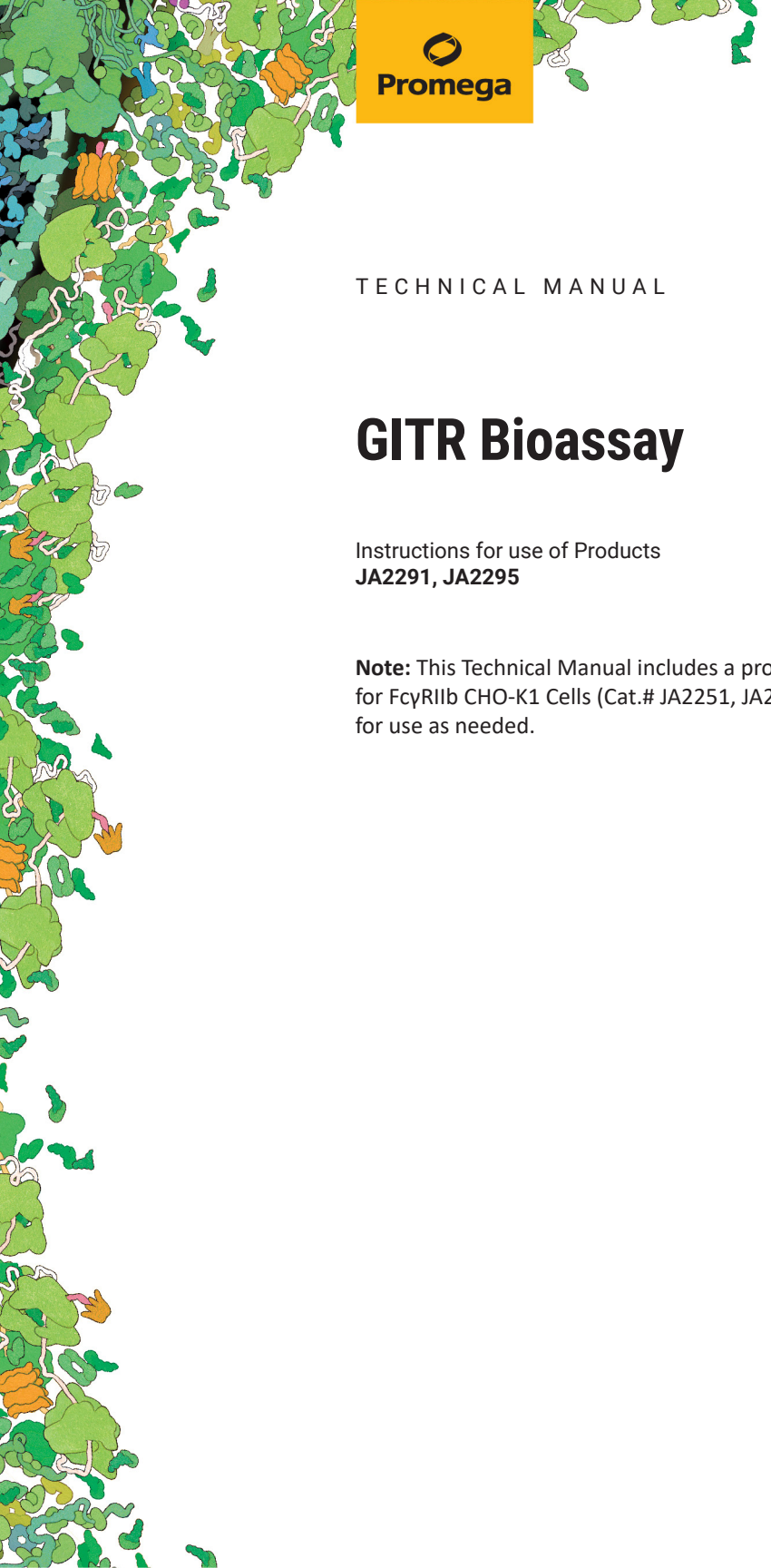

TECHNICAL MANUAL

GITR Bioassay

Instructions for use of Products
JA2291, JA2295

Note: This Technical Manual includes a protocol for FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255) for use as needed.

