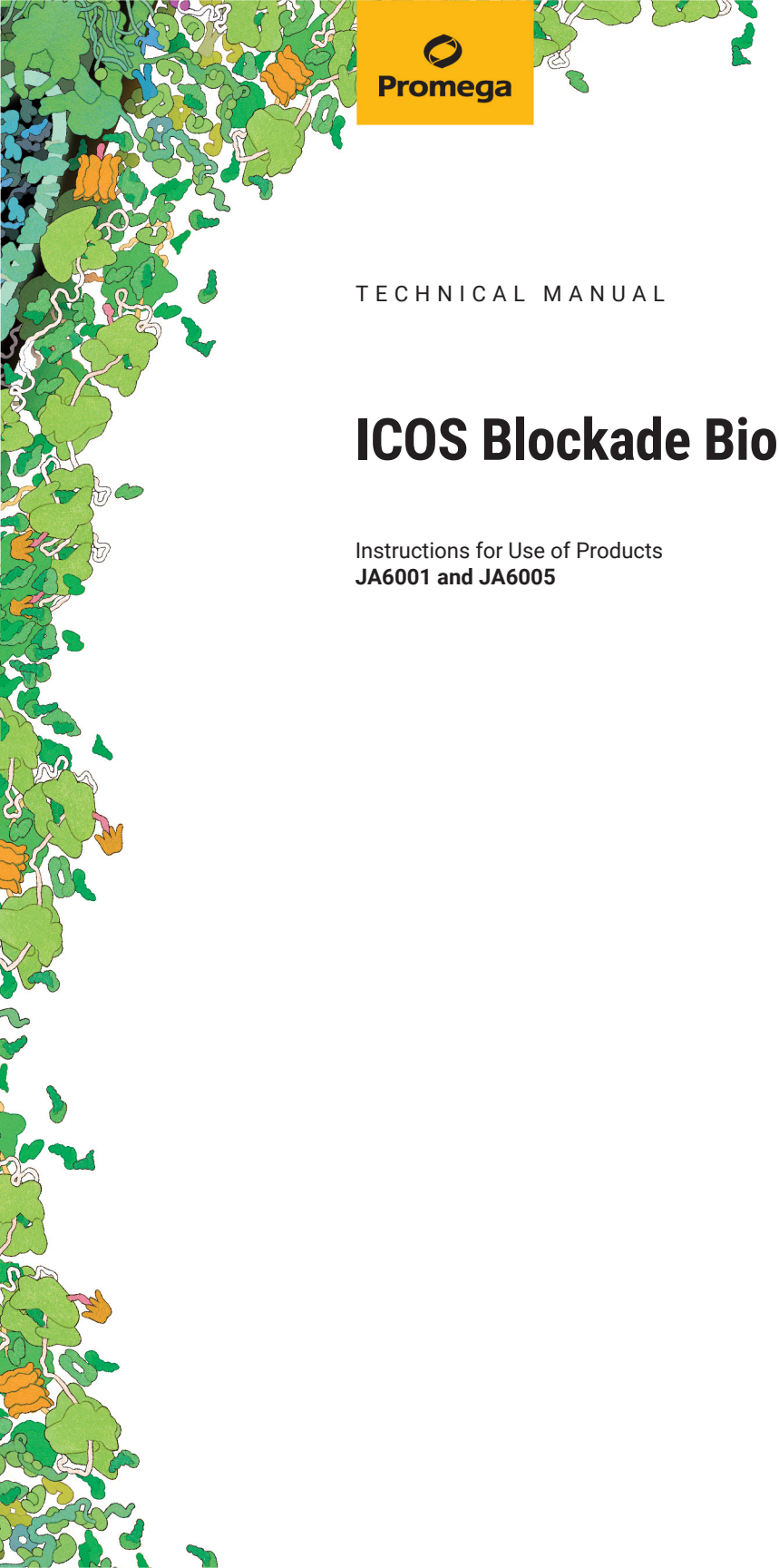

TECHNICAL MANUAL

ICOS Blockade Bioassay

Instructions for Use of Products
JA6001 and JA6005

Revised 5/25
TM629

ICOS Blockade Bioassay

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 T-cell activation 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[a–e] (Cat.# JA6001, JA6005), 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 thaw-and-use format as cryopreserved cells that can be thawed, plated and used in an assay without the need for cell culture and propagation.

