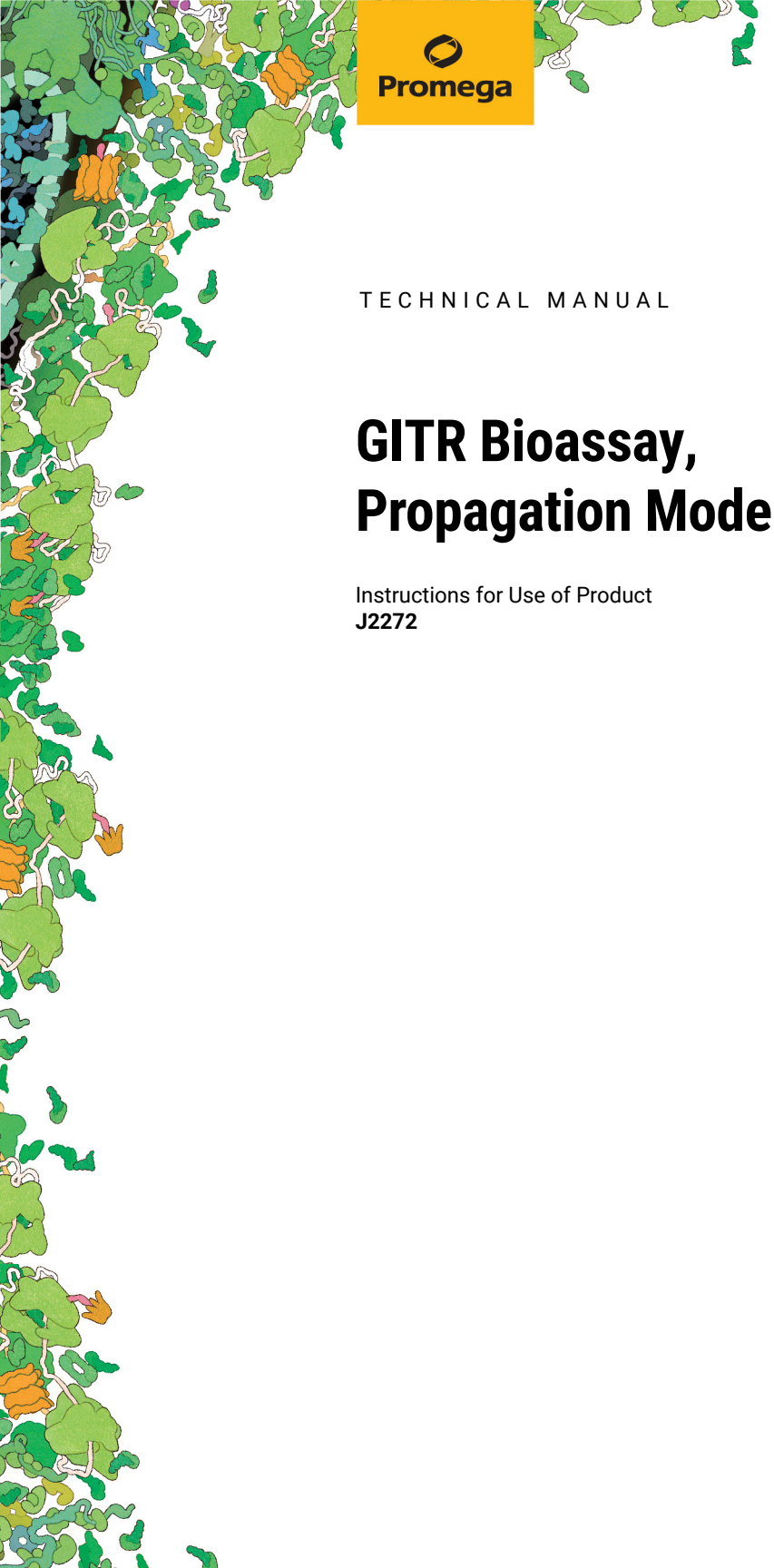

TECHNICAL MANUAL

GITR Bioassay, Propagation Model

Instructions for Use of Product
J2272

Revised 5/26
TM583

Promega

GITR 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. Stimulatory immune checkpoint receptors appear to have a significant role in cancer progression and autoimmune disease. Several costimulatory immune checkpoint receptors such as glucocorticoid-induced tumor necrosis factor (TNF) receptor family-related protein (GITR), 4-1BB, OX40, CD40 and inducible T-cell costimulator (ICOS) have been identified. Activating these receptors with ligands or agonist antibodies has emerged to be the next generation of immunotherapeutic strategies to enhance anti-tumor immune responses and promote immune-mediated tumor rejection (1–3).

GITR (CD357/TNFRSF18), a member of the tumor necrosis factor receptor superfamily, is a costimulatory receptor widely expressed on most immune cells, and further upregulated on activated T cells (3, 4). When engaged with GITR ligand (GITRL) on the cell surface, GITR enhances subsequent T cell expansion and cytokine production including interleukin-2 (IL-2) and IL-9 (5,6).

Current methods used to measure the activity of biologic drugs targeting GITR rely on primary human T cells and measurement of functional endpoints such as cell proliferation, cell surface marker expression, and IL-2 production. These assays are laborious and highly variable due to their reliance on donor primary cells, complex assay protocols and unqualified assay reagents. As a result, these assays are difficult to establish in a quality controlled drug-development setting.

The GITR Bioassay, Propagation Model[a–d] (Cat.# J2272), is a bioluminescent cell-based assay that overcomes the limitations of existing assays and can be used to measure the potency and stability of ligands or agonist antibodies that can bind and activate GITR (7). The assay consists of a genetically engineered Jurkat T cell line that expresses human GITR and a luciferase reporter driven by a response element that can respond to GITR ligand/agonist antibody stimulation. The GITR Effector Cells are provided in Cell Propagation Model (CPM) format, as cryopreserved cells that can be thawed, propagated and banked for long-term use.

