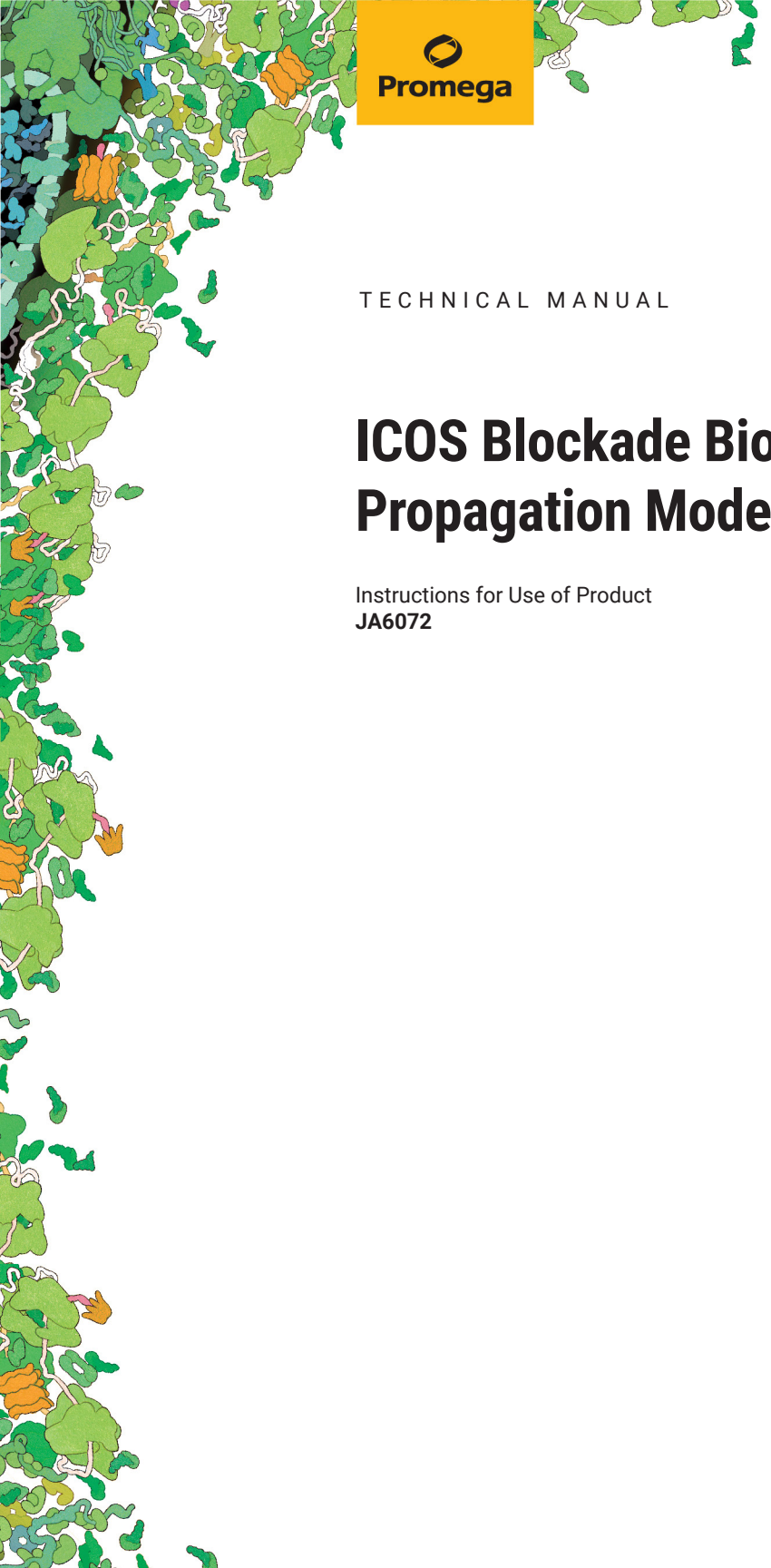

TECHNICAL MANUAL

ICOS Blockade Bioassay, Propagation Model

Instructions for Use of Product
JA6072

Revised 4/25
TM630

Promega

ICOS Blockade Bioassay, Propagation Model

All technical literature is available at: www.promega.com/protocols/
Visit the website to verify that you are using the most current version of this Technical Manual.
Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

The human immune system is regulated by a complex network of inhibitory and stimulatory receptors that facilitate the elimination of pathogens, while maintaining tolerance to self-antigens. T cells play a central role in cell-mediated immunity against pathogens; however, T cells also contribute to the pathogenesis and exacerbation of autoimmune disorders.

Optimal activation of T cells is initiated by engagement of the T cell antigen receptor (TCR)/CD3 complex and activation of a costimulatory receptor such as Inducible T Cell Costimulator (ICOS, CD278) (1–3). ICOS binds to its ligand ICOSL (B7-H2, CD275), which is constitutively expressed on B cells, monocytes and dendritic cells, and can be induced on endothelial and epithelial cells during inflammation (4–7). ICOS costimulation induces the production of effector T cell cytokines such as interferon (IFN)-γ, interleukin (IL)-4 and IL-10 (1–3).

Blockade of ICOS or ICOSL has been investigated in preclinical models of allergy, autoimmunity and alloimmunity. Specifically, blocking antibodies directed to ICOS reduced experimental graft-versus-host disease (GVHD) and graft rejection (8,9). In addition, ICOS blockade reduced the severity of experimental autoimmune arthritis and experimental allergic encephalomyelitis (10,11). An inhibitor of ICOSL is currently in clinical trials for the treatment of systemic lupus erythematosus.

There are no easy-to-use, quantitative, functional bioassays available to measure the in vitro potency of biologics designed to block ICOS/ICOSL. Current methods rely on primary human T cells and antigen-presenting cells (APCs), and measurement of functional endpoints such as cell proliferation, cell surface marker expression and cytokine production. These assays are laborious and highly variable due to their reliance on donor cells, complex assay protocols and unqualified assay reagents. Current methods are, as a result, difficult to establish in a quality-controlled setting.

The ICOS Blockade Bioassay, Propagation Model[a–c] (Cat.# JA6072), is a bioluminescent cell-based assay that overcomes the limitations of existing assays. It can be used to measure the potency and stability of antibodies and other biologics that block ICOS/ICOSL. The assay consists of two genetically engineered cell lines:

- **ICOS Effector Cells:** Jurkat T cells expressing ICOS and endogenous TCR/CD3 and a NanoLuc® (NL) luciferase reporter driven by ICOS and TCR/CD3 pathway-dependent response elements.
- **ICOSL aAPC/CHO-K1 Cells:** CHO-K1 cells expressing an engineered cell surface protein designed to activate TCR/CD3 in an antigen-independent manner, and ICOSL.