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[a,b,d] (Cat.# J3081), and a standard luminometer such as the GloMax® Discover System (see Related Products, Section 7.B).

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). 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. The ICOS Blockade Bioassay is not designed to detect activation of ICOS antibodies. We offer separately the ICOS Bioassay, Propagation Model (CPM; Cat.# JA3072), and thaw-and-use (Cat.# JA6801, JA6805) formats for screening and potency testing of ICOS agonists.

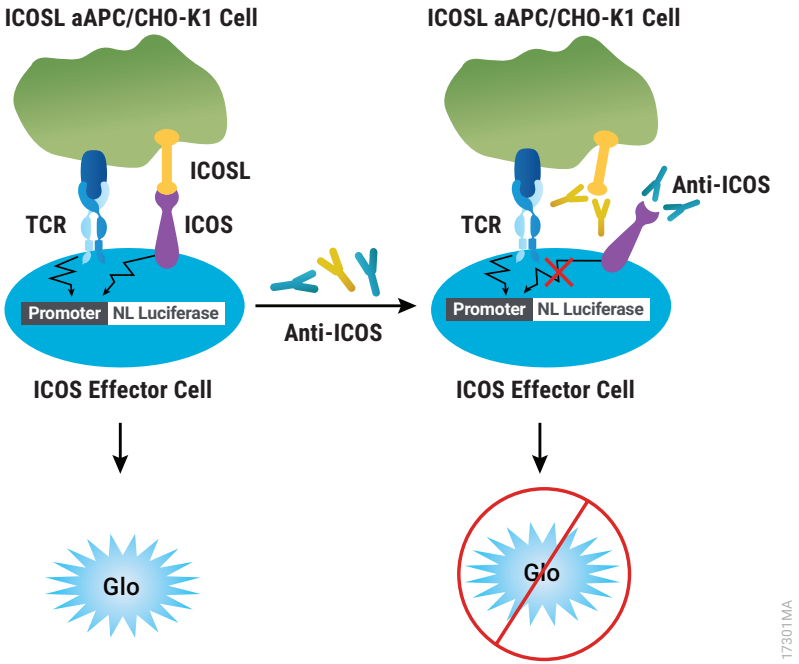

Figure 1. Representation of the ICOS Blockade Bioassay. The ICOS Blockade Bioassay consists of two cell lines, ICOS Effector Cells and ICOSL aAPC/CHO-K1 Cells. When cocultured, ICOSL aAPC/CHO-K1 Cells activate TCR/CD3 and ICOS on the ICOS Effector Cells to induce maximum promoter-mediated luminescence. 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)

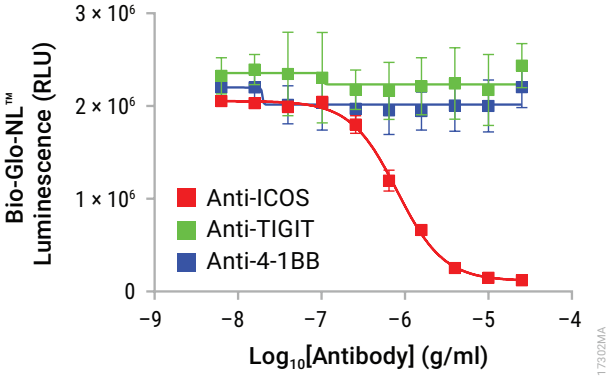

Figure 2. The ICOS Blockade Bioassay reflects the mechanism of action (MOA) and shows specificity for antibodies designed to block ICOS/ICOSL interaction. ICOS Effector Cells were incubated with ICOSL aAPC/CHO-K1 Cells in the presence of serial titrations of blocking antibodies as indicated. After a 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.