The GITR Bioassay should be conducted with FcγRIIb CHO-K1 Cells (Cat.# J2232) to test whether agonist antibodies activate GITR in an FcγRIIb-dependent manner. FcγRIIb CHO-K1 Cells may be required to crosslink agonist antibodies but are not required for testing ligands. It is recommended that when screening for agonist antibodies of costimulatory immune checkpoints, you perform the assay both with and without FcγRIIb CHO-K1 Cells to ascertain the need for these cells in enhancing the effect of the agonist antibodies raised against the costimulatory immune checkpoint targets.

Induction of the GITR Effector Cells with a GITR ligand or agonist antibody results in response element-mediated luminescence (Figure 1). The bioluminescent signal is quantified using the Bio-Glo™ Luciferase Assay System and a standard luminometer such as the GloMax® Discover System.

The GITR Bioassay, Propagation Model, reflects the mechanism of action (MOA) of biologics designed to activate GITR. Specifically, GITR-mediated luminescence is detected following the addition of GITR agonist antibodies and GITR ligand, respectively (Figure 2). The bioassay is prequalified according to ICH guidelines and shows the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 3). The assay can be performed in a one-day or two-day time frame depending on antibody properties. The bioassay workflow is simple and 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.

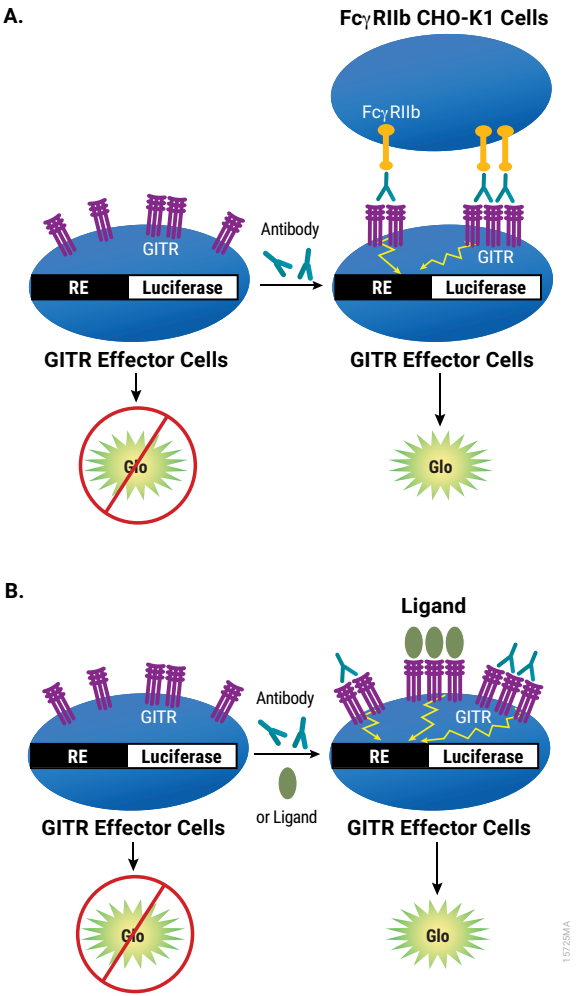

Figure 1. Representation of the GITR Bioassay. Panel A. Assay with FcγRIIb-dependent agonist antibody. The bioassay consists of two engineered cell lines, GITR Effector Cells and FcγRIIb CHO-K1 Cells. In the presence of FcγRIIb CHO-K1 Cells, the anti-GITR antibody can be crosslinked, thereby inducing GITR pathway-activated luminescence. **Panel B.** Assay with FcγRIIb-independent agonist antibody or ligand. The bioassay consists of one engineered cell line, GITR Effector Cells. In the absence of agonist antibody or GITR ligand, the GITR receptor is not activated and luminescence signal is low. The addition of agonist antibody or GITR ligand induces the GITR pathway-activated luminescence, which can be detected in a dose-dependent manner.

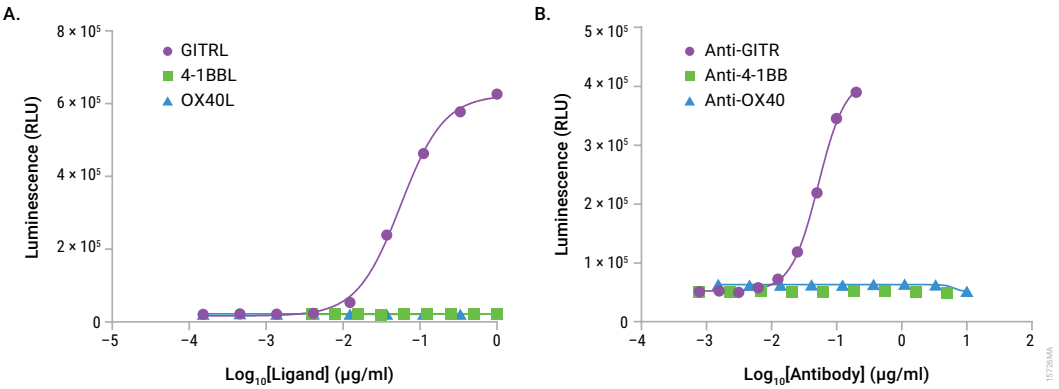

Figure 2. The GITR Bioassay reflects the mechanism of action (MOA) and shows specificity of biologics designed to activate GITR. Panel A. GITR Effector Cells were induced, respectively, with a serial titration of ligands: GITRL, 4-1BBL or OX40L as indicated. **Panel B.** GITR Effector Cells were induced with a serial titration of anti-GITR, anti-4-1BB or anti-OX40 antibody, as indicated, in the presence of FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255). After a 6-hour induction at 37°C, Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were generated using thaw-and-use cells.