Revised 6/25
TM582

Promega

GITR 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. 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 strategy 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[a–e] (Cat.# JA2291, JA2295), 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 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.

The GITR Bioassay should be conducted with FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255) 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[e] and a standard luminometer such as the GloMax® Discover System (see Section 8.C, Related Products).

The GITR Bioassay 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.

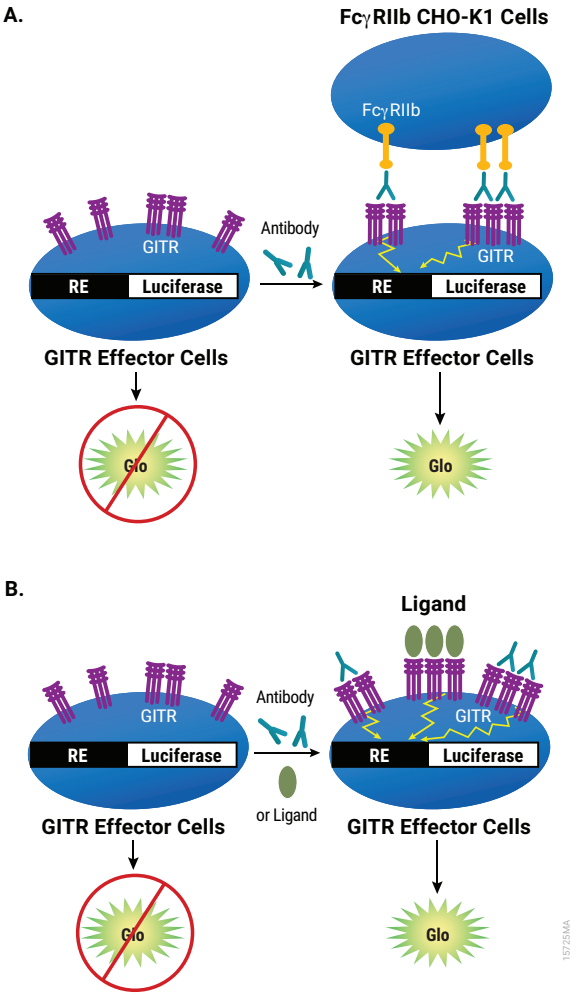

Figure 1. Representation of the GITR Bioassay. Panel A. Assay with FcγRIIb-dependent agonist antibody. The bioassay consists of two engineered cell lines, GITR Effector Cells and FcγRIIb CHO-K1 Cells. In the presence of FcγRIIb CHO-K1 Cells, the anti-GITR antibody can be crosslinked, thereby inducing 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.

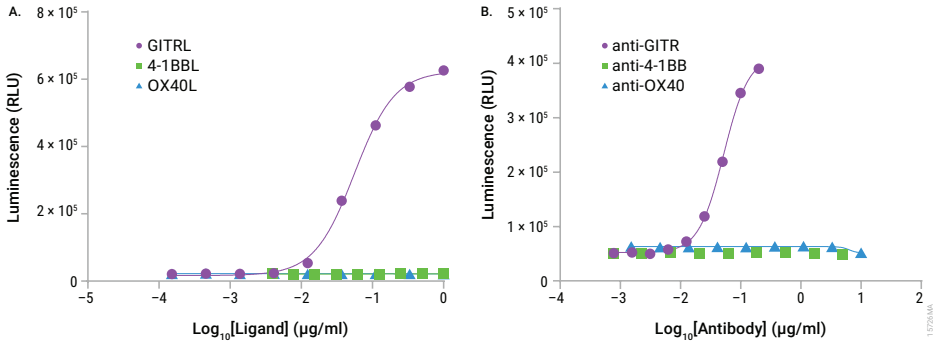

Figure 2. The GITR Bioassay reflects the mechanism of action (MOA) and shows specificity of biologics designed to activate GITR. Panel A. GITR Effector Cells were induced, respectively, with a serial titration of ligands: GITRL, 4-1BBL or OX40L as indicated. **Panel B.** GITR Effector Cells were induced with a serial titration of anti-GITR, anti-4-1BB or anti-OX40 antibodies, 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)

