removal Wash to the binding column, and allow the vacuum to pull the solution through the column.
11. Add 20ml of Column Wash Solution to the binding column, and allow the vacuum to draw the solution through.
12. Dry the membrane by applying a vacuum for 30 seconds. Repeat this step for an additional 30 seconds if the top of the binding membrane appears wet or there is a detectable ethanol odor.
13. Remove the binding column from the vacuum manifold, and tap it on a paper towel to remove excess ethanol.

Elute by Vacuum (alternatively, see Elute by Centrifugation below)

14. Place a 1.5ml microcentrifuge tube into the base of the Eluator™ Vacuum Elution Device, securing the tube cap as shown in Figure 9.9, Panel A.

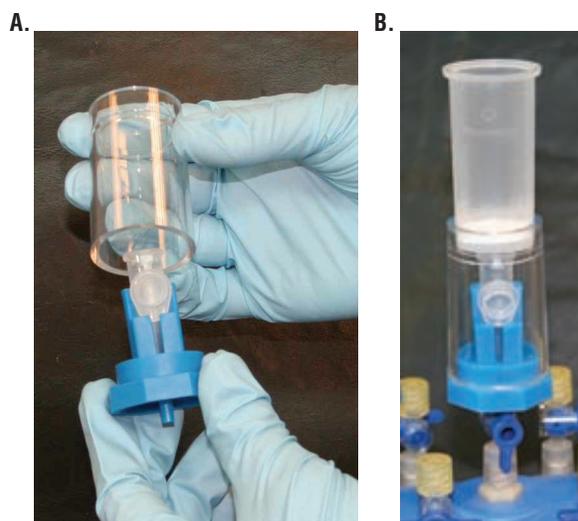


Figure 9.9. The Eluator™ Vacuum Elution Device for elution by vacuum. Panel A. A 1.5ml microcentrifuge tube is placed into the base of the Eluator™ Device, and the tube cap is secured in an open position, as shown. Panel B. The Eluator™ Vacuum Elution Device assembly, including the binding column, on a vacuum manifold.

15. Assemble the Eluator™ Vacuum Elution Device, and insert the DNA binding column into the device, making sure that the column is fully seated on the collar.
16. Place the elution device assembly, including the binding column, onto a vacuum manifold (Figure 9.9, Panel B).
17. Add 400–600µl of Nuclease-Free Water to the DNA binding membrane in the binding column. Wait for 1 minute. Apply maximum vacuum for 1 minute or until all liquid has passed through the column.
18. Remove the microcentrifuge tube and save for DNA quantitation and gel analysis.

Elute by Centrifugation

19. Place the binding column into a new 50ml disposable plastic tube.
20. Add 600µl of Nuclease-Free Water to the DNA binding membrane in the binding column. Wait for 1 minute. Centrifuge the binding column at 1,500–2,000 × g for 5 minutes using a **swinging bucket rotor**, and collect the filtrate.

Note: Do not cap the 50ml tube during centrifugation.

For complete protocol information, see the *PureYield™ Plasmid Midiprep System Technical Manual #TM253* (www.promega.com/tbs/tm253/tm253.html).

VI. Genomic DNA Isolation Systems

Purified genomic DNA is necessary for further analysis of disease states, single nucleotide polymorphisms (SNPs) and for many other multiplex and real-time PCR applications. Many methods exist for isolation of chromosomal DNA, and Promega has genomic purification systems that are both general (able to isolate from many source materials) or specialized (primarily used for one source type). The source types range from bacteria to humans and can encompass tissues from blood to muscle and from leaf to seed.

A. Wizard® Genomic DNA Purification Kit

The Wizard® Genomic DNA Purification Kit (Cat.# A1120, A1125, A1620) is both a versatile and scalable system for isolating genomic DNA. With this system alone, chromosomal DNA can be isolated from whole blood (Walker *et al.* 2003), plant leaf (Zhang *et al.* 2004), Gram-positive (van Schaik *et al.* 2004) and Gram-negative bacteria (Flashner *et al.* 2004), mouse tail (Lee *et al.* 2005) and yeast (Martinez *et al.* 2004). Additional sample types like fungus (Ahmed *et al.* 2003), infected frog tissues embedded in paraffin (Pereira *et al.* 2005), saliva (Cox *et al.* 2004) and flour beetles (Lorenzen *et al.* 2002) have also been used successfully with the Wizard® Genomic DNA Purification Kit. Not only is this genomic purification system successful with many sample types, it is also easily scaled for the quantity of starting material by adjusting reagent volumes to accommodate your needs. Additional references for the Wizard® Genomic DNA Purification Kit or any of the Promega DNA isolation systems can be found on our Citations web site (www.promega.com/citations/).

Additional Resources for the Wizard® Genomic DNA Purification Kit

Technical Bulletins and Manuals

TM050 *Wizard® Genomic DNA Purification Kit Technical Manual*
(www.promega.com/tbs/tm050/tm050.html)

Promega Publications

PN073 Using the Wizard® Genomic DNA Purification Kit with 96-well plates
(www.promega.com/pnotes/73/8235_20/8235_20.html)

PN068 Isolation of Spirochete DNA using the Wizard® Genomic DNA Purification Kit
(www.promega.com/pnotes/68/7381_30/7381_30.html)

eNotes Wizard® Genomic DNA Purification Kit provides high-quality genomic DNA template for molecular phylogenetic studies on Copepod crustaceans
(www.promega.com/enotes/applications/ap0063.htm)

eNotes Wizard® Genomic DNA Purification Kit and the isolation of plant genomic DNA
(www.promega.com/enotes/applications/ap0027_tabs.htm)

eNotes Isolation of genomic DNA from small volumes of whole blood using the Wizard® Genomic DNA Purification Kit
(www.promega.com/enotes/applications/ap0011_tabs.htm)

eNotes Extraction and amplification of DNA from an ancient moss
(www.promega.com/enotes/applications/ap0004_tabs.htm)

Online Tools

Sample Types Processed with the Wizard® Genomic DNA Purification Kit (www.promega.com/applications/dna_rna/sampletypes/wizgensampletypes.htm)

Citations

El Sheikh, A.F., Poret-Peterson, A.T. and Klotz, M.G. (2008) Characterization of two new genes, *amoR* and *amoD*, in the *amo* operon of the marine ammonia oxidizer *Nitrosococcus oceani* ATCC 19707. *Appl. Environ. Microbiol.* **74**, 312–8.

These authors investigated the *amo* operon of the marine ammonia oxidizer *Nitrosococcus oceani*. The bacteria were grown at 30°C for 3 weeks in 200–400ml batch cultures in artificial seawater in the dark without shaking. Genomic DNA was isolated from cells in stationary phase using the Wizard® Genomic DNA Purification Kit. The isolated DNA was then used for PCR analysis.

PubMed Number: 17993553

Gomes, R. *et al.* (2008) Immunity to a salivary protein of a sand fly vector protects against the fatal outcome of visceral leishmaniasis in a hamster model. *Proc. Natl. Acad. Sci. USA* **105**, 7845–50.

The authors tested the ability of the salivary proteins of sand fly, the *Leishmania* vector, to protect against cutaneous leishmaniasis in hamsters. Genomic DNA from 300µl of blood and 100mg of spleen and liver tissue of intradermal inoculated (infected) hamsters or control hamsters was isolated using the Wizard® Genomic DNA Purification Kit. PCR determined the parasite burden using primers that amplified a 120bp conserved region of the *Leishmania* kDNA minicircle.

PubMed Number: 18509051

B. Wizard® SV Genomic DNA Purification System

The Wizard® SV Genomic DNA Purification System (Cat.# A2360, A2361) provides a fast, simple technique for the preparation of purified and intact DNA from mouse tails, tissues and cultured cells in as little as 20 minutes, depending on the number of samples processed (up to 24 by centrifugation, depending on the rotor size, or up to 20 by vacuum). With some modifications, whole blood can also be used with this isolation system (Promega Corporation, 2002). This is a silica membrane-based system,

meaning there are limitations to the amount of material that can be loaded onto a single SV column; up to 20mg of tissue (mouse tail or animal tissue) or between 1×10^4 and 5×10^6 tissue culture cells can be processed per purification. With more sample, the prepared lysate may need to be split among two or more columns to avoid clogging the column.

For the single-column isolation, a vacuum manifold or a microcentrifuge can be used for sample processing. The technology is based on binding of the DNA to silica under high-salt conditions. In the case of the Wizard® SV Genomic DNA Purification System, the silica is present in a membrane format in a small column. The key to isolating any nucleic acid with silica is the presence of a chaotropic salt like guanidine hydrochloride. Chaotropic salt present in high quantities is able to disrupt cells, deactivate nucleases and allow nucleic acid to bind to silica. Once the genomic DNA is bound to the silica membrane, the nucleic acid is washed with a salt/ethanol solution. These washes remove contaminating proteins, lipopolysaccharides and small RNAs to increase purity while keeping the DNA bound to the membrane. Once the washes are finished, the genomic DNA is eluted under low-salt conditions using either nuclease-free water or TE buffer.

The genomic DNA isolated with the Wizard® SV Genomic DNA Purification System is of high quality and serves as an excellent template for agarose gel analysis, restriction enzyme digestion and PCR analysis as seen in Figure 9.10. Table 9.3 provides typical yields of genomic DNA purified from a variety of sources.

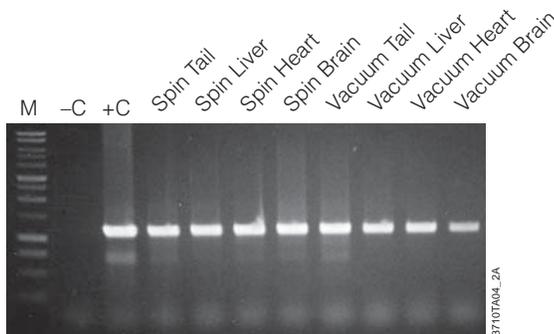


Figure 9.10. Amplification of genomic DNA isolated from various tissue sources using the Wizard® SV Genomic DNA Purification System. One microliter of purified genomic DNA was amplified using PCR Master Mix (Cat.# M7502) and mouse-specific IL-1 β primers (1.2kb product). Reactions with Mouse Genomic DNA (Cat.# G3091; +C) and without DNA (-C) were performed as positive and negative controls, respectively. Thermal cycling conditions were: one cycle of 3 minutes at 95°C; followed by 30 cycles of: 95°C for 30 seconds, 60°C for 1 minute, 70°C for 1 minute and 30 seconds; final extension at 70°C for 7 minutes; 4°C soak. All lanes contained 10 μ l of reaction product separated on a 1% agarose gel. PCR products were visualized by ethidium bromide staining. "Spin" and "Vacuum" designations indicate the protocol used for genomic DNA isolation.

Table 9.3. Typical Genomic DNA Yield From Various Tissues using the Wizard® SV Genomic DNA Purification System.

Sample	Amount	Average Yield
Tail Clipping	20mg	20 μ g
Liver	20mg	15 μ g
Heart	20mg	10 μ g
Brain	20mg	6 μ g
CHO cells	1×10^6	5 μ g
NIH/3T3 cells	1×10^6	9 μ g
293 cells	1×10^6	8 μ g

Researchers have used this simple and rapid system for many additional sample types and applications including mosquitoes (Stump *et al.* 2005), mammary stem cells followed by STR analysis (Dontu *et al.* 2003), *Bacillus subtilis* (Park *et al.* 2004), *Escherichia coli* (Teresa Pellicer *et al.* 2003), the larval form of the *Schistosoma mansoni* parasite (Smith *et al.* 2004) and viral DNA from Kaposi's sarcoma herpes virus-infected BC3 cells (Ohsaki *et al.* 2004).

Additional Resources for the Wizard® SV Genomic DNA Purification System

Technical Bulletins and Manuals

- TB302 [Wizard® SV Genomic DNA Purification System Technical Bulletin \(www.promega.com/tbs/tb302/tb302.html\)](http://www.promega.com/tbs/tb302/tb302.html)

Promega Publications

- PN081 [Introducing the Wizard® SV and SV 96 Genomic DNA Purification Systems \(www.promega.com/pnotes/81/9939_09/9939_09.html\)](http://www.promega.com/pnotes/81/9939_09/9939_09.html)
- eNotes [A modified Wizard® SV Genomic DNA Purification System protocol to purify genomic DNA from shed reptile skin \(www.promega.com/enotes/applications/ap0089.htm\)](http://www.promega.com/enotes/applications/ap0089.htm)
- eNotes [Isolation of genomic DNA from bacterial cells using the Wizard® SV Genomic DNA Purification System \(www.promega.com/enotes/applications/ap0051_tabs.htm\)](http://www.promega.com/enotes/applications/ap0051_tabs.htm)

Online Tools

Sample Types Processed with the Wizard® SV Genomic DNA Purification System (www.promega.com/applications/dna_rna/sampletypes/wizSVgensampletypes.htm)

Citations

Mancuso, M. *et al.* (2008) Oncogenic bystander radiation effects in *Patched* heterozygous mouse cerebellum *Proc. Natl. Acad. Sci. USA* **105**, 12445–50.

To examine radiation-bystander responses in neonatal mouse cerebellum, heterozygous radiosensitive *Patched-1* (*Ptch1*) mice were exposed to either whole body (WB) X-rays or shielded head/rest of body (SH) irradiation. Genomic DNA was isolated from tumors and normal tissue

using Wizard® SV Genomic DNA Purification System. Loss of heterozygosity was tested using PCR and sequencing of exon 23 of the *Ptch1* gene.

PubMed Number: 18711141

C. Wizard® SV 96 Genomic DNA Purification System

The Wizard® SV 96 Genomic DNA Purification System (Cat.# A2370, A2371) is available for high-throughput, 96-well isolation. Amplifiable genomic DNA can be isolated from up to 5×10^6 cells per prep, from 20mg of tissue or from up to 1.2cm of a mouse tail tip without centrifugation of the lysate prior to purification. This multiwell system requires a vacuum manifold (Vac-Man® 96 Vacuum Manifold) and a vacuum pump capable of generating 15–20 inches of mercury or the equivalent. Genomic DNA was isolated from three different source types then used in a monoplex PCR and run on an agarose gel as shown in Figure 9.11. Figure 9.12 compares the yield from the three Wizard® SV Genomic DNA purification methods (96-well plate, vacuum and centrifugation).

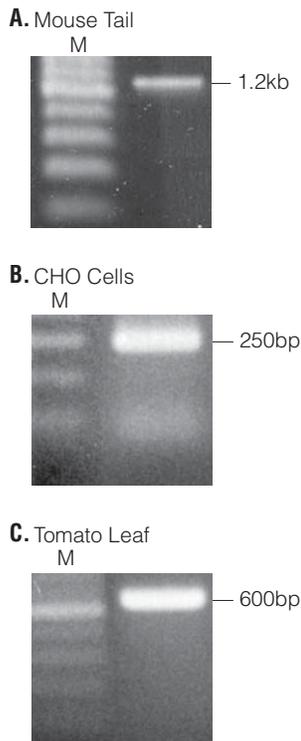


Figure 9.11. Agarose gel electrophoresis of PCR products amplified from 1 μ l of mouse tail, CHO cells and tomato leaf sample genomic DNA isolated using the Wizard® SV 96 Genomic DNA Purification System. A total of 10 μ l of PCR product is visualized on a 1.5% agarose gel stained with ethidium bromide. **Panel A.** IL-1 β (1.2kb) amplified from mouse tail. **Panel B.** β -actin (250bp) amplified from CHO cells. **Panel C.** Chloroplast DNA (600bp) amplified from tomato leaf. Lane M, 1kb DNA Ladder (Cat.# G5711).

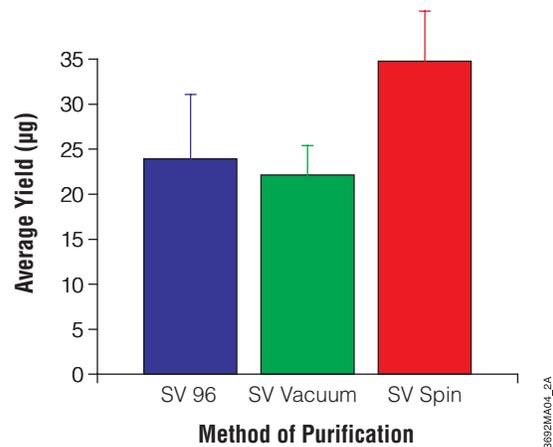


Figure 9.12. Comparison of DNA yields using the Wizard® SV and SV 96 Genomic DNA Purification Systems. Average yield of genomic DNA in micrograms purified from 20mg mouse tail clippings. The average A_{260}/A_{280} ratios are: SV 96, 1.7 ± 0.08 ; SV vacuum method, 1.7 ± 0.14 ; SV spin method, 1.7 ± 0.14 .

Additional Resources for the Wizard® SV 96 Genomic DNA Purification System

Technical Bulletins and Manuals

TB303 *Wizard® SV 96 Genomic DNA Purification System Technical Bulletin*
(www.promega.com/tbs/tb303/tb303.html)

Promega Publications

PN081 *Introducing the Wizard® SV and SV 96 Genomic DNA Purification Systems*
(www.promega.com/pnotes/81/9939_09/9939_09.html)

PN078 *Automated isolation of genomic DNA using Promega's DNA binding plates on the Beckman Biomek® 2000*
(www.promega.com/pnotes/78/9186_06/9186_06.html)

Citations

Yamada, M. *et al.* (2008) Mutations in the quinolone resistance determining region in *Staphylococcus epidermidis* recovered from conjunctiva and their association with susceptibility to various fluoroquinolones. *Br. J. Ophthalmol.* **92**, 848–51.

To study how mutations in quinolone resistance determining region (QRDR) of *Staphylococcus epidermidis*, a pathogen that infects eyes, may have a role in fluoroquinolone resistance, 138 samples of *S. epidermidis* were swabbed from the conjunctival sac of 129 patients. These samples were cultured overnight in tryptic soy broth, and genomic DNA isolated using the Wizard® SV 96 Genomic DNA Purification System. One microliter of the isolated DNA was used in PCR for the QRDR genes (*gyrA*, *gyrB*, *parC* and *parE*).

PubMed Number: 18460536

D. MagneSil® Genomic Systems for Blood Isolation

Promega offers several MagneSil® paramagnetic silica-based systems for specialized sample-type use. Three systems address isolation of DNA from whole blood on automated platforms: MagneSil® ONE, Fixed Yield Blood Genomic System; MagneSil® Blood Genomic, Max Yield System and MagneSil® Genomic, Large Volume System. These DNA purification systems are for automated use and require hardware accessories in addition to the instrument workstation. A list of the essential accessories for use with a robotic setup are included on the online catalog pages for each DNA isolation system at: www.promega.com. The main differences in these three DNA purification systems are format, the blood volume processed and DNA yield recovered. The MagneSil® ONE, Fixed Yield Blood Genomic System (Cat.# MD1370) purifies 1 μ g of DNA (\pm 50%) from 60 μ l of anticoagulated whole blood in a 96-well plate format. Purification of a "fixed yield" of DNA eliminates the need to quantitate and normalize concentrations post-purification.

To maximize the quantity of DNA recovered from 200 μ l blood, use the MagneSil® Blood Genomic, Max Yield System (Cat.# MD1360). The methodology is the same—lysing the cells and capturing the genomic DNA from the solution—but the recovered yield is more variable, between 4–9 μ g, depending on the number of white cells. Figure 9.13 shows a multiplex PCR using DNA isolated from both the MagneSil® Blood Genomic, Max Yield System, and the MagneSil® ONE, Fixed Yield Blood Genomic System.

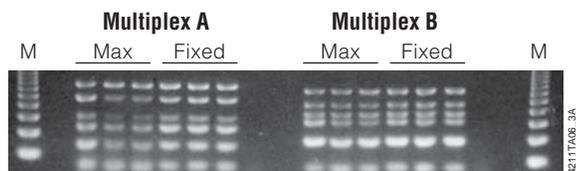


Figure 9.13. Multiplex PCR analysis on genomic DNA purified from blood. Genomic DNA purified with either the MagneSil® Blood Genomic System, Max Yield System (Max) or the MagneSil® ONE, Fixed Yield Blood Genomic System (Fixed) was amplified using the Y Chromosome Deletion Detection System, Version 1.1. Ten microliters of the amplification reactions for Multiplex A and B was run on a gel and visualized by ethidium bromide staining.

For the ability to isolate large quantities of genomic DNA from large-volume tubes including blood samples (1–10ml), the MagneSil® Genomic, Large Volume System (Cat.# A4080, A4082, A4085) may meet your needs. There are several accessories needed in order to use this system for isolation of genomic DNA (see the Specialized Genomic DNA Purification Protocol for a listing), but the MagneSil® Genomic, Large Volume System, can process even mishandled blood samples and, depending on the white cell count, may yield ~450 μ g genomic DNA/10ml blood. These DNA isolation systems produce high-quality DNA suitable for use in PCR, multiplex PCR and SNP genotyping

applications. As seen in Figure 9.14, genomic DNA isolated using the MagneSil® Genomic, Large Volume System, works well in real-time PCR analysis.

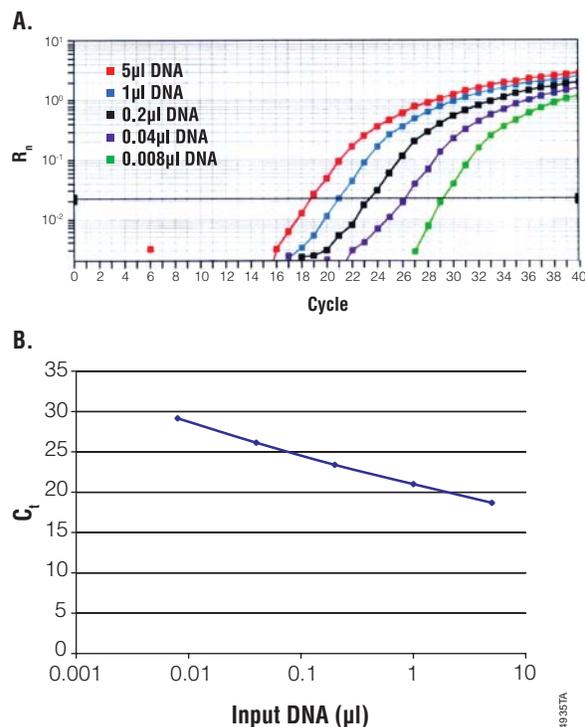


Figure 9.14. Real-time PCR assay for quality of genomic DNA purified by the MagneSil® Genomic, Large Volume System. Decreasing volumes of isolated human DNA were analyzed using β -actin real-time PCR control reagents from Applied Biosystems. **Panel A.** Amplification curve for the DNA volume range tested. **Panel B.** Linear detection for the amplification.

While these MagneSil® Genomic Systems are primarily designed for use with whole blood, other sample types can be used. However, there are no specific protocols associated with other sample types. Visit our Citations database (www.promega.com/citations/) or contact Promega Technical Services (techserv@promega.com) to learn about other possible sample types used for genomic DNA purification.

Additional Resources for the MagneSil® Genomic Systems for Blood Isolation

Technical Bulletins and Manuals

- TB313 *MagneSil® ONE, Fixed Yield Blood Genomic System Technical Bulletin*
(www.promega.com/tbs/tb313/tb313.html)
- TB312 *MagneSil® Blood Genomic, Max Yield System Technical Bulletin*
(www.promega.com/tbs/tb312/tb312.html)
- TB549 *MagneSil® Genomic, Large Volume System Technical Bulletin*
(www.promega.com/tbs/tb549/tb549.html)

Promega Publications

- PN090 MagneSil® Genomic, Large Volume System, for large-sample genomic DNA isolation (www.promega.com/pnotes/90/12727_22/12727_22.html)
- PN085 Automated 96-well purification of genomic DNA from whole blood (www.promega.com/pnotes/85/10904_07/10904_07.html)
- PN083 Expanding the capabilities of plant genomic DNA purification (www.promega.com/pnotes/83/10492_25/10492_25.html)

Citations

Bailey, A.M. *et al.* (2003) Robotic nucleic acid isolation using a magnetic bead resin and an automated liquid handler for biological agent simulants. *J. Assoc. Lab. Automation* **8**, 113–20.

This study describes the development of a system that can rapidly and accurately detect traces of biological agents from environmental samples. Using *Erwinia herbicola* and *Bacillus subtilis* var. *niger* as models for potential biological warfare agents, a method for DNA extraction using the Wizard® Magnetic DNA Purification System for Food, MagneSil® Blood Genomic, Max Yield System, and a combination of the two was automated on a Beckman Coulter Biomek® FX robotic liquid handling system. The isolated DNA was used in a TaqMan® real-time PCR assay that specifically amplified and identified DNA species. The ability of the MagneSil®-based DNA purification technology to eliminate PCR inhibitors was also evaluated. Various soil samples, surface swabs and air samples were mixed with bacterial cultures to see if any contaminants present in the samples inhibited PCR. The authors found that the modified MagneSil® method described here eliminated many PCR inhibitors.

E. Maxwell® 16 System

As laboratories try to improve productivity, the need has increased for easy-to-use, low- to moderate-throughput automation of purification processes. The Maxwell® 16 Instrument is designed for efficient, automated purification from a wide range of sample types. The instrument is supplied with preprogrammed automated purification methods and is designed for use with prefilled reagent cartridges, maximizing simplicity and convenience. The instrument can process up to 16 samples in approximately 30–40 minutes (depending on sample type and method). Purified concentrated products are high quality and obtained at high yield to be used directly in a variety of downstream applications.

The Maxwell® 16 System purifies samples using paramagnetic particles (PMPs), which provide a mobile solid phase that optimizes capture, washing and elution of the target material. The Maxwell® 16 Instrument is a magnetic-particle-handling instrument that efficiently preprocesses liquid and solid samples, transports the PMPs through purification reagents in the prefilled cartridges, and mixes efficiently during processing. The efficient magnetic particle-based methodology used by the Maxwell® 16 Instrument avoids common problems associated with automated purification systems, such as clogged tips or partial reagent transfers, which can result in suboptimal purification processing. Several Maxwell® 16 reagent kits are available and allow optimal purification from a variety of sample types including blood, FTA® paper, formalin-fixed, paraffin-embedded tissue, bacteria, plant and animal tissue (see Figures 9.15 and 9.16).

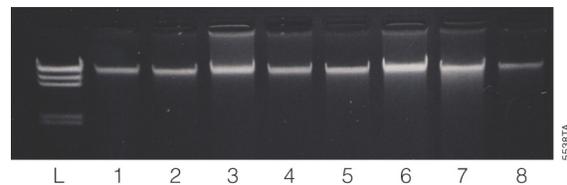


Figure 9.15. A panel of murine tissues purified using the Maxwell® 16 Tissue DNA Purification Kit on the Maxwell® 16 Instrument. Five microliters of genomic DNA was purified from 50mg of the following mouse tissues: Lane 1, brain; lane 2, heart; lane 3, intestine; lane 4, liver; lane 5, pancreas; lane 6, spleen; lane 7, 1cm mouse tail clipping; lane 8, 0.5cm mouse tail clipping; lane L, Lambda DNA/HindIII Marker (Cat.# G1711) All tissue samples were added directly to the reagent cartridge without preprocessing.

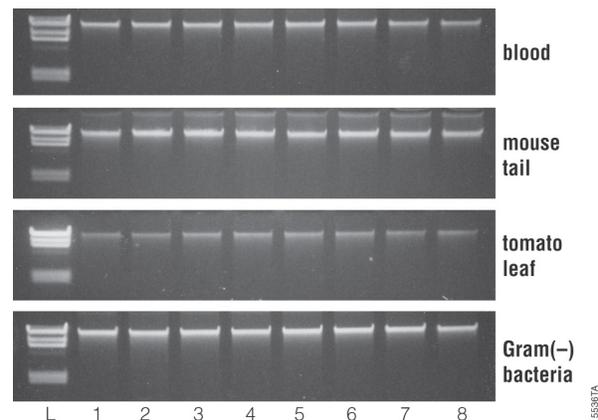


Figure 9.16. Consistent purification using the Maxwell® 16 Instrument. Five microliters of genomic DNA was purified from 400µl of human whole blood, 1cm mouse tail, 25mg tomato leaf or 400µl of an overnight culture of Gram-negative bacteria. Lane L, Lambda DNA/HindIII Marker (Cat.# G1711).

The Maxwell® 16 Instrument is easy to set up—just unpack and begin to use. No training or external computer required, so the instrument is ready for immediate use. Optimized automated methods are preloaded, the prefilled reagent cartridges are snapped into place, your sample is added and you press "Start" to begin the appropriate method.

For challenging sample types or more control over the DNA purification application, the Maxwell® 16 Flexi Method Firmware (Cat.# AS6411) allows you to create your own program by optimizing the lysis, binding, drying, elution and paramagnetic particle capture parameters. Parameters are entered using the on-screen prompts on the Maxwell® 16 Instrument. The Maxwell® 16 Flexi Method Firmware is available as an option for new Maxwell® 16 Instruments and can be installed on existing instruments.

In addition to kits and methods developed by Promega [e.g., the Maxwell® 16 Cell LEV DNA Purification Kit (Cat.# AS1140) and the Maxwell® 16 FFPE Tissue LEV DNA Purification Kit (Cat.# AS1130)], customers and Promega applications scientists are continuously developing new applications for the Maxwell® 16 System. To facilitate sharing information about samples tested by both Promega scientists and Maxwell® 16 users throughout the world, the Maxwell® 16 Applications Database (www.promega.com/maxwell16/applications) is available. This online resource allows you to search by keyword or browse through folders organized by sample type and applications, and contains information on samples processed, yields achieved and downstream applications tested. You can submit entries to the database using the provided submission form (www.promega.com/maxwell16/applications/submission.asp). Recent applications include RNA and DNA from *Spirulina* (blue-green algae) and total RNA from Gram Negative bacterial culture.

Automation eliminates the hands-on time and labor of manual purification, giving you the time to focus on your research. In addition, the Maxwell® 16 Instrument design, optimized reagents and automated methods provide consistent yield and purity for your downstream applications. The instrument is benchtop compact and can purify from many sample types (Table 9.4). Future automated method updates can be added through the instrument communications port.

Table 9.4. DNA yield from various sample types after purification using the Maxwell® 16 Instrument and DNA Purification Kits.

Sample Type	Sample Size	Yield
Whole blood	200µl	4–9µg (>3pg/white blood cell)
Whole blood	400µl	8–15µg (>3pg/white blood cell)
Mouse tail	1.2cm	≥20µg
Animal tissue	20–25mg	60–100µg (mouse liver)
Tissue culture cells	5 × 10 ⁶	15–20µg (HeLa)
Gram–bacteria	2 × 10 ⁹	25–30µg (<i>E. coli</i> BL21)
Gram+ bacteria ¹	2 × 10 ⁹	15–25µg (<i>B. cereus</i>)
Plant leaf (tomato) ¹	25mg	9–13µg
<i>Drosophila melanogaster</i>	1 fly	0.32µg
<i>Drosophila melanogaster</i>	5 flies	1.52µg
<i>Caenorhabditis elegans</i>	~50,000 worms	0.08µg
<i>Danio rerio</i>	50mg	24.8µg
<i>Saccharomyces cerevisiae</i> ²	1 colony (3mm)	0.72µg
<i>Arabidopsis thaliana</i>	1 leaf	0.13µg

¹With optional pretreatment.

²Includes 3 hour digestion with lyticase prior to DNA isolation.

Additional Resources for Maxwell® 16 System

Technical Bulletins and Manuals

TM295	<i>Maxwell® 16 Instrument Operating Manual</i> (www.promega.com/tbs/tm295/tm295.html)
TM284	<i>Maxwell® 16 DNA Purification Kits Technical Manual</i> (www.promega.com/tbs/tm284/tm284.html)
TM309	<i>Maxwell® 16 Mouse Tail DNA Purification Kit Technical Manual</i> (www.promega.com/tbs/tm309/tm309.html)
TB383	<i>Maxwell® 16 Cell LEV DNA Purification Kit Technical Bulletin</i> (www.promega.com/tbs/tb383/tb383.html)
TB382	<i>Maxwell® 16 FFPE Tissue LEV DNA Purification Kit Technical Bulletin</i> (www.promega.com/tbs/tb382/tb382.html)

- TB347 DNA IQ™ Reference Sample Kit for Maxwell® 16 Technical Bulletin (www.promega.com/tbs/tb347/tb347.html)
- TB354 DNA IQ™ Casework Sample Kit for Maxwell® 16 Technical Bulletin (www.promega.com/tbs/tb354/tb354.html)
- TB381 Maxwell® 16 Flexi Method Firmware Technical Bulletin (www.promega.com/tbs/tb381/tb381.html)

Promega Publications

- PN098 Have you seen the Maxwell® 16 applications database? (www.promega.com/pnotes/98/15963_11/15963_11.html)
- PN097 Personal Automation™ for increased productivity (www.promega.com/pnotes/97/15377_03/15377_03.html)
- PN097 The versatility of the Maxwell® 16 System for genomic DNA extraction (www.promega.com/pnotes/97/15377_12/15377_12.html)
- PN097 New Maxwell® 16 Instrument accessories increase the versatility of your Maxwell® 16 System (www.promega.com/pnotes/97/15377_04/15377_04.html)
- PN097 Maxwell® 16 buffy coat genomic DNA application (www.promega.com/pnotes/97/15377_06/15377_06.html)
- PN092 Introducing the Maxwell® 16 Instrument: A simple, robust and flexible tool for DNA purification (www.promega.com/pnotes/92/13408_20/13408_20.html)
- GIN021 Forensic application of the Maxwell® 16 Instrument (www.promega.com/profiles/901/901_03.html)
- eNotes Automated DNA purification from Oragene•DNA/saliva samples using the Maxwell® 16 System (www.promega.com/enotes/applications/ap0091.htm)

Online Tools

Introducing Personal Automation™ from Promega (video and web site) (www.promega.com/maxwell16/)

Citations

Ansell, A. *et al.* (2009) Polymorphism of FGFR4 in cancer development and sensitivity to cisplatin and radiation in head and neck cancer. *Oral Oncol.* **45**, 23–9.

In this study, the Maxwell® 16 Instrument and

Maxwell® 16 DNA Purification Kit were used to isolate DNA from cell lines derived from head and neck squamous cell carcinomas.

PubMed Number: 18487077

Suzuki, H. *et al.* (2008) Interaction of the cytochrome P4501A2, SULT1A1 and NAT gene polymorphisms with smoking and dietary mutagen intake in modification of the risk of pancreatic cancer. *Carcinogenesis* **29**, 1184–91.

In this study, the Maxwell® 16 Instrument and Maxwell® 16 Blood DNA Purification Kit were used to purify genomic DNA from blood samples. The extracted DNA was amplified by PCR for subsequent genotype analysis.

PubMed Number: 18499698

F. Plant Genomic DNA Isolation

The Wizard® Magnetic 96 DNA Plant System (Cat.# FF3760, FF3761) is designed for manual or automated 96-well purification of DNA from plant leaf and seed tissue. The Wizard® Magnetic 96 DNA Plant System has been validated with corn and tomato leaf as well as with canola and sunflower seeds. The DNA purified from these samples can be used in PCR and other more demanding applications, such as RAPD analysis. Additional required equipment includes not only a magnet (MagnaBot® 96 Magnetic Separation Device) but a device capable of breaking up seed or leaf material (e.g., Geno/Grinder® 2000 from SPEX CertiPrep, Inc.). The yield depends on the source material and how well the seeds or leaf disks are pulverized prior to the genomic DNA isolation. Yield may range from 10–100ng from a single 8mm leaf punch. To increase the yield from the Wizard® Magnetic 96 DNA Plant System, a scale up in volume with up to 5 leaf punches can be used [as demonstrated in *Promega Notes* 79 (www.promega.com/pnotes/79/9492_25/9492_25.html)]. The potential scale-up is limited by the volume in a deep-well, 96-well plate.

Additional Resources for Plant Genomic DNA Isolation**Technical Bulletins and Manuals**

- TB289 *Wizard® Magnetic 96 DNA Plant System Technical Bulletin* (www.promega.com/tbs/tb289/tb289.html)

Promega Publications

- PN083 Expanding the capabilities of plant genomic DNA purification (www.promega.com/pnotes/83/10492_25/10492_25.html)
- PN079 Automated genomic DNA purification using the Wizard® Magnetic 96 DNA Plant System (www.promega.com/pnotes/79/9492_25/9492_25.html)

Citations

el-Lithy, M.E. *et al.* (2006) New *Arabidopsis* recombinant inbred line populations genotyped using SNPWave and their use for mapping flowering-time quantitative trait loci. *Genetics* **172**, 1867–76.

To examine the flowering time for three new *Arabidopsis thaliana* recombinant inbred lines (RIL), genomic DNA was isolated from leaves of 92 *Arabidopsis* accessions and from flower buds of the three RILs using the Wizard® Magnetic 96 DNA Plant System. The purified DNA was used for single sequence length polymorphisms (SSLP) genotyping.

PubMed Number: 16361234

G. Food DNA Isolation

Another specialized genomic DNA isolation system is the Wizard® Magnetic DNA Purification System for Food (Cat.# FF3750, FF3751). It is designed for manual purification of DNA from a variety of food samples including corn seeds, cornmeal, soybeans, soy flour and soy milk. In addition, DNA can be purified from processed food such as corn chips, chocolate and chocolate-containing foods, lecithin and vegetable oils if used with the appropriate optimized protocols. The DNA purified from many of these samples can be used in PCR-based testing for Genetically Modified Organism (GMO) DNA sequences including quantitative analysis using TaqMan® assays. As with all isolation systems using the MagneSil® PMPs, a magnetic separation stand is needed (can process from 2–12 samples). With samples containing highly processed food, the genomic DNA isolated will be fragmented and better suited for analysis using amplification rather than a Southern blot. The yield of DNA from this system will vary depending on source type and extent of food processing.

Additional Resources for Food DNA Isolation**Technical Bulletins and Manuals**

TB284 *Wizard® Magnetic DNA Purification System for Food Technical Bulletin*
(www.promega.com/tbs/tb284/tb284.html)

Promega Publications

PN076 *Wizard® Magnetic DNA Purification System for Food: Part I. DNA isolation and analysis of GMO foods by PCR*
(www.promega.com/pnotes/76/8840_14/8840_14.html)

PN076 *Wizard® Magnetic DNA Purification System for Food: Part II. Semi-automated DNA isolation and analysis of GMO foods*
(www.promega.com/pnotes/76/8840_19/8840_19.html)

Citations

Fumière, O. *et al.* (2006) Effective PCR detection of animal species in highly processed animal byproducts and compound feeds. *Anal. Bioanal. Chem.* **385**, 1045–54.

The authors developed a PCR method to detect the presence of meat and bone meal (MBM) in animal feed even if the MBM had been heat-treated, and discern whether the animal component is bovine or porcine in origin. The genomic DNA from 100mg of various feedstuffs with known and unknown amounts of MBM, fishmeal or poultry feed or a combination of these compounds was isolated using the Wizard® Magnetic DNA Purification System for Food with the KingFisher® System. Real-time PCR was performed using 5µl of extracted DNA.

PubMed Number: 16761123

H. Fixed-Tissue Genomic DNA Isolation

The MagneSil® Genomic, Fixed Tissue System (Cat.# MD1490), provides a fast, simple technique for the preparation of genomic DNA from formalin-fixed, paraffin-embedded tissue. After an overnight Proteinase K digestion, genomic DNA can be manually purified from formalin-fixed, paraffin-embedded thin tissue sections in less than an hour. Amplifiable genomic DNA can be isolated from 10µm sections without centrifugation of the lysate prior to purification. Up to 12 samples can be processed in the manual format using the MagneSphere® Technology Magnetic Separation Stand (Cat.# Z5342). One advantage this system has over other purification methods, such as phenol:chloroform extraction, is its ability to remove most inhibitors of amplification, including very small fragments of DNA. Tissue that has been stored in formalin for extended periods of time may be too cross-linked or too degraded to perform well as template for amplification. Figure 9.17 shows an amplification of 16 short tandem repeat (STR) loci and demonstrates how well the isolated DNA can work in multiplex PCR using the PowerPlex® 16 System.

The Maxwell® 16 FFPE Tissue LEV DNA Purification Kit (Cat.# AS1130) is an automated method for purifying up to 16 samples of one to ten 5µm sections of FFPE tissue samples on the Maxwell® 16 Instrument. The DNA-binding capacity of the system is limited to a few hundred nanograms of pure DNA, which is suitable for amplification applications including qPCR and multiplex PCR.

Additional Resources for Fixed-Tissue Genomic DNA Isolation

Technical Bulletins and Manuals

- TB319 *MagneSil® Genomic, Fixed Tissue System Technical Bulletin*
www.promega.com/tbs/tb319/tb319.html
- TB382 *Maxwell® 16 FFPE Tissue LEV DNA Purification Kit Technical Bulletin*
www.promega.com/tbs/tb382/tb382.html

Citations

Oliveira, L.R. *et al.* (2008) HPV infection in Brazilian oral squamous cell carcinoma patients and its correlation with clinicopathological outcomes. *Mol. Med. Reports Atenas* **1**, 123–9.

To study the frequency of human papilloma virus (HPV) in Brazilian patients with oral squamous cell carcinoma (OSCC), DNA was isolated from formalin-fixed paraffin-embedded tissue samples of primary tumors and their matched samples using the MagneSil® Genomic, Fixed Tissue System. Five microliters of genomic DNA was amplified using PCR Master Mix and primers for both a 110bp fragment of human β -globin gene and HPV

genotype. After PCR, the product was analyzed on an 8% nondenaturing polyacrylamide gel and stained with AgNO₃.

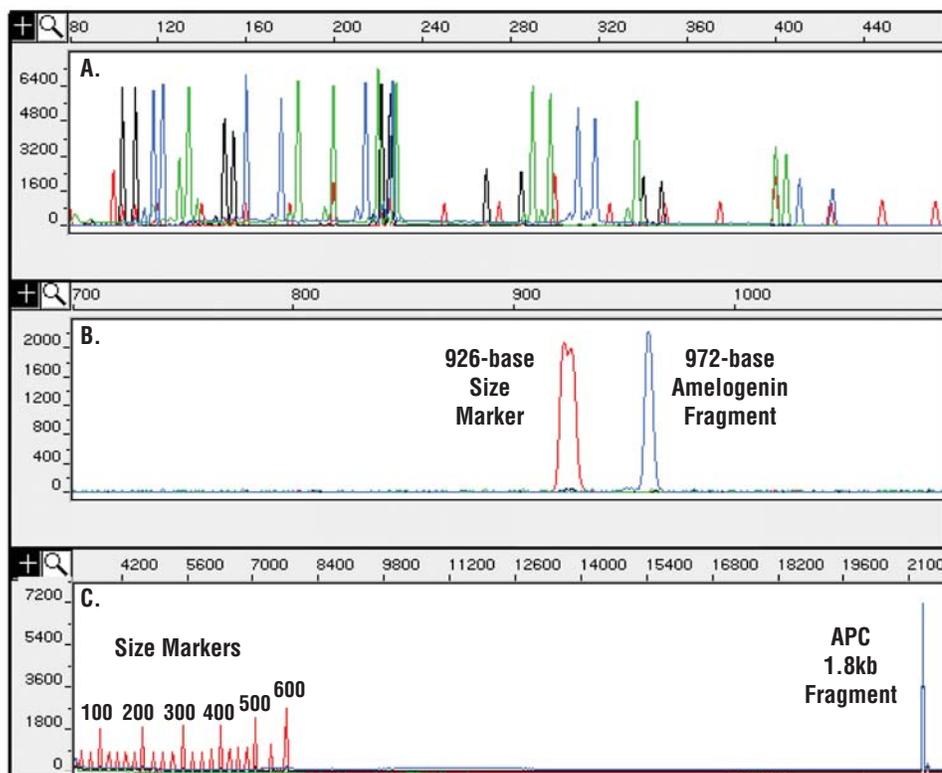


Figure 9.17. Analysis of DNA purified from paraffin-embedded, formalin-fixed 10µm thin sections using the MagneSil® Genomic, Fixed Tissue System. Purified DNA was amplified, and the amplification products were analyzed on an ABI PRISM® 310 or 3100 genetic analyzer. **Panel A.** Amplification with a set of 16 fluorescently labeled primers. Amplification products range in size from 104 to 420 bases. **Panel B.** A 972-base fragment amplified using an amelogenin primer set. **Panel C.** A 1.8kb fragment amplified from the *Adenomatosis polyposis coli* (APC) gene. Increasing the extension time during amplification may help to balance yields between small and large amplification products and increase yields for large amplification products. Results will vary depending on the degree of cross-linking due to formalin fixation.

VII. Genomic DNA Purification Protocols Featuring the Wizard® Genomic DNA Purification Kit

The Wizard® Genomic DNA Purification Kit can isolate genomic DNA from many source types. The four purification protocols detailed below can be used for whole blood, tissues, bacteria, yeast and plants. Table 9.5 lists typical yields for specific source types.

Table 9.5. DNA Yields from Various Starting Materials.

Material	Amount of Starting Material	Typical DNA Yield
Human Whole Blood	300µl	5–15µg
	1ml	25–50µg
	10ml	250–500µg
Mouse Whole Blood	300µl	6–7µg
K562 (human)	3 × 10 ⁶ cells	15–30µg
COS (African green monkey)	1.5 × 10 ⁶ cells	10µg
NIH/3T3 (mouse)	2.25 × 10 ⁶ cells	12.5µg
CHO (Chinese hamster ovary)	1–2 × 10 ⁶ cells	6–7µg
Sf9 Insect	5 × 10 ⁶ cells	16µg
Mouse Liver	11mg	15–20µg
Mouse Tail	0.5–1cm tail	10–30µg
Tomato Leaf	40mg	7–12µg
<i>Escherichia coli</i> JM109, overnight culture, ~2 × 10 ⁹ cells/ml	1ml	20µg
<i>Staphylococcus epidermis</i> , overnight culture, ~3.5 × 10 ⁸ cells/ml	1ml	6–13µg
<i>Saccharomyces cerevisiae</i> , overnight culture, ~1.9 × 10 ⁸ cells/ml	1ml	4.5–6.5µg

A. Isolation of Genomic DNA from Whole Blood

Materials Required:

- Wizard® Genomic DNA Purification Kit (Cat.# A1120)
- sterile 1.5ml microcentrifuge tubes (for 300µl blood samples)
- sterile 15ml centrifuge tubes (for 3ml blood samples)
- sterile 50ml centrifuge tubes (for 10ml blood samples)
- water bath, 37°C
- isopropanol, room temperature
- 70% ethanol, room temperature
- water bath, 65°C (optional; for rapid DNA rehydration)

Red Blood Cell Lysis

1. Using volumes from Table 9.6, combine the appropriate volumes of Cell Lysis Solution and blood. Mix by inversion.
2. Incubate for 10 minutes at room temperature.
3. Centrifuge:
≤300µl sample: 13,000–16,000 × g ; 20 seconds
1–10ml: sample 2,000 × g; 10 minutes
4. Discard supernatant. Vortex pellet.

Nuclei Lysis and Protein Precipitation

5. Using volumes from Table 9.6, add Nuclei Lysis Solution and mix by inversion.
6. Add Protein Precipitation Solution; vortex for 20 seconds.
7. Centrifuge:
≤300µl sample: 13,000–16,000 × g ; 3 minutes
1–10ml: sample 2,000 × g; 10 minutes

DNA Precipitation and Rehydration

8. Transfer supernatant to a new tube containing isopropanol (using volumes from Table 9.6). Mix by inversion.
9. Centrifuge as in Step 7.
10. Discard supernatant. Add 70% ethanol (same volume as isopropanol).
11. Centrifuge as in Step 7.
12. Aspirate the ethanol and air-dry the pellet (10–15 minutes).

Table 9.6. Solution Volumes for Whole Blood Genomic DNA Isolation.

Sample Size	Lysis Solution		Protein Precipitation Solution	Isopropanol	DNA Rehydration Solution
	Cell	Nuclei			
300µl	900µl	300µl	100µl	300µl	100µl
1ml	3ml	1ml	330µl	1ml	150µl
3ml	9ml	3ml	1ml	3ml	250µl
10ml	30ml	10ml	3.3ml	10ml	800µl

- Rehydrate the DNA in the appropriate volume of DNA Rehydration Solution for 1 hour at 65°C or overnight at 4°C.

B. Isolation of Genomic DNA from Tissue Culture Cells and Animal Tissue

Materials Required:

- Wizard® Genomic DNA Purification Kit (Cat.# A1120)
- 1.5ml microcentrifuge tubes
- 15ml centrifuge tubes
- small homogenizer (Fisher Tissue-Tearor™, Cat.# 15-338-55, or equivalent; for animal tissue)
- trypsin (for adherent tissue culture cells only)
- PBS
- 0.5M EDTA (pH 8.0; for mouse tail)
- Proteinase K [20mg/ml in water (Cat.# V3021); for mouse tail]
- water bath, 37°C
- isopropanol, room temperature
- 70% ethanol, room temperature
- water bath, 65°C (optional; for rapid DNA rehydration)

Prepare Tissues

Tissue Culture Cells: Centrifuge at 13,000–16,000 × g for 10 seconds. Wash the cell pellet with PBS, vortex and then add 600µl of Nuclei Lysis Solution and mix by pipetting.

Animal Tissue: Add 10–20mg of fresh or thawed tissue to 600µl of chilled Nuclei Lysis Solution and homogenize for 10 seconds. Alternatively, use 10–20mg of ground tissue. Incubate at 65°C for 15–30 minutes.

Mouse Tail: Add 600µl of chilled EDTA/Nuclei Lysis Solution to 0.5–1cm of fresh or thawed mouse tail. Add 17.5µl of 10mg/ml Proteinase K and incubate overnight at 55°C with gentle shaking.

Lysis and Protein Precipitation

- Add 3µl of RNase Solution to the cell or animal tissue nuclei lysate and mix. Incubate for 15–30 minutes at 37°C. Cool to room temperature.
- Add 200µl of Protein Precipitation Solution. Vortex and chill on ice for 5 minutes.
- Centrifuge at 13,000–16,000 × g for 4 minutes.

DNA Precipitation and Rehydration

- Transfer supernatant to a fresh tube containing 600µl of room temperature isopropanol.
- Mix gently by inversion.
- Centrifuge at 13,000–16,000 × g for 1 minute.
- Remove supernatant and add 600µl of room temperature 70% ethanol. Mix.
- Centrifuge as in Step 6.
- Aspirate the ethanol and air-dry the pellet for 15 minutes.

- Rehydrate the DNA in 100µl of DNA Rehydration Solution for 1 hour at 65°C or overnight at 4°C.

For additional protocol information, see Technical Manual #TM050 (www.promega.com/tbs/tm050/tm050.html).

C. Isolation of Genomic DNA from Gram-Positive and Gram-Negative Bacteria

Materials Required:

- Wizard® Genomic DNA Purification Kit (Cat.# A1120)
- 1.5ml microcentrifuge tubes
- water bath, 80°C
- water bath, 37°C
- isopropanol, room temperature
- 70% ethanol, room temperature
- water bath, 65°C (optional; for rapid DNA rehydration)
- 50mM EDTA (pH 8.0; for Gram-positive bacteria)
- 10mg/ml lysozyme (Sigma Cat.# L7651; for Gram-positive bacteria)
- 10mg/ml lysostaphin (Sigma Cat.# L7386; for Gram-positive bacteria)

Pellet Cells

Centrifuge 1ml of overnight culture for 2 minutes at 13,000–16,000 × g. Discard the supernatant.

For Gram-Positive Bacteria

- Suspend cells in 480µl 50mM EDTA.
- Add lytic enzyme(s) [120µl (lysozyme and/or lysostaphin)].
- Incubate at 37°C for 30–60 minutes.
- Centrifuge for 2 minutes at 13,000–16,000 × g and remove supernatant.
- Go to Step 1, **Lyse Cells** (below).

For Gram-Negative Bacteria

Go to Step 1, **Lyse Cells** (below).

Lyse Cells

- Add 600µl Nuclei Lysis Solution. Pipet gently to mix.
- Incubate for 5 minutes at 80°C, then cool to room temperature.
- Add 3µl of RNase Solution. Mix, incubate at 37°C for 15–60 minutes, then cool to room temperature.

Protein Precipitation

- Add 200µl of Protein Precipitation Solution. Vortex.
- Incubate on ice for 5 minutes.
- Centrifuge at 13,000–16,000 × g for 3 minutes.

DNA Precipitation and Rehydration

- Transfer the supernatant to a clean tube containing 600µl of room temperature isopropanol. Mix by inversion.

8. Centrifuge as in "Pellet Cells" above, and decant the supernatant.
9. Add 600µl of room temperature 70% ethanol. Mix.
10. Centrifuge for 2 minutes at 13,000–16,000 × g.
11. Aspirate the ethanol and air-dry the pellet for 10–15 minutes.
12. Rehydrate the DNA pellet in 100µl of Rehydration Solution for 1 hour at 65°C or overnight at 4°C.

D. Isolation of Genomic DNA from Yeast Cultures or Plant Tissue

Materials Required:

- Wizard® Genomic DNA Purification Kit (Cat.# A1120)
- 1.5ml microcentrifuge tubes
- water bath, 37°C
- isopropanol, room temperature
- 70% ethanol, room temperature
- water bath, 65°C (optional; for rapid DNA rehydration)
- microcentrifuge tube pestle or mortar and pestle (for plant tissue)
- YPD broth (for yeast)
- 50mM EDTA (pH 8.0; for yeast)
- 20mg/ml lyticase (Sigma Cat.# L2524; for yeast)

Prepare Yeast Lysate

1. Pellet cells from 1ml of culture by centrifugation at 13,000–16,000 × g for 2 minutes.
2. Suspend the cell pellet in 293µl of 50mM EDTA.
3. Add 7.5µl of 20mg/ml lyticase and mix gently.
4. Incubate for 30–60 minutes at 37°C. Cool to room temperature.
5. Centrifuge as in Step 1. Discard the supernatant.
6. Add 300µl of Nuclei Lysis Solution. Proceed to **Protein Precipitation and DNA Rehydration** Table 9.7, Step 1.

Prepare Plant Lysate

1. Grind approximately 40mg of leaf tissue in liquid nitrogen.
2. Add 600µl of Nuclei Lysis Solution. Incubate at 65°C for 15 minutes.
3. Add 3µl of RNase Solution. Incubate at 37°C for 15 minutes. Cool sample to room temperature for 5 minutes. Proceed to **Protein Precipitation and DNA Rehydration** Table 9.7, Step 1.

Table 9.7. Protein Precipitation and DNA Rehydration.

	Yeast	Plant
1. Add Protein Precipitation Solution. Vortex. For yeast only: Incubate 5 minutes on ice.	100µl	200µl
2. Centrifuge at 13,000–16,000 × g.	3 minutes	3 minutes
3. Transfer supernatant to clean tube containing room temperature isopropanol.	300µl	600µl
4. Mix by inversion and centrifuge at 13,000–16,000 × g.	2 minutes	1 minute
5. Decant supernatant and add room temperature 70% ethanol.	300µl	600µl
6. Centrifuge at 13,000–16,000 × g.	2 minutes	1 minute
7. Aspirate the ethanol and air-dry the pellet.		
8. Add DNA Rehydration Solution.	50µl	100µl
9. For yeast only: Add RNase. Incubate at 37°C for 15 minutes.	1.5µl	—
10. Rehydrate at 65°C for 1 hour or overnight at 4°C.		

Additional Resources for the Wizard® Genomic DNA Purification Kit

Technical Bulletins and Manuals

TM050 *Wizard® Genomic DNA Purification Kit Technical Manual*
www.promega.com/tbs/tm050/tm050.html

Online Tools

Sample Types Processed with the Wizard® Genomic DNA Purification Kit (www.promega.com/techserv/apps/dna_rna/wizgensampletypes.htm)

VII. Specialized Genomic DNA Purification Protocol Featuring the MagneSil® Genomic, Large Volume System

This overview describes the automated liquid-handling and purification steps required for genomic DNA isolation using the MagneSil® Genomic, Large Volume System. This protocol can be performed either manually using an e-protocol available from Promega or on an automated liquid-handling workstation, such as the Tecan Freedom EVO® Instrument. Shaker speeds and times will be set automatically by the automated method or the e-protocol.

The protocol below describes use of an IKA Works KS 130 Control Shaker. This is the only shaker currently validated for use with the MagneSil® Genomic, Large Volume System. For optimal mixing performance, the shaker must have a 4mm orbit. Use of a shaker other than the IKA Works KS

130 Control Shaker will result in inefficient MagneSil® PMP washing and poor genomic DNA purification. During all mixing steps, the shaker should generate a vortex to ensure efficient mixing and washing of the MagneSil® PMPs.

Materials Required:

- MagneSil® Genomic, Large Volume System (Cat.# A4080)
- Tecan Freedom EVO® liquid-handling instrument
- MagnaBot® Large Volume Magnetic Separation Device (Cat.# V3471)
- Tube Holder, 50ml Tubes (Cat.# Z3631)
- Heat Block Insert (Cat.# Z3651)
- Heat Block Adapter, 50ml Tubes (Cat.# Z3661)
- Shaker Top Adaptor (Cat.# Z3671)
- heat block set to 90–95°C (Fisher Cat.# 11-718-2 or 11-715-125D; VWR Cat.# 13259-032 or 13259-052)
- IKA KS 130 Control Orbital Shaker (IKA Works Cat.# 2980100; **no other** shaker can substitute)
- 50ml conical tubes (1 per purification; Corning® 50ml orange-cap tubes recommended)
- ethanol (95%)
- isopropanol (99%)

A. Sample Lysis and DNA Binding

1. Place the empty, uncapped 50ml conical tubes into the 50ml tube holder.
2. Add 1–10ml of sample to the 50ml conical tubes in the tube holder and place the tube holder onto the shaker.
3. Add the indicated volume of eLysis Buffer to the sample, depending on the sample starting volume (Table 9.8) and shake at 600rpm for 1.5 minutes to lyse the samples.

Table 9.8. Volume of eLysis Buffer and MagneSil® PMPs to Add to Various Volumes of Whole Blood Sample.