Table 1. The GITR Bioassay Shows Precision, Accuracy and Linearity.

Parameter	**Results**	
Accuracy	% Expected Relative Potency	% Recovery
	50	99.3
	75	98.9
	125	102.5
	150	102.9
Repeatability (% CV)	100% (Reference)	1.3
Intermediate Precision (% CV)		6.6
Linearity (r^2)		0.997
Linearity (y = mx + b)		y = 1.053x – 3.742
A 50–150% theoretical potency series of Control Ab, Anti-GITR, was analyzed in triplicate in three independent experiments performed on three days by two analysts. Bio-Glo™ 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.		

1. Description (continued)

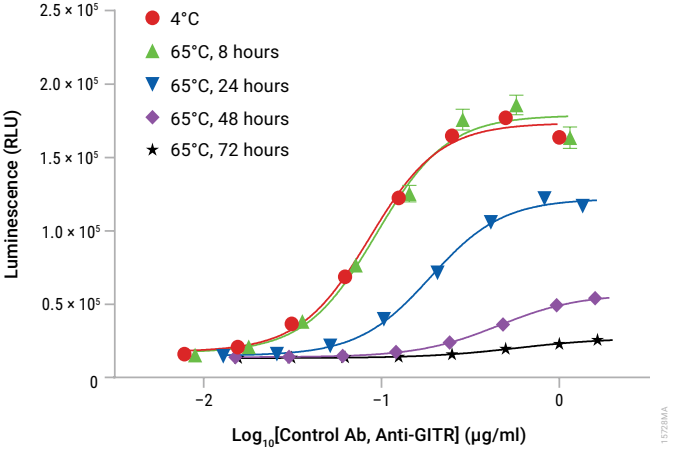

Figure 3. The GITR Bioassay is stability-indicating. Samples of Control Ab, Anti-GITR (Cat.# K1171), were maintained at 4°C (control) or heat-treated at 65°C for the indicated times, then analyzed using the GITR Bioassay with FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255). After a 6-hour induction at 37°C, Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were generated using thaw-and-use cells and fitted to a four-parameter logistic curve using GraphPad Prism® software.

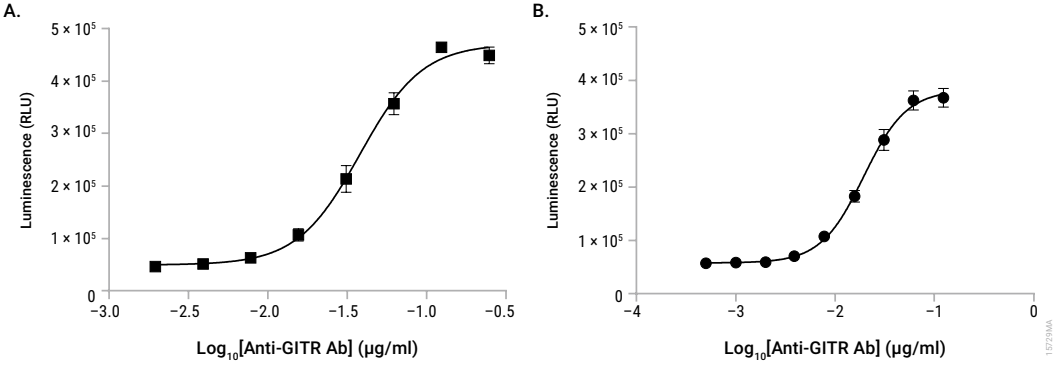

Figure 4. The GITR Bioassay is amenable to 384-well plate format and compatible with laboratory automation. Panel A. The GITR Bioassay was performed in 96-well plates as described in this technical manual using anti-GITR antibody. **Panel B.** The GITR Bioassay was performed in 384-well format using a Mantis® liquid handler to dispense the cells and Echo® Acoustic liquid handler for antibody handling. On the day before the assay, FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255) were plated at 8×10^3 cells/10µl/well. On the day of the assay, anti-GITR antibody was serially diluted and added to the plate at 0.2µl/well. Finally, GITR Effector Cells were added at 1.0×10^4 cells/10µl/well. After a 6-hour incubation, 20µl of Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The EC_{50} values were 0.04µg/ml and 0.02µg/ml, and the fold inductions were 10.1 and 7.1 for 96-well and 384-well format, respectively. Data were generated using thaw-and-use cells.