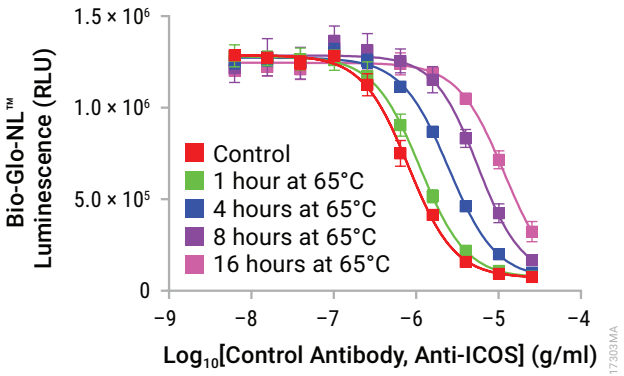

Figure 3. The ICOS Blockade Bioassay is stability-indicating. Samples of Control Ab, Anti-ICOS (Cat.# K1241), were maintained at 4°C (control) or heat-treated at the indicated times and temperatures, then analyzed using the ICOS Blockade Bioassay. Bio-Glo-NL™ Reagent was added and luminescence quantified 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)

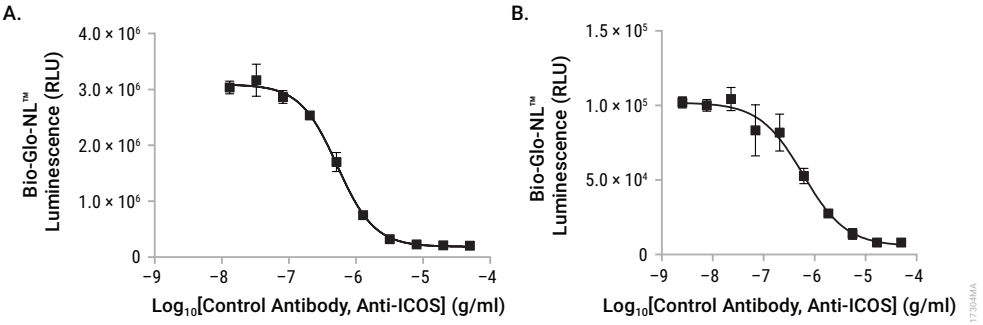

Figure 4. The ICOS Blockade Bioassay is amenable to 384-well plate format. Panel A. The ICOS Blockade Bioassay was performed in 96-well plates as described in this technical manual with a titration of Control Ab, Anti-ICOS (Cat.# K1241). **Panel B.** The ICOS Blockade Bioassay was performed in 384-well format as described here. ICOSL aAPC/CHO-K1 cells were thawed, and 15µl 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 of 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.

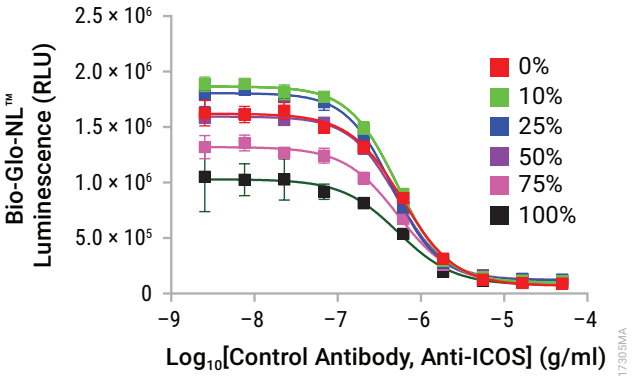

Figure 5. The ICOS Blockade Bioassay is tolerant to human serum. Control Ab, Anti-ICOS (Cat.# K1241), was analyzed in the absence or presence of pooled normal human serum (0–100% in the antibody sample). After the 6-hour assay induction at 37°C, Bio-Glo-NL™ Reagent was added and luminescence quantified 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).

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2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
ICOS Blockade Bioassay	**1 each**	**JA6001**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 120 assays using the inner 60 wells of two 96-well plates. Includes:

- 1 vial ICOS Effector Cells, 3.3 × 10^7 cells/ml (1.0ml per vial)
- 1 vial ICOSL aAPC/CHO-K1 Cells, 1.2 × 10^7 cells/ml (0.5ml per vial)
- 25ml Ham's F12 Medium
- 36ml RPMI 1640 Medium
- 4ml Fetal Bovine Serum
- 1 vial Bio-Glo-NL™ Luciferase Assay Substrate
- 10ml Bio-Glo-NL™ Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
ICOS Blockade Bioassay 5X	**1 each**	**JA6005**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 600 assays using the inner 60 wells of ten 96-well plates. Includes:

- 5 vials ICOS Effector Cells, 3.3 × 10^7 cells/ml (1.0ml per vial)
- 5 vials ICOSL aAPC/CHO-K1 Cells, 1.2 × 10^7 cells/ml (0.5ml per vial)
- 5 × 25ml Ham's F12 Medium
- 5 × 36ml RPMI 1640 Medium
- 5 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo-NL™ Luciferase Assay Substrate
- 5 × 10ml Bio-Glo-NL™ Luciferase Assay Buffer

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 negatively impact cell viability and cell performance.
- Store Bio-Glo-NL™ Luciferase Assay Substrate, Bio-Glo-NL™ Luciferase Assay Buffer and Fetal Bovine Serum at –30°C to –10°C. The Bio-Glo-NL™ Luciferase Assay Substrate stays liquid and does not freeze. Avoid multiple freeze-thaw cycles of the serum.
- Store RPMI 1640 and Ham's F12 Medium at 4°C, protected from fluorescent light.

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 Certificates 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 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 7.A, Representative Assay Results.

The ICOS Effector Cells and ICOSL aAPC/CHO-K1 Cells are provided in frozen, thaw-and-use format and are ready to be used without any additional cell culture or propagation. When thawed and diluted as instructed, the cells will be at the appropriate concentration for the assay. The cells are sensitive, and care should be taken to follow cell thawing and plating procedures as described. Do not overmix or overwarm the cell reagents.

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 7.B, 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 light 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

- user-defined anti-ICOS antibodies or other biologics samples
- 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 system)
- **optional:** Control Ab, Anti-ICOS (Cat.# K1241)

4. 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).

4.A. Preparing Cell Recovery Medium, Assay Buffer and Bio-Glo-NL™ Reagent

1. **ICOSL aAPC/CHO-K1 Cell Recovery Medium:** On the day before the assay, prepare 14.5ml of cell recovery 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. To prepare cell recovery medium, add 1.5ml of FBS to 13ml of Ham's F-12 medium. For reference, 14.5ml of cell recovery medium is sufficient to thaw and plate 1 vial of ICOSL aAPC/CHO-K1 Cells. If multiple vials will be thawed, then scale the amount of cell recovery medium appropriately. Warm the remaining Ham's F12 Medium to 37°C. Store the remaining FBS at 4°C for use in preparing the assay buffer on the day of the assay.
2. **Assay Buffer:** On the day of the assay, prepare 20ml of assay buffer (99% RPMI 1640/1% FBS). Add 0.2ml of FBS to 19.8ml RPMI 1640 Medium. Mix well and warm to 37°C before use. Warm the remaining RPMI 1640 Medium to 37°C.

   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™ Luciferase 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™ Luciferase Assay 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 Reagent (Cat.# J3081, J3082, J3083). **Do not** use the Bio-Glo™ Luciferase Assay Reagent (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.

   Note: If you are using Control Ab, Anti-ICOS (Cat.#K1241), as a reference antibody in your assay, prepare a 500µl starting dilution of 100µg/ml of Control Ab, Anti-ICOS (dilu1, 2X final concentration) by adding 50µl of Control Ab, Anti-ICOS (1mg/ml stock) to 450µl of assay buffer.

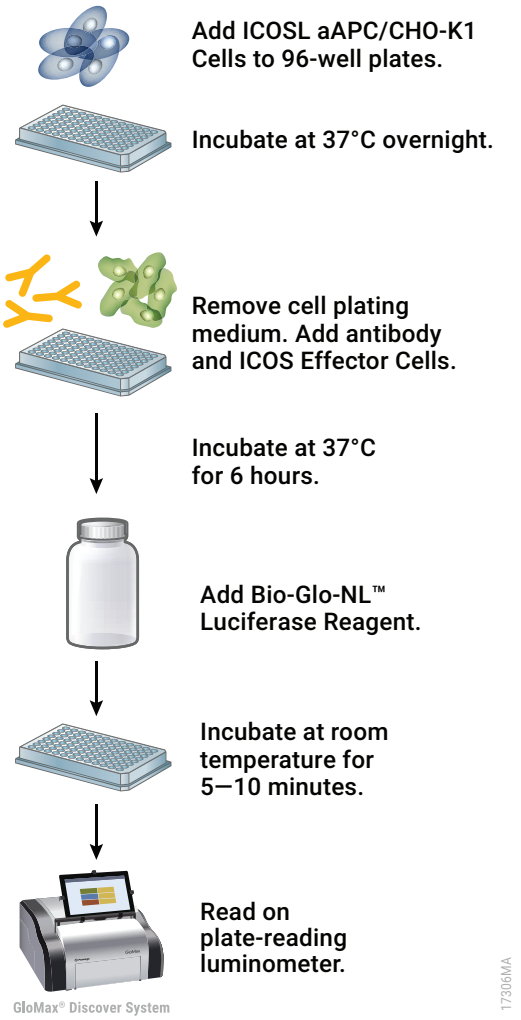

Figure 6. Schematic protocol for the ICOS Blockade Bioassay.