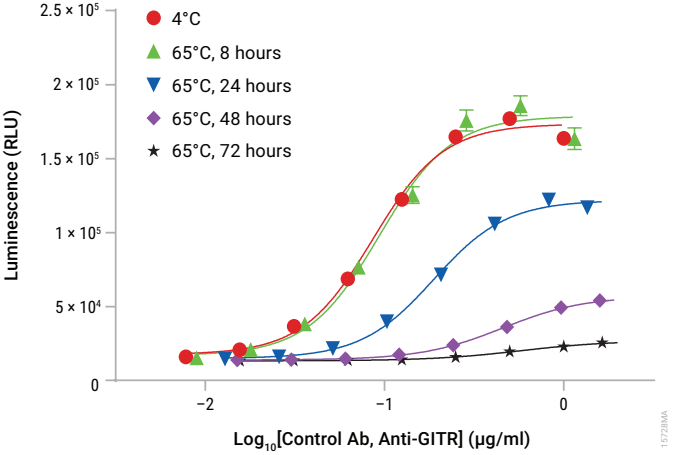

Figure 3. The GITR Bioassay is stability-indicating. Samples of Control Ab, Anti-GITR (Cat.# K1171), were maintained at 4°C (control) or heat-treated at 65°C for the indicated times, then analyzed using the GITR Bioassay with FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255). After a 6-hour induction at 37°C, 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.

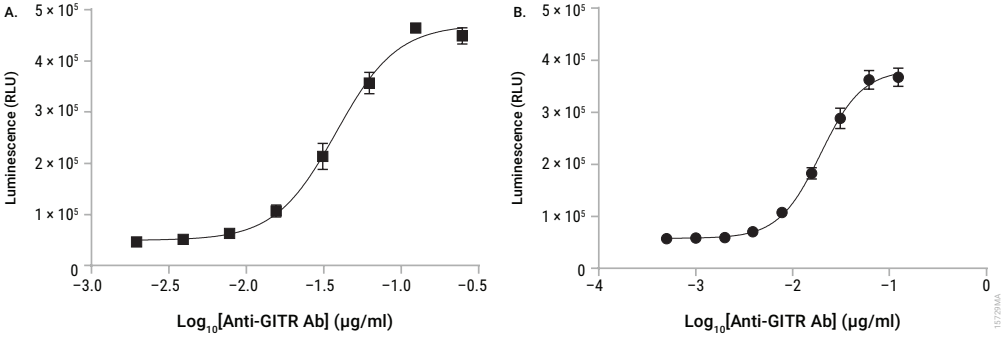

Figure 4. The GITR Bioassay is amenable to 384-well plate format and compatible with laboratory automation. Panel A. The GITR Bioassay was performed in 96-well plates as described in this technical manual using anti-GITR antibody. **Panel B.** The GITR Bioassay was performed in 384-well format using 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)