1. Description (continued)

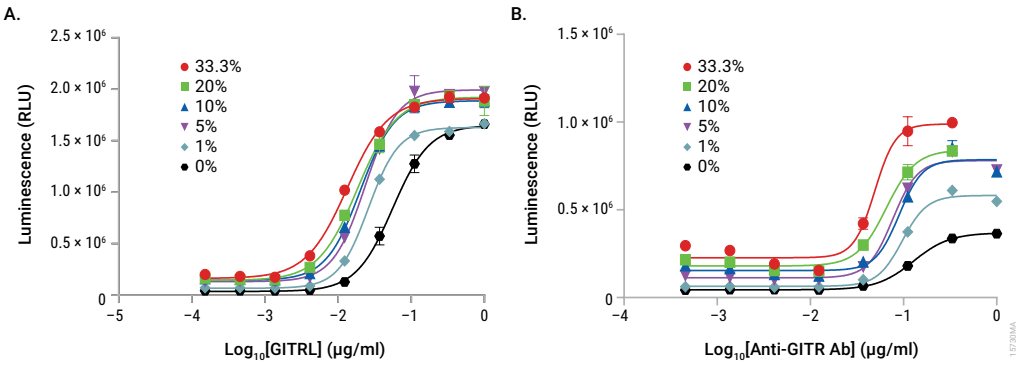

Figure 5. The GITR Bioassay is tolerant to human serum. Panel A. GITR ligand (GITRL, R&D Systems Cat.#6987-GL-025/CF) was analyzed in the presence of increasing concentrations of pooled normal human serum (0–100% in the ligand sample), resulting in final assay concentration of human serum of 0–33.3%. **Panel B.** Anti-GITR antibody was analyzed in the presence of FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255) and increasing concentrations of pooled normal human serum (0–100% in the antibody sample), resulting in final assay concentration of human serum of 0–33.3%. After a 6-hour induction at 37°C, Bio-Glo™ Reagent was added, and luminescence was 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 GITR Bioassay is tolerant to serum with 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.#
GITR Bioassay, Propagation Model	**1 each**	**J2272**

Not for Medical Diagnostic Use.

Includes:

- 2 vials GITR Effector Cells (CPM), 2.0 × 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.

The GITR Bioassay, Propagation Model, is intended to be used with user-provided antibodies or ligands designed to activate GITR. Control Ab, Anti-GITR (Cat.# K1171) and FcγRIIb CHO-K1 Cells (Cat.# J2232) are available separately for use in assay optimization and routine quality control. We strongly recommend including GITR Ligand or Control Ab, Anti-GITR, and FcγRIIb CHO-K1 Cells as a positive control in the first few assays to gain familiarity with the assay. Data generated using these reagents are shown in Figures 2–5 and Sections 9.A and B, Representative Assay Results.

Cell thawing, propagation and banking should be performed exactly as described in Section 4. 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 Sections 5 and 6 were established using GITR Ligand and Control Ab, Anti-GITR, respectively. You may need to adjust the parameters provided here and optimize assay conditions for your own antibody or biologic.

The GITR Bioassay, Propagation Model, produces a bioluminescent signal and should work with all major luminometers or luminescence plate readers for the detection of luminescence. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System. An integration time of 0.5 seconds/well was used for all readings.

3.A. Materials to Be Supplied by the User

(Composition of buffers and solutions is provided in Section 9.C.)

Reagents

- user-defined anti-GITR 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., HyClone Cat.# SH30070.03 or GIBCO® Cat.# 16000044)
- hygromycin B (e.g., GIBCO® Cat.# 10687010)
- G418 sulfate solution (e.g., GIBCO® Cat.# 10131035)
- sodium pyruvate (e.g., GIBCO® Cat.# 11360070)
- MEM nonessential amino acids, 100X (e.g., GIBCO® Cat.# 11140050)
- DMSO (e.g., Sigma Cat.# D2650)
- DPBS (e.g., GIBCO® Cat.# 14190)
- Accutase® solution (e.g., Sigma Cat.# A6964)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941)
- **optional:** FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255; We recommend this cell line when using this assay for the first time and/or testing an Ab that may be dependent on crosslinking FcγRIIb.)
- **optional:** control GITR ligand/TNFSF18 with HA tag (R&D Systems, Cat.# 6987-GL-025/CF)
- **optional:** anti-hemagglutinin/HA tag Ab for crosslinking control GITR ligand (R&D Systems, Cat.# MAB060)
- **optional:** Control Ab, Anti-GITR (Cat.# K1171)

Supplies and Equipment

- solid-white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917) for plating and reading luminescence
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896 or Linbro Cat.# 76-223-05) 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., Costar®/Corning® Cat.# 4870)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent system)

4. Preparing GITR Effector Cells

4.A. Cell Thawing and Initial Cell Culture

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

1. Prepare 50ml of initial cell culture medium by adding 5ml of FBS, 0.5ml of 100X MEM nonessential amino acids (NEAA) and 0.5ml of 100mM sodium pyruvate to 44ml 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 GITR 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 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 ~27 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, measure cell viability and density by Trypan blue staining.
2. Seed the cells at a density of 5×10^5 cells/ml if passaging every two days (e.g., Monday-Wednesday or Wednesday-Friday) or 3×10^5 cells/ml if passaging every three days (e.g., Friday-Monday). Maintain the cell culture at between 2.0×10^5 cells/ml and 2.0×10^6 viable cells/ml. Do not allow cells to grow to a density greater than 2.3×10^6 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 37°C, 5% CO_2 incubator.

4.C. Cell Freezing and Banking

1. On the day of cell freezing, make 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 5×10^6–1×10^7 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. Carefully aspirate the supernatant. Avoid disturbing the cell pellet.
6. Gently resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of 5×10^6–1×10^7 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 at or below –140°C for long-term storage.

5. Assay Protocol for FcγRIIb-Dependent Antibodies

This assay protocol requires two engineered cell lines: GITR Effector Cells and FcγRIIb CHO-K1 Cells. The FcγRIIb CHO-K1 Cells are provided in thaw-and-use format (*Technical Manual* #TM570) and CPM format (see *FcγRIIb CHO-K1 Propagation Model Technical Manual*, #TM569, for details). Either cell format can be used in this assay.

The following procedure illustrates the use of the GITR Bioassay to test two FcγRIIb-dependent antibody samples against a reference sample in a single assay run using the FcγRIIb CHO-K1 Cells in CPM format. 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 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 1µg/ml as a starting concentration (1X) and twofold serial dilution when testing Control Ab, Anti-GITR.

5.A. Preparing Assay Buffer, Bio-Glo™ Reagent and Antibody Samples

1. **FcγRIIb CHO-K1 Cell Plating Medium:** On the day before the assay, prepare 40ml of cell plating medium (90% Ham's F-12/10% FBS) in a 50ml conical tube. Thaw the FBS overnight at 4°C or in a 37°C water bath on the day of use. Add 4ml of FBS to 36ml of Ham's F-12 medium. Mix well and warm to 37°C before use.

