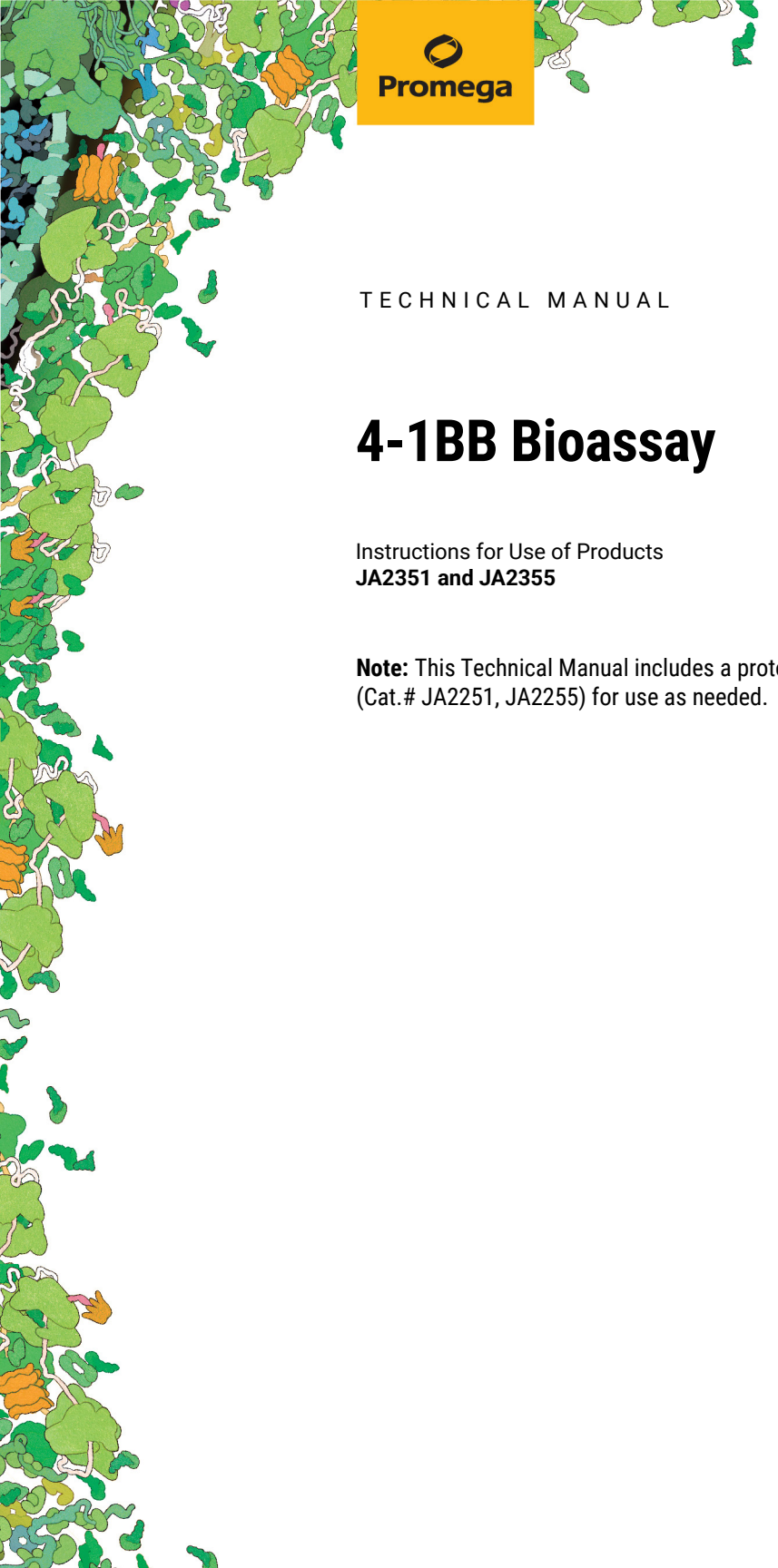


TECHNICAL MANUAL

4-1BB Bioassay

Instructions for Use of Products
JA2351 and JA2355

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

Revised 8/26
TM570

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4-1BB 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 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).