The ICOS Effector Cells and ICOSL aAPC/CHO-K1 Cells are provided in Cell Propagation Model (CPM) format, which includes cryopreserved cells that can be thawed, propagated and banked for long-term use.

When the two cell types are cocultured, the ICOSL aAPC/CHO-K1 Cells activate TCR/CD3 and ICOS on the ICOS Effector Cells to induce maximum promoter-mediated luminescence. Adding a biologic that blocks ICOS/ICOSL inhibits costimulation by ICOS and results in decreased promoter-mediated luminescence (Figure 1). The bioluminescent signal is quantified using the Bio-Glo-NL™ Luciferase Assay System (Cat.# J3081), and a standard luminometer such as the GloMax® Discover System (see Related Products, Section 9.C).

The ICOS Blockade Bioassay reflects the mechanism of action (MOA) of biologics designed to block ICOS/ICOSL interactions (Figure 1). Specifically, ICOS-mediated luminescence activation is reduced following the addition of an ICOS blocking biologic but not following addition of anti-TIGIT or anti-4-1BB blocking Abs (Figure 2). The bioassay is prequalified following International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines and shows the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 3). The bioassay is performed in a two-day timeframe, and the workflow is simple, robust and compatible with both 96-well and 384-well plate formats used for antibody screening in early drug discovery (Figure 4). In addition, the bioassay can be used with up to 100% human serum (in antibody samples; Figure 5), indicating potential for further development into a neutralizing antibody bioassay.

In addition to the ICOS Blockade Bioassay, we offer Control Ab, Anti-ICOS (Cat.# K1241) for use as a positive control.

Activation of ICOS by agonist antibodies is a separate immunotherapy strategy to activate the immune system. The ICOS Blockade Bioassay is not designed to detect activation of ICOS antibodies. We also offer the ICOS Bioassay, Propagation Model in cell propagation model (Cat.# JA3072) and thaw-and-use (Cat.# JA6801, JA6805) formats for screening and potency testing of ICOS agonists.

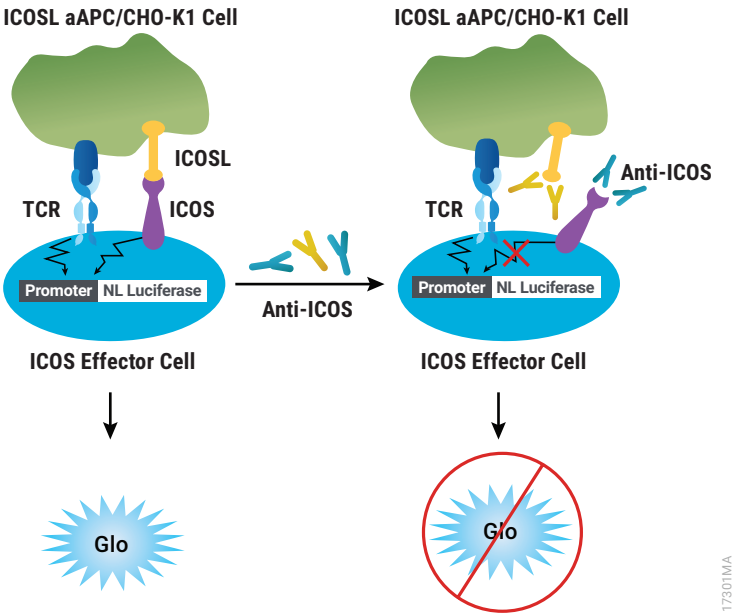

Figure 1. Representation of the ICOS Blockade Bioassay. The ICOS Blockade Bioassay consists of two cell lines, ICOS Effector Cells and ICOSL aAPC/CHO-K1 Cells. When cocultured, ICOSL aAPC/CHO-K1 Cells activate TCR/CD3 and ICOS on the ICOS Effector Cells to induce maximum promoter-mediated luminescence. Adding a biologic that blocks ICOS/ICOSL inhibits T cell costimulation by ICOS and results in decreased promoter-mediated luminescence. This decrease in luminescence can be detected in a dose-dependent manner by adding Bio-Glo-NL™ Reagent and quantitating with a luminometer.

1. Description (continued)

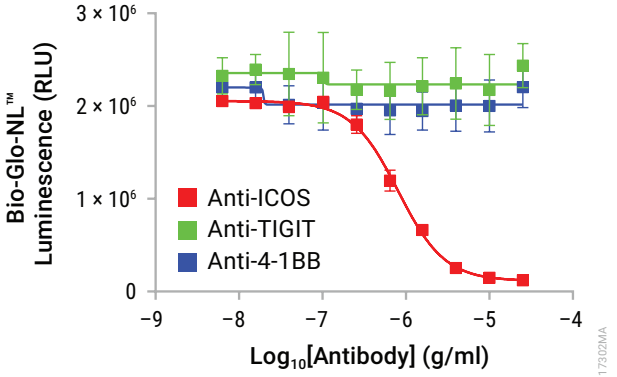

Figure 2. The ICOS Blockade Bioassay reflects the mechanism of action (MOA) and shows specificity for antibodies designed to block ICOS/ICOSL interaction. ICOS Effector Cells were incubated with ICOSL aAPC/CHO-K1 Cells in the presence of serial titrations of blocking Abs as indicated. After a 6-hour induction, Bio-Glo-NL™ Reagent was added, and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

Table 1. The ICOS Blockade Bioassay Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	53.0
	70	71.0
	100	101.5
	140	145.1
	200	205.2
Repeatability (% CV)	100% (Reference)	5.6
Intermediate Precision (% CV)		6.5
Linearity (r^2)		0.9996
Linearity (y = mx + b)		y = 1.025x – 0.3804

A 50–200% theoretical potency series of Control Ab, Anti-ICOS, was analyzed in triplicate in three independent experiments performed on three days by two analysts using the ICOS Blockade Bioassay. Bio-Glo-NL™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were analyzed and relative potencies were calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.