2. **Assay Buffer:** On the day of the assay, prepare an appropriate amount of assay buffer (99% RPMI 1640/1% 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, 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-GITR, that we tested.

3. **Bio-Glo™ Reagent:** For reference, 10ml of Bio-Glo™ Reagent is sufficient to assay 120 wells in a 96-well assay format. Thaw the Bio-Glo™ Luciferase Assay Buffer at 4°C overnight or in a room temperature water bath on the day of assay. Equilibrate the Bio-Glo™ Luciferase Assay Buffer to ambient temperature, protected from light.

   Transfer all of the Bio-Glo™ Luciferase Assay Buffer into the amber bottle containing the Bio-Glo™ Luciferase Assay Substrate and mix by inversion until the Substrate is thoroughly dissolved. Equilibrate and store the reconstituted Bio-Glo™ Reagent at ambient temperature (22–25°C) protected from light before adding to assay plates.

   If you are using a large (100ml) size of Bio-Glo™ Luciferase Assay System, dispense the reconstituted Bio-Glo™ Reagent into 10ml aliquots and store at –20°C for up to six weeks. Avoid repeated freeze-thaw cycles. On the day of the assay, thaw an appropriate amount of reconstituted Bio-Glo™ Reagent in a room temperature water bath for at least 1–2 hours before use. The Bio-Glo™ Reagent can be stored at room temperature after reconstitution with ~18% loss in luminescence after 24 hours.

4. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 3X final concentration) of two test antibodies (240µl each) and one reference antibody (500µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Notes:

   a. If you are using Control Ab, Anti-GITR (Cat.# K1171), as a reference antibody in your assay, prepare a 500µl starting dilution with 3µg/ml of anti-GITR antibody (dilu1, 3X final concentration) by adding 1.5µl of anti-GITR stock (1,000µg/ml) to 498.5µl of assay buffer. Store the antibody starting dilution on ice until ready to use in the assay.

   b. To streamline assay setup, prepare antibody serial dilutions prior to harvesting and plating GITR Effector Cells.

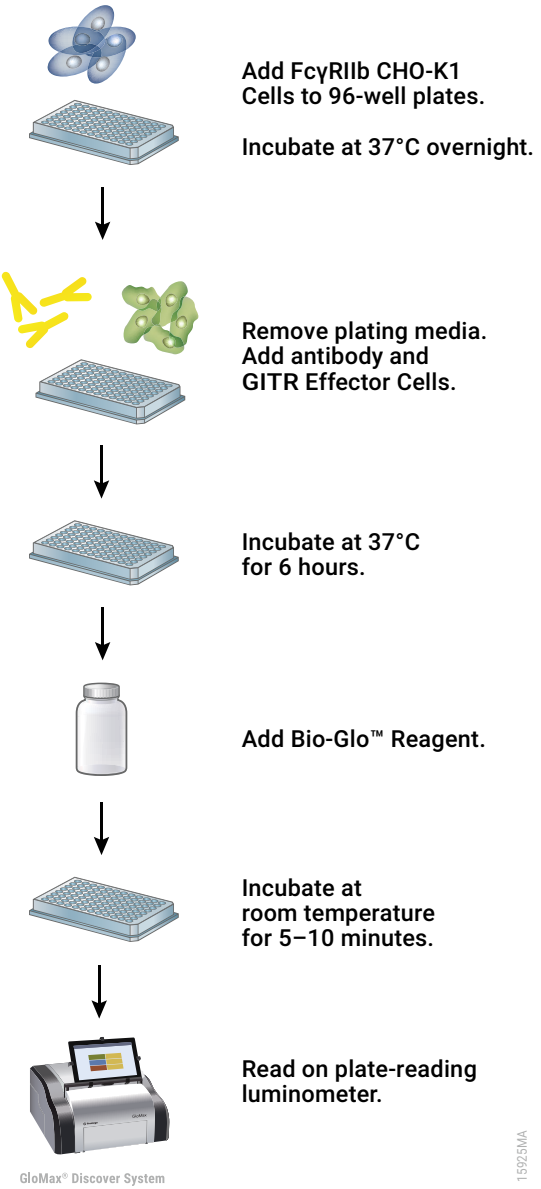

Figure 6. Schematic protocol for GITR Bioassay, Propagation Model, with FcγRIIb-dependent antibody.

5.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 alone (denoted by "B").

5.C. Preparing and Plating FcγRIIb CHO-K1 Cells

While maintaining the FcγRIIb CHO-K1 Cells (Cat.# J2232), follow the cell seeding density recommendations in the *FcγRIIb CHO-K1 Cells Propagation Model Technical Manual,* #TM569. 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 FcγRIIb CHO-K1 Cells two days before plating for the assay to ensure optimal and consistent assay performance.
2. On the day before performing the assay, prepare new FcγRIIb CHO-K1 cell plating medium (Ham's F-12/10% FBS) for the FcγRIIb CHO-K1 Cells.
3. Aspirate the cell culture medium from the FcγRIIb CHO-K1 Cells and wash with DPBS.

5.C. Preparing and Plating FcγRIIb 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 FcγRIIb 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 FcγRIIb CHO-K1 Cells by Trypan blue staining.
7. Centrifuge at 230 × *g* for 5 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).

5.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparation of a single stock of twofold serial dilutions of a single antibody for analysis in triplicate (120µl of each antibody 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 twofold serial dilutions, you will need 500µl of reference antibody at 3X the highest antibody concentration in your dose-response curve. You will need 240µl of each test antibody at 3X the highest antibody concentration for test antibody dose-response curves. For other dilution schemes, adjust the volumes accordingly.

