How to Choose a Cell Health Assay

Choosing the Right Cell Health Assay Depends on What You Want to Measure

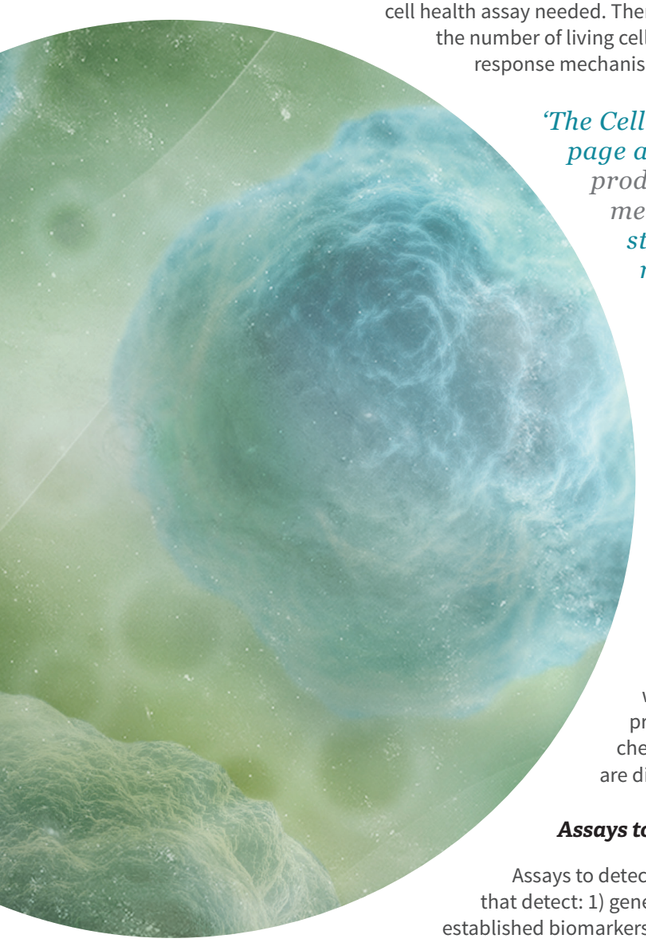
by Terry Riss

Introduction

Promega has a large portfolio of cell health assays, which has grown significantly since the introduction of the CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) in 1991. With so many excellent choices in plate-based assays, choosing the most appropriate one for your experiment can be difficult. Once you have identified the question you are trying to answer, selecting the appropriate assay is much easier.

What Question Are you Asking?

The first, and perhaps most important, factor to consider when choosing a cell health assay is: What is it exactly that you want to know at the end of the experiment? This answer will dictate at least the class of cell health assay needed. There are cell health assays available to specifically detect the number of living cells, the number of dead cells, and for assessing stress response mechanisms or pathways that may eventually lead to cell death.



'The Cell Health and Metabolism page at www.promega.com/products/cell-health-and-metabolism is an excellent starting point when you are ready to choose an assay.'

The second most important factor is having an understanding of the model system to be used. This is critical to designing an effective and informative assay protocol. Cells in culture represent a heterogeneous population of individuals in different phases of the cell cycle. Exposing a population of cells in culture to a toxin or other treatment may result in the appearance of markers of cell stress or death that transiently appear and then disappear. Using known positive and negative controls helps to establish a general understanding of the physiological condition of the cells throughout the course of the experiment. Periodic observation of cell morphology or measurement of markers using reagents compatible with living cultures (real-time analysis reagents) can provide early hints for when to implement endpoint assay chemistries (1). Cells in culture are only a model system and are different than cells in their normal in vivo environment.

Assays to Detect the Number of Living Cells

Assays to detect living cells fall into three general categories: Assays that detect: 1) general metabolic activity, 2) enzymatic activity, and 3) established biomarkers of viable cells. These assays are summarized in Table 1.