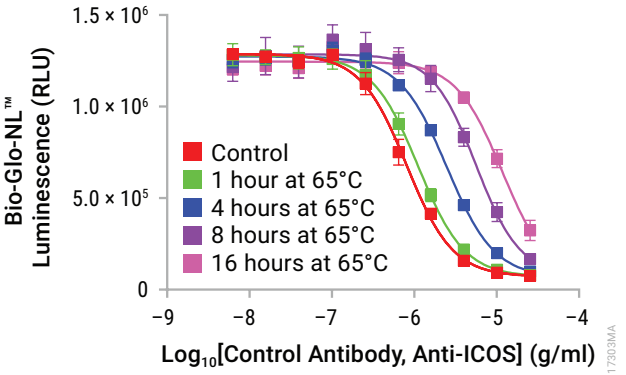

Figure 3. The ICOS Blockade Bioassay is stability-indicating. Samples of Control Ab, Anti-ICOS (Cat.# K1241) were maintained at 4°C (control) or heat-treated at the indicated times and temperatures, then analyzed using the ICOS Blockade Bioassay. Bio-Glo-NL™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

1. Description (continued)

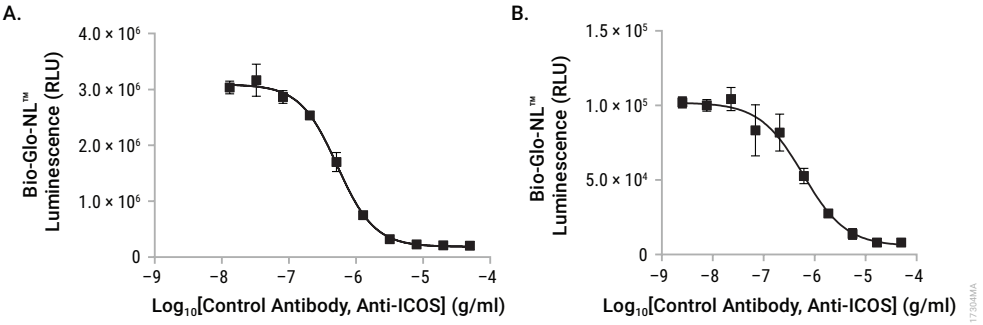

Figure 4. The ICOS Blockade Bioassay is amenable to 384-well plate format. Panel A. The ICOS Blockade Bioassay was performed in 96-well plates as described in this technical manual with a titration of Control Ab, Anti-ICOS (Cat.# K1241). **Panel B.** The ICOS Blockade Bioassay was performed in 384-well format as described here. ICOSL aAPC/CHO-K1 cells were harvested, and 20µl of cells plated at 8×10^3 cells/well 16–24 hours prior to assay, in a 384-well white assay plate (e.g., Corning® Cat.# 3570). On the day of the assay, 5µl of 5X serially diluted Control Ab, Anti-ICOS was added, followed by addition of 5µl of ICOS Effector Cells at 2×10^4 cells/well. After a 6-hour incubation at 37°C, 5% CO_2, 25µl Bio-Glo-NL™ Reagent was added per well and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The IC_{50} values were 0.53 and 0.59µg/ml for 96-well and 384-well format, respectively, and the percent maximal blocking was 94% and 93% for 96-well and 384-well format, respectively. Data were generated using thaw-and-use cells.

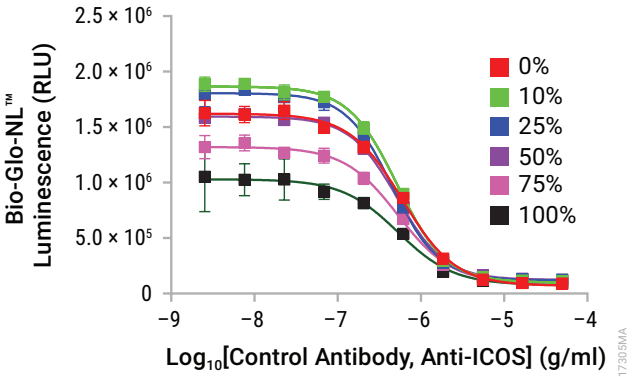

Figure 5. The ICOS Blockade Bioassay is tolerant to human serum. Control Ab, Anti-ICOS (Cat.#K1241) was analyzed in the absence or presence of pooled normal human serum (0–100% in the antibody sample). After the 6-hour assay induction at 37°C, Bio-Glo-NL™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells. The ICOS Blockade Bioassay is tolerant to this human serum pool. A different human serum pool showed similar effects on the assay (data not shown).

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
ICOS Blockade Bioassay, Propagation Model	**1 each**	**JA6072**

Not for Medical Diagnostic Use. Includes:

- 2 vials ICOS Effector Cells (CPM), 3.2×10^7 cells/ml (1.0ml per vial)
- 2 vials ICOSL aAPC/CHO-K1 Cells (CPM), 1.2×10^7 cells/ml (1.0ml per vial)

Note: Thaw and propagate one vial to create frozen cell banks before use in an assay. The second vial should be reserved for future use.

Storage Conditions: Upon arrival, immediately transfer the cell vials to below –140°C (freezer or liquid nitrogen vapor phase) for long-term storage. Do not store cell vials submerged in liquid nitrogen. **Do not** store cell vials at –80°C because this will decrease cell viability and cell performance.

3. Before You Begin

Please read through the entire protocol to become familiar with the components and the assay procedure before beginning.

Note the catalog number and lot number from the cell vial box label. This information can be used to download documents for the specified product from the website, such as Certificate of Analysis.

Note: The ICOS Blockade Bioassay uses Bio-Glo-NL™ Luciferase Assay System (Cat.# J3081, J3082, J3083) for detection. **Do not** use Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941).

The ICOS Blockade Bioassay, Propagation Model, is intended to be used with user-provided antibodies or other biologics designed to block ICOS/ICOSL. Control Ab, Anti-ICOS (Cat.# K1241), is available separately for use in assay optimization and routine quality control. We strongly recommend including Control Ab, Anti-ICOS as a positive control in the first few assays to gain familiarity with the assay. Data generated using Control Ab, Anti-ICOS are shown in Section 9.A, Representative Assay Results.

Cell thawing, propagation and banking should be performed exactly as described in Sections 4 and 5. Cell seeding and propagation densities have been optimized to ensure stable cell growth, which is reflected in a steady cell doubling rate, to achieve optimal, consistent performance. An accurate, reliable and reproducible cell counting method is required for routine cell culturing and optimal bioassay performance.

The recommended cell plating densities, induction time and assay buffer components described in Section 6 were established using Control Ab, Anti-ICOS. You may need to adjust the parameters provided here and optimize assay conditions for your own antibody or biologic samples.