Sample Volume	Volume of eLysis Buffer	Volume of MagneSil® PMPs
1ml	0.9ml	0.35ml
2ml	1.8ml	0.7ml
3ml	2.7ml	1.05ml
4ml	3.6ml	1.4ml
5ml	4.5ml	1.75ml
6ml	5.4ml	2.1ml
7ml	6.3ml	2.45ml
8ml	7.2ml	2.8ml
9ml	8.1ml	3.15ml
10ml	9ml	3.5ml

4. Thoroughly resuspend the MagneSil® PMPs by vigorously shaking the bottle by hand. Add the indicated volume of thoroughly resuspended MagneSil® PMPs (Table 9.8) to the lysed sample.

5. Start the shaker and shake at 600rpm for 30 seconds. After 30 seconds, reduce the shaker speed to 400rpm. Shake at 400rpm for 4 minutes.
6. Stop the shaker. Remove the 50ml tube holder containing the sample lysate and MagneSil® PMPs and place onto the magnetic base.
7. Wait for 4 minutes to capture the MagneSil® PMPs. Remove supernatant and discard.

B. Sample Washing

Combined eLysis Buffer/Alcohol Wash #1

1. Remove the 50ml tube holder from the magnetic base and place on the shaker.
2. Add 4.5ml of eLysis Buffer to each 50ml conical tube containing the MagneSil® PMPs.
3. Add 1.5ml of Alcohol Wash Solution (ethanol and isopropanol added) to each 50ml conical tube containing MagneSil® PMPs and eLysis Buffer.
4. Shake at 700rpm for 30 seconds.
5. Stop the shaker. Remove the 50ml tube holder containing the MagneSil® PMPs and wash solutions and place onto the magnetic base.
6. Wait for 1 minute to capture the MagneSil® PMPs. Remove supernatant and discard.

Combined eLysis Buffer/Alcohol Wash #2

7. Remove the 50ml tube holder from the magnetic base and place on the shaker.
8. Add 3ml of eLysis Buffer to each 50ml conical tube containing the MagneSil® PMPs.
9. Add 3ml of Alcohol Wash Solution (ethanol and isopropanol added) to each 50ml conical tube containing MagneSil® PMPs and eLysis Buffer.
10. Shake at 700rpm for 30 seconds.
11. Stop the shaker. Remove the 50ml tube holder containing the MagneSil® PMPs and wash solutions and place onto the magnetic base.
12. Wait for 1 minute to capture the MagneSil® PMPs. Remove supernatant and discard.
13. Repeat the combined eLysis Buffer/Alcohol Wash #2 for a total of 3 wash steps.

Combined eLysis Buffer/Alcohol Wash #3

14. Remove the 50ml tube holder from the magnetic base and place on the shaker.
15. Add 9ml of Alcohol Wash Solution (ethanol and isopropanol added) to each 50ml conical tube containing MagneSil® PMPs and shake at 750rpm for 30 seconds.
16. Stop the shaker. Remove the 50ml tube holder containing the MagneSil® PMPs and wash solutions and place onto the magnetic base.
17. Wait for 1 minute to capture the MagneSil® PMPs. Remove supernatant and discard.
18. Repeat for a total of 3 alcohol washes.

C. Elution of Purified Genomic DNA

1. Ensure that all the Alcohol Wash Solution has been aspirated away from the MagneSil® PMPs. Do not dry the MagneSil® PMPs.
2. Remove the 50ml tube holder from the magnetic base and place on the shaker.
3. Add 2.5ml room temperature Elution Buffer to the tubes containing the MagneSil® PMPs. Shake at 800rpm for 2 minutes.
Note: Elution volume may be adapted to meet your requirements [see Technical Bulletin #TB549 (www.promega.com/tbs/tb549/tb549.html)].
4. Stop the shaker. Remove the 50ml tube holder containing the MagneSil® PMPs and Elution Buffer to a heat block (with heat block adapter) set to 90–95°C. Heat the sample on the heat block for 15 minutes.
5. Remove the 50ml tube holder containing the MagneSil® PMPs and Elution Buffer from the heat block and place back onto the shaker.
6. Shake at 800rpm for 2 minutes.
7. Stop the shaker. Remove the 50ml tube holder containing the MagneSil® PMPs and Elution Buffer from the shaker back onto the heat block. Heat the sample for 5 minutes.
8. Repeat Steps 5 through 7 of Elution of Purified Genomic DNA for a total of two cycles of 5 minutes of heating followed by 2 minutes of shaking.
9. After the final shaking step, remove the 50ml tube holder containing the MagneSil® PMPs and Elution Buffer from the shaker and place onto the magnetic base.
10. Wait for 5 minutes or until all the MagneSil® PMPs are captured to the side of the tubes.

11. Slowly aspirate supernatant containing purified genomic DNA to a new tube.
12. Repeat the elution procedure (Steps 1–11) for maximal yield.

Additional Resources for the MagneSil® Genomic, Large Volume System**Technical Bulletins and Manuals**

TB549 *MagneSil® Genomic, Large Volume System Technical Bulletin*
(www.promega.com/tbs/tb549/tb549.html)

Promega Publications

PN090 *MagneSil® Genomic, Large Volume System, for large-sample genomic DNA isolation*
(www.promega.com/pnotes/90/12727_22/12727_22.html)

IX. Fragment/PCR Product Purification Systems

Generally, purification of DNA fragments or PCR products does not involve disruption of cellular structures but rather separation of DNA from in vitro reactions or agarose gel slices. In many cases, after a PCR amplification or restriction enzyme digestion, the reaction components include protein and salts that may inhibit subsequent applications and will need to be removed from the DNA fragments. An agarose gel may be run to isolate a fragment of the correct size if there is more than one product present. Fragment DNA purification can improve efficiency in subsequent reactions.

For example, PCR products can be used directly in T-vector cloning. However, nonspecific products and primer dimers can compete for ligation with the desired PCR product, resulting in low frequency of positive clones. Also, removing the reaction components prior to sequencing will ensure the right primers are used for sequencing and the fluorescently labeled nucleotides are not competing with the unlabeled dNTPs remaining from the PCR amplification.

A. Wizard® SV Gel and PCR Clean-Up System

The Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281, A9282) is designed to extract and purify DNA fragments of 100bp to 10kb from standard or low-melting point agarose gels in either Tris acetate (TAE) or Tris borate (TBE) buffer, or to purify PCR products directly from an amplification reaction, using the SV silica membrane column. This purification kit is a single column system that can be used with a vacuum manifold [e.g., Vac-Man® Laboratory Vacuum Manifold (Cat.# A7231)] or a standard microcentrifuge. Up to 95% recovery is achieved, depending upon the DNA fragment size (see Table 9.9). PCR products are commonly purified to remove excess nucleotides, primers and PCR additives like DMSO and betaine (Table 9.10). This membrane-based system, which can bind up to 40µg DNA, allows recovery of isolated DNA fragments or PCR products in as little as 20 minutes,

depending on the number of samples processed and the protocol used. The purified DNA can be used for automated fluorescent DNA sequencing, cloning, labeling, restriction enzyme digestion or in vitro transcription/translation without further manipulation.

Table 9.9. Percent Recovery Versus Double-Stranded DNA Fragment Size Using the Wizard® SV Gel and PCR Clean-Up System.

DNA Fragment Size	Percent Recovery
55bp	26%
70bp	39%
85bp	55%
100bp	84%
500bp	89%
1,000bp	92%
3,199bp	95%
9,416bp	95%
23,130bp	47%

Table 9.10. Effect of Various PCR Additives on Percent Recovery of a 1,000bp PCR Product Using the Direct Purification Method and the Wizard® SV Gel and PCR Clean-Up System.

PCR Additive	Percent Recovery ¹
no additive	100%
1M betaine	94%
1M Q-Solution	97%
0.1% Triton® X-100	92%
0.1% Tween®-20	87%
0.1% NP-40	82%
5% glycerol	87%
5% formamide	90%
5% DMSO	87%
0.5M tetramethylene sulfoxide	94%
0.4M sulfolane	94%
0.4M 2-pyrrolidone	95%
1mM tartrazine	100%
1% Ficoll®-400	100%

¹Percent recovery shown is relative to the “no additive” recovery. For direct purification from a reaction, note that any nucleic acid present in solution will be isolated. Therefore, if an amplification reaction has more than one product, all fragments will be present in the eluted DNA. If you are interested in isolating a single amplicon, separate the reaction products on an agarose gel and cut out the band desired prior to purification.

When purifying DNA from an agarose slice, the primary consideration is to melt the agarose so the DNA is available for binding to the silica membrane. The purified DNA can then be used for cloning or sequencing.

Additional Resources for the Wizard® SV Gel and PCR Clean-Up System

Technical Bulletins and Manuals

TB308 *Wizard® SV Gel and PCR Clean-Up System Technical Bulletin*
(www.promega.com/tbs/tb308/tb308.html)

Promega Publications

PN082 *Wizard® SV Gel and PCR Clean-Up System*
(www.promega.com/pnotes/82/10203_02/10203_02.html)

eNotes *Removal of ethidium bromide and calf intestinal alkaline phosphatase using the Wizard® SV Gel and PCR Clean-Up System*
(www.promega.com/enotes/applications/ap0045_tabs.htm)

Citations

Suzuki, A. *et al.* (2006) NDR2 acts as the upstream kinase of ARK5 during insulin-like growth factor-1 signaling. *J. Biol. Chem.* **281**, 13915–21.

A deletion mutation of the serine/threonine protein kinase NDR2 was created by PCR using two mutagenesis primers and two plasmid-based primers of the clone. After amplification, the two products were run on a 1% agarose gel and extracted using the Wizard® SV Gel and PCR Clean-Up System. The purified fragments were mixed, annealed, reamplified and then digested prior to cloning into an expression vector. The human colorectal cancer cell lines HCT-116, DLD-1, and SW480 used in the study were seeded into a 24-well plate at 5×10^4 cells/well and transfected using the TransFast™ Transfection Reagent. The transfection was assessed with a green fluorescent protein expression vector.

PubMed Number: 16488889

B. Wizard® SV 96 PCR Clean-Up System

To purify 96 amplification reactions at once, use the Wizard® SV 96 PCR Clean-Up System (Cat.# A9340, A9341, A9342, A9345) with a 96-well vacuum manifold (Vac-Man® 96 Vacuum Manifold) and a vacuum pump capable of generating 15–20 inches of mercury or the equivalent. This system is designed to purify 100bp to 10kb PCR products directly from a reaction with typical recovery >90% as seen in Figure 9.18.

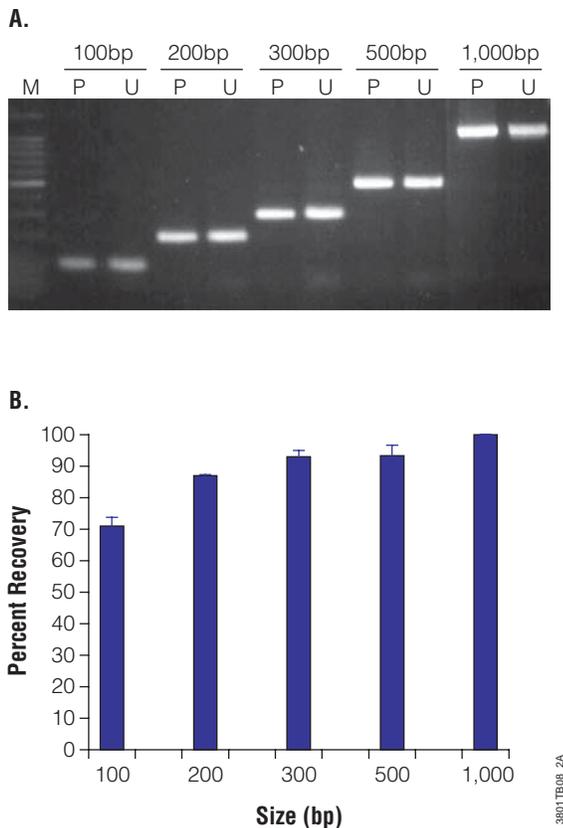


Figure 9.18. Purification and recovery of PCR products using the Wizard® SV 96 PCR Clean-Up System. PCR fragments of 100, 200, 300, 500 and 1,000 base pairs were purified using the Wizard® SV 96 PCR Clean-Up System on the Biomek® 2000 robotic workstation. **Panel A.** Agarose gel analysis. Purified (P) and unpurified (U) fragments were separated on an ethidium bromide-stained, 2% agarose gel. **Panel B.** Percent recovery of purified PCR products. Percent recovery was quantitated using a Hitachi FMBIO® Fluorescent Scanner. Results show the mean and standard deviation for 6 purified fragments of each size. For small PCR fragments (<500bp), optimal recovery requires a 95% ethanol wash. For larger fragments (>500bp), optimal results are achieved using an 80% ethanol wash.

The technology is the same as the single-column system, utilizing the SV silica membrane and chaotropic salts to purify the nucleotides and primers from the PCR product(s). This system allows recovery of 96 PCR fragments in as little as 20 minutes in multiwell plate format. The DNA can be used for automated fluorescent DNA sequencing, cloning, labeling, restriction enzyme digestion or DNA microarray analysis without further manipulation.

Additional Resources for the Wizard® SV 96 PCR Clean-Up System

Technical Bulletins and Manuals

- TB311 *Wizard® SV 96 PCR Clean-Up System Technical Bulletin*
(www.promega.com/tbs/tb311/tb311.html)

Promega Publications

- PN082 *Introducing the Wizard® SV 96 PCR Clean-Up System*
(www.promega.com/pnotes/82/10203_06/10203_06.html)

Citations

Nagase, T. *et al.* (2008) Exploration of human ORFeome: High-throughput preparation of ORF clones and efficient characterization of their protein products. *DNA Research* **15**, 137–49.

To clone ORFs into the Flexi® Vector System, 1–4ng of a plasmid containing the ORF was amplified and the Wizard® SV 96 PCR Clean-Up System was used for purifying the PCR products. The cleaned up amplicons were then digested with SgfI and PmeI prior to cloning into the pF1K T7 Flexi® Vector.

PubMed Number: 18316326

C. BigDye® Sequencing Clean-Up

Designed for BigDye® sequencing reaction clean-up, the Wizard® MagneSil® Sequencing Reaction Clean-Up System (Cat.# A1830, A1831, A1832, A1835) can be placed on a robotic platform and purified using the MagneSil® PMPs to clean up sequencing reaction products prior to analysis. We have developed procedures for use on several robotic workstations with standard 96- and 384-well amplification plates. The Plate Clamp 96 (Cat.# V8251) is recommended for automated protocols and is designed to ensure PCR plates are uniformly flat for liquid transfer on a robotic platform. No user intervention is required from the time the multiwell plates are placed on the robot deck until the samples are loaded onto the DNA sequencer. For further information on robotic platforms and required hardware, visit: Automated Methods (www.promega.com/automethods/).

Additional Resources for the Wizard® MagneSil® Sequencing Reaction Clean-Up System

Technical Bulletins and Manuals

- TB287 *Wizard® MagneSil® Sequencing Reaction Clean-Up System Technical Bulletin*
(www.promega.com/tbs/tb287/tb287.html)

Promega Publications

- PN085 *High-throughput DNA fragment purification using the MagneSil® Automated 384-Well Clean-Up Systems*
(www.promega.com/pnotes/85/10904_03/10904_03.html)

Citations

O'Leary, V.B. *et al.* (2005) Screening for new MTHFR polymorphisms and NTD risk. *Am. J. Med. Genet.* **138A**, 99–106.

The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) has at least one polymorphism that is a neural

tube defect (NTD) risk factor within the Irish population. To survey for common variations in MTHFR, genomic DNA was extracted from blood, and exons 1–11 of MTHFR were amplified and sequenced with BigDye® Terminator mix. The Wizard® MagneSil® Sequencing Reaction Clean-Up System was used to purify the sequencing reactions prior to analysis on an ABI PRISM® 377 DNA sequencer.

PubMed Number: 16145688

X. Fragment/PCR Product Purification Protocol Featuring the Wizard® SV Gel and PCR Clean-Up System

Materials Required:

- Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281)
- 1.5ml microcentrifuge tubes
- ethanol (95%)
- Vacuum Adapters (Cat.# A1331; only for vacuum purification)
- agarose gel (standard or low-melting point; only for gel purification)
- 1X TAE or TBE electrophoresis buffer (only for gel purification)
- 50–65°C heating block (only for gel purification)

A. Preparing the Membrane Wash Solution

Add the indicated volume of 95% ethanol to the Membrane Wash Solution prior to beginning the procedure (see Table 9.11). Mark the bottle label to record that this addition was made. Tightly close the bottle cap after each use to prevent evaporation.

Table 9.11. Volume of 95% Ethanol to Add to Membrane Wash Solution for Each System Size.

System Size	Volume of 95% Ethanol
10 preps	15ml
50 preps	75ml
250 preps	375ml

B. DNA Purification by Centrifugation

Gel Slice and PCR Product Preparation

Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
2. Add 10µl of Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65°C until gel slice is completely dissolved.

Processing PCR Amplifications

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA

1. Insert SV Minicolumn into Collection Tube.
2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

Note: If the volume of the DNA solution is >700µl, repeat Steps 2 and 3, transferring ≤700µl until all the solution has been processed.

Washing

4. Add 700µl of Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5. Repeat Step 4 with 500µl of Membrane Wash Solution. Centrifuge at 16,000 × g for 5 minutes.
6. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

7. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
8. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × g for 1 minute.
9. Discard Minicolumn and store DNA at 4°C or –20°C.

C. DNA Purification by Vacuum

Gel Slice and PCR Product Preparation

Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
2. Add 10µl of Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65°C until gel slice is completely dissolved.

Processing PCR Amplifications

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA

1. Attach Vacuum Adapter to manifold port and insert SV Minicolumn into Adapter.
2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn. Incubate at room temperature for 1 minute.

- Apply vacuum to pull liquid through Minicolumn. Release vacuum when all liquid has passed through Minicolumn.

Washing

- Add 700µl of Membrane Wash Solution (ethanol added). Apply a vacuum to pull solution through Minicolumn.
- Turn off vacuum and repeat Step 4 with 500µl of Membrane Wash Solution. Apply a vacuum to pull solution through Minicolumn.
- Transfer Minicolumn to a Collection Tube. Centrifuge at 16,000 × g for 5 minutes.
- Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

- Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
- Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × g for 1 minute.
- Discard Minicolumn and store DNA at 4°C or -20°C.

XI. Composition of Solutions

LB (Luria-Bertani) medium (1 liter)

- 10g Bacto®-tryptone
- 5g Bacto®-yeast extract
- 5g NaCl

Adjust pH to 7.5 with NaOH. Autoclave.

LB-Miller medium (1 liter)

- 10g Bacto®-tryptone
- 5g Bacto®-yeast extract
- 10g NaCl

Adjust pH to 7.0 with NaOH. Autoclave.

Membrane Wash Solution

(Wizard® SV Gel and PCR Clean-Up System)

- 10mM potassium acetate (pH 5.0)
- 80% ethanol (after ethanol addition)
- 16.7µM EDTA (pH 8.0)

To prepare this solution, add 95% ethanol to the supplied Membrane Wash Solution (concentrated) as described in Table 9.11 in the Fragment/PCR Product Purification protocol section.

Membrane Binding Solution (Wizard® SV Gel and PCR Clean-Up System)

- 4.5M guanidine isothiocyanate
- 0.5M potassium acetate (pH 5.0)

1X TE buffer

- 10mM Tris-HCl (pH 7.5)
- 1mM EDTA (pH 8.0)

1X TBE buffer

- 89mM Tris base
- 89mM boric acid
- 2mM EDTA (pH 8.0)

1X TAE buffer

- 40mM Tris base
- 5mM sodium acetate
- 1mM EDTA (pH 8.0)

Terrific Broth (1 liter)

- 12g Bacto®-tryptone
- 24g Bacto®-yeast extract
- 4ml glycerol

Add components to 900ml deionized water. Autoclave and allow solution to cool to ~60°C. Add 100ml of a sterile solution of 0.17M KH₂PO₄, 0.72M K₂HPO₄ and mix to disperse evenly.

0.17M KH₂PO₄, 0.72M K₂HPO₄ sterile solution

- 2.31g KH₂PO₄
- 12.54g K₂HPO₄

Dissolve in 90ml deionized water. Adjust volume to 100ml and sterilize by autoclaving.

YPD broth (1 liter)

- 10g yeast extract
- 20g peptone
- 20g dextrose

Autoclave. Final pH 6.5±0.2 at 25°C.

2X YT medium (1 liter)

- 16g Bacto®-tryptone
- 10g Bacto®-yeast extract
- 5g NaCl

Adjust pH to 7.0 with NaOH. Autoclave.

XII. References

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I. Introduction

Researchers are increasingly adding imaging analyses to their repertoire of experimental methods for understanding the structure and function of biological systems. New methods and instrumentation for imaging have improved resolution, signal detection, data collection and manipulation for virtually every sample type. Live-cell and in vivo imaging have benefited from the availability of reagents such as vital dyes that have minimal toxicity, the discovery of intrinsically fluorescent proteins (IFPs; Stephens and Allan, 2003; Sullivan and Kay, 1999) and the development of imaging systems that do not damage biological samples (Stephens and Allan, 2003). Techniques such as fluorescent analog chemistry have allowed scientists to study the cytoskeleton in living cells, to follow the recycling of cell-surface components, and to look at movement and distribution of cellular proteins (Wang, 1989). Additionally, techniques for culturing cells on the microscope stage and maintaining a constant focal plane continue to improve (Stephens and Allan, 2003; McKenna and Wang, 1989) so that cellular processes can be followed in real time. New technologies such as magnetic resonance imaging have allowed in vivo imaging of processes, such as brain activity, in whole organisms (Check, 2005).

Here we present a summary of reagents that researchers can use for imaging studies in virtually any field of investigation. HaloTag® technology allows researchers to image live cells, perform multicolor imaging experiments and immunocytochemistry, or potentially immunohistochemistry. We also present the Monster Green® Fluorescent Protein, an engineered intrinsically fluorescent protein (IFP) with improved signal and expression for imaging studies in mammalian cells. Additionally, we describe antibody reagents that label protein markers of specific cell types, such as the Anti-βIII Tubulin mAb. For researchers dissecting apoptosis, imaging reagents for multiple points in the pathway are also discussed, including a phosphorylation-specific antibody to caspase-3, an antibody against the p85-fragment of PARP, and an anti-cytochrome c. Finally, we present phosphorylation-specific antibodies for studying a variety of cell-signaling pathways.

II. HaloTag® Technology

A. Overview

HaloTag® Technology provides new options for rapid, site-specific labeling of HaloTag® fusion proteins in living cells and in vitro [Animation (www.promega.com/paguide/animation/selector.htm?coreName=halotag01)]. The technology is based on the formation of a covalent bond between the HaloTag® protein and synthetic ligands that carry a variety of functionalities, including fluorescent labels, affinity tags and attachments to solid supports. The covalent bond forms rapidly under physiological conditions, is highly specific and essentially irreversible, yielding a complex that is stable even under denaturing conditions. Therefore, using HaloTag® requires only a

single fusion construct that can be expressed, labeled with any of a variety of fluorescent moieties, and imaged in live or fixed cells.

Components of the HaloTag® Technology

The HaloTag® protein is a genetically engineered derivative of a hydrolase that efficiently forms a covalent bond with the HaloTag® Ligands (Figure 10.1). This 34kDa monomeric protein can be used to generate N- or C-terminal fusions that can be expressed in a variety of cell types. Since the HaloTag® protein is rare and of prokaryotic origin, endogenous activity is absent from *E.coli*, plant and mammalian cells. This technology has been used successfully in many systems ranging from plant to human stem cells (Eisenstein, 2006; Zhang, 2006; Lang, 2006; and Shinohara and Matsubayahshi, 2007).

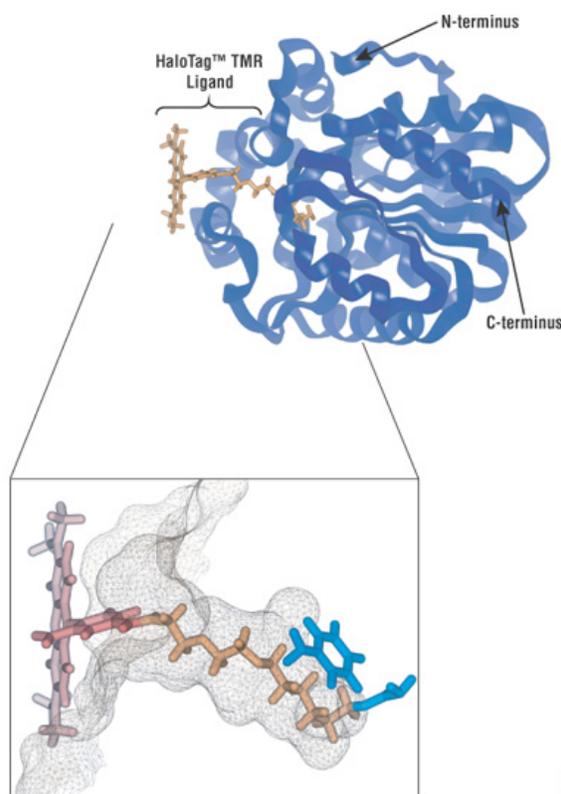


Figure 10.1. Molecular model of the HaloTag® protein with a covalently bound HaloTag® TMR Ligand. Overview of the protein structure (top right) with close-up of the ligand tunnel outlined by a mesh Connolly surface (lower left). The HaloTag® TMR Ligand (fluorescent moiety in red, reactive linker in orange) is shown covalently bound to the aspartate nucleophile (shown in blue). Replacement of the catalytic base (histidine) with a phenylalanine (also shown in blue) makes the protein incapable of hydrolyzing the formed covalent bond, leading to the formation of a stable covalent bond.

A variety of HaloTag® Flexi® Vectors for mammalian, *E. coli* and cell-free protein expression are available. These vectors are compatible with the Flexi® Vector System, a directional cloning method for protein-coding sequences. The Flexi® Vector System provides a rapid, efficient and

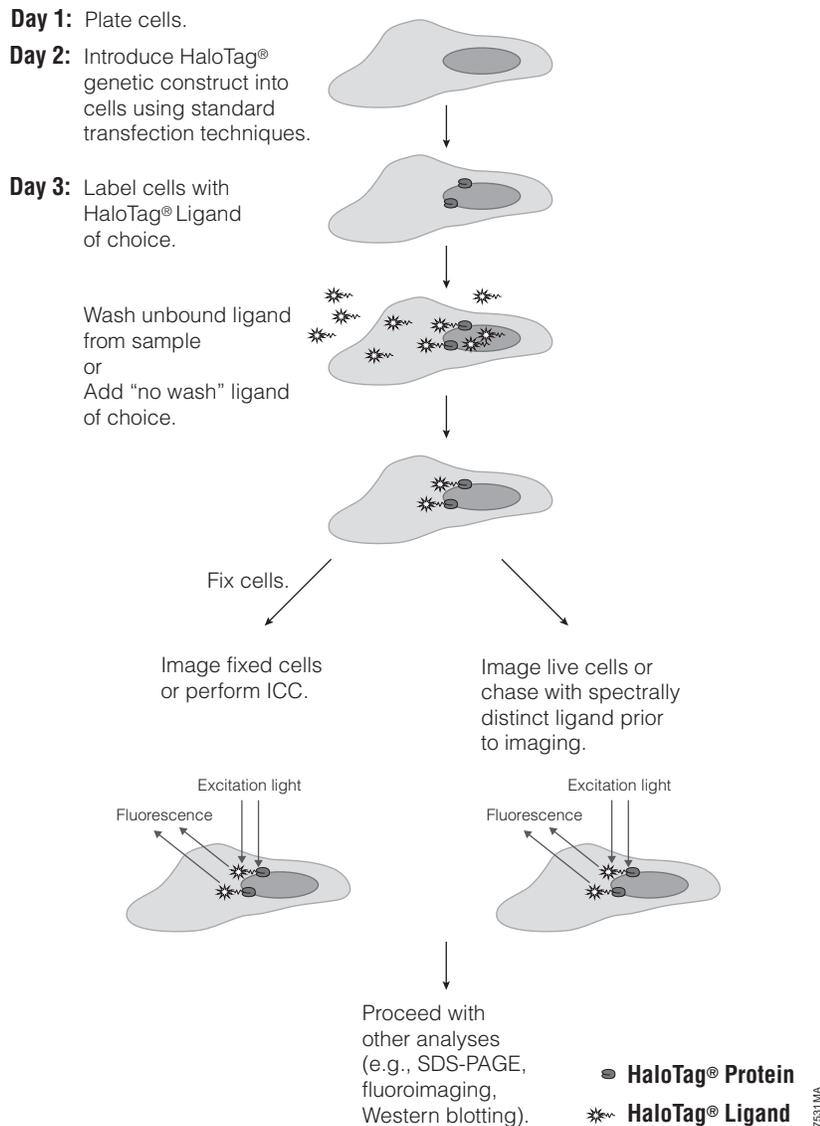


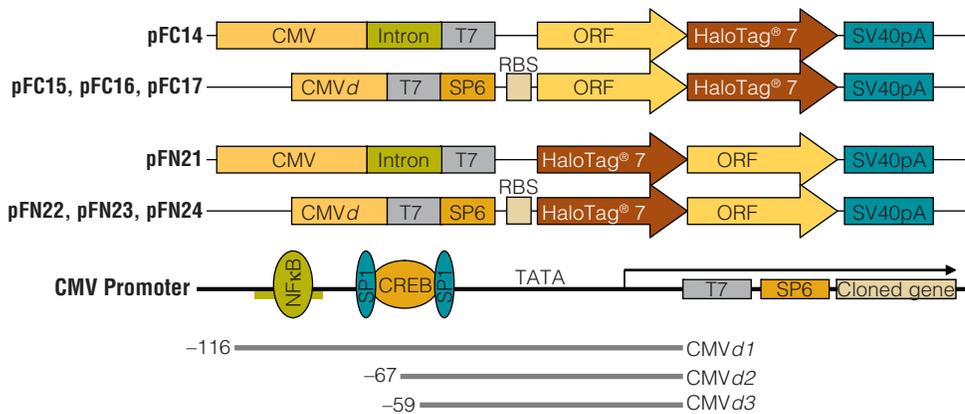
Figure 10.2. Overview of protocol for live-cell and fixed-cell imaging using the HaloTag® Technology. This technology allows labeling and analysis of HaloTag® fusion proteins by live- or fixed-cell imaging, gel-based fluoroimaging, Western blotting and capture of HaloTag® fusion proteins for proteomics applications. Note that, although this technology provides multiple alternatives to the use of antibodies, the Anti-HaloTag® pAb adds further flexibility by enabling immunocytochemistry and Western blot analysis.

high-fidelity way to transfer these sequences between a variety of Flexi® Vectors. These vectors contain various expression or peptide tag options to enable expression of native or fusion proteins for studies of protein structure and function or protein:protein or protein:DNA interactions.

We developed the HaloTag® 7 Flexi® Vector CMV deletion series, a series of human CMV promoter deletions that offer different constitutive expression levels for HaloTag® fusion proteins (Figure 10.2). This allows researchers to express just enough protein for their application. The vectors are available with kanamycin or ampicillin resistance markers, and vectors are available for generating either N-terminal or C-terminal fusions to the HaloTag® 7 protein. For more

information on HaloTag® Flexi® Vectors see the Promega Notes article (www.promega.com/pnotes/100/16620_13/16620_13.html).

HaloTag® Ligands are small chemical tags that are capable of covalently binding the HaloTag® protein. These ligands contain two crucial components: 1) a common HaloTag® Reactive Linker that ensures formation of a covalent bond with the HaloTag® protein, and 2) a functional group : fluorescent or affinity tag. The HaloTag® Ligands for imaging contain fluorophores that span the visible spectrum and include cell-permeant and impermeant options.



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Figure 10.3. The HaloTag® 7 Flexi® Mammalian Vectors This series of vectors for creating N-terminal or C-terminal HaloTag® fusion proteins gives researchers the ability to choose a vector with the ideal expression levels for their experimental needs. The vectors are available with either Kanamycin or Ampicillin resistance. For more details see the Promega Notes (www.promega.com/pnotes/100/16620_16/16620_16.html) article.

B. Live-Cell Imaging Using the HaloTag® Technology

With the HaloTag® technology, researchers clone their gene of interest into a HaloTag® Flexi® vector. When expressed this fusion protein can be labeled specifically and efficiently with a variety of spectrally distinct fluorescent tags. These small tags, called HaloTag® Ligands, are comprised of a linker that covalently binds to the HaloTag® protein and a fluorescent moiety. Importantly the HaloTag® protein and these ligands are completely nontoxic to cells. The HaloTag® protein itself has no endogenous eukaryotic equivalent and does not interfere with the proper cellular functioning of fusion partners.

The HaloTag® labeling technology for imaging offers a quick and simple way to label expressed HaloTag® fusions within live cells. Using this strategy, termed “Rapid” Labeling, the HaloTag® fusion can be labeled with any of a variety of cell-permeant ligands and/or an impermeant one. During the recommended short incubation in the presence of ligand, cell-permeant ligands freely enter cells and their subcellular compartments, covalently attaching to the HaloTag® fusion protein (Figure 10.4). A subsequent wash step allows the unbound ligand to exit cells, resulting in a highly specific signal with very low background noise (Figure 10.4).

C. Live-Cell Labeling (Rapid Protocol)

This protocol is intended for labeling live cells with the cell-permeant HaloTag® TMR, diAcFAM, Oregon Green®, or Coumarin Ligands or the cell-impermeant Alexa Fluor® 488 Ligand.

Materials Required:

- HaloTag® pHT2 Vector or HaloTag® Flexi® Vectors, desired ligands and protocol #TM260 (www.promega.com/tbs/tm260/tm260.html)
- chambered cover glass with cells expressing HaloTag® fusion protein
- complete culture medium appropriate for your cells at 37°C
- culture medium lacking phenol red at 37°C (optional)
- 1X PBS (pH 7.5, optional for washes)
- confocal microscope or wide-field fluorescent microscope equipped with appropriate filter sets and lasers
- 37°C + CO₂ cell culture incubator

Rapid Labeling Procedure

1. Prepare a 1:200 dilution of HaloTag® TMR, diAcFAM, Oregon Green®, Coumarin or Alexa Fluor® 488 Ligand in warm culture medium just prior to addition to cells. This is a 5X working stock solution.
2. Label cells by replacing one-fifth of the existing volume of medium with the 5X HaloTag® ligand working stock solution, and mix gently. This results in the recommended final labeling concentrations of 5µM TMR; 1µM diAcFAM, Oregon Green® or Alexa Fluor® 488; and 10µM Coumarin.
3. Incubate for 15 minutes in a 37°C + CO₂ cell culture incubator.

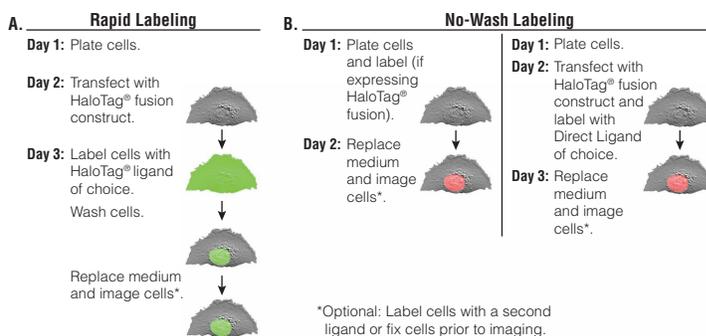


Figure 10.4. Live-cell labeling with the HaloTag® Technology. Schematics show live-cell labeling options. **Panel A.** Rapid labeling. **Panel B.** No-Wash labeling.

- Gently replace the ligand-containing medium with an equal (or greater) volume of warm fresh medium (or 1X PBS [pH 7.5]). Repeat this two times ending with warm complete medium, for a total of three complete rinses.
- Incubate cells in complete culture medium at 37°C + CO₂ in a cell culture incubator for 30 minutes to wash out unbound ligand (TMR and Alexa Fluor® 488 ligands may need only 15 minutes).
- Replace the medium with an equal volume of fresh warm culture medium (use of medium lacking phenol red may improve imaging).
- Transfer to a microscope and capture images.



Figure 10.5. HaloTag® rapid labeling results in signal that is robust and specific. Confocal image of U2OS cells expressing HaloTag®-NLS (nuclear localization sequence) and labeled with the HaloTag® TMR Ligand using the rapid labeling protocol clearly shows a strong fluorescent signal that is restricted to the nucleus. Panels (left to right) show fluorescence, DIC image and overlay. The image was acquired on a confocal microscope equipped with fluorophore-appropriate filter sets and an environmental chamber in which the cells remained at 37°C + CO₂ throughout imaging.

D. Live-Cell Labeling (No-Wash Protocol)

This protocol is intended for labeling of live cells with the cell-permeant HaloTag® TMRDirect™ or R110Direct™ Ligand. This protocol can be used to label cells that are adherent or nonadherent, and adherent cells can already

be plated or still be in suspension. Note: We do not recommend using the HaloTag® TMR Ligand (Cat.# G8251, G8252) for this protocol.

Materials Required:

- HaloTag® TMRDirect™ (Cat.# G2991) or R110Direct™ Ligand (Cat.# G3221) and protocol #TM260 (www.promega.com/tbs/tm260/tm260.html)
 - cells expressing HaloTag® fusion protein (in suspension or plated)
 - chambered cover glass or other cell culture device
 - complete culture medium appropriate for your cells at 37°C
 - culture medium lacking phenol red (optional) at 37°C
 - imaging device equipped with appropriate filter sets and lasers
 - 37°C + CO₂ cell culture incubator
- Prepare a 1:200 dilution of HaloTag® TMRDirect™ or R110Direct™ Ligand in warm culture medium just prior to addition to cells. This is a 5X working stock solution.
 - For adherent cells: Replace one-fifth of the existing volume of medium with the 5X HaloTag® ligand working stock solution, and mix gently. For cell suspensions: Add 5X ligand working stock to existing cell suspension, resulting in a 1X final concentration. Step 2 results in the recommended final labeling concentration of 100nM HaloTag® TMRDirect™ or R110Direct™ Ligand.
 - Plate cells (if necessary), and incubate overnight in a 37°C + CO₂ cell culture incubator.
 - Gently replace the ligand-containing medium with an equal (or greater) volume of warm fresh medium, or fix cells.
 - Transfer to an imaging device, and capture images.

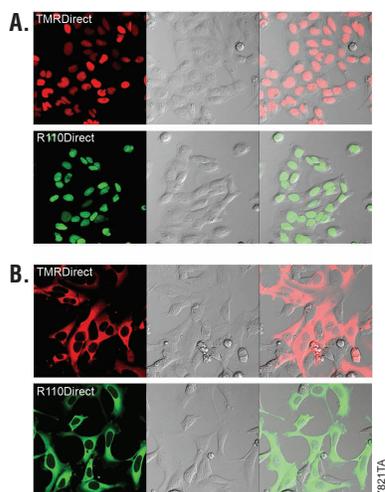


Figure 10.6. HaloTag® No-Wash Labeling results in signal that is robust and specific. Panel A. Confocal images of U2OS cells expressing HaloTag®-NLS₃ (nuclear localization sequence) and labeled with either the TMRDirect™ or R110Direct™ Ligand using the No-Wash Labeling protocol clearly show strong fluorescent signal that is restricted to the nucleus. Panel B. Confocal images of HEK 293 cells expressing HaloTag®-p65 (cytoplasmic in cells at rest) and labeled with the TMRDirect™ or R110Direct™ Ligand using the No-Wash Labeling protocol clearly show strong fluorescent signal that is restricted to the cytoplasm. All panels (left to right) show fluorescence, DIC image and overlay. Images were acquired on a confocal microscope equipped with fluorophore-appropriate filter sets and an environmental chamber in which cells remained at 37 °C + CO₂ throughout imaging.

E. Fluorescence Activated Cell Sorting (FACS®)

Materials Required:

- large-well format plate of HaloTag®-expressing cells and negative control cells that do not express the HaloTag® fusion protein

Materials Required:

- complete culture medium appropriate for your cells at 37°C

Materials Required:

- For adherent cells: 1X PBS (pH 7.5) and 1X PBS containing 3mM EDTA or trypsin solution at 37 °C (see Step 2 below)

Materials Required:

- cell sorting machine with appropriate filter sets and lasers for ligand used

Materials Required:

- 37°C + CO₂ cell culture incubator

1. Label cells expressing HaloTag® protein with ligand of choice. Recommended controls: (a) Label cells that are not expressing HaloTag® protein to assess background with chosen ligand; (b) prepare unlabeled cells expressing HaloTag® protein to assess background from endogenous cell fluorescence.

2. For adherent cells with HaloTag® fusion protein expressed on the cell surface: Rinse cells twice with an equal volume of 1X PBS, incubate in 1X PBS with 3mM EDTA at 37°C for 5 minutes, then gently scrape the cells off of the plate. For adherent cells with HaloTag® fusion protein expressed *inside the cell*: Suspend cells using trypsin-containing solution in a manner appropriate for the cell line.
3. Collect, pellet, count and resuspend cells in medium at 37°C to a concentration of 0.5 to 1 x 10⁶ cells/ml.
4. Sort cells.
5. Place sorted cells in 37°C + CO₂ incubator, using a format appropriate for later application.
6. Transfer to a microscope and image, perform SDS-PAGE, or relabel cells (e.g., with a different ligand color) and image.

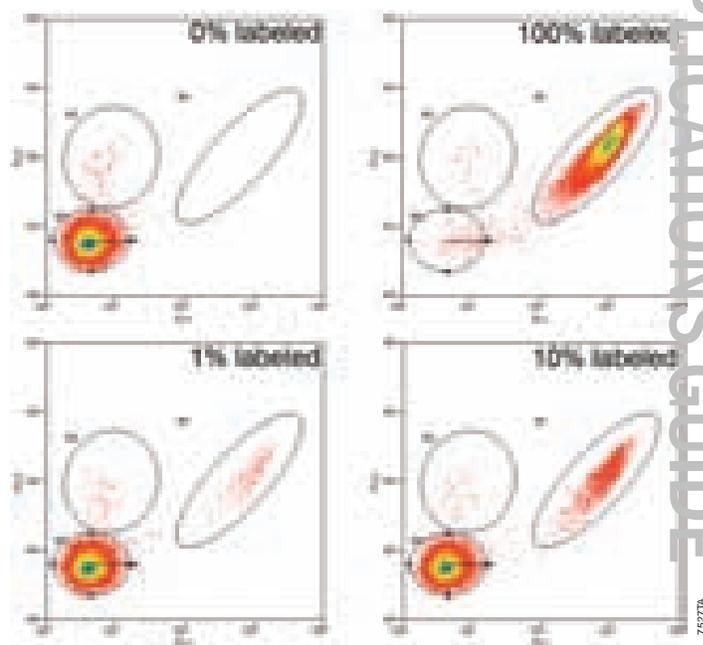


Figure 10.7. Successful FACS® of labeled HaloTag®-expressing cells. U2OS cells stably expressing HaloTag® 2 protein fused to three copies of a nuclear localization sequence were labeled with the HaloTag® R110Direct™ Ligand as described in Technical Manual #TM260, and known numbers (1%, 10% or 100% labeled) were sorted from nonlabeled cells. FACS® was performed using a MoFlo® instrument with approximately 20,000 cells represented on each graph. R1 indicates cells labeled with R110, R2 are dead cells (by propidium iodide), and R3 indicates unlabeled cells. Results show that labeled cells are successfully sorted from nonlabeled cells.

F. Fixed-Cell Labeling Protocol

This protocol is intended to serve as a guide to fix cells expressing a HaloTag® fusion protein. The covalent bond that forms between the ligand and HaloTag® protein during

live-cell labeling allows you to subsequently fix, permeabilize and wash the cells under stringent conditions without significant loss of the specific fluorescent signal. We recommend the use of paraformaldehyde (PFA) as a fixative because it crosslinks proteins in cells and at the membrane and has the added benefit of reducing cell loss from the growth surface. Fixed cells can be treated with detergents, such as Triton® X-100, to further eliminate nonspecific labeling and permeabilize cells for downstream immunocytochemical applications. The conditions here are sufficient to permeabilize the plasma membrane. Alternative or additional detergents might be necessary to permeabilize other structures.

Materials Required:

- HaloTag® pHT2 Vector or HaloTag® Flexi® Vectors, desired ligands, and protocol #TM260 (www.promega.com/tbs/tm260/tm260.html)
 - 4% paraformaldehyde/0.2M sucrose/1X PBS (pH 7.5)
 - 1X PBS buffer (pH 7.5)
 - 1X PBS + 0.1% Triton® X-100
 - confocal microscope or wide-field fluorescent microscope equipped with appropriate filter sets and lasers
1. Follow Steps 1–5 of Rapid Labeling or No-Wash Labeling protocol to label cells, if desired.
 2. Replace the medium with an equal volume of warm 4% paraformaldehyde/0.2M sucrose/1X PBS (pH 7.5), and incubate for 10 minutes at room temperature.
 3. Replace fixative with an equal volume of 1X PBS + 0.1% Triton®X-100, and incubate for 10 minutes at room temperature.
 4. Replace the detergent-containing solution with an equal volume of 1X PBS.
 5. Transfer to a microscope and capture images, or proceed to the next section for a immunocytochemistry protocol.

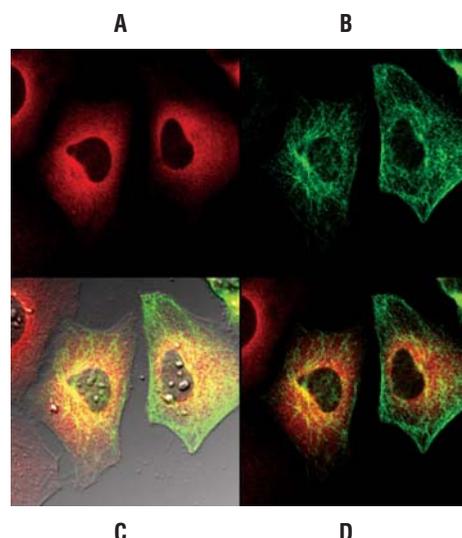


Figure 10.8. Multiplexing HaloTag® labeling with antibody staining. HeLa cells transiently transfected with the p65-HaloTag® construct and labeled with TMR Ligand were fixed and stained with mouse Anti-βIII Tubulin Antibody followed by incubation with Alexa Fluor® 488-conjugated goat anti-mouse IgG (Molecular Probes). Images were generated on an Olympus FV500 confocal microscope in sequential mode using appropriate filter sets. **Panel A.** TMR fluorescence. **Panel B.** Alexa Fluor® 488 fluorescence. **Panel C.** Overlay of Alexa Fluor® 488 and TMR fluorescence and transmitted light. **Panel D.** Overlay of Alexa Fluor® 488 and TMR fluorescence.

G. Immunocytochemistry Using the Anti-HaloTag® Polyclonal Antibody

This protocol is intended to serve as a guide for immunocytochemistry using Anti-HaloTag® Polyclonal Antibody. This antibody is a purified rabbit polyclonal antibody raised against the HaloTag® protein. The antibody was purified using Protein G affinity resin and has been shown to detect HaloTag® fusion proteins in both immunocytochemistry and Western blot applications with high sensitivity and specificity. Further, this antibody labels HaloTag® fusion proteins independently of HaloTag® ligands. As these labels do not interfere with one another, it is possible to colabel HaloTag® fusion proteins with a fluorescent ligand and the anti-HaloTag® pAb in conjunction with an anti-rabbit secondary antibody bearing a spectrally distinct fluorescent tag (Figure 10.9).

Materials Required:

- 1X PBS buffer (pH 7.5)
- fluorophore-conjugated anti-rabbit secondary antibody of choice
- PBS + 2% donkey serum + 0.01% Triton® X-100
- PBS + 1% donkey serum
- confocal or wide-field fluorescent microscope equipped with appropriate filter sets and lasers
- PBS + 0.1% sodium azide (optional, for storage)

1. Cells expressing a HaloTag[®] fusion protein, whether labeled or unlabeled, should be fixed and permeabilized as described in Steps 2–5 above.
2. Replace the 1X PBS with an equal volume of PBS + 2% donkey serum + 0.01% Triton[®] X-100, and block for 1 hour at room temperature.
3. Dilute the Anti-HaloTag[®] pAb 1:500 in PBS + 1% donkey serum, to final labeling concentration of 2 μ g/ml.
4. Replace the blocking solution with the antibody solution, and incubate for 1 hour at room temperature.
5. Wash cells twice with PBS + 1% donkey serum for 10 minutes at room temperature.
6. Dilute the secondary antibody according to the manufacturer's recommendations in PBS + 1% donkey serum.
7. Replace the wash solution with the secondary antibody solution, and incubate for 30 minutes at room temperature.
8. Wash cells twice with PBS + 1% donkey serum for 10 minutes each wash at room temperature.
9. Replace wash solution with 1X PBS.
10. Transfer to a microscope, and capture images. Store cells in PBS + 0.1% sodium azide.

H. Multiplexing HaloTag[®] protein, other reporters and/or Antibodies in Imaging Experiments

HaloTag[®] Technology can simplify multicolor/multiplex labeling experiments. The HaloTag[®] protein is not an intrinsically fluorescent protein (IFP), and the choice of fluorescent labels can be made after creating the HaloTag[®] fusion protein. This feature allows flexibility in experimental design for multicolor labeling as well as immunocytochemistry experiments.

To demonstrate this flexibility, we labeled cells expressing HaloTag[®]- α -tubulin fusion protein with the TMR Ligand or the diAcFAM Ligand and processed the cells for ICC using Anti- β III Tubulin mAb (Cat.# G7121) and Alexa Fluor[®] 488 or Cy[®]3-conjugated secondary antibodies (Figure 10.10). All HeLa cells expressed β III-tubulin in the cytoplasm. The HaloTag[®]- α -tubulin reporter was localized to the cytoplasm in the subpopulation of successfully transfected cells.

Additional Resources for HaloTag[®] Interchangeable Labeling Technology

Technical Bulletins and Manuals

- TM260 *HaloTag[®] Technology: Focus on Imaging Technical Manual*
www.promega.com/tbs/tm260/tm260.html

- TM254 *Flexi[®] Vector Systems Technical Manual*
www.promega.com/tbs/tm254/tm254.html

Promega Publications

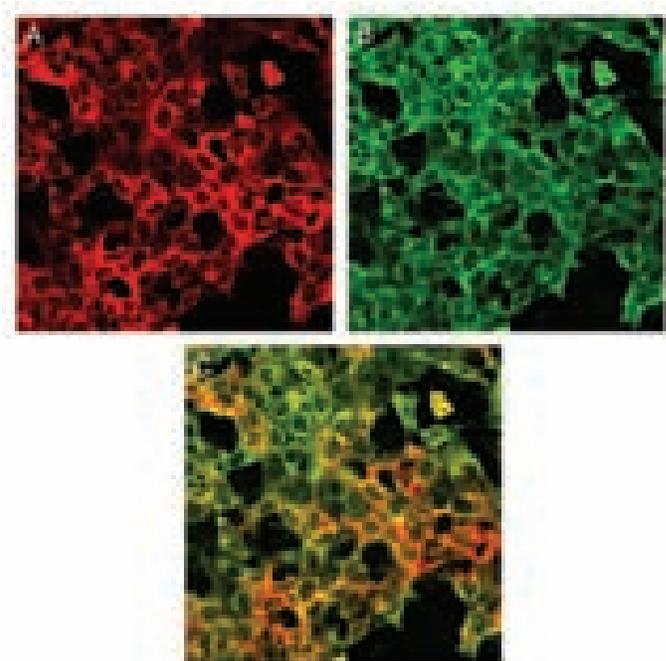
- PN101 HaloTag[®] Technology: Convenient, simple and reliable labeling from single wells to high-content screens
www.promega.com/pnotes/101/17252_05/17252_05.html
- PN100 Expression of Fusion Proteins: How to get started with the HaloTag[®] Technology
www.promega.com/pnotes/100/16620_13/16620_13.html
- PN100 Achieve the protein expression level you need with the mammalian HaloTag[®] 7 Flexi[®] Vectors
www.promega.com/pnotes/100/16620_16/16620_16.html
- PN089 HaloTag[®] Interchangeable Labeling Technology for cell imaging, protein capture and immobilization
www.promega.com/pnotes/89/12416_02/12416_02.html
- CN014 HaloTag[®] Technology: Cell imaging and protein analysis
www.promega.com/cnotes/cn014/cn014_10.htm
- CN011 HaloTag[®] Interchangeable Labeling Technology for cell imaging and protein capture
www.promega.com/cnotes/cn011/cn011_02.htm
- CN012 Perform multicolor live- and fixed-cell imaging applications with the HaloTag[®] Interchangeable Labeling Technology
www.promega.com/cnotes/cn012/cn012_04.htm

HaloTag[®] Vector Maps (www.promega.com/vectors/mammalian_express_vectors.htm#halo1)

Citations

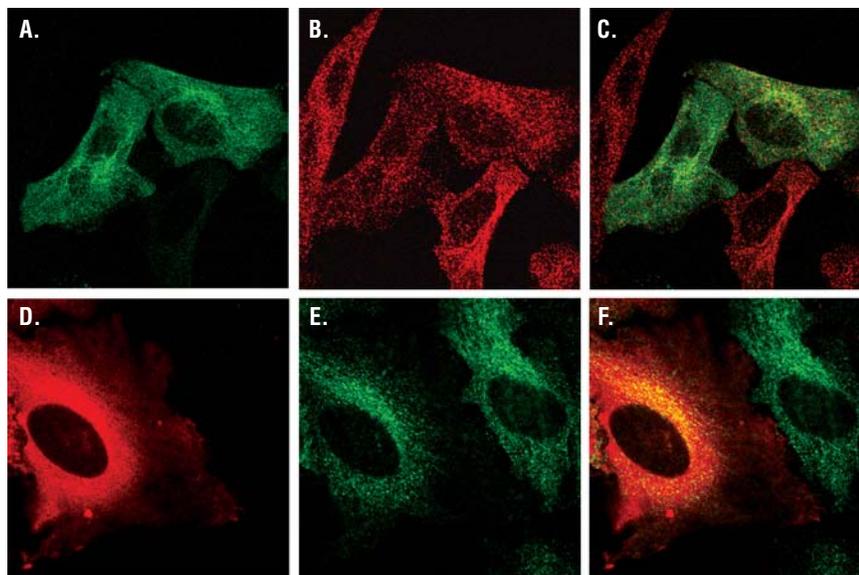
Schröder, J. *et al.* (2009) In vivo labeling method using genetic construct for nanoscale resolution microscopy. *Biophysical J.* **96**, L1-L3.

Traditionally light microscopy resolution has been limited by the diffraction of light. However several new technologies have emerged that partially overcome that limitation. One of these, stimulated emission depletion (STED) microscopy, is now commercially available and has been integrated into confocal microscope platforms. Because STED depends on fluorescent markers that fulfill specific spectroscopic needs, its uses have been limited. The authors of this paper demonstrate successful high resolution of β 1-integrin-HaloTag[®]-fusion protein imaging using STED microscopy. This was possible because HaloTag[®]



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Figure 10.9. Distinct labeling of a single HaloTag® 7 fusion by a HaloTag® Ligand and Anti-HaloTag® pAb. HEK 293 cells stably transfected with a p65-HaloTag® 7 construct (p65 is a cytoplasmic signaling protein) were labeled with HaloTag® TMR Ligand, then processed for immunocytochemistry using the Anti-HaloTag® pAb. The secondary antibody used was Alexa Fluor® 488-conjugated anti-rabbit-IgG (Jackson ImmunoResearch). Distinct labeling is demonstrated by the fact that the red fluorescence associated with specific HaloTag® TMR Ligand labeling (**Panel A**) does not prevent green fluorescence associated with specific Anti-HaloTag® pAb labeling (**Panel B**). Merging the fluorescent images (**Panel C**) confirms that the HaloTag® fusion protein is labeled specifically (restricted to the cytoplasm), as expected. Images were collected on an Olympus FV500 confocal microscope in sequential mode using appropriate filter sets.



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Figure 10.10. Multiplexing HaloTag® labeling and immunocytochemistry. HeLa cells were transfected with HaloTag®- α -tubulin fusion protein, labeled with HaloTag® diAcFAM (**Panels A–C**) or TMR Ligand (**Panels D–F**), washed and fixed as described in Los *et al.* 2005. Cells were immunolabeled with Anti- β III tubulin mAb (1:5,000 dilution, Cat.# G7121) followed by labeling with an Alexa Fluor® 488-conjugated secondary antibody (**Panels A–C**) or Cy®3-conjugated secondary antibody (**Panels D–F**). **Panels A and D** show cells the HaloTag® Ligands only; **Panel A**, diAcFAM Ligand; **Panel D**, TMR Ligand. **Panels B and E** show labeling for β III tubulin. **Panels C and F** show the overlay of HaloTag® protein and β III tubulin labeling.

technology allows researchers to create a reporter that can be labeled with STED-optimized fluorescent tags.

PubMed Number: 19134467

Citations

Svensden, S. *et al.* (2008) Spatial separation and bidirectional trafficking of proteins using a multi-functional reporter. *BMC Cell Biology*, 9, 17.

This paper demonstrates use of HaloTag® technology to study expression and trafficking of an integrin-HaloTag® fusion protein. The authors fused the HaloTag® reporter protein to a truncated integrin. They then labeled live cells with different in a pulse-chase format using a green cell-impermeant ligand (Alexa Fluor® 488) followed by a cell-permeant TMR Ligand (red). This allowed direct monitoring of protein trafficking because pools of the same transmembrane protein were labeled with distinct fluorescent ligands.

PubMed Number: 18384686

Los, G.V. *et al.* (2008) HaloTag: A Novel Protein Labeling Technology for Cell Imaging and Protein Analysis *ACS Chemical Biology* 3, 373–82.

The authors of this study describe the HaloTag® reporter system and use it to characterize a protein known to influence DNA transcription. The researchers create one genetic construct and demonstrate its use in determining protein localization and dynamics through imaging as well as biologically relevant interactions with a protein partner and DNA promoter.