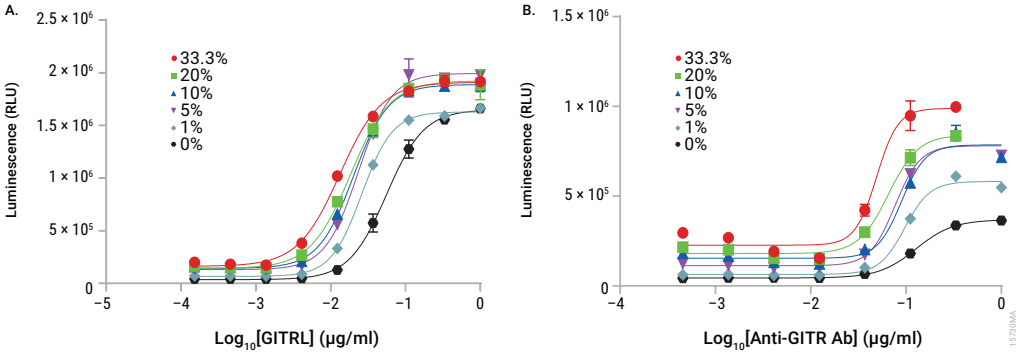

Figure 5. The GITR Bioassay is tolerant to human serum. Panel A. GITR ligand (GITRL, R&D Systems Cat.#6987-GL-025/CF) was analyzed in the presence of increasing concentrations of pooled normal human serum (0–100% in the ligand sample), resulting in final assay concentration of human serum (0–33.3%). **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 concentrations of human serum (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	**1 each**	**JA2291**

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 GITR Effector Cells (0.5ml)
- 36ml RPMI 1640 Medium
- 4ml Fetal Bovine Serum
- 1 vial Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 10ml Bio-Glo™ Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
GITR Bioassay 5X	**1 each**	**JA2295**

Not for Medical Diagnostic Use.

Each kit contains sufficient reagents for 600 assays using the inner 60 wells of two 96-well plates. Includes:

- 5 vials GITR Effector Cells (0.5ml)
- 5 × 36ml RPMI 1640 Medium
- 5 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 5 × 10ml Bio-Glo™ Luciferase Assay Buffer

Note: The GITR Bioassay components are shipped separately because of different temperature requirements. The GITR Effector Cells are shipped on dry ice. The Bio-Glo™ Luciferase Assay Substrate and Buffer and Fetal Bovine Serum are shipped on dry ice, separately from the cells. The RPMI 1640 Medium is shipped at ambient temperature.

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.
- Store Bio-Glo™ Luciferase Assay Substrate, Bio-Glo™ Luciferase Assay Buffer and Fetal Bovine Serum at –20°C. Avoid multiple freeze-thaw cycles of the serum.
- For optimal performance, use reconstituted Bio-Glo™ Reagent on the day of preparation. However, once reconstituted, Bio-Glo™ Reagent can be stored at –20°C for up to 6 weeks.
- Store RPMI 1640 Medium at 4°C protected from fluorescent light.

Available Separately

PRODUCT	SIZE	CAT.#
FcγRIIb CHO-K1 Cells	**1 each**	**JA2251**
FcγRIIb CHO-K1 Cells 5X	**1 each**	**JA2255**

Not for Medical Diagnostic Use.

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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 the Certificate of Analysis.

The GITR Bioassay 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.# JA2251, JA2255) 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. Representative data are shown Figures 2–5 and Sections 8.A and B, Representative Assay Results.

The GITR Effector 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.

The GITR Bioassay 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 (see Section 8.C, Related Products). An integration time of 0.5 seconds/well was used for all readings.

Materials to Be Supplied by the User

- user-defined anti-GITR antibodies or other biologics samples
- 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)
- **optional:** Control Ab, Anti-GITR (Cat.# K1171)
- **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)

4. 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 (Cat.# JA2251, JA2255) and cell propagation model (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 thaw-and-use format. Each test and reference antibody is run in triplicate, in a ten-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.

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

1. **FcγRIIb CHO-K1 Cell Recovery Medium:** On the day before the assay, prepare 30ml of cell recovery medium (95% RPMI 1640/5% 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 1.5ml of FBS to 28.5ml of RPMI 1640 medium. Mix well and warm to 37°C before use. Store the remaining FBS at 4°C overnight for use on the assay day.

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-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.

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:

   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.