The ICOS Blockade Bioassay produces a bioluminescent signal and requires a sensitive luminescence plate reader. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System (see Section 9.C, Related Products). An integration time of 0.5 second/well was used for all readings. The bioassay is compatible with most other plate-reading luminometers, though relative luminescence unit (RLU) readings

will vary with the sensitivity and settings of each instrument. If using a reader with adjustable gain, we recommend a high-gain setting. The use of different instruments and gain adjustment will affect the magnitude of the raw data but should not affect the measured relative potency of test samples.

3.A. Materials to Be Supplied by the User

(Composition of Buffers and Solutions is provided in Section 8.C.)

Reagents

- user-defined anti-ICOS antibodies or other biologics samples
- RPMI 1640 Medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO® Cat.# 22400105)
- Ham's F-12 Medium with L-glutamine (e.g., GIBCO® Cat.# 11765062)
- fetal bovine serum (e.g., VWR Cat.# 89510-194, GIBCO® Cat.# 35-015-CV or HyClone Cat.# SH30071.03)
- hygromycin B (e.g., GIBCO® Cat.# 10687010)
- G418 Geneticin (e.g., GIBCO® Cat.# 10131035)
- blasticidin S HCl (e.g., GIBCO® Cat.# A11139)
- sodium pyruvate (e.g., GIBCO® Cat.# 11360070)
- MEM nonessential amino acids, 100X (e.g., GIBCO® Cat.# 11140050)
- DMSO (e.g., Sigma Cat.# D2650)
- Accutase® solution (e.g., Sigma Cat.# A6964) for lifting CHO-K1 Cells
- DPBS (e.g., GIBCO® Cat.# 14190144) for washing CHO-K1 Cells
- Bio-Glo-NL™ Luciferase Assay System (Cat.# J3081, J3082, J3083)
- **optional:** Control Ab, Anti-ICOS (Cat.# K1241)

Supplies and Equipment

- solid-white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917) or 384-well assay plates (e.g., Corning® Cat.# 3570) for plating and reading luminescence
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896) for preparing antibody dilutions
- pipettes (single-channel and 12-channel; for best results use both manual and electronic pipettes as needed)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning®/Costar® Cat.# 4870)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent)

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4. Preparing ICOS Effector Cells

Follow institutional guidelines for handling, including use of personal protective equipment (PPE) and waste disposal for biohazardous material.

4.A. Cell Thawing and Initial Cell Culture

1. Prepare 40ml of initial cell culture medium by adding 4ml of FBS to 36ml of RPMI 1640 medium prewarmed to 37°C. This initial cell culture medium will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of prewarmed initial cell culture medium to a 50ml conical tube.
3. Remove one vial of ICOS Effector Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed (typically 2–3 minutes).
4. Transfer all of the cells (approximately 1ml) to the 50ml conical tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 90 × *g* for 10 minutes.
6. Carefully aspirate the medium and resuspend the cell pellet in 25ml of prewarmed initial cell culture medium.
7. Transfer the cell suspension to a T75 tissue culture flask and place the flask horizontally in a humidified 37°C, 5% CO_2 incubator.
8. Incubate for approximately 48 hours before passaging the cells.

4.B. Cell Maintenance and Propagation

Note: For cell maintenance and propagation starting from the second cell passage, use the cell growth medium containing antibiotics and monitor cell viability and doubling rate during propagation. The cell growth rate will stabilize by 7–10 days after thawing, at which time cell viability is typically >90%, and the average cell doubling rate is 22–26 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 25 passages, or 46 cell doublings, if passaging is performed on a Monday-Wednesday-Friday schedule.

1. On the day of cell passage, measure cell viability and density by Trypan blue staining.
2. Seed the cells at a density of 4.5×10^5 cells/ml if passaging every two days (e.g., Monday-Wednesday or Wednesday-Friday) or 2.5×10^5 cells/ml if passaging every three days (e.g., Friday-Monday). Always maintain the flasks in a horizontal position in the incubator. Do not allow the cells to grow to a density greater than 2×10^6 cells/ml.
3. Maintain the cell culture by adding fresh cell growth medium to the cell suspension in the original flask or by transferring the cells to a new flask while maintaining a consistent ratio of culture volume to flask surface area (e.g., 25ml volume per T75 flask or 50ml volume per T150 flask).
4. Place the flasks horizontally in a humidified, 37°C, 5% CO_2 incubator.

4.C. Cell Freezing and Banking

1. On the day of cell freezing, prepare fresh cell freezing medium and keep on ice.
2. Gently mix the cells with a pipette to create a homogenous cell suspension.
3. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of cell freezing medium needed based on desired cell freezing densities of 1×10^7–2×10^7 cells/ml.
4. Transfer the cell suspension to 50ml sterile conical tubes or larger sized centrifuge tubes, and centrifuge at 130 × *g*, 4°C, for 10–15 minutes.
5. Gently aspirate the medium, taking care not to disturb the cell pellet.
6. Carefully resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of 1×10^7– 3×10^7 cells/ml. Combine the cell suspensions into a single tube and dispense into cryovials.
7. Freeze the cells using a controlled-rate freezer (preferred), or a Mr. Frosty® or a Styrofoam® rack in a –80°C freezer overnight. Transfer the vials to –140°C or below for long-term storage.

5. Preparing ICOSL aAPC/CHO-K1 Cells

Note: This assay requires the ICOSL aAPC/CHO-K1 cell line, and is incompatible with any other aAPC/CHO-K1 cell line.

5.A. Cell Thawing and Initial Cell Culture

1. Prepare 50ml of initial cell culture medium by adding 5ml of FBS to 45ml of Ham's F12 medium prewarmed to 37°C. This initial cell culture medium will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of prewarmed initial cell culture medium to a 50ml conical tube.
3. Remove one vial of ICOSL aAPC/CHO-K1 cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed (typically 2–3 minutes).
4. Transfer all the cells (approximately 1ml) to the 50ml conical tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 90 × *g* for 10 minutes.
6. Carefully aspirate the medium and resuspend the cell pellet in 40ml of pre-warmed initial cell culture medium.
7. Transfer the cell suspension to a T150 tissue culture flask and place the flask horizontally in a 37°C, 5% CO_2 incubator.
8. Incubate for approximately 24 hours before passaging the cells.

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5.B. Cell Maintenance and Propagation

Note: For cell maintenance and propagation starting from the second cell passage, use the cell growth medium containing antibiotics and monitor cell viability and doubling rate during propagation. The cell growth rate will stabilize by 7–10 days post-thaw, at which time cell viability is typically >95%, and the average cell doubling rate is ~24 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 25 passages if passaging is performed on a Monday-Wednesday-Friday schedule.