4-1BB (CD137/TNFRSF9), a member of the tumor necrosis factor receptor superfamily, is an inducible costimulatory receptor expressed on T cells, natural killer (NK) cells and innate immune cell populations (4). When present on the cell surface, 4-1BB interacts with 4-1BB ligand (4-1BBL) and induces subsequent cell proliferation and production of interferon gamma (IFNγ) and IL-2, particularly in T and NK cells (5,6).

Current methods used to measure the activity of biologic drugs targeting 4-1BB rely on primary human T cells and measurement of functional endpoints such as cell proliferation, cell surface marker expression, and IFNγ and interleukin-2 (IL-2) production. These assays are laborious and highly variable due to their reliance on donor primary cells, complex assay protocols and nonqualified assay reagents. As a result, these assays are difficult to establish in a quality-controlled, drug-development setting.

The 4-1BB Bioassay[a,c] (Cat.# JA2351, JA2355) 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 4-1BB (6,7). The assay consists of a genetically engineered Jurkat T cell line that expresses human 4-1BB and a luciferase reporter driven by a response element that can respond to 4-1BB ligand/agonist antibody stimulation. The 4-1BB Effector Cells[a,b,c] 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 antibodies used to stimulate T cell activation via interaction with co-stimulatory receptors on the effector cells, can be crosslinked via binding to FcγRIIb receptors. This crosslinking increases avidity, stabilizes the interaction of the antibody to the receptor, enhances receptor clustering and downstream signaling (8,9).

The 4-1BB Bioassay can be conducted with FcγRIIb CHO-K1 Cells[a,c] (Cat.# JA2251, JA2255) to test whether agonist antibodies activate 4-1BB 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 co-stimulatory 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 co-stimulatory immune checkpoint targets.

Induction of the 4-1BB Effector Cells with a 4-1BB 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 (Cat.# GM3000).

The 4-1BB Bioassay reflects the mechanism of action (MOA) of biologics designed to activate 4-1BB. Specifically, 4-1BB-mediated luminescence is detected following the incubation of 4-1BB agonist antibodies or 4-1BB ligand (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 is 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 ligand or antibody samples) with minimal impact on fold induction (Figure 5), indicating potential for further development into a neutralizing antibody bioassay.