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

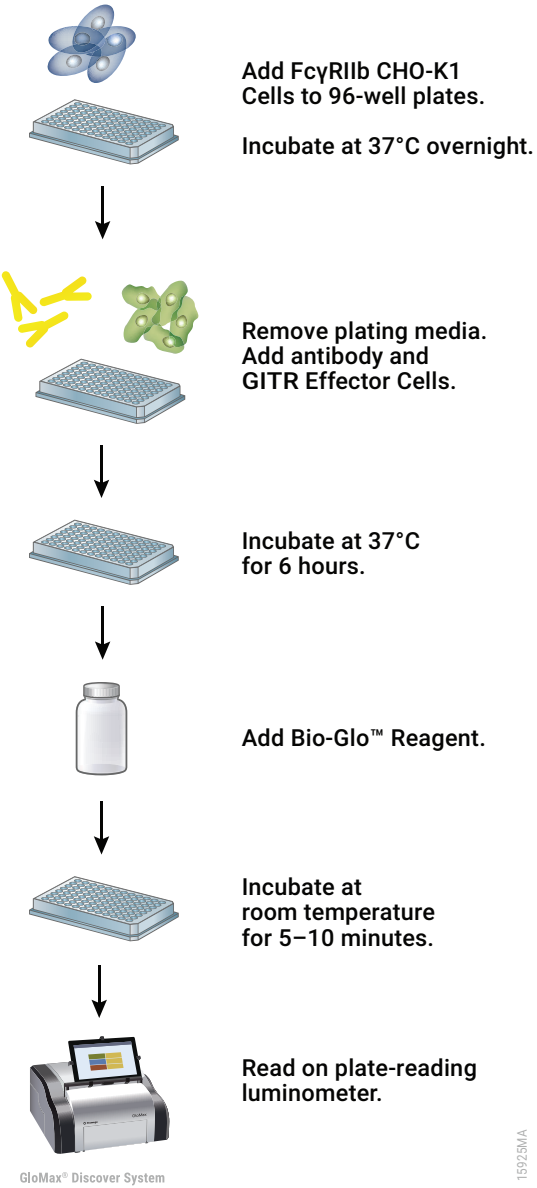

Figure 6. Schematic protocol for GITR Bioassay with FcγRIIb-dependent antibody.

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 alone (denoted by "B").

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

The thaw-and-use FcγRIIb CHO-K1 Cells not 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 cell manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at one time.

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

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

1. On the day before performing the assay, prepare 30ml of cell recovery medium (RPMI 1640/5% FBS) as described in Section 4.A.
2. Warm the cell recovery medium in a 37°C water bath for 15 minutes and transfer 14.5ml of cell recovery medium to a 15ml conical tube.
3. Remove one vial of thaw-and-use FcγRIIb CHO-K1 Cells from –140°C storage and transfer to the bench on dry ice. Thaw the vial in a 37°C water bath until cells are just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the cells.
4. Gently mix the cell suspension in the vial by pipetting, then transfer 0.5ml of cells to the tube containing 14.5ml of cell recovery medium. Mix well by gently inverting 1–2 times.
5. Transfer the cell suspension to a sterile reagent reservoir. Immediately, using a multichannel pipette, dispense 100µl of cell suspension to each well of the inner 60-wells of two 96-well, white, flat-bottom assay plates.
6. Add 100µl of cell recovery medium per well to the outside wells of the assay plates.
7. Cover the assay plates with a lid and incubate the cells overnight in a 37°C, 5% CO_2 incubator.

4.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 4.A.
2. Warm the assay buffer in a 37°C water bath for 15 minutes then transfer 9.5ml of assay buffer to a 15ml conical tube. Set tube aside to be used in Section 4.E.
3. 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).
4. Add 240µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and G11, respectively (see Figure 8).
5. Add 120µl of assay buffer to the other wells in these four rows, from column 10 to column 2.
6. Transfer 120µl of the antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
7. Repeat equivalent twofold 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 120µl of assay buffer without antibody as a negative control.
8. Remove the 96-well assay plates containing FcγRIIb CHO-K1 Cells from the incubator, then remove 95µl of medium per well from the inner 60 wells using manual multichannel pipette.
9. Using an electronic multichannel pipette, add 25µl of the appropriate antibody dilution (see Figure 8) to the preplated FcγRIIb CHO-K1 Cells according to the plate layout in Figure 7.
10. Cover the assay plate with a lid and keep at ambient temperature (22–25°C) while preparing the GITR 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.

4.E. Preparing GITR Effector Cells and Setting Up Assay

Note: The thaw-and-use GITR 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 cell manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at one time.