4.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 ten-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.

4.C. Plating ICOSL aAPC/CHO-K1 Cells

Thaw-and-use ICOSL aAPC/CHO-K1 Cells are sensitive and care should be taken to follow the cell thawing and plating procedures **exactly** as described. Do not overmix or overwarm the cell reagents. No additional cell culture or manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

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

Perform the following steps in a sterile cell culture hood.

1. On the day before performing the assay, in a sterile 50ml conical tube, prepare 14.5ml of cell recovery medium (90% Ham's F12/10% FBS) as described in Section 4.A.
2. Remove one vial of ICOSL aAPC/CHO-K1 Cells from storage at –140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial. Do not invert.
3. Gently mix the cell suspension by pipetting, then transfer 0.5ml cells to the tube containing 14.5ml of cell recovery medium. Mix well by gently inverting the tube 1–2 times.
4. 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 two 96-well white flat-bottom assay plates.
5. Add 100µl of prewarmed (37°C) Ham's F12 Medium to each of the outside wells of the assay plates.
6. Place lids on the assay plates and incubate in a 37°C, 5% CO_2 incubator overnight (18–22 hours).

4.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 4.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.

4.D. Preparing Antibody Serial Dilutions (continued)

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.

4.E. Preparing ICOS Effector Cells

The thaw-and-use ICOS Effector Cells included in this kit are sensitive and care should be taken to follow the cell thawing and plating procedures **exactly** as described. Do not overmix or overwarm the cell reagents. No additional cell culture or manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

1. Label a sterile 15ml conical tube "Effector Cells". Add 6ml of prewarmed (37°C) assay buffer to the 15ml conical tube.
2. Remove one vial of ICOS Effector Cells from storage at –140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial.
3. Gently mix the cell suspension by pipetting, then transfer 0.5ml cells to the 15ml conical tube containing 6ml of assay buffer. Mix well by gently inverting the tube.

4.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 inverting the tube 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.

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

We recommend preparing the Bio-Glo-NL™ Luciferase Assay 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 Bio-Glo-NL™ Luciferase Assay Reagent (Cat.# J3081, J3082, J3083). **Do not** use Bio-Glo™ Luciferase Assay Reagent (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™ Luciferase Assay Reagent by combining one volume of substrate with 50 volumes of 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™ Luciferase Assay 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™ incubation time will affect the raw relative light unit (RLU) values but should not significantly change the IC_{50} value and maximum percent blocking.

4.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).

5. 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.
Variability in assay performance	Ensure that you are using Bio-Glo-NL™ Reagent in the assay. The ICOS Blockade Bioassay is **not** compatible with Bio-Glo™ Reagent.