Note: If you are using Control Ab, Anti-GITR, as a control in the assay, follow the instructions below to prepare twofold serial dilutions. A twofold serial dilution for test antibodies is listed as an example below as well.

1. On the day of assay, prepare an appropriate amount of assay buffer as described in Section 5.A.
2. To a sterile, clear V-bottom 96-well plate, add 240µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 8).
3. Add 240µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and G11, respectively (see Figure 8).
4. Add 120µl of assay buffer to the other wells in these four rows, from column 10 to column 2.
5. Transfer 120µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent twofold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.
7. Cover the plate with a lid and keep at ambient temperature (22–25°C) while preparing the GITR Effector Cells.

   Note: Wells A2, B2, E2 and G2 contain 120µl of assay buffer without antibody as a negative control.

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.

5.E. Preparing GITR Effector Cells

While maintaining the GITR 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.

1. Passage the cells two or three days before performing the assay as described in Section 4. To ensure optimal and consistent assay performance, maintain the cell density, upon harvest, in the range of 1.5–2.0 × 10^6 cells/ml and cell viability greater than 90%.
2. Count the GITR Effector Cells by Trypan blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of GITR Effector Cells from the culture vessel to a 50ml conical tube or larger sized centrifuge tube.
4. Collect the cells at 130 × *g* for 10 minutes at ambient temperature, and resuspend the pellet in assay buffer to generate the targeted cell density of 1.0 × 10^6 cells/ml.
5. You will need at least 8ml of GITR Effector Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.

5.F. Adding Antibody Samples and GITR Effector Cells to Assay Plates

1. Remove the 96-well assay plates containing FcγRIIb CHO-K1 Cells from the incubator. Using a manual multichannel pipette, remove 95µl of medium from each of the wells. Alternatively, 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.
2. Using an electronic multichannel pipette, add 25µl of the appropriate antibody titration to the assay plates according to the plate layout in Figure 7.
3. Add 75µl of assay buffer to the outside wells of the 96-well assay plates.
4. Transfer the GITR Effector Cells prepared in Section 5.E to a sterile reagent reservoir. Using a multichannel pipette, dispense 50µl (0.5×10^5 cells) of GITR Effector Cells into the wells containing antibody and FcγRIIb CHO-K1 Cells.
5. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 6 hours.

5.G. Adding Bio-Glo™ Reagent

Note: Bio-Glo™ Reagent should be at ambient temperature (22–25°C) when added to assay plates.

1. Remove the assay plates from the incubator and equilibrate to ambient temperature for 10–15 minutes.
2. Using a manual multichannel pipette, add 75µl of Bio-Glo™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, C1 and D1 of each assay plate to measure the background signal.
4. Incubate at ambient temperature for 5–15 minutes.

   Note: Varying the incubation time will affect the raw relative light unit (RLU) values but should not significantly change the EC_{50} value and fold induction.
5. Measure luminescence using a luminometer or luminescence plate reader.

5.H. Data Analysis

1. Determine the plate background by calculating the average RLU from wells B1, C1 and D1.
2. Calculate fold induction = $$\frac{\text{RLU (induced–background)}}{\text{RLU (no antibody control–background)}}$$
3. Graph data as RLU versus Log_{10} [antibody] and fold induction versus Log_{10} [antibody]. Fit curves and determine the EC_{50} value of antibody response using appropriate curve fitting software (such as GraphPad Prism® software).

6. Assay Protocol for Ligand or FcγRIIb-Independent Antibodies

This assay protocol illustrates the use of the GITR Bioassay, Propagation Model, 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 antibodies or ligand, choose an appropriate starting concentration and dilution scheme to achieve a full dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 1µg/ml as a starting concentration (1X) and a threefold dilution when testing GITR Ligand.

6.A. Preparing Assay Buffer, Bio-Glo™ Reagent and Antibody Samples

1. **Assay Buffer:** On the day of the assay, prepare an appropriate amount of assay buffer (99% RPMI 1640/1% 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, 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 with the crosslinked control ligand, GITRL, that we tested.

2. **Bio-Glo™ Reagent:** For reference, 10ml of Bio-Glo™ Reagent is sufficient to assay 120 wells in a 96-well assay format. Thaw the Bio-Glo™ Luciferase Assay Buffer at 4°C overnight or in a room temperature water bath on the day of assay. Equilibrate the Bio-Glo™ Luciferase Assay Buffer to ambient temperature, protected from light.

   Transfer all of the Bio-Glo™ Luciferase Assay Buffer into the amber bottle containing the Bio-Glo™ Luciferase Assay Substrate and mix by inversion until the substrate is thoroughly dissolved. Equilibrate and store the reconstituted Bio-Glo™ Reagent at ambient temperature (22–25°C) protected from light before adding to assay plates.

   If you are using a large (100ml) size of Bio-Glo™ Luciferase Assay System, dispense the reconstituted Bio-Glo™ Reagent into 10ml aliquots and store at –20°C for up to six weeks. Avoid repeated freeze-thaw cycles. On the day of the assay, thaw an appropriate amount of reconstituted Bio-Glo™ Reagent in a room temperature water bath for at least 1–2 hours before use. The Bio-Glo™ Reagent can be stored at room temperature after reconstitution with ~18% loss in luminescence after 24 hours.

3. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 3X final concentration) of two test antibodies (180µl each) and one reference crosslinked ligand (400µl) in 1.5ml tubes. Store the tubes containing ligand or antibody starting dilutions appropriately before making antibody serial dilutions.