PubMed Number: 18533659

III. Monster Green® Fluorescent Protein phMGFP Vector

With the discovery of intrinsically fluorescent proteins (IFPs) such as the green fluorescent protein (GFP) and the subsequent creation of a full-color spectrum of these IFPs, researchers can now fuse a protein of interest to IFPs with a variety of properties. IFPs are commonly used reporter molecules that can be visualized without cell lysis using standard fluorescence microscopy. They are often used to monitor gene expression but also can be used to monitor intracellular protein trafficking by creating C- and N-terminal protein fusions.

The Monster Green® Fluorescent Protein (Cat.# E6421) is an improved synthetic version of the green fluorescent protein gene originally cloned from *Montastrea cavernosa* (Great Star Coral). The synthetic gene (hMGFP) expresses a 26kDa protein that shows improved fluorescence intensity compared to the native gene. Peak excitation occurs at 505nm, and peak emission occurs at 515nm. Standard FITC filters may be used to visualize hMGFP fluorescence. The hMGFP gene is codon optimized and cleared of most consensus sequence transcription factor binding sites to ensure reliability and high levels of expression.

The Monster Green® Fluorescent Protein encoded by the hMGFP gene is an ideal fluorescent reporter, providing high-level fluorescence and reduced cytotoxicity. Monster Green® Protein generally fluoresces at least 20% brighter than other commercially available green fluorescent proteins (GFPs) and also reduces cytotoxicity, offering flexibility when working with transient and stable expression assays (Figure 10.11).

Additional Resources for the Monster Green® Fluorescent Protein

Technical Bulletins and Manuals

TB320 *Monster Green® Fluorescent Protein phMGFP Vector Technical Bulletin*
(www.promega.com/tbs/tb320/tb320.html)

Promega Publications

CN007 Transfecting a human neuroblastoma cell line with Monster Green® Fluorescent Protein
(www.promega.com/notes/cn007/cn007_14.htm)

PN084 Monster Green® Protein: a brighter, longer-expressing green fluorescent protein
(www.promega.com/notes/84/10705_12/10705_12.html)

Vector Maps

phMGFP Vector Map (www.promega.com/figures/popup.asp?fn=3898ma)

Citations

Inman, M. *et al.* (2004) Identification of a novel bovine herpesvirus 1 transcript containing a small open reading frame that is expressed in trigeminal ganglia of latently infected cattle. *J. Virol.* 78, 5438–47.

A fusion protein construct was made using the Monster Green® Fluorescent Protein phMGFP Vector and the PCR-amplified Open Reading Frame E (ORF-E) from Bovine herpesvirus 1. Transfected human neuroblastoma (SK-N-SH) cells, rabbit skin cells and bovine kidney cells were visualized using an Olympus FV500/BX60 confocal microscope with 488nm excitation laser and 522nm emission filter set. The ORF-E-MGFP protein was localized in discrete domains within the nucleus of Neuro-2A and SK-N-SH cells. In rabbit skin cells and bovine kidney cells the ORF-E-MGFP protein was detected in the cytoplasm and nucleus.

PubMed Number: 15113922

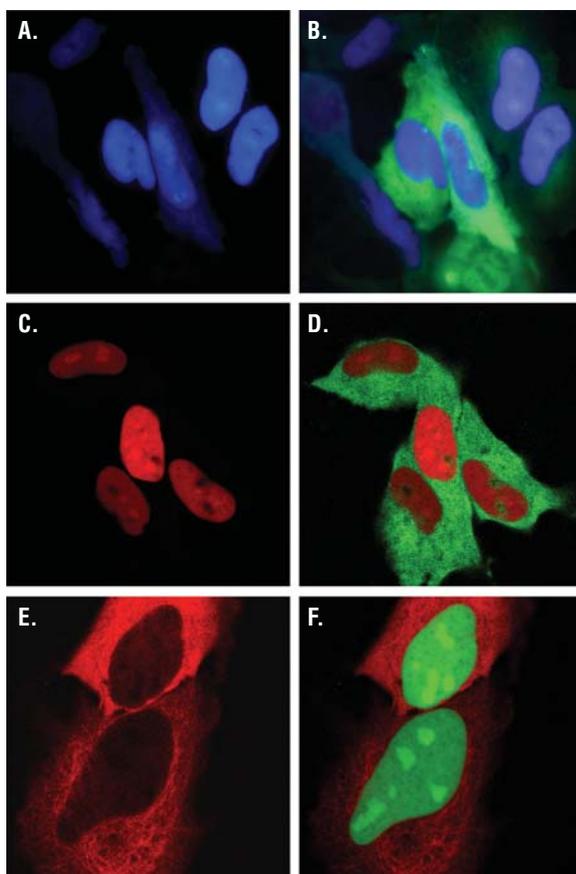


Figure 10.11. Expression of Monster Green® Fluorescent Protein in HeLa Cells. HeLa cells were transiently co-transfected with either HaloTag®-(NLS)₃ plus hMGFP- α -tubulin (**Panels A–D**) or HaloTag®- α -tubulin plus hMGFP-(NLS)₃ (**Panels E and F**). Twenty-four hours later, cells expressing HaloTag®-(NLS)₃ were incubated with either 25 μ M Coumarin Ligand (Cat.# G8581, **Panels A and B**) or 5 μ M HaloTag® TMR Ligand (**Panels C and D**) for 15 minutes; cells expressing HaloTag®- α -tubulin were incubated with 5 μ M TMR Ligand for 15 minutes at 37°/5%CO₂ (**Panels E and F**). Cells were washed and incubated for 30 minutes. In Panels A and B, cells were imaged with an Olympus IX81 epifluorescent microscope equipped with Chroma filter sets (#31000 DAPI for the Coumarin Ligand and #41001 FITC for hMGFP), a Hamamatsu Orca CCD camera and environmental controls. Images in Panels C–F were captured with an Olympus FV500 confocal attachment with sequential two-laser scanning and filters appropriate for TMR and FITC fluorescence.

IV. Antibodies and Other Cellular Markers

Promega offers a variety of antibodies for detecting markers of apoptosis, assessing activation of cellular signaling pathways, and monitoring indicators of cell type. These antibodies include antibodies raised against phosphorylated proteins including kinases as well as antibodies against growth factors and growth-factor receptors.

A. Antibodies and Markers of Apoptosis

In Situ Marker for Caspase-3: FITC-VAD-FMK

CaspACE™ FITC-VAD-FMK In Situ Marker (Cat.# G7461) is a fluorescent analog of the pan caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone). The fluorescein isothiocyanate (FITC) group has been substituted for the carbobenzoxy (Z) N-terminal blocking group to create the fluorescent apoptosis marker. This structure allows delivery of the inhibitor into the cell where it irreversibly binds to activated caspases. The FITC label allows for a single-reagent addition to assay for caspase activity in situ. The FITC-VAD-FMK Marker is supplied as a 5mM solution in DMSO and is intended for in situ monitoring of caspase activity by fluorescence detection. The suggested concentration for use in anti-Fas-treated Jurkat cell culture is 10 μ M.

Method for Detecting Apoptosis in Jurkat Cells

Materials Required:

- CaspACE™ FITC-VAD-FMK In Situ Marker (Cat.# G7461, G7462)
- poly-L-lysine-coated slides
- anti-Fas mAb (Clone CH-11 MBL International Cat.# SY-100)
- PBS
- formalin
- mounting medium
- fluorescence microscope

1. Seed Jurkat cells at 1×10^5 cells/ml and grow in RPMI-1640 + 10% FBS in a 37°C, 5% CO₂ incubator for 2–3 days until they reach a density of 5×10^5 cells/ml.
2. Prepare poly-L-lysine-coated slides. Coat each chamber of multichamber slides with 0.01% poly-L-lysine solution. When partially dry, rinse the slides in NANOpure® water and then air-dry. Poly-L-lysine-coated slides can be prepared in advance and stored at 4°C for up to 7 days before use.
3. To induce apoptosis, add anti-Fas mAb (Clone CH-11, MBL International Cat.# SY-100) to a final concentration of 0.1 μ g/ml. Do not add to controls. Incubate for 3–4 hours at 37°C.
4. Add CaspACE™ FITC-VAD-FMK In Situ Marker to the Jurkat cells at a final concentration of 10 μ M. Protect cells from light and incubate for 20 minutes in the incubator. Keep cells protected from light for the remaining steps.
5. Centrifuge at $300 \times g$ for 5 minutes.
6. Wash cells with PBS, then centrifuge at $300 \times g$ for 5 minutes.
7. Suspend cells in PBS to 1.5×10^6 cells/ml.

8. Add cells to poly-L-lysine-coated slides and incubate at room temperature for 5 minutes to allow the cells to adhere to the poly-L-lysine.
9. Fix in 10% buffered formalin for 30 minutes at room temperature (protected from light).
10. Rinse 3 times for 5 minutes each time in PBS.
11. Add mounting medium and coverslips to the slides. Analyze under a fluorescence microscope.

Additional Resources for the CaspACE™ FITC-VAD-FMK In Situ Marker

Technical Bulletins and Manuals

9PIG746 *CaspACE™ FITC-VAD-FMK In Situ Marker Product Information*
www.promega.com/tbs/9pig746/9pig746.html

Promega Publications

eNotes *CaspACE™ FITC-VAD-FMK In Situ Marker as a probe for flow cytometry detection of apoptotic cells*
www.promega.com/enotes/applications/ap0020_tabs.htm

PN075 *CaspACE™ FITC-VAD-FMK In Situ Marker for Apoptosis: Applications for flow cytometry*
www.promega.com/pnotes/75/8554_20/8554_20.html

NN016 *Live/Dead Assay: In situ labeling of apoptotic neurons with CaspACE™ FITC-VAD-FMK Marker*
www.promega.com/nnotes/nn503/503_14.htm

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)

Citations

Rouet-Benzineb, P. *et al.* (2004) TOrexins acting at native OX1 receptor in colon cancer and neuroblastoma cells or at recombinant OX1 receptor suppress cell growth by inducing apoptosis. *J. Biol. Chem.* **279**, 45875–86.

Human colon cancer (HT29-D4) cells were analyzed for activated caspases using the CaspACE™ FITC-VAD-FMK In Situ Marker. HT29-D4 cells (7×10^5) were cultured in the presence or absence of 1 μ M orexins, peptide growth inhibitors. Cells were washed, and bound CaspACE™ FITC-VAD-FMK In Situ Marker was visualized by confocal microscopy.

PubMed Number: 15310763

Detecting Active Caspase-3 Using an Antibody

Anti-ACTIVE® Caspase-3 pAb (Cat.# G7481) is intended for use as a marker of apoptosis; it specifically stains apoptotic human cells without staining nonapoptotic cells. All known caspases are synthesized as pro-enzymes activated by proteolytic cleavage. Anti-ACTIVE® Caspase-3

pAb is an affinity-purified rabbit polyclonal antibody directed against a peptide from the p18 fragment of human caspase-3. The antibody is affinity purified using a peptide corresponding to the cleaved region of caspase-3.

General Immunochemical Staining Protocol

Materials Required:

- Anti-ACTIVE® Caspase-3 pAb (Cat.# G7481)
 - prepared, fixed samples on slides
 - Triton® X-100
 - PBS
 - blocking buffer (PBS/0.1% Tween® 20 + 5% horse serum)
 - donkey anti-rabbit Cy®3 conjugate secondary antibody (Jackson Laboratories Cat.# 711-165-152)
 - mounting medium
 - humidified chamber
1. Permeabilize the fixed cells by incubating in PBS/0.2% Triton® X-100 for 5 minutes at room temperature. Wash three times in PBS, in Coplin jars, for 5 minutes at room temperature.
 2. Drain the slide and add 200 μ l of blocking buffer (PBS/0.1% Tween® 20 + 5% horse serum). Use of serum from the host species of the conjugate antibody (or closely related species) is suggested. Lay the slides flat in a humidified chamber and incubate for 2 hours at room temperature. Rinse once in PBS.
 3. Add 100 μ l of the Anti-ACTIVE® Caspase-3 pAb diluted 1:250 in blocking buffer. Prepare a slide with no Anti-ACTIVE® Caspase-3 pAb as a negative control. Incubate slides in a humidified chamber overnight at 4°C.
 4. The following day, wash the slides twice for 10 minutes in PBS, twice for 10 minutes in PBS/0.1% Tween® 20 and twice for 10 minutes in PBS at room temperature.
 5. Drain slides and add 100 μ l of donkey anti-rabbit Cy®3 conjugate diluted 1:500 in PBS. (We recommend Jackson ImmunoResearch Cat.# 711-165-152.) Lay the slides flat in a humidified chamber, protected from light, and incubate for 2 hours at room temperature. Wash twice in PBS for 5 minutes, once in PBS/0.1% Tween® 20 for 5 minutes and once in PBS for 5 minutes, protected from light.
 6. Drain the liquid, mount the slides in a permanent or aqueous mounting medium and observe with a fluorescence microscope.

Additional Resources for the Anti-ACTIVE® Caspase-3 pAb

Technical Bulletins and Manuals

9PIG748 *Anti-ACTIVE® Caspase-3 pAb Product Information*
www.promega.com/tbs/9pig748/9pig748.html

Promega Publications

- CN001 Immunohistochemical staining using Promega Anti-ACTIVE® and apoptosis antibodies
(www.promega.com/cnotes/cn001/cn001_4.htm)
- PN075 Anti-ACTIVE® Caspase-3 pAb for the detection of apoptosis
(www.promega.com/pnotes/75/8554_17/8554_17.html)

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)
Antibody Assistant (www.promega.com/techserv/tools/abasst/)

Citations

Debacq-Chainiaux, F. *et al.* (2005) Repeated exposure of human skin fibroblasts to UVB at subcytotoxic level triggers premature senescence through the TGF- β 1 signaling pathway. *J. Cell Science* **118**, 743–58.

The authors of this study developed a model for UVB-induced premature senescence of skin human diploid fibroblasts. Markers of senescence were expressed in the system, and the authors were able to detect a common mitochondrial DNA 4,977-bp deletion that is associated with oxidative damage. After a series of ten UVB stresses, total RNA was prepared from the cells using the RNAgents® Total RNA Isolation System and used for RT-PCR to detect differentially expressed genes. Forty-four stress or senescence-associated genes were identified that were differentially expressed between UVB irradiated and untreated cells including c-fos, c-jun, insulin-like growth factor binding protein 3, several HSPs, genes involved in protection from oxidative stress, and the type II receptor of TGF- β . The Anti-ACTIVE® Caspase-3 pAb was used to assess whether the UVB treatment or incubation with TGF- β 1 led to apoptosis in this system.

PubMed Number: 15671065

Using an Antibody Against a Cleaved Caspase-3 Substrate (Anti-PARP p85 Fragment pAb)

Poly (ADP-ribose) polymerase (PARP), a nuclear enzyme involved in DNA repair, is a well-known substrate for caspase-3 cleavage during apoptosis. Anti-PARP p85 Fragment pAb (Cat.# G7341) is a rabbit polyclonal antibody specific for the p85 fragment of PARP that results from caspase cleavage of the 116kDa intact molecule and thus provides an in situ marker for apoptosis. Each batch of antibody is tested for use in immunostaining applications and contains sufficient antibody for 50 immunocytochemical reactions at a working dilution of 1:100.

General Immunocytochemistry Protocol**Materials Required:**

- Anti-PARP p85 Fragment pAb (Cat.# G7341)
- cells fixed on slides
- PBS

- blocking buffer (PBS/0.1% Tween® 20 + 5% horse serum)
 - donkey anti-rabbit biotin conjugate (Jackson Cat.# 711-065-152) **or** donkey anti-rabbit Cy®3 conjugate (Jackson Cat.# 711-165-152)
 - H₂O₂ (if using biotin conjugate)
 - DAB solution (if using biotin conjugate)
 - ultrapure water
 - humidified chamber
 - peroxidase-labeled streptavidin (eg., KPL Cat.# 14-300-00, diluted 1 μ g/ml in PBS)
1. Permeabilize cells fixed on slides in 0.2% Triton® X-100/PBS for 5 minutes at room temperature.
 2. Wash in 1X PBS in coplin jars for 5 minutes at room temperature. Repeat twice for a total of 3 washes.
 3. Drain the slides and add blocking buffer (PBS/0.1% Tween® 20 + 5% normal serum). Cover cells with blocking buffer (200 μ l per slide). Lay the slides flat in a humidified chamber and incubate for 2 hours at room temperature.
 4. Rinse once in PBS.
 5. Add 100 μ l of the Anti-PARP p85 Fragment pAb diluted in blocking buffer. We recommend a starting dilution of 1:100. Include a slide with no Anti-PARP p85 Fragment pAb as a negative control. Incubate slides in a humidified chamber overnight at 4°C.
 6. The following day, wash the slides twice for 10 minutes in 1X PBS, twice for 10 minutes in PBS/0.1% Tween® 20, and twice for 10 minutes in 1X PBS at room temperature.
 7. If the secondary antibody is a horseradish peroxidase (HRP) conjugate, block endogenous peroxidases by incubating with 0.3% hydrogen peroxide for 4–5 minutes at room temperature. If you are using a different method of detection with a secondary antibody, proceed to Step 9.
 8. Wash in 1X PBS in Coplin jars for 5 minutes. Repeat twice for a total of 3 washes.

9. Drain slides and add 100–200µl of diluted secondary antibody to each slide. We recommend donkey anti-rabbit biotin conjugate (Jackson Cat.# 711-065-152) or donkey anti-rabbit Cy[®]3 conjugate (Jackson Cat.# 711-165-152) diluted 1:500 in PBS/0.1% Tween[®] 20. Lay the slides flat in a humidified chamber and incubate for 2 hours at room temperature.
10. Wash several times in 1X PBS.
11. For the biotin conjugate, drain the slides and add 100–200µl of Streptavidin-HRP solution to each slide. Lay the slides flat in a humidified chamber and incubate for 45 minutes at room temperature. For HRP-conjugated secondary antibodies, proceed to Step 13. For other secondary antibodies, proceed to Step 15.
12. Wash in 1X PBS in Coplin jars for 5 minutes. Repeat twice for a total of 3 washes.
13. Add 100–200µl of freshly made diaminobenzidine (DAB) solution to each slide. Lay the slides flat and incubate for ~10 minutes at room temperature.
14. Rinse the slides in NANOpure[®] water. Bleach is frequently used to inactivate the DAB before disposal; however, local requirements for hazardous waste should be followed.
15. Drain the liquid and mount the slides in a permanent or aqueous mounting medium (slides mounted in 70% glycerol can be stored for several weeks at 4°C or –20°C).

Method for Staining Postnatal Day 0 Mouse Brain, Paraffin-Embedded Sections. (All steps are performed at room temperature.)

Materials Required:

- Anti-PARP p85 Fragment, pAb (Cat.# G7341)
 - paraffin-embedded, fixed sample
 - Hemo-De[®] (Fisher Scientific) or xylene
 - ethanol (100, 95 and 70%)
 - PBS
 - Triton[®] X-100
 - H₂O₂
 - biotin-conjugated donkey anti-rabbit pAb
 - RTU ABC reagent (Vector Laboratories)
 - DAB substrate kit (Vector Laboratories)
 - VECTASHIELD[®] DAPI anti-fade Reagent (Vector Laboratories)
1. Embed tissue in paraffin after fixation in 4% paraformaldehyde. Six micron sections are used for this protocol.
Note: Best results will be obtained if the animal is perfused with fix and postfixed after dissection.
 2. Deparaffinize by washing tissue 3 times for 5 minutes each in Hemo-De[®] (Fisher Scientific) or xylene. Rinse tissue sections for 2 minutes in 100% ethanol. Transfer

sections to 95% ethanol for 2 minutes, then transfer them to 70% ethanol for 2 minutes. Finally, rinse tissue sections 2 times for 2 minutes each in PBS.

3. Permeabilize for 10 minutes in PBS + 0.1% Triton[®] X-100.
4. Wash sections 2 times for 5 minutes each in PBS.
5. Block endogenous peroxide activity by incubating sections in 0.3% H₂O₂ in PBS for 30 minutes.
6. Wash sections 2 times for 5 minutes each in PBS.
7. Block for 45 minutes in PBS + 5% donkey serum
8. Incubate with Anti-PARP p85 Fragment pAb diluted 1:50 in PBS + 1.0 % donkey serum for 60 minutes.
9. Wash sections 3 times for 5 minutes each in PBS.
10. Incubate with biotin-conjugated donkey anti-rabbit pAb (Jackson ImmunoResearch) diluted 1:500 in PBS for 60 minutes.
11. Wash sections 3 times for 5 minutes each in PBS.
12. Incubate in R.T.U. (Ready-To-Use) ABC reagent (Vector Laboratories) for 60 minutes.
13. Wash sections 3 times for 5 minutes each in PBS.
14. Develop with DAB substrate kit (Vector Laboratories) for 10 minutes.
15. Wash 3 times for 5 minutes each in water.
16. Mount in VECTASHIELD[®] + DAPI anti-fade reagent (Vector Laboratories).
17. Analyze samples immediately using a fluorescence microscope.

Additional Resources for the Anti-PARP p85 Fragment pAb

Technical Bulletins and Manuals

TB273 *Anti-PARP p85 Fragment pAb Technical Bulletin*
(www.promega.com/tbs/tb273/tb273.html)

Promega Publications

PN072 Cleaved PARP as a marker for apoptosis in tissue sections
(www.promega.com/pnotes/72/8094_07/8094_07.html)

CN001 Immunohistochemical staining using Promega Anti-ACTIVE[®] and apoptosis antibodies
(www.promega.com/cnotes/cn001/cn001_4.htm)

Online Tools

Apoptosis Assistant (www.promega.com/apoasst/)

Antibody Assistant (www.promega.com/techserv/tools/abasst/)

Citations

Davidson, B. *et al.* (2003) Expression and activation of the nerve growth factor receptor TrkA in serous ovarian carcinoma. *Clin. Cancer Res.* **9**, 2248–59.

Anti-PARP p85 Fragment pAb was used to stain human peritoneal and pleural effusions.

PubMed Number: 12796393

O'Brien, M.A., Moravec, R.A. and Riss, T. (2001) Poly (ADP-ribose) polymerase cleavage monitored in situ in apoptotic cells. *Biotechniques*. **30**, 886–91.

The authors demonstrate specificity of an affinity-purified polyclonal antibody to the p85 fragment of PARP with Western blots that show that the antibody recognizes the 85kDa (p85) fragment of PARP but not full-length PARP.

PubMed Number: 11314271

B. Antibodies and Cellular Markers for Studying Cell Signaling Pathways

Promega provides a variety of phosphorylation-specific antibodies for studying cell signaling pathways. These antibodies and example protocols are discussed in detail in the *Protocols and Applications Guide* chapter on Cell Signaling (www.promega.com/paguide/chap7.htm#title4). Brief descriptions of these products are provided below.

Anti-ACTIVE® Phosphorylation-Specific Antibodies

The Anti-ACTIVE® phosphorylation-specific antibodies were developed to provide an accurate measure of enzyme activation. These antibodies specifically recognize the active, phosphorylated form of a given kinase. The Anti-ACTIVE® Antibodies are raised against phosphorylated peptide sequences present in the activating loop of a number of protein kinases. Whether used in Western analysis, immunocytochemistry or immunohistochemical staining, the Anti-ACTIVE® MAPK, JNK, p38 and CaM KII Antibodies will recognize only the active form of the enzyme.

Anti-ACTIVE® MAPK, pAb, Rabbit, (pTEpY)

This antibody is an affinity-purified polyclonal antibody that specifically recognizes the dually phosphorylated, active form of MAPK. The antibody is raised against a dually phosphorylated peptide sequence representing the catalytic core of the active ERK enzyme and recognizes the active forms of ERK1, ERK2 and ERK7.

Anti-ACTIVE® JNK pAb, Rabbit, (pTPpY)

Anti-ACTIVE® JNK pAb is an affinity-purified polyclonal antibody that recognizes the dually phosphorylated, active form of cJun N-terminal protein Kinase (JNK).

Anti-ACTIVE® JNK pAb is raised against a dually

phosphorylated peptide sequence representing the catalytic core of the active JNK enzyme. The antibody recognizes the active forms of JNK1, JNK2, and JNK3 isoforms.

Anti-ACTIVE® p38 pAb, Rabbit, (pTGpY)

Anti-ACTIVE® p38 Ab, Rabbit, is an affinity-purified polyclonal antibody that recognizes the active form of p38 kinase. The Anti-ACTIVE® p38 pAb is raised against the dually phosphorylated peptide sequence representing the catalytic core of the active p38 enzyme. The Anti-ACTIVE® p38 pAb recognizes the active forms of p38 α , γ , and δ isoforms.

Additional Resources for the Anti-ACTIVE® Antibodies**Technical Bulletins and Manuals**

TB262 *Anti-ACTIVE® MAPK, p38 and JNK Polyclonal Antibodies and Anti-ACTIVE® Qualified Secondary Antibody Conjugates* (www.promega.com/tbs/tb262/tb262.html)

Promega Publications

PN069 New Anti-ACTIVE® MAPK and 'pan ERK 1/2' antibodies for Western analysis (www.promega.com/pnotes/69/7542_09/7542_09.html)

PN076 Technically speaking: Anti-ACTIVE® Antibodies and MAPK signaling pathways (www.promega.com/pnotes/76/8840_23/8840_23.html)

PN080 Demonstration of immunohistochemical staining using Promega's Anti-ACTIVE® and apoptosis Antibodies (www.promega.com/pnotes/80/9748_20/9748_20.html)

Online Tools

Antibody Assistant (www.promega.com/techserv/tools/abasst/)

Citations

Hsu, C.Y. *et al.* (2004) Characterization of active mitogen-activated protein kinase in ovarian serous carcinomas *Clin. Can. Res.* **10**, 6432–6.

The Anti-ACTIVE® MAPK polyclonal antibody was used to immunohistochemically stain and type patient ovarian serous carcinomas using paraffin-fixed tissue sections on tissue microarrays. Western blots were also performed on tissue lysates using a 1:3,000 dilution of the antibody.

PubMed Number: 15475429

Le'Negrata, G. *et al.* (2003) Downregulation of caspases and Fas ligand expression, and increased lifespan of neutrophils after transmigration across intestinal epithelium *Cell Death Differ.* **10**, 153–62.

Anti-ACTIVE® JNK pAb was used in immunoblot analysis of human polymorphonuclear leukocyte protein lysates.

PubMed Number: 12700643

Aballay, A. *et al.* (2003) *Caenorhabditis elegans* innate immune response triggered by *Salmonella enterica* requires intact LPS

and is mediated by a MAPK signaling pathway *Curr. Biol.* **13**, 47–52.

Activation of the p38 homolog in the worm was monitored by Western analysis using the Anti-ACTIVE® p38 pAb.

PubMed Number: 12526744

Phosphorylation-Specific CaM KII Antibody

This antibody recognizes calcium/calmodulin-dependent protein kinase, CaM KII, that is phosphorylated on threonine 286. The Anti-ACTIVE® CaM KII pAb (Cat.# V1111) was raised against the phosphothreonine-containing peptide derived from this region.

Additional Information for the Anti-ACTIVE® CaM KII pAb

Technical Bulletins and Manuals

- TB264 *Anti-ACTIVE® CaM KII pAb, (pT²⁸⁶) and Anti-ACTIVE® Qualified Secondary Antibody Conjugates Technical Bulletin*
(www.promega.com/tbs/tb264/tb264.html)

Promega Publications

- PN067 *Anti-ACTIVE® Antibody for specific detection of phosphorylated CaM KII protein kinase*
(www.promega.com/pnotes/67/7201_09/7201_09.html)
- BR095 *Signal Transduction Resource*
(www.promega.com/guides/sigtrans_guide/default.htm)

Online Tools

Antibody Assistant (www.promega.com/techserv/tools/abasst/)

Citations

Matsumoto, Y. and Maller, J.L. (2002) Calcium, calmodulin and CaM KII requirement for initiation of centrosome duplication in *Xenopus* egg extracts *Science* **295**, 499–502. CaM KII (281-309) was added to metaphase-arrested extracts. After adding calcium, the extracts were incubated at room temperature. Anti-ACTIVE® CaM KII pAb and Anti-ACTIVE® Qualified HRP secondary antibodies were used to probe immunoblots for phospho-T²⁸⁶ CaM KII α .

PubMed Number: 11799245

C. Marker Antibodies

Anti- β III Tubulin mAb

Anti- β III Tubulin mAb (Cat.# G7121) is a protein G-purified IgG₁ monoclonal antibody (from clone 5G8) raised in mice against a peptide (EAQGPK) corresponding to the C-terminus of β III tubulin. It is directed against β III tubulin, a specific marker for neurons. The major use of this antibody is for labeling neurons in tissue sections and cell culture. The antibody performs in frozen and paraffin-embedded sections of rat brain, cerebellum and

spinal cord, human and rat fetal CNS progenitor cell cultures and adult human paraffin-embedded brain (Figure 10.12).

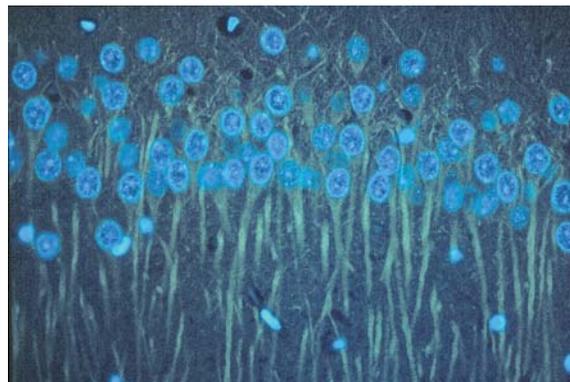


Figure 10.12. Immunostaining for β III tubulin in rat cerebellum using Anti- β III Tubulin mAb. Paraffin-embedded rat brain section double-immunofluorescence-labeled with the primary antibody and detected using an anti-mouse Cy³-conjugated secondary antibody (yellow-green). Nuclei were stained with DAPI (blue). Protocols developed and performed at Promega.

- **Immunogen:** Peptide corresponding to the C-terminus (EAQGPK) of β III tubulin.
- **Antibody Form:** Mouse monoclonal IgG1 (clone 5G8), 1mg/ml in PBS containing no preservatives.
- **Specificity:** Cross-reacts with most mammalian species. Does not label nonneuronal cells (e.g., astrocytes).
- **Suggested Working Dilutions:** Immunocytochemistry (1:2,000), Immunohistochemistry (1:2,000), Western blotting (1:1,000 dilution).

Additional Resources for β -III Tubulin mAb

Promega Publications

- CN012 *Perform multicolor live- and fixed-cell imaging applications with the HaloTag® Interchangeable Labeling Technology*
(www.promega.com/cnotes/cn012/cn012_04.htm)
- NN020 *CellTiter-Glo® Luminescent Cell Viability Assay: Primary neurons and human neuroblastoma SH-SY5Y cells*
(www.promega.com/nnotes/nn020/20_05.htm)
- NN019 *Rat neurospheres express mRNAs for TrkB, BDNF, NT-3 and p75*
(www.promega.com/nnotes/nn019/19_18.htm)
- NN018 *Specific labeling of neurons and glia in mixed cerebrocortical cultures*
(www.promega.com/nnotes/nn018/018_10.htm)
- NN018 *Imaging with Promega Reagents: Anti- β III Tubulin mAb (1,439kb PDF)*
(www.promega.com/nnotes/nn018/18_8.pdf)

- NN016 Imaging with Promega Reagents: Anti- β III Tubulin mAb (293kb PDF) (www.promega.com/nnotes/nn503/503_10.pdf)
- NN015 Imaging with Promega Reagents: Anti- β III Tubulin mAb (417kb PDF) (www.promega.com/nnotes/nn502/502_08.pdf)
- NN014 Imaging with Promega Reagents: Anti- β III Tubulin mAb (187kb PDF) (www.promega.com/nnotes/nn501/501_10.pdf)

Online Tools

Antibody Assistant (www.promega.com/techserv/tools/abasst/antibodypages/antib3tubmab.htm)

Citations

Brunelli, G. *et al.* (2005) Glutamatergic reinnervation through peripheral nerve graft dictates assembly of glutamatergic synapses at rat skeletal muscle *Proc. Natl. Acad. Sci. USA* **102**, 8152–7.

PubMed Number: 15937120

Citations

Walker, K. *et al.* (2001) mGlu5 receptors and nociceptive function II. mGlu5 receptors functionally expressed on peripheral sensory neurons mediate inflammatory hyperalgesia *Neuropharmacology* **40**, 10–19.

Rat skin sections were subjected to immunohistochemistry with the Anti- β III Tubulin mAb to detect metabolic glutamate receptor expressing neurons. Twenty micron sections were fixed in acetone, permeabilized with 0.1% Triton® X-100, and incubated with the Anti- β III Tubulin mAb at a final concentration of 1 μ g/ml.

PubMed Number: 11077066

Anti-GFAP pAb

Anti-GFAP pAb (Cat.# G5601) is a polyclonal antibody against glial fibrillary acidic protein (GFAP), a specific marker of astrocytes in the central nervous system and is qualified for immunostaining applications (Figure 10.13)

- **Immunogen:** Purified glial fibrillary acidic protein from bovine spinal cord.
- **Antibody Form:** Purified rabbit IgG; supplied at 1mg/ml in PBS containing 50 μ g/ml gentamicin.
- **Specificity:** Human, bovine and rat GFAP; not recommended for mouse.
- **Suggested Dilutions:** 1:1,000 for Western blotting, immunocytochemistry and immunohistochemistry.

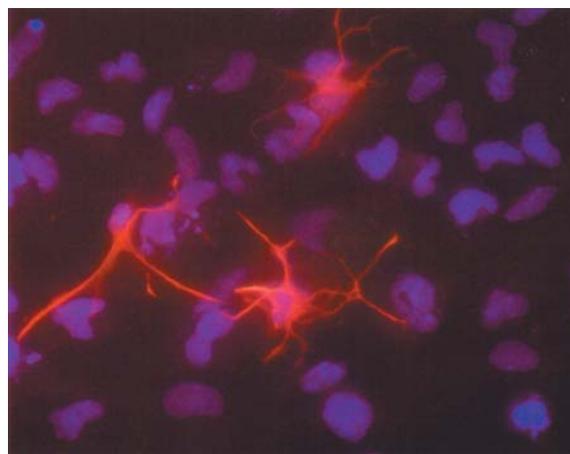


Figure 10.13. Anti-GFAP-labeled astrocytes in mixed-rate neural progenitor cultures. DAPI staining (blue) and Anti-GFAP pAb with Cy[®]-3-conjugated secondary (red) were used. Protocols developed and performed at Promega.

Additional Resources for Anti-GFAP pAb**Promega Publications**

- NN018 Specific labeling of neurons and glia in mixed cerebrocortical cultures (www.promega.com/nnotes/nn018/018_10.htm)
- CN001 Immunohistochemical staining using Promega Anti-ACTIVE[®] and apoptosis antibodies (www.promega.com/cnotes/cn001/cn001_4.htm)
- NN016 Imaging with Promega Reagents: Anti-GFAP pAb (293kb pdf) (www.promega.com/nnotes/nn503/503_10.pdf)

Citations

Moreno-Flores, M.T. *et al.* (2003) Immortalized olfactory ensheathing glia promote axonal regeneration of rat retinal ganglion neurons. *J. Neurochem.* **85**, 861–71.

This paper describes the development of an immortalized line of olfactory bulb ensheathing glia (OEG) from rat olfactory bulbs. Immortalized lines were established by transfection of primary OEG with the plasmid pEF321-T, which expressed the viral oncogene SV40 large T antigen. The starting primary cell culture was characterized by immunocytochemistry for OEG-specific markers such as p75-NGFr, S100, neuroligin, vimentin and GFAP. Western blotting of p75-NGFr and GFAP was performed on the established cell lines to determine levels of these markers. The Anti-GFAP pAb was used at a concentration of 1:200 for immunocytochemistry and at 1:1,000 for Western blotting.

PubMed Number: 12716418

Anti-VACHT pAb

The purified Anti-VACHT (Vesicular Acetylcholine Transporter) pAb (Cat.# G4481) is raised in goats against a peptide (CSPPGPFDCEDDYNYSRS) corresponding to amino acids 511–530 of the carboxy terminus of the cloned rat VACHT. It is a novel tool to identify functional cholinergic neurons in the central and peripheral nervous system where the antibody stains fibers and neuronal cell bodies. This antibody has application for the study of the pathophysiology of neurodegenerative diseases involving the cholinergic system and for mapping cholinergic neurons in the nervous system.

- **Immunogen:** Carboxy-terminal peptide sequence 511–530 corresponding to cloned rat VACHT protein.
- **Antibody Form:** Purified goat polyclonal IgG; 0.5mg/ml in PBS containing no preservatives.
- **Specificity:** Cross-reacts with VACHT in rat and mouse, but not in human, guinea pig, rabbit or cat.
- **Suggested Dilution:** 1:500 for immunohistochemistry.

Additional Resources for Anti-VACHT Antibody**Promega Publications**

NN010 Immunostaining with Promega Reagents: Anti-VACHT pAb (514kb pdf) (www.promega.com/nnotes/nn303/303_12.pdf)

Online Tools

Antibody Assistant (www.promega.com/techserv/tools/abasst/antibodypages/antivachtpab.htm)

D. Other Antibodies

Promega offers a variety of antibodies against growth factors, neurotrophic factor receptors and other molecules. Usage information for these antibodies is summarized in Table 10.1. For additional information on these antibodies, please visit the Antibody Assistant (www.promega.com/techserv/tools/abasst/).

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Zhang, Y. *et al.* (2006) HaloTag® protein-mediated site-specific conjugation of bioluminescent proteins to quantum dots. *Angew. Chem. Int. Ed. Engl.* **45** 4936–40.

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Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.

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Table 10.1. Additional Antibodies Available From Promega.

Antibody	Species (subclass)	Application and Recommended Dilution ¹	Known Species Cross-Reactivity	Catalog Number
Anti-Human BDNF pAb	Chicken (IgY)	Western 1µg/ml; ELISA 1µg/ml; ICC 1–10µg/ml; IHC 10–15µg/ml; BN 10µg/ml	Human, mouse, rat, rabbit ² and quail ²	Cat.# G1641
Anti-Rat CNTF pAb	Chicken (IgY)	Western 1µg/ml; ELISA 1µg/ml; ICC 1–10µg/ml	Rat, mouse, human and cow ²	Cat.# G1631
Anti-Human GDNF pAb	Chicken (IgY)	Western 1µg/ml; ELISA 1µg/ml; ICC 1–10µg/ml	Human, mouse, rat and Rhesus monkey	Cat.# G2791
Anti-NGF mAb	Rat (IgG)	Western 1µg/ml; ELISA 10.5µg/ml; ICC, IHC 0.5–1.0µg/ml; BN not recommended	Human, mouse, guinea pig, rat, rabbit, goat, sheep, cow, pig, horse, cat ²	Cat.# G1132; Cat.# G1131
Anti-Human NT-3 pAb	Chicken (IgY)	Western 1µg/ml; ELISA 1µg/ml; ICC 1–10µg/ml; IHC ² ; BN 1–10µg/ml	Human, mouse, rat and cat ²	Cat.# G1651
Anti-TBFB β 1 pAb	Rabbit (IgG)	Western 1:1,000 dilution; ELISA not recommended; IHC 1:50 dilution; BN ²	Human, mouse, rat and cat ²	Cat.# G1221
Anti-Human p75 pAb	Rabbit (IgG)	Western not recommended; IP 1–10µg/ml; ICC, IHC 1–10µg/ml; BN 1–10µg/ml	Human, mouse, rat and chicken (species cross-reactivity due to high conservation of cytoplasmic domain)	Cat.# G3231
Anti-TrkB In pAb	Chicken (IgY)	ICC, IHC 1–10µg/ml; BN not recommended	Human, rat and mouse ²	Cat.# G1561
Anti-Pan Trk pAb	Chicken (IgY)	Western 1µg/ml; ELISA 1µg/ml; ICC, IHC 1–10µg/ml; IP 0.5µg/ml; BN not recommended	TrkA, TrkB and TrkC due to the highly conserved ALAQAPPVLDVL sequence of human, rat and mouse	Cat.# G1581
Anti-Human Trypsin mAb Biotin	Mouse	Western 1:10,000 dilution; ELISA 1:2,000–1:5,000 dilution; ICC, IHC 1:1,000 dilution	Human and nonhuman primates; marker for mast cells	Cat.# G3361

¹ Investigators should optimize concentrations for their specific applications and conditions.

² This information was not generated by Promega scientists but has been published in the scientific literature. Please contact Promega Technical Services or refer to the Promega Antibody Assistant (www.promega.com/techserv/tools/abasst/) for additional information.

³ Key: ICC = immunocytochemistry; IHC = immunohistochemistry; BN = biological neutralization; IP = immunoprecipitation



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I. Introduction

Information about the regulation of protein expression, protein modification, protein:protein interactions and protein function during different stages of cell development is needed to understand the development and physiology of organisms. This complex analysis of protein function is a major task facing scientists today. Although the field of proteomics was first described only as the study of proteins encoded by the genome, it has now expanded to include the function of all expressed proteins. Thus it is not just the study of all proteins expressed in a cell but also all protein isoforms and modifications, interactions, structure and high-order complexes (Tyers and Mann, 2003).

A fundamental step for studying individual proteins is the purification of the protein of interest. A variety of strategies have been developed for purifying proteins. These strategies address different requirements of downstream applications including scale and throughput. There are four basic steps required for protein purification: 1) cell lysis; 2) binding to a matrix; 3) washing; and 4) elution. Cell lysis can be accomplished a number of ways, including nonenzymatic methods (e.g., sonication or French press) or use of hydrolytic enzymes such as lysozyme or a detergent reagent such as FastBreak™ Cell Lysis Reagent (Cat.# V8571). FastBreak™ Cell Lysis Reagent offers a convenient format for the in-media lysis of *E. coli* cells expressing recombinant proteins without interfering with downstream purification of tagged proteins (Stevens and Kobs, 2004). In addition, the FastBreak™ Reagent requires only minor modifications to be used with mammalian and insect cell lines (Betz, 2004).

Affinity purification tags can be fused to any recombinant protein of interest, allowing fast, easy purification using the affinity properties of the tag (Nilsson *et al.* 1997). Certain tags are used because they encode an epitope that can be purified or detected by a specific antibody or because they enable simplified purification of a desired protein.

Since protein is directly involved in biological function, a great deal of emphasis has been placed on developing new tools for proteomic studies (Zhu *et al.* 2003). A number of methods are available for functional protein interaction studies. These include protein pull-down assays, yeast two-hybrid systems (Fields and Song, 1989; Chien *et al.* 1991) and mammalian two-hybrid systems (Giniger *et al.* 1985; Lin *et al.* 1988) to identify protein:protein interactions, as well as protein-chip technology, mass spectrometry and traditional one- or two-dimensional gel electrophoresis for protein identification.

II. Affinity Tags

Researchers often need to purify a single protein for further study. One method for isolating a specific protein is the use of affinity tags. Affinity purification tags can be fused to any recombinant protein of interest, allowing fast and easy purification following a procedure that is based on the affinity properties of the tag (Nilsson *et al.* 1997). Many different affinity tags have been developed to simplify

protein purification (Terpe, 2002). Fusion tags are polypeptides, small proteins or enzymes added to the N- or C-terminus of a recombinant protein. The biochemical features of different tags influence the stability, solubility and expression of proteins to which they are attached (Stevens *et al.* 2001). Using expression vectors that include a fusion tag facilitates recombinant protein purification.

A. Polyhistidine

The most commonly used tag to purify and detect recombinant expressed proteins is the polyhistidine tag (Yip *et al.* 1989). Protein purification using polyhistidine tags relies on the affinity of histidine residues for immobilized metal such as nickel, which allows selective protein purification (Yip *et al.* 1989; Hutchens and Yip, 1990). This affinity interaction is believed to be a result of coordination of a nitrogen on the imidazole moiety of polyhistidine with a vacant coordination site on the metal. The metal is immobilized to a support through complex formation with a chelate that is covalently attached to the support.

Polyhistidine tags offer several advantages for protein purification. The small size of the polyhistidine tag renders it less immunogenic than other larger tags. Therefore, the tag usually does not need to be removed for downstream applications following purification. A large number of commercial expression vectors that contain polyhistidine are available. The polyhistidine tag may be placed on either the N- or C-terminus of the protein of interest. And finally, the interaction of the polyhistidine tag with the metal does not depend on the tertiary structure of the tag, making it possible to purify otherwise insoluble proteins using denaturing conditions.

B. Glutathione-S-Transferase

The use of the affinity tag glutathione-S-transferase (GST) is based on the strong affinity of GST for immobilized glutathione-covered matrices (Smith and Johnson, 1988). Glutathione-S-transferases are a family of multifunctional cytosolic proteins that are present in eukaryotic organisms (Mannervik and Danielson, 1988; Armstrong, 1997). GST isoforms are not normally found in bacteria; thus endogenous bacterial proteins don't compete with the GST-fusion proteins for binding to purification resin. The 26kDa GST affinity tag enhances the solubility of many eukaryotic proteins expressed in bacteria.

III. Purification of Polyhistidine-Tagged Proteins

A. Rapid Purification of Polyhistidine-Tagged Proteins Using Magnetic Resins

There is a growing need for high-throughput protein purification methods. Magnetic resins enable affinity-tagged protein purification without the need for multiple centrifugation steps and transfer of samples to multiple tubes. There are several criteria that define a good protein purification resin: minimal nonspecific protein binding, high binding capacity for the fusion protein and efficient recovery of the fusion protein. The MagneHis™ Protein Purification System meets these criteria, enabling

purification of proteins with a broad range of molecular weights and different expression levels. The magnetic nature of the binding particles allows purification from crude lysates to be performed in a single tube. In addition, the system can be used on automated liquid-handling platforms for high-throughput applications.

MagneHis™ Protein Purification System

The MagneHis™ Protein Purification System (Cat.# V8500, V8550) uses paramagnetic precharged nickel particles (MagneHis™ Ni-Particles) to isolate polyhistidine-tagged protein directly from a crude cell lysate. Figure 11.1 shows a schematic diagram of the MagneHis™ Protein Purification System protocol. Using a tube format, polyhistidine-tagged protein can be purified on a small scale using less than 1ml of culture or on a large scale using more than 1 liter of culture. Samples can be processed in a high-throughput manner using a robotic platform such as the Beckman Coulter Biomek® 2000 or Biomek® FX or Tecan Freedom EVO® instrument. Polyhistidine-tagged proteins can be purified under native or denaturing (2–8M urea or guanidine-HCl) conditions. The presence of serum in mammalian and insect cell culture medium does not interfere with purification. For more information and a detailed protocol, see Technical Manual #TM060. (www.promega.com/tbs/tm060/tm060.html)

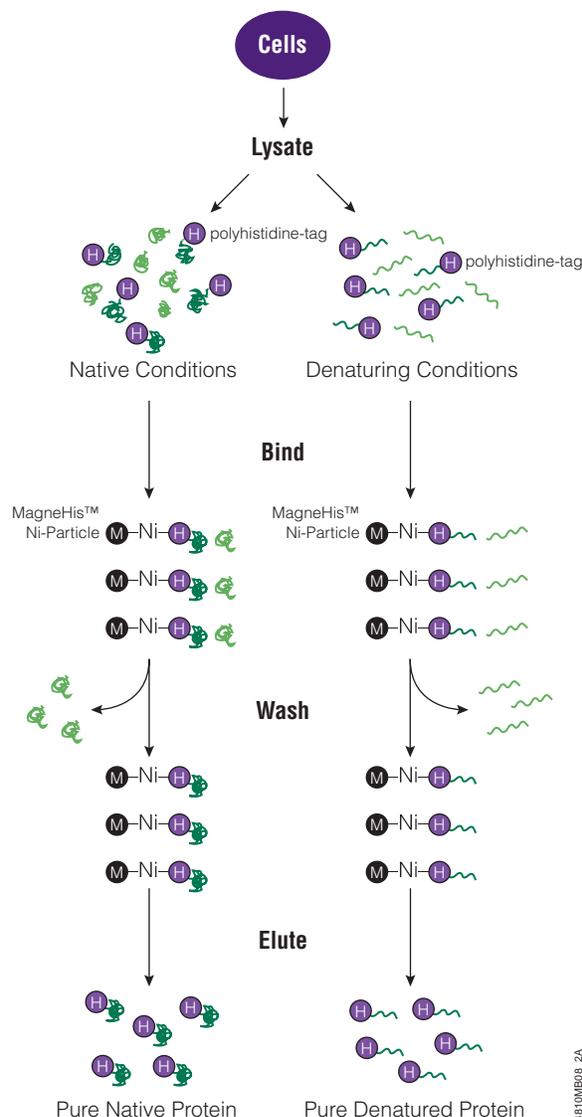


Figure 11.1. Diagram of the MagneHis™ Protein Purification System protocol.

Example Protocol for the MagneHis™ Protein Purification System for Bacterial Expression

Materials Required:

(see Composition of Solutions section)

- MagneHis™ Protein Purification System (Cat.# V8500, V8550) and protocol
- 37°C incubator for flasks/tubes
- shaker
- magnetic separation stand
- 1M imidazole solution (pH 8.0; for insect or mammalian cells or culture medium)
- additional binding/wash buffer (may be required if processing numerous insect cell, mammalian cell or culture medium samples)
- solid NaCl (for insect or mammalian cells or culture medium)

1. Add 110µl FastBreak™ Cell Lysis Reagent, 10X, to 1ml of fresh bacterial culture.
2. Resuspend DNase I as indicated on the vial. Add 1µl to the lysed culture.
3. Incubate with shaking for 10–20 minutes at room temperature.
4. Vortex the MagneHis™ Ni-Particles to a uniform suspension.
5. Add 30µl MagneHis™ Ni-Particles to 1.1ml of cell lysate.
6. Pipet to mix, and incubate for 2 minutes at room temperature.
7. Place the tube in the appropriate magnetic stand for approximately 30 seconds to capture the MagneHis™ Ni-Particles. Carefully remove supernatant.
8. Remove the tube from the magnet. Add 150µl of MagneHis™ Binding/Wash Buffer to the MagneHis™ Ni-Particles, and pipet to mix. Make sure that particles are resuspended well.
9. Place the tube in the appropriate magnetic stand for approximately 30 seconds to capture the MagneHis™ Ni-Particles. Carefully remove supernatant. Repeat Steps 8 and 9 for a total of three washes.
10. Add 100µl of MagneHis™ Elution Buffer, and pipet to mix. Incubate for 1–2 minutes at room temperature. Place the tube in the appropriate magnetic stand, and capture the particles. Carefully remove the supernatant, which now contains the fusion protein.

Purification using Denaturing Conditions. Proteins expressed in bacterial cells may be present in insoluble inclusion bodies. To determine if your protein is located in an inclusion body, perform the lysis step using FastBreak™ Cell Lysis Reagent, 10X, as described in Technical Manual #TM060. Pellet cellular debris by centrifugation, and check the supernatant and pellet for the polyhistidine-tagged protein by gel analysis. Insoluble proteins need to be purified under denaturing conditions. Since the interaction of polyhistidine-tagged fusion proteins and MagneHis™ Ni-Particles does not depend on tertiary structure, fusion proteins can be captured and purified using denaturing conditions by adding a strong denaturant such as 2–8M guanidine hydrochloride or urea to the cells. Denaturing conditions need to be used throughout the procedure; otherwise the proteins may aggregate. We recommend preparing denaturing buffers by adding solid guanidine-HCl or urea directly to the MagneHis™ Binding/Wash and Elution Buffers. For more detail, see Technical Manual #TM060 (www.promega.com/tbs/tm060/tm060.html).

Note: Do not combine FastBreak™ Cell Lysis Reagent and denaturants. Cells can be lysed directly using denaturants such as urea or guanidine-HCl.

Purification from Insect and Mammalian Cells. Process cells at a cell density of 2×10^6 cells/ml of culture. Adherent cells may be removed from the tissue culture vessel by scraping and resuspending in culture medium to this density. Cells may be processed in culture medium containing up to 10% serum. Processing more than the indicated number of cells per milliliter of sample may result in reduced protein yield and increased nonspecific binding. For proteins that are secreted into the cell culture medium, cells should be removed from the medium prior to purification. For more detail, see Technical Manual #TM060 (www.promega.com/tbs/tm060/tm060.html).

Additional Resources for the MagneHis™ Protein Purification System

Technical Bulletins and Manuals

TM060 *MagneHis™ Protein Purification System Technical Manual*
(www.promega.com/tbs/tm060/tm060.html)

Promega Publications

PN087 Efficient purification of his-tagged proteins from insect and mammalian cells
(www.promega.com/pnotes/87/11527_29/11527_29.html)

PN086 Technically speaking: Choosing the right protein purification system
(www.promega.com/pnotes/86/11217_28/11217_28.html)

CN009 Purifying his-tagged proteins from insect and mammalian cells
(www.promega.com/cnotes/cn009/cn009_06.htm)

PN084 Rapid detection and quantitation of his-tagged proteins purified by MagneHis™ Ni-Particles
(www.promega.com/pnotes/84/10705_27/10705_27.html)

PN083 MagneHis™ Protein Purification System: Purification of his-tagged proteins in multiple formats
(www.promega.com/pnotes/83/10492_02/10492_02.html)

PN083 Automated polyhistidine-tagged protein purification using the MagneHis™ Protein Purification System
(www.promega.com/pnotes/83/10492_06/10492_06.html)

Citations

Lee, M. *et al.* (2004) Peptidoglycan recognition proteins involved in 1,3-beta-D-glucan-dependent prophenoloxidase activation system of insect. *J. Biol. Chem.* **279**, 3218–27.

Researchers used MagneHis™ Ni-Particles to purify polyhistidine-tagged peptidoglycan recognition protein-1 (PGRP1 and PGRP2) that had been excreted into medium supernatants. The polyhistidine-tagged proteins were created by making fusion-protein expression vectors from isolated *H. diomphalia* larvae cDNA and the pMT/Bip/V5-His vector (Invitrogen). The construct was then stably transfected into *Drosophila* Schneider S2 cells, and the medium was monitored for secreted protein by Western blot analysis.

PubMed Number: 14583608

MagZ™ Protein Purification System for Purification of Proteins Expressed in Rabbit Reticulocyte Lysate

Purification of a polyhistidine-tagged protein that has been expressed in rabbit reticulocyte lysate is complicated by hemoglobin in the lysate copurifying with the protein of interest. Hemoglobin copurification limits downstream applications (e.g., fluorescence-based functional assays, protein:protein interaction studies) and reduces the amount of protein purified. The MagZ™ Protein Purification System provides a simple, rapid and reliable method to purify expressed polyhistidine-tagged protein from rabbit reticulocyte lysate. Paramagnetic precharged particles can be used to isolate polyhistidine-tagged protein from 50–500 µl of TNT® Rabbit Reticulocyte Lysate with minimal copurification of hemoglobin. These polyhistidine-tagged proteins are 99% free of contaminating hemoglobin.

The MagZ™ System is flexible enough to be used with different labeling and detection methods.

Polyhistidine-tagged proteins expressed in rabbit reticulocyte lysate can be labeled with [³⁵S]methionine or the FluoroTect™ Green_{Lys} in vitro Translation Labeling System. FluoroTect™-labeled polyhistidine-tagged proteins can be visualized by gel analysis and analyzed using a FluorImager® instrument. Figure 11.2 shows a schematic diagram of the MagZ™ Protein Purification System protocol. For more detail, see Technical Bulletin #TB336 (www.promega.com/tbs/tb336/tb336.html).

Materials Required:

(see Composition of Solutions section)

- MagZ™ Protein Purification System (Cat.# V8830) and protocol
- platform shaker or rocker, rotary platform or rotator
- MagneSphere® Technology Magnetic Separation Stand (Cat.# Z5331, Z5332, Z5341, Z5342)

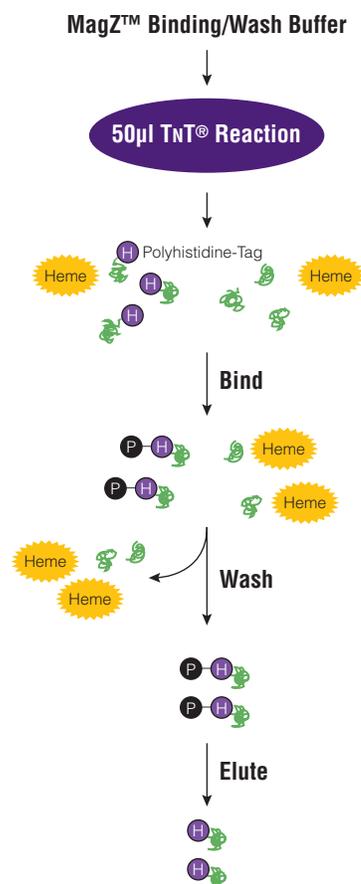


Figure 11.2. Schematic diagram of the MagZ™ Protein Purification System. A TNT® reaction expressing polyhistidine-tagged proteins is diluted with MagZ™ Binding/Wash Buffer and added to MagZ™ Particles. The polyhistidine-tagged proteins bind to the particles during incubation, then are washed to remove unbound and nonspecifically bound proteins.

Additional Resources for the MagZ™ Protein Purification System

Technical Bulletins and Manuals

- | | |
|-------|---|
| TB336 | <i>MagZ™ Protein Purification System Technical Bulletin</i>
(www.promega.com/tbs/tb336/tb336.html) |
|-------|---|

Promega Publications

- | | |
|-------|--|
| PN088 | The MagZ™ System: His-tagged protein purification without hemoglobin contamination
(www.promega.com/pnotes/88/12162_09/12162_09.html) |
| PN088 | In vitro his-tag pull-down assay using MagZ™ Particles
(www.promega.com/pnotes/88/12162_13/12162_13.html) |

B. Medium- to Large-Scale Purification of Polyhistidine-Tagged Proteins in Column or Batch Formats

The two most common support materials for resin-based, affinity-tagged protein purification are agarose and silica gel. As a chromatographic support, silica is advantageous because it has a rigid mechanical structure that is not vulnerable to swelling and can withstand large changes in pressure and flow rate without disintegrating or deforming. Silica is available in a wide range of pore and particle sizes including macroporous silica, providing a higher capacity for large biomolecules such as proteins. However, two of the drawbacks of silica as a solid support for affinity purification are the limited reagent chemistry that is available and the relatively low efficiency of surface modification.