1. Remove one vial of thaw-and-use GITR Effector Cells from –140°C storage and transfer to the bench on dry ice. Thaw the vial in a 37°C water bath until cells are just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the cells.
2. Gently mix the cell suspension in the vial by pipetting, and then transfer 0.5ml of cells to the 15ml conical tube containing 9.5ml of assay buffer (from Section 4.D). Mix well by gently inverting 1–2 times.
3. Transfer the GITR Effector Cell suspension to a sterile reagent reservoir. Immediately, using a multichannel pipette, dispense 50µl of cell suspension to each well of the inner 60-wells of the plates containing preplated FcγRIIb CHO-K1 Cells and anti-GITR antibody.
4. Add 75µl of assay buffer to the outside wells of the 96-well assay plates.
5. Place lids on the plates and incubate the plates in a CO_2 incubator at 37°C for 6 hours.

4.F. Adding Bio-Glo™ Reagent

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

1. Take the assay plates out of 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.

4.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).

5. Assay Protocol for Ligand or FcγRIIb-Independent Antibodies

This assay protocol illustrates the use of the GITR 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 ten-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.

5.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. 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:

   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.

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

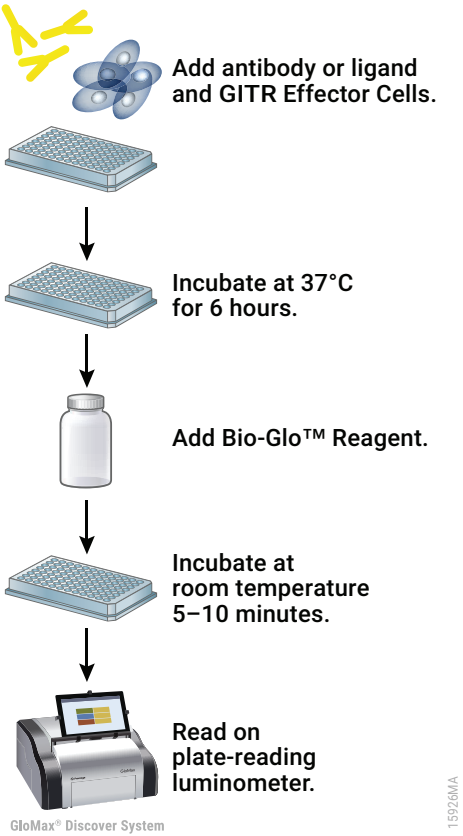

Figure 9. Schematic protocol for GITR Bioassay with GITR ligand or FcγRIIb-independent antibody.

5.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").

5.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.

5.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 5.A.
2. Warm the assay buffer in a 37°C water bath for 15 minutes and transfer 9.5ml of assay buffer to a 15ml conical tube. Set the tube aside to be used in Section 5.D.
3. 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).
4. Add 180µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and G11, respectively (Figure 11).
5. Add 120µl of assay buffer to other wells in these four rows, from column 10 to column 2.
6. Transfer 60µl of the ligand or antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
7. Repeat equivalent threefold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.
8. 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.

5.D. Preparing GITR Effector Cells and Setting Up Assay

Note: The thaw-and-use GITR 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 cell manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at one time.

1. Remove one vial of thaw-and-use GITR Effector Cells from –140°C storage and transfer to the bench on dry ice. Thaw the vial in a 37°C water bath until cells are just thawed (about 2 minutes). While thawing, gently agitate and visually inspect.
2. Gently mix the cell suspension in the vial by pipetting, then transfer 0.5ml of cells to the 15ml conical tube containing 9.5ml of assay buffer (from Section 5.C). Mix well by gently inverting 1–2 times.
3. Transfer the GITR cell suspension to a sterile reagent reservoir. Immediately, using a multichannel pipette, dispense 50µl of cell suspension to each well of the inner 60 wells of two 96-well, white, flat-bottom assay plates.
4. Using an electronic multichannel pipette, add 25µl of the appropriate antibody or ligand titrations, prepared in Section 5.C, to the assay plates according to the plate layout in Figure 10.
5. Add 75µl of assay buffer to the outside wells of the 96-well assay plates.
6. Cover each assay plate with a lid and incubate the plates in a 37°C, 5% CO_2 incubator at 37°C for 6 hours.