Table 1. Assays to Detect the Number of Living Cells.

	Definition	Comments
General Metabolic Activity	A property of viable cells that can be detected by the conversion of tetrazolium or resazurin substrates into colored or fluorescent products.	The **CellTiter 96® AQ_{ueous} One Solution Cell Proliferation** and **CellTiter-Blue® Cell Viability Assays** are suitable for many situations. The classic MTT and MTS tetrazolium assays and resazurin-based assays are widely used and suitable for many applications. Disadvantages that are often overlooked include interference by reducing compounds and general toxicity to cells due to the nature of their mechanism of action, which uses up cellular reducing equivalents (e.g., NADH) to convert the substrate into the colored or fluorescent product. The MTT approach also requires more than one addition of reagent to samples and subsequent incubation of cells to generate a signal (2).
Enzymatic Activity	Detecting an enzymatic activity that is present only when cells are alive. An example of this approach is using a cell-permeable protease substrate conjugated to a fluorophore (Gly-Phe-AFC) that only viable cells process to release the fluorescent product aminofluorocoumarin (AFC, 3). When cells die, the aminopeptidase activity disappears; thus only viable cells generate a signal.	This approach, exemplified by the **CellTiter-Fluor™ Cell Viability Assay**, is homogeneous, more sensitive, less toxic than tetrazolium or resazurin reduction assays (3), and requires a shorter incubation period with cells to generate a signal. In addition, because the cells remain viable after exposure to GF-AFC, the samples can be processed with additional assay chemistries for multiplex detection of other parameters (4).
Established Viable Cell Biomarkers	The luminescent ATP quantitation assay is an established approach for measuring viable cells.	**The CellTiter-Glo® Luminescent Cell Viability Assay**: This is often the best choice for measuring cell viability in multiwell plates. It has a single-reagent addition, homogeneous protocol, fast 10-minute incubation, gives the greatest sensitivity due to the luminescent nature of the detection system, and has the least interference from chemical compounds (5). A major advantage of the ATP assay is addition of detergent-and ATPase-containng reagent that immediately lyses the cells and stabilizes the ATP. There is no need for a 37°C incubation period with viable cells to allow for conversion of a substrate into a measurable product like the above-mentioned viability assays. See the article on page 10 describing the convenient new CellTiter-Glo® Reagent that eliminates reagent preparation.

Assays to Detect the Number of Dead Cells

Assays to detect dead cells fall into two general categories: Assays that detect: 1) enzymatic activity in the culture medium due to a compromised ('leaky') cytoplasmic membrane, and 2) fluorescent probes that only enter and stain dead cells with a compromised membrane. These assays are summarized in Table 2.

Table 2. Assays to Detect the Number of Dead Cells.

	Definition	Comments
Enzymatic Activity from Compromised ('Leaky') Cytoplasmic Membranes	Assay approaches are based on leakage of enzymes such as lactate dehydrogenase (LDH) or protease into culture medium. The disadvantage of enzyme release assays is the inherent instability of the marker enzymes after release into the culture medium. Appropriate controls should be used to account for the loss of released enzyme activity over time, especially for protocols where the endpoint is measured days after beginning experimental treatment.	**CytoTox-ONE™ Homogeneous Membrane Integrity Assay** (fluorescent) and **CytoTox 96® Non-Radioactive Cytotoxicity Assay** (colorimetric): In this format, a reagent containing an excess of pyruvate and NAD+ drives the LDH reaction to form NADH. This metabolite in turn is used to reduce a redox indicator molecule into a colored or fluorescent product (6). **CytoTox-Fluor™** and **CytoTox-Glo™ Cytotoxicity Assays:** Similarly, the leakage of cytoplasmic protease activity into the culture medium can be measured by using an impermeable protease substrate (such as bis-Ala-Ala-Phe-R110 or Ala-Ala-Phe-aminoluciferin) that does not enter the cytoplasm of viable cells to detect intracellular activity. Protease that leaks from cells with compromised membranes generates free rhodamine 110 (R110) or aminoluciferin, which can be measured using a plate reader (7).
Dead Cell Fluorescent Probes	Assay approaches are based on non-permeable fluorescent dyes that selectively stain dead cells (cannot penetrate cytoplasmic membrane of living cells). DNA staining is the predominant target because of the relatively constant amount in normal somatic cells.	**CellTox™ Green Cytotoxicity Assay:** Uses a non-permeable dye that is nontoxic to viable cells. The environmentally sensitive dye enters cells with a compromised membrane, binds to DNA, and results in a fluorescent signal. This dye has been developed into a "real-time" detection reagent that can be added to cells in culture for at least three days and fluorescence monitored repeatedly over time to detect appearance of dead cells in the population (1). The first appearance of fluorescence in samples indicates the onset of a cell death process in the population of cells. The fluorescent assay is compatible with many multiplexing options. The mechanism leading to cell death can be detected with a variety of other compatible assay chemistries applied to the same sample.