The HisLink™ Protein Purification Resin (Cat.# V8821) and HisLink™ 96 Protein Purification System (Cat.# V3680, V3681) overcome these limitations using a new modification process for silica surfaces that provides a tetradentate metal-chelated solid support with a high binding capacity and concomitantly eliminates the nonspecific binding that is characteristic of unmodified silica. HisLink™ Resin is a macroporous silica resin modified to contain a high level of tetradentate-chelated nickel (>20mmol Ni/ml settled resin). Figure 11.3 show a schematic diagram of HisLink™ Resin and polyhistidine-tag interaction. The HisLink™ Resin has a pore size that results in binding capacities as high as 35mg of polyhistidine-tagged protein per milliliter of resin.

The HisLink™ Resin enables efficient capture and purification of bacterially expressed polyhistidine-tagged proteins. This resin may also be used for general applications that require an immobilized metal affinity chromatography (IMAC) matrix (Porath *et al.* 1975; Lonnerdal and Keen, 1982). HisLink™ Resin may be used in either column or batch purification formats. For a detailed protocol see Technical Bulletin #TB327 (www.promega.com/tbs/tb327/tb327.html).

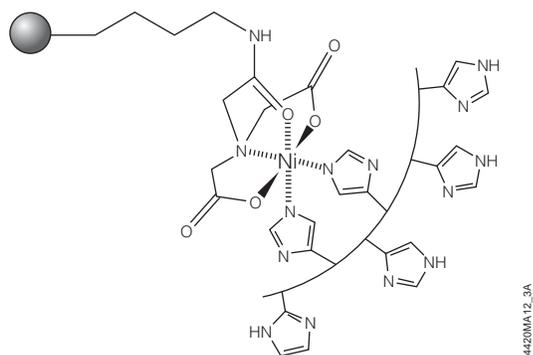


Figure 11.3. Schematic diagram of HisLink™ Resin and polyhistidine interaction. Two sites are available for polyhistidine-tag binding and are rapidly coordinated with histidine in the presence of a polyhistidine-tagged polypeptide.

Column-Based Purification using HisLink™ Resin

The HisLink™ Resin provides a conventional means to purify polyhistidine-tagged proteins and requires only a column that can be packed to the appropriate bed volume. If packed to 1ml under gravity-driven flow, HisLink™ Resin shows an average flow rate of approximately 1ml/minute. In general a flow rate of 1–2ml/minute per milliliter of resin is optimal for efficient capture of polyhistidine-tagged protein. Gravity flow of a cleared lysate over a HisLink™ column will result in complete capture and efficient elution of polyhistidine-tagged proteins; however, the resin may also be used with vacuum filtration devices (e.g., Vac-Man® Vacuum Manifold, Cat.# A7231) to allow simultaneous processing of multiple columns. HisLink™ Resin is also an excellent choice for affinity purification using low- to medium-pressure liquid chromatography systems such as fast performance liquid chromatography (FPLC).

Example Protocol Using the HisLink™ Resin to Purify Proteins from Cleared Lysate by Gravity-Flow Column Chromatography

Materials Required:

(see Composition of Solutions section)

- HisLink™ Protein Purification Resin (Cat.# V8821) and protocol
- HEPES buffer (pH 7.5)
- imidazole
- binding buffer
- wash buffer
- elution buffer
- column [e.g., Fisher PrepSep Extraction Column (Cat.# P446) or Bio-Rad Poly-Prep® Chromatography Column (Cat.# 731-1550)]

Cell Lysis: Cells may be lysed using any number of methods including sonication, French press, bead milling, treatment with lytic enzymes (e.g., lysozyme) or use of a commercially available cell lysis reagent such as the FastBreak™ Cell Lysis Reagent (Cat.# V8571). If lysozyme is used to prepare a lysate, add salt (>300mM NaCl) to the binding and wash buffers to prevent the lysozyme binding to the resin. Finally, adding protease inhibitors such as 1mM PMSF to cell lysates does not inhibit binding or elution of polyhistidine-tagged proteins with the HisLink™ Resin and is highly recommended. When preparing cell lysates from high-density cultures, adding DNase and RNase (concentrations up to 20µg/ml) will reduce the lysate viscosity and aid in purification.

Example Protocol

1. Prepare the binding, wash and elution buffers.
2. Determine the column volume required to purify the protein of interest. In most cases 1ml of settled resin is sufficient to purify the amount of protein typically found in up to 1 liter of culture (cell density of O.D.₆₀₀ < 6.0). In cases of very high expression levels (e.g., 50mg protein/liter), up to 2ml of resin per liter of culture may be needed.

3. Once you have determined the volume of settled resin required, precalibrate this amount directly in the column by pipetting the equivalent volume of water into the column and marking the column to indicate the top of the water. This mark indicates the top of the settled resin bed. Remove the water before adding resin to the column.
4. Make sure that the resin is fully suspended; fill the column with resin to the line marked on the column by transferring the resin with a pipette. Allow the resin to settle, and adjust the level of the resin by adding or removing resin as necessary.
Note: If the resin cannot be pipetted within 10–15 seconds of mixing, significant settling will occur, and the resin will need to be resuspended. Alternatively, a magnetic stir bar may be used to keep the resin in suspension during transfer. To avoid fracturing the resin, do not leave the resin stirring any longer than the time required to pipet and transfer the resin.
5. Allow the column to drain, and equilibrate the resin with five column volumes of binding buffer, allowing the buffer to completely enter the resin bed.
6. Gently add the cleared lysate to the resin until the lysate has completely entered the column. The rate of flow through the column should not exceed 1–2ml/minute for every 1ml of column volume. Under normal gravity flow conditions the rate is typically about 1ml/minute. The actual flow rate will depend on the type of column used and the extent to which the lysate was cleared and filtered. Do not let the resin dry out after you have applied the lysate to the column.
7. Wash unbound proteins from the resin using at least 10–20 column volumes of wash buffer. Divide the total volume of wash buffer into two or three aliquots, and allow each aliquot to completely enter the resin bed before adding the next aliquot.
8. Once the wash buffer has completely entered the resin bed, add elution buffer and begin collecting fractions (0.5–5ml fractions). Elution profiles are protein-dependent, but polyhistidine-tagged proteins will generally elute in the first 1ml. Elution is usually complete after 3–5ml of buffer have been collected per 1.0ml of settled resin, provided the imidazole concentration is high enough to efficiently elute the protein of interest.

Batch Purification Using HisLink™ Resin

One of the primary advantages of the HisLink™ Resin is its use in batch purification. In batch mode, the protein of interest is bound to the resin by mixing lysate with the resin for approximately 30 minutes at a temperature range of 4–22°C. Once bound with protein, the resin is allowed to settle to the bottom of the container, and the spent lysate is poured off. Washing only requires resuspension of the resin in an appropriate wash buffer followed by a brief

period to allow the resin to settle. The wash buffer is then carefully poured off. This process is repeated as many times as desired. Final elution is best achieved by transferring the HisLink™ Resin to a column to elute the protein in fractions. The advantages of batch purification are: 1) less time is required to perform the purification; 2) large amounts of lysate can be processed; and 3) clearing the lysate prior to purification is not required.

Purification of Polyhistidine-Tagged Proteins by FPLC

The rigid particle structure of the silica base used in the HisLink™ Resin make this material an excellent choice for applications that require applied pressure to load the lysate, wash or elute protein from the resin. These applications involve both manual and automated systems that operate under positive or negative pressure (e.g., FPLC and vacuum systems, respectively). To demonstrate the use of HisLink™ Resin on an automated platform we used an AKTA explorer from GE Healthcare. Milligram quantities of polyhistidine-tagged protein were purified from one liter of culture. The culture was lysed in 20ml of binding/wash buffer and loaded onto a column containing 1ml of HisLink™ Resin. We estimate the total amount of protein recovered to be 75–90% of the protein expressed in the original lysate.

Purification under denaturing conditions: Proteins that are expressed as an inclusion body and have been solubilized with chaotropic agents such as guanidine-HCl or urea can be purified by modifying the protocols to include the appropriate amount of denaturant (up to 6M guanidine-HCl or up to 8M urea) in the binding, wash and elution buffers.

Adjuncts for lysis or purification: The materials shown in Table 11.1 may be used without adversely affecting the ability of HisLink™ Resin to bind and elute polyhistidine-tagged proteins.

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Table 11.1. Additives That Will Not Affect Binding or Elution of Polyhistidine-Tagged Proteins Using HisLink™ Resin.

Additive	Concentration
HEPES, Tris or sodium phosphate buffers	≤100mM
NaCl	≤1M
β-mercaptoethanol	≤100mM
DTT	≤10mM
Triton® X-100	≤2%
Tween®	≤2%
glycerol	≤20%
guanidine-HCl	≤6M
urea	≤8M
RQ1 RNase-Free DNase	≤5µl/1ml original culture

Additional Resources for the HisLink™ Protein Purification Resin

Technical Bulletins and Manuals

- TB327 *HisLink™ Protein Purification Resin Technical Bulletin*
www.promega.com/tbs/tb327/tb327.html

Promega Publications

- PN090 HisLink™ 96 Protein Purification System: Fast purification of polyhistidine-tagged proteins
www.promega.com/pnotes/90/12727_15/12727_15.html
- PN086 Finding the right protein purification system
www.promega.com/pnotes/86/11217_11/11217_11.html
- CN009 Finding the right protein purification system
www.promega.com/cnotes/cn009/cn009_02.htm

Manifold (Cat.# A2291, Figure 11.5) or a compatible vacuum manifold. For more detailed protocol information, see Technical Bulletin #TB342 (www.promega.com/tbs/tb342/tb342.html).

Manual Protocol

Materials Required:

(see Composition of Solutions section)

- HisLink™ 96 Protein Purification System (Cat.# V3680, V3681) and protocol
- Nuclease-Free Water (Cat.# P1195)
- Vac-Man® 96 Vacuum Manifold (Cat.# A2291)
- plate shaker (manual) or multichannel pipette
- wide-bore tips (Racked, Sterile, Yellow Lift Top Racks; E&K Scientific Cat.# 3502-R96S)
- 96-well, deep-well plates (e.g., ABgene 2.2ml storage plate, Marsh Bio Products Cat.# AB-0932)
- 96-well sealing mats (Phenix Research Products Cat.# M-0662)
- 96-well plate adhesive sealers
- reservoir boats (Diversified Biotech Cat.# RESE-3000)

Automated Purification

The manual protocol described in Section III.C can be used as a guide to develop protocols for automated workstations. The protocol may require optimization, depending on the instrument used.

Additional Resources for HisLink™ 96 Protein Purification System

Technical Bulletins and Manuals

- TB342 *HisLink™ 96 Protein Purification System Technical Bulletin*
www.promega.com/tbs/tb342/tb342.html

Promega Publications

- PN090 HisLink™ 96 Protein Purification System: Fast purification of polyhistidine-tagged proteins
www.promega.com/pnotes/90/12727_15/12727_15.html

C. 96-Well Format For Purification of Polyhistidine-Tagged Proteins

The HisLink™ 96 Protein Purification System (Cat.# V3680, V3681) uses a vacuum-based method to purify polyhistidine-tagged expressed proteins directly from *E. coli* cultures grown in deep-well, 96-well plates. The HisLink™ 96 System is amenable to manual or automated methods for high-throughput applications. In preparation for protein purification, bacterial cells expressing a polyhistidine-tagged protein are lysed directly in culture using the provided FastBreak™ Cell Lysis Reagent. The HisLink™ Resin is added directly to the lysate and mixed, and the polyhistidine-tagged proteins bind within 30 minutes. The samples are then transferred to a filtration plate. Unbound proteins are washed away, and the target protein is recovered by elution. Figure 11.4 describes protein purification using the HisLink™ 96 System. This system requires the use of the Vac-Man® 96 Vacuum

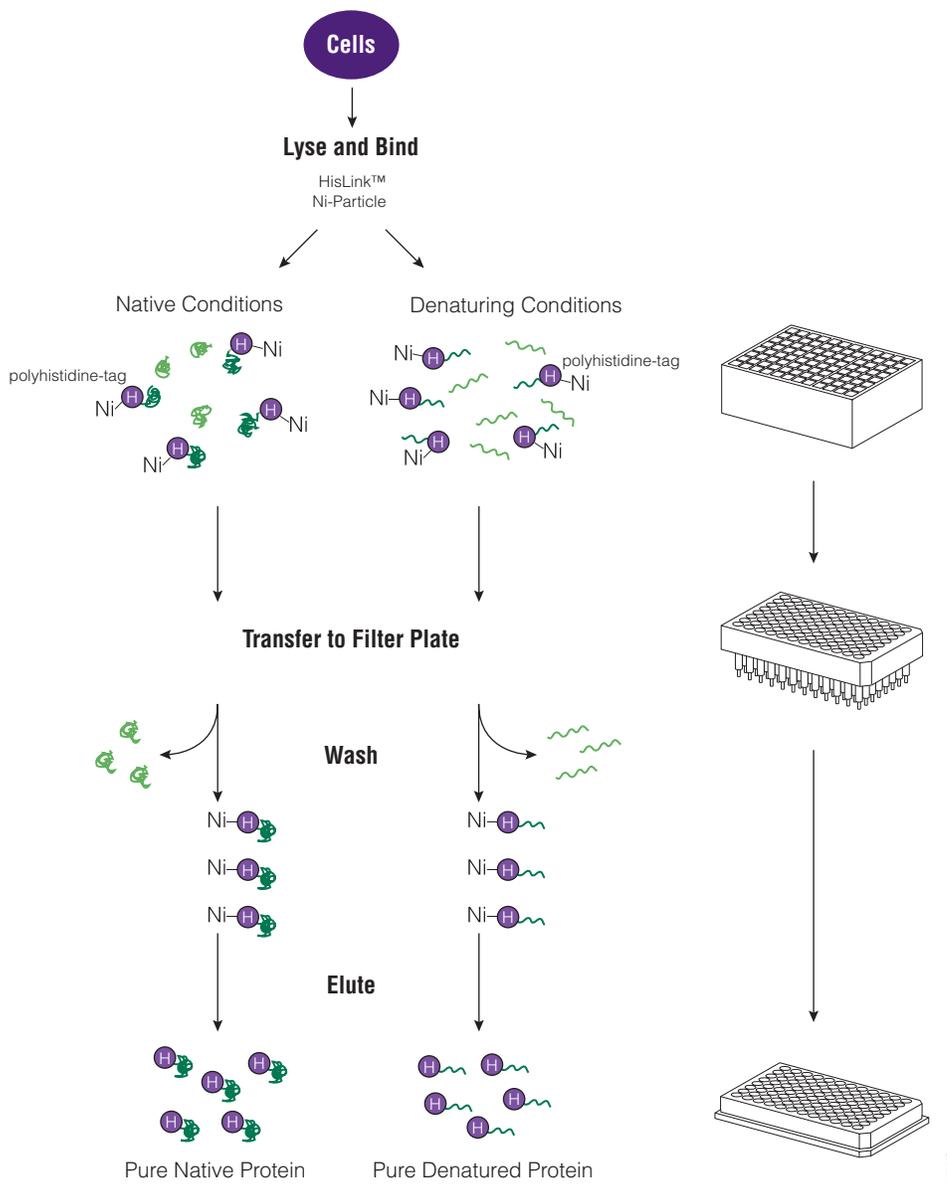


Figure 11.4. A schematic representation of the HisLink™ 96 Protein Purification protocol.

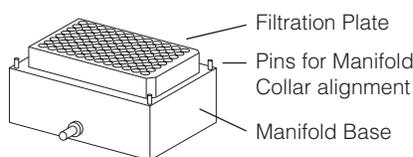
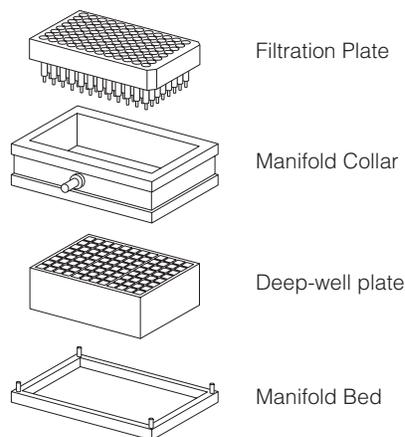
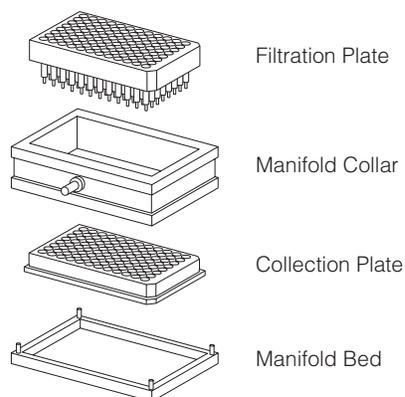
A. Protein Wash Apparatus**B. Flowthrough Collection****C. Elution Apparatus**

Figure 11.5. Flow diagram of vacuum apparatus assembly for polyhistidine-tagged protein purification using the HisLink™ 96 Protein Purification System.

IV. Purification of GST-Tagged Proteins**A. Rapid Purification of GST-Tagged Proteins Using Magnetic Resins**

There is a growing need for protein purification methods that are amenable to high-throughput screening. Magnetic resins enable affinity-tagged protein purification without the need for multiple centrifugation steps and transfer of samples to multiple tubes. There are several criteria that define a good protein purification resin: minimal nonspecific protein binding, high binding capacity for the fusion protein and efficient recovery of the fusion protein.

The MagneGST™ Protein Purification System (Cat.# V8600, V8603) meets these criteria, enabling purification of proteins with a broad range of molecular weights and different expression levels. The magnetic nature of the binding particles allows purification from a crude lysate in a single tube. In addition, the system can be used with automated liquid-handling platforms for high-throughput applications.

MagneGST™ Protein Purification System for Purification of GST-Tagged Proteins

The MagneGST™ Protein Purification System provides a simple, rapid and reliable method to purify glutathione-S-transferase (GST) fusion proteins. Glutathione immobilized on paramagnetic particles (MagneGST™ Glutathione Particles; Cat.# V8611, V8612) is used to isolate GST-fusion proteins directly from a crude cell lysate using a manual or automated procedure. The use of paramagnetic particles eliminates several centrifugation steps and the need for multiple tubes. It also minimizes the loss of sample material. Although the MagneGST™ System is designed for manual applications, samples can also be processed using a robotic platform, such as the Beckman Coulter Biomek® 2000 or Biomek® FX workstation, for high-throughput applications. Visit the Promega web site (www.promega.com/automethods/) for more information about using the MagneGST™ System in an automated format.

Bacterial cells containing a GST-fusion protein are lysed using the provided MagneGST™ Cell Lysis Reagent or an alternative lysis method, and the MagneGST™ Particles are added directly to the crude lysate. GST-fusion proteins bind to the MagneGST™ Particles. Unbound proteins are washed away, and the GST-fusion target protein is recovered by elution with 50mM glutathione. Figure 11.6 shows a schematic diagram of the MagneGST™ Protein Purification System protocol. For more detailed information about the protocol, see Technical Manual #TM240 (www.promega.com/tbs/tm240/tm240.html).

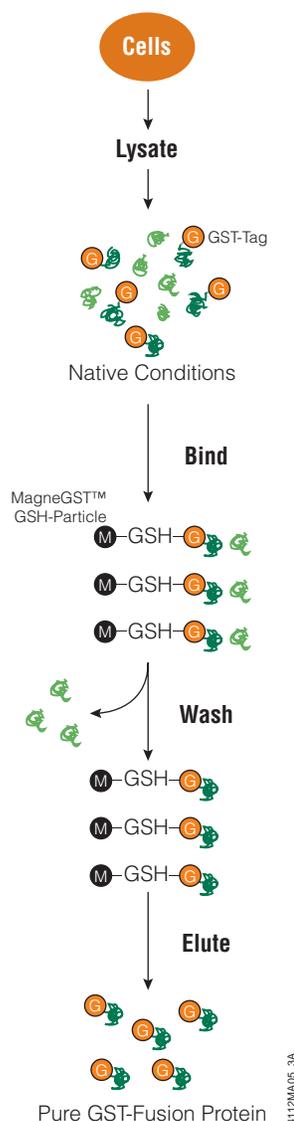


Figure 11.6. Schematic diagram of the MagneGST™ Protein Purification System. A bacterial culture expressing GST-fusion proteins is pelleted and lysed by enzymatic or mechanical methods. MagneGST™ Glutathione Particles are added directly to cleared or crude lysate. GST-fusion proteins bind to the particles during incubation at room temperature or 4°C, then are washed to remove unbound and nonspecifically bound proteins; three wash steps are performed. GST-fusion protein is eluted from the particles by adding 10–50mM reduced glutathione at pH 8.

Additionally, we have used the MagneGST™ Particles to purify GST-fusion protein generated *in vitro* using the *E. coli* S30 Extract System for Circular DNA (Cat.# L1020). When eluted protein was analyzed by SDS polyacrylamide gel electrophoresis, no major contaminating proteins were found to copurify with the GST-fusion proteins.

Example Protocol for the MagneGST™ Protein Purification System

Materials Required:

(see Composition of Solutions section)

- MagneGST™ Protein Purification System (Cat.# V8600, V8603) and protocol
- 1.5ml microcentrifuge tubes for small-scale protein purifications or 15ml or 50ml conical tubes for large-scale protein purifications
- magnetic separation stand
- RQ-1 RNase-Free DNase (Cat.# M6101)
- shaker or rotating platform
- centrifuge

Cell Lysis

1. Prepare cell pellets from 1ml of bacterial culture.
2. Add 200µl of MagneGST™ Cell Lysis Reagent to each fresh or frozen cell pellet. Resuspend the cell pellet at room temperature (20–25°C) by pipetting or gentle mixing.
3. Add 2µl of RQ1 RNase-Free DNase.
4. Incubate the cell suspension at room temperature for 20–30 minutes on a rotating platform or shaker.

Equilibrate Particles

1. Thoroughly resuspend the MagneGST™ Particles by inverting the bottle to obtain a uniform suspension.
2. Pipet 100µl of MagneGST™ Particles into a 1.5ml tube.
3. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Magnetic capture of the particles will typically occur within a few seconds.
4. Carefully remove and discard the supernatant.
5. Remove the tube from the magnetic stand. Add 250µl of MagneGST™ Binding/Wash Buffer to the particles, and resuspend by pipetting or inverting.
6. Repeat Particle Equilibration Steps 3–5 twice for a total of three washes.

Bind Proteins

1. After the final wash, gently resuspend the particles in 100µl of MagneGST™ Binding/Wash Buffer.
2. Add 200µl of cell lysate, prepared as described above, to the particles.
3. Mix gently by pipetting or inverting. If the combined volume of cell lysate and MagneGST™ Particles is less than 300µl, add additional MagneGST™ Binding/Wash Buffer so that the final volume is 300µl.
4. Incubate with gentle mixing on a rotating platform or shaker for 30 minutes at 4°C or room temperature.

Wash

1. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet.
2. Carefully remove the supernatant. Save the supernatant (flowthrough) for SDS-PAGE analysis, if desired.
3. Remove the tube from the magnetic stand. Add 250µl of MagneGST™ Binding/Wash Buffer to the particles, and mix gently by pipetting or inverting. Incubate at room temperature or 4°C for 5 minutes. Occasionally mix by inverting the tube.
4. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet.
5. Carefully remove the supernatant. Save the supernatant if analysis of wash solution is desired.
6. Remove the tube from the magnetic stand. Add 250µl of MagneGST™ Binding/Wash Buffer to the particles, and mix gently by pipetting or inverting. Incubation is not necessary at this step.
7. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet.
8. Carefully remove the supernatant. Save the supernatant if analysis of wash solution is desired.
9. Repeat Wash Steps 6–8 once for a total of three washes.

Elution

1. After the final wash, add 200µl of elution buffer.
2. Incubate at room temperature or 4°C for 15 minutes with gentle mixing.
3. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet.
4. Carefully remove the supernatant, and transfer it to a clean tube. The supernatant contains the eluted GST-fusion protein.
5. If a second elution is desired, repeat Elution Steps 1–4.

Compatibility with Common Buffer Components: The MagneGST™ Particles have been shown to be compatible with many common buffer components (Table 11.2).

Table 11.2. Buffer Components Compatible with the MagneGST™ Particles.

Buffer Component	Concentration
DTT	≤10mM
NaCl	≤0.64M
Tris, HEPES, sodium phosphate, potassium phosphate	≤100mM
Triton® X-100	≤1%
Tween®	≤1%
MAZU	≤1%
cetyltrimethylammonium bromide (CTAB)	≤1%
ethanol	20%
protease inhibitor cocktail (Roche Molecular Systems, Inc. Cat.# 1836170)	1X

Additional Resources for the MagneGST™ Protein Purification System

Technical Bulletins and Manuals

- TM240 *MagneGST™ Protein Purification System Technical Manual*
www.promega.com/tbs/tm240/tm240.html

Promega Publications

- PN086 Purification of GST-fusion proteins by magnetic resin-based MagneGST™ Particles
www.promega.com/pnotes/86/11217_06/11217_06.html
- CN009 Finding the right protein purification system
www.promega.com/cnotes/cn009/cn009_02.htm

V. Purification of Biotinylated Proteins

A. PinPoint™ Xa System and SoftLink™ Resin for Purification of Biotinylated Protein

Biotinylated fusion proteins such as those produced with the PinPoint™ Xa Protein Purification System (Cat.# V2020) can be affinity-purified using the SoftLink™ Soft Release Avidin Resin (Cat.# V2011). This proprietary resin allows elution of a fusion protein under native conditions by adding exogenous biotin. The TetraLink™ Tetrameric Avidin Resin (Cat.# V2591) can be used for irreversible capture of biotinylated proteins to generate affinity resins to purify other proteins that interact with the fusion protein. This approach is particularly useful in the affinity purification of antibodies.

The PinPoint™ Xa Protein Purification System is designed to produce and purify fusion proteins that are biotinylated in vivo. The biotinylation reaction in *E. coli* is catalyzed by biotin ligase holoenzyme and results in a fusion purification tag that carries a single biotin specifically on one lysine residue (Wilson *et al.* 1992; Xu and Beckett, 1994; Cronan,

11 Protein Purification and Analysis

1990). The biotin moiety is accessible to avidin or streptavidin, as demonstrated by binding to resins containing either molecule, and serves as a tag for detection and purification. *E. coli* produces a single endogenous biotinylated protein that, in its native conformation, does not bind to avidin, rendering the affinity purification highly specific for the recombinant fusion protein.

The system contains vectors in all possible reading frames, an avidin-conjugated resin, Streptavidin-Alkaline Phosphatase, a purification column and biotin. The PinPoint™ Xa Control Vector contains the chloramphenicol acetyltransferase (CAT) gene and is provided as a means of monitoring protein expression, purification and processing conditions. The PinPoint™ Vectors feature the encoded endoproteinase Factor Xa proteolytic site that provides a way to separate the purification tag from the native protein. These vectors also carry a convenient multiple cloning region for ease in construction of fusion proteins.

Biotinylated proteins synthesized using the PinPoint™ Xa System can be affinity-purified using the SoftLink™ Soft Release Avidin Resin. Avidin-biotin interactions are so strong that elution of biotin-tagged proteins from avidin-conjugated resins usually requires denaturing conditions. In contrast, the SoftLink™ Soft Release Avidin Resin, which uses monomeric avidin, allows the protein to be eluted with a nondenaturing 5mM biotin solution. The rate of dissociation of the monomeric avidin-biotin complex is sufficiently fast to effectively allow recovery of all bound protein in neutral pH and low salt conditions. The diagram in Figure 11.7 outlines the expression and purification system procedure.

The SoftLink™ Soft Release Avidin Resin is highly resistant to many chemical reagents (e.g., 0.1N NaOH, 50mM acetic acid and nonionic detergents), permitting stringent wash conditions. The TetraLink™ Tetrameric Avidin Resin (Cat.# V2591) can be used in place of the SoftLink™ Soft Release Avidin Resin to create affinity resins for purifying antibodies that recognize the antigenic portion of a fusion protein. Therefore, the TetraLink™ Resin is useful for immobilization and not necessarily the recovery of the fusion protein.

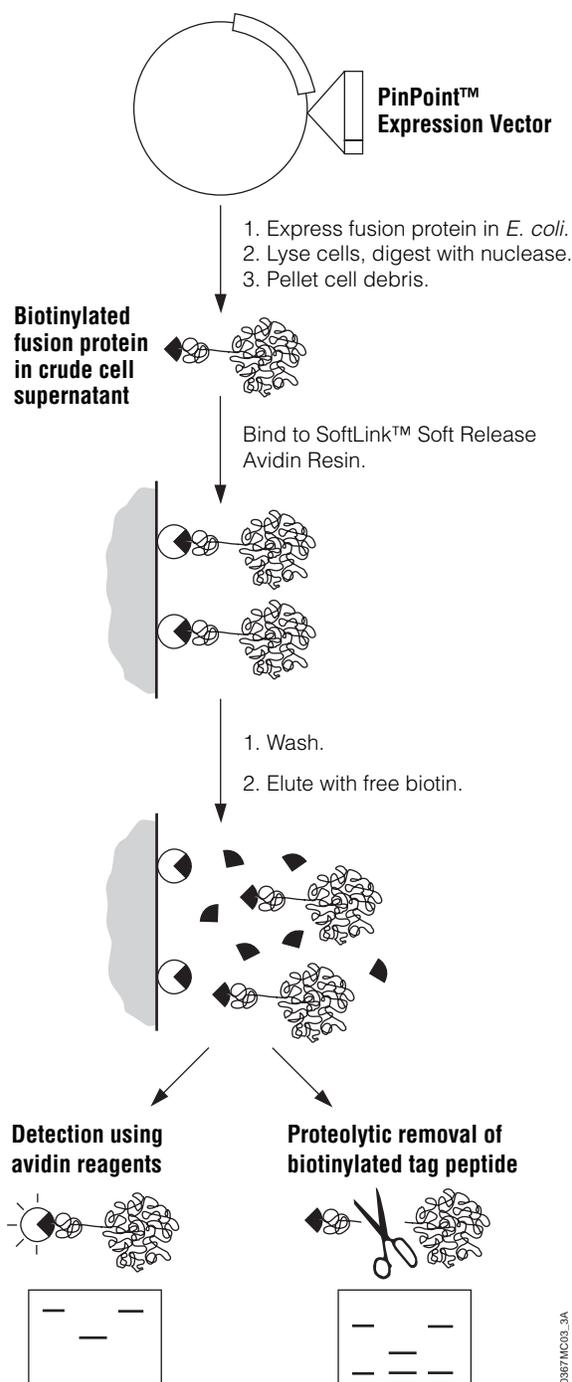


Figure 11.7. Schematic diagram of recombinant protein expression and purification using the PinPoint™ Xa Protein Purification System.

Additional Resources for the PinPoint™ Xa Protein Purification System

Technical Bulletins and Manuals

- TM028 *PinPoint™ Xa Protein Purification System Technical Manual*
www.promega.com/tbs/tm028/tm028.html

Promega Publications

- PN070 Development of a rapid capture ELISA using PCR products and the PinPoint™ System
www.promega.com/pnotes/70/7618_14/7618_14.html
- PN048 Technically speaking: PinPoint™ Xa Protein Purification System
www.promega.com/pnotes/48/techspk/techspk.html
- PN042 SoftLink™ Soft Release Avidin Resin
www.promega.com/pnotes/42/softlk4/softlk4.html

Citations

Kawasaki, H. *et al.* (2003) siRNAs generated by recombinant human Dicer induce specific and significant but target site-independent gene silencing in human cells. *Nucleic Acids Res.* **31**, 981–7.

The PinPoint™ Xa Protein Purification System was used to clone and produce recombinant human Dicer (re-hDicer). Blunt-end cDNA coding hDicer was cloned into one of the PinPoint™ Xa Vectors. Biotinylated re-hDicer was produced in *E. coli* by inducing cultures with 100µM IPTG in the presence of 2µM biotin. Recombinant hDicer was purified from *E. coli* lysates with SoftLink™ Soft Release Avidin Resin. The purified re-hDicer was demonstrated to have a putative molecular weight of ~220kDa. Recombinant-hDicer protein was also demonstrated to have RNase III-like activity in a processing assay using double-stranded puromycin resistance gene mRNA.

PubMed Number: 12560494

VI. Protein:Protein Interaction Analysis: In Vivo and In Vitro Methods

Determining the protein:protein interaction map (“interactome”) of the whole proteome is one major focus of functional proteomics (Li *et al.* 2004; Huzbun *et al.* 2003). Various methods have been used for studying protein:protein interactions, including yeast, bacterial and mammalian two- and three-hybrid systems, immunoaffinity purifications, affinity tag-based methods and mass spectrometry (reviewed in Li *et al.* 2004; Huzbun *et al.* 2003; Zhu *et al.* 2003). Moreover, in vitro pull-down-based techniques such as tandem affinity purification (TAP) are being widely used for isolating protein complexes (Forler *et al.* 2003).

In vitro protein pull-down assays can be performed using cell lysates, cell-free lysates, tissue samples, etc. These options are not possible with two-hybrid approaches. There are several reports describing the use of in vitro pull-down assays for analyzing protein:protein interactions using proteins translated in vitro using cell-free expression systems such as rabbit reticulocyte lysate-based expression systems (Charron *et al.* 1999; Wang *et al.* 2001; Pflieger *et al.* 2001). Cell-free expression is a powerful method for expressing cDNA libraries. This technique is also amenable to high-throughput protein expression and identification. Cell-free expression systems, especially rabbit reticulocyte lysate-based methods, have been extensively used for in vitro pull-down assays because of the ease of performing these experiments (Charron *et al.* 1999; Wang *et al.* 2001; Pflieger *et al.* 2001). There are also reports describing high-throughput identification of protein:protein interactions using TNT® Rabbit Reticulocyte Lysate (Pflieger *et al.* 2001).

A. Mammalian Two-Hybrid Systems

Two-hybrid systems are powerful methods to detect protein:protein interactions in vivo. The basis of two-hybrid systems is the modular nature of some transcription factor domains: a DNA-binding domain, which binds to a specific DNA sequence, and a transcriptional activation domain, which interacts with the basal transcriptional machinery (Sadowski *et al.* 1988). A transcriptional activation domain in association with a DNA-binding domain promotes the assembly of RNA polymerase II complexes at the TATA box and increases transcription. In the CheckMate™ Mammalian Two-Hybrid System (Cat.# E2440), the DNA-binding domain and transcriptional activation domain, produced by separate plasmids, are closely associated when one protein (“X”) fused to a DNA-binding domain interacts with a second protein (“Y”) fused to a transcriptional activation domain. In this system, interaction between proteins X and Y results in transcription of a reporter gene or selectable marker gene (Figure 11.8).

Originally developed in yeast (Fields and Song, 1989; Chien *et al.* 1991), the two-hybrid system has been adapted for use in mammalian cells (Dang *et al.* 1991; Fearon *et al.* 1992). One major advantage of the CheckMate™ Mammalian Two-Hybrid System over yeast systems is that the protein:protein interaction can be studied in the cell line of choice. The CheckMate™ System also uses the Dual-Luciferase® Reporter Assay System for rapid and easy quantitation of luciferase reporter gene expression.

Application of the CheckMate™ Mammalian Two-Hybrid System confirms suspected interactions between two proteins and identifies residues or domains involved in protein:protein interactions. When identifying residues or domains involved in an interaction, the GeneEditor™ in vitro Site-Directed Mutagenesis System (Cat.# Q9280) for making site-directed mutants and Erase-a-Base® Systems for deletion analysis are useful tools. These products are fully compatible with the CheckMate™ Mammalian

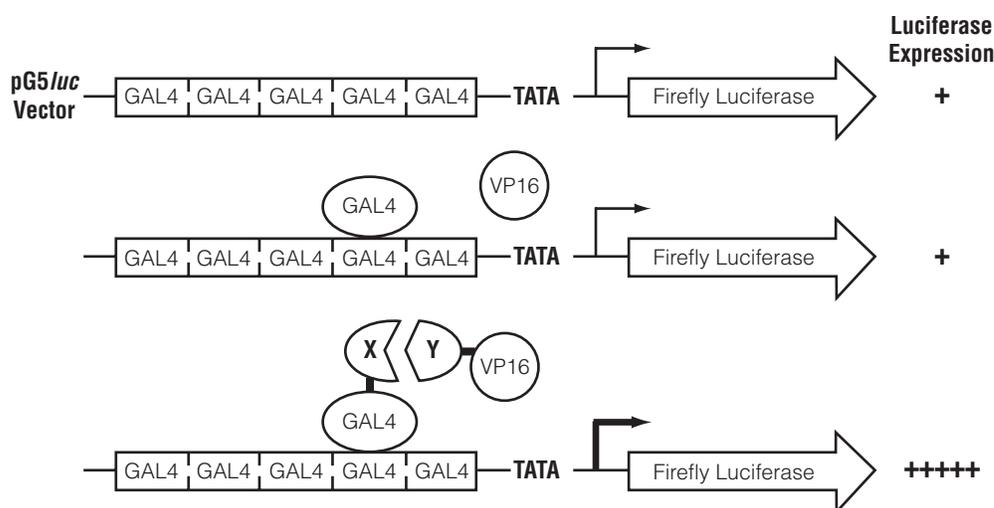


Figure 11.8. Schematic representation of the CheckMate™ Mammalian Two-Hybrid System. The pG5*luc* Vector contains five GAL4 binding sites upstream of a minimal TATA box, which in turn, is upstream of the firefly luciferase gene. In negative controls, the background level of luciferase is measured in the presence of GAL4 (from the pBIND Vector) and VP16 (from the pACT Vector). Interaction between the two test proteins, as GAL4-X and VP16-Y fusion constructs, results in an increase in luciferase expression over the negative controls.

Two-Hybrid System. Detailed protocol information is available in Technical Manual #TM049 (www.promega.com/tbs/tm049/tm049.html).

Assessing Protein:Protein Interactions

cDNA sequences encoding the polypeptides of interest are subcloned into pBIND and pACT Vectors. The insert in each vector must be in the correct orientation and reading frame. See the *CheckMate™ System Technical Manual #TM049* (www.promega.com/tbs/tm049/tm049.html) for the multiple cloning region following the 3' end of the GAL4 fragment for pBIND Vector and for the multiple cloning region following the 3' end of the VP16 fragment for pACT Vector. All vectors in the CheckMate™ Mammalian Two-Hybrid System confer ampicillin resistance and are compatible with *E. coli* strains such as JM109. We **strongly recommend** sequencing the 5' junction between the insert and vector to ensure that the insert is subcloned properly. The T3 Promoter Primer (Cat.# Q5741) can be used for sequence verification.

Certain inserts appear to show vector “directionality” (or preference) in which the interaction between a pair of proteins is fusion vector-dependent (Finkel *et al.* 1993). Protein:protein interactions may appear stronger given a particular vector context for the inserts. Because of this

phenomenon, we advise subcloning each cDNA of interest into both the pBIND and pACT Vectors and testing the two possible fusion protein interactions.

Following the successful subcloning of the test cDNAs into the pBIND and pACT Vectors, the resultant plasmids should be purified such that the DNA is free of protein, RNA and chemical contamination. Before completing any experiments with the CheckMate™ System, optimize the transfection method for the cell type being transfected. The optimization process is easier using a reporter gene and assay system. Many DNA delivery agents exist for transfecting mammalian cells. Transfection of DNA into mammalian cells may be mediated by cationic lipids, calcium phosphate, DEAE-dextran or electroporation. Transfection systems based on cationic lipids (TransFast™ Transfection Reagent, Transfectam® Reagent, Tfx™-20, Tfx™-50 Reagents) and calcium phosphate (ProFection® Mammalian Transfection System) are available from Promega. The efficiency of each transfection method is highly dependent upon the cell type. When optimizing a transfection method for a particular cell type, use a reporter gene such as the firefly luciferase gene whose activity is easily and rapidly assayed. The pGL3-Control Vector (Cat.# E1741) expresses the firefly luciferase gene from the SV40 early promoter.

Table 11.3 presents the recommended combinations of vectors to properly control an experiment when using the CheckMate™ System to determine the extent to which two proteins interact in a two-hybrid assay.

The amount of vector DNA to use will depend upon the method of transfection. However, we recommend that the molar ratio of pBIND:pACT Vector constructs be 1:1. We have varied the amount of pG5luc Vector in the positive control experiment and have found that the signal-to-noise ratio of firefly luciferase expression does not differ significantly. We routinely use a molar ratio of 1:1:1 for pBIND:pACT:pG5luc Vector in the CheckMate™ Mammalian Two-Hybrid System. Maintain a constant amount of DNA for each transfection reaction within an experiment by adding plasmid DNA such as pGEM®-3Zf(+) Vector (Cat.# P2271).

We recommend testing a specific cell line with positive and negative control transfection reactions before initiating test experiments. The pBIND Vector encodes the *Renilla* luciferase gene to normalize for transfection efficiency. Replication of pBIND and pACT Vectors and their recombinants is expected in COS cells or other types of cells that express the SV40 large T antigen. Use the Dual-Luciferase® Reporter Assay System (Cat.# E1910) to quantitate *Renilla* luciferase and firefly luciferase activities.

Additional Resources for the CheckMate™ Mammalian Two-Hybrid System

Technical Bulletins and Manuals

TM049 *CheckMate™ Mammalian Two-Hybrid System Technical Manual*
(www.promega.com/tbs/tm049/tm049.html)

Promega Publications

PN066 The CheckMate™ Mammalian Two-Hybrid System
(www.promega.com/pnotes/66/7014_02/7014_02.html)

Vector Maps

pACT Vector and pACT-MyoD Control Vector
(www.promega.com/vectors/mammalian_express_vectors.htm#b01)

pBIND Vector and pBIND-Id Control Vector
(www.promega.com/vectors/mammalian_express_vectors.htm#b02)

pG5luc Vector (www.promega.com/vectors/reporter_vectors.htm#b03)

Citations

Suico, M. *et al.* (2004) Myeloid Elf-1-like factor, an ETS transcription factor, up-regulates lysozyme transcription in epithelial cells through interaction with promyelocytic leukemia protein. *J. Biol. Chem.* **279**, 19091–8.

The authors investigated the role of the promyelocytic leukemia (PML) nuclear body in transactivation of myeloid elf-1-like factor (MEF), a transcription factor that upregulates lysozyme transcription. To determine if the nuclear factors affected MEF, HeLa cells were cotransfected with 0.2µg of a pGL2 Vector construct with a lysozyme promoter and various combinations of 0.1µg of MEF, 0.5µg of PML and 1µg of Sp100 (another nuclear body factor) plasmids. Expression was normalized to 10ng of pHRG-TK Vector. Forty-eight hours post-transfection, the cells were harvested and luciferase activity measured using the Dual-Luciferase® Reporter Assay System. In addition, MEF mutants were made and tested in the same dual-reporter system to determine if transactivation was affected by the various deletion mutations. These MEF mutants were also cloned into a vector with the yeast GAL4 DNA-binding domain to help determine which domain of MEF was interacting with PML nuclear body in a mammalian two-hybrid system. This was done using the CheckMate™ Mammalian Two-Hybrid System.

PubMed Number: 14976184

B. In Vitro Pull-Down Assays

Glutathione-S-Transferase (GST) Pull-Down Assays

The glutathione-S-transferase (GST) pull-down assay (Kaelin *et al.* 1991) is an important tool to validate suspected protein:protein interactions and identify new interacting partners (Benard and Bokoch, 2002; Wang *et al.* 2000; Wada *et al.* 1998; Malloy *et al.* 2001). GST pull-down assays use a GST-fusion protein (bait) bound to glutathione (GST)-coupled particles to affinity purify any proteins that interact with the bait from a pool of proteins (prey) in

Table 11.3. Recommended Experimental Design to Determine the Magnitude of Interaction Between Two Proteins.

Transfection	pBIND Vector	pACT Vector	pG5luc Vector
1	pBIND Vector	pACT Vector	pG5luc Vector
2	pBIND-Id Control Vector	pACT-MyoD Control Vector	pG5luc Vector
3	–	–	–
4	pBIND-X Vector	pACT Vector	pG5luc Vector
5	pBIND Vector;	pACT-Y Vector	pG5luc Vector
6	pBIND-X Vector;	pACT-Y Vector	pG5luc Vector

solution. Bait and prey proteins can be obtained from multiple sources, including cell lysates, purified proteins and in vitro transcription/translation systems.

The MagneGST™ Pull-Down System (Cat.# V8870) is optimized for detection of protein:protein interactions where the bait protein is prepared from an *E. coli* lysate and mixed with prey protein synthesized in the TNT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170). The magnetic nature of the MagneGST™ GSH-linked particles in this system offers significant advantages over traditional resins, which require lengthy preparation and equilibration and are hard to dispense accurately in small amounts. The MagneGST™ Particles are easy to dispense in volumes less than 5µl, and equilibration is quick and easy and does not require any centrifugation steps. Another advantage of this system is that the pull-down reaction is performed in one tube. The particles are easily and efficiently separated from supernatants using a magnetic stand without centrifugation, increasing reproducibility and reducing sample loss. The flexible format of the MagneGST™ Pull-Down System allows optimization of experimental conditions, including modification of particle volume, to fit specific requirements of each unique protein:protein interaction. Additionally, the system allows easy processing of multiple samples at once.

The MagneGST™ Pull-Down System provides GST-linked magnetic particles that enable simple immobilization of bait proteins from bacterial lysates and an in vitro transcription/translation system for expressing prey proteins. The MagneGST™ Pull-Down protocol can be divided into three phases: 1) the prey protein is expressed in the TNT® T7 Quick Coupled System; 2) bait protein present in crude *E. coli* lysate is immobilized on the MagneGST™ Particles; and 3) the prey protein is mixed with MagneGST™ Particles carrying the bait protein and captured through bait:prey interaction. Nonspecifically bound proteins are washed away, and the prey and bait proteins are eluted with SDS loading buffer. Prey proteins can be analyzed by SDS-PAGE and autoradiography if the prey protein was radioactively labeled during synthesis.

The transcription/translation component of the MagneGST™ Pull-Down System is the TNT® T7 Quick Master Mix, which allows convenient, single-tube, coupled transcription/translation of genes cloned downstream from a T7 RNA polymerase promoter. The TNT® System is compatible with circular (plasmid) or linear (plasmid or PCR product) templates. For more information on the TNT® T7 Quick Coupled Transcription/Translation System, refer to Technical Manual #TM045 (www.promega.com/tbs/tm045/tm045.html). An overview of the MagneGST™ Pull-Down System is depicted in Figure 11.9. An animated presentation (www.promega.com/paguide/animation/selector.htm?coreName=tnt01) (requires Flash plug in) of the MagneGST™ pull-down process using the TNT® T7 Quick Coupled System is

available. More information and detailed protocol information is available in Technical Manual #TM249 (www.promega.com/tbs/tm249/tm249.html).

Example Protein Pull-Down Protocol Using the MagneGST™ Pull-Down System

Materials Required:

(see Composition of Solutions section)

- MagneGST™ Pull-Down System (Cat.# V8870) and protocol
- Magnetic Separation Stand (Cat.# Z5342, Z5343, Z5332, Z5333 or A2231)
- radiolabeled methionine (e.g., [³⁵S]Met, 10–40µCi per TNT® reaction) for radioactive detection of prey protein or specific antibodies for detection using Western blot analysis
- RQ1 RNase-Free DNase (Cat.# M6101)
- NANOpure® or double-distilled water
- SDS loading buffer
- BSA (Cat.# W3841) or IGEPAL® CA-630 (Sigma Cat.# I3021)

Express Prey Protein using a TNT® T7 Quick Coupled Transcription/Translation Reaction

1. Remove the reagents from storage at –70°C. (Store the RQ1 DNase at –20°C after first use.) Thaw the TNT® T7 Quick Master Mix by hand-warming or on ice. The other components can be thawed at room temperature and stored on ice.
2. Assemble the reaction components as shown in the table below using template DNA encoding your prey protein of interest. Incubate the reaction at 30°C for 60–90 minutes. During this incubation period, prepare the MagneGST™ Particles.

Example of a TNT® T7 Quick Reaction Using Plasmid DNA.

Components	Reaction Using [³⁵ S]Methionine	Reaction Using Unlabeled Methionine
TNT® T7 Quick Master Mix	40µl	40µl
Methionine, 1mM	–	1µl
[³⁵ S]methionine (1,000Ci/mmol at 10mCi/ml)	2µl	–
plasmid DNA template(s) (0.5µg/µl)	2µl	2µl
Nuclease-Free Water to a final volume of	50µl	50µl

Immobilize GST-Fusion Proteins onto MagneGST™ Particles

Lyse Cells

1. Harvest cells from 1ml of bacterial culture.

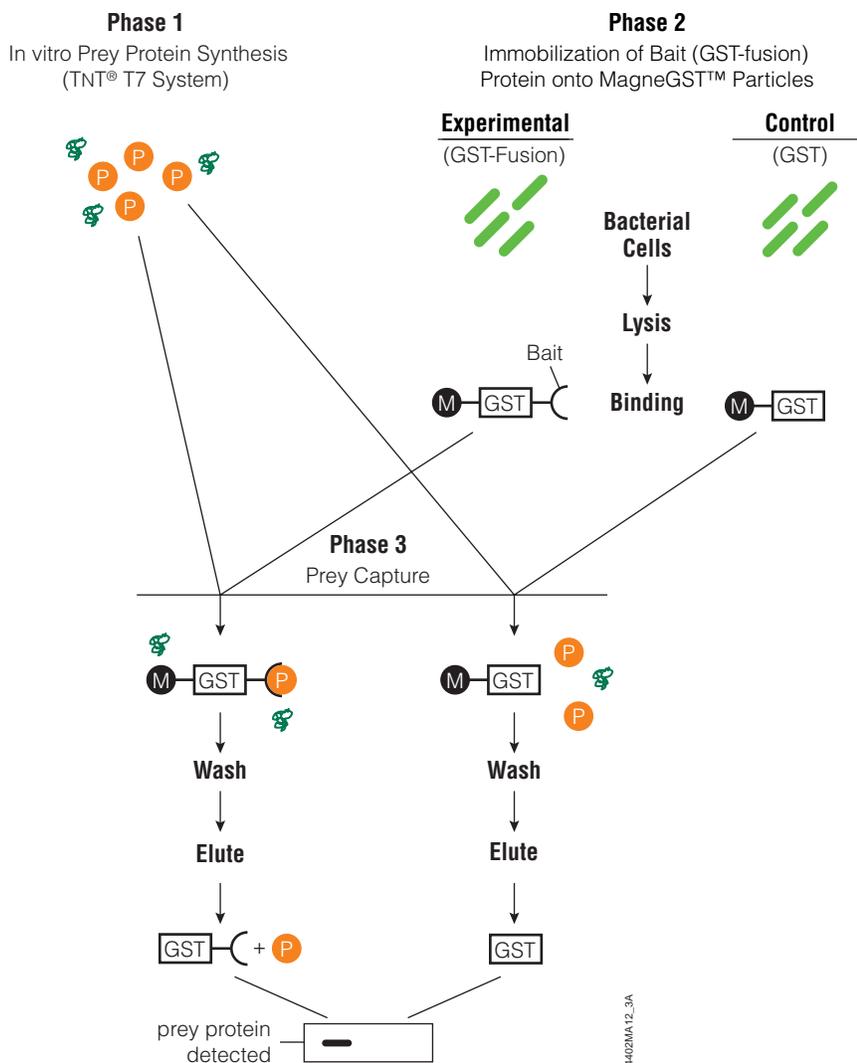


Figure 11.9. Schematic diagram of the MagneGST™ Pull-Down System protocol. P = prey protein, M = MagneGST™ Particle.

2. Add 200µl of MagneGST™ Cell Lysis Reagent to each fresh or frozen cell pellet. Resuspend the cell pellet at room temperature (20–25°C) by pipetting or gentle mixing.
3. Add 2µl of RQ1 RNase-Free DNase.

Note: Addition of DNase reduces viscosity and can increase the purity of GST-fusion proteins. Up to 5µl of RQ1 RNase-Free DNase can be added to reduce viscosity. The DNase can be omitted, if desired.
4. Incubate the cell suspension at room temperature for 20–30 minutes on a rotating platform or shaker. During this incubation, begin the particle equilibration procedure.
2. Pipet 20µl of MagneGST™ Particles into a 1.5ml tube.
3. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Magnetic capture will typically occur within a few seconds.
4. Carefully remove and discard the supernatant.
5. Remove the tube from the magnetic stand. Add 250µl of MagneGST™ Binding/Wash Buffer to the particles, and resuspend by pipetting or inverting.
6. Repeat Steps 3–5 two more times for a total of three washes.

Bind Protein

1. After the final wash, resuspend the particles in 100µl of MagneGST™ Binding/Wash Buffer.

Note: Adding up to 1% BSA may reduce nonspecific binding and potential problems with background.



11 Protein Purification and Analysis

IGEPAL® CA-630 (NP40 analog) at final concentration 0.5% may have the same effect. The amount of BSA used may need to be optimized for your particular protein.

2. Add 200µl of cell lysate containing the GST-fusion protein or GST control to the MagneGST™ Particles.
3. Incubate (with constant gentle mixing) for 30 minutes at room temperature on a rotating platform.

Note: Do not allow the MagneGST™ Particles to settle for more than a few minutes during capture of the bait protein as this will reduce binding efficiency.

Wash

1. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Carefully remove the supernatant, and save for gel analysis (optional).
2. Add 250µl of MagneGST™ Binding/Wash Buffer to the particles, and gently mix. Incubate at room temperature for 5 minutes while mixing occasionally by tapping or inverting the tube.
3. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Carefully remove the supernatant, and discard (or save if analysis of wash is desired).
4. Add 250µl of MagneGST™ Binding/Wash Buffer to the particles, and mix gently by inverting the tube. (The 5-minute incubation is not required at this wash step.)
5. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Carefully remove the supernatant, and discard (or save if analysis of wash is desired).
6. Repeat Steps 4–5 for a total of three washes.
7. After the last wash, resuspend the particles in 20µl of MagneGST™ Binding/ Wash Buffer.
8. We recommend using 5µl of the immobilized GST-fusion or GST control for the pull-down assay. Thus, 20µl of particles will provide sufficient material for more than one set of pull-down reactions. However, in some cases more than 5µl may be required for one pull-down reaction.

Capture, Wash and Analysis of Prey Protein

Capture

1. Add 20µl of the TNT® T7 Quick coupled transcription/translation reaction from Phase 1 to each 5µl aliquot of particles carrying GST-fusion protein (or GST control).

2. Add 155µl MagneGST™ Binding/Wash Buffer and 20µl 10% BSA (or 175µl MagneGST™ Binding/Wash Buffer if BSA is omitted) to a final volume of 200µl for each pull-down reaction.

Note: MagneGST™ Binding/Wash Buffer is a neutral PBS buffer, allowing the user to optimize buffer conditions for each specific protein:protein interaction. Some protein interactions will require the presence of various cofactors, salts and detergents.

3. Incubate for 1 hour (with gentle mixing) at room temperature on a rotating platform.

Note: Do not allow the MagneGST™ Particles to settle for more than a few minutes during capture of the prey protein, as this will reduce binding efficiency.

4. Place the tube in a magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet.

Washing

1. Add 400µl of MagneGST™ Binding/Wash Buffer, and mix gently by inverting the tube.
2. Incubate at room temperature for 5 minutes while mixing occasionally by tapping or inverting the tube.
3. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Remove the supernatant, and save for analysis (it is especially important to keep this fraction during initial optimization).
4. Add 400µl of MagneGST™ Binding/Wash Buffer, and mix gently by inverting the tube. (The 5-minute incubation is not required at this wash step.)
5. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet.
6. Repeat Steps 4 and 5 three more times for a total of five washes.

Elution

1. Add 20µl of 1X SDS loading buffer.
2. Incubate for 5 minutes at room temperature with mixing.
3. Place the tube in the magnetic stand, and allow the MagneGST™ Particles to be captured by the magnet. Remove the eluate for analysis.

Analysis

Prepare samples for SDS-PAGE analysis. For radioactively labeled prey proteins, we recommend loading 1–2% of each sample volume.

Additional Resources for the MagneGST™ Pull-Down System

Technical Bulletins and Manuals

- TM249 *MagneGST™ Pull-Down System Technical Manual*
www.promega.com/tbs/tm249/tm249.html

Promega Publications

- PN087 Detection of protein:protein interactions using the MagneGST™ Pull-Down System
www.promega.com/pnotes/87/11527_23/11527_23.html
-

VII. Analysis of DNA:Protein Interactions

Regulation of chromatin structure and gene expression is essential for normal development and cellular growth. Transcriptional events are tightly controlled both spatially and temporally by specific protein:DNA interactions. Currently there is a rapidly growing trend toward genome-wide identification of protein-binding sites on chromatin to characterize regulatory protein:DNA interactions that govern the transcriptome. Common methods to examine protein:DNA interactions include the electrophoretic mobility shift assay, also known as the gel shift assay, and chromatin immunoprecipitation (Solomon *et al.* 1985; Solomon *et al.* 1988) coupled with DNA microarray or ultrahigh-throughput sequencing analysis.

A. Gel Shift Assays

Electrophoretic mobility shift assays (EMSA) or gel shift assays can be used to analyze protein:DNA complexes expressed *in vitro*. The proteins are incubated with an oligonucleotide containing a target consensus sequence site, and DNA binding is detected by gel shift. An animated presentation (www.promega.com/paguide/animation/selector.htm?coreName=tnt02) (requires Flash plug in) of protein:DNA interaction detection using the TNT® Systems and Gel Shift Assay is available. The gel shift assay provides a simple and rapid method to detect DNA-binding proteins (Ausubel *et al.* 1989). This method is used widely in the study of sequence-specific DNA-binding proteins such as transcription factors. The assay is based on the observation that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides. The gel shift assay is performed by incubating a purified protein, or a complex mixture of proteins (such as nuclear or cell extract preparations), with a ³²P end-labeled DNA fragment containing the putative protein-binding site. The reaction products are then analyzed on a nondenaturing polyacrylamide gel. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using DNA fragments or oligonucleotides containing a binding site for the protein of interest or other unrelated DNA sequences.