1. On the day of cell passage, aspirate the cell culture medium and wash the cells with DPBS.
2. Add 2ml of Accutase® solution to each T75 flask and place in a 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.
3. Add 8ml of cell growth medium to each T75 flask. Transfer the cell suspension to a sterile 15ml or 50ml conical tube.
4. Count the cells by Trypan blue staining. We suggest seeding the cells at a density of 4×10^4 cells/cm^2 if passaging every two days (e.g., Monday-Wednesday or Wednesday-Friday) or 2×10^4 cells/cm^2 if passaging every three days (e.g., Friday-Monday)
5. Add an appropriate amount of cell growth medium to a new flask.
6. Transfer the appropriate volume of cell suspension to achieve the desired cell seeding density per area.
7. Place the flasks horizontally in a humidified 37°C, 5% CO_2 incubator.

5.C. Cell Freezing and Banking

1. On the day of cell freezing, make fresh cell freezing medium and keep on ice.
2. Aspirate the cell culture medium and wash the cells with DPBS.
3. Add 2ml of Accutase® solution to each T75 flask and place in a 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.
4. Add 8ml of cell growth medium to each T75 flask. Transfer the cell suspension to a sterile 15ml or 50ml conical tube.
5. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of cell freezing medium needed based on desired cell freezing densities of 3×10^6–2×10^7 cells/ml.
6. Transfer the cell suspension to 50ml sterile conical tubes or larger centrifuge tubes, and centrifuge for 10 minutes at 180 × *g*, 4°C.
7. Carefully aspirate the supernatant and avoid disturbing the cell pellet.
8. Gently resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of 3×10^6–1.2×10^7 cells/ml. Combine the cell suspensions into a single tube and dispense into cryovials.
9. Freeze the cells using a controlled-rate freezer (preferred), or a Mr. Frosty® or a Styrofoam® rack in a –80°C freezer overnight. Transfer the vials to at or below –140°C for long-term storage.

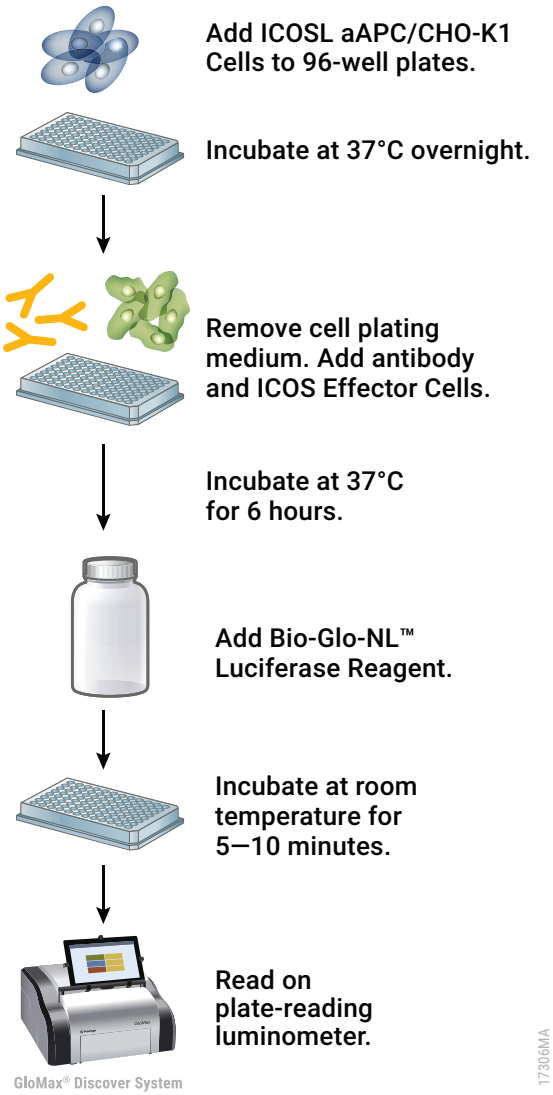

Figure 6. Schematic protocol for the ICOS Blockade Bioassay, Propagation Model.

6. Assay Protocol

The procedure below illustrates the use of the ICOS Blockade Bioassay to test two antibody samples against a reference sample in a single assay run. Each test and reference antibody is run in triplicate, in a 10-point dilution series, in a single 96-well assay plate using the inner 60 wells. Other experimental and plate layouts are possible but may require further optimization.

Note: When preparing test and reference antibodies, choose an appropriate starting concentration and dilution scheme to achieve a complete dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 50µg/ml as a starting concentration (1X) and 2.5-fold serial dilution when testing Control Ab, Anti-ICOS (Cat.# K1241).

6.A. Preparing Cell Plating Medium, Assay Buffer and Bio-Glo-NL™ Reagent

1. **ICOSL aAPC/CHO-K1 Cell Plating Medium:** On the day before the assay, prepare an appropriate amount of cell plating medium (90% Ham's F-12/10% FBS). Thaw the FBS overnight at 4°C or in a 37°C water bath on the day of use. Mix well and warm to 37°C before use. For reference, 40ml of cell plating medium is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

2. **Assay Buffer:** On the day of the assay, prepare an appropriate amount of assay buffer (99% RPMI 1640/1% FBS). Mix well and warm to 37°C before use. For reference, 30ml of assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

   Note: The recommended assay buffer contains 1% FBS. This concentration of FBS works well for the Control Ab, Anti-ICOS, that we tested. If you experience assay performance issues when using this assay buffer, we recommend testing different serum concentrations in the range of 0.5–10%.

3. **Bio-Glo-NL™ Luciferase Reagent:** For reference, 10ml of Bio-Glo-NL™ Reagent is sufficient to assay 120 wells in a 96-well assay format. The Bio-Glo-NL™ Luciferase Assay Substrate should always be stored at –20°C. Thaw the Bio-Glo-NL™ Luciferase Assay Buffer at room temperature (do not exceed 25°C) during the 6-hour induction period of the assay. We recommend preparing the reconstituted Bio-Glo-NL™ Reagent immediately before use. For instructions on use of Bio-Glo-NL™ Reagent, please refer to the *Bio-Glo-NL™ Luciferase Assay System Quick Protocol,* #FB227.

   Note: The ICOS Blockade Bioassay is compatible only with Bio-Glo-NL™ Luciferase Assay System (Cat.# J3081, J3082, J3083). **Do not** use the Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941) with the ICOS Blockade Bioassay.