   Notes:

   a. If you are using GITRL as a reference ligand, prepare a 400µl starting dilution with 3µg/ml of GITRL (HA-tagged) and 15µg/ml of crosslinking antibody, anti-HA (dilu1, 3X final concentration) by adding 12µl of GITRL, stock (100µg/ml) and 12µl of crosslinking antibody, anti-HA stock (500µg/ml) to 376µl of assay buffer. The final (1X) starting concentration is 1µg/ml of GITRL and 5µg/ml of anti-HA Ab. Store the antibody starting dilution on ice until ready to use in the assay.

   b. To streamline assay setup, prepare antibody or ligand serial dilutions prior to harvesting and plating cells.

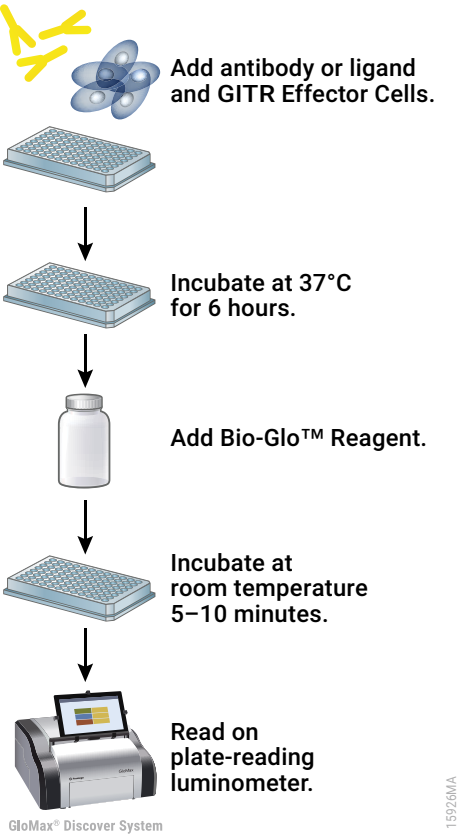

Figure 9. Schematic protocol for GITR Bioassay, Propagation Model, with GITR Ligand or FcγRIIb-independent antibody.

6.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 10 as a guide. The protocol describes serial replicate dilutions (n = 3) of test antibody and reference ligand 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 Ligand
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 Ligand
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 Ligand
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 10. Example plate layout showing nonclustered sample locations of test antibody and reference ligand dilution series and wells containing assay buffer alone (denoted by "B").

6.C. Preparing Ligand or Antibody Serial Dilutions

The instructions described here are for preparation of a single stock of threefold serial dilutions of a ligand for analysis in triplicate (120µl of each ligand 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 threefold serial dilutions for ligand, you will need 400µl of reference ligand at 3X the highest concentration in your dose response curve. To prepare threefold serial dilutions for test antibodies, you will need 180µl of each test antibody at 3X 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 GITRL (see Section 3) as a control in the assay, use the following instructions to prepare threefold serial dilutions. A threefold serial dilution for test antibodies is shown as an example on the next page.

6.C. Preparing Ligand or Antibody Serial Dilutions (continued)

1. On the day of 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 180µl of reference ligand starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 11).
3. Add 180µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and G11, respectively (Figure 11).
4. Add 120µl of assay buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 60µl of the ligand or antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent threefold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.
7. Cover the plate with a lid and keep at ambient temperature (22–25°C) while preparing the GITR Effector Cells.

   Note: Wells A2, B2, E2 and G2 contain 120µl of assay buffer without antibody as a negative control.

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 ligand	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ligand
B		no ligand	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ligand
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 11. Example plate layout showing antibody serial dilutions.

6.D. Preparing GITR Effector Cells

While maintaining the GITR 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.

1. Passage the cells two days or three days before performing the assay as described in Section 4. To ensure optimal and consistent assay performance, maintain the cell density, upon harvest, in the range of $1.5–2.0 \times 10^6$ cells/ml and cell viability at greater than 90%.
2. Count the GITR Effector Cells by Trypan blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of GITR Effector Cells from the culture vessel to a 50ml conical tube or larger sized centrifuge tube.
4. Collect the cells at 130 × *g* for 10 minutes at ambient temperature, and resuspend the pellet in assay buffer to generate the targeted cell density of 1.0×10^6 cells/ml.
5. You will need at least 8ml of GITR Effector Cells to fill 120 assay wells, using the inner 60 wells of two assay plates.

6.E. Adding Ligand or Antibody Samples and GITR Effector Cells to Assay Plates

1. Using an electronic multichannel pipette, add 25µl of the appropriate antibody or ligand titration to the assay plates according to the plate layout in Figure 10.
2. Add 75µl of assay buffer to the outside wells of the 96-well assay plates.
3. Transfer the GITR Effector Cells prepared in Section 6.D to a sterile reagent reservoir. Using a multichannel pipette, dispense 50µl (0.5×10^5 cells) of GITR Effector Cells into the wells containing antibody or ligand.
4. Cover each assay plate with a lid and incubate in a 37°C, 5% CO_2 incubator for 6 hours.

6.F. Adding Bio-Glo™ Reagent

Note: Bio-Glo™ Reagent should be at ambient temperature (22–25°C) when added to assay plates.

1. Remove the assay plates from the incubator and equilibrate to ambient temperature for 10–15 minutes.
2. Using a manual multichannel pipette, add 75µl of Bio-Glo™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, C1 and D1 of each assay plate to measure the background signal.
4. Incubate at ambient temperature for 5–15 minutes.

   Note: Varying the incubation time will affect the raw relative light unit (RLU) values but should not significantly change the EC_{50} value and fold induction.
5. Measure luminescence using a luminometer or luminescence plate reader.