Promega gel shift assay systems contain target oligonucleotides, a control extract containing DNA-binding proteins, binding buffer and reagents for phosphorylating oligonucleotides. The Gel Shift Assay Core System (Cat.# E3050) includes sufficient HeLa nuclear extract to perform 20 control reactions, Gel Shift Binding 5X Buffer, an SP1 Consensus Oligo and an AP2 Consensus Oligo. The complete Gel Shift Assay System (Cat.# E3300) contains five additional double-stranded oligonucleotides that represent consensus binding sites for AP1, NF-κB, OCT1, CREB and TFIID. These oligonucleotides can be end-labeled and used as protein-specific probes or as specific or nonspecific competitor DNA in competition assays. A detailed protocol is available in Technical Bulletin #TB110 (www.promega.com/tbs/tb110/tb110.html).

Additional Resources for Gel Shift Assay Systems

Technical Bulletins and Manuals

- TB110 *Gel Shift Assay Systems Technical Bulletin*
www.promega.com/tbs/tb110/tb110.html

Promega Publications

- PN037 Gel shift analysis with Human Recombinant AP1 (c-jun): The effect of poly d(I-C) on specific complex formation
www.promega.com/pnotes/37/37_14/37_14.htm
- PN045 Technically speaking: Gel Shift Assay System
www.promega.com/pnotes/45/45p25/45p25.html

Citations

Lee, J. *et al.* (2002) Kaurane diterpene, kamebakaurin, inhibits NF-kappa B by directly targeting the DNA-binding activity of p50 and blocks the expression of antiapoptotic NF-kappa B target genes *J. Biol. Chem.* **277**, 18411–20.

To investigate the effect of the compound kamebakaurin (KA) on NF-κB, an NF-κB-responsive firefly luciferase vector was transfected into HeLa, Jurkat and THP-1 cells. The Luciferase Assay System was used to assay the level of NF-κB induction after treatment of cells with various concentrations of KA. To determine if KA influenced the DNA-binding activity of NF-κB, nuclear extracts of HeLa, Jurkat and THP-1 cells were prepared after preincubation with KA and stimulation of NF-κB activity. Control nuclear extracts were prepared from unstimulated p50- or RelA-overexpressed MCF-7 cells. In addition, the wildtype and DNA-binding mutant RelA and p50 (NF-κB) His-tagged proteins were translated using the TNT® Quick Coupled Transcription/Translation System and subsequently purified. Using the Gel Shift Assay System, the NF-κB and AP1 oligos were tested for electromobility shifts with the prepared nuclear extracts or with purified wildtype and mutant proteins. Supershift studies using anti-p50 or anti-RelA antibodies were also performed

PubMed Number: 11877450

B. Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) is an experimental method used to determine whether DNA-binding proteins, such as transcription factors, associate with a specific genomic region in living cells or tissues. Cells are treated with formaldehyde to form covalent crosslinks between interacting proteins and DNA. Following crosslinking, cells are lysed, and the crude cell extracts are sonicated to shear the DNA. The DNA:protein complex is immunoprecipitated using an antibody that recognizes the protein of interest. The isolated complexes are washed, then eluted. The DNA:protein crosslinks are reversed by heating and the proteins removed by proteinase K treatment. The remaining DNA is purified and analyzed by various ways, including PCR, microarray analysis or direct sequencing.

Antibody-Based ChIP

The standard ChIP assay requires 3–4 days for completion (Figure 11.10). The procedure requires antibodies highly specific to the protein of interest to immunoprecipitate the DNA:protein complex. The success of the procedure relies on the ability of the antibody to bind to the target protein after crosslinking, cell lysis and sonication, all of which can negatively affect epitope recognition by the antibody.

HaloCHIP™ System—an Antibody-Free Approach

To address the difficulties that arise when performing ChIP, a novel method that does not require the use of antibodies, the HaloCHIP™ System, has been devised for the covalent capture of protein:DNA complexes. DNA-binding proteins of interest are expressed in cells as HaloTag® fusion proteins, crosslinked to DNA, then captured on the HaloLink™ Resin, which forms a highly specific, covalent interaction with HaloTag® proteins. Due to the covalent linkage between the resin and crosslinked protein:DNA complexes, the resin can be stringently washed to remove nonspecifically bound DNA and protein more efficiently than co-immunoprecipitation. The crosslinks are reversed to release purified DNA fragments from the resin. By improving specificity and reducing background during the isolation of protein:DNA complexes, the HaloCHIP™ approach effectively increases the signal-to-noise ratio to permit detection of small changes in protein binding within a genome. The HaloCHIP™ System (Cat.# G9410) is currently available. An animation of this procedure (www.promega.com/multimedia/halochip01.htm) (requires Flash plug in) is available.

Additional Resources for the HaloCHIP™ System**Technical Bulletins and Manuals**

- TM075 *HaloCHIP™ System Technical Manual*
(www.promega.com/tbs/tm075/tm075.html)

Promega Publications

- PN097 HaloCHIP™ System: Mapping intracellular protein:DNA interactions using HaloTag® technology
(www.promega.com/pnotes/97/15377_18/15377_18.html)

Additional Resources for the HaloTag® Technology**Technical Bulletins and Manuals**

- TM260 *HaloTag® Technology: Focus on Imaging Technical Manual*
(www.promega.com/tbs/tm260/tm260.html)

Promega Publications

- CN014 HaloTag™ technology: Cell imaging and protein analysis
(www.promega.com/cnotes/cn014/cn014_10.htm)
- CN012 Perform multicolor live- and fixed-cell imaging applications with the HaloTag™ interchangeable labeling technology
(www.promega.com/cnotes/cn012/cn012_04.htm)
- CN011 HaloTag™ interchangeable labeling technology for cell imaging and protein capture
(www.promega.com/cnotes/cn011/cn011_02.htm)
- PN095 HaloTag® protein: A novel reporter protein for human neural stem cells
(www.promega.com/pnotes/95/14867_20/14867_20.html)
- PN095 Cell surface HaloTag® technology: Spatial separation and bidirectional trafficking of proteins
(www.promega.com/pnotes/95/14867_16/14867_16.html)
- PN092 HaloLink™ Resin: High capacity, specificity and scalable throughput for protein analysis
(www.promega.com/pnotes/92/13408_24/13408_24.html)
- PN089 HaloTag™ interchangeable labeling technology for cell imaging, protein capture and immobilization
(www.promega.com/pnotes/89/12416_02/12416_02.html)

VIII. Proteomics Approaches for the Analysis of Complex Mixtures of Proteins

Mass spectrometry is a powerful tool for protein analysis and the major technique used to study proteins in the field of proteomics (Mann *et al.* 2001). Mass spectrometry can be used to characterize recombinant proteins, identify proteins and detect and characterize posttranslational modifications.

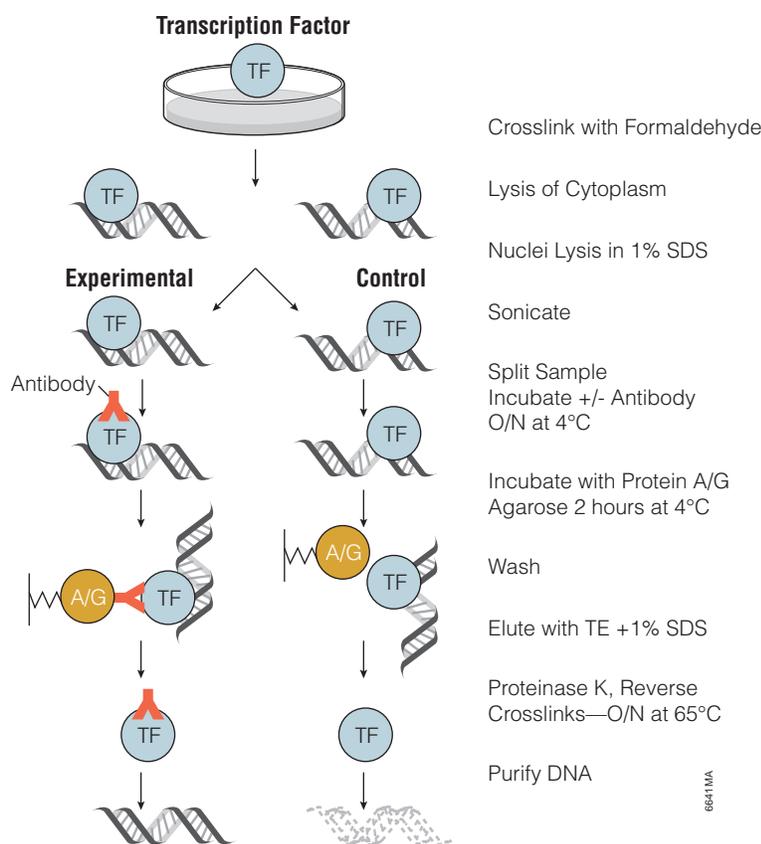


Figure 11.10. Overview of chromatin immunoprecipitation using antibodies. Cells are grown using the appropriate conditions to form an interaction between the transcription factor (TF) of interest and DNA. To preserve the DNA:protein association during cell lysis, formaldehyde is added, resulting in crosslinks between the DNA and protein. A whole-cell extract is prepared, and the crosslinked chromatin is sheared by sonication to reduce the average DNA fragment size. A polyclonal or monoclonal antibody that recognizes the target protein is added, then incubated overnight. Protein A or Protein G agarose beads are added to capture the complex, then washed. The antibody must specifically and tightly bind its target protein under the wash conditions used. Finally, reversal of the formaldehyde crosslinking by heating permits the recovery and quantitative analysis of the immunoprecipitated DNA.

One method of protein identification uses enzymatic digestion followed by mass spectrometry analysis. In this procedure, complex protein mixtures such as cell extracts are resolved by gel electrophoresis, and the band or spot of interest is excised from the gel and digested with trypsin. Trypsin is a serine protease that specifically cleaves at the carboxylic side of lysine and arginine residues. The distribution of Lys and Arg residues in proteins is such that trypsin digestion yields peptides of molecular weights that can be analyzed by mass spectrometry. The pattern of peptides obtained is used to identify the protein. Database searches can then be performed, using the mass of the peptides to identify the protein(s) resolved on the gel (Mann *et al.* 2001).

The stringent specificity of trypsin is essential for protein identification. Native trypsin is subject to autolysis, generating pseudotrypsin, which exhibits a broadened specificity, including a chymotrypsin-like activity (Keil-Dlouha *et al.* 1971). Such autolysis products would result in additional peptide fragments that could interfere with database analysis of the mass of fragments detected by mass spectrometry.

Trypsin Gold, Mass Spectrometry Grade (Cat.# V5280), provides maximum specificity. Lysine residues in the porcine trypsin are modified by reductive methylation, yielding a highly active and stable molecule that is extremely resistant to autolytic digestion (Rice *et al.* 1977). The specificity of the purified trypsin is further improved by TPCK treatment, which inactivates chymotrypsin. The treated trypsin is then purified by affinity chromatography and lyophilized to yield Trypsin Gold, Mass Spectrometry Grade. More information and a detailed protocol are available in Technical Bulletin TB309 (www.promega.com/tbs/tb309/tb309.html).

A. In-Gel Trypsin Digestion of Proteins

Numerous protocols for in-gel protein digestion have been described (Flannery *et al.* 1989; Shevchenko *et al.* 1996; Rosenfeld *et al.* 1992). The following procedure has been used successfully by Promega scientists.

Materials Required:

(see Composition of Solutions section)

- Trypsin Gold, Mass Spectrometry Grade (Cat.# V5280) and protocol

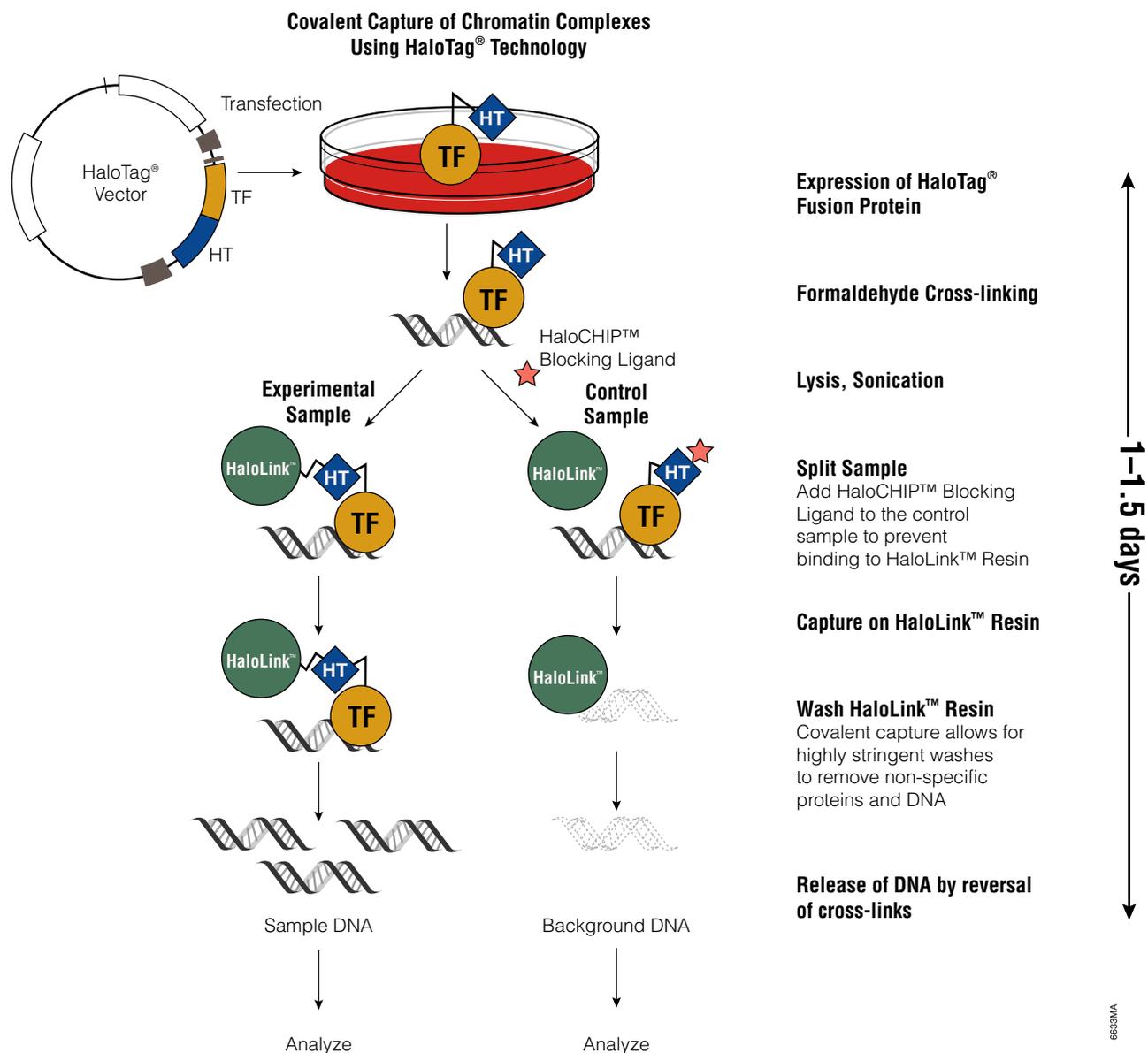


Figure 11.11. Capture of DNA:protein interactions using the HaloTag® technology. The protein-coding sequence of a transcription factor (TF) is cloned into a HaloTag® (HT) mammalian expression vector. This recombinant vector is transfected into mammalian cells, and the cells are grown under the appropriate conditions to allow formation of DNA:protein interactions. To preserve the DNA:protein association, formaldehyde is added, resulting in crosslinks between DNA and protein. A whole-cell extract is prepared, and the crosslinked chromatin is sheared by sonication to reduce the average DNA fragment size. The complex is then immobilized by adding the HaloLink™ Resin, followed by a short incubation. Reversal of the formaldehyde crosslinking by heating permits the recovery and quantitative analysis of immunoprecipitated DNA.

- SimplyBlue™ SafeStain (Invitrogen Cat.# LC6060)
- trifluoroacetic acid (TFA)
- acetonitrile (ACN)
- 200mM NH₄HCO₃ buffer (pH 7.8)
- NANOpure® water
- ZipTip® scx pipette tips (Millipore Cat.# ZTSCXS096)
- α-cyano-4-hydroxycinnamic acid (CHCA)
- MALDI target
- ZipTip® C18 pipette tips (Millipore Cat.# ZTC18S096)

1. Separate protein samples by electrophoresis on an SDS-Tris-Glycine gel.

Note: Other gel systems and staining reagents can be used for in-gel digestions but should be tested to ensure compatibility with the protein of interest and detection system being used.

2. Rinse the gel three times, for 5 minutes each rinse, in NANOpure® water. Stain for 1 hour in SimplyBlue™ SafeStain (Invitrogen Corporation) at room temperature with gentle agitation. When staining is complete, discard the staining solution.
3. Destain the gel for 1 hour in NANOpure® water at room temperature with gentle agitation. When destaining is complete, discard the solution.
4. Using a clean razor blade, cut the protein bands of interest from the gel, eliminating as much polyacrylamide as possible. Place the gel slices into a 0.5ml microcentrifuge tube that has been prewashed twice with 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA).
5. Destain the gel slices twice with 0.2ml of 100mM NH₄HCO₃/50% ACN for 45 minutes each treatment, at 37°C to remove the SimplyBlue™ SafeStain.
6. Dehydrate the gel slices for 5 minutes at room temperature in 100µl of 100% ACN. At this point the gel slices will be much smaller than their original size and will be whitish or opaque in appearance.
7. Dry the gel slices in a Speed Vac® concentrator for 10–15 minutes at room temperature to remove the ACN.
8. Resuspend the Trypsin Gold at 1µg/µl in 50mM acetic acid, then dilute in 40mM NH₄HCO₃/10% ACN to 20µg/ml. Preincubate the gel slices in a minimal volume (10–20µl) of the trypsin solution at room temperature (do not exceed 30°C) for 1 hour. The slices will rehydrate during this time. If the gel slices appear white or opaque after one hour, add an additional 10–20µl of trypsin and incubate for another hour at room temperature.
9. Add enough digestion buffer (40mM NH₄HCO₃/10% ACN) to completely cover the gel slices. Cap the tubes tightly to avoid evaporation. Incubate overnight at 37°C.
10. Incubate the gel slice digests with 150µl of NANOpure® water for 10 minutes, with frequent vortex mixing. Remove and save the liquid in a new microcentrifuge tube.
11. Extract the gel slice digests twice, with 50µl of 50% ACN/5% TFA (with mixing) for 60 minutes each time, at room temperature.
12. Pool all extracts (from Steps 10 and 11), and dry in a Speed Vac® concentrator at room temperature for 2–4 hours (do not exceed 30°C).
13. Purify and concentrate the extracted peptides using ZipTip® pipette tips (Millipore Corporation) following the manufacturer's directions.

14. The peptides eluted from the ZipTip® tips are now ready for mass spectrometric analysis.

B. In-Solution Trypsin Digestion of Proteins

In general, proteins require denaturation and disulfide bond cleavage for enzymatic digestion to reach completion (Wilkinson, 1986). If partial digestion of a native protein is desired, begin this protocol at Step 3.

1. Dissolve the target protein in 6M guanidine HCl (or 6–8M urea or 0.1% SDS), 50mM Tris-HCl (pH 8), 2–5mM DTT (or β-mercaptoethanol).
2. Heat at 95°C for 15–20 minutes or at 60°C for 45–60 minutes. Allow the reaction to cool.
3. For denatured proteins, add 50mM NH₄HCO₃ (pH 7.8) or 50mM Tris-HCl, 1mM CaCl₂ (pH 7.6), until the guanidine HCl or urea concentration is less than 1M. If SDS is used, dilution is not necessary. For digestion of native proteins, dissolve in buffer with a pH between 7 and 9.
4. Add Trypsin Gold to a final protease:protein ratio of 1:100 to 1:20 (w/w). Incubate at 37°C for at least 1 hour. Remove an aliquot, and chill the remainder of the reaction on ice or freeze at –20°C.
5. Terminate the protease activity in the aliquot from Step 4 by adding an inhibitor. Alternatively, precipitate the aliquot by adding TCA to 10% final concentration. The reaction can also be terminated by freezing at –20°C. Trypsin can also be inactivated by lowering the pH of the reaction below pH 4. Trypsin will regain activity as the pH is raised above pH 4 (Wilkinson, 1986).

Note: The following are general trypsin inhibitors: Antipain (50µg/ml), antithrombin (1unit/ml), APMSF (0.01–0.04mg/ml), aprotinin (0.06–2µg/ml), leupeptin (0.5µg/ml), PMSF (17–170µg/ml), TLCK (37–50µg/ml), trypsin inhibitors (10–100µg/ml).
6. Determine the extent of digestion by subjecting the aliquot in Steps 4 and 5 to reverse phase HPLC or SDS-PAGE.
7. If no inhibitors were added to the remainder of the reaction and further proteolysis is required, incubate at 37°C until the desired digestion is obtained (Sheer, 1994). Reducing the temperature will decrease the digestion rate. Incubations of up to 24 hours may be required, depending on the nature of the protein. With long incubations, take precautions to avoid bacterial contamination.

Additional Literature for Trypsin Gold, Mass Spectrometry Grade

Technical Bulletins and Manuals

TB309 *Trypsin Gold, Mass Spectrometry Grade, Technical bBulletin*
(www.promega.com/tbs/tb309/tb309.html)

C. Affinity Tag In Vitro Pull-Down Assay with Trypsin Digestion and Protein Analysis

Markillie and associates describe a simple exogenous protein complex purification and identification method that can be easily automated (Markillie *et al.* 2005). The method uses MagneHis™ Ni Particles (Cat.# V8560, V8565) to pull down target proteins, followed by denaturing elution, trypsin digestion and mass spectrometry analysis (Figure 11.12).

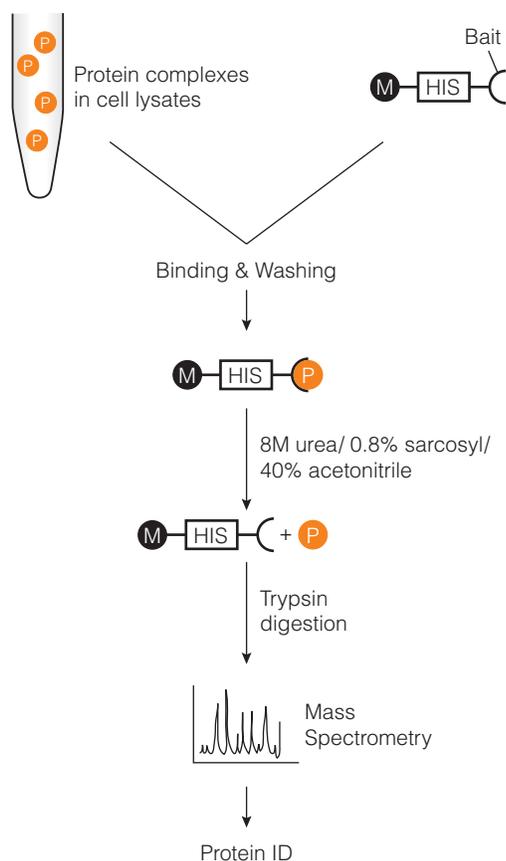


Figure 11.12. Schematic diagram of affinity tag in vitro pull-down with trypsin digestion and mass spectrometry analysis.

IX. SDS-PAGE Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a method used to analyze and isolate small amounts of protein. In this technique, the sample to be fractionated is denatured and coated with detergent by heating in the presence of SDS and a reducing agent. The SDS coating gives the protein a high net negative charge that is proportional to the length of the polypeptide chain.

The sample is loaded on a polyacrylamide gel, and high voltage is applied, causing the proteins to migrate toward the positive electrode (anode).

Since the proteins have a net negative charge that is proportional to their size, proteins are separated solely on the basis of their molecular mass—a result of the sieving effect of the gel matrix. The molecular mass of a protein can be estimated by comparing the gel mobility of a band with those of protein standards. Sharp protein bands are achieved by using a discontinuous gel system, having stacking and separating gel layers that differ in either salt concentration or pH or both (Hanes, 1981).

Materials Required:

(see Composition of Solutions section)

SDS-PAGE

- acrylamide solution, 40%
- upper gel 4X buffer
- lower gel 4X buffer
- ammonium persulfate, 10%
- TEMED
- SDS-polyacrylamide gel running 1X buffer
- loading 2X buffer
- trichloroacetic acid (TCA) (optional)
- acetone, ice-cold (optional)

This gel system uses the method described by Laemmli (Laemmli, 1970). Formulations for preparing resolving and stacking minigels are provided in Tables 11.4 and 11.5. The amounts of reagents indicated in Tables 11.4 and 11.5 are sufficient to prepare two 7 × 10cm gels, 0.75–1.00mm thick. Add ammonium persulfate and TEMED just prior to pouring the gel, as these reagents promote and catalyze polymerization of acrylamide. Pour the resolving gel mix into assembled gel plates, leaving sufficient space at the top for the stacking gel to be added later. Gently overlay the gel mix with 0.1% SDS, and allow the gel to polymerize for at least 15–30 minutes. After polymerization, remove the SDS overlay, and rinse the surface of the resolving gel with water to remove any unpolymerized acrylamide. Rinse one more time with a small volume of stacking gel buffer. Fill the remaining space with the stacking gel solution, and insert the comb immediately. After the stacking gel has polymerized, remove the comb, and rinse the wells with water to remove unpolymerized acrylamide. At least 1cm of stacking gel should be present between the bottom of the loading wells and the resolving gel.

Table 11.4. Formulation for Stacking Gel.

Component	Volume
upper gel 4X buffer	2.5ml
water	6.6ml
acrylamide solution, 40%	0.8ml
APS, 10% ¹	100µl
TEMED ²	10µl

¹ ammonium persulfate (always prepare fresh)

² N,N,N',N'-tetramethylethylenediamine

Prepare Samples

1. Add an equal volume of loading 2X buffer to the sample.
2. Incubate the sample at 95°C for 2–5 minutes, mix by vortexing and load onto the gel.

Optional

1. If the sample is very dilute or contains salts that may interfere with gel analysis, add TCA to a final concentration of 10% (w/v).
2. Place the sample on ice for 5 minutes, centrifuge at 4°C for 2 minutes at 12,000 × g in a microcentrifuge and discard the supernatant.
3. Wash the protein pellet with ice-cold acetone, and resuspend it in a suitable volume (generally <20µl) of loading 1X buffer (prepared by adding an equal volume of water to loading 2X buffer).
4. Incubate the sample at 95°C for 2–5 minutes, mix by vortexing and load onto the gel.

X. Composition of Solutions**MagneHis™ Binding/Wash Buffer (pH 7.5)**

100mM HEPES
10mM imidazole

MagneHis™ Elution Buffer (pH 7.5)

100mM HEPES
500mM imidazole

MagneHis™ Binding/Wash Buffer for Denaturing Conditions (pH 7.5)

100mM HEPES
10mM imidazole
2–8M guanidine-HCl or urea

MagneHis™ Elution Buffer for Denaturing Conditions (pH 7.5)

100mM HEPES
500mM imidazole
2–8M guanidine-HCl or urea

4X SDS gel-loading buffer

0.24M Tris-HCl (pH 6.8)
2% SDS
3mM bromophenol blue
50.4% glycerol
0.4M dithiothreitol

SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should be added from a 1M stock just before the buffer is used.

MagZ™ Binding/Wash Buffer (pH 7.4)

20mM sodium phosphate
500mM NaCl

MagZ™ Elution Buffer

1M imidazole (pH 7.5)

Binding Buffer (HisLink™)

100mM HEPES (pH 7.5)
10mM imidazole

Wash Buffer (HisLink™)

100mM HEPES (pH 7.5)
10–100mM imidazole

Elution Buffer (pH 7.5) (HisLink™)

100mM HEPES
500mM imidazole

MagneGST™ Binding/Wash Buffer

4.2mM Na₂HPO₄
2mM K₂HPO₄
140mM NaCl
10mM KCl

Elution Buffer (MagneGST™ System)

50mM glutathione (pH 7.0–8.0)
50mM Tris-HCl (pH 8.1)

The glutathione provided has a pH value between 7.0 and 8.0. If a different source of glutathione is being used, adjust the pH to 7.0–8.0 before adding the Tris-HCl (pH 8.1). The glutathione solution has little buffering capacity at pH 7.0–8.0, so take care when adjusting the pH. Failure to adjust the pH of glutathione will decrease the pH of the elution buffer, especially when final glutathione concentrations are ≥50mM.

Table 11.5. Formulation for Resolving Gel.

Component	Volume for Different Percentages of Acrylamide				
	8%	10%	12%	15%	20%
lower gel 4X buffer	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml
water	5.4ml	4.9ml	4.4ml	3.65ml	2.4ml
acrylamide solution, 40%	2.0ml	2.5ml	3.0ml	3.75ml	5.0ml
APS, 10% ¹	50.0µl	50.0µl	50.0µl	50.0µl	50.0µl
TEMED ²	5.0µl	5.0µl	5.0µl	5.0µl	5.0µl

¹ ammonium persulfate (always prepare fresh)

² N,N,N',N'-tetramethylethylenediamine

1X SDS gel-loading buffer

50mM Tris-HCl (pH 6.8)
 2% SDS
 0.1% bromophenol blue
 10% glycerol
 10mM dithiothreitol

SDS gel-loading buffer lacking dithiothreitol can be stored at room temperature. Dithiothreitol should be added from a 1M stock just before the buffer is used.

acrylamide solution, 40%

38.9g acrylamide
 1.1g bisacrylamide

Dissolve in 100ml of water.

upper gel 4X buffer

0.5M Tris-HCl (pH 6.8)
 0.4% SDS

lower gel 4X buffer

1.5M Tris-HCl (pH 8.8)
 0.4% SDS

gel running buffer

25mM Tris base
 192mM glycine
 0.1% SDS

Adjust pH to 8.3.

TBE 10X buffer (1L)

107.80g Tris base
 ~55g boric acid
 7.44g disodium EDTA•2H₂O

Add components in the order listed above to ~800ml of distilled water. Add slightly less than the total amount of boric acid. Mix until completely dissolved, check pH and adjust to 8.3 with boric acid. Bring final volume to 1L with distilled water.

TE buffer

10mM Tris-HCl (pH 8.0)
 1mM EDTA

T4 Polynucleotide Kinase 10X Buffer

700mM Tris-HCl (pH 7.6)
 100mM MgCl₂
 50mM DTT

Coomassie® Blue staining solution

50% (v/v) methanol
 10% (v/v) acetic acid
 0.25% (w/v) Coomassie® Blue R-250

destaining solution

10% (v/v) methanol
 5% acetic acid

gel loading 10X buffer

250mM Tris-HCl (pH 7.5)
 0.2% bromophenol blue
 40% glycerol

Gel Shift Binding 5X Buffer

20% glycerol
 5mM MgCl₂
 2.5mM EDTA
 2.5mM DTT
 250mM NaCl
 50mM Tris-HCl (pH 7.5)
 0.25mg/ml poly(dI-dC)•poly(dI-dC)

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11 Protein Purification and Analysis

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I. Introduction

The process of introducing nucleic acids into eukaryotic cells by nonviral methods is defined as transfection. Using various chemical, lipid or physical methods, this gene transfer technology is a powerful tool to study gene function and protein expression in the context of a cell. Development of reporter gene systems and selection methods for stable maintenance and expression of transferred DNA have greatly expanded the applications for transfection. Assay-based reporter technology, together with the availability of transfection reagents, provides the foundation to study mammalian promoter and enhancer sequences, *trans*-acting proteins such as transcription factors, mRNA processing, protein:protein interactions, translation and recombination events (Groskreutz and Schenborn, 1997).

Transfection is a method that neutralizes or obviates the issue of introducing negatively charged molecules (e.g., phosphate backbones of DNA and RNA) into cells with a negatively charged membrane. Chemicals like calcium phosphate and DEAE-dextran or cationic lipid-based reagents coat the DNA, neutralizing or even creating an overall positive charge to the molecule (Figure 12.1). This makes it easier for the DNA:transfection reagent complex to cross the membrane, especially for lipids that have a “fusogenic” component, which enhances fusion with the lipid bilayer. Physical methods like microinjection or electroporation simply punch through the membrane and introduce DNA directly into the cytoplasm. Each of these transfection technologies is discussed in the following sections.

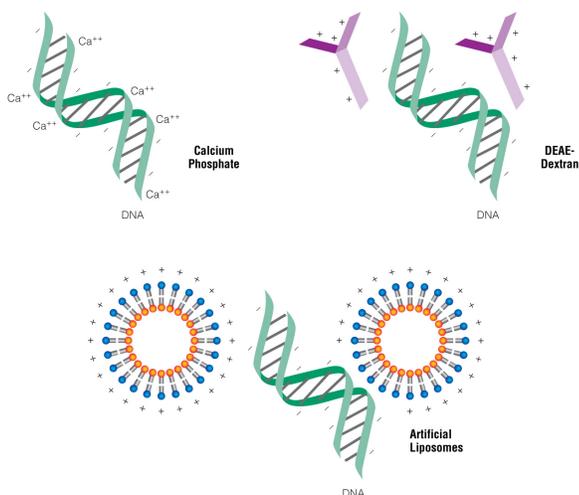


Figure 12.1. Schematic representation of various transfection technologies and how they neutralize the negatively charged DNA. Note that lipid-based reagents also can coat DNA in addition to forming micelles and associating with DNA by attraction as depicted.

This chapter covers general information on transfection techniques and considerations for transfection efficiency and optimization. In addition, we discuss various transfection agents available from Promega as well as

general protocols for transfection and specific examples using our transfection reagents. Finally, we review stable transfection and outline a protocol using drug selection.

A. Chemical Reagents

One of the first chemical reagents used to transfer nucleic acids into cultured mammalian cells was DEAE-dextran (Vaheri and Pagano, 1965; McCutchan and Pagano, 1968). DEAE-dextran is a cationic polymer that tightly associates with negatively charged nucleic acids. An excess of positive charge, contributed by the polymer in the DNA:polymer complex, allows the complex to come into closer association with the negatively charged cell membrane. Uptake of the complex is presumably by endocytosis. This method successfully delivers nucleic acids into cells for transient expression; that is, for short-term expression studies of a few days in duration. However, this technique is not generally useful for stable or long-term transfection studies that rely upon integration of transferred DNA into the chromosome (Gluzman, 1981). Other synthetic cationic polymers have been used to transfer DNA into cells, including polybrene (Kawai and Nishizawa, 1984), polyethyleneimine (Boussif *et al.* 1995) and dendrimers (Haensler and Szoka, 1993; Kukowska-Latallo *et al.* 1996).

Calcium phosphate co-precipitation became a popular transfection technique following the systematic examination of this method in the early 1970s (Graham and van der Eb, 1973). The authors examined the performance of various cations and effects of cation concentration, phosphate concentration and pH on transfection. Calcium phosphate co-precipitation is widely used because the components are easily available and inexpensive, the protocol is easy-to-use, and it is effective with many different types of cultured cells. The protocol involves mixing DNA with calcium chloride, adding this in a controlled manner to a buffered saline/phosphate solution and allowing the mixture to incubate at room temperature. The controlled addition generates a precipitate that is dispersed onto the cultured cells. The precipitate is taken up by cells via endocytosis or phagocytosis. Calcium phosphate transfection is routinely used for both transient and stable transfection of a variety of cell types. In addition, calcium phosphate appears to provide protection against intracellular and serum nucleases (Loyter *et al.* 1982).

However, calcium phosphate co-precipitation is prone to variability and is not suited for *in vivo* gene transfer to whole animals. In addition, small pH changes (± 0.1) can compromise the efficacy of calcium phosphate transfection (Felgner, 1990). Promega offers the calcium phosphate reagent as part of the ProFection[®] Mammalian Transfection System – Calcium Phosphate (Cat.# E1200).

B. Cationic Lipids

The term “liposome” refers to lipid bilayers that form colloidal particles in an aqueous medium (Sessa and Weissmann, 1968). By 1980, artificial liposomes were being used to deliver DNA into cells (Fraleley *et al.* 1980). The next advance in liposomal vehicles was development of synthetic

cationic lipids by Felgner and colleagues (Felgner *et al.* 1987). The cationic head group of the lipid compound associates with negatively charged phosphates on the nucleic acid. Liposome-mediated delivery offers advantages such as relatively high efficiency of gene transfer, ability to transfect certain cell types that are resistant to calcium phosphate or DEAE-dextran, *in vitro* and *in vivo* applications, successful delivery of DNA of all sizes from oligonucleotides to yeast artificial chromosomes (Felgner *et al.* 1987; Capaccioli *et al.* 1993; Felgner *et al.* 1993; Haensler and Szoka, 1993; Lee and Jaenisch, 1996; Lamb and Gearhart, 1995), delivery of RNA (Malone *et al.* 1989; Wilson *et al.* 1979), and delivery of protein (Debs *et al.* 1990). Cells transfected by liposome techniques can be used for transient expression studies and long-term experiments that rely on integration of DNA into the chromosome or episomal maintenance. Unlike DEAE-dextran or calcium phosphate chemical methods, liposome-mediated nucleic acid delivery can be used for *in vivo* transfer of DNA and RNA to animals and humans (Felgner *et al.* 1995).

A lipid with overall net positive charge at physiological pH is the most common synthetic lipid component of liposomes developed for gene delivery (Figure 12.2). Often the cationic lipid is mixed with a neutral lipid such as L-dioleoyl phosphatidylethanolamine (DOPE; Figure 12.3), which can enhance the gene transfer ability of certain synthetic cationic lipids (Felgner *et al.* 1994; Wheeler *et al.* 1996). The cationic portion of the lipid molecule associates with negatively charged nucleic acids, resulting in compaction of the nucleic acid in a liposome/nucleic acid complex (Kabanov and Kabanov, 1995; Labat-Moleur *et al.* 1996), presumably from electrostatic interactions between the negatively charged nucleic acid and positively charged head group of the synthetic lipid. For cultured cells, an overall net positive charge of the liposome/nucleic acid complex generally results in higher transfer efficiencies, presumably because this allows closer association of the complex with the negatively charged cell membrane. Entry of the liposome complex into the cell may occur by endocytosis or fusion with the plasma membrane via the lipid moieties of the liposome (Gao and Huang, 1995). Following cellular internalization, the complexes appear in the endosomes and later in the nucleus. It is unclear how the nucleic acids are released from the endosomes and lysosomes and traverse the nuclear membrane. DOPE is considered a “fusogenic” lipid (Farhood *et al.* 1995), and its role may be to release these complexes from endosomes as well as to facilitate fusion of the outer cell membrane with liposome/nucleic acid complexes. While DNA will need to enter the nucleus, the cytoplasm is the site of action for RNA, protein or antisense oligonucleotides delivered via liposomes.

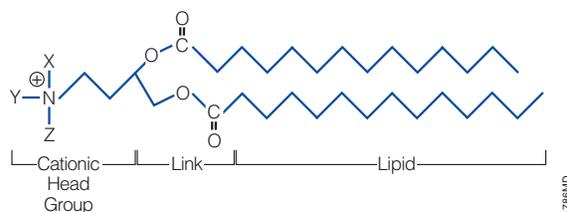


Figure 12.2. The general structure of a synthetic cationic lipid. X, Y and Z represent a number of possible chemical moieties, which can differ, depending on the specific lipid.

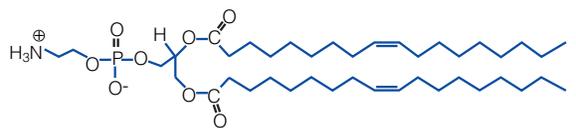


Figure 12.3. Structure of the neutral lipid DOPE.

Promega offers the FuGENE® HD Transfection Reagent (Cat.# E2311), a novel nonliposomal transfection reagent with wide application in different cell types and low toxicity, and the TransFast™ Transfection Reagent (Cat.# E2431), which uses a polycationic head group attached to a lipid backbone structure to deliver nucleic acids into eukaryotic cell. The best transfection reagent and conditions for a particular cell type must be empirically and systematically determined because inherent properties of the cell influence the success of any specific transfection method.

C. Physical Methods

Physical methods for gene transfer were developed and used beginning in the early 1980s. Direct microinjection into cultured cells or nuclei is an effective although laborious technique to deliver nucleic acids into cells by means of a fine needle (Cappuchi, 1980). This method has been used to transfer DNA into embryonic stem cells that are used to produce transgenic organisms (Bockamp *et al.* 2002) and to introduce antisense RNA into *C. elegans* (Wu *et al.* 1998). However, the apparatus is costly and the technique extremely labor-intensive, thus it is not an appropriate method for studies that require a large number of transfected cells.

Electroporation was first reported for gene transfer studies in mouse cells (Wong and Neumann, 1982). This technique is often used for cell types such as plant protoplasts, which are difficult to transfect by other methods. The mechanism is based on the use of an electrical pulse to perturb the cell membrane and form transient pores that allow passage of nucleic acids into the cell (Shigekawa and Dower, 1988). The technique requires fine-tuning and optimization of pulse duration and strength for each type of cell used. In addition, electroporation often requires more cells than chemical methods because of substantial cell death, and extensive optimization often is required to balance transfection efficiency and cell viability. More modern instrumentation allows nucleic acid delivery to the nucleus and successful transfer of DNA and RNA to primary and stem cells.

Another physical method of gene delivery is biolistic particle delivery, also known as particle bombardment. This method relies upon high-velocity delivery of nucleic acids on microprojectiles to recipient cells by membrane penetration (Ye *et al.* 1990). This method is successfully employed to deliver nucleic acid to cultured cells as well as to cells *in vivo* (Klein *et al.* 1987; Burkholder *et al.* 1993; Ogura *et al.* 2005). Biolistic particle delivery is relatively costly for many research applications, but the technology also can be used for genetic vaccination and agricultural applications.

D. Viral Methods

While transfection has been used successfully for gene transfer, the use of viruses as vectors has been explored as an alternative method to deliver foreign genes into cells and as a possible *in vivo* option. Adenoviral vectors are useful for gene transfer due to a number of key features: 1) they rapidly infect a broad range of human cells and can achieve high levels of gene transfer compared to other available vectors; 2) adenoviral vectors can accommodate relatively large segments of DNA (up to 7.5kb) and transduce these transgenes in nonproliferating cells; and 3) adenoviral vectors are relatively easy to manipulate using recombinant DNA techniques (Vorburger and Hunt, 2002). Other vectors of interest include adeno-associated virus, herpes simplex virus, retroviruses and lentiviruses, a subset of the retrovirus family. Lentiviruses (e.g., HIV-1) are of particular interest because they are well studied, can infect quiescent cells, and can integrate into the host cell genome to allow stable, long-term transgene expression (Anson, 2004).

As with all gene transfer methods, there are drawbacks. For adenoviral vectors, packaging capacity is low, and production is labor-intensive (Vorburger and Hunt, 2002). With retroviral vectors, there is the potential for activation of latent disease and, if there are replication-competent viruses present, activation of endogenous retroviruses and limited transgene expression (Vorburger and Hunt, 2002; Anson, 2004).

II. General Considerations

A. Reagent Selection

With so many different methods of gene transfer, how do you choose the right transfection reagent or technique for your needs? Any time a new parameter, like a new cell line, is introduced, the optimal conditions for transfection will need to be determined. This may involve choosing a new transfection reagent. For example, one reagent may work well with HEK-293 cells, but a second reagent is a better choice when using HepG2 cells. Promega offers several online resources to help identify a transfection reagent and protocol for your cell line, including the Transfection Assistant (www.promega.com/transfectionasst/) and FuGENE® HD Protocol Database (www.promega.com/techserv/tools/FugeneHD/). A drop-down menu allows you to search the databases by cell line, transfection type and transfection reagent. The conditions listed should be

considered only guidelines since you may need to optimize the transfection conditions for your specific application. See Optimization of Transfection Efficiency (Section IV) and General Transfection Protocol (Section VI) for details.

B. Transient Expression versus Stable Transfection

Another parameter to consider is the time frame of the experiment you wish to conduct. Is it short- or long-term? For instance, determining which promoter deletion constructs can still function as a promoter can be accomplished with a transient transfection experiment, while establishing stable expression of an exogenously introduced gene construct will require a longer term experiment.

Transient Expression

Cells are typically harvested 24–72 hours post-transfection for studies designed to analyze transient expression of transfected genes. The optimal time interval depends on the cell type, research goals and specific expression characteristics of the transferred gene. Analysis of gene products may require isolation of RNA or protein for enzymatic activity assays or immunoassays. The method used for cell harvest will depend on the end product assayed. For example, expression of the firefly luciferase gene in the pGL4.10[*luc2*] Vector (Cat.# E6651) is generally assayed 24–48 hours post-transfection, whereas the pGL4.12[*luc2CP*] Vector (Cat.# E6671) with its protein degradation sequences can be assayed in a shorter time frame (e.g., 3–12 hours), depending on the research goals and the time it takes for the reporter gene to reach steady state. For more information on luminescent reporter genes like firefly luciferase, see the *Protocols and Applications Guide* chapter on bioluminescent reporters (www.promega.com/paguide/chap8.htm).

Stable Transfection

The goal of stable, long-term transfection is to isolate and propagate individual clones containing transfected DNA that has integrated into the cellular genome. Distinguishing nontransfected cells from those that have taken up exogenous DNA involves selective screening. This screening can be accomplished by drug selection when an appropriate drug-resistance marker is included in the transfected DNA. Alternatively, morphological transformation can be used as a selectable trait in certain cases. For example, bovine papilloma virus vectors produce a morphological change in transfected mouse CI127 cells (Sarver *et al.* 1981).

Before using a particular drug for selection purposes, you will need to determine the amount of drug necessary to kill untransfected cells. This may vary greatly among cell types. Consult Ausubel *et al.* 1995 for additional information about designing experiments to test various drug concentrations and determine the amount needed to select resistant clones (i.e., generate a kill curve).

When drug selection is used, cells are maintained in nonselective medium for 1–2 days post-transfection, then replated in selective medium containing the drug. The use of selective medium is continued for 2–3 weeks, with

frequent changes of medium to eliminate dead cells and debris, until distinct colonies can be visualized. Individual colonies can be isolated by cloning cylinders, selected and transferred to multiwell plates for further propagation in the presence of selective medium. Individual cells that survive the drug treatment expand into clonal groups that can be individually propagated and characterized. For a protocol to select transfected cells by antibiotics, see Stable Transfection (Section VII).

Several different drug-selection markers are commonly used for long-term transfection studies. For example, cells transfected with recombinant vectors containing the bacterial gene for neomycin phosphotransferase [e.g., pCI-neo Mammalian Expression Vector (Cat.# E1841)] can be selected for stable transformation in the presence of the neomycin analog G-418 (Cat.# V8091; Southern and Berg, 1982). Similarly, expression of the hygromycin B phosphotransferase gene from the transfected vector [e.g., pGL4.14 [*luc2/Hygro*] Vector (Cat.# E6691)] will confer resistance to the drug hygromycin B (Blochlinger and Diggelmann, 1984).

An alternative strategy is to use a vector carrying an essential gene that is defective in a given cell line. For example, CHO cells deficient in dihydrofolate reductase (DHFR) gene expression do not survive without added nucleosides. However, these cells, when stably transfected with DNA expressing the DHFR gene, will synthesize the required nucleosides and survive (Stark and Wahl, 1984). An additional advantage of using DHFR as a marker is that gene amplification of DHFR and associated transfected DNA occurs when cells are exposed to increasing doses of methotrexate, resulting in multiple copies of the plasmid in the transfected cell (Schimke, 1988).

C. Type of Molecule Transfected

Plasmid DNA is most commonly transfected into cells, but other macromolecules can be transferred as well. For example, short interfering RNA (siRNA; Hong *et al.* 2004; Snyder *et al.* 2004; Klampfer *et al.* 2004), oligonucleotides (Labroille *et al.* 1996; Berasain *et al.* 2003; Lin *et al.* 2004), RNA (Shimoike *et al.* 1999; Ray and Das, 2004) and even proteins (Debs *et al.* 1990; Lin *et al.* 1993) have been successfully introduced into cells via transfection methods. However, conditions that work for plasmid DNA transfer will likely need to be optimized when using other macromolecules. In all cases, the agent transfected needs to be of high quality and relatively pure. Nucleic acids need to be free of proteins, other contaminating nucleic acids and chemicals (e.g., salts from oligo synthesis). Protein should be pure and in a solvent that is not detrimental to cell health. For additional information on plasmid DNA quality, see DNA Quality and Quantity (Section III.D).

D. Assay for Transfection

After cells are transfected, how will you determine success? Plasmids containing reporter genes can be used to easily monitor transfection efficiencies and expression levels in the cells. An ideal reporter gene product is one that is

unique to the cell, can be expressed from plasmid DNA and can be assayed conveniently. Generally, reporter gene assays are performed 1–3 days after transfection; the optimal time should be determined empirically. For a discussion of luminescent reporter gene options, see the *Protocols and Applications Guide* chapter on bioluminescence reporters (www.promega.com/paguide/chap8.htm). A direct test for the protein of interest, such as an enzymatic assay, may be another method to assess transfection success.

In the case of siRNA, success may be measured using a reporter gene or assaying mRNA (e.g., RT-PCR) or protein target levels (e.g., Western blotting). For additional siRNA-specific reporter options, see the *Protocols and Applications Guide* chapter on RNA interference (www.promega.com/paguide/chap2.htm).

If multiple assays will be performed, make sure the techniques you choose are compatible with all assay chemistries. For example, if lysates are made from transfected cells, the lysis buffer used ideally would be compatible with all subsequent assays. In addition, if cells are needed for propagation after assessment, make sure to retain some viable cells for passage after the assay.

III. Factors Influencing Transfection Efficiency

With any transfection reagent or method, cell health, degree of confluency, number of passages, contamination, and DNA quality and quantity are important parameters that can greatly influence transfection efficiency. Note that with any transfection reagent or method used, some cell death will occur.

A. Cell Health

Cells should be grown in medium appropriate for the cell line and supplemented with serum or growth factors as needed for viability. Contaminated cells and media (e.g., contaminated with yeast or mycoplasma) should never be used for transfection. If cells have been compromised in any way, discard them and reseed from a frozen, uncontaminated stock. Make sure the medium is fresh if any components are unstable. Medium lacking necessary factors can harm cell growth. Be sure the 37°C incubator is supplied with CO₂ at the correct percentage (usually 5–10%) and kept at 100% relative humidity.

If there are any concerns about what type of culture medium or CO₂ levels are needed for your cell line of interest, consult the American Type Culture Collection [ATCC] web site (<http://www.atcc.org/>).

B. Confluency

As a general guideline, transfect cells at 40–80% confluency. Too few cells will cause the culture to grow poorly without cell-to-cell contact. Too many cells results in contact inhibition, making cells resistant to uptake of foreign DNA. Actively dividing cells take up introduced DNA better than quiescent cells.

C. Number of Passages

Keep the number of passages low (<50). In addition, the number of passages for cells used in a variety of experiments should be consistent. Cell characteristics can change over time with immortalized cell lines, and cells may not respond to the same transfection conditions after repeated passages, resulting in poor expression.

D. DNA Quality and Quantity

Plasmid DNA for transfections should be free of protein, RNA, chemical and microbial contamination. Suspend ethanol-precipitated DNA in sterile water or TE buffer to a final concentration of 0.2–1mg/ml. The optimal amount of DNA to use in the transfection will vary widely, depending on the type of DNA, transfection reagent, target cell line and number of cells.

IV. Optimization of Transfection Efficiency

You will need to optimize specific transfection conditions to achieve the desired transfection efficiencies. Important parameters to consider are the charge ratio of cationic lipid transfection reagent to DNA, amount of transfected nucleic acid, length of time cells are exposed to the transfection reagent and presence or absence of serum. Reporter genes are useful to determine optimal conditions. The transfection efficiency achieved using any transfection reagents varies depending on the cell type being transfected and transfection conditions used.

A. Charge Ratio of Cationic Transfection Reagent to DNA

The amount of positive charge contributed by the cationic lipid component of the transfection reagent should equal or exceed the amount of negative charge contributed by the phosphates on the DNA backbone, resulting in a net neutral or positive charge on the multilamellar vesicles associating with the DNA. Charge ratios of 1:1 to 2:1 TransFast™ Reagent:DNA have worked well with various cultured cells, but ratios outside of this range may be optimal for other cell types or applications. See the *TransFast™ Transfection Reagent Technical Bulletin #TB260* (www.promega.com/tbs/tb260/tb260.html) for more details.

B. DNA or RNA

The optimal amount of DNA or RNA will vary depending on the type of nucleic acid, number of cells, culture dish size and target cell line used. For example, HEK-293 cells are optimally transfected with 0.25µg of pGL3-Control Vector (Cat.# E1741) using TransFast™ Reagent at a 2:1 ratio in a 24-well plate. In contrast, the same cells are optimally transfected with 0.55µg of DNA using the FuGENE® HD Transfection Reagent at a 3:1 ratio in the same well size. For other cell lines, we suggest testing the DNA amounts given in Table 12.1.

Table 12.1. Suggested DNA Amounts to Use for Optimization.

Transfection Reagent	DNA Amount to Test	Reagent:DNA Ratios to Test	Culture Dish Size
FuGENE® HD Transfection Reagent	0.04–0.2µg	4:1, 3.5:1, 3:1, 2.5:1, 2:1 and 1.5:1	96-well plate
TransFast™ Reagent	0.25, 0.50, 0.75, 1µg	2:1 and 1:1	24-well plate

Increasing the quantity of transfected DNA significantly may not yield better results. In fact, if initial transfection results are satisfactory, a reduced DNA quantity can be tested (while keeping the optimal reagent:DNA ratio constant). Often a range of DNA concentration is suitable for transfection. However, if the DNA concentration is below or above this range, transfection efficiencies will decrease. If there is too little DNA, the experimental response may not be present. If there is too much DNA, the excess can be toxic to cells. Calibrate the system using a test plasmid with reporter gene function.

C. Time

Traditionally, transfection reagents must be in contact with cells for some period of time, then additional medium is added or the medium is replaced to help minimize toxic effects of the reagent. The optimal transfection time depends on the cell line, transfection reagent and nucleic acid used. For the FuGENE® HD Transfection Reagent, which is one of the more gentle methods of DNA transfection into cells, there is no need to add additional medium or replace the medium after transfection.

For initial tests with liposomal reagents that require adding or replacing the medium, use a one-hour transfection interval, and test transfection times of 30 minutes to 4 hours (Figure 12.4) or even overnight, depending on the reagent used. Monitor cell morphology during the transfection interval, particularly when cells are maintained in serum-free medium because some cell lines lose viability under these conditions. The transfection time with the TransFast™ Reagent is usually significantly shorter than that required with other cationic lipid compounds and can be decreased to as little as 30 minutes with certain cell lines. In addition to saving time, this shortened transfection time may significantly reduce the risk of cell death.

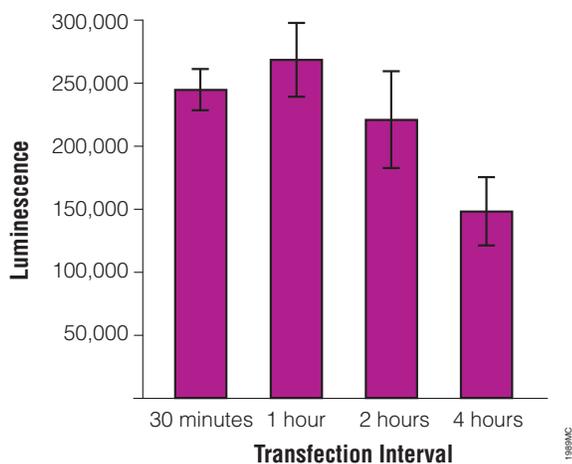


Figure 12.4. Effect of transfection interval on transfection of CHO cells using TransFast™ Reagent. CHO cells were transfected with 250ng of pGL3-Control DNA using TransFast™ Reagent at a 2:1 reagent:DNA charge ratio for various times in the absence of serum. All transfections were performed in 24-well plates, and cell lysates were harvested 2 days post-transfection. The results represent the mean of 6 replicates and are expressed as relative light units (RLU) per well.

D. Serum

Transfection protocols often require serum-free conditions for optimal performance because serum can interfere with many commercially available transfection reagents. The TransFast™ and FuGENE® HD Transfection Reagents can be used in transfection protocols in the presence of serum, allowing transfection of cell types or applications that require continuous exposure to serum (e.g., primary cells). Note that best results are obtained when variability is minimized among lots of serum.

E. Co-Transfection and Dual-Reporter Assays

While many people use a single reporter gene for their experimental system, a dual-reporter system has distinct advantages. A second reporter gene allows expression to be normalized for transfection efficiency and cell number. Small perturbations in growth conditions for transfected cells can dramatically affect gene expression. A second reporter helps to determine if the effects are due to the treatment of the cells or a response from the experimental reporter.

The Dual-Glo™ Luciferase Assay System (Cat.# E2920, E2940, E2980) is an efficient means of quantitating luminescent signal from two reporter genes in the same sample. In this system, firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*) luciferase activities are measured sequentially from a single sample in a homogeneous format. In the Dual-Glo™ System, both reporters yield linear assay responses (with respect to the amount of enzyme) and exhibit no endogenous activity in experimental host cells. In addition, the extended half-life of the reporter signals are ideal for use with multiwell assay formats.

The various Promega *Renilla* luciferase vectors can be used as control vectors when co-transfected with a firefly luciferase vector into which the promoter of interest is cloned. Alternatively, the firefly vector may be used as the control vector and the *Renilla* luciferase vector as the experimental construct. In a co-transfection experiment, it is important to realize that *trans* effects between promoters on co-transfected plasmids can potentially affect reporter gene expression (Farr and Roman, 1992). This is primarily of concern when either the control or experimental reporter vector or both contain very strong promoter/enhancer elements. The occurrence and magnitude of such effects will depend on several factors: 1) the combination and activities of genetic regulatory elements present on the co-transfected vectors; 2) the amount and relative ratio of experimental vector to control vector introduced into cells; and 3) the cell type transfected.