5.E. 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.F. 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. 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 FcγRIIb CHO-K1 Cells, as your antibody of interest may be dependent on crosslinking by FcγRIIb. In the case of ligands, crosslinking by an antibody may be necessary.
Variability in assay performance	Inappropriate cell handling during cell plating, including long water bath incubation time, may cause low assay performance. We recommend dispensing cells into plates immediately after thawing cell vials.
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 CHO-K1 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.

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7. References

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5. Kim, I.K. *et al.* (2015) Glucocorticoid-induced tumor necrosis factor receptor–related protein co-stimulation facilitates tumor regression by inducing IL-9–producing helper T cells. *Nat. Med.* **21**, 1010–17.
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8. Appendix

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

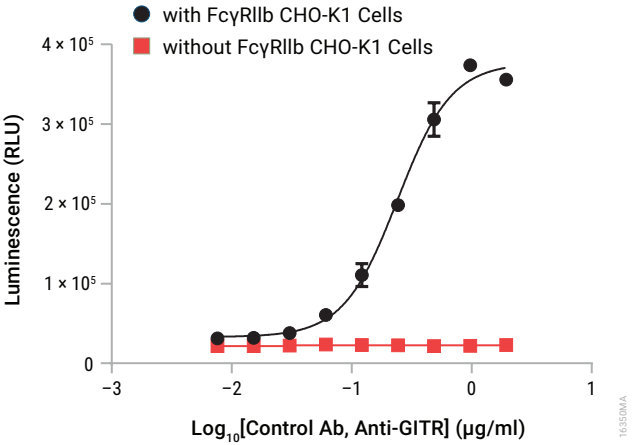

Figure 12. The GITR Bioassay measures the activity of Control Antibody, Anti-GITR. FcγRIIb CHO-K1 Cells were plated overnight. The following day, a titration of Control Ab, Anti-GITR, (Section 4.D) was added followed by addition of GITR Effector Cells. After a 6-hour induction at 37°C, Bio-Glo™ Reagent 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 ~0.25µg/ml, and the fold induction was ~12.

8.B. Representative Assay Results with GITR Ligand

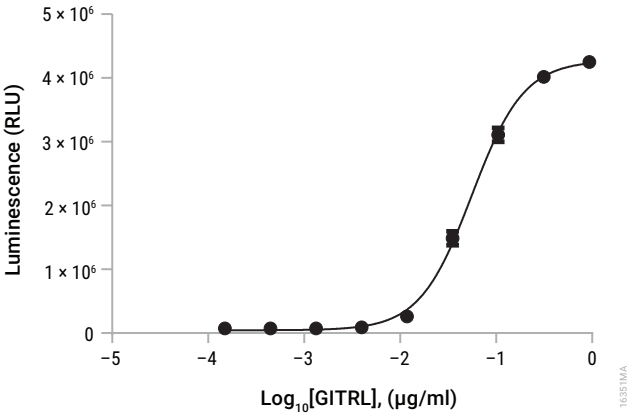

Figure 13. The GITR Bioassay measures the activity of GITR Ligand. On the assay day, GITR Effector Cells were plated in a 96-well plate. Cells were incubated with various concentrations of GITRL (Section 5.C). After a 6-hour induction at 37°C, Bio-Glo™ Reagent was added, and luminescence was determined using a GloMax® Discover System. Four-parameter logistic curve analysis was performed with GraphPad Prism® software. The EC_{50} response determined was ~0.06µg/ml, and the fold induction was ~50.