1. Description (continued)

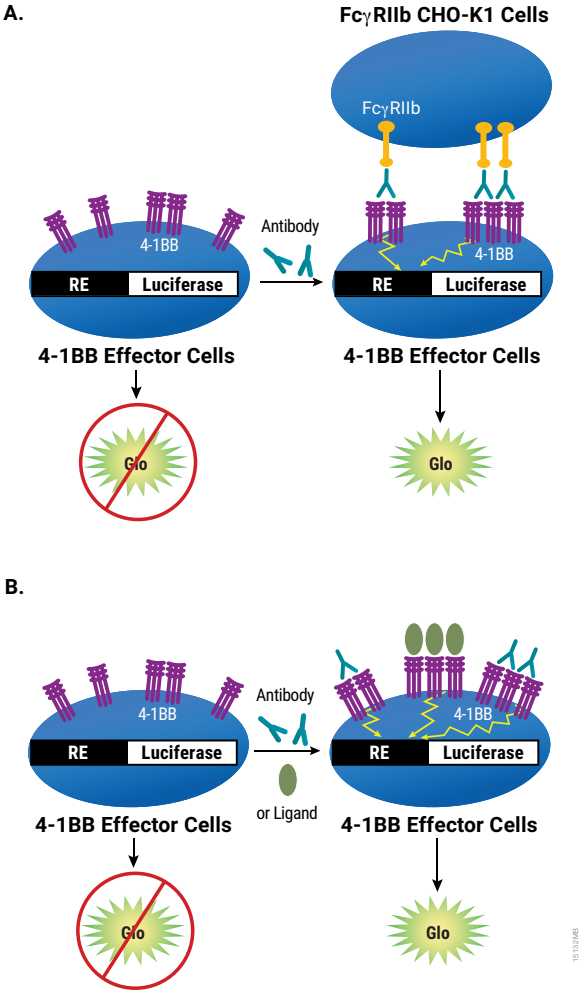


Figure 1. Representation of the 4-1BB Bioassay. Panel A. Assay with FcγRIIb-dependent agonist antibody. The bioassay consists of two engineered cell lines, 4-1BB Effector Cells and FcγRIIb CHO-K1 Cells. In the presence of FcγRIIb CHO-K1 Cells, the anti-4-1BB antibody can be crosslinked, thereby inducing 4-1BB pathway-activated luminescence. **Panel B.** Assay with FcγRIIb-independent agonist antibody or ligand. The bioassay consists of one engineered cell line, 4-1BB Effector Cells. In the absence of agonist antibody or 4-1BB ligand, the 4-1BB receptor is not activated and luminescence signal is low. The addition of agonist antibody or 4-1BB ligand induces the 4-1BB pathway-activated luminescence, which can be detected in a dose-dependent manner.

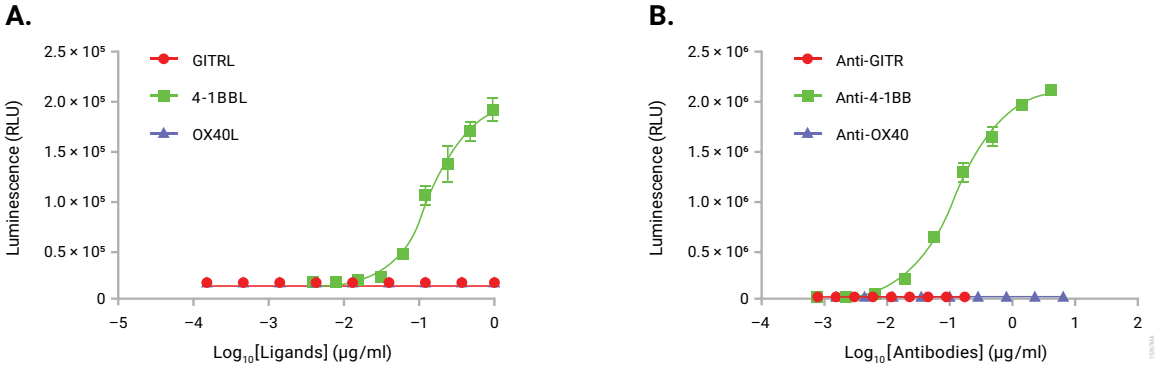


Figure 2. The 4-1BB Bioassay reflects the mechanism of action (MOA) and shows specificity of biologics designed to activate 4-1BB. Panel A. 4-1BB Effector Cells were induced, respectively, with a serial titration of ligands: GITRL, 4-1BBL and OX40L as indicated. **Panel B.** 4-1BB Effector Cells were induced with a serial titration of anti-GITR antibody, Control Ab, Anti-4-1BB (Cat.# K1161) and anti-OX40 antibody, as indicated, in the presence of FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255). After a 6-hour incubation, 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 4-parameter logistic curve using GraphPad Prism® software.

Table 1. The 4-1BB Bioassay Shows Precision, Accuracy and Linearity.

Parameter	**Results**	
Accuracy	% Expected Relative Potency	% Recovery
	50	106.0
	70	96.2
	140	97.3
	200	103.2
Repeatability (% CV)	100% (Reference)	3.5
Intermediate Precision (% CV)		6.6
Linearity (r^2)		0.996
Linearity (y = mx + b)		y = 1.025x–1.835
A 50–200% theoretical potency series of Control Ab, Anti-4-1BB, was analyzed in triplicate in three independent experiments performed on three days by two analysts (for a total of six independent experiments). Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were analyzed and relative potencies calculated after parallelism determination using JMP® software. Data were generated using thaw-and- use cells.		