To help ensure independent genetic expression of experimental and control reporter genes, preliminary co-transfection experiments should be performed to optimize both the vector DNA amount and ratio of co-reporter vectors. Because the Promega *Renilla* luciferase vectors are designed for optimal expression, it is possible to use very small quantities of these vectors to provide low-level, constitutive co-expression of *Renilla* luciferase activity. This means that the ratio of firefly and *Renilla* luciferase vectors to test can range from 1:1 to 100:1 (or greater) to determine the optimal expression. The key to a dual-reporter system is to maximize expression of the experimental reporter while minimizing that of the control reporter. However, the expression level of the control reporter should be three standard deviations above background to be significant.

Additionally, experimental treatments may sometimes undesirably affect control reporter expression. This compromises the accuracy of experimental data interpretation; typically this occurs through sequences in the vector backbone, promoter or reporter gene itself. For this reason, Promega offers different promoter elements with either the same vector backbone, such as that of the pGL4.7 Vector series, or a choice of vector backbones, available with the synthetic *Renilla* luciferase vectors (phRL and phRG), to select the most reliable co-reporter vector for your system. In fact, due to extremely complicated cellular experimental conditions, testing several vectors is sometimes required before finding the best internal control for a particular experimental situation.

The strength of the promoter in your cell system is an important consideration. A more moderately expressing promoter like thymidine kinase [TK; e.g., pGL4.74[*hRluc*/TK] Vector (Cat.# E6921)] may be preferable to SV40 or CMV. Stronger promoters may exhibit more *trans* effects, cross-talk or regulatory problems. However, adjusting the ratio of experimental vector to control vector (e.g., using 100:1 or 200:1) may eliminate some of these issues.

For a discussion of other dual-reporter assays and vector offerings, see the *Protocols and Applications Guide* chapter on bioluminescent reporters (www.promega.com/paguide/chap8.htm) and the complete listing of Promega vectors (www.promega.com/vectors/). Information on how to normalize dual-reporter assay data can be found in *Promega Notes 72* (www.promega.com/pnotes/72/8094_17/8094_17.html).

V. Promega Transfection Products

The following sections discuss the various Promega transfection reagents.

A. ProFection® Mammalian Transfection System

The ProFection® Mammalian Transfection System—Calcium Phosphate (Cat.# E1200) is a simple system containing two buffers: CaCl₂ and HEPES-buffered saline. A precipitate containing calcium phosphate and DNA is formed by slowly mixing a HEPES-buffered phosphate solution with a solution containing calcium chloride and DNA. The DNA precipitate is distributed onto eukaryotic cells and enters cells through an endocytic-type mechanism. Calcium phosphate transfection may be used to produce long-term stable transfectants. It also works well for transient expression of transfected genes and can be used with most adherent cell lines.

For a list of references using the ProFection® Mammalian Transfection System with a variety of cell lines, see the online Transfection Assistant (www.promega.com/transfectionasst/).

Special Usage Notes:

- To increase transfection efficiency for some cell types, additional treatments such as glycerol (Frost and Williams, 1978; Wilson and Smith, 1997), dimethyl sulfoxide (DMSO; Lowy *et al.* 1978; Lewis *et al.* 1980), chloroquine (Luthman and Magnusson, 1983) and sodium butyrate (Gorman *et al.* 1983) may be added during incubation with the calcium phosphate/DNA precipitate. These treatments are thought to disrupt the phagocytic vacuole membrane, allowing the DNA to be released to the cytoplasm (Felgner, 1990). Since each of these chemicals is toxic to cells, transfection conditions for individual cell types, including reagent concentration and exposure time, must be carefully optimized.
- Alternatively, glycerol or DMSO may be added briefly (4–6 hours) after adding the DNA precipitate (Ausubel *et al.* 1995).

Additional Resources for the ProFection® Mammalian Transfection System

Technical Bulletins and Manuals

- TM012 *ProFection® Mammalian Transfection System Technical Manual*
(www.promega.com/tbs/tm012/tm012.html)

Citations

Hay, E. *et al.* (2004) Bone morphogenetic protein receptor IB signaling mediates apoptosis independently of differentiation in osteoblastic cells. *J. Biol. Chem.* **279**, 1650–58.

MC3T3-E1 (mouse preosteoblast) cells were plated at 5,000 cells/cm² in 100mm dishes the day before transfection. Cells were co-transfected with 15µg of plasmids expressing either wildtype or dominant negative bone morphogenetic protein-2 (BMP-2) and pSV-β-Galactosidase Control Vector at a 10:1 ratio in the presence of serum for 16 hours using the ProFection® Mammalian Transfection System—Calcium Phosphate. After the incubation, the medium was changed and the expression assessed 72 hours post-transfection by counting the number of β-galactosidase-positive cells and assessing the expression of BMP-2 by Western blot analysis.

PubMed Number: 14576167

McNamara-Schroeder, K.J. *et al.* (2001) The *Drosophila* U1 and U6 gene proximal sequence elements act as important determinants of the RNA polymerase specificity of small nuclear RNA gene promoters in vitro and in vivo. *J. Biol. Chem.* **276**, 31786–92.

Drosophila S2 cells were transfected with a U6 maxigene plasmid and a control firefly luciferase construct (derived from the pGL2 Basic Vector) using the ProFection® Mammalian Transfection System—Calcium Phosphate. Expression of the maxigene was confirmed by isolating total RNA and performing primer extension analysis with the aid of the Primer Extension System. Luciferase assays were performed on an aliquot of cells after lysis with Reporter Lysis Buffer.

PubMed Number: 11431466

B. TransFast™ Transfection Reagent

The TransFast™ Transfection Reagent (Cat.# E2431) comprises the synthetic cationic lipid, (+)-N,N [bis (2-hydroxyethyl)-N-methyl-N- [2,3-di(tetradecanoyloxy) propyl] ammonium iodide (Figure 12.5), and the neutral lipid DOPE.

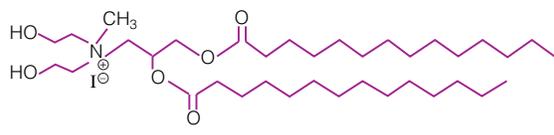


Figure 12.5. Structure of the TransFast™ cationic lipid.

The TransFast™ Reagent is supplied as a dried lipid film that forms multilamellar vesicles upon hydration with water. The TransFast™ Transfection Reagent delivers nucleic acid into eukaryotic cells in vitro and in vivo (Bennett *et al.* 1997) and performs well with many cell lines including NIH/3T3, CHO, HEK-293, K562, PC12, Jurkat and insect Sf9 cells. The TransFast™ Reagent combines the advantages of cationic liposome-mediated transfection with speed and ease-of-use to transfect cells for transient and stable expression. For a list of conditions

that have been used successfully to transfect various cell types using TransFast™ Reagent, visit our Transfection Assistant (www.promega.com/transfectionasst/).

Special Usage Notes:

- The TransFast™ Reagent can be used in the presence of serum, allowing transfection of cell types that are serum-sensitive, such as primary cell cultures.
- Prepare the TransFast™ Reagent the day before transfection, because it needs to be frozen before the initial use.
- There are separate protocols to transfect adherent and suspension cells using the TransFast™ Reagent.

Additional Resources for TransFast™ Transfection Reagent

Technical Bulletins and Manuals

- TB260 *TransFast™ Transfection Reagent Technical Bulletin*
(www.promega.com/tbs/tb260/tb260.html)

Promega Publications

- CN007 Transfecting a human neuroblastoma cell line with Monster Green™ Fluorescent Protein
(www.promega.com/cnotes/cn007/cn007_14.htm)
- PN090 Using bioluminescent reporter genes to optimize shRNA target sites for RNAi of the *bcr/abl* gene
(www.promega.com/pnotes/90/12727_26/12727_26.html)
- PN075 A comparison of pCI-neo Vector and pcDNA4/HisMax Vector
(www.promega.com/pnotes/75/8554_33/8554_33.html)
- PN071 TransFast™ Transfection Reagent update
(www.promega.com/pnotes/71/7807_18/7807_18.html)
- PN065 An efficient new transfection reagent for eukaryotic cells: TransFast™ Transfection Reagent
(www.promega.com/pnotes/65/6921_02/default.html)

Citations

Boggs, K. and Reisman, D. (2007) C/EBPbeta participates in regulating transcription of the p53 gene in response to mitogen stimulation. *J. Biol. Chem.* **282**, 7982–90.

To explore further the role of C/EBPb isoforms in regulating p53 expression during the cell cycle, the 1.7kb murine p53 promoter was cloned into the pGL3-Basic Vector. Using TransFast™ Reagent, Swiss3T3 and 6629 (C/EBPb-null) cells were transfected using 0.1–0.75µg of pGL3-1.7-kb p53 promoter construct with or without co-transfection of 0.25µg of C/EBPb-2 and 50ng of pRL-TK Vector as an internal control. Twenty-four hours post-transfection, cells were harvested and assayed for luciferase activity, normalizing reporter activity to *Renilla* luciferase.

Site-directed mutagenesis was used to mutate or delete the –972/–953 *cis*-acting element carrying the C/EBPb-binding site within the p53 promoter, and 0.1–0.75µg of each mutant construct was transfected into Swiss3T3 cells with or without co-transfection of 0.25µg of C/EBPb-2 and 50ng of pRL-TK Vector. The cells were harvested 24 hours post-transfection and assayed for reporter activity, normalizing to pRL-TK Vector activity.

PubMed Number: 17244625

Guindalini, C. *et al.* (2006) A dopamine transporter gene functional variant associated with cocaine abuse in a Brazilian sample. *Proc. Natl. Acad. Sci. U S A* **103**, 4552–7.

The authors investigated the effect of various polymorphisms in the dopamine transporter gene (*SLC6A3*) on susceptibility to cocaine addiction. Genotyping of various polymorphisms in cocaine abusers and control subjects revealed a potential association of the int8 VNTR with cocaine abuse. Seven alleles of the int8 VNTR were sequenced. Various allelic sequences then were cloned into a modified phRL-SV40 *Renilla* luciferase reporter vector and transfected into the mouse SN4741 cell line, which expresses the dopamine transporter, and the effects on reporter activity were monitored. Sequences of two alleles then were cloned into a pGL3 Promoter Vector construct and transfected into JAP cells. The cells were challenged with various amounts of cocaine or KCl and forskolin, and the effect on reporter activity was monitored. The TransFast™ Reagent was used for transfections at a 2:1 reagent:DNA ratio.

PubMed Number: 16537431

C. FuGENE® HD Transfection Reagent

The FuGENE® HD Transfection Reagent (Cat.# E2311) is a novel, nonliposomal formulation to transfect DNA into a wide variety of cell lines with high efficiency and low toxicity. The protocol does not require removal of serum (including up to 100% serum) or culture medium and does not require washing or changing of medium after introducing the reagent:DNA complex. Additionally, the FuGENE® HD reagent supports transfection in chemically defined media and does not contain any animal derived components. For more information about transfection conditions using the FuGENE® HD Transfection Reagent, visit the FuGENE® HD Protocol Database (www.promega.com/techserv/tools/FugeneHD/).

VI. General Transfection Protocol

A. Preparation of Cells for Transfection

Trypsinization Procedure to Remove Adherent Cells

Trypsinizing cells prior to subculturing or cell counting is an important technique for successful cell culture. The following technique works consistently well when passaging cells.

Materials Required:

- 1X trypsin-EDTA solution
- 1X PBS or 1X HBSS

- adherent cells to be subcultured
 - appropriate growth medium (e.g., DMEM) with serum or growth factors or both added
 - culture dishes, flasks or multiwell plates, as needed
 - hemocytometer
1. Prepare a sterile trypsin-EDTA solution in a calcium- and magnesium-free salt solution such as 1X PBS or 1X HBSS. The 1X solution can be frozen and thawed for future use, but trypsin activity will decline with each freeze-thaw cycle. The trypsin-EDTA solution may be stored for up to 1 month at 4°C.
 2. Remove medium from the tissue culture dish. Add enough PBS or HBSS to cover the cell monolayer: 2ml for a 150mm flask, 1ml for a 100mm plate. Rock the plates to distribute the solution evenly. Remove and repeat the wash. Remove the final wash. Add enough trypsin solution to cover the cell monolayer.
 3. Place plates in a 37°C incubator until cells just begin to detach (usually 1–2 minutes).
 4. Remove the flask from the incubator. Strike the bottom and sides of the culture vessel sharply with the palm of your hand to help dislodge the remaining adherent cells. View the cells under a microscope to check whether all cells have detached from the growth surface. If necessary, cells may be returned to the incubator for an additional 1–2 minutes.
 5. When all cells have detached, add medium containing serum to cells to inactivate the trypsin. Gently pipet cells to break up cell clumps. Cells may be counted using a hemocytometer and/or distributed to fresh plates for subculturing.

Typically, cells are subcultured in preparation for transfection the next day. The subculture should bring the cells of interest to the desired confluency for transfection. As a general guideline, plate 5×10^4 cells per well in a 24-well plate or 5.5×10^5 cells for a 60mm culture dish for ~80% confluency the day of transfection. Change cell numbers proportionally for different size plates (see Table 12.2).

Table 12.2. Area of Culture Plates for Cell Growth.

Size of Plate	Growth Area ^a (cm ²)	Relative Area ^b
24-well	1.88	1X
96-well	0.32	0.2X
12-well	3.83	2X
6-well	9.4	5X
35mm	8.0	4.2X
60mm	21	11X
100mm	55	29X

^aThis information was calculated for Corning® culture dishes.

^bRelative area is expressed as a factor of the total growth area of the 24-well plate recommended for optimization studies. To determine the proper plating density, multiply 5×10^4 cells by this factor.

B. Preparation of DNA for Transfection

High-quality DNA free of nucleases, RNA and chemicals is as important for successful transfection as the reagent chosen. See the *Protocols and Applications Guide* chapter on DNA purification (www.promega.com/paguide/chap9.htm) for information about purifying transfection-quality DNA.

In the case of a reporter gene carried on a plasmid, a promoter appropriate to the cell line is needed for gene expression. For example, the CMV promoter works well in many mammalian cell lines but has little functionality in plants. The best reporter gene is one that is not endogenously expressed in the cells. Firefly luciferase, *Renilla* luciferase, click beetle luciferase, chloramphenicol acetyltransferase and β -galactosidase fall into this category. Vectors for all five reporters are available from Promega. See the Reporter Vectors web page (www.promega.com/vectors/reporter_vectors.htm) for more information on our wide array of reporter plasmids.

C. Optimization of Transfection

In previous sections, we discussed factors that influence transfection success. Here we present a method to optimize transfection of a particular cell line with a single transfection reagent. For more modern lipid-based reagents such as the FuGENE® HD Transfection Reagent, we recommend using 100ng of DNA per well of a 96-well plate at reagent:DNA ratios of 4:1, 3.5:1, 3:1, 2.5:1, 2:1 and 1.5:1. Figure 12.6 outlines a typical optimization matrix. When preparing the FuGENE® HD Transfection Reagent:DNA complex, the incubation time may require optimization; we recommend 0–15 minutes. Incubations longer than 30 minutes may result in decreased transfection efficiency. See Technical Manual #TM328.

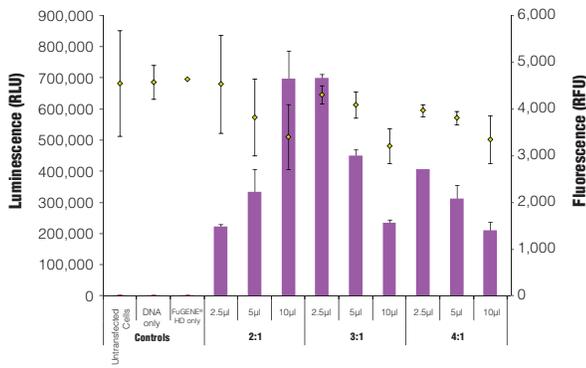


Figure 12.6. Transfection optimization using the FuGENE® HD Transfection Reagent. FuGENE® HD Transfection Reagent: DNA complexes were formed by combining 2µg of pGL4.13[*luc2/SV40*] Vector, enough FuGENE® Reagent to achieve the indicated ratios and cell culture medium to a final volume of 100µl. To HEK-293 cells in a 96-well plate (2×10^4 cells/100µl/well), 2µl, 5µl or 10µl of this complex was added per well. Control wells contained untransfected cells, cells transfected with DNA only (no FuGENE® HD Reagent) or cells transfected with FuGENE® HD Transfection Reagent only (no DNA). Firefly luciferase activity was measured using the ONE-Glo™ Luciferase Assay System, and cell viability was determined using the CellTiter-Fluor™ Cell Viability Assay. Luminescence and fluorescence were quantified using the GloMax®-Multi+ Detection System, and results are expressed as relative light units and relative fluorescence units (RFU), respectively.

For traditional reagents, such as the TransFast™ Reagent, we recommend testing various amounts of transfected DNA (0.25, 0.5, 0.75 and 1µg per well in a 24-well plate) at two charge ratios of lipid reagent to DNA (2:1 and 4:1; see Figure 12.7 and Technical Bulletin #TB260). This brief optimization can be performed using a transfection interval of one hour under serum-free conditions. One 24-well culture plate per reagent is required for the brief optimization with adherent cells (3 replicates per DNA amount).

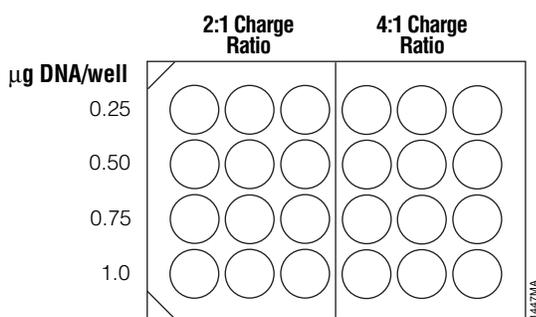


Figure 12.7. Suggested plating format for initial optimization of cationic lipid transfection conditions.

A more thorough optimization can be performed to screen additional charge ratios, time points and effects of serum-containing medium at the DNA amounts found to be optimal during initial optimization studies. One hour or two hours for the transfection interval is optimal for

many cell lines. In some cases, however, it may be necessary to test charge ratios and transfection intervals outside of these ranges to achieve optimal gene transfer.

Both DEAE-dextran and calcium phosphate work well with larger cell cultures (e.g., 100mm culture dish or T75 flask). General guidelines for DNA amount and time for calcium phosphate-mediated transfection are given in Table 12.3.

Table 12.3. Guidelines for Calcium Phosphate Transfection.

Size of Culture Dish	Amount of DNA Transfected	Incubation Time with Transfection Complex ^a
60mm	6–12µg DNA	4–16 hours
100mm	10–20µg	4–16 hours

^aIf the cells are sensitive to the reagent, incubate for no more than 4 hours. Incubation time can be longer but will need to be optimized for the individual cell line.

Some transfection methods require removal of medium with reagent after incubation; others do not. Read the technical literature accompanying the selected transfection reagent to learn which method is appropriate for your system. However, if there is excessive cell death during transfection, consider decreasing time of exposure to the transfection reagent, decreasing the amounts of DNA and reagent added to cells, plating additional cells and removing the reagent after the incubation period and adding complete medium.

D. Endpoint Assay

Many transient expression assays use lytic reporter assays like the Dual-Luciferase® Assay System (Cat.# E1910) or Bright-Glo™ Assay System (Cat.# E2610) 24 hours post-transfection. However, the assay time frame can vary (24–72 hours after transfection), depending on protein expression levels. Reporter-protein assays use colorimetric, radioactive or luminescent methods to measure enzyme activity present in a cell lysate. Some assays (e.g., Luciferase Assay System) require that cells are lysed in a buffer after removing the medium, then mixed with a separate assay reagent to determine luciferase activity. Others are homogeneous assays (e.g., Bright-Glo™ Assay System) that include the lysis reagent and assay reagent in the same solution and can be added directly to cells in medium. Examine the reporter assay results and determine where the greatest expression (highest reading) occurred. These are the conditions to use with your constructs of interest.

Other assays include histochemical staining of cells (determining the percentage of cells that are stained in the presence of the reporter gene substrate; Figure 12.8), fluorescence microscopy (Figure 12.9) or cell sorting if using a fluorescent reporter like the Monster Green® Fluorescent Protein pHMGFP Vector (Cat.# E6421).

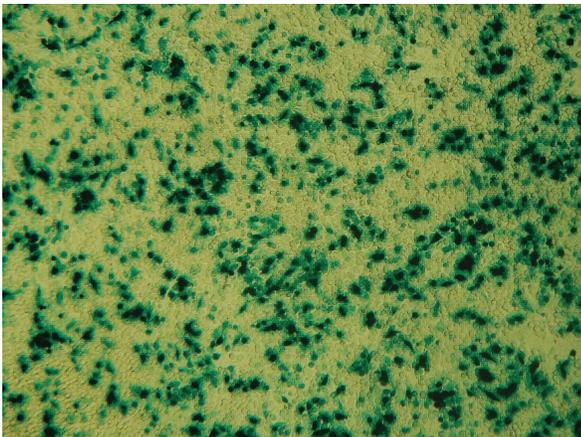


Figure 12.8. Histochemical staining of RAW 264.7 cells for β -galactosidase activity. RAW 264.7 cells were transfected using 0.1 μ g DNA per well and a 3:1 ratio of FuGENE® HD to DNA. Complexes were formed for 5 minutes prior to applying 5 μ l of the complex mixture to 50,000 cells/well in a 96-well plate. Twenty-four hours post-transfection, cells were stained for β -galactosidase activity using X-gal. Data courtesy of Fugent, LLC.

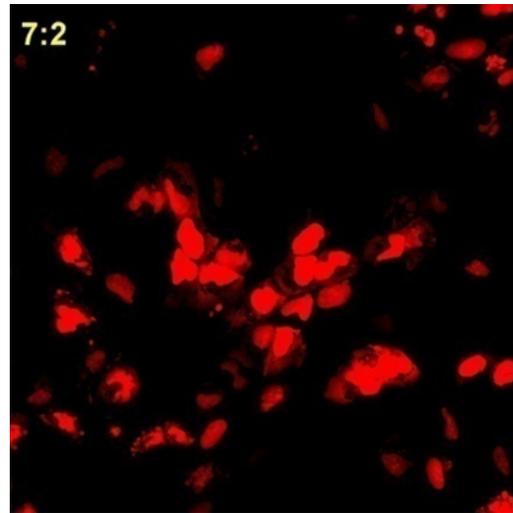


Figure 12.9. Fluorescent microscopy of U-2 OS cells transfected with a HaloTag®-NLS₃ Vector using the FuGENE® HD Transfection Reagent. U-2 OS cells were transiently transfected with 0.5 μ g of the HaloTag®-NLS₃ Vector, which encodes the HaloTag® protein with three copies of a nuclear localization signal (NLS), at a 3.5:1 FuGENE® HD Reagent:DNA ratio. Twenty-four hours post-transfection, cells were labeled using the HaloTag® TMR Ligand and the live-cell imaging protocol described in the *HaloTag® Technology: Focus on Imaging Technical Manual #TM260*. The resulting fluorescence was visualized by microscopy.

Assaying relative expression using the HaloTag® technology provides new options for rapid, site-specific labeling of proteins in living cells and in vitro. The ability to create labeled HaloTag® fusion proteins with a wide range of optical properties and functions allows researchers to image and localize labeled HaloTag® protein fusions in live- or fixed-cell populations and isolate and analyze HaloTag® protein fusions and protein complexes. Several ligands are available for this system with new options being added regularly. For more information on this labeling technology, see the *Protocols and Applications Guide* chapter on cell labeling (www.promega.com/paguide/chap10.htm).

VII. Stable Transfection

A. Selection of Stably Transfected Cells

Optimization for stable transfection begins with successful transient transfection. However, cells should be transfected with a plasmid containing a gene for drug resistance, such as neomycin phosphotransferase (*neo*). As a negative control, transfect cells using DNA that does not contain the drug-resistance marker.

1. Prior to transfection, determine the selective drug concentration required to kill untransfected cells (kill curve; Ausubel *et al.* 1995).
2. Forty-eight hours after transfection, trypsinize adherent cells and replate at several different dilutions (e.g., 1:100, 1:500) in medium containing the appropriate selection drug. For effective selection, cells should be subconfluent since confluent, nongrowing cells are very resistant to the effects of antibiotics like G-418.
3. For the next 14 days, replace the drug-containing medium every 3 to 4 days.
4. During the second week, monitor cells for distinct "islands" of surviving cells. Drug-resistant clones can appear in 2–5 weeks, depending on the cell type. Cell death should occur after 3–9 days in cultures transfected with the negative control plasmid.
5. Transfer individual clones by standard techniques (e.g., using cloning cylinders) to 96-well plates, and continue to maintain cultures in medium containing the appropriate drug.

Table 12.4 provides an overview of commonly used antibiotics to select and maintain stable transfectants.

B. Calculating Stable Transfection Efficiency

The following procedure may be used to determine the percentage of stable transfectants obtained.

Note: The stained cells will not be viable after this procedure.

Materials Required:

- methylene blue
 - methanol
 - cold deionized water
 - light microscope
1. After approximately 14 days of selection in the appropriate drug, monitor the cultures microscopically for the presence of viable cell clones. When distinct "islands" of surviving cells are visible and nontransfected cells have died out, proceed with Step 2.
 2. Prepare stain containing 2% methylene blue in 50–70% methanol.
 3. Remove the growth medium from cells by aspiration.
 4. Add to cells sufficient stain to cover the bottom of the dish.
 5. Incubate for 5 minutes.
 6. Remove the stain, and rinse gently under deionized cold water. Shake off excess moisture.
 7. Allow the plates to air-dry. The plates can be stored at room temperature.
 8. Count the number of colonies, and calculate the percent of transfectants based on the cell dilution and original cell number.

For further information on stable transfections, see Ausubel *et al.* 1995.

VIII. Composition of Solutions

1X HBSS (Hanks Balanced Salt Solution)

- 5mM KCl
- 0.3mM KH₂PO₄
- 138mM NaCl
- 4mM NaHCO₃
- 0.3mM Na₂HPO₄
- 5.6mM D-glucose

The final pH should be 7.1.

Table 12.4. Antibiotics Used to Select Stable Transfectants.

Antibiotic	Resistance Gene	Working Concentration	Stock Solution
G-418 or Geneticin®	aminoglycoside (e.g., neomycin) phosphotransferase	G-418 is often used for initial selection at 500µg/ml with a range of 50–1,000µg/ml	50mg/ml in either water or 100mM HEPES (pH 7.3); the latter buffer helps maintain culture medium pH
Hygromycin (Hygro)	<i>hph</i>	10–400µg/ml	100mg/ml in water
Puromycin (Puro)	<i>pac</i>	1–10µg/ml	10mg/ml in water or HEPES buffer (pH 7.0)

1X PBS

137mM NaCl
2.7mM KCl
4.3mM Na₂HPO₄
1.47mM KH₂PO₄

The final pH should be 7.1.

1X Trypsin-EDTA solution

0.05% trypsin (w/v)
0.53mM EDTA

Dissolve in a calcium- and magnesium-free salt solution such as 1X PBS or 1X HBSS.

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I. Introduction

The cloning of genes, gene fragments and other DNA sequences is a fundamental part of molecular biology. To study the function of a particular DNA sequence, you must be able to manipulate that sequence. There are two main ways to achieve this: the polymerase chain reaction (PCR) and the more traditional use of restriction enzymes and modifying enzymes to “cut and paste” the desired DNA fragments into cloning vectors, which can then be replicated using live cells, most commonly *E. coli*. The use of PCR has an advantage in that it gives you the option to re-amplify the target DNA each time your DNA supplies dwindle without ligation into a vector or transformation into *E. coli*. Alternatively, PCR products can be ligated into a suitable vector, which can then be transformed into and replicated by *E. coli*. This chapter covers the basics of cloning using PCR and restriction enzymes, including DNA cleanup prior to ligation, ligation, transformation and screening to identify recombinant clones.

The PCR process is a useful tool to quickly and easily amplify the desired sequences. With the successful sequencing of whole and partial genomes of organisms across all biological kingdoms, DNA cloning by PCR is an easily attainable option. Public DNA databases allow researchers to design primers to amplify their DNA fragment of interest directly from the genomic DNA of the desired organism. With the simple addition of a reverse transcription step prior to PCR, RNA sequences can be converted to cDNA, which can then be cloned into a suitable vector. For additional information about amplification of DNA and RNA sequences using PCR, see the *Protocols and Applications Guide* chapter on PCR Applications (www.promega.com/paguide/chap1.htm).

PCR products generated using a nonproofreading DNA polymerase such as *Taq* DNA polymerase, which lacks 3'→5' exonuclease activity, have a single template-independent nucleotide at the 3' end of the DNA strand (Clark, 1988; Newton and Graham, 1994). This single-nucleotide overhang, which is most commonly an A residue, allows hybridization with and cloning into T vectors, which have a complementary 3' single T overhang. PCR products generated using a proofreading DNA polymerase, such as *Pfu* DNA polymerase, have blunt ends and must be cloned into a blunt-ended vector or need a single 3' A overhang added to ligate into a T vector (Knoche and Kephart, 1999).

If PCR amplification of the desired DNA fragment is not possible or desirable, restriction enzyme digestion of the target DNA can be employed. The desired fragment may need to be separated from other DNA fragments in the reaction, so the size of the desired DNA fragment should be known. Once isolated, the fragment is cloned into a vector with compatible ends. If the vector ends are capable of religating (e.g., the vector has blunt ends or is cut with a single restriction enzyme), the vector is often treated with alkaline phosphatase to discourage recircularization and maximize ligation between the insert and vector.

Following transformation into *E. coli*, the resulting bacterial colonies are screened by PCR for the correct recombinant vector using primers to amplify the insert. Alternatively, the recombinant vector can be identified by performing a restriction enzyme digestion to determine the presence of the correct insert. Screening is often simplified by using vectors that contain an antibiotic-resistance gene, so cells containing the vector will survive on medium supplemented with the appropriate antibiotic. Screening can be further simplified by choosing a vector and *E. coli* strain that are compatible with blue/white screening, which takes advantage of intracistronic α -complementation to regenerate β -galactosidase activity. Many *E. coli* strains used for cloning and propagation of plasmids contain a chromosomal deletion of the *lac* operon but carry an F' episome that provides the remaining coding sequence of the *lacZ* gene. The functional *lacZ* gene product, β -galactosidase, is produced when the *lacZ* coding information missing on the F' episome is provided by the information contained in the plasmid. This activity is detected by plating bacteria transformed by plasmids on plates containing isopropyl β -D-thiogalactopyranoside (IPTG; an inducer of the *lac* promoter) and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal; a dye that produces a blue color when hydrolyzed by β -galactosidase). When the reading frame of the α peptide is disrupted by insertion of a foreign DNA fragment or deletion of vector sequences, α -complementation does not occur, and the bacterial colonies remain white or occasionally light blue.

II. Promega Products for Cloning

A. Thermostable DNA Polymerases

The use of amplification enzymes is the first step in cloning by PCR. Most people use PCR for cloning, taking advantage of the single nucleotide A overhang left after amplification with a nonproofreading DNA polymerase to ligate the amplicon to a vector containing T overhangs. However, products will be blunt-ended if the DNA polymerase has 3'→5' exonuclease activity, also known as proofreading activity. Alternatively, PCR primers can add sequences for restriction enzyme sites, and these resulting products can be digested and ligated into a vector with compatible ends. Promega provides several thermostable DNA polymerases. These include the GoTaq® Amplification Family, *Tfi* DNA polymerase and proofreading polymerases. A detailed listing of the various enzymes for use in PCR can be found in the *Protocol and Applications Guide* chapter on PCR Applications (www.promega.com/paguide/chap1.htm), in the section "Thermostable DNA Polymerases". The GoTaq® Amplification Family of products is highlighted in the following section.

GoTaq® Amplification Family

GoTaq® DNA Polymerase is available in various formulations to suit your needs: the standard GoTaq® DNA Polymerase, which is supplied with 1.5mM MgCl₂ in the 1X reaction buffer; GoTaq® Flexi DNA Polymerase, which

allows a range of MgCl₂ to be added for PCR; and GoTaq® Green Master Mix, which is a premixed, ready-to-use solution containing GoTaq® DNA Polymerase, dNTPs, MgCl₂ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. All GoTaq® products contain *Taq* DNA polymerase in a proprietary formulation that offer enhanced amplification over conventional *Taq* DNA polymerase. Each member of the GoTaq® family has a reaction buffer that contains two dyes (a blue dye and a yellow dye) that separate during electrophoresis to show migration progress as well as a compound that increases sample density. Samples can be loaded directly onto gels without the need to add a separate loading dye. If the dyes interfere with your downstream applications, GoTaq® DNA Polymerases are supplied with a 5X Colorless Reaction Buffer. Alternatively, the PCR Master Mix offers a ready-to-use formulation without any dyes. Reaction products generated with these systems contain A overhangs and are ready for T-vector cloning.

Additional Resources for GoTaq® DNA Polymerase

Promega Publications

- PN083 Introducing GoTaq® DNA Polymerase: Improved amplification with a choice of buffers
(www.promega.com/pnotes/83/)

Citations

Theodoropoulos, G. *et al.* (2006) Determination of prevalence and risk factors of infection with *Babesia* in small ruminants from Greece by polymerase chain reaction amplification. *Vet. Parasitol.* **135**, 99–104.

Researchers used GoTaq® DNA Polymerase to test sheep and goat blood samples for the presence of *Babesia* DNA. Primers were designed around the 18S rRNA sequence of *Babesia* sp. PCR was performed in a 50µl reaction volume using 1 unit of GoTaq® DNA Polymerase. Ten microliters of each amplification reaction were loaded on gels and subjected to electrophoresis.

PubMed Number: 16139956

Weinberg, J.B. *et al.* (2005) Acute respiratory infection with mouse adenovirus type 1. *Virology* **340**, 245–54.

Mouse adenovirus type 1 (MAV-1) was detected in DNA extracted from the lungs of mice by PCR amplification of the E1A region of MAV-1. For these assays, 80ng of total DNA was added to a 20µl PCR reaction containing 0.5 units of GoTaq® DNA Polymerase, 4µl of 5X GoTaq® Buffer, dNTPs and primers for MAV-1 E1A. The amplified products were separated on a 1.8% agarose gel and stained with ethidium bromide.

PubMed Number: 16054189

Additional Resources for GoTaq® Flexi DNA Polymerase

Promega Publications

- PN089 GoTaq® Flexi DNA Polymerase: Robust performance with magnesium optimization
(www.promega.com/pnotes/89/)

Additional Resources for GoTaq® Green Master Mix

Promega Publications

- eNotes Activity of Promega Restriction Enzymes in GoTaq® Green Master Mix and PCR Master Mix
(www.promega.com/notes/applications/ap0075.htm)
- eNotes Analyses of gene disruption by whole-cell PCR using the GoTaq® Green Master Mix
(www.promega.com/notes/applications/ap0065.htm)
- PN091 GoTaq® Green Master Mix: From amplification to analysis
(www.promega.com/pnotes/91/)
- PN101 Recombinant clone screening using the GoTaq® Hot Start Green Master Mix
(www.promega.com/pnotes/101/17252_13/17252_13.html)

Citations

Rendón, M.A. *et al.* (2007) Commensal and pathogenic *Escherichia coli* use a common pilus adherence factor for epithelial cell colonization. *Proc. Natl. Acad. Sci. USA.* **104**, 10637–42.

The authors identified an adherence factor of enterohemorrhagic *E. coli* that is involved in colonization of cultured epithelial cells. This factor, named *E. coli* common pilus (ECP), is encoded by the *ecpA* gene, which is present 96% of *E. coli* strains tested, as determined by PCR. The remaining 4% of the strains were deficient in the ECP operon, as determined by multiplex PCR amplification of *ecpR*, *ecpA*, *ecpB* and *ecpC* sequences. PCR were performed using GoTaq® Green Master Mix. An *ecpA* deletion mutant exhibited impaired adherence compared to the wildtype *E. coli* strain. Complementation of the mutant strain with the plasmid pMR13, the pGEM®-T Vector containing the *ecpA* gene, restored the strain's ability to adhere to epithelial cells.

PubMed Number: 17563352

Song, J.H. *et al.* (2006) Human astrocytes are resistant to Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *J. Neurosci.* **26**, 3299–308.

Total RNA was extracted from human astrocytes and control A549 cells. First strand cDNA was synthesized from 3µg of total RNA using random hexamers. PCR was performed on the cDNA samples using primers for DR4, DR5 and GAPDH with GoTaq® Green Master Mix. The

PCR products were analyzed on a 1.5% agarose gel and stained with ethidium bromide.

PubMed Number: 16554480

Additional Resources for PCR Master Mix

Promega Publications

eNotes	Activity of Promega Restriction Enzymes in GoTaq® Green Master Mix and PCR Master Mix www.promega.com/enotes/applications/ap0075.htm
PN078	Performance advantages designed into Promega's PCR Master Mix www.promega.com/pnotes/78/

B. T-Cloning Vectors

T vectors are a specific type of cloning vector that get their name from the T overhangs added to a linearized plasmid. These vectors take advantage of the A overhangs on PCR products after amplification with *Taq* DNA polymerase by providing compatible ends for ligation (Mezei and Storts, 1994; Robles and Doers, 1994). There are three different T-cloning vectors from Promega: two are basic cloning vectors, and the third is a mammalian expression vector.

pGEM®-T and pGEM®-T Easy Vector Systems

The pGEM®-T (Cat.# A3600, A3610) and pGEM®-T Easy Vector Systems (Cat.# A1360, A1380) are convenient systems for the cloning of PCR products. The vectors are prepared by cutting with a blunt-ended restriction endonuclease and adding a 3' terminal thymidine to both ends (Figures 13.1 and 13.2). These single 3' T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products with 5' A overhangs.

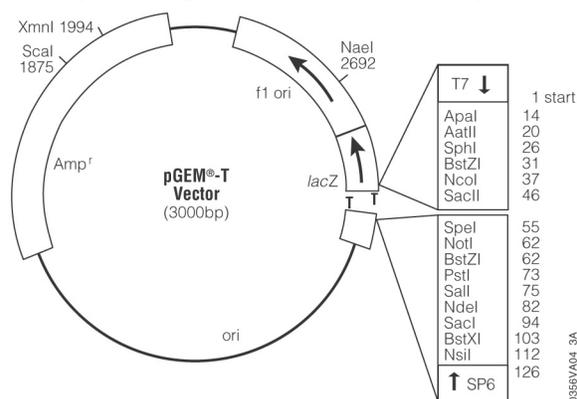


Figure 13.1. pGEM®-T Vector circle map.

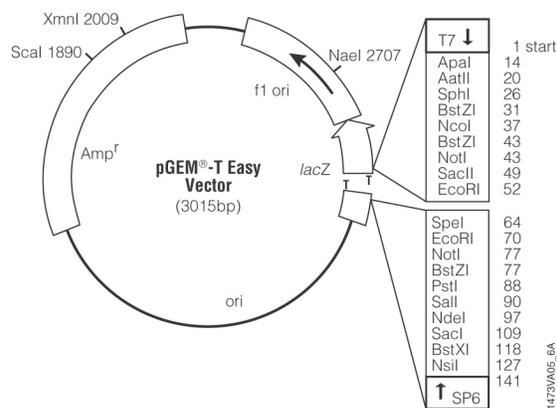


Figure 13.2. pGEM®-T Easy Vector circle map.

The high-copy-number pGEM®-T and pGEM®-T Easy Vectors contain T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the coding region for the α -peptide of β -galactosidase. Insertional inactivation of the α -peptide allows recombinant clones to be directly identified by color screening on indicator plates containing

X-Gal (Cat.# V3941) and IPTG (Cat.# V3955). Both the pGEM[®]-T and pGEM[®]-T Easy Vectors contain numerous restriction sites within the multiple cloning region. The pGEM[®]-T Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzymes EcoRI, BstZI and NotI, thus providing three single-enzyme digestions for release of the insert. The pGEM[®]-T Vector cloning region is flanked by recognition sites for the enzyme BstZI. Alternatively, a double-digestion may be used to release the insert from either vector.

The pGEM[®]-T and pGEM[®]-T Easy Vectors also contain the origin of replication of the filamentous phage f1 for the preparation of single-stranded DNA (ssDNA). Both pGEM[®]-T vector systems include a 2X Rapid Ligation Buffer for ligation of PCR products, which requires only a 1-hour incubation at room temperature. The incubation period may be extended to increase the number of colonies after transformation. Generally, an overnight incubation at 4°C will produce the maximum number of transformants.

Inserts of several kilobases have been successfully cloned into the pGEM[®]-T and pGEM[®]-T Easy Vectors (D'Avino *et al.* 2004). However, as the insert gets larger, the ratio of vector to insert may need to be optimized further to maximize ligation efficiency (see Ligation and Transformation in the section "Vector:Insert Ratio").

One of the disadvantages of PCR cloning into a T vector is that the insert can be cloned in either direction. Analysis of recombinant vectors by PCR or restriction enzyme digestion can be used to determine not only the success of cloning, but in which direction the insert was cloned. To verify the direction of the insert, amplify recombinant plasmids using one of the gene specific PCR primers and each of the phage promoter primers, which are present on the pGEM[®]-T Vector (Knoche and Kephart, 1999). The correct orientation is important for transcription or translation or both.

Additional Resources for the pGEM[®]-T and pGEM[®]-T Easy Vector Systems

Technical Bulletins and Manuals

- TM042 *pGEM[®]-T and pGEM[®]-T Easy Vector Systems Technical Bulletin*
(www.promega.com/tbs/tm042/tm042.html)

Promega Publications

- eNotes pGEM[®]-T Easy Vector System is an easy tool for preparing gel shift probes
(www.promega.com/enotes/applications/ap0039_tabs.htm)
- eNotes Cloning differential display-PCR products with pGEM[®]-T Easy Vector System
(www.promega.com/enotes/applications/ap0025_tabs.htm)

- PN082 Technically Speaking: T-vector cloning
(www.promega.com/pnotes/82/10203_24/10203_24.html)
- PN073 Constructing genomic libraries using the pGEM[®]-T Vector
(www.promega.com/pnotes/73/8235_26/8235_26.html)
- PN071 Cloning blunt-end *Pfu* DNA Polymerase-generated PCR fragments into pGEM[®]-T Vector Systems
(www.promega.com/pnotes/71/7807_10/7807_10.html)
- PN065 Stability of pGEM[®]-T Vectors
(www.promega.com/pnotes/65/6921_25/default.html)
- PN062 Cloning blunt-end DNA fragments into the pGEM[®]-T Vector Systems
(www.promega.com/pnotes/62/7807_15/promega.html)
- PN061 Amplification of flanking regions: New applications and performance optimization of single specific primer PCR and T-vector cloning
(www.promega.com/pnotes/61/6222_26/promega.html)

Online Tools

- pGEM[®]-T Easy Vector sequence (www.promega.com/vectors/pgemtez.txt)
- pGEM[®]-T Vector sequence (www.promega.com/vectors/pgemt.txt)

Citations

Renaud, S. *et al.* (2007) Dual role of DNA methylation inside and outside of CTCF-binding regions in the transcriptional regulation of the telomerase hTERT gene. *Nucleic Acids Res.* **35**, 1245–56.

Telomeres shorten by 50–100 bases with each cell division, making the telomere a "mitotic counter" that can limit cellular lifespan. Telomerase is a two-component protein consisting of a reverse transcriptase (hTERT) bound to its own RNA template that can act to maintain telomere length in dividing cells. This paper investigated the role of methylation of the hTERT promoter and the transcription factor CTCF in regulation of telomerase activity. LacZ reporter plasmids driven by the hTERT minimal promoter were transiently transfected into HeLa cells, and reporter assays were performed on lysate generated using Passive Lysis Buffer. The hTERT minimal promoter did not show activity if all of the CpG sites were methylated. The promoter and first exon of hTERT were amplified using PCR Master Mix from sodium bisulfite-treated genomic DNA isolated from telomerase-positive cell lines and tissues. The resulting fragments were cloned using the pGEM[®]-T Vector System II. For the methylation cassette assay, methylated and unmethylated fragments were cloned into a methylated or unmethylated vector using the LigaFast[™] Rapid DNA Ligation System. The authors

conclude that methylation plays a dual role in regulating hTERT expression. CTCF will bind to the first exon of hTERT when the hTERT CpG island is not methylated, resulting in downregulation of hTERT expression.

PubMed Number: 17267411

Wu, S. *et al.* (2006) Reversal of the malignant phenotype of cervical cancer CaSki cells through adeno-associated virus-mediated delivery of HPV16 E7 antisense RNA. *Cancer Res.* **12**, 2032–7.

The coding sequence of the Human Papilloma Virus (HPV16) E7 oncogene was isolated after purification of total RNA from CaSki cells, RT-PCR, subsequent PCR and cloning into the pGEM®-T Easy Vector. To test the effectiveness of antisense HPV16 E7 therapy against cervical cancer, an adeno-associated virus vector was constructed using this coding sequence and used to transfer the antisense construct of the E7 coding sequence into CaSki cervical cancer cells.

PubMed Number: 16609012

pTARGET™ Mammalian Expression Vector System

The pTARGET™ Mammalian Expression Vector System (Cat.# A1410) is a convenient system to clone PCR products and express cloned PCR products in mammalian cells. As with the pGEM®-T and pGEM®-T Easy Vector Systems, the pTARGET™ Vector is supplied already linearized with single T overhangs (Figure 13.3). These single 3' T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmid. The pTARGET™ Vector also contains a modified version of the coding sequence of the α peptide of β -galactosidase, which allows recombinants to be selected using blue/white screening.

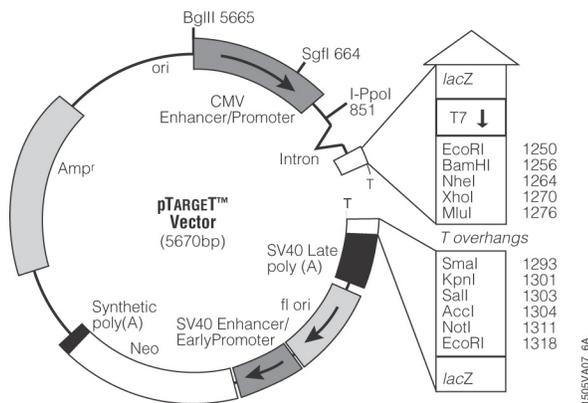


Figure 13.3. pTARGET™ Vector circle map.

The pTARGET™ Vector carries the human cytomegalovirus (CMV) immediate-early enhancer/promoter region to promote constitutive expression of cloned DNA inserts in mammalian cells. This vector also contains the neomycin phosphotransferase gene, a selectable marker for mammalian cells. The pTARGET™ Vector can be used for transient expression or for stable expression by selecting transfected cells with the antibiotic G-418. Like the

pGEM®-T or pGEM®-T Easy Vectors, inserts of several kilobases can be cloned in and expressed from the pTARGET™ Vector (Sakakida *et al.* 2005; Le Gall *et al.* 2003).

Additional Resources for the pTARGET™ Mammalian Expression Vector System

Technical Bulletins and Manuals

- TM044 *pTARGET™ Mammalian Expression Vector System Technical Manual*
(www.promega.com/tbs/tm044/tm044.html)

Promega Publications

- PN082 *Technically Speaking: T-vector cloning*
(www.promega.com/pnotes/82/10203_24/10203_24.html)
- PN058 *pTARGET™ Vector: A new mammalian expression T-vector*
(www.promega.com/pnotes/58/5189a/5189a.html)

Online Tools

pTARGET™ Mammalian Expression Vector sequence
(www.promega.com/vectors/ptarget.txt)

Citations

Treeck, O. *et al.* (2007) Novel estrogen receptor beta transcript variants identified in human breast cancer cells affect cell growth and apoptosis of COS-1 cells. *Mol. Cell. Endocrinol.* **264**, 50–60.

This study identified two novel transcript variants of the estrogen receptor ER β that were expressed in the ER α -negative breast cancer cell line MDA-MD-231. These variants were identified after amplification of ER β transcripts from the breast cancer cell line by RT-PCR. The amplification products were then excised from gels and subcloned into the pTARGET™ Mammalian Expression Vector prior to sequencing. COS1 cells, which do not express the estrogen receptor, were then stably transfected with full-length ER β or one of the splice variants, and the effects on cell proliferation, apoptosis and estrogen response were evaluated. In COS1 cells expressing either ER β or the transcript variants, cell proliferation decreased and basal apoptosis (caspase-3/7 activity) increased, compared to cells transfected with vector alone. Exposure to therapeutic doses of tamoxifen induced apoptosis in cells expressing the full-length ER β but not in cells expressing either of the variant isoforms.

PubMed Number: 17095148

Guyonnet-Duperat, V. *et al.* (2006) Functional implication of an ARG307GLY substitution in corticosteroid binding globulin, a candidate gene for a QTL associated with cortisol variability and obesity. *Genetics* **173**, 2143–9.

In this study, the effects of amino acid substitutions in porcine corticosteroid-binding globulin gene (*Cbg*) were tested on CBG binding and affinity. *Cbg* cDNA was obtained by reverse transcribing pig liver total RNA using M-MLV Reverse Transcriptase followed by PCR. The

1257bp PCR product was ligated into the pTARGET™ Mammalian Expression Vector. The GeneEditor™ in vitro Site-Directed Mutagenesis System was used to introduce four different codon substitutions in the *Cbg* cDNA. Once created, the mutated and unmodified *Cbg* cDNA constructs were transfected into HEK 293T (human embryonic kidney) cells. After 48 hours, the supernatant was collected to analyze secreted CBG.

PubMed Number: 16702435

C. Flexi® Vector Systems

The Flexi® Vector Systems (Cat.# C8640, C8820, C9320) are based on a simple, yet powerful, directional cloning method for protein-coding sequences. First, a PCR product is generated by primers designed with two rare-cutting restriction enzymes, SgfI and PmeI. Then, after restriction enzyme digestion, the insert can ligate in a single orientation. All Flexi® Vectors carry the lethal barnase gene, which is replaced by the DNA fragment of interest and acts as a positive selection for the successful ligation of the insert. The two restriction enzymes provide a rapid, efficient and high-fidelity way to transfer protein-coding regions between a variety of Flexi® Vectors without the need to resequence while maintaining the reading frame (see Figure 13.4 for system overview and Figure 13.5 for list of example vectors). A current list of available vectors can be found at:

www.promega.com/catalog/catalogredirect.asp?partno=c8640.

To design PCR primers appropriate for your insert and with SgfI and PmeI restriction sites, visit the Flexi® Vector Primer Design Tool (www.promega.com/techserv/tools/flexivector/).

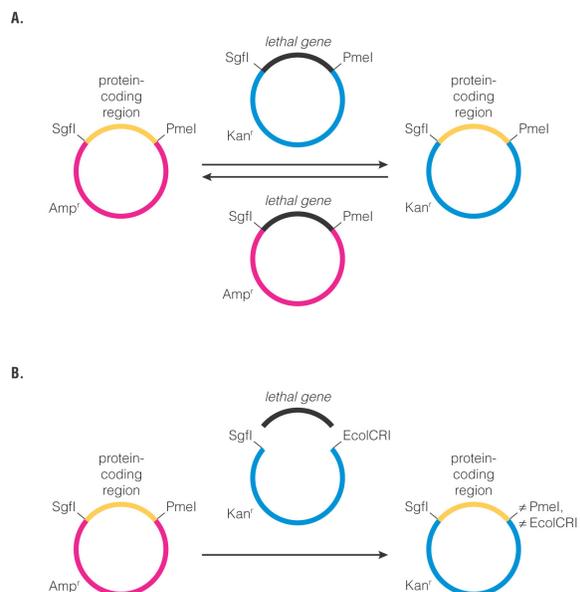


Figure 13.4. Transferring protein-coding regions in the Flexi® Vector Systems. Panel A. The Flexi® Vector Systems employ a flexible, directional cloning method to create plasmids to express protein-coding regions with or without peptide fusion tags. The features necessary for expression and the options for protein fusion tags are carried on the vector backbone, and the protein-coding region can be shuttled between vectors using two rare-cutting restriction endonucleases, SgfI and PmeI. The Flexi® Vectors contain a lethal gene, barnase, for positive selection of the protein-coding sequence and an antibiotic resistance marker for selection of colonies containing the Flexi® Vector. Transfer between Flexi® Vectors for expression of native or N-terminal-tagged fusion proteins is reversible (i.e., it is a two-way exchange). Panel B. C-terminal Flexi® Vectors contain SgfI and EcoICRI sites and are designed to allow expression of C-terminal-tagged proteins. Joining PmeI and EcoICRI blunt ends eliminates the stop codon present in the PmeI site and allows readthrough to the C-terminal protein-coding sequences in the C-terminal Flexi® Vectors. Since both restriction sites are destroyed by joining, transfer into C-terminal Flexi® Vectors is not reversible (i.e., it is a one-way exchange).

Flexi® Vector Name ¹	Expression Application	Promoter for Expression			Peptide Fusion Tag	
		<i>E. coli</i>	Mammalian Cells	in vitro Transcription/ Translation	N-terminus	C-terminus
pF1A	Inducible expression of native protein	T7		T7		
pF1K						
pFN2A	Solubilization and purification via an N-terminal GST tag	T7		T7	GST	
pFN2K						
pF3A WG	In vitro wheat germ expression of native protein			T7, SP6		
pF3K WG						
pF4A CMV	Constitutive expression of native protein		CMV	T7		
pF4K CMV						
pF5A CMV-neo	Constitutive expression of native protein with selection for stable transfectants		CMV	T7		
pF5K CMV-neo						
pFN6A (HQ)	Protein purification via a metal affinity resin	T7		T7	HQHQHQ	
pFN6K (HQ)						
pFC7A (HQ)	Protein purification via a metal affinity resin	T7		T7		HQHQHQ
pFC7K (HQ)						
pFC8A	Protein labeling, cell imaging and surface immobilization		CMV	T7		HaloTag™
pFC8K						
pF9A CMV <i>hRluc</i> -neo	Constitutive expression of native protein with selection and reporter screening for stable transfectants		CMV	T7		
pFN10A (ACT)	Mammalian in vivo protein:protein interaction		CMV	T7	HSV VP16 activation domain	
pFN11A (BIND)	Mammalian in vivo protein:protein interaction		CMV	T7	GAL4 DNA-binding domain	

¹The "pF" indicates that the vector is a Flexi® Vector. The letter after "pF" indicates the position of any expression tags (e.g., "N" for an N-terminal expression tag; "C" for a C-terminal expression tag). The number associated with the vector specifies the type of expression and application. The letters "A" and "K" designate the bacterial drug selection for the vector (A = ampicillin and K = kanamycin).

Figure 13.5. Features of the Flexi® Vectors.

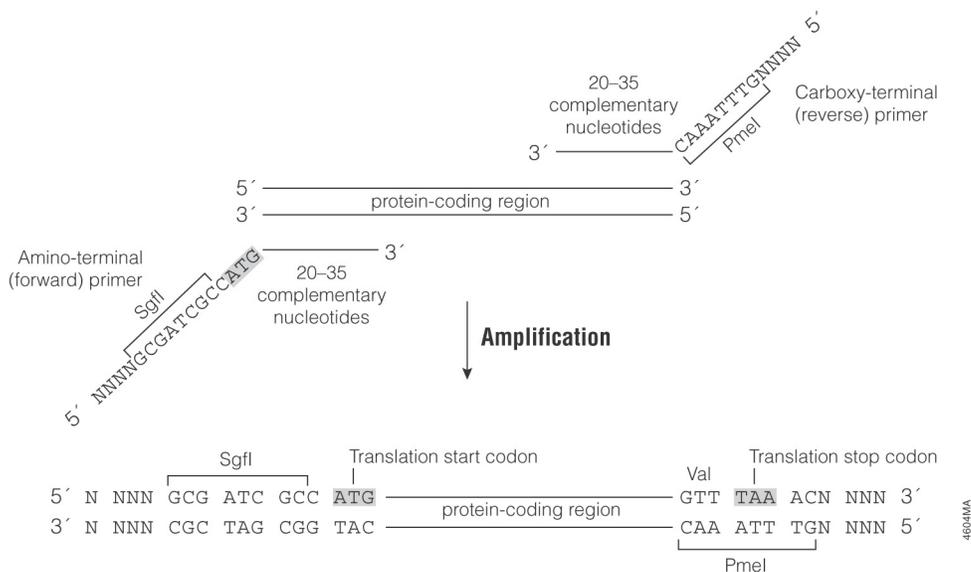


Figure 13.6. PCR primer design. The PmeI site appends a single valine codon at the 3' end of the protein-coding region and allows either termination or readthrough to append a carboxy-terminal peptide, depending on the vector backbone.

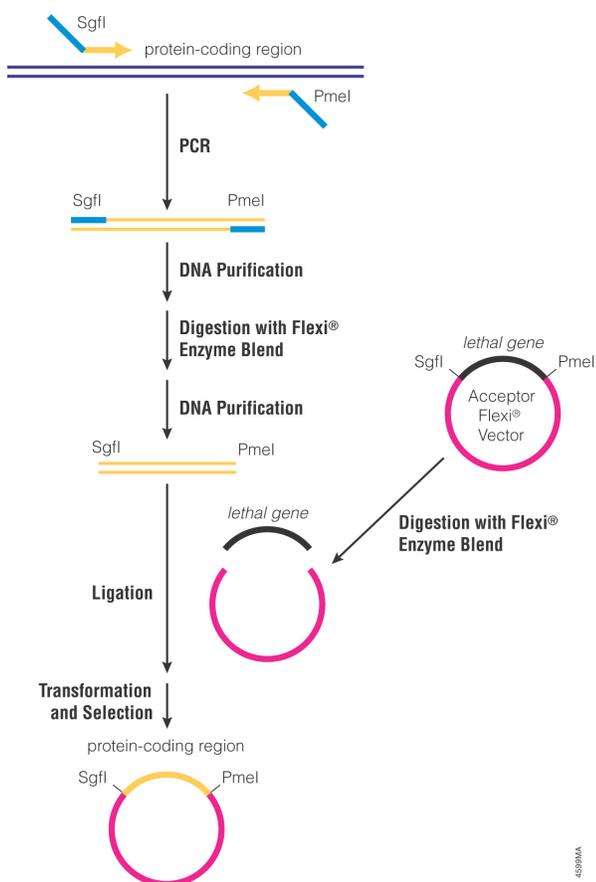


Figure 13.7. Cloning a protein-coding region into the Flexi® Vectors. PCR primers are designed to append SgfI and PmeI sites onto the protein-coding region. After amplification, the PCR product is purified to remove the DNA polymerase and primers and digested with SgfI and PmeI. The DNA is purified again to remove the small oligonucleotides released by the restriction enzymes. The digested PCR product is ligated into an acceptor Flexi® Vector that has been digested with SgfI and PmeI. Following transformation, the cells are selected with the appropriate antibiotic for the particular Flexi® Vector used.