8.C. Related Products

Immune Checkpoint Bioassays

Product	Size	Cat.#
CD40 Bioassay	1 each	JA2151
CD40 Bioassay 5X	1 each	JA2155
CD40 Bioassay, Propagation Model	1 each	J2132
4-1BB Bioassay	1 each	JA2351
4-1BB Bioassay 5X	1 each	JA2355
4-1BB Bioassay, Propagation Model	1 each	J2332
LAG-3/MHCII Blockade Bioassay	1 each	JA1111
LAG-3/MHCII Blockade Bioassay 5X	1 each	JA1115
LAG-3/MHCII Blockade Bioassay, Propagation Model	1 each	JA1112
PD-1/PD-L1 Blockade Bioassay	1 each	J1250
PD-1/PD-L1 Blockade Bioassay 5X	1 each	J1255
PD-L1 Negative Cells	1 each	J1191
CTLA-4 Blockade Bioassay	1 each	JA3001
CTLA-4 Blockade Bioassay 5X	1 each	JA3005
Control Ab, Anti-CTLA-4	100µg	JA1020
Control Ab, Anti-4-1BB	50µg	K1161
Control Ab, Anti-CD40	50µg	K1181
Control Ab, Anti-GITR	50µg	K1171
Control Ab, Anti-LAG-3	100µg	K1150
Control Ab, Anti-OX40	50µg	K1191
TIGIT/CD155 Blockade Bioassay	1 each	J2201
TIGIT/CD155 Blockade Bioassay 5X	1 each	J2205
TIGIT/CD155 Blockade Bioassay, Propagation Model	1 each	J2092
PD-1+TIGIT Combination Bioassay	1 each	J2211
PD-1+TIGIT Combination Bioassay 5X	1 each	J2215
PD-1+TIGIT Combination Bioassay, Propagation Model	1 each	J2102
FcγRIIb CHO-K1 Cells	1 each	JA2251
FcγRIIb CHO-K1 Cells 5X	1 each	JA2255
FcγRIIb CHO-K1 Cells (CPM)	1 each	J2232
OX40 Bioassay	1 each	JA2191
OX40 Bioassay 5X	1 each	JA2195

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Additional kit formats are available.

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8.C. Related Products (continued)

Cytokine and Growth Factor Bioassays

Product	Size	Cat.#
IL-2 Bioassay	1 each	JA2201
IL-2 Bioassay 5X	1 each	JA2205
IL-2 Bioassay, Propagation Model	1 each	J2952
IL-15 Bioassay	1 each	JA2011
IL-15 Bioassay 5X	1 each	JA2015
IL-15 Bioassay, Propagation Model	1 each	J2962

Fc Effector Bioassays

Product	Size	Cat.#
ADCC Reporter Bioassay, Complete Kit (Raji)*	1 each	G7015
ADCC Reporter Bioassay, Target Kit (Raji)*	1 each	G7016
ADCC Reporter Bioassay, Core Kit*	1 each	G7010
ADCC Reporter Bioassay, F Variant, Core Kit**	1 each	G9790
FcγRIIa-H ADCP Reporter Bioassay, Complete Kit**	1 each	G9901
FcγRIIa-H ADCP Reporter Bioassay, Core Kit**	1 each	G9991

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T Cell Activation Bioassays

Product	Size	Cat.#
T Cell Activation Bioassay (NFAT)	1 each	J1621
T Cell Activation Bioassay (NFAT) 5X	1 each	J1625
T Cell Activation Bioassay (NFAT), Propagation Model	1 each	J1601
T Cell Activation Bioassay (IL-2)	1 each	J1651
T Cell Activation Bioassay (IL-2) 5X	1 each	J1655
T Cell Activation Bioassay (IL-2), Propagation Model	1 each	J1631

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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/**

Detection Reagent

Product	Size	Cat.#
Bio-Glo™ Luciferase Assay System	10ml	G7941
	100ml	G7940

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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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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/**

9. Summary of Changes

The following changes were made to the 6/25 revision of this document:

1. Revised text about the label in Section 3.
2. Removed an expired patent statement and revised another patent.
3. Updated the fonts and the cover image.

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[d]U.S. Pat. No. 8,008,006.

[e]U.S. Pat. No. 10,077,244 and other patents.

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