1. Description (continued)

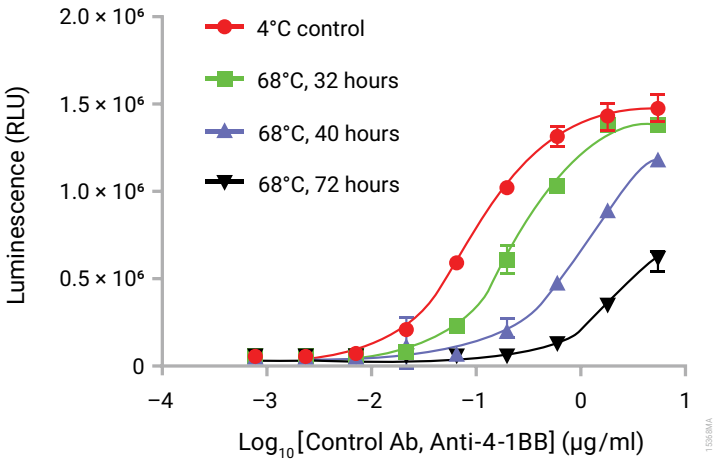


Figure 3. The 4-1BB Bioassay is stability-indicating. Samples of Control Ab, Anti-4-1BB (Cat.# K1161), were maintained at 4°C (control) or heat-treated at 68°C for indicated times, and then analyzed using the 4-1BB Bioassay with FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255). After a 6-hour incubation, 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.

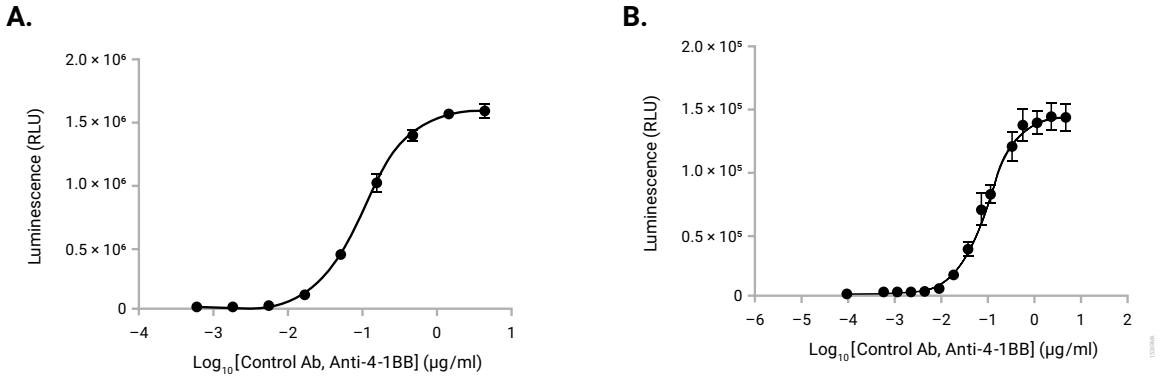


Figure 4. The 4-1BB Bioassay is amenable to 384-well plate format and compatible with laboratory automation. Panel A. The 4-1BB Bioassay was performed in 96-well plates as described in this technical manual using Control Ab, Anti-4-1BB. **Panel B.** The 4-1BB Bioassay was performed in 384-well format using a Mantis® liquid handler to dispense the cells and Echo® Acoustic liquid handler for antibody handling. On the day before assay, FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255) were plated at 8×10^3 cells/10µl/well. On the day of the assay, Control Ab, Anti-4-1BB (Cat.# K1161), was serially diluted and added to the plate at 0.2µl/well. Finally, 4-1BB 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 0.13µg/ml and 0.095µg/ml, and the fold inductions were 103 and 58 for 96-well and 384-well format, respectively. Data were generated using thaw-and-use cells.