Unlike site-specific recombination vector systems, the Flexi® Vector Systems do not require appending multiple amino acids to the amino or carboxy termini of the protein of interest (Figure 13.6). In addition, the systems do not require an archival entry vector, and most applications allow direct entry into the vector suited to the experimental design (e.g., mammalian expression or N-terminal, glutathione-S-transferase (GST) fusion vectors). For instance, you might clone your PCR product into the pFN2A (GST) Flexi® Vector to express your GST-tagged protein in *E. coli* for purification. However, an easy transfer of your insert after SgfI/PmeI digest followed by ligation into the pF4K CMV Flexi® Vector will allow you to transfect the same protein-coding region into a mammalian cell and determine its expression level.

Any Flexi® Vector can act as an acceptor of a protein-coding region flanked by SgfI and PmeI sites (Figure 13.7). The SgfI site is upstream of the start codon of the protein-coding region, and depending upon the Flexi® Vector used for cloning, this allows the expression of a native (untagged) protein or an amino (N)-terminal-tagged protein by readthrough of the SgfI site. The PmeI site contains the stop codon for the protein-coding region and appends a single valine residue to the carboxy (C)-terminus of the protein (Figure 13.6).

The C-terminal Flexi® Vectors allow expression of C-terminal-tagged proteins. While these vectors can act as acceptors of protein-coding regions flanked by SgfI and PmeI, they lack a PmeI site and contain a different blunt-ended site, EcoICRI. Inserts cloned using these sites cannot be removed from the C-terminal Flexi® Vectors and transferred to other Flexi® Vectors (Figure 13.4, Panel B).

Additional Resources for the Flexi® Vector Systems

Technical Bulletins and Manuals

- TM254 *Flexi® Vector Systems Technical Manual*
www.promega.com/tbs/tm254/tm254.html

Promega Publications

- CN011 Clone and express protein-coding regions using the Flexi® Vector Systems
www.promega.com/cnotes/cn011/cn011_13.htm
- PN094 The next-generation assay for mammalian protein interactions: The CheckMate™/Flexi® Vector Mammalian Two-Hybrid System
www.promega.com/pnotes/94/14410_12/14410_12.html
- PN093 The Flexi® Vector Systems: The easy way to clone
www.promega.com/pnotes/93/14023_08/14023_08.html
- PN091 Metal affinity tag for protein expression and purification using the Flexi® Vectors
www.promega.com/pnotes/91/12972_17/12972_17.html
- PN089 A new system for cloning and expressing protein-coding regions
www.promega.com/pnotes/89/12416_11/12416_11.html

Online Tools

- Flexi® Vector Systems Animation (www.promega.com/paguide/animation/selector.htm?coreName=flexi01)
 Flexi® Vector Primer Design Tool (www.promega.com/techserv/tools/flexivector/)

Citations

Blommel, P.G. *et al.* (2006) High efficiency single step production of expression plasmids from cDNA clones using the Flexi Vector cloning system. *Protein Expr. Purif.* **47**, 562–70.

In this study, the Flexi® Vector Systems was compared with the Gateway® Cloning System to determine its utility in high-throughput expression cloning by subcloning 96 human target genes. A direct comparison between pVP16, the Gateway vector, and the equivalent Flexi® Vector, pVP33A or K, was achieved by modifying pVP16 with the barnase gene and PmeI/SgfI restriction sites, duplicating the design available in the commercial Flexi® Vectors. Capture of genes by PCR amplification of the cDNAs was similar for both systems, but the timeline for the Flexi® Vector system was shorter at 6–8 days compared to 12 days for the Gateway® system. They also found the Flexi® Vector System was lower cost and more accurate due to the shorter primers required for the Flexi® Vector cloning. Since the amplification primers were shorter, the authors found nearly twofold fewer missense errors in the Flexi® Vector system. In their protocol, 96 cDNAs were amplified simultaneously and the PCR products were cleaned up using either the MagneSil® PCR Clean-Up System or Wizard® SV 96 PCR Clean-Up, ligated into the Flexi® Vector, and transformed into Select96™ Competent Cells. They also compared transfer of cDNA inserts between different Flexi® Vectors and transfer of cDNA inserts between different Gateway® vectors and found similar performance in the two systems. For the Flexi® Vector test set, the authors sequenced the clones, validating the high fidelity transfer of cDNA inserts between Flexi® Vectors.

PubMed Number: 16377204

Temple, G. *et al.* (2006) From genome to proteome: Developing expression clone resources for the human genome. *Hum. Mol. Genet.* **15**, R31–R43.

The Kazusa cDNA Project is constructing a library of more than 1,000 “full ORF” (F-ORF) clones in the Flexi® Vector System to characterize the function of proteins that are larger than 50kDa.

PubMed Number: 16651367

D. Modifying and Restriction Enzymes

Promega offers a vast array of both modifying enzymes (e.g., ligase or phosphatase) and restriction endonucleases for use in cloning. This section is an overview of the products available from Promega to enhance your cloning results and highlights the enzymes that may be most useful to you. For example, ligase is a key enzyme in cloning as this enzyme joins the vector and insert to create a circular recombinant plasmid. Restriction enzymes (REs) are used to cut a vector and a PCR product, or other type of insert, to generate compatible ends for ligation. REs can also evaluate the success of the ligation by screening the

recombinant plasmid for the correct restriction sites. To explore strategies for subcloning, visit the Subcloning Notebook (www.promega.com/guides/subcloning_guide/).

DNA Ligase

DNA ligase catalyzes the joining of two strands of DNA using the 5′-phosphate and the 3′-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration (Engler and Richardson, 1982). This allows the “pasting” together of inserts and receptive vectors (e.g., A-tailed product into T vectors).

T4 DNA Ligase (Cat.# M1801, M1804, M1794) can join DNA strands together and has also been shown to catalyze the joining of RNA to a DNA or RNA strand in a duplex molecule. However, DNA ligase will not join single-stranded nucleic acids (Engler and Richardson, 1982).

Additional Resources for T4 DNA Ligase**Technical Bulletins and Manuals**

9PIM180 *T4 DNA Ligase Promega Product Information*
(www.promega.com/tbs/9pim180/9pim180.html)

The **LigaFast™ Rapid DNA Ligation System** (Cat.# M8221, M8225) is designed for the efficient ligation of sticky-ended DNA inserts into plasmid vectors in just 5 minutes (blunt-ended inserts in as little as 15 minutes). Rapid ligation is based on the combination of T4 DNA Ligase with a unique 2X Rapid Ligation Buffer. The LigaFast™ System is designed to eliminate any further purification prior to transformation of ligated DNA. The specially formulated 2X Rapid Ligation Buffer requires no additional ATP or Mg²⁺ prior to use.

Additional Resources for the LigaFast™ Rapid DNA Ligation System**Technical Bulletins and Manuals**

9PIM822 *LigaFast™ Rapid DNA Ligation System Promega Product Information*
(www.promega.com/tbs/9pim822/9pim822.html)

Promega Publications

eNotes Cloning differential display-PCR products with pGEM®-T Easy Vector System
(www.promega.com/enotes/applications/ap0025_tabs.htm)

PN077 Technically Speaking: Subcloning plasmid DNA constructs
(www.promega.com/pnotes/77/9028_28/9028_28.html)

PN071 Rapid ligation for the pGEM®-T and pGEM®-T Easy Vector Systems
(www.promega.com/pnotes/71/7807_08/7807_08.html)

Alkaline Phosphatases

Alkaline phosphatases catalyze the dephosphorylation of 5' phosphates from DNA. These enzymes are used to prevent recircularization and religation of linearized vector DNA by removing 5' phosphate groups from both termini and may also be used to dephosphorylate 5' phosphorylated ends of DNA for subsequent labeling with [³²P]ATP and T4 Polynucleotide Kinase. Unit usage guidelines are usually included with the alkaline phosphatase (e.g., 0.01 units per picomole ends). For assistance in calculating picomoles of vector or insert ends for dephosphorylation, visit the BioMath Calculators (www.promega.com/biomath/).

TSAP Thermosensitive Alkaline Phosphatase (Cat.# M9910) catalyzes the removal of 5' phosphate groups from DNA and is effective on 3' overhangs, 5' overhangs and blunt ends. TSAP is active in all Promega restriction enzyme buffers, a convenience that allows a single, streamlined restriction enzyme digestion-dephosphorylation step. TSAP is also effectively and irreversibly inactivated by heating at 74°C for 15 minutes. Therefore, a DNA cleanup step is not required before ligation.

Additional Resources for TSAP Thermosensitive Alkaline Phosphatase**Technical Bulletins and Manuals**

9PIM991 *TSAP Thermosensitive Alkaline Phosphatase Promega Product Information*
(www.promega.com/tbs/9pim991/9pim991.html)

Promega Publications

eNotes TSAP Thermosensitive Alkaline Phosphatase activity in restriction enzyme buffers from New England Biolabs
(www.promega.com/enotes/applications/ap0080.htm)

PN095 TSAP: A new thermosensitive alkaline phosphatase
(www.promega.com/pnotes/95/14867_03/14867_03.html)

Alkaline Phosphatase, Calf Intestinal (CIAP; Cat.# M1821, M2825), catalyzes the hydrolysis of 5'-phosphate groups from DNA, RNA, and ribo- and deoxyribonucleoside triphosphates. This enzyme is not inactivated by heat but can be denatured and removed by phenol extraction. CIAP is active on 5' overhangs and 5' recessed and blunt ends (Sambrook *et al.* 1989; Seeburg *et al.* 1977; Ullrich *et al.* 1977; Meyerowitz *et al.* 1980; Grosveld *et al.* 1981).

Additional Resources for Alkaline Phosphatase, Calf Intestinal**Technical Bulletins and Manuals**

9PIM182 *Alkaline Phosphatase, Calf Intestinal Promega Product Information*
(www.promega.com/tbs/9pim182/9pim182.html)

Promega Publications

PN077 Technically Speaking: Subcloning plasmid DNA constructs
(www.promega.com/pnotes/77/9028_28/9028_28.html)

Shrimp Alkaline Phosphatase (SAP; Cat.# M8201) catalyzes the dephosphorylation of 5' phosphates from DNA and RNA. Unlike Calf Intestinal Alkaline Phosphatase, SAP is completely and irreversibly inactivated by heating at 65°C for 15 minutes. SAP is active on 5' overhangs and 5' recessed and blunt ends (Sambrook *et al.* 1989).

Additional Resources for Shrimp Alkaline Phosphatase**Technical Bulletins and Manuals**

9PIM820 *Shrimp Alkaline Phosphatase Promega Product Information*
(www.promega.com/tbs/9pim820/9pim820.html)

Restriction Enzymes

Restriction enzymes, also referred to as restriction endonucleases, are enzymes that recognize short, specific (often palindromic) DNA sequences. They cleave double-stranded DNA (dsDNA) at specific sites within or adjacent to their recognition sequences. Most restriction enzymes (REs) will not cut DNA that is methylated on one or both strands of their recognition site, although some require substrate methylation. A complete listing of restriction enzymes available from Promega can be found on the web (www.promega.com/techserv/enzymes/res.htm).

Additional Resources for Restriction Enzymes**Promega Publications**

PN081 Work smarter using isoschizomers and neoschizomers
(www.promega.com/pnotes/81/9939_27/9939_27.html)

PN042 A practical guide to DNA methylation
(www.promega.com/pnotes/42/methv3/methv3.html)

Online Tools

Restriction Enzyme Resource Guide (www.promega.com/guides/re_guide/)

Citations

Capozzo, A.V.E. *et al.* (2003) Development of DNA vaccines against hemolytic-uremic syndrome in a murine model. *Infect. Immun.* **71**, 3971–8.

Researchers used the pGEM®-T Vector System to clone the entire 1.4kb Shiga toxin type 2 gene (Stx2) from *E. coli* O157-H7 C600 (933W). The resultant construct, named pGEMTStx2, was used as a template in PCR to amplify each region of the gene corresponding to Shiga toxin type 2 subunits A and B. Each PCR product was digested with BamHI and EcoRI before ligation into pCDNA 3.1+ (Invitrogen) to create pStx2ΔA and pStx2B. Mice were then

immunized with either one or both of these constructs and another construct expressing murine granulocyte-macrophage colony-stimulating factor. Expression of each subunit in mouse tissue was verified by RT-PCR with specific primers and the AccessQuick™ RT-PCR System.

PubMed Number: 12819084

Jordan, R.A. *et al.* (2003) Production of genetically engineered biotinylated interleukin-2 and its application in a rapid nonradioactive assay for T-cell activation. *Clin. Diagn. Lab. Immunol.* **10**, 339–44.

Researchers used PCR primers with KpnI and HindIII restriction sites to clone human IL-2 from a known rhIL-2 *E. coli* clone containing the PTCGF-11 vector. The PCR product was digested with KpnI and HindIII and cloned into the PinPoint™ Xa-3 Vector. The vector was transformed into competent *E. coli* cells, the cells were induced to express the cloned protein, and the protein was isolated and determined to be biotinylated rhIL-2.

PubMed Number: 12738628

E. Competent Cells

Transforming a newly constructed plasmid into competent *E. coli* cells is the primary method to propagate and select for the clone or clones of interest. Competent bacterial cells are receptive to importing foreign DNA and replicating it. High-quality competent *E. coli* is an integral part of a successful cloning protocol.

JM109 Competent Cells

JM109 Competent Cells (Cat.# L2001) are prepared according to a modified procedure of Hanahan, 1985. These cells are transformed with plasmid DNA via the heat-shock method. JM109 cells (Yanisch-Perron *et al.* 1985) are an ideal host for many molecular biology applications and can be used for α -complementation of β -galactosidase for blue/white screening.

Additional Resources for JM109 Competent Cells

Technical Bulletins and Manuals

TB095 *E. coli* Competent Cells Technical Bulletin
(www.promega.com/tbs/tb095/tb095.html)

Promega Publications

eNotes What are the effects of the bacterial DNA restriction-modification systems on cloning and manipulations of DNA in *E. coli*?
(www.promega.com/enotes/faqspeak/0011/fq0026.htm)

Citations

Tambong, J.T. *et al.* (2006) Oligonucleotide array for identification and detection of *Pythium* species. *Appl. Environ. Microbiol.* **72**, 2691–706.

This study compared detection of *Pythium* species in soil samples by DNA array hybridization and PCR cloning. DNA fragments from three *Pythium* species were amplified from purified total DNA in soil samples, a single 3' A

overhang was added to the resulting PCR product, and the DNA ligated into the pGEM®-T Easy Vector overnight at 4°C. The ligated vector was transformed into JM109 Competent Cells, and 100 colonies were chosen and grown overnight in LB broth. The plasmid DNA was isolated using the Wizard® SV 96 Plasmid DNA Purification System and then sequenced.

PubMed Number: 16597974

Jordan, R.A. *et al.* (2003) Production of genetically engineered biotinylated interleukin-2 and its application in a rapid nonradioactive assay for T-cell activation. *Clin. Diagn. Lab. Immunol.* **10**, 339–44.

Researchers used PCR to subclone human IL-2 from a known rhIL-2 *E. coli* clone into the PinPoint™ Xa-3 Vector. Transformed *E. coli* JM109 clones were then pre-incubated in the presence of 8 μ M biotin for 2 hours before being induced with 100 μ M IPTG for an additional 2 hours. After induction, the cells were collected and resuspended before mechanical lysis with a French press. The lysate was then passed over a SoftLink™ Soft Release Avidin Resin column and the biotinylated rhIL-2 eluted. The resultant purified biotinylated rhIL-2 displayed similar properties and biological activity to native IL-2. Elutants from cells transformed with the PinPoint™ Xa Control Vector produced no biotinylated rhIL-2 and did not display any properties indicating that IL-2 was present.

PubMed Number: 12738628

Single Step (KRX) Competent Cells

Single Step (KRX) Competent Cells (Cat.# L3001, L3002) are not only highly competent and compatible with blue/white screening but can be used for tightly controlled protein expression. KRX incorporates a chromosomal copy of the T7 RNA polymerase gene driven by a rhamnose promoter (*rhaBAD*). T7 RNA polymerase-based systems (Studier and Moffat, 1986) are one of the most widely used protein expression systems by virtue of its well-defined promoter, which is completely independent of *E. coli* RNA polymerase promoters, and its rapid elongation rate, about five times that of *E. coli* RNA polymerases. The *rhaBAD* promoter is subject to catabolite repression by glucose, is activated by addition of rhamnose to the medium, and provides precise control of T7 RNA polymerase abundance and thereby precise control of recombinant protein production.

Additional Resources for Single Step (KRX) Competent Cells

Technical Bulletins and Manuals

TB352 *Single Step (KRX) Competent Cells Technical Bulletin*
(www.promega.com/tbs/tb352/tb352.html)

Promega Publications

PN097	¹⁵ N protein labeling using <i>Escherichia coli</i> strain KRX (www.promega.com/pnotes/97/15377_28/15377_28.html)
PN096	Compatibility of Single Step (KRX) Competent Cells with the MagneGST™ Pull-Down System (www.promega.com/pnotes/96/15080_22/15080_22.html)
PN096	Selenomethionine protein labeling using the <i>Escherichia coli</i> strain KRX (www.promega.com/pnotes/96/15080_24/15080_24.html)
PN094	The Single Step (KRX) Competent Cells: Efficient cloning and high protein yields (www.promega.com/pnotes/94/14410_27/14410_27.html)

HB101 Competent Cells

HB101 Competent Cells (Cat.# L2011) are prepared according to a modified procedure of Hanahan, 1985. HB101 cells (Yanisch-Perron *et al.* 1985) are useful for cloning with vectors that do not require α -complementation for blue/white screening.

Additional Resources for HB101 Competent Cells**Technical Bulletins and Manuals**

TB095	<i>E. coli</i> Competent Cells Technical Bulletin (www.promega.com/tbs/tb095/tb095.html)
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III. PCR Cloning Protocols**A. Amplification, Analysis and PCR Cleanup**

The following protocol is a general procedure to analyze and purify a PCR fragment. Protocols for amplification can be found in the *Protocols and Applications Guide* chapter on PCR Applications. Additional information regarding PCR, analysis and product purification can be found in the following resources:

- *Protocols and Applications Guide* chapter on PCR Applications (www.promega.com/paguide/chap1.htm)
- *Protocols and Applications Guide* chapter on DNA Purification (www.promega.com/paguide/chap9.htm)

Amplification

A basic protocol for amplifying genomic DNA by PCR can be found in the *Protocols and Applications Guide* chapter on PCR Applications (www.promega.com/paguide/chap1.htm), in the section "Example of a PCR Protocol".

Analysis**Materials Required:**

(see Composition of Solutions section)

- aliquot of amplification reaction (usually 5–10 μ l)
 - **Optional:** Blue/Orange Loading Dye, 6X (Cat.# G1881) if GoTaq® Green Reaction Buffer is not used
 - appropriately sized DNA marker
 - appropriate percentage agarose gel (typically 0.8–1.2%; see Table 13.1 for guidelines)
 - gel running buffer (1X TAE or 0.5X TBE)
 - 10mg/ml ethidium bromide
1. Analyze 5–10 μ l of the amplification reaction using agarose gel electrophoresis. Include at least one lane containing a DNA size marker to determine if the PCR products are of the correct size. The products should be readily visible by UV transillumination of the ethidium bromide-stained gel (50 μ g/ml final concentration in the agarose).

2. Store reaction products at –20°C until needed.

Table 13.1. Gel Percentages: Resolution of Linear DNA on Agarose Gels.

Recommended % Agarose	Optimum Resolution for Linear DNA
0.5	1,000–30,000bp
0.7	800–12,000bp
1.0	500–10,000bp
1.2	400–7,000bp
1.5	200–3,000bp
2.0	50–2,000bp

If there are primer dimers or at least two PCR products present, the band of interest will need to be removed and purified (see the next section, PCR Cleanup, for more information). To minimize the number of extraneous amplimers, the PCR conditions may need to be optimized. For suggestions on troubleshooting PCR, visit the *Protocols and Applications Guide* chapter on PCR Applications (www.promega.com/paguide/chap1.htm).

PCR Cleanup

Once you have determined the PCR was successful, you can purify the desired product from the rest of the reaction components. This can be accomplished using a number of procedures including the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9280, A9281, A9282) or agarose gel isolation. Alternatively, you can use a portion of the amplification reaction directly in a ligation. However, the presence of primer dimers or other amplimers present can cause false-positive reactions or yield an incorrect clone (see Figure 13.8). If the reaction is clean (i.e., a single band seen on an analytical gel) and is a minimum of 100bp in size, you can use the Wizard® SV Gel and PCR Clean-Up System to directly purify the PCR product [see the *Protocols and Applications Guide* chapter on DNA Purification (www.promega.com/paguide/chap9.htm) for product protocol].

If there are other bands or a large primer-dimer band present, we recommend gel electrophoresis to separate the products so the desired band can be excised. The DNA can be recovered using an agarose-digesting enzyme such as AgarACE™ Enzyme (Cat.# M1741, M1743) or by melting the excised agarose and using the Wizard® SV Gel and PCR Clean-Up System.

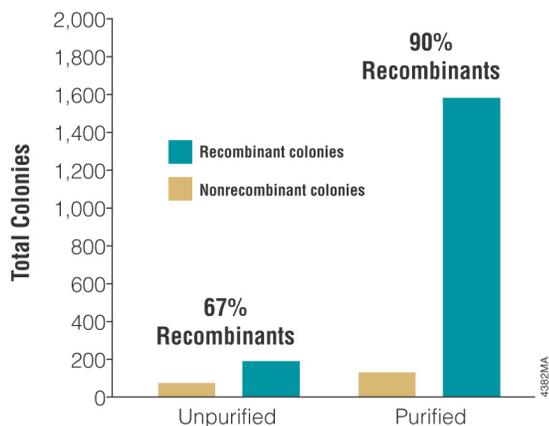


Figure 13.8. Purification of PCR products enhance cloning success. A 500bp PCR product was purified with Wizard® SV Gel and PCR Clean-Up System and cloned into the pGEM®-T Easy Vector. Both the percent recombinants and total number of colonies increase with a pure PCR product.

Optional: A-Tailing Reaction for Blunt-Ended Products:

If a proofreading DNA polymerase was used for amplification and you want to clone into a T vector, an adenosine residue must be added onto the PCR product. This can be accomplished by incubating the DNA fragment with dATP and a nonproofreading DNA polymerase, which will add a single 3' A residue. Blunt DNA fragments resulting from restriction enzyme digestion can also be cloned into T vector after adding an adenosine residue.

Materials Required:

- blunt-ended product (from PCR or restriction enzyme digestion), purified
- GoTaq® Flexi DNA Polymerase
- 25mM MgCl₂
- 5X GoTaq® Colorless or Green Reaction Buffer
- 1mM dATP (Cat.# U1205; diluted 1:100 in nuclease-free water)

1. Set up the following reaction in a thin-walled PCR tube:

Purified DNA fragment	1–4.4µl
5X GoTaq® Reaction Buffer (Colorless or Green)	2µl
1mM dATP (0.2mM final concentration)	2µl
GoTaq® Flexi DNA Polymerase (5u/µl)	1µl
25mM MgCl ₂ (1.5mM final concentration)	0.6µl
Nuclease-free water to	10µl

2. Incubate at 70°C for 15–30 minutes in a water bath or thermal cycler. After the tailing reaction is finished, 1–2µl can be used without further cleanup for ligation with pGEM®-T or pGEM®-T Easy Vector Systems.

B. Ligation and Transformation

Materials Required:

(see Composition of Solutions section)

- PCR product (has an A overhang and optional purification) or blunt DNA fragment with an A residue added
- pGEM®-T Easy Vector System (Cat.# A1380) or pGEM®-T Easy Vector System (Cat.# A3610) Both systems include T4 DNA ligase and chemically competent JM109 cells.
- Nuclease-Free Water (Cat.# P1193)
- **Optional:** 4°C water bath
- LB-Ampicillin plates containing X-Gal and IPTG
- high-efficiency competent cells [e.g., JM109 Competent Cells (Cat.# L2001) or Single Step KRX Competent Cells (Cat.# L3001)]
- SOC medium
- 42°C water bath
- ice

Vector:Insert Ratio

After the insert DNA has been prepared for ligation, estimate the concentration by comparing the staining intensity with that of DNA molecular weight standards of known concentrations on an ethidium bromide-stained agarose gel. If the vector DNA concentration is unknown, estimate the vector concentration by the same method. Test various vector:insert DNA ratios to determine the optimal ratio for a particular vector and insert. In most cases, either a 1:1 or a 1:3 molar ratio of vector:insert works well. The following example illustrates the calculation of the amount of insert required at a specific molar ratio of vector.

$$[\text{ng of vector} \times \text{size of insert (in kb)}] \div \text{size of vector (in kb)} \times \text{molar amount of (insert} \div \text{vector)} = \text{ng of insert}$$

Example:

How much 500bp insert DNA needs to be added to 100ng of 3.0kb vector in a ligation reaction for a desired vector:insert ratio of 1:3?

$$[(100\text{ng vector} \times 0.5\text{kb insert}) \div 3.0\text{kb vector}] \times (3 \div 1) = 50\text{ng insert}$$

Ligation

- Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect contents at the bottom of the tube.
- Set up ligation reactions as described below. Vortex the 2X Rapid Ligation Buffer vigorously before each use. Use 0.5ml tubes known to have low DNA-binding capacity.

Reagents	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer	5µl	5µl	5µl
pGEM®-T or pGEM®-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	Xµl	–	–
Control Insert DNA	–	2µl	–
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
Nuclease-Free Water to a final volume of	10µl	10µl	10µl

- Mix the reactions by pipetting. Incubate the reactions for 1 hour at room temperature. Alternatively, incubate the reactions overnight at 4°C for the maximum number of transformants.

Transformation

- Prepare LB/ampicillin/IPTG/X-Gal plates (see Composition of Solutions).
- Centrifuge the ligation reactions briefly. Add 2µl of each ligation reaction to a sterile 1.5 ml microcentrifuge tube on ice. Prepare a transformation control tube with 0.1ng of an uncut plasmid. pGEM®-T Vectors are **not** suitable for the transformation control as they are linear, not circular.

Note: In our experience, the use of larger (17 × 100mm) polypropylene tubes (e.g., BD Falcon Cat.# 352059) has been observed to increase transformation efficiency. Tubes from some manufacturers bind DNA, thereby decreasing the colony number, and should be avoided.

- Place the high-efficiency JM109 Competent Cells in an ice bath until just thawed (5 minutes). Mix cells by gently flicking the tube.
- Carefully transfer 50µl of cells to the ligation reaction tubes prepared in Step 2. Use 100µl of cells for the transformation control tube. Gently flick the tubes, and incubate on ice for 20 minutes.

- Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C. **DO NOT SHAKE**. Immediately return the tubes to ice for 2 minutes.
- Add 950µl of room temperature SOC medium to the ligation reaction transformations and 900µl to the transformation control tube. Incubate for 1.5 hours at 37°C with shaking (~150rpm).
- Plate 100µl of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC is recommended prior to plating.
- Incubate plates overnight at 37°C. Select white colonies.

Calculation of Transformation Efficiency

For every transformation with competent cells, we recommend performing a transformation control experiment using a known quantity of a purified, supercoiled plasmid DNA (e.g., pGEM®-3Z Vector, Cat.# P2151). Calculate the transformation efficiency as described below.

transformation efficiency (cfu/µg) = (cfu on control plate ÷ ng of supercoiled vector plated) × (10³ng/µg) × final dilution factor

cfu = colony forming units

Example:

A 100µl aliquot of competent cells is transformed with 1ng of supercoiled pGEM®-3Z Vector DNA. Ten microliters of the transformation reaction (0.1ng total DNA) is added to 990µl of SOC medium (1:100 dilution). Of that volume (1,000µl), a 100µl aliquot is plated (1:1,000 final dilution), and 100 colonies are obtained on the plate. What is the transformation efficiency?

(100cfu ÷ 0.1ng of supercoiled vector plated) × (10³ng/µg) × 1,000 = 1 × 10⁹ cfu/µg

C. Screening of Transformants

To determine if the insert was successfully cloned, there are two methods for screening the transformed bacteria: colony PCR or plasmid miniprep followed by restriction enzyme digestion.

Successful cloning of an insert into the pGEM®-T and pGEM®-T Easy Vectors disrupts the coding sequence of the β-galactosidase α peptide. Recombinant clones can usually be identified by color screening on X-Gal/IPTG indicator plates following transformation of competent cells. However, the characteristics of PCR products cloned into these T vectors can significantly affect the ratio of blue:white colonies obtained. Clones that contain PCR products, in most cases, produce white colonies, but blue colonies can result from PCR fragments that are cloned in-frame with the *lacZ* gene. Such fragments are usually a multiple of 3 base pairs long (including the 3'-A overhangs)

and do not contain in-frame stop codons. There have been reports of DNA fragments of up to 2kb that have been cloned in-frame and have produced blue colonies.

Even if your PCR product is not a multiple of 3 bases long, the amplification process can introduce mutations (e.g., deletions or point mutations) that may result in blue colonies when competent cells are transformed with the fragment inserted into the pGEM®-T or pGEM®-T Easy Vectors.

Screening of recombinant clones using restriction enzymes is more time consuming than that of colony PCR. It involves isolating the plasmid DNA from liquid cultures of individual *E. coli* colonies, performing the enzyme digestion and determining if the insert is of the correct size. To learn more about screening by restriction enzyme digestion, visit the Subcloning Notebook (www.promega.com/guides/subcloning_guide/).

The following protocol is for colony PCR analysis of transformants.

Materials Required:

(see Composition of Solutions section)

- plate of colonies containing the recombinant plasmid
- toothpicks or sterile bacterial loop
- LB Broth (optional)
- upstream screening primer
- downstream screening primer
- GoTaq® Flexi DNA Polymerase
- 5X Green GoTaq® Flexi Buffer
- 25mM MgCl₂
- Nuclease-Free Water (Cat.# P1193)
- Nuclease-Free Light Mineral Oil (e.g., Sigma Cat.# M5904 or Promega Cat.# DY1151) if you are using a thermal cycler without a heated lid; do **not** autoclave
- dNTP Mix (10mM of each dNTP; Cat.# U1511, U1515)

1. Pick a well-isolated colony using either a sterile toothpick or a flamed and cooled bacterial loop, and transfer to 50µl of sterile water. Part of the colony may be transferred to LB medium containing the appropriate antibiotic for overnight culture and plasmid miniprep, if desired.
2. Boil for 10 minutes to break open the bacterial cell wall and release the DNA.
3. Centrifuge at 16,000 × *g* for 5 minutes to pellet the cell debris.
4. Use 5µl of the supernatant in a 50µl amplification reaction (see Table 13.2 for a sample reaction).

Table 13.2. Colony PCR using GoTaq® Flexi DNA Polymerase.

Components	Volume	Final Concentration
Nuclease-Free Water (to a final volume of 50µl)	Xµl	
5X Reaction Buffer	10µl	1X
dNTP mix (10mM of each dNTP)	1µl	0.2mM each
GoTaq® DNA polymerase (5u/µl)	0.25µl	0.025u/µl
25mM MgCl ₂	3µl	1.5mM
Downstream screening primer	50pmol ¹	1µM
Upstream screening primer	50pmol ¹	1µM
Boiled colony supernatant	5µl	

¹A general formula for calculating the number of nanograms of primer equivalent to 50pmol is: 50pmol = 16.3ng × *b*; where *b* is the number of bases in the primer.

5. Amplify the target DNA using cycling conditions appropriate for your screening primers and size of amplicon (see Table 13.3 for suggestions). Place reactions in a thermal cycler that has been preheated to 94°C.

Table 13.3. Suggested Amplification Conditions.

Step	Temperature	Time (minutes)	Cycles
Initial denaturation	94°C	2	1
Denaturation	94°C	0.5–1.0	25–35
Annealing	42–65°C ¹	0.5–1.0	
Extension	72°C	1 minute/ kilobase ²	
Final extension	72°C	5	1
Soak/Hold	4°C	Indefinite	1

¹Annealing temperature should be optimized for each primer set based on the primer melting temperature (*T_m*). To calculate melting temperatures of primers in GoTaq® Reaction Buffer, go to BioMath Calculators (www.promega.com/biomath/).

²The extension time should be at least 1 minute per kilobase of target. Typically, amplicons smaller than 1kb use a 1-minute extension.

6. Remove an aliquot of the completed PCR and analyze by agarose gel electrophoresis for the appropriate size product, which indicates the correct insert is present in the clone.

7. **Recommended:** Culture the appropriate colony or colonies to create a glycerol stock of your recombinant plasmid or plasmids, and purify the plasmids in larger quantities [e.g., PureYield™ Plasmid Systems (Cat.# A2492, A2495)] for downstream applications or further manipulation.

D. Subcloning

Classic subcloning involves restriction digestion of the plasmid of interest to remove the desired DNA fragment followed by ligation into a second vector with compatible ends. PCR can be used for subcloning as well, using the A overhangs to clone into another T vector. Alternatively, the PCR product can be generated using primers with restriction enzyme sites, cut with the appropriate enzymes, then cloned into a vector with compatible ends. Further information on subcloning can be found by visiting the Subcloning Notebook (www.promega.com/guides/subcloning_guide/).

IV. Cloning Protocol for the Flexi® Vector Systems

A. PCR Primer Design, Amplification and Cleanup

The desired protein-coding region must be amplified by PCR before being cloned into the Flexi® Vectors (Figures 13.6 and 13.7). The optimal conditions for amplifying the protein-coding region will depend on the DNA template, DNA polymerase, PCR primers and other reaction parameters. We recommend following the protocol provided with the DNA polymerase to generate the PCR product. For protein-coding regions less than 700bp, consider using GoTaq® DNA Polymerase to amplify your protein-coding region. For regions greater than 700bp, we recommend the use of a high-fidelity DNA polymerase, such as *Pfu* DNA polymerase. To facilitate cloning, the PCR primers used to amplify the protein-coding region must append an SgfI site and a PmeI site to the PCR product. To append these sites, incorporate an SgfI site in your amino-terminal PCR primer and a PmeI site in your carboxy-terminal PCR primer. Transfer of protein-coding regions into N-terminal fusion vectors results in translational readthrough of the SgfI site, which encodes the peptide sequence Ala-Ile-Ala. The PmeI site is placed at the carboxy terminus, appending a single valine residue to the last amino acid of the protein-coding region. The valine codon, GTT, is immediately followed by an ochre stop codon, TAA. Primer design guidelines are provided in Technical Manual #TM254 (www.promega.com/tbs/tm254/tm254.html) and the Flexi® Vector Primer Design Tool (www.promega.com/techserv/tools/flexivector/).

To cleanup the PCR product, refer to Amplification, Analysis and PCR Cleanup.

B. Restriction Digest of PCR Product and Acceptor Flexi® Vector

Digestion reactions for the PCR product and the acceptor Flexi® Vector can be performed concurrently.

Note: Do not use C-terminal Flexi® Vectors, which have names starting with "pFC", as acceptors for PCR products if you plan to transfer the protein-coding region to a different Flexi® Vector in the future. C-terminal Flexi® Vectors lack PmeI sites and cannot serve as donors for other Flexi® Vectors.

Materials Required:

- Flexi® System, Entry/Transfer (Cat.# C8640)
 - chosen acceptor Flexi® Vector
 - purified PCR product
1. Thaw the 5X Flexi® Digest Buffer, the acceptor Flexi® Vector and Nuclease- Free Water, and store on ice. Vortex the 5X Flexi® Digest Buffer and the acceptor Flexi® Vector before use.
 2. Assemble the following reaction components to cut the PCR product with SgfI and PmeI.

Component	Volume
5X Flexi® Digest Buffer	4µl
Purified PCR product (up to 500ng)	Xµl
Flexi® Enzyme Blend (SgfI and PmeI)	4µl
Nuclease-Free Water to a final volume of	20µl

3. Assemble the following reaction components to cut the acceptor Flexi® Vector with SgfI and PmeI.

Component	Volume
Nuclease-Free Water	12µl
5X Flexi® Digest Buffer	4µl
Acceptor Flexi® Vector (200ng)	2µl
Flexi® Enzyme Blend (SgfI and PmeI)	2µl
Final Volume of	20µl

Note: Take care when pipetting solutions that contain glycerol, such as the Flexi® Enzyme Blend, because small volumes are difficult to pipet accurately.

4. Incubate both reactions (Steps 2 and 3) at 37°C for 30 minutes.
5. Heat the reaction with the Flexi® Vector (Step 3) at 65°C for 20 minutes to inactivate the restriction enzymes. Store on ice until the PCR product and vector are ligated in Ligation of PCR Product and Acceptor Flexi® Vector.
6. Directly purify the digested PCR product using the Wizard® SV Gel and PCR Cleanup System (Cat.# A9281).

C. Ligation of PCR Product and Acceptor Flexi® Vector

1. Assemble the following reaction components:

Component	Volume
2X Flexi® Ligase Buffer	10µl
Acceptor Flexi® Vector from Restriction Digest of PCR Product and Acceptor Flexi® Vector, Step 5 (50ng)	2µl
PCR product (approximately 100ng)	Xµl
T4 DNA Ligase (HC; 20u/µl)	1µl
Nuclease-Free Water to a final volume of	20µl

2. Incubate at room temperature for 1 hour.

Note: The 2X Flexi® Ligase Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single-use aliquots of the buffer.

D. Screening Clones with SgfI and PmeI

Note: Do not use this protocol to screen for inserts in C-terminal Flexi® Vectors, which have names starting with “pFC”, since these clones lack PmeI sites.

Materials Required:

(see Composition of Solutions section)

- 10X Flexi® Enzyme Blend (SgfI and PmeI; Cat.# R1851, R1852)
- Blue/Orange 6X Loading Dye (Cat.# G1881)
- plasmid isolated from colonies

1. Chill reaction components and reaction tubes or plates.
2. Prepare a master mix by combining the components listed below. Increase volumes proportionately depending on the number of reactions.

Component	Volume per Reaction
Nuclease-Free Water	10.5µl
5X Flexi® Digest Buffer	4µl
10X Flexi® Enzyme Blend (SgfI and PmeI)	2µl
Final Volume of	15µl

3. Add 15µl of master mix to 5µl (200–500ng) of plasmid DNA. Mix thoroughly by pipetting.
4. Incubate for 2 hours at 37°C.
5. Add 5µl of loading dye (Blue/Orange Loading Dye, 6X, Cat.# G1881). Incubate at 65°C for 10 minutes.
6. Load 20µl of the reaction onto a 1% agarose gel and separate fragments by electrophoresis. Visualize the fragments by ethidium bromide staining.

E. Transfer of Protein-Coding Region

Transfer refers to moving your protein-coding region from one Flexi® Vector (donor) to another Flexi® Vector (acceptor). Choose an appropriate acceptor vector with the desired expression and tag options and a different antibiotic resistance marker than the donor because antibiotic selection is the basis for selecting the desired clone (Figures 13.5, 13.9 and 13.10).

There are two basic categories of Flexi® Vectors, those containing SgfI and PmeI sites and expressing either a native (untagged) protein or an N-terminal-tagged protein, and those containing SgfI and EcoICRI sites and expressing a C-terminal-tagged protein. Flexi® Vectors for expressing C-terminal-tagged proteins act only as acceptors, never as donor vectors. To transfer protein-coding regions between Flexi® Vectors expressing native protein or an N-terminal-tagged protein, the donor and acceptor vectors are digested with SgfI and PmeI simultaneously, prior to ligation of the insert, transformation and selection of the cells (Figure 13.9).

To create a C-terminal-tagged protein, the donor plasmid expressing native protein or an N-terminal-tagged protein is digested with SgfI and PmeI. Because EcoICRI cuts frequently in protein-coding regions, the acceptor plasmid containing the C-terminal tag is digested with SgfI and EcoICRI in a separate reaction. The two separate digests are combined for ligation of the insert, transformation and selection of the cells (Figure 13.10).

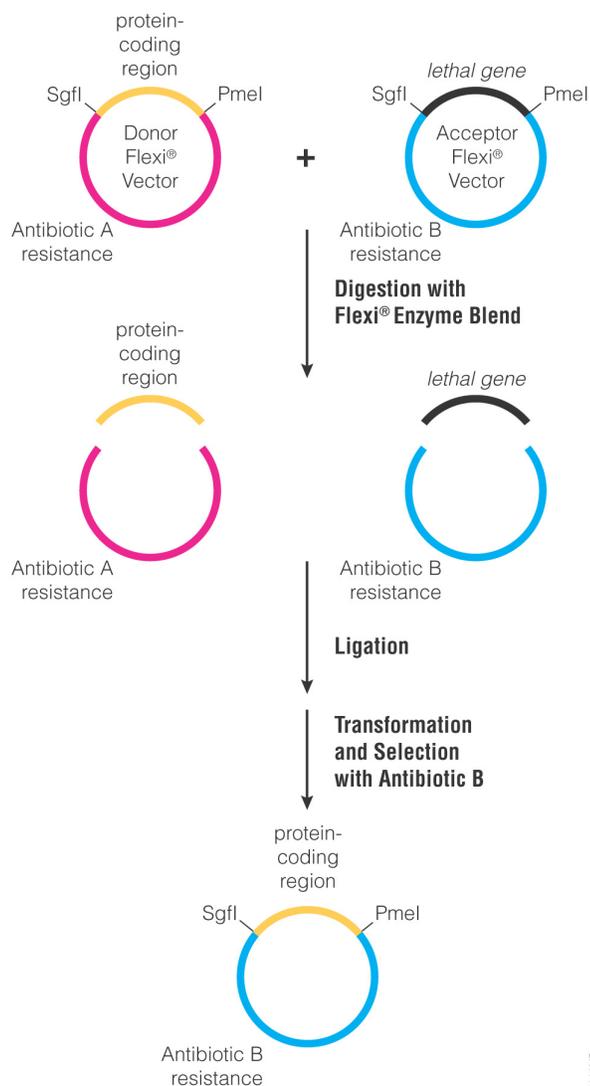


Figure 13.9. Transfer of a protein-coding region between N-terminal or native Flexi® Vectors. The donor Flexi® Vector containing the protein-coding region is mixed with an acceptor Flexi® Vector that has a different antibiotic resistance. The two plasmids are digested with SgfI and PmeI, and the mixture is ligated and transformed into *E. coli*. The cells are plated on the appropriate selective media for the acceptor Flexi® Vector. Protein-coding regions transferred into N-terminal fusion vectors allow translational readthrough of the SgfI site, which encodes the peptide sequence Ala-Ile-Ala.

Transfer of Protein-Coding Regions Between Flexi® Vectors Expressing Native or N-Terminal Fusion Proteins

Materials Required:

- Flexi® System, Transfer (Cat.# C8820)
 - competent *E. coli* cells [e.g., JM109 Competent Cells (Cat.# L2001) or Single Step (KRX) Competent Cells (Cat.# L3001)]
 - LB plates supplemented with the appropriate antibiotic at the appropriate concentration used (see Composition of Solutions and Figure 13.5)
1. Use the Wizard® Plus SV Minipreps DNA Purification System (Cat.# A1330), Wizard® SV 96 Plasmid DNA Purification System (Cat.# A2250) or a similar method to prepare the donor Flexi® Vector DNA [see the *Protocols and Applications Guide* chapter on DNA Purification (www.promega.com/paguide/chap9.htm)]. Adjust the volume, so the final DNA concentration is 50–100ng/μl.

2. Assemble the following reaction components to cut the Flexi® Vectors:

Component	Volume
5X Flexi® Digest Buffer	4μl
Acceptor Flexi® Vector (100ng)	1μl
Donor Flexi® Vector (100ng)	Xμl
Flexi® Enzyme Blend (SgfI and PmeI)	2μl
Nuclease-Free Water to a final volume of	20μl

Note: Take care when pipetting solutions that contain glycerol, such as the Flexi® Enzyme Blend, because small volumes are difficult to pipet accurately.

3. Incubate at 37°C for 15–30 minutes.
4. Heat the reaction at 65°C for 20 minutes to inactivate the restriction enzymes. Store the reaction on ice while assembling the ligation reaction in Step 5.
5. Assemble the following ligation reaction components:

Component	Volume
2X Flexi® Ligase Buffer	10μl
Digested DNA from Step 4 (100ng total)	10μl
T4 DNA Ligase (HC; 20u/μl)	1μl
Final volume of	21μl

6. Incubate at room temperature for 1 hour.
7. Transform the ligation reaction into high-efficiency, *E. coli* competent cells ($\geq 1 \times 10^8$ cfu/μg DNA). If you are using competent cells other than high-efficiency JM109 Competent Cells (Cat.# L2001) purchased from Promega, it is important to follow the appropriate transformation protocol. The recommended transformation protocol for our high-efficiency JM109 Competent Cells is provided in Ligation and Transformation. Selection for transformants should be on LB plates supplemented with 100μg/ml ampicillin

for Flexi® Vectors with the letter "A" in the name or 30µg/ml kanamycin for Flexi® Vectors with the letter "K" in the name, as appropriate for the acceptor Flexi® Vector. See Figure 13.5 for a list of antibiotic-resistance genes carried on the various vectors.

- Screen at least 4 colonies for each protein-coding region. Digest the plasmid to ensure that SgfI and PmeI cleave their recognition sites flanking the protein-coding region, so the insert can be cloned into other Flexi® Vectors.

Screen at least 8 colonies for each protein-coding region transferred to the pF3A WG (BYDV) or pF3K WG (BYDV) Flexi® Vectors. Lower transfer frequencies with these vectors are due to a higher background of plasmid backbone heterodimers between the WG (BYDV) Vectors and other Flexi® Vectors. Other Flexi® Vectors share common regions flanking the SgfI and PmeI sites, such that plasmid backbone dimers are unstable (Yoshimura *et al.* 1986). The pF3A and pF3K WG (BYDV) Flexi® Vectors lack these common flanking regions because of the inclusion of the BYDV translation-enhancing sequences.

If you are using the pF3A WG (BYDV) or pF3K WG (BYDV) Flexi® Vectors, the number of minipreps performed can be reduced by prescreening colonies to identify those harboring plasmid backbone heterodimers. Colonies containing such heterodimers can be identified by their ability to grow on both antibiotics. Pick individual colonies and restreak on an ampicillin plate and a kanamycin plate, or inoculate two culture plates for overnight growth in media: one with ampicillin and the other with kanamycin. Colonies containing the clone of interest will grow only in the antibiotic associated with the acceptor plasmid.

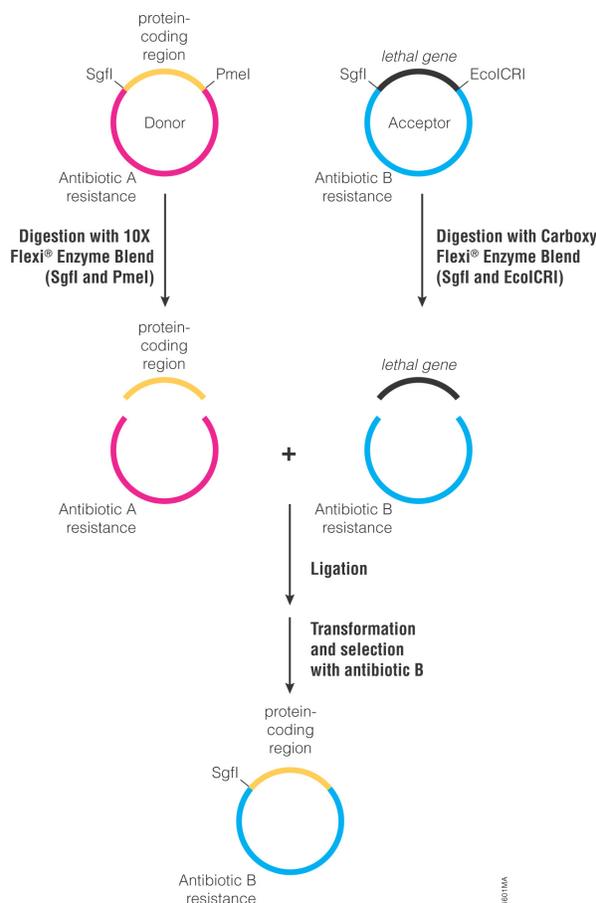


Figure 13.10. Transfer of a protein coding region into the C-terminal Flexi® Vectors. The donor Flexi® Vector containing the protein-coding region is digested with SgfI and PmeI. The acceptor Flexi® Vector, which has a different antibiotic resistance, is digested with SgfI and EcoICRI in a separate reaction. The two digested plasmids are combined, the mixture is ligated and transformed into *E. coli*, and cells are plated on the appropriate selective media for the acceptor Flexi® Vector. When the blunt ends of PmeI and EcoICRI are joined, an in-frame Ser codon, which is appended to the downstream C-terminal protein-coding region contained on the Flexi® Vector backbone, is created.

Transfer of Protein-Coding Regions from Native or N-Terminal Flexi® Vectors to C-Terminal Flexi® Vectors

Use the C-terminal Flexi® Vectors, which have names starting with "pFC", as acceptors but not as donors since they lack PmeI sites.

Materials Required:

- Carboxy Flexi® System, Transfer (Cat.# C9320)
- competent *E. coli* cells [e.g., JM109 Competent Cells (Cat.# L2001) or Single Step (KRX) Competent Cells (Cat.# L3001)]
- LB plates supplemented with the appropriate antibiotic at the appropriate concentration used (see Composition of Solutions and Figure 13.5)

1. Use the Wizard® *Plus* SV Minipreps DNA Purification System (Cat.# A1330), Wizard® SV 96 Plasmid DNA Purification System (Cat.# A2250) or a similar method to prepare the donor Flexi® Vector DNA [see the *Protocols and Applications Guide* chapter on DNA Purification (www.promega.com/paguide/chap9.htm)]. Adjust the volume, so the final DNA concentration is 50–100ng/μl.

2. Assemble the following reaction components to cut the donor Flexi® Vector:

Component	Volume
5X Flexi® Digest Buffer	2μl
Donor Flexi® Vector (100ng)	Xμl
Flexi® Enzyme Blend (SgfI and PmeI)	1μl
Nuclease-Free Water to a final volume of	10μl

Note: Take care when pipetting solutions that contain glycerol, such as the Flexi® Enzyme Blend, because small volumes are difficult to pipet accurately.

3. In a separate tube, assemble the following reaction components to cut the acceptor C-terminal Flexi® Vector:

Component	Volume
Nuclease-Free Water	6μl
5X Flexi® Digest Buffer	2μl
Acceptor C-Terminal Flexi® Vector (100ng) ¹	1μl
Carboxy Flexi® Enzyme Blend (SgfI and EcoICRI)	1μl
Final volume	10μl

¹ Acceptor C-terminal Flexi® Vectors will have names starting with "pFC".

4. Incubate both reactions at 37°C for 15–30 minutes.
5. Heat both reactions at 65°C for 20 minutes to inactivate the restriction enzymes. Store the reactions on ice while assembling the ligation reaction in Step 6.
6. Assemble the following ligation reaction components:

Component	Volume
2X Flexi® Ligase Buffer	10μl
Digested donor Flexi® Vector prepared in Step 2 (approximately 50ng)	5μl
Digested acceptor C-terminal Flexi® Vector prepared in Step 3 (50ng)	5μl
T4 DNA Ligase (HC; 20u/μl)	1μl
Nuclease-Free Water to a final volume of	21μl

7. Incubate at room temperature for 1 hour.

8. Transform the ligation reaction into high-efficiency, *E. coli* competent cells ($\geq 1 \times 10^8$ cfu/μg DNA). If you are using competent cells other than high-efficiency JM109 Competent Cells (Cat.# L2001) purchased from Promega, it is important to follow the appropriate transformation protocol. The recommended transformation protocol for our high-efficiency JM109 Competent Cells is provided in Ligation and Transformation. Selection for transformants should be on LB plates supplemented with 100μg/ml ampicillin for Flexi® Vectors with the letter "A" in the name or 30μg/ml kanamycin for Flexi® Vectors with the letter "K" in the name, as appropriate for the acceptor Flexi® Vector. See Figure 13.5 for a list of antibiotic-resistance genes carried on the various vectors.
9. Screen at least 8 colonies for each protein-coding region. Successful plasmid constructs will not cut with PmeI but will cut with SgfI. Lower transfer frequencies are due to a higher background of plasmid backbone heterodimers between the C-terminal Flexi® Vectors and other Flexi® Vectors. Other Flexi® Vectors share common regions flanking the SgfI and PmeI sites, such that plasmid backbone dimers are unstable (Yoshimura *et al.* 1986). The C-terminal Flexi® Vectors may lack these common flanking regions because of the inclusion of the protein fusion tag sequence.

V. Supplemental Cloning Techniques

A. Converting a 5' Overhang to a Blunt End

Materials Required:

- Nuclease-Free Water (Cat.# P1193)
- DNA Polymerase I Large (Klenow) Fragment and 10X Reaction Buffer (Cat.# M2201) or T4 DNA Polymerase (Cat.# M4211)
- T4 DNA Polymerase 10X buffer (optional)
- Bovine Serum Albumin (BSA), Acetylated, 1mg/ml (Cat.# R9461)
- dNTPs, 100mM (Cat.# U1240)

Both Klenow (DNA Polymerase I Large Fragment) and T4 DNA Polymerase can be used to fill 5'-protruding ends with deoxynucleotide triphosphates (dNTPs). Properties of these enzymes are discussed in Anderson *et al.* 1980 and Challberg and Englund, 1980.

Klenow Polymerase Method

For optimal activity, use the Klenow 10X Buffer supplied with the enzyme. DNA Polymerase I Large (Klenow) Fragment is also active in many restriction enzyme buffers, and some users may choose to perform the fill-in reaction directly in the restriction buffer.

1. Following the restriction enzyme digestion that generated the 5'-protruding ends, the DNA can be purified rapidly using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281) or other DNA

purification system. Alternatively, the DNA can be extracted with phenol:chloroform, ethanol precipitated and rehydrated for use in the conversion reaction.

- Proceed with one of the following for the fill-in reaction (total reaction volume can be between 10–100 μ l.):

For DNA purified over a column: The optimal reaction conditions for filling in are: 1X Klenow Reaction Buffer [50mM Tris- HCl (pH 7.2), 10mM MgSO₄, 0.1mM DTT], 40 μ M of each dNTP, 20 μ g/ml acetylated BSA and 1 unit of Klenow Fragment per microgram of DNA.

For digested DNA in restriction enzyme buffer: Klenow Polymerase is also partially active in many restriction enzyme buffers (such as our 4-CORE® Buffers), and the fill-in reaction may be performed directly in the restriction enzyme buffer supplemented with 40 μ M of each dNTP, thereby eliminating the clean-up step. Add 1 unit of Klenow Polymerase per microgram of DNA.

For ethanol-precipitated DNA: Resuspend DNA in Klenow 1X Buffer containing 40 μ M of each dNTP and 20 μ g/ml of Acetylated Bovine Serum Albumin (BSA). Add 1 unit of Klenow Polymerase per microgram of DNA.

- Incubate the reaction at room temperature for 10 minutes.
- Stop the reaction by heating at 75°C for 10 minutes.

T4 DNA Polymerase Method

Prepare the DNA as described for the Klenow Polymerase method. Like Klenow Fragment, T4 DNA Polymerase functions well in many restriction enzyme buffers. Add 5 units of T4 DNA Polymerase per microgram of DNA, 100 μ M of each dNTP and 0.1mg/ml Acetylated BSA. The recommended reaction buffer for T4 DNA Polymerase is 1X T4 DNA Polymerase Buffer. Incubate the reaction at 37°C for 5 minutes. Stop the reaction by heating at 75°C for 10 minutes or by adding 2 μ l of 0.5M EDTA.

B. Converting a 3' Overhang to a Blunt End

Materials Required:

- Nuclease-Free Water (Cat.# P1193)
- Bovine Serum Albumin (BSA), Acetylated, 1mg/ml (Cat.# R9461)
- dNTPs, 100mM (Cat.# U1240)
- T4 DNA Polymerase (Cat.# M4211)
- T4 DNA Polymerase 10X buffer (optional)

Note: T4 DNA Polymerase has a 3'→5' exonuclease activity that will, in the presence of excess dNTPs, convert a 3'-protruding end to a blunt end (Burd and Wells, 1974).

- Following the restriction enzyme digestion that generates 3'-protruding ends, leave the DNA in restriction enzyme buffer, exchange the buffer for 1X T4 DNA Polymerase Buffer or gel purify the desired fragment (see the PCR Cleanup section of Amplification, Analysis and PCR Cleanup).

- Add 5 units of T4 DNA Polymerase per microgram of DNA and 100 μ M of each dNTP.
- Incubate at 37°C for 5 minutes.
Note: With high concentrations of dNTPs (100 μ M), degradation of the DNA will stop at duplex DNA; however, if the dNTP supply is exhausted, the very active exonuclease activity (200 times more active than that of DNA polymerase I) will degrade the double-stranded DNA (Sambrook *et al.* 1989).
- Stop the reaction by heating at 75°C for 10 minutes or by adding 2 μ l of 0.5M EDTA.

C. Dephosphorylation of 5' Ends

If the ends of the prepared vector are identical (e.g., following a single digestion), it is advantageous to treat the vector with TSAP Thermosensitive Alkaline Phosphatase (Cat.# M9910) to remove the phosphate groups from the 5' ends to prevent self-ligation of the vector (Sambrook *et al.* 1989). For linear vectors with unique 5' ends, TSAP treatment is not necessary.

Note: Since TSAP is active in all Promega restriction enzyme buffers, the vector DNA easily can be restriction digested and dephosphorylated at the same time. The following protocol reflects this streamlined method. See the *TSAP Thermosensitive Alkaline Phosphatase Product Information* #9PIM991 (www.promega.com/tbs/9pim991/9pim991.html) for alternative protocols.