4. **Test and Reference Samples:** Using assay buffer as the diluent, prepare stock starting dilutions (dilu1, 2X final concentration) of two test antibodies (250µl each) and one reference antibody (500µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Notes:

- If you are using Control Ab, Anti-ICOS (Cat.# K1241), as a reference antibody in your assay, prepare 500µl of starting dilution of 100µg/ml of Control Ab, Anti-ICOS (dilu1, 2X final concentration) by adding 50µl of Control Ab, Anti-ICOS (1 mg/ml stock) to 450µl of assay buffer.
- To streamline assay setup, prepare antibody serial dilutions prior to harvesting and plating ICOS Effector Cells (Figure 7).

6.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 7 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibody to generate two 10-point dose-response curves for each plate.

Recommended Plate Layout Design

	1	2	3	4	5	6	7	8	9	10	11	12	
A	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)
B	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
C	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
D	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
G	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 7. Example plate layout showing nonclustered sample locations of test antibody and reference antibody dilution series, and wells containing assay buffer (denoted by "B") alone.

6.C. Preparing and Plating ICOSL aAPC/CHO-K1 Cells

While maintaining the ICOSL aAPC/CHO-K1 Cells, follow the recommended cell seeding density (refer to Section 5 for culture instructions for ICOSL aAPC/CHO-K1 Cells, Propagation Model). Changes in cell culture volume or seeding density will affect cell growth rate and assay performance. Do not allow the cells to grow to 100% confluence. Only use the cells in the assay after the cell doubling rate has stabilized during propagation.

Note: Perform the following steps in a sterile cell culture hood.

1. We recommend passaging the ICOSL aAPC/CHO-K1 Cells two days before plating for the assay (as described in Section 5) to ensure optimal and consistent assay performance.
2. On the day before performing the assay, prepare ICOSL aAPC/CHO-K1 cell plating medium (90% Ham's F-12/10% FBS) for the ICOSL aAPC/CHO-K1 Cells.
3. Aspirate the cell culture medium from the ICOSL aAPC/CHO-K1 Cells and wash with DPBS.

6.C. Preparing and Plating ICOSL aAPC/CHO-K1 Cells (continued)

4. Add 2ml of Accutase® solution to each T75 flask, and place the flask in a 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.
5. Add 8ml of ICOSL aAPC/CHO-K1 cell plating medium to the flask. Transfer the cell suspension to a 50ml (or larger) conical centrifuge tube.
6. Gently mix and count the ICOSL aAPC/CHO-K1 Cells by Trypan blue staining.
7. Centrifuge at 230 × *g* for 10 minutes.
8. Gently resuspend the cell pellet in cell plating medium to achieve a concentration of 4×10^5 viable cells/ml.
9. Transfer the suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 100µl of the cell suspension to each of the inner 60 wells of a 96-well white flat-bottom assay plate. The final cell number in each well should be 4×10^4 cells/well.
10. Add 100µl of cell plating medium to each of the outside wells of the assay plates.
11. Place lids on the assay plates and incubate in a 37°C, 5% CO_2 incubator overnight (18–22 hours).

6.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparation of a single stock of 2.5-fold serial dilutions of a single antibody for analysis in triplicate (150µl of each dilution provides a sufficient volume for analysis in triplicate). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples. To prepare 2.5-fold serial dilutions, you will need 500µl of reference antibody at 2X the highest antibody concentration in your dose-response curve. You will need 250µl of each test antibody at 2X the highest antibody concentration in each of the test antibody dose-response curves. For other dilution schemes, adjust the volumes accordingly.

Note: If you are using Control Ab, Anti-ICOS (Cat.# K1241) as a control in the assay, follow the instructions below to prepare 2.5- fold serial dilutions. A 2.5-fold serial dilution for test antibodies is listed as an example below as well.

1. On the day of the assay, prepare an appropriate amount of assay buffer as described in Section 6.A.
2. To a sterile clear V-bottom 96-well plate, add 250µl of reference antibody starting dilution (dilu1, 2X final concentration) to wells A11 and B11 (see Figure 8).
3. Add 250µl of test antibodies 1 and 2 starting dilution (dilu1, 2X final concentration) to wells E11 and G11, respectively (see Figure 8).
4. Add 150µl of assay buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 100µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent 2.5-fold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.

   Note: Wells A2, B2, E2 and G2 contain 150µl of assay buffer without antibody as a negative control.
7. Cover the antibody dilution plate with a lid and keep at ambient temperature (22–25°C) while preparing the ICOS Effector Cells.

Recommended Plate Layout for Antibody Dilutions Prepared from a Single Antibody Stock													
	1	2	3	4	5	6	7	8	9	10	11	12	
A		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ab
B		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ab
C													
D													
E		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 1
F													
G		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 2
H													

Figure 8. Example plate layout showing antibody serial dilutions.

6.E. Preparing ICOS Effector Cells

While maintaining the ICOS Effector Cells, it is important to follow the recommended cell seeding density. Changes in cell culture volume or seeding density will affect cell growth rate and assay performance. Only use the cells in the assay after the cell doubling rate has stabilized during propagation and cell viability is greater than 95%.

1. Passage the cells two days before performing the assay as described in Section 4.B.
2. Count the ICOS Effector Cells by Trypan blue staining and calculate the cell density and viability.
3. Transfer an appropriate amount of ICOS Effector Cells from the culture vessel to a 50ml conical tube or larger sized centrifuge tube.
4. Pellet the cells at 130 × *g* for 10 minutes at ambient temperature, and resuspend the pellet in assay buffer at 70% of the full volume needed to generate the targeted final cell density of 2.5×10^6 cells/ml.
5. Count the cells again and adjust the volume of assay buffer to achieve a final cell density of 2.5×10^6 cells/ml. You will need at least 6ml of ICOS Effector Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.

6.F. Adding ICOS Effector Cells and Antibody to Assay Plates

1. Take the 96-well assay plates containing ICOSL aAPC/CHO-K1 Cells out of the incubator. Invert the assay plate above a sink to remove the medium. Then, place the inverted plate on paper towels for 5–10 seconds to drain any remaining medium. Alternatively, remove 95µl of medium from each of the wells using a manual multichannel pipette.
2. Using a multichannel pipette, add 40µl of the appropriate antibody dilution (Figure 8) to the assay plates according to the plate layout in Figure 7.
3. Mix the ICOS Effector Cells by tube inversion and transfer the suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 40µl of the cell suspension to each of the inner 60 wells of the assay plates. Gently swirl the assay plates to ensure mixing of the Effector Cells and antibody.
4. Add 80µl of assay buffer to each of the outside wells of the assay plates.
5. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 6 hours.