1. Description (continued)

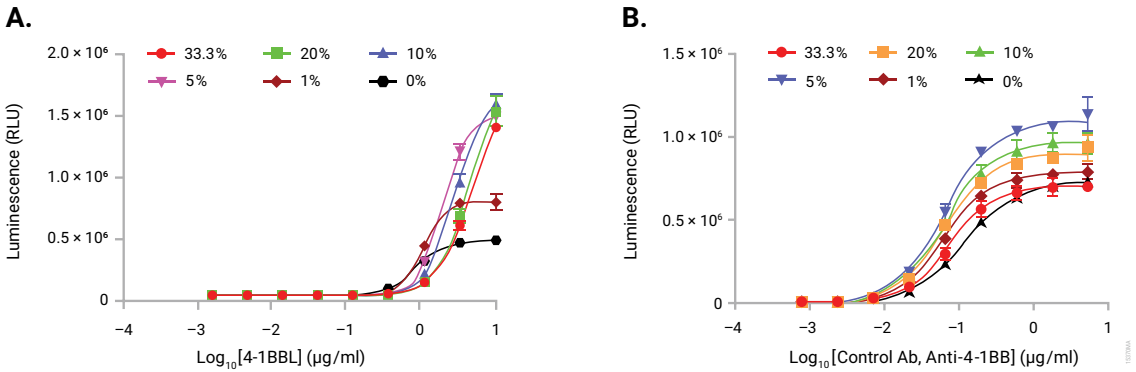


Figure 5. The 4-1BB Bioassay is tolerant to human serum. Panel A. 4-1BB ligand (4-1BBL, R&D Systems Cat.#2295-4L-025/CF) was analyzed in the presence of increasing concentrations of pooled normal human serum (0–100% in the ligand sample), resulting in final assay concentration of human serum (0–33.3%). **Panel B.** Control Ab, Anti-4-1BB 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 (0–33.3%). After a 6-hour incubation, Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells. The 4-1BB 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.#
4-1BB Bioassay	**1 each**	**JA2351**

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

PRODUCT	SIZE	CAT.#
4-1BB Bioassay 5X	**5 each**	**JA2355**

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 4-1BB Effector Cells (0.5ml per vial)
- 5 × 36ml RPMI 1640 Medium
- 5 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo™ Luciferase Assay System (lyophilized)
- 5 × 10ml Bio-Glo™ Luciferase Assay Buffer

Available Separately

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

Not for Medical Diagnostic Use.

Note: The 4-1BB Bioassay components are shipped separately because of different temperature requirements. The 4-1BB 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 –30°C to –10°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 –30°C to –10°C for up to 6 weeks.

Store RPMI 1640 Medium at +2°C to +10°C protected from fluorescent light.

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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 kit 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 4-1BB Bioassay is intended to be used with user-provided antibodies or ligands designed to activate 4-1BB. Control Ab, Anti-4-1BB (Cat.# K1161) and FcγRIIb CHO-K1 Cells Cat.# JA2251, JA2255) are available separately for use in assay optimization and routine quality control. We strongly recommend including 4-1BB Ligand or Control Ab, Anti-4-1BB, along with 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 8.A and B, Representative Assay Results.

The 4-1BB 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 appropriated concentration for the assay. The cells are sensitive, and care should be taken to follow cell thawing and plating procedures as described in Section 4.E.

The 4-1BB 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 (Cat.# GM3000). An integration time of 0.5 second/well was used for all readings.

3.A. Materials to Be Supplied by the User

- user-defined anti-4-1BB 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., Corning® 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., 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:** FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255; If using this assay for the first time and/or testing an Ab that might be dependent on crosslinking FcγRIIb, use this cell line.)
- **optional:** Control Ab, Anti-4-1BB (Cat.# K1161)
- **optional:** control 4-1BB ligand/TNFSF9 with His tag (R&D Systems Cat.# 2295-4L-025/CF)
- **optional:** anti-His tag Ab for crosslinking control 4-1BB ligand (Biolegend Cat.# 652502)

4. Assay Protocol for FcγRIIb-Dependent Antibodies

This assay protocol requires two engineered cell lines: 4-1BB Effector Cells and FcγRIIb CHO-K1 Cells. The FcγRIIb CHO-K1 Cells are provided in Thaw-and-Use format (Cat.# JA2251, JA2255) and CPM format (Cat.# JA2252; see *FcγRIIb CHO-K1 Cells Propagation Model Technical Manual*, #TM569, for details).

The procedure below illustrates the use of the 4-1BB 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 10-point dilution series, in a single 96-well assay plate using the inner 60 wells. Other experimental and plate layouts are possible but may require further optimization.

Note: When preparing test and reference antibodies, choose an appropriate starting concentration and dilution scheme to achieve a complete dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 10µg/ml as a starting concentration (1X) and threefold serial dilution when testing Control Ab, Anti-4-1BB.