6.G. Data Analysis

1. Determine the plate background by calculating the average RLU from wells B1, C1 and D1.
2. Calculate fold induction = $\frac{\text{RLU (induced–background)}}{\text{RLU (no antibody control–background)}}$
3. Graph data as RLU versus Log_{10} [antibody] and fold induction versus Log_{10} [antibody]. Fit curves and determine the EC_{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.
	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™ Reagent leads to low RLU. Store and handle the Bio-Glo™ Reagent according to the instructions.
	If performing the assay for the first time, we recommend that you use the FcγRIIb Cells since your Ab of your interest may be dependent on crosslinking by FcγRIIb. In the case of ligands, crosslinking by an antibody may be necessary.
Variability in assay performance	Variations in cell growth conditions including cell plating and harvest density, cell viability and cell doubling time. Ensure consistent cell growth by handling the cells exactly according to the instructions.
	Inappropriate cell handling during cell harvest, including long centrifuge times and high centrifuge speeds, may cause low assay performance and high assay variation.
	Inappropriate cell handling during cell freezing, including long DMSO exposure times before freezing may cause low assay performance and high assay variation.
	Inappropriate cell counting methods may lead to variation in cell numbers in culture and assays and cause high assay variation. Ensure accurate and consistent cell counting methods.

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7. Troubleshooting (continued)

Symptoms	Causes and Comments
Weak assay response (low fold induction)	Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The EC_{50} value obtained in the GITR Bioassay may vary from the EC_{50} value obtained using other methods such as primary T cell-based assays.
	Determine if the antibody used is dependent on crosslinking for performance by testing in the presence of FcγRIIb cells.
	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.

8. References

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9. Appendix

9.A. Representative Assay Results with FcγRIIb-Dependent Antibody

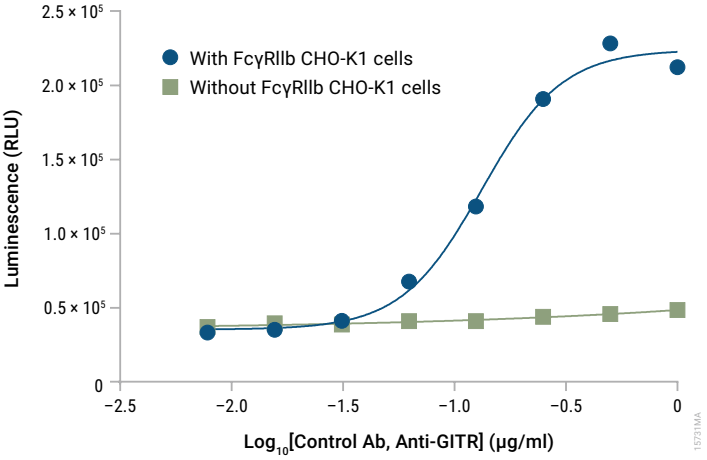

Figure 12. The GITR Bioassay measures the activity of Control Antibody, Anti-GITR. FcγRIIb CHO-K1 Cells were plated overnight. The following day, a titration of Control Ab, Anti-GITR, (Section 5.C) was added followed by GITR Effector Cells. After a 6-hour induction at 37°C, Bio-Glo™ Reagent was added, and luminescence was determined using a GloMax® Discover System. Four-parameter logistic curve analysis was performed with GraphPad Prism® software. The EC_{50} response determined was ~0.13µg/ml, and the fold induction was ~7.

9.B. Representative Assay Results with GITR Ligand

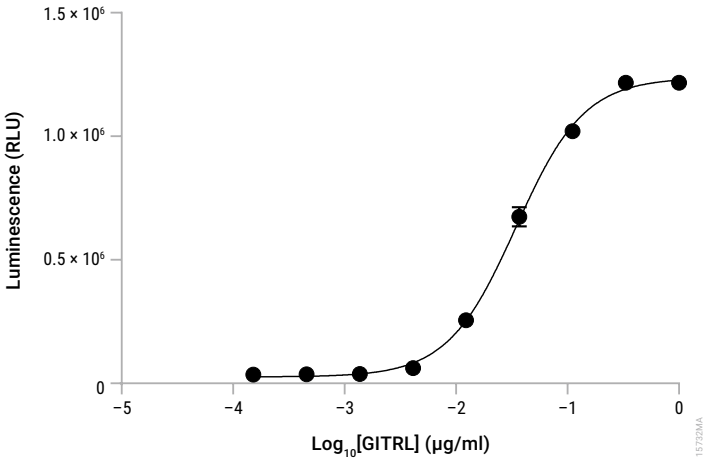

Figure 13. The GITR Bioassay measures the activity of GITR Ligand. On the assay day, GITR Effector Cells were plated in a 96-well plate at 50,000 cells/well. Cells were incubated with various concentrations of GITRL (Section 6.C). After a 6-hour induction at 37°C, Bio-Glo™ Reagent was added, and luminescence was determined using a GloMax® Discover System. Four-parameter logistic curve analysis was performed with GraphPad Prism® software. The EC_{50} response determined was ~0.035µg/ml, and the fold induction was ~34.

9.C. Composition of Buffers and Solutions

initial cell culture medium for GITR Effector Cells

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS
1mm	sodium pyruvate
0.1mm	MEM nonessential amino acids

cell growth medium for GITR Effector Cells

90%	RPMI 1640 with L-glutamine and HEPES
10%	FBS
500µg/ml	hygromycin B
800µg/ml	G418 sulfate solution
1mm	sodium pyruvate
0.1mm	MEM nonessential amino acids

cell freezing medium for GITR Effector Cells

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

cell plating medium for FcγRIIb CHO-K1 Cells

90%	Ham's F12
10%	FBS

assay buffer

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

10. Summary of Changes

The following changes were made to the 5/26 revision of this document:

1. Removed Section 9.D, Related Products.
2. Made minor text and formatting edits.

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