HOW TO CHOOSE A CELL HEALTH ASSAY

How Did My Cells Die? (Methods of Detecting Mechanisms That Lead to Cell Death)

Multiple assays have been developed to detect the cellular mechanisms that lead to cell death. The predominant classes of assays are based on the detection of: 1) apoptosis, 2) oxidative stress, 3) mitochondrial selective toxins, and 4) toxicity pathway analysis (gene expression resulting from initiation of stress response pathways). Approaches to measuring the mechanism of cell death are summarized in Table 3.

Table 3. Methods of Detecting Mechanisms That Lead to Cell Death.

	Definition	Comments
Apoptosis	Measurement of caspase 3/7 activity is the most widely used plate-based marker for measuring apoptosis. Homogeneous "add-mix-measure" assays based on cleavage of a four amino acid peptide (DEVD) from an indicator molecule are available with fluorescent or luminescent output.	**Caspase-Glo® 3/7 Assay Systems:** The luminescent assay is the fastest, has the least amount of interference, and the sensitivity is adequate for 96-, 384-, or 1536-well plate formats (8). Multiplex options exist with fluorescent caspase, viability or cytotoxicity assays. **Caspase-Glo® 8** and **9** assays are also available for determining the pathway of apoptosis induction. **ApoONE® Caspase-3/7 Assay:** Based on the cleavage of a bis-DEVD-R110 substrate, resulting in fluorescence. It is the most sensitive fluorescent caspase-3/7 assay and can be multiplexed with other compatible luminescent or fluorescent assays.
Oxidative Stress	Oxidative stress is an imbalance between the production of ROS and the cell's capacity to detoxify the ROS or to repair the oxidative damage. Most cellular ROS (super oxide, hydroxyl radical, nitric oxide, hypochlorite) are converted to hydrogen peroxide (H_2O_2), which is more stable and often measured as a general marker of oxidative stress. The reduced form of glutathione (GSH) serves as an antioxidant in cells, and detecting a decrease in GSH concentration is often used as a marker of oxidative stress (9). Rather than simply measuring a decrease in the level of GSH, the ratio of the reduced and oxidized forms of glutathione (GSH:GSSG) can be measured to more accurately reflect the extent of oxidative stress.	**ROS-Glo™ H_2O_2 Assay:** A luminogenic assay chemistry has recently been developed that is based on processing of a H_2O_2 sensing pro-luciferin substrate molecule that is converted to luciferin used to generate a signal. The luminescent assay is the method of choice to measure ROS because it is more robust and has far less chemical interference compared to fluorescent methods using Amplex Red and horseradish peroxidase (10). The cell-based ROS assay is performed directly in culture wells and also can be performed by sampling the culture medium, which provides flexibility for measuring other parameters from the same sample (e.g., cell viability). **GSH-Glo™ Glutathione Assay:** This luminescent assay procedure enables measurement (9). **GSH/GSSG-Glo™ Assay:** This luminescent assay measures and can be used to calculate the GSH:GSSG ratio (11).