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6. References

1. Hutloff, A. *et al.* (1999) ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* **397**, 263–6.
2. Yoshinaga, S. *et al.* (1999) T-cell co-stimulation through B7RP-1 and ICOS. *Nature* **402**, 827–32.
3. Coyle, A. *et al.* (2000) The CD28-related molecule ICOS is required for effective T cell-dependent immune responses. *Immunity* **13**, 95–105.
4. Wang, S. *et al.* (2000) Costimulation of T cells by B7-H2, a B7-like molecule that binds ICOS. *Blood* **96**, 2808–13.
5. Aicher, A. *et al.* (2000) Characterization of human inducible costimulatory ligand expression and function. *J. Immunol.* **164**, 4689–96.
6. Khayyamian, S. *et al.* (2002) ICOSLigand expressed on human endothelial cells, costimulates Th1 and Th2 cytokine secretion by memory CD4+ T cells. *Proc. Natl. Acad. Sci. USA* **99**, 6198–203.
7. Wahl, P. *et al.* (2002) Renal tubular epithelial expression of the costimulatory molecule B7RP-1 (inducible costimulatory ligand). *J. Am. Soc. Nephrol.* **13**, 1517–26.
8. Taylor, P. *et al.* (2005) Targeting of inducible costimulator (ICOS) expressed on alloreactive T cells down-regulates graft-versus-host disease (GVHD) and facilitates engraftment of allogeneic bone marrow (BM). *Blood* **105**, 3372–80.
9. Ozkaynak, E. *et al.* (2001) Importance of ICOS-B7RP-1 costimulation in acute chronic allograft rejection. *Nat. Immunol.* **2**, 591–6.
10. Frey, O. *et al.* (2010) Inducible costimulator (ICOS) blockade inhibits accumulation of polyfunctional T helper 1/T helper 17 cells and mitigates autoimmune arthritis. *Ann. Rheum. Dis.* **69**, 1495–501.
11. Rottman, B. *et al.* (2001) The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. *Nat. Immunol.* **2**, 605–11.

7. Appendix

7.A. Representative Assay Results

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

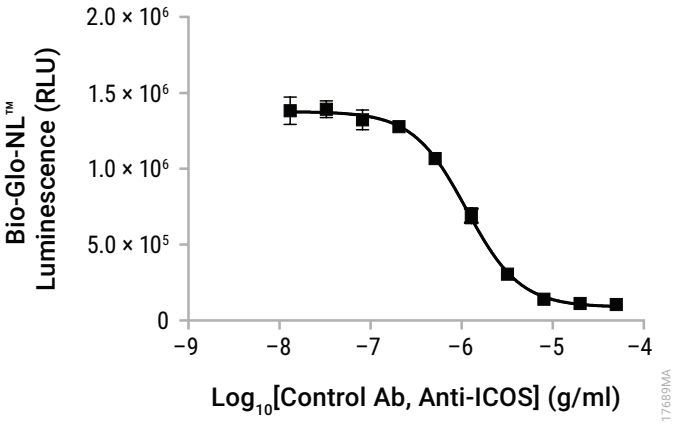

Figure 9. The ICOS Blockade Bioassay measures the activity of Control Ab, Anti-ICOS. ICOSL aAPC/CHO-K1 Cells were added to a 96-well assay plate 18 hours prior to the assay. On the day of assay, ICOS Effector Cells and a titration of Control Ab, Anti-ICOS (Cat.# K1241), were added. After a 6-hour induction at 37°C, Bio-Glo-NL™ 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 1.13µg/ml and the percent maximal blocking was 93.4%.

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7.B. Related Products

T Cell Activation Bioassays

Product	Size	Cat.#
T Cell Activation Bioassay (IL-2)	1 each	J1651
T Cell Activation Bioassay (NFAT)	1 each	J1621

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

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

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

Fc Effector Immunoassays

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

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

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
PD-L1 Negative Cells	1 each	J1191
PD-1+TIGIT Combination Bioassay	1 each	J2211
TIGIT/CD155 Blockade Bioassay	1 each	J2201
TIM-3 Bioassay	1 each	JA2211

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

Luminometers

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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7.B. Related Products (continued)

Control Antibodies and Proteins

Product	Size	Cat.#
Control Ab, Anti-4-1BB	50µg	K1161
Control Ab, Anti-CD-20	5 µg	GA1130
Control Ab, Anti-OX40	50µg	K1191
Control Ab, Anti-CD40	50µg	K1181
Control Ab, Anti-CTLA-4	100µg	JA1020
Control Ab, Anti-LAG-3	100µg	K1150
Control Ab, Anti-PD-1	100µg	J1201
Control Ab, Anti-TIGIT	100µg	J2051
Control Ab, Anti-TIM-3	100µg	K1210
Recombinant VEGF ligand	10µg	J2371

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

Detection Reagent

Product	Size	Cat.#
Bio-Glo-NL™ Luciferase Assay System	10ml	J3081
	100ml	J3082
	1,000ml	J3083
Bio-Glo™ Luciferase Assay System	10ml	G7941
	100ml	G7940

Not for Medical Diagnostic Use.

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/**

8. Summary of Changes

The following change was made to the 5/25 revision of this document:

1. Revised text about the label, in Section 3.

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