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.
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-4-1BB, 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. The Bio-Glo™ Reagent can be stored at room temperature after reconstitution with only ~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 (180µl each) and one reference antibody (400µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Note: If you are using Control Ab, Anti-4-1BB, as a reference antibody in your assay, prepare a 400µl starting dilution with 30µg/ml of anti-4-1BB antibody (dilu1, 3X final concentration) by adding 12µl of anti-4-1BB stock (1,000µg/ml) to 388µl of assay buffer. Store the antibody starting dilution on ice until ready to use in the assay.

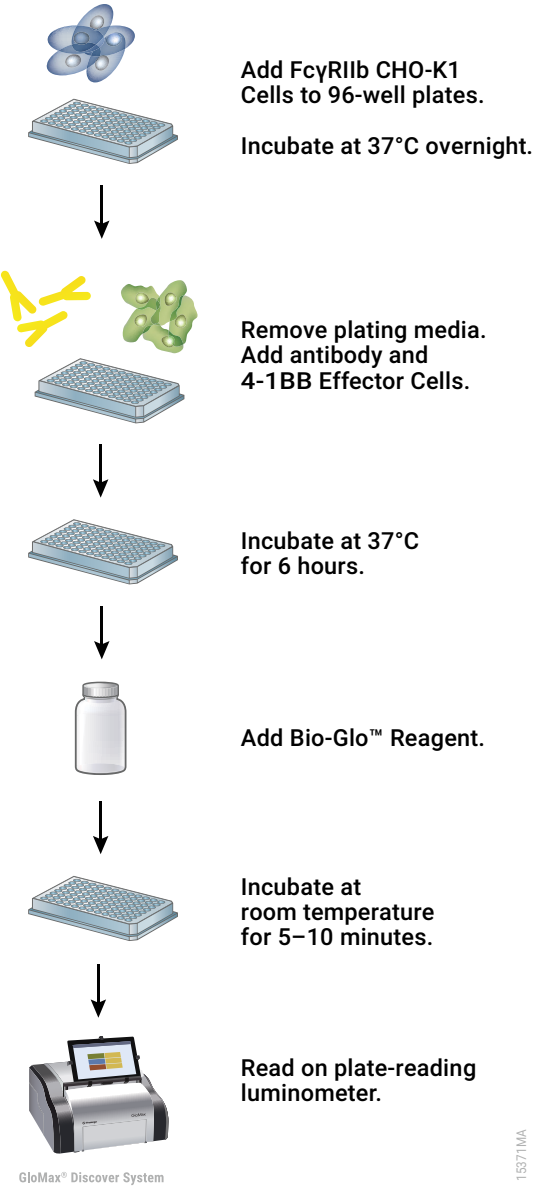


Figure 6. Schematic protocol for 4-1BB Bioassay with FcγRIIb-dependent antibody.

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 (denoted by "B") alone.

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.

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

4.C. Preparing and Plating FcγRIIb CHO-K1 Cells (continued)

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

1. On the day before performing the assay, prepare 30ml of FcγRIIb CHO-K1 cell recovery medium (RPMI 1640/5% FBS) as described in Section 4.A
2. Warm the recovery medium in a 37°C water bath for 15 minutes and transfer 14.5ml of 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 lab 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.
4. Gently mix the cell suspension in the vial by pipetting, and then transfer 0.5ml of cells to the tube containing 14.5ml of 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 assay medium per well to 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 preparing a single stock of threefold 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 threefold serial dilutions, you will need 400µl of reference antibody at 3X the highest antibody concentration in your dose-response curve. You will need 180µl 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-4-1BB, as a control in the assay, follow the instructions below to prepare threefold serial dilutions. A threefold 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 and 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 180µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 8).

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. Add 180µl 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 other wells in these four rows, from column 10 to column 2.
6. Transfer 60µl of the 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.

   Note: Wells A2, B2, E2 and G2 contain 120µl of assay buffer without antibody as a negative control.
8. Take the 96-well assay plates containing FcγRIIb CHO-K1 Cells out of the incubator and remove 95µl of medium per well from the inner 60 wells using manual multichannel pipette.
9. Using an electronic multichannel pipette, immediately 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 4-1BB Effector Cells.