Mitochondrial Selective Toxins	Mitochondrial selective toxins can be detected by measuring a decrease in ATP production without loss of membrane integrity (i.e., cell death).	**Mitochondrial ToxGlo™ Assay:** In order to eliminate the net contribution of ATP from glycolysis, cells are incubated in glucose-free, serum-free medium and exposed to test compounds for short exposure periods (up to 4 hours). Cell membrane integrity is measured first by detecting leakage of protease from cells with damaged membranes. Next, ATP is measured using the luminescent assay. The two sets of data can be combined to produce profiles representative of mitochondrial dysfunction or non-mitochondrial-related cytotoxic mechanisms (12).
Toxicity Pathway Analysis	Measuring the expression of various genes resulting from initiation of stress response pathways is a common approach to assessing the mechanism of cell death. When cells detect a sub-lethal toxic insult from the environment, signaling pathways are triggered that result in gene transcription and expression of proteins to react to the toxin. The pathways triggered depend on the class of toxic event, and thus measurement of various response elements and pathways can provide useful information on the class of toxin.	Bioluminescence-based reporter gene assays that measure genetic response element activity are well suited to this approach. A variety of vectors are available that report the activity of signaling pathways using the *luc2* firefly luciferase gene. Promega has designed and validated a panel of ready-to-use response element-containing reporter vectors that target the key pathways involved in cellular stress response. See the article “Using pGL4 Reporter Vector Panels to Profile Chemical Toxicity” in this book on page 14.

What Else Must Be Considered?

There are several basic parameters that need to be considered when choosing the most appropriate assay to measure cell health. The main parameters include: 1) the number of samples being tested, 2) the sensitivity required, 3) the nature of the sample, 4) plates and plate readers, and 5) reagent costs. These additional parameters are summarized in Table 4.

Table 4. Additional Requirements to Consider When Choosing a Cellular Health Assay.

Requirement	Description
Number of Samples	If only one or a few samples need to be measured, manual counting of live or dead cells using a hemacytometer may be adequate. If large numbers of samples will be measured, assay procedures that use a homogeneous add-mix-measure approach are the most efficient. For screening thousands of samples, it is more efficient to choose assays with adequate sensitivity to allow miniaturization into high-density plate formats (384- or 1536-well).
Sensitivity	The sensitivity required is closely linked to the plate format (96-, 384-, 1536-well) and ultimately the number of cells used per sample. In general, the luminescent endpoint assays are more sensitive than assays based on detecting fluorescence or absorbance because of the minimal background luminescence that results in high signal:noise ratios.
Assay Volume	The volume of assay reagent added to cell culture needs to be considered. It should fit the plate well size, and if desired for HTS formats, the chemistry should be scalable.
Nature of the Sample	What is the sample and in vitro culture model system being tested? Most currently available assays were designed using cells grown as monolayers or in suspension culture. However, there is a growing trend toward culturing cells in 3D model systems to achieve a more physiologically relevant environment. Culturing cells in a 3D architecture and co-culture of interacting cell types are approaches to improve the physiological relevance of model systems. Microtissues composed of cells grown in 3D culture may require special procedures or reagent formulations to ensure complete cell lysis prior to assay. Modified assay protocols that incorporate a plate shaking step to accelerate physical disruption of large microtissues have been developed for use with ATP and caspase assays. Because the nature of the sample can vary depending on cell type and size of microtissue, the assay procedure should be validated for each culture model system.
Plates and Plate Readers	**Plates:** If microscopic observation of cells is desired, clear-bottom plates are required. In general, opaque black plates are used for reduced background for fluorescent assays, and opaque white plates are used for optimum light output for luminescent assays. However, the signal strength from most luminescent assays is adequate such that black plates can be used. Using black plates for luminescent assays provides the most flexibility for combining fluorescent and luminescent assays from the same sample (‘multiplexing’, 13). **Plate Readers:** Instruments recording absorbance, fluorescence or luminescence are the most commonly used with cell health assays. Many modern plate readers have the capability of recording multiple modalities (13). Having an appropriate instrument available is occasionally overlooked the first time a new assay is evaluated. In addition, having the correct excitation and emission filter set(s) to achieve optimum performance of fluorescent assays is also necessary when performing cell-based assays. See the article “Getting the Most From Your Plate-Based Assays” on page 20.)
Reagent Costs	Usually there is a trade-off between the cost of the reagent and the quality of the assay or the convenience it provides the end user. Less costly reagents often have more complex procedures, limited sensitivity or toxicity to cells in culture, and take longer to perform. Despite those general disadvantages, there may be many applications where less costly assays are suitable; however, care should be taken to avoid reagents that are cytotoxic where they can affect the outcome or limit the ability to multiplex with other assays (2). quality controlled reagents and assay kits are generally more expensive but often save time and expense.