   Note: The 6-hour assay time was optimized using the Control Ab, Anti-ICOS. We recommend optimizing assay time (5–24 hours) with your own antibody or other biologic samples.

6.G. Preparing and Adding Bio-Glo-NL™ Reagent

We recommend preparing the Bio-Glo-NL™ Reagent immediately before use. Ensure that the Bio-Glo-NL™ Luciferase Assay Buffer is equilibrated to room temperature (do not exceed 25°C) before reconstituting the reagent. **Do not** store the reconstituted reagent. Once reconstituted, the reagent will lose 10% activity in approximately 8 hours at room temperature.

Note: The ICOS Blockade Bioassay is compatible only with the Bio-Glo-NL™ Luciferase Assay System (Cat.# J3081, J3082, J3083). **Do not** use the Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941) with the ICOS Blockade Bioassay.

1. Remove the Bio-Glo-NL™ Luciferase Assay Substrate from –20°C storage and mix by pipetting. Briefly centrifuge the tubes if the substrate has collected in the cap or on the sides of the tubes.
2. Prepare the desired amount of reconstituted Bio-Glo-NL™ Reagent by combining one volume of Bio-Glo-NL™ Luciferase Assay Substrate with 50 volumes of Bio-Glo-NL™ Luciferase Assay Buffer. For example, if the experiment requires 10ml of reagent add 200µl of substrate to 10ml of buffer. Ten milliliters (10ml) of Bio-Glo-NL™ Reagent is sufficient for 120 wells (two assay plates, using the inner 60 wells of each plate).
3. Remove assay plates from the incubator after the incubation period and equilibrate to room temperature for 10–15 minutes.
4. Using a manual multichannel pipette, add 80µl of Bio-Glo-NL™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
5. Add 80µl of Bio-Glo-NL™ Reagent to wells B1, D1 and F1 of each assay plate to measure background signal.
6. Incubate at room temperature for 5–10 minutes, then measure the luminescence in a GloMax® Discover System or a plate reader with glow-type luminescence reading capabilities. The luminescence intensity will decay gradually, with a signal half-life of approximately 120 minutes at room temperature.

   Note: Varying the Bio-Glo-NL™ Reagent incubation time will affect the raw relative light unit (RLU) values but should not significantly change the IC_{50} value and maximum percent blocking.

6.H. Data Analysis

1. Determine the plate background by calculating the average RLU from wells B1, D1 and F1.

2. Calculate percent blocking = $1 - \frac{\text{RLU (antibody–background)}}{\text{RLU (no antibody control–background)}} \times 100$

3. Graph data as RLU versus Log_{10} [antibody] and percent blocking versus Log_{10} [antibody]. Fit curves and determine the IC_{50} value of antibody response using appropriate curve fitting software (such as GraphPad Prism® software).

7. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com** Email: **techserv@promega.com**

Symptoms	Causes and Comments
Low luminescence measurements (RLU readout)	Choose an instrument designed for plate-reading luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual RLU numbers will vary between instruments.
	Some models of luminometers with low sensitivity should be avoided. If using a reader with an adjustable gain, we recommend a high gain setting.
	Insufficient cells per well can lead to low RLU. Handle and plate cells according to the instructions to ensure a sufficient number of viable cells per well.
	Low activity of Bio-Glo-NL™ Reagent leads to low RLU. Store and handle the Bio-Glo-NL™ Reagent according to the instructions.
Weak assay response (low percent blocking)	Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The IC_{50} value obtained in the ICOS Blockade Bioassay may vary from the IC_{50} value obtained using other methods such as primary T cell-based assays.
	The assay is sensitive to the concentration of FBS in assay buffer. Optimize the FBS concentration from 0.5%-10% in assay buffer if assay performance is not ideal.
	Optimize the assay incubation time within a range of 5–24 hours.
	If untreated control RLU is less than 100-fold above plate reader background RLU, subtract plate background RLU from all samples before calculating fold induction.

7. Troubleshooting (continued)

Symptoms	Causes and Comments
Variability in assay performance	Variations in cell growth conditions including cell plating, harvest density, cell viability and cell doubling time may cause low assay performance and high assay variation. Avoid one-day cell passages whenever possible, especially with the ICOS Effector Cells. Use high-quality cell culture reagents (especially serum) and plasticware for maintaining cells in culture. Ensure consistent cell growth by handling the cells exactly according to the instructions.
Variability in assay performance (continued)	Ensure that you are using Bio-Glo-NL™ Reagent in the assay. The ICOS Blockade Bioassay, Propagation Model, is not compatible with Bio-Glo™ Reagent.
	Inappropriate cell handling during cell harvest, including long centrifuge times and high centrifuge speeds, may cause low assay performance and high assay variation. Centrifuge the cells exactly according to the instructions.
	Inappropriate cell freezing/DMSO exposure may cause low assay performance and high assay variation. Freeze the cells exactly according to the instructions.
	Inappropriate cell counting methods may lead to variation in cell numbers in culture and assays and cause high assay variation. Ensure consistent and accurate cell counting methods.

8. References

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9. Appendix

9.A. Representative Assay Results

The following data were generated using the ICOS Blockade Bioassay, Propagation Model, using Control Ab, Anti-ICOS (Figure 9).

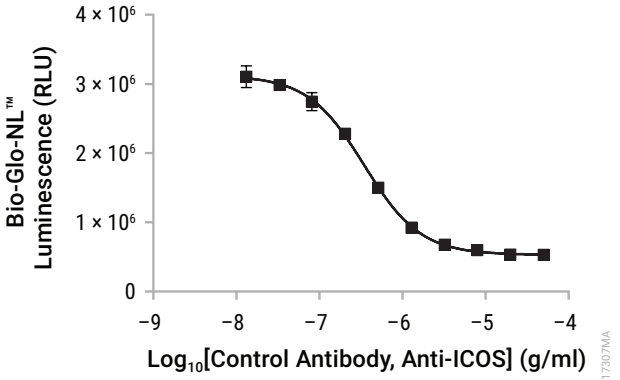

Figure 9. The ICOS Blockade Bioassay measures the activity of Control Ab, Anti-ICOS. ICOSL aAPC/CHO-K1 Cells were added to a 96-well assay plate 18 hours prior to the assay. On the day of assay, ICOS Effector Cells and a titration of Control Ab, Anti-ICOS (Cat. #K1241) were added. After a 6-hour induction at 37°C, Bio-Glo-NL™ Luciferase Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The IC_{50} value was 0.35 µg/ml and the percent maximal blocking was 82.8%.