- As a general guideline, for reactions containing up to 1 μ g of DNA, add 15 units of restriction enzyme and the amount of TSAP listed below to the vector DNA in a total reaction volume of 20–50 μ l. Set up the reaction in the appropriate 1X Promega restriction enzyme reaction buffer.

**Amount of TSAP
for Reactions
Containing \leq 1 μ g
DNA**

Reaction Buffer

Promega 10X Reaction Buffers A–L (except F)	1 μ l
Promega 10X Reaction Buffer F	2 μ l
MULTI-CORE™ 10X Buffer	1 μ l

- Incubate the reaction at 37°C for 15 minutes. This is a sufficient amount of time to digest and dephosphorylate all vector DNA overhang types (3', 5' or blunt).
- Heat-inactivate the TSAP and the restriction enzyme by incubating the reaction at 74°C for 15 minutes.

Note: Not all restriction enzymes can be heat-inactivated. If the restriction enzyme cannot be heat-inactivated, clean up the digest using the Wizard® SV Gel and PCR Clean-Up System (Cat.# A9281).

- Briefly centrifuge the reaction, and use approximately 40ng of dephosphorylated vector in a ligation reaction containing DNA insert, 1X Rapid Ligation Buffer, and 2µl (6 units) of T4 DNA Ligase (LigaFast™ Rapid DNA Ligation System, Cat.# M8221). Incubate ligation reactions containing vector with 5' or 3' overhangs at 25°C for 5 minutes. Incubate ligation reactions containing vector with blunt ends at 25°C for 15 minutes.

Note: Optimal vector-to-insert ratios may need to be determined. We recommend using a 1:2 molar ratio of vector to insert DNA as a starting point. See the *LigaFast™ Rapid DNA Ligation System Product Information* #9PIM822 (www.promega.com/tbs/9pim822/9pim822.html) for additional information.

- Transform the ligated material directly into *E. coli* competent cells following the recommended transformation protocol provided with the cells.

VI. Composition of Solutions

antibiotic stock solutions

100mg/ml ampicillin in deionized water (sterile filtered)
25mg/ml kanamycin; kanamycin sulfate in deionized water (sterile filtered)

Store at -20°C.

Blue/Orange 6X Loading Dye

0.03% bromophenol blue
0.03% xylene cyanol FF
0.4% orange G
15% Ficoll® 400
10mM Tris-HCl (pH 7.5)
50mM EDTA (pH 8.0)

5X Flexi® Digest Buffer

50mM Tris-HCl (pH 7.9 at 37°C)
250mM NaCl
50mM MgCl₂
5mM DTT
0.5mg/ml acetylated BSA

2X Flexi® Ligase Buffer

60mM Tris-HCl (pH 7.8 at 25°C)
20mM MgCl₂
20mM DTT
2mM ATP

Store in single-use aliquots at -20°C. Avoid multiple freeze-thaw cycles.

IPTG stock solution (0.1M)

1.2g isopropyl β-D-thiogalactopyranoside

Add deionized water to 50ml final volume. Filter sterilize and store at 4°C.

LB medium

10g Bacto®-tryptone
5g Bacto®-yeast extract
5g NaCl

Add deionized water to approximately 1L. Adjust pH to 7.5 with 10N NaOH and autoclave. For LB plates, include 15g agar prior to autoclaving.

LB plates with antibiotic

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100µg/ml or kanamycin to a final concentration of 30µg/ml, as appropriate for the acceptor Flexi® Vector. Pour 30–35ml of medium into 85mm petri dishes. Let the agar harden. Store at 4°C for up to 1 month or at room temperature for up to 1 week.

LB plates with ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as described above, then supplement with 0.5mM IPTG and 80µg/ml X-Gal and pour the plates. Alternatively, spread 100µl of 100mM IPTG and 20µl of 50mg/ml X-Gal over the surface of an LB-ampicillin plate and allow to absorb for 30 minutes at 37°C prior to use.

2M Mg²⁺ stock

20.33g MgCl₂ • 6H₂O
24.65g MgSO₄ • 7H₂O

Add distilled water to 100ml. Filter sterilize.

SOC medium (100ml)

2.0g Bacto®-tryptone
0.5g Bacto®-yeast extract
1ml 1M NaCl
0.25ml 1M KCl
1ml 2M Mg²⁺ stock, filter-sterilized
1ml 2M glucose, filter-sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml of distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg²⁺ stock and 2M glucose, each to a final concentration of 20mM. Bring the volume to 100ml with sterile, distilled water. The final pH should be 7.0.

1X TBE buffer

89mM Tris base
89mM boric acid
2mM EDTA (pH 8.0)

1X TAE buffer

40mM Tris base
5mM sodium acetate
1mM EDTA (pH 8.0)

X-Gal (2ml)

100mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside
Dissolve in 2ml N,N'-dimethyl-formamide. Cover with aluminum foil and store at -20°C. Alternatively, use 50mg/ml X-Gal (Cat.# V3941).

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I. Introduction

The human genome is peppered with regions of repetitive DNA—hypervariable regions consisting of a short DNA sequence repeated in tandem. These regions are polymorphic in that the sequence varies in the number of copies of the repeat unit. The number of repeat units is indicated by the allele designation. For example, 14 copies of the repeat unit would be referred to as allele 14. In the early 1980s, these regions were investigated as informative markers to map the human genome (Wyman and White, 1980; Jeffreys *et al.*, 1985a), but one of the founding fathers of DNA typing, Alec Jeffreys, quickly realized that these markers could be employed for DNA-based human identification (Jeffreys *et al.*, 1985b).

A. Variable Number Tandem Repeats and Restriction Fragment Length Polymorphisms

In the early days of DNA-based identification, the hypervariable regions of interest were variable number tandem repeat (VNTR) loci, which had a high level of heterozygosity and were relatively large in size (300–10,000bp) (Nakamura *et al.*, 1987; Budowle *et al.*, 1991). VNTRs were analyzed using restriction fragment length polymorphism (RFLP), where high-molecular-weight target DNA is digested with a restriction enzyme that has recognition sites at both ends of the hypervariable region. The size of the DNA fragment resulting from the restriction enzyme digestion is dictated by the number of repeat elements. These fragments are separated by size using agarose or polyacrylamide gel electrophoresis and detected using a labeled VNTR probe. Analysis of multiple VNTR loci results in a unique pattern of DNA fragments on the gel. The patterns generated from a DNA sample of unknown origin and DNA of known origin are compared. Matching patterns indicate that the source of the unknown and known DNA samples are likely the same. RFLP analysis of VNTR loci works well to resolve immigration and paternity disputes and for other applications where large amounts of intact DNA can be collected. However, RFLP is not ideally suited to forensic investigations because microgram amounts of high-molecular-weight DNA are required. Thus, VNTR analysis is limited to investigations where large amounts of DNA are recovered.

B. Short Tandem Repeats

Many samples recovered from crime scenes yield only nanogram or picogram amounts of DNA that is sometimes degraded, and thus not suitable for VNTR analysis. To overcome this limitation, scientists harnessed the polymerase chain reaction (www.promega.com/paguide/chap1.htm) (PCR; Saiki *et al.*, 1985) to amplify shorter hypervariable regions known as short tandem repeat (STR) loci. STR loci consist of short, repetitive sequence elements 3–7 base pairs in length (Edwards *et al.*, 1991a; Edwards *et al.*, 1991b; Edwards *et al.*, 1992; Warne *et al.*, 1991). By using PCR, minute amounts of DNA can be amplified and analyzed and, because STR loci are much shorter than VNTR loci, degraded DNA can be successfully analyzed. This amplified fragment length polymorphism

(AmpFLP) technique allows generation of full DNA profiles from trace biological samples, such as blood spatter, fingerprints, saliva, semen and other body fluids, bone, tissue and hair collected during forensic casework and missing persons investigations. In addition, PCR-based STR analysis is higher throughput so that more samples can be analyzed in less time. For these reasons, STR analysis has replaced VNTR analysis for human identification purposes.

The human genome is organized as 23 pairs of chromosomes: the X and Y sex chromosomes and the remaining autosomal chromosome pairs, numbered 1 through 22. STR loci exist on both autosomes and sex chromosomes. Ideally STR loci for forensic use are physically separated enough so that they are inherited independently of each other (i.e., not genetically linked). Sequences on different autosome pairs are not usually linked due to independent assortment of chromosomes during meiosis. Females possess two X chromosomes, which can recombine during meiosis. However, X-STR loci can show linkage disequilibrium (Tillmar, *et al.*, 2008) and tend to fall within four linkage groups. In males, neither X-STR nor Y-STR loci are linked because these chromosomes do not pair during meiosis and cannot undergo recombination, except for a small pseudoautosomal region of the X-Y pair (Gusmão and Carracedo, 2003). This difference in genetic inheritance becomes important during data analysis. Data generated using autosomal STR loci and X- and Y-STR loci cannot be used in the same way (see the Data Analysis section). A more detailed discussion of STR analysis is provided below.

C. Single Nucleotide Polymorphisms

A single nucleotide polymorphism (SNP) is a variation in DNA sequence at a single base. SNPs can be used for human identification, although studies suggest that 50–80 loci are required to achieve the same discrimination level of 16 STR loci [Gill, 2001]. There are nearly 6 million SNPs in numerous online databases, but only a fraction of these are useful for human identification because some are less polymorphic than others or difficult to amplify in multiplex PCR. The requirement for small amplicon sizes (60–120 bp) and robust multiplex PCR with minimal nonspecific amplification further reduces the number of SNPs suitable for forensic applications. Appropriate criteria are being identified to select the most useful and robust loci and develop a core panel of SNPs for optimum discrimination in forensic applications (Phillips, 2003).

Analysis of large numbers of SNPs by direct sequencing is expensive and time-consuming, so in addition to multiplex PCR, SNPs are typed by minisequencing, which is simple, non-radioactive and easily multiplexed. Minisequencing involves amplification of an unlabeled PCR product that spans the SNP, then interrogation using a probe that anneals immediately adjacent to the SNP. This probe is allowed to hybridize to the PCR product in the presence of labeled dideoxynucleotides (ddNTPs) and DNA polymerase. The probe is extended at the 3'-end through

addition of the ddNTP that is complementary to the single nucleotide polymorphism. The extended probe is separated by electrophoresis, and the incorporated ddNTP is detected. Because each ddNTP is labeled with a different fluor, the color of the peak denotes the SNP allele. Multiplex SNP analysis is made possible by adding a defined number of nucleotides to the 5'-end of each probe so that the SNP probes can be resolved electrophoretically.

D. Mitochondrial DNA Analysis

While analysis of nuclear DNA provides full STR profiles in many situations, ancient or degraded samples often yield partial profiles or no profile. In these cases, analysis of mitochondrial DNA (mtDNA) may provide information where nuclear DNA analysis cannot. Mitochondria are cellular organelles that provide most of the energy required for various cellular functions. The number of mitochondria per cell varies with the cell type, ranging from hundreds to thousands of mitochondria per cell, and each mitochondrion contains many copies of its own DNA. Thus, mtDNA has a much higher copy number per cell than nuclear DNA. In addition, human mtDNA is a circular molecule of 16,569bp (Anderson *et al.*, 1981), and this circular nature makes mtDNA more resistant to exonucleases. For these reasons, there is often enough mtDNA, even in degraded samples, for analysis.

Samples well suited to mtDNA analysis include bones, teeth, hair and old or degraded samples, which often have little high-molecular-weight nuclear DNA. Hair is a common sample type discovered at crime scenes, but hair presents a problem to forensic examiners because, as hair is shed, genomic DNA in the root cells undergoes programmed degradation (Linch, 1998). Also, cells within the hair shaft lose their nuclei, but not mitochondria, during development. As a result, nuclear DNA analysis of hair is frequently unsuccessful. Sequence analysis of hypervariable regions within mtDNA from shed hair shafts offer an alternative, but less discriminating, approach.

mtDNA contains two hypervariable regions that are used for human identification purposes: the 342bp HVI region and 268bp HVII region. HVI and HVII polymorphisms arise through random mutation and are inherited through the maternal lineage. Thus, mtDNA analysis cannot distinguish between people of the same maternal lineage. mtDNA sequence variations, or haplotypes, are identified by sequencing the HVI and HVII regions and comparing these sequences to a reference sequence. Any nucleotides that differ from this standard are noted. However, mtDNA analysis is complicated by the fact that not all mitochondria within an organism or even a single cell have exactly the same mtDNA sequence. This heterogeneity, known as heteroplasmy, may be present as single nucleotide substitutions or variations in the length of the hypervariable region.

II. DNA Purification

Any biological material is a potential source of DNA for analysis. However, the success of DNA typing often depends on the quality and nature of the sample. In the past, DNA-typing efforts focused on samples that had a high probability of providing relatively large amounts of intact DNA and yielding a full DNA profile. However, trace evidence samples, which have limited amounts of biological material, are increasingly common in forensic laboratories due to the sensitive nature of STR typing. DNA profiles can be successfully generated from trace samples such as fingerprints, saliva and sweat stains. While success rates for analysis of these trace samples are increasing, some samples still do not yield adequate DNA amounts for analysis, and even if DNA yields appear high, the DNA may be degraded to the point where amplification is impossible. This is often the case when samples are exposed to the environment for long periods of time. Environmental exposure is not kind to DNA. Many biological samples are ideal substrates for the growth of bacteria and other microorganisms, which can degrade DNA. Exposure to ultraviolet light in the form of sunlight can induce pyrimidine dimers, which can inhibit PCR. Other PCR inhibitors can be introduced by the environment (e.g., humic acid in soil), by the substrate on which the sample is deposited (e.g., indigo dye from denim) or by the sample itself (e.g., hematin from blood samples). The ability to purify DNA free of these inhibitors can be critical to STR analysis success.

Samples collected during an investigation are often collected on a cotton swab or other solid support. The first step to purify DNA from these samples involves removing the biological material from the solid support, typically by soaking the material in an aqueous buffer. Some samples, such as blood and semen, require a proteinase K digestion at this step for maximum DNA yield and quality. The solid support is removed by placing the entire sample, including the solid support, into a spin basket assembly and centrifuging so that the liquid flows through the spin basket into a collection tube and the solid support remains in the spin basket. The solid support is discarded, and the aqueous DNA-containing fraction undergoes subsequent purification steps to remove PCR inhibitors and other components that may interfere with DNA quantitation and amplification.

Sample transfer and assembly and disassembly of spin baskets can be tedious and prone to error when large numbers of samples are processed and individual spin baskets are used. The Slicprep™ 96 Device (Cat.# V1391) offers a higher throughput option that allows the simultaneous centrifugation of 96 samples. The device is designed so that both the digestion or cell lysis step and centrifugation are performed in the same device. The Slicprep™ 96 Device consists of 3 components: a 2.2ml 96 Deep Well Plate, a 96 Spin Basket and a U-Shaped Collar. In the digestion position, the 96 Spin Basket is fully inserted into the 96 Deep Well Plate, allowing space for approximately 165µl of solution below the basket in each

well. After the incubation, the baskets are raised approximately 1cm (the spin position) by inserting the U-Shaped Collar to create space for an additional 500µl of solution (Figure 14.1). The device is centrifuged, and the collar and 96 Spin Basket are discarded, leaving the DNA-containing solution in the 96 Deep Well plate. The 96 Deep Well Plate then can be used for manual DNA purification or transferred to an automated workstation for DNA purification.

More information on the Slicprep™ 96 Device... (www.promega.com/profiles/802/802_03.html).

Common DNA purification methods include organic extraction using phenol:chloroform, Chelex® extraction and the use of a silica magnetic resin. Phenol:chloroform extraction is considered by many DNA analysts to be the “gold standard” of DNA isolation methods. However, this method uses hazardous organic chemicals, is time-consuming, requires multiple centrifugations, may result in significant loss of material, is not amenable to automation and can introduce amplification inhibitors. DNA extraction using Chelex® resin is rapid but does a poor job of removing organic inhibitors. In contrast, magnetic resin-based DNA purification systems are effective at removing PCR inhibitors, do not require organic solvents and can be automated to increase throughput.

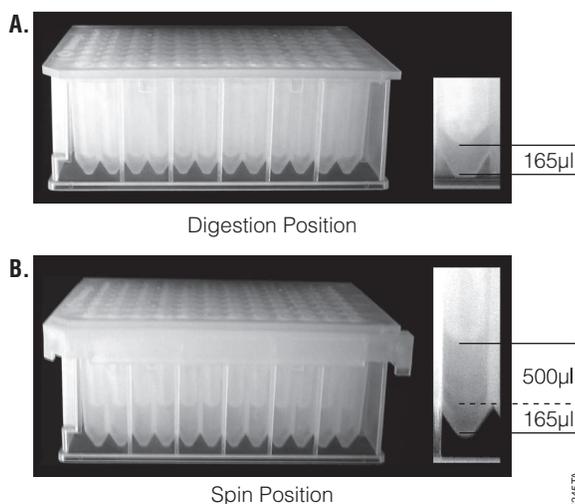


Figure 14.1. Operational modes of the Slicprep™ 96 Device. **Panel A.** The digestion position. The 96 Spin Basket is fully inserted into the 96 Deep Well Plate to allow incubation of solid supports with digestion or lysis buffer. **Panel B.** The spin position. The U-Shaped Collar is inserted between the 96 Deep Well Plate and 96 Spin Basket. This raises the baskets and allows an additional 500µl below the baskets.

A. DNA IQ™ System—An Example of a Magnetic Resin

The DNA IQ™ System uses a silica-based paramagnetic resin to isolate DNA from liquid samples and samples on solid supports. The DNA IQ™ System will isolate all DNA types present in a sample, including nuclear and mitochondrial DNA. Two protocols exist for the manual format, one for database samples (www.promega.com

[/tbs/tb297/tb297.html](http://tbs/tb297/tb297.html)) and another for forensic casework samples (www.promega.com/tbs/tb296/tb296.html). For samples with abundant DNA, such as buccal swabs collected as reference samples, the amount of DNA IQ™ Resin used is limiting so that the resin becomes saturated and any excess DNA is not bound, leading to the isolation of a consistent amount of DNA from all samples. For casework samples with limited DNA amounts, the resin is not saturated, and the amount of DNA isolated is limited only by the amount of starting material.

The DNA IQ™ System allows DNA isolation from a wide variety of sample types, including blood and blood stains, semen and semen stains, chewing gum, hair, bone, urine, cigarette butts (www.promega.com/appnotes/an106/an106.html), buccal swabs and blood card punches (www.promega.com/appnotes/an102/an102.html).

View a more comprehensive list of sample types processed using the DNA IQ™ System... (www.promega.com/dnaiqsamples/)

Hair, bone, sperm and tissue masses, including formalin-fixed, paraffin-embedded tissue, require proteinase K digestion to obtain reliable amounts of DNA. The Tissue and Hair Extraction Kit (for use with DNA IQ™) (Cat.# DC6740) includes proteinase K and DTT, which aid in the break up of tissue, hair and bone samples (www.promega.com/profiles/502/502_11.html) prior to DNA purification using the DNA IQ™ System.

The paramagnetic DNA IQ™ Resin is amenable to automation, and the DNA IQ™ System has been automated on a number of robotic platforms. More information about automated DNA purification can be found below

Additional Resources for DNA IQ™ System

Technical Bulletins and Manuals

- TB296 *DNA IQ™ System—Small Sample Casework Protocol* (www.promega.com/tbs/tb296/tb296.html)
- TB297 *DNA IQ™ System—Database Protocol* (www.promega.com/tbs/tb297/tb297.html)
- TB307 *Tissue and Hair Extraction Kit (for use with DNA IQ™) Technical Bulletin* (www.promega.com/tbs/tb307/tb307.html)

Promega Publications

- GIN014 Forensic extraction and isolation of DNA From hair, tissue and bone (www.promega.com/profiles/502/502_11.html)
- GIN013 DNA IQ™ System “frequently asked questions” (www.promega.com/profiles/501/501_11.html)
- GIN012 DNA IQ™: The intelligent way to purify DNA (www.promega.com/profiles/403/403_16.html)

- AN106 Genomic DNA purification from cigarette butts and buccal swabs using the DNA IQ™ System
(www.promega.com/appnotes/an106/an106.html)

III. Differential Extraction

Sexual assault swabs make up a large percentage of samples submitted for DNA analysis. Processing of these samples involves separating sperm cells, which contain male DNA, and epithelial cells, which are mostly female-derived so that female DNA will not obscure the profile of the male DNA contributor. In 1985, Gill *et al.* (Gill *et al.*, 1985) developed a method to selectively enrich for sperm cells in the presence of an excess of epithelial cells. In the absence of dithiothreitol (DTT), detergent/proteinase K treatment preferentially lyses epithelial cells, converting the large excess of epithelial cells into soluble DNA. Sperm are resistant to lysis under these conditions. Proteinase K digestion also loosens the attachment of sperm to solid supports and reduces the degree of sperm and epithelial cell clumping, increasing yields and allowing better separation (Tereba *et al.*, 2004). The proteinase K-digested sample is centrifuged to efficiently pull sperm out of the cotton fibers, extract the entire volume of epithelial DNA-containing buffer from the matrix and pellet intact sperm at the bottom of the tube. The aqueous phase is removed as the epithelial fraction, and the sperm pellet is washed and recentrifuged to remove any residual epithelial DNA-containing buffer prior to sperm lysis. Multiple rounds of washes and centrifugations may be required to obtain a sperm pellet relatively free of epithelial DNA.

One limitation of this traditional method is the inability to efficiently separate soluble DNA from the cell pellet. In addition, the high number of washes and centrifugations is laborious and time-consuming and necessitates a delicate balance between clean separation and loss of sperm. In spite of these issues, this method remains the preferred method in many forensic laboratories. However, the amount of time required to process sexual assault samples using this method has resulted in a steady increase in sample backlogs. Unfortunately, traditional differential extraction methods to process sexual assault samples are not amenable to automation.

To increase the throughput of differential extraction, Promega has developed the Differex™ System (Cat.# DC6800). Whereas traditional differential extraction methods use serial washes and centrifugations to separate sperm and epithelial cells, the Differex™ System uses a combination of phase separation and differential centrifugation. This protocol uses only one centrifugation instead of four or more for traditional methods, and because fewer sperm cells are lost, DNA yields are higher.

The Differex™ protocol starts by placing the entire proteinase K-digested sample in a spin basket seated in a tube containing a nonaqueous Separation Solution.

Alternatively, for large numbers of samples, the Slicprep™ 96 Device can be used instead of individual spin baskets. The Separation Solution is not miscible with water and is more dense than water but less dense than sperm. During centrifugation, the sperm are pulled from the solid matrix and form a tight pellet at the bottom of the tube under the Separation Solution. The soluble DNA remains in the aqueous buffer, which forms a layer on top of the Separation Solution. The aqueous buffer containing the epithelial DNA is removed, and the DNA purified using the DNA IQ™ chemistry. The Separation Solution is washed to eliminate the thin film of epithelial DNA-containing buffer at the interface between the two layers and any droplets on the side of the tube. Up to half of the Separation Solution can be removed to eliminate any cell debris that is present at the interface between the two solutions. After washing, the tube contains the sperm pellet and remaining Separation Solution. Two or more volumes of DNA IQ™ Lysis Buffer containing DTT are added to lyse the sperm and solubilize the Separation Solution. The DNA IQ™ Resin is added, and the sperm DNA is purified using the DNA IQ™ chemistry. The Differex™ separation and subsequent DNA purification require as little as 2 hours. An adaptation of this process for automated differential extraction is described below.

Additional Resources for Differex™ System

Technical Bulletins and Manuals

- TBD020 *Differex™ System Technical Bulletin*
(www.promega.com/tbs/tbd020/tbd020.html)

Promega Publications

- GIN019 Tech Tips: The Differex™ System
(www.promega.com/profiles/801/801_14.html)
- GIN018 A new, rapid method to separate sperm and epithelial cells
(www.promega.com/profiles/702/702_08.html)

IV. DNA Quantitation

DNA purification methods cannot differentiate between human DNA and other DNA (e.g., bacterial and fungal DNA). Therefore, if the sample is not pristine and you want to determine the concentration of human genomic DNA present, you will need to use a human-specific DNA quantitation system. In forensics, the most common systems are based on real-time PCR and harness fluorescently labeled oligonucleotide probes or primers to detect and quantitate a PCR product in real time. These systems employ two different fluorescent reporters and rely on energy transfer from one reporter (the energy donor) to a second reporter (the energy acceptor) when the reporters are in close proximity. The second reporter can be a quencher or a fluor. Most commonly, the second reporter is a quencher, and the energy from the first reporter is absorbed but re-emitted as heat rather than light. The

progress of the reaction can be monitored as the change in fluorescence of the energy donor. During the exponential phase of PCR, the change in fluorescence is proportional to the accumulation of PCR product. To simplify quantitation, specially designed instruments perform both the thermal cycling steps to amplify the target and fluorescence detection steps to measure the change in fluorescence in real time.

More information about quantitative real-time PCR... (www.promega.com/paguide/chap1.htm#title1.f)

One quantitative PCR (qPCR) approach employs hydrolysis probes that are complementary to the accumulating PCR product and are labeled with a fluor at the 5'-end and a quencher at the 3'-end. Because the two reporters are in close proximity, the fluorescent signal is quenched. During the annealing step, the probe hybridizes to PCR product generated in previous amplification cycles. The resulting probe:target hybrid is a substrate for the 5'→3' exonuclease activity of the DNA polymerase, which degrades the annealed probe and liberates the fluor (Holland *et al.* 1991). The fluor is freed from the effects of the energy-absorbing quencher, and the progress of the reaction and accumulation of PCR product is monitored by the resulting increase in fluorescence.

The Plexor® HY System takes a different approach, one that does not require a hydrolysis probe, to determine the concentration of total human DNA and male human DNA simultaneously in one reaction. This qPCR system takes advantage of two novel bases, isoguanine (iso-G) and 5'-methylisocytosine (iso-C), which form a unique base pair in double-stranded DNA. Amplification of each target uses two primers: one primer is synthesized with an iso-C residue as the 5'-terminal nucleotide and a fluorescent label at the 5'-end; the second primer is unlabeled. The reaction contains a fluorescent quencher covalently linked to iso-G. As amplification proceeds, fluorescence is reduced by site-specific incorporation of the fluorescent quencher opposite the iso-C and in close proximity to the fluorescent dye located on the end of the primer.

By measuring the reduction in signal during each PCR cycle, an amplification curve is generated. An amplification threshold is set within the exponential phase of PCR at a fluorescence level where all amplification curves exhibit the most significant signal decrease. To calculate the DNA concentration of an unknown sample, the cycle threshold (C_T), the point at which the amplification curve crosses the amplification threshold, is determined and compared to a standard curve. The standard curve is generated by determining the C_T values for a dilution series of a sample of known DNA quantity and plotting the log concentration on the X axis and cycle threshold on the Y axis. After amplification, a melt analysis can be performed to confirm that the correct product was amplified and expedite troubleshooting, if necessary. The kit contains an internal PCR control (IPC) to test for false-negative results that may occur in the presence of PCR inhibitors.

The Plexor® HY System is a sensitive multiplex kit that routinely detects approximately 6.4pg of total DNA. PCR setup is performed at room temperature and is compatible with automated platforms. The Plexor® HY System is optimized for use on the Applied Biosystems 7500 and 7500 FAST real-time PCR systems and Stratagene Mx3005P® and Mx3000P® qPCR systems. Protocols (www.promega.com/plexorhy/techmanuals.htm) also exist for other qPCR instrumentation. These real-time PCR instruments are not designed to interpret a decrease in fluorescence, so specific software is required to make sense of raw Plexor® HY data. The Plexor® Analysis Software interprets these data and allows you to quickly and easily review data and create reports. Replicate samples are automatically averaged, template amounts are calculated and the necessary volume of DNA is displayed for your optimized STR amplification conditions in the Forensic Report. The forensic release of the Plexor® Analysis Software is available for free download (www.promega.com/plexorhy/software.htm).

Additional Resources for DNA Quantitation

Technical Bulletins and Manuals

- | | |
|-------|---|
| TM293 | <i>Plexor® HY System for the Applied Biosystems 7500 and 7500 FAST Real-Time PCR Systems Technical Manual</i>
(www.promega.com/tbs/tm293/tm293.html) |
| TM294 | <i>Plexor® HY System for the Stratagene Mx3000P® and Mx3005P® Quantitative PCR Systems Technical Manual</i>
(www.promega.com/tbs/tm294/tm294.html) |
| TM296 | <i>Plexor® HY System for the Bio-Rad iQ™5 Real-Time PCR Detection System Technical Manual</i>
(www.promega.com/tbs/tm296/tm296.html) |
| TM299 | <i>Plexor® HY System for the Corbett Rotor-Gene 6000 Series Detection System Technical Manual</i>
(www.promega.com/tbs/tm299/tm299.html) |

Promega Publications

GIN024	The Plexor® HY System: Not solely a quantitation technique (www.promega.com/profiles/1002/1002_06.html)
GIN023	The hidden benefits of real-time PCR: Assessing and addressing qualitative challenges (www.promega.com/profiles/1001/1001_06.html)
GIN020	The Plexor™ Analysis Software (www.promega.com/profiles/802/802_06.html)
GIN019	Development of a novel, fluorescent, two-primer approach to quantitative PCR (www.promega.com/profiles/801/801_03.html)
AN157	Developmental validation of a real-time PCR assay for the simultaneous quantification of total human and male DNA (www.promega.com/plexorhy/an157.pdf)
GE295	Validation Guide for the Plexor® HY System (www.promega.com/plexorhy/plexorhy_validationguide.pdf)

V. STR Analysis**A. STR Amplification and Detection**

STR loci are amplified using fluorescently labeled PCR primers that flank the hypervariable regions. One of the biggest strengths of PCR-based DNA typing is the degree to which DNA can be amplified. Starting with a single DNA molecule, millions or billions of DNA molecules can be synthesized after 32 cycles of amplification. This level of sensitivity allows scientists to extract and amplify DNA from minute or damaged samples and obtain useful DNA profiles.

STR amplification systems can accommodate a range of template DNA concentrations. Most of the Promega PowerPlex® STR systems provide optimal sister allele balance and locus-to-locus balance with 0.5–1.0ng of DNA template, and studies performed at Promega show that full profiles can be observed with less than 100pg (Ensenberger and Fulmer, 2009; McLaren, 2007; Krenke *et al.* 2005; Krenke *et al.* 2002). However, amplification and detection instrumentation can vary. You may need to optimize protocols, including cycle number and detection conditions (e.g., injection time or loading volume), for each laboratory instrument. Most of the PowerPlex® systems use a thermal cycling program with 30 or 32 cycles for 0.5–1ng of purified DNA template. For larger amounts of input DNA (i.e., FTA® paper) or to decrease sensitivity, fewer cycles should

be evaluated. In-house validation should be performed to determine the optimal amplification and detection conditions.

The sensitive nature of PCR works in a lab's favor, but it can cause problems if great care is not taken to avoid contaminating the reaction with exogenous DNA. Three main categories of exogenous DNA have the biggest impact on DNA-typing laboratories: 1) DNA from the analyst, 2) DNA from other samples in the lab and 3) allelic ladder fragments. DNA from nonhuman sources, such as bacteria and fungi, will not be amplified and detected because STR systems are species-specific. Extreme care must be taken to avoid cross-contamination when preparing sample DNA, handling primer pairs, assembling amplification reactions and analyzing amplification products. Reagents and materials used prior to amplification should be stored separately from those used following amplification. Amplification reactions should be assembled in a room dedicated for reaction setup, using equipment and supplies reserved for amplification setup. We highly recommend the use of gloves and aerosol-resistant pipette tips. To detect reagent contamination, assemble a negative control reaction (i.e., no template) for every set of reactions, and scrutinize the reactions for the presence of unexpected peaks. After setting up reactions, wash all surfaces with a dilute bleach solution.

After assembly, reactions are subjected to thermal cycling, and PCR products are separated by size using specialized polyacrylamide gel electrophoresis or capillary electrophoresis (CE) instruments. During electrophoresis, DNA molecules move through a polymer matrix in response to an electric field. The rate of migration depends on fragment size, with smaller DNA fragments migrating more quickly through the porous matrix than larger fragments. The instruments use a laser near the anode of the capillary or polyacrylamide gel to excite and detect the fluorescent PCR products, which appear as peaks on the resulting electropherogram (Figure 14.2). Each fluor is detected in a separate dye channel. To reduce background, spectral calibration must be performed to correct spectral overlap of dyes.

Size separation of amplification products in parallel with separation of an allelic ladder, which consists of all major alleles at a particular locus, allows positive identification of each allele that makes up the DNA profile. An internal size standard is included in each analysis to control for run-to-run variation in migration.

More information about the background theory and principles of capillary electrophoresis... (http://www.csl.nist.gov/strbase/pub_pres/AAFS2006_CEttheory.pdf)

B. Data Analysis

Specialized allele-calling software eliminates the need for visual comparisons to assign alleles, making the process faster, easier and more accurate. However, the software does not address data quality, making a review of each sample necessary to judge data quality and detect DNA

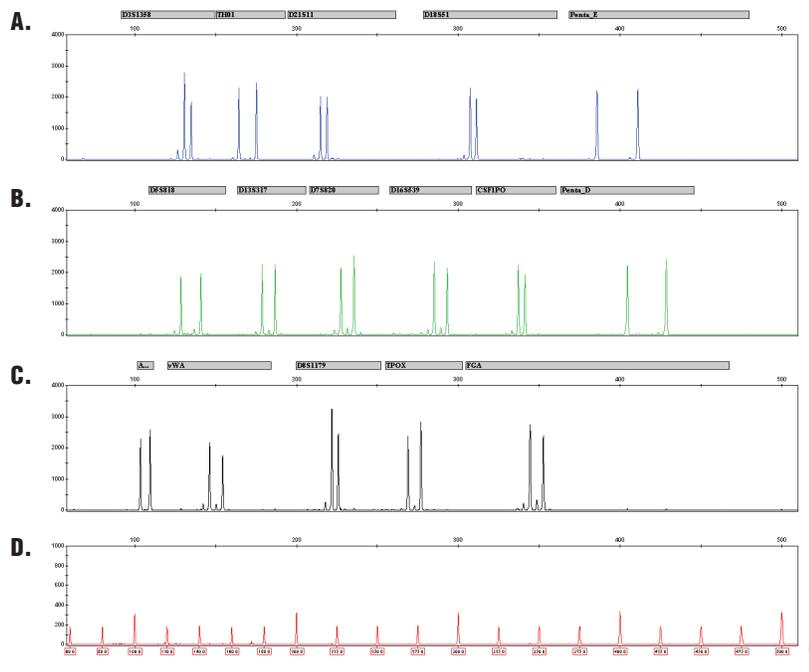


Figure 14.2. Representative PowerPlex® 16 HS System data. A single template DNA (0.5ng) was amplified using the PowerPlex® 16 HS 10X Primer Pair Mix. Amplification products were mixed with Internal Lane Standard 600 and analyzed with an Applied Biosystems 3130 Genetic Analyzer using a 3kV, 5-second injection. Results were analyzed using GeneMapper® ID software, version 3.2. **Panel A.** An electropherogram showing the peaks of the fluorescein-labeled loci: D3S1358, TH01, D21S11, D18S51 and Penta E. **Panel B.** An electropherogram showing the peaks of the JOE-labeled loci: D5S818, D13S317, D7S820, D16S539, CSF1PO and Penta D. **Panel C.** An electropherogram showing the peaks of the TMR-labeled loci: Amelogenin, vWA, D8S1179, TPOX and FGA. **Panel D.** An electropherogram showing the 60bp to 500bp fragments of the Internal Lane Standard 600.

mixtures or contamination. These reviews can be performed manually by DNA analysts, but this is often tedious and time-consuming. In many laboratories, data review has become the bottleneck for STR analysis now that other DNA-typing steps are being automated. This has resulted in an increased interest in expert system software. Expert systems for DNA-typing laboratories are defined as systems that increase the efficiency or effectiveness of genotyping samples by automatically interpreting STR data. An expert system can provide a second set of independent allele calls and allow the analyst to skip the review of sample data that the software has judged to be of high quality and focus on problematic samples. Expert systems can objectively evaluate DNA mixtures in seconds and present an analyst with the best-fit combinations of DNA profiles. Expert systems also can compare all alleles called in a sample to the alleles in every other sample in the batch and flag potential contamination events. Finally the software can summarize the data and generate reports that contain only the necessary information.

C. Statistical Analysis

Once a DNA profile is generated, statistical analysis is performed to determine if the profile matches, with a reasonable degree of scientific certainty, that of a known reference sample. The analyses performed depend on the type of STR locus.

Autosomal STR analysis employs the product rule, which calculates the probability of finding a specific DNA profile within a population by multiplying the frequencies of the alleles at each STR locus analyzed. This probability represents the number of randomly selected individuals that theoretically must be surveyed before finding the same DNA pattern in a population. To use the product rule, the STR loci must be unlinked.

Autosomal STR analysis has a high statistical power of discrimination. For example, when analyzing the 15 STR loci amplified by the PowerPlex® 16 System, the power of discrimination is as high as 1 in 1.42×10^{18} for some ethnic groups; this exceeds the current global human population, making it unlikely that two DNA profiles will match at random.

Statistical analysis can be used to determine if two individuals are related, as is done for paternity testing and in missing persons investigations where a reference sample from the missing person is not available but reference samples from close relatives exist. For paternity testing, the paternity index is calculated to estimate how many more times likely it is that the person being tested is the biological father, rather than a randomly selected individual. The typical paternity index is assigned to a locus rather than an individual case. The PI_{typical} of several loci is the product of the individual PI_{typical} values. Generally, a PI_{typical} of less than one is indicative of nonrelatedness.

The PI_{typical} is represented by the following equation:

$$PI_{\text{typical}} = X/Y$$

where X represents the chance that the alleged father transmitted the allele to the child and Y represents the chance that some other man transmitted the allele.

For more information about statistical analysis of DNA testing results, refer to *Forensic DNA Typing* (Butler, 2005).

Y-STR loci used in forensic and paternity applications are located in the nonrecombining region of the human Y chromosome (NRY) and are faithfully transmitted from father to son, unchanged except for rare mutations. Because Y-STR loci are not inherited independently, statistical analysis cannot proceed by the product rule. Instead, the frequency of a particular Y-STR profile (haplotype) is estimated by the counting method, which is based on the frequency of a specific haplotype in a population. To be useful, Y-STR databases must include thousands of Y-STR haplotypes, enough that a sufficient number of haplotypes is represented and a meaningful estimate of the frequency of a specific haplotype can be made. Several online databases are available to assess Y-STR haplotype frequency:

U.S. Y-STR database (<http://www.usystrdatabase.com>)

Y-HRD database (<http://www.yhrd.org>)

Additional Resources for STR Analysis

Technical Bulletins and Manuals

TMD022	<i>PowerPlex® 16 HS System Technical Manual</i> (www.promega.com/tbs/tmd022/tmd022.html)
TMD021	<i>PowerPlex® S5 System Technical Manual</i> (www.promega.com/tbs/tmd021/tmd021.html)
TMD018	<i>PowerPlex® Y System Technical Manual</i> (www.promega.com/tbs/tmd018/tmd018.html)
TMD017	<i>PowerPlex® ES System Technical Manual</i> (www.promega.com/tbs/tmd017/tmd017.html)
TMD016	<i>PowerPlex® 16 BIO System Technical Manual</i> (www.promega.com/tbs/tmd016/tmd016.html)
TMD012	<i>PowerPlex® 16 System Technical Manual</i> (www.promega.com/tbs/tmd012/tmd012.html)
TMD011	<i>PowerPlex® 2.1 System Technical Manual</i> (www.promega.com/tbs/tmd011/tmd011.html)
TMD009	<i>PowerPlex® 1.2 System Technical Manual</i> (www.promega.com/tbs/tmd009/tmd009.html)

TMD008 *PowerPlex® 1.1 System Technical Manual*
(www.promega.com/tbs/tmd008/tmd008.html)

TMD006 *GenePrint® Fluorescent STR Systems Technical Manual*
(www.promega.com/tbs/tmd006/tmd006.html)

Promega Publications

GIN027 The PowerPlex® 16 HS System.
(www.promega.com/profiles/1201/1201_09.html)

GIN026 Bringing laser microdissection and on-chip PCR together.
(www.promega.com/profiles/1102/1102_06.html)

GIN026 A solution for the split peak and n-10 artifacts at the vWA locus in PowerPlex® 16 and PowerPlex® ES Systems.
(www.promega.com/profiles/1102/1102_13.html)

GIN026 Useful resources for your laboratory.
(www.promega.com/profiles/1102/1102_16.html)

GIN024 An interview with Sir Alec Jeffreys.
(www.promega.com/profiles/1002/1002_03.html)

GIN024 Validating expert systems: Examples with the FSS-i3™ Expert Systems Software.
(www.promega.com/profiles/1002/1002_13.html)

GIN023 An introduction to PCR inhibitors.
(www.promega.com/profiles/1001/1001_09.html)

GIN023 Tech Tips: FSS-i3™ Expert Systems Software.
(www.promega.com/profiles/1001/1001_11.html)

GIN022 Debunking some urban legends surrounding validation within the forensic DNA community.
(www.promega.com/profiles/902/902_03.html)

GIN022 Nanotechnology and its potential in forensic DNA analysis.
(www.promega.com/profiles/902/902_07.html)

GIN020 FSS-i3™ expert systems.
(www.promega.com/profiles/802/802_08.html)

GIN020 Identifying and preventing DNA contamination in a DNA-typing laboratory.
(www.promega.com/profiles/802/802_11.html)

GIN020	Using GeneMapper® ID with Promega STR systems. (www.promega.com/profiles/802/802_14.html)
GIN018	Comparison of multiple STR platforms and instrumentation. (www.promega.com/profiles/702/702_03.html)
GIN017	The Penta BEC multiplex primers from Promega: Additional loci available for identity testing. (www.promega.com/profiles/701/701_09.html)
GIN017	Tech Tips: The PowerPlex® Y System. (www.promega.com/profiles/701/701_12.html)
GIN016	Validation questions and answers. (www.promega.com/profiles/602/602_13.html)
GIN016	Troubleshooting capillary electrophoresis systems. (www.promega.com/profiles/602/602_10.html)
More articles	(www.promega.com/profiles/)
AN126	PowerPlex® 16 System validation. (www.promega.com/apnotes/an126/an126.pdf)

VI. Automation

In the past, law enforcement officers often limited the forensic casework samples submitted for analysis to samples that had a good chance of producing a DNA profile. More recently, realizing the sensitivity of STR analysis, officers are submitting more trace samples with the hope that a DNA profile can be generated. Also, many law enforcement agencies now are collecting database samples from an increasing number of individuals such as arrestees in addition to convicted offenders. These factors increase the number of samples that require processing. To keep pace with the increased workload, many laboratories are automating key, potentially rate-limiting steps to increase throughput. Such steps include differential extraction, DNA isolation, DNA quantitation and normalization, and PCR setup.

Automation workstations vary in their capacities, from the low-throughput Biorobot EZ1™ (Qiagen), which can purify DNA from up to six samples simultaneously in approximately 20 minutes, and medium-throughput Maxwell® 16 Instrument (Cat.# AS2000), which can process up to 16 samples simultaneously in the same amount of time, to high-throughput workstations, such as the Beckman Coulter Biomek® laboratory automation workstations and Tecan Freedom EVO® liquid handlers, which can process up to 96 samples simultaneously in as little as 40 minutes.

A. Maxwell® 16 Instrument

The Maxwell® 16 Instrument is a magnetic-particle-handling device designed for low- to medium-throughput automated DNA purification from a wide range of sample types. Unlike liquid handlers, which move samples and reagents to the purification medium, magnetic-particle handlers move the purification medium through each step in the purification process. Using magnets to move the purification medium eliminates issues with clogged tips and incomplete liquid transfers. The instrument is preprogrammed with purification protocols and uses prefilled reagent cartridges, maximizing simplicity and convenience. The instrument processes up to 16 samples per instrument run. There are two configurations of the Maxwell® 16 Instrument. The SEV (standard-elution volume) system elutes DNA in 300µl of elution buffer and is suitable for DNA isolation from reference samples. The LEV (low-elution volume) system can elute DNA in 25–50µl of elution buffer and is suitable for forensic casework samples. The instrument can be converted easily from one configuration to another by installing the Standard (SEV) Hardware Kit (Cat.# AS1200) or LEV Hardware Kit (Cat.# AS1250).

The DNA IQ™ Reference Sample Kit for Maxwell® 16 (Cat.# AS1040) allows DNA extraction from buccal swabs, FTA® blood card punches, liquid blood or other DNA reference samples using the Maxwell® 16 SEV Instrument. The kit contains the trusted DNA IQ™ System reagents and is optimized to yield a final DNA concentration that minimizes the need for concentration or dilution prior to amplification. Liquid samples are placed directly into the cartridges, while samples on solid supports require a preprocessing step in DNA IQ™ Lysis Buffer and removal of the solid support before the samples are placed into the cartridges. High-quality genomic DNA is obtained in approximately 20 minutes, at a concentration suitable for direct use in STR analysis.

The DNA IQ™ Casework Sample Kit for Maxwell® 16 (Cat.# AS1210) allows DNA extraction from forensic casework samples, including blood stains, semen stains, hairs, cigarette butts, tissue samples, and trace or "touch" DNA samples regularly encountered in forensic DNA analysis. The kit contains the DNA IQ™ reagents and uses the Maxwell® 16 LEV Instrument with plastic cartridges and plungers that allow DNA elution in a final volume of up to 50µl, providing a final DNA extract in a concentrated format.

B. High-Throughput Automation Workstations

There are many robotic platforms that can dispense reagents, aspirate liquids and perform manipulations of multiwell plates, including the Beckman Coulter Biomek® laboratory automation workstations and Tecan Freedom EVO® automated liquid-handlers. These high-throughput instruments are significantly larger and more expensive than low- and medium-throughput instruments. They also are complex to use and require detailed knowledge of how

the instrument operates, but their increased flexibility makes them adaptable to a greater variety of tasks in the laboratory.

The Biomek® workstations use multichannel tools to pipet volumes from 1µl to 1ml with high precision. Disposable pipette tips address concerns about contamination during liquid-transfer steps. Beckman Coulter offers a range of instruments, including the Biomek® 3000, Biomek® NXP and Biomek® FXP, with a range of options, capabilities, throughput levels and price points for laboratories looking to automate their processes. With single-channel and eight-channel pipetting, plate-moving capabilities, sample-tracking functionalities and a rich programming language, the Biomek® 3000 is the lowest cost option of these three instruments. Both the Biomek® NXP and FXP workstations offer independent eight-channel pipetting and gripping capabilities as well as flexible instrument configuration. The Biomek® NXP represents an intermediate cost and throughput level; the Biomek® FXP platform adds a 96-well pipetting head to the capabilities of the Biomek® NXP. With increased size and pipetting capabilities, the Biomek® FXP offers the highest flexibility and throughput of the Beckman Coulter workstations.

The Tecan Freedom EVO® automated liquid-handler boasts independent liquid-handling and gripping arms, adjustable tip spacing, independent volume control on each tip and disposable-tip volumes of up to one milliliter. In addition, the Freedom EVO® supports multiple deck sizes and tip configurations, from a lower cost, 4-tip format to a higher throughput, 8-tip instrument.

Promega has developed methods for these instruments to automate several steps of the DNA-typing process, including differential extraction, DNA purification using the DNA IQ™ System, DNA quantitation, DNA normalization and PCR setup.

C. Automated DNA Purification

Magnetic beads, such as the silica-coated DNA IQ™ Resin, can be used to separate DNA from cellular debris and are ideally suited to automation. In addition to the standard pipetting tools, robotic workstations can be equipped with a magnet, shaking platform and thermal exchange unit to perform DNA-binding steps, washes and heated elution. DNA can be isolated from both database and casework samples without detectable cross-contamination using the DNA IQ™ System in combination with the Biomek® or Freedom EVO® workstation. Database samples that have been automated successfully include buccal swabs, blood punches (FTA® and S&S 903) and liquid blood; casework samples include sperm and epithelial fractions from differential extractions, tissue samples, hairs and blood spots on blue jeans and underwear, among others.

The Biomek® 2000 and 3000 instruments enable the user to purify DNA from 8 to 96 samples (in increments of 8) from either “aqueous” or “lysis” samples. Aqueous sample types are typically those that are preprocessed by

incubating with proteinase K prior to extraction, whereas lysis samples are preprocessed by incubating in DNA IQ™ Lysis Buffer. For the Biomek® 2000, a different method has been developed depending on the number of samples being processed (i.e., one method for 8 samples, one for 16, etc.) as well as for each sample type. The net result is that 24 methods exist for 8 to 96 aqueous or lysis samples. For the Biomek® 3000 this number is reduced to one method. This single method allows the user to choose between aqueous and lysis sample types and designate the number of samples being processed. Promega also has developed and optimized scripts for DNA isolation on the 4-tip and 8-tip Freedom EVO® instruments using the DNA IQ™ System. These scripts are able to process 1–96 aqueous samples and lysis samples.

Additional Resources for Automated DNA Purification Technical Bulletins and Manuals

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| TB347 | <i>DNA IQ™ Reference Sample Kit for Maxwell® 16 Technical Bulletin</i>
(www.promega.com/tbs/tb347/tb347.html) |
| TB354 | <i>DNA IQ™ Casework Sample Kit for Maxwell® 16 Technical Bulletin</i>
(www.promega.com/tbs/tb354/tb354.html) |

Promega Publications

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|--------|---|
| GIN026 | The Maxwell® 16 Low Elution Volume System for forensic casework: Implementation and routine use in a forensic laboratory.
(www.promega.com/profiles/1102/1102_09.html) |
| GIN025 | DNA extraction using the Tecan Freedom EVO® 200 and DNA IQ™ System.
(www.promega.com/profiles/1101/1101_07.html) |
| GIN025 | The Maxwell® 16 applications database.
(www.promega.com/profiles/1101/1101_15.html) |
| GIN022 | Automation in a forensic laboratory: An update.
(www.promega.com/profiles/902/902_19.html) |
| GIN021 | Forensic application of the Maxwell™ 16 Instrument.
(www.promega.com/profiles/901/901_03.html) |
| GIN021 | The DNA IQ™ System on the Tecan Freedom EVO® 100
(www.promega.com/profiles/901/901_08.html) |
| GIN021 | Automating the DNA IQ™ System on the Biomek® 3000 laboratory automation workstation
(www.promega.com/profiles/901/901_11.html) |

- GIN018 Automation in a forensic laboratory
(www.promega.com/profiles/702/702_12.html)
- GIN013 Robotic extraction of mock sexual assault samples using the Biomek® 2000 and the DNA IQ™ System
(www.promega.com/profiles/501/501_03.html)
- AN102 Extraction and isolation of DNA from blood cards and buccal swabs in a 96-well format
(www.promega.com/appnotes/an102/an102.html)
- GE181 Validation Guide for the DNA IQ™ Reference Sample Kit for Maxwell® 16.
(www.promega.com/maxwell16/maxwell_dna_iq_reference_manual.pdf)

D. Automated Differential Extraction

The Automated Differex™ System allows high-throughput, automated differential extraction of sexual assault samples and integrates with the DNA IQ™ System for subsequent DNA purification. Up to 48 samples can be processed in less than 5 hours using the automated Differex™ protocol; 40 samples can be processed if wells are reserved for DNA standards in downstream DNA quantitation. Two independent, but related, automated methods are used to process differential extractions: the automated Differex™ method and automated DNA IQ™ for Differex™ method. The automated Differex™ method involves separating samples into epithelial and sperm fractions, while the automated DNA IQ™ for Differex™ method purifies genomic DNA from each fraction. The protocol begins with a proteinase K digestion of swab samples in a Slicprep™ 96 Device, followed by centrifugation to pellet the sperm and separate digestion products from the solid support material. The plate containing the sperm pellets and supernatants is placed onto the deck of a robotic workstation, and the automated Differex™ method begins. Automated Differex™ protocols exist for the Biomek® 2000 and Biomek® 3000 workstations and Tecan Freedom EVO® 100 liquid-handler.

The automated Differex™ method combines the Differex™ System reagents and DNA IQ™ Resin in a novel pellet-capping process. First, the robot dispenses DNA IQ™ Resin directly on top of each sperm pellet. Upon application of a magnetic field, the paramagnetic resin particles form a capping layer, which allows supernatant manipulation without pellet disruption (Figure 14.3). A portion of the supernatant is removed from each sample well and moved to an adjacent well for downstream processing as the epithelial fraction. The remaining supernatant is removed from the pellet and discarded or, if desired, archived. The resin-capped sperm pellets are washed four times to dilute and remove any residual epithelial DNA-containing supernatant. During the third wash, the resin-capped sperm pellets are resuspended to release any trapped epithelial

material. Resuspending the pellets requires a manual step to centrifuge the sample plate and pellet the sperm. Following this centrifugation the robot adds a second aliquot of DNA IQ™ Resin to re-establish the pellet cap. The Differex™ Separation Solution is used to float the epithelial DNA-containing wash buffer away from the capped pellet to further improve epithelial DNA removal. The fourth and final wash is performed to complete the automated Differex™ extraction. The sperm and epithelial fractions then undergo automated DNA isolation using the DNA IQ™ System. Integration of DNA IQ™ and Differex™ System components results in higher sperm fraction yields and reproducible, automated sexual assault sample processing.

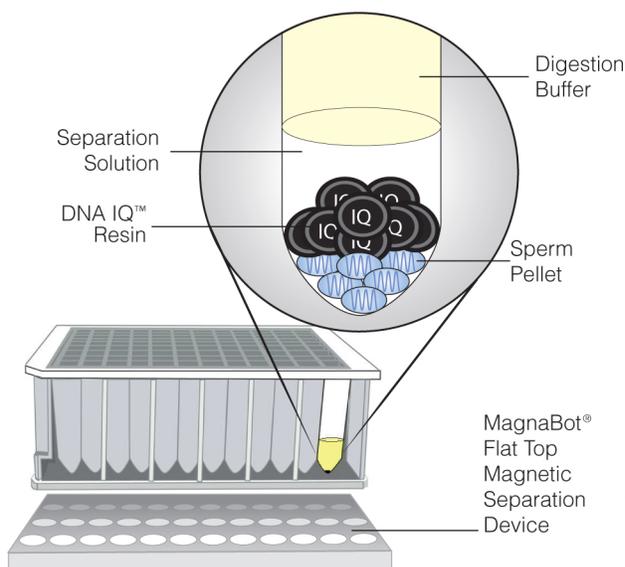


Figure 14.3. The Automated Differex™ System. The automated Differex™ method uses DNA IQ™ Resin to cap the sperm pellet after differential lysis and centrifugation. The MagnaBot® Flat Top Magnetic Separation Device immobilizes the DNA IQ™ Resin, holding the sperm pellet in place during automated wash steps. The Separation Solution helps remove any remaining digestion buffer and epithelial DNA from the sperm pellet.

Additional Resources for Automated Differex™ System

Technical Bulletins and Manuals

- EP030 *Automated Differex™ System Protocol for the Tecan Freedom EVO® System*
(www.promega.com/tbs/ep030/ep030.html)
- EP031 *Automated Differex™ System Protocol for the Beckman Coulter Biomek® 2000*
(www.promega.com/tbs/ep031/ep031.html)
- EP032 *Automated Differex™ System Protocol for the Beckman Coulter Biomek® 3000*
(www.promega.com/tbs/ep032/ep032.html)

Promega Publications

GIN024 Automating the Differex™ System.
(www.promega.com/profiles/1002/1002_19.html)

E. Automated DNA Normalization and PCR Setup

Promega has worked in conjunction with Beckman Coulter to develop a tool for DNA normalization and PCR setup on the Biomek® 2000 workstation. The Genetic Identity version of the Normalization Wizard, available from Beckman Coulter, dilutes DNA samples at different concentrations to the desired final concentration, then assembles the amplification reactions. The robot transfers PCR master mix to strip tubes or plates, then adds a fixed volume of the normalized DNA. The samples are capped manually and placed directly in a thermal cycler for amplification.

While this automation section focuses on the Beckman Coulter Biomek® 2000 and 3000 laboratory automation workstations and Tecan Freedom EVO® liquid handlers, automation users and other instrument suppliers have developed their own methods for human DNA quantitation, normalization and PCR setup using other instruments (www.promega.com/applications/hmndid/automation/automation_sp.htm). Though these methods have not been validated by Promega scientists, they might be of interest to those involved in automation. For example, PerkinElmer has developed methods for DNA quantitation, subsequent normalization and PCR setup on the JANUS® Automated Workstation (www.promega.com/applications/hmndid/automation/automation_qnp.htm).

Additional Resources for DNA Normalization and PCR Setup**Promega Publications**

GIN018 Automated DNA normalization and STR multiplex setup methods.
(www.promega.com/profiles/702/702_11.html)

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STR loci are the subject of U.S. Pat. No. RE 37,984, German Pat. No. DE 38 34 636 C2 and other patents issued to the Max-Planck-Gesellschaft zur Förderung der Wissenschaften, e.V., Germany. The development and use of STR loci are covered by U.S. Pat. No. 5,364,759, Australian Pat. No. 670231 and other pending patents assigned to Baylor College of Medicine, Houston, Texas. Patents for the foundational PCR process, European Pat. Nos. 201,184 and 200,362, expired on March 28, 2006. In the U.S., the patents covering the foundational PCR process expired on March 29, 2005.

The purchase of this product does not convey a license to use AmpliTaq Gold® DNA polymerase. You should purchase AmpliTaq Gold® DNA polymerase licensed for the forensic and human identity field directly from your authorized enzyme supplier.

U.S. Pat. Nos. 6,238,863 and 6,767,703, Korean Pat. No. 691195 and other patents pending.

U.S. Pat. Nos. 5,843,660, 6,479,235, 6,221,598 and 7,008,771, Australian Pat. No. 724531, Canadian Pat. No. 2,118,048, Korean Pat. No. 290332, Singapore Pat. No. 57050, Japanese Pat. No. 3602142, Chinese Pat. No. 10366753, European Pat. No. 0960207 and other patents pending.

Allele sequences for one or more of the loci vWA, FGA, D8S1179, D21S11 and D18S51 in allelic ladder mixtures is licensed under U.S. Pat. No. 7,087,380, Australia Pat. No. 2003200444 and corresponding patent claims outside the US.

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