Table 5. Multiplexing Compatibilities of Promega Cell Health Assays.

Downstream Assay	*CellTiter-Fluor™ Cell Viability Assay*	*CytoTox-Fluor™ Cytotoxicity Assay*	*MultiTox-Fluor Multiplex Cytotoxicity Assay*	*CellTox™ Green Cytotoxicity Assay*
ONE-Glo™ Luciferase Assay System	ONE-Glo™ + Tox Luciferase Reporter and Cell Viability Assay	Yes	Yes	Yes
Bright-Glo™ Luciferase Assay System	Yes	Yes	Yes	Yes
Steady-Glo® Luciferase Assay System	Yes	Yes	Yes	Yes
Renilla-Glo® Luciferase Assay System	Yes	Yes	Yes	Yes
Beta-Glo® Assay System	Yes	Yes	Yes	Yes
Caspase-Glo® 3/7 Assay	ApoLive-Glo™ Multiplex Assay	Yes	ApoTox-Glo™ Triplex Assay	Yes
Caspase-Glo® 8 or 9 Assay	Yes	Yes	Yes	Yes
Apo-ONE® Caspase-3/7 Assay	Yes	No	No	No
HDAC-Glo™ I/II Assay	Yes	Yes	Yes	Yes
GSH/GSSG-Glo™ Assay	Yes	Yes	Yes	Yes
P450-Glo™ Cell-Based Assays	Yes	Yes	Yes	Yes
CellTiter-Glo® Cell Viability Assay	Yes	Mitochondrial ToxGlo™ Assay	Yes	Yes
Ros-Glo™ H_2O_2 Assay	Yes	Yes	Yes	Yes

Can I Combine Assays to Get More Information from a Single Experiment?

Multiplexing more than one assay is a versatile approach that can provide more information from a single sample such as simultaneously measuring cell stress response pathway events and the mechanism of cell death. Multiplexing requires compatibility of the assay chemistries being used and the ability to distinguish separate signals using different detection wavelengths or detecting different modalities such as fluorescence and luminescence. For compatible assay chemistries, multiplexing can be accomplished simultaneously in the same sample well. For other assay combinations, measurements must be done in a sequential manner. Still other multiplexing options can be enabled by separating a portion of the sample to a different container to overcome assay chemistry incompatibility, instrument requirements or plate format (e.g., clear vs. opaque plastic). A summary of Promega cell health assays that are compatible for multiplexing is shown in Table 5.

Summary

Selecting the most appropriate cell health assay can appear complicated given all the possible choices available. To simplify the selection process, we suggest having a clear idea of exactly what data you require at the end of the experiment and a general understanding of how the various assay chemistries work. Availability of an appropriate instrument, desired throughput, the nature of the sample and cost are additional key considerations.

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