4.E. Preparing 4-1BB Effector Cells and Setting Up Assay

The thaw-and-use 4-1BB 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 4-1BB 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 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 (see Section 4.D). Mix well by gently inverting the tube 1–2 times.
3. Transfer the 4-1BB 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 FcγRIIb CHO-K1 Cells and anti-4-1BB antibody.
4. Add 75µl of assay buffer to the outside wells of the 96-well assay plates.
5. Place a lid 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. 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.

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 4-1BB Bioassay to test two antibody samples against a reference sample in a single assay run. Each test and reference antibody is run in triplicate, in a 10-point dilution series, in a single 96-well assay plate using the inner 60 wells. Other experimental and plate layouts are possible but may require further optimization.

Note: When preparing test 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 2-fold dilution when testing 4-1BB 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, 4-1BBL, that we tested. If you experience assay performance issues when using this assay buffer, we recommend testing different serum concentrations in the range of 0.5–10%.

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 only ~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 (500µl) in 1.5ml tubes. Store the tubes containing ligand or antibody starting dilutions appropriately before making antibody serial dilutions.

   Notes:

   - If you are using 4-1BBL as a reference ligand, prepare a 500µl starting dilution with 3µg/ml 4-1BBL (His-tagged) and 15µg/ml crosslinking antibody, anti-His (dilu1, 3X final concentration) by adding 15µl of 4-1BBL, stock (100µg/ml) and 15µl of crosslinking antibody, anti-His stock (500µg/ml) to 470µl of assay buffer. The final (1X) starting concentration is 1µg/ml of 4-1BBL and 5µg/ml of anti-His 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.

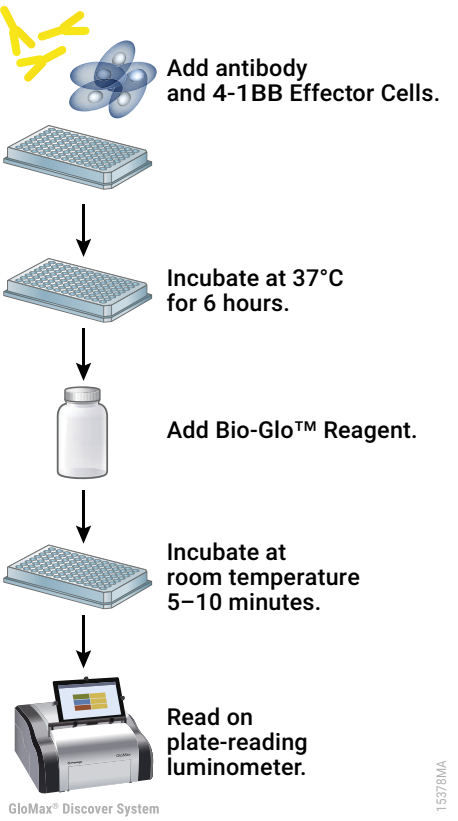


Figure 9. Schematic protocol for 4-1BB Bioassay with 4-1BB Ligand or FcγRIIb-independent antibody.

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 (denoted by "B") alone.

5.C. Preparing Ligand or Antibody Serial Dilutions

The instructions described here are for preparation of a single stock of twofold serial dilutions of a crosslinked ligand for analysis in triplicate (120µl of each crosslinked 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 twofold serial dilutions for crosslinked ligand, you will need 500µl of reference crosslinked 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 4-1BBL as a control in the assay, use the following instructions to prepare twofold 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 240µl of reference crosslinked 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 120µl of the crosslinked ligand or 60µl test antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
7. Repeat equivalent twofold or threefold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.
8. Cover the assay plate with a lid and keep at ambient temperature (22–25°C) while preparing the 4-1BB 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 Ligand
B		no Ab	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 4-1BB Effector Cells and Setting Up Assay

The thaw-and-use 4-1BB 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 4-1BB 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, and 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 the tube 1–2 times.
3. Transfer the 4-1BB 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 in a 37°C, 5% CO_2 incubator 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.