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9.B. Composition of Buffers and Solutions

initial cell culture medium for ICOS Effector Cells

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS

cell growth medium for ICOS Effector Cells

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS
400µg/ml	hygromycin B
1mg/ml	G418 Geneticin
1mm	sodium pyruvate
0.1mm	MEM nonessential amino acids

cell freezing medium for ICOS Effector Cells

85%	RPMI 1640 with L-glutamine and HEPES
10%	FBS
5%	DMSO

initial cell culture medium for ICOSL aAPC/CHO-K1 cells

90%	Ham's F12
10%	FBS

cell growth medium for ICOSL aAPC/CHO-K1 Cells

90%	Ham's F12
10%	FBS
10µg/ml	blasticidin
200µg/ml	hygromycin

cell freezing medium for ICOSL aAPC/CHO-K1 Cells

85%	Ham's F-12
10%	FBS
5%	DMSO

cell plating medium for ICOSL aAPC/CHO-K1 Cells

90%	Ham's F12
10%	FBS

assay buffer

99%	RPMI 1640 with L-glutamine and HEPES
1%	FBS

9.C. Related Products

Fc Effector Bioassays

Product	Size	Cat.#
ADCC Reporter Bioassay, Complete Kit (Raji)*	1 each	G7015
ADCC Reporter Bioassay, Core Kit*	1 each	G7010
ADCC Reporter Bioassay, F Variant, Core Kit**	1 each	G9790
ADCC Reporter Bioassay, Target Kit (Raji)*	1 each	G7016
FcγRIIa-H ADCP Reporter Bioassay, Complete Kit**	1 each	G9901
FcγRIIa-H ADCP Reporter Bioassay, Core Kit**	1 each	G9991
Mouse FcγRIV ADCC Bioassay, Complete Kit**	1 each	M1201
Mouse FcγRIV ADCC Bioassay, Core Kit**	1 each	M1211
Membrane TNFα Target Cells**	1 each	J3331
Membrane RANKL Target Cells**	1 each	J3381

*For Research Use Only. Not for use in diagnostic procedures.
**Not for Medical Diagnostic Use.
Additional kit formats are available.

Fc Effector Immunoassay

Product	Size	Cat.#
Lumit® FcRn Binding Immunoassay	100 assays	W1151

Not for Medical Diagnostic Use. Additional kit formats and sizes are available.

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9.C. Related Products (continued)

Immune Checkpoint Bioassays

Product	Size	Cat.#
4-1BB Bioassay	1 each	JA2351
CD28 Bioassay	1 each	JA6701
CD28 Blockade Bioassay	1 each	JA6101
CD40 Bioassay	1 each	JA2151
CTLA-4 Blockade Bioassay	1 each	JA3001
GITR Bioassay	1 each	JA2291
ICOS Bioassay	1 each	JA6801
ICOS Blockade Bioassay	1 each	JA6001
LAG-3/MHCII Blockade Bioassay	1 each	JA1111
OX40 Bioassay	1 each	JA2191
PD-1/PD-L1 Blockade Bioassay	1 each	J1250

Not for Medical Diagnostic Use. Additional kit formats and sizes are available.

Product	Size	Cat.#
PD-1+TIGIT Combination Bioassay	1 each	J2211
PD-L1 Negative Cells	1 each	J1191
TIGIT/CD155 Blockade Bioassay	1 each	J2201

Not for Medical Diagnostic Use. Additional kit formats and sizes are available.

T Cell Activation Bioassays

Product	Size	Cat.#
T Cell Activation Bioassay (IL-2)	1 each	J1651
T Cell Activation Bioassay (NFAT)	1 each	J1621
T Cell Activation Bioassay (TCRαβ-KO, CD4+)	1 each	GA1172
T Cell Activation Bioassay (TCRαβ-KO, CD8+)	1 each	GA1162
T Cell Activation Bioassay (TCRαβ-KO, CD4+, CD8+)	1 each	GA1182

Not for Medical Diagnostic Use. Additional kit formats and sizes are available.

Cytokine and Growth Factor Bioassays

Product	Size	Cat.#
IL-2 Bioassay	1 each	JA2201
IL-6 Bioassay	1 each	JA2501
IL-12 Bioassay	1 each	JA2601
IL-15 Bioassay	1 each	JA2011
IL-23 Bioassay	1 each	JA2511
RANKL Bioassay	1 each	JA2701
VEGF Bioassay	1 each	GA2001

Not for Medical Diagnostic Use. Additional kit formats and sizes are available.

Macrophage-Directed Bioassays

Product	Size	Cat.#
SIRPα/CD47 Blockade Bioassay	1 each	JA6011
SIRPα/CD47 Blockade Bioassay, Fc-dependent	1 each	JA4801
TLR Bioassay	1 each	JA9011
ADCP Reporter Bioassay (THP-1)	1 each	JA9411

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Control Antibodies and Proteins

Product	Size	Cat.#
Control Ab, Anti-4-1BB	50µg	K1161
Control Ab, Anti-CD20	5µg	GA1130
Control Ab, Anti-CD40	50µg	K1181
Control Ab, Anti-CTLA-4	100µg	JA1020
Control Ab, Anti-LAG-3	100µg	K1150
Control Ab, Anti-OX40	50µg	K1191
Control Ab, Anti-PD-1	100µg	J1201
Control Ab, Anti-SIRPα	50µg	K1251
Control Ab, Anti-TIGIT	100µg	J2051
Control Ab, Anti-TIM-3	100µg	K1210
Recombinant VEGF ligand	10µg	J2371

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9.C. Related Products (continued)

Detection Reagents

Product	Size	Cat.#
Bio-Glo™ Luciferase Assay System	10ml	G7941
Bio-Glo-NL™ Luciferase Assay System	10ml	J3081
Bio-Glo-NB™ TCK Luciferase Assay System	10ml	JB1001

Not for Medical Diagnostic Use. Additional sizes are available.

Detection Instruments

Product	Size	Cat.#
GloMax® Navigator System	1 each	GM2000
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500

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Note: Additional Fc Effector, Immune Checkpoint, T Cell Activation, Cytokine, Macrophage, Primary Cell and Target Cell Killing Bioassays are available. To view and order Promega Bioassay products visit: **www.promega.com/products/reporter-bioassays/** or email: **EarlyAccess@promega.com**.

For information on custom bioassay development and services visit the Promega Tailored R&D Solutions website: **www.promega.com/custom-solutions/tailored-solutions/**

10. Summary of Changes

The following changes were made to the 4/25 revision of this document:

1. Removed an expired patent statement.
2. Revised text about the label in Section 3.

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