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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.
	If performing the assay for the first time, we recommend that you try using 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.
Weak assay response (low fold induction)	Low activity of Bio-Glo™ Reagent leads to low RLU. Store and handle the Bio-Glo™ Reagent according to instructions.
	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 4-1BB 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.

7. References

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8. Appendix

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

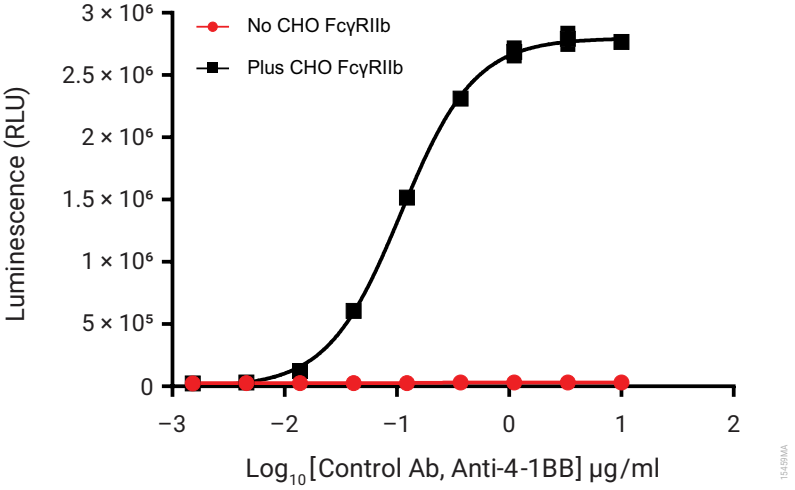


Figure 12. The 4-1BB Bioassay measures the activity of Control Antibody, Anti-4-1BB. Thaw-and-use FcγRIIb CHO-K1 Cells were plated overnight. On the following day, a titration of Control Ab, Anti-4-1BB, (Section 4.C) was added, followed by addition of thaw-and-use 4-1BB Effector Cells. After 6 hours of induction at 37°C, Bio-Glo™ Luciferase Assay 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 0.11µg/ml, and the fold induction was ~110.

8.B. Representative Assay Results with Crosslinked Ligand or FcγRIIb-Independent Antibody

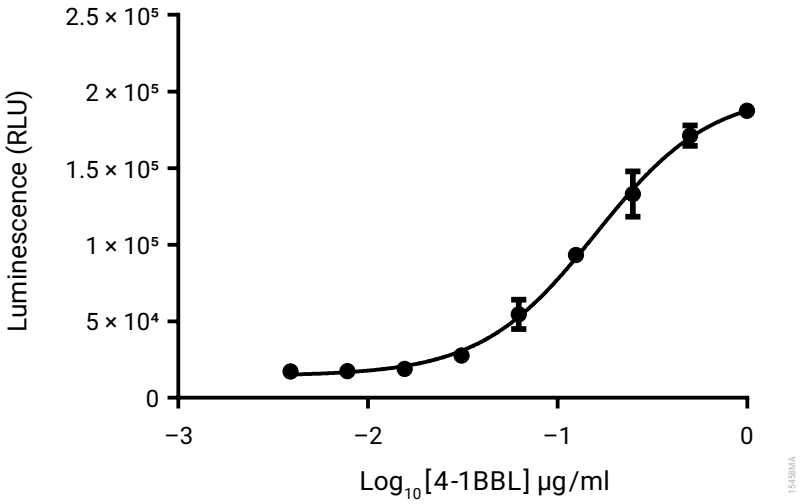


Figure 13. The 4-1BB Bioassay measures the activity of crosslinked 4-1BB Ligand. On the assay day, thaw-and-use 4-1BB Effector Cells were plated in a 96-well plate at 50µl/well. Cells were incubated with various concentrations of crosslinked 4-1BBL (Section 5.C). After 6 hours of induction at 37°C, Bio-Glo™ Luciferase Assay 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 ~0.16µg/ml, and the fold induction was 12.

9. Summary of Changes

The following changes were made to the 8/26 revision of this document:

1. Revised text about the antibody used in Table 1.
2. Deleted Section 8.C, Related Products.
3. Added a second licensing statement and removed an expired patent statement.
4. Made minor text edits.

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