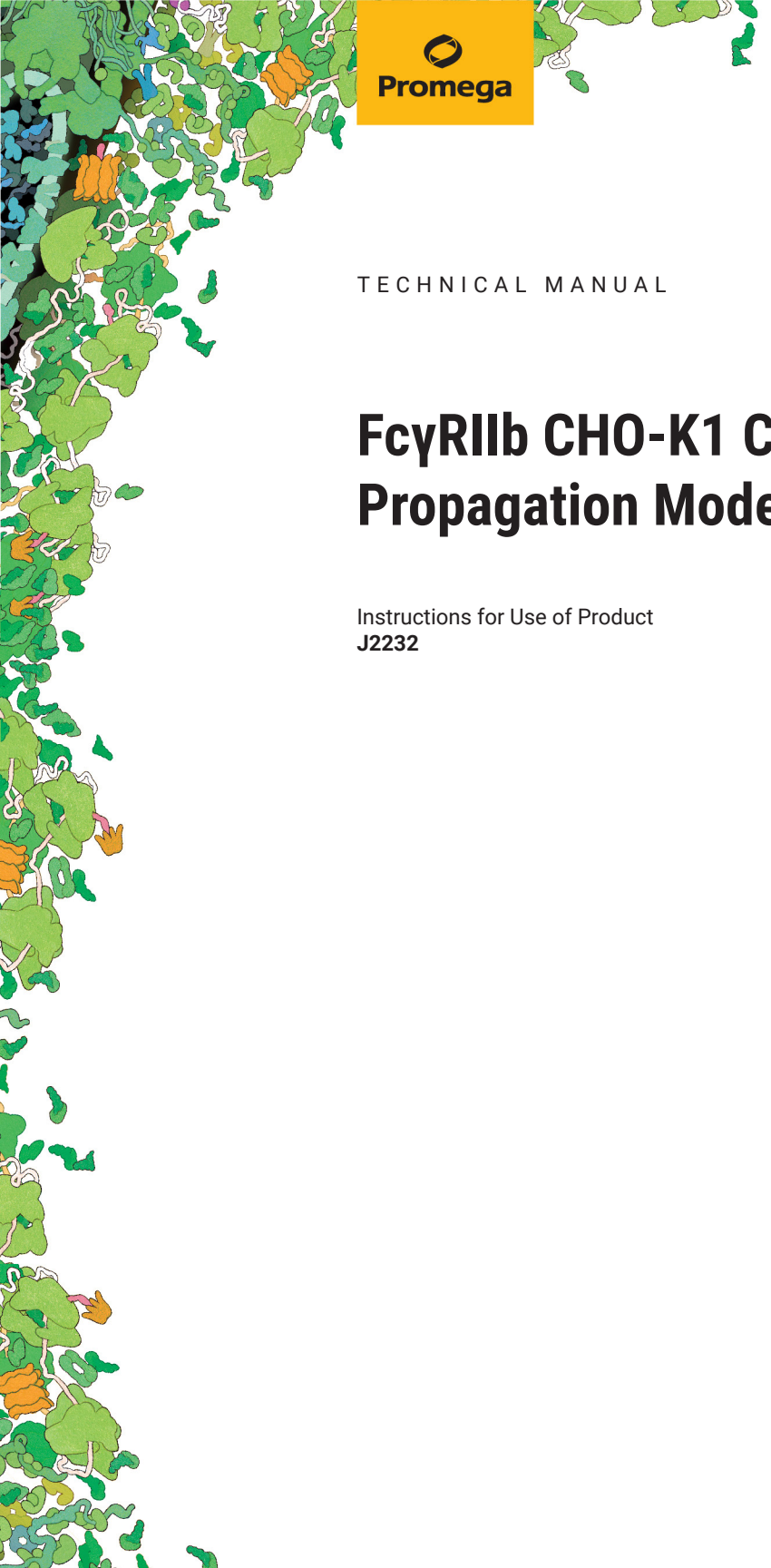

TECHNICAL MANUAL

FcγRIIb CHO-K1 Cells, Propagation Model

Instructions for Use of Product
J2232

Revised 6/25
TM569

FcγRIIb CHO-K1 Cells, 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

Promega

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 receptor (TNFR) family-related protein (GITR), 4-1BB (CD137/TNFRSF9), OX40 (CD134) and CD40 have been identified. Activating these receptors with agonist monoclonal antibodies has emerged to be a novel strategy to enhance anti-tumor immune responses and promote immune-mediated tumor rejection (1,2).

CD40 is a member of the TNFR superfamily expressed by B cells, dendritic cells, monocytes, normal cells and some malignant cells. CD40 agonists have been shown to rescue function of antigen presenting cells (APC) in tumor-bearing hosts and restore effective immune responses against tumor antigens.

4-1BB, a member of the TNFR superfamily, is an inducible costimulatory receptor expressed on T cells, natural killer (NK) cells and innate immune cell populations (3). When present on the cell surface, 4-1BB interacts with 4-1BB ligand and induces subsequent cell proliferation and production of interferon gamma (IFNγ) and interleukin-2 (IL-2), particularly in T and NK cells (4,5).

GITR is a member of the TNF receptor superfamily and can be modulated by agonist antibodies or natural ligand to serve as an effective anti-tumor therapy. Preclinical studies have demonstrated that signaling through GITR can enhance activation of T effector cells (Teff) and modulate the activity of intra-tumor T regulatory cells (Tregs) during immunotherapy.

OX40 is a member of the TNFR superfamily. OX40 signaling has also been shown to inhibit the production of IL-10 by suppressing the function of Treg cells. Preclinical studies have demonstrated that treatment of tumor bearing hosts with OX40 agonists, including both agonist antibody (Ab) and fusion proteins, results in T cell differentiation and cytolytic function leading to enhanced anti-tumor immunity against a variety of tumors. Multiple preclinical investigations have unveiled a new strategy of targeting multiple immune checkpoints with different mechanisms for improved anti-tumor responses in a variety of cancers (1).

Current methods used to measure the activity of biologic drugs targeting costimulatory immune checkpoint molecules rely on primary human T cells and measurement of functional endpoints such as cell proliferation, cell surface marker expression, and IFNγ 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 drug-development setting.

The CD40 Bioassay, Propagation Model (Cat.# J2132), 4-1BB Bioassay, Propagation Model (Cat.# J2332), OX40 Biossay and GITR Bioassay are bioluminescent cell-based assays that overcome the limitations of existing assays and can be used to measure the potency and stability of antibodies and other biologics targeting CD40, 4-1BB, OX40 and GITR, respectively (6, 7).

The antibodies used to stimulate T cell activation via interaction with costimulatory receptors on the effector cells can be cross-linked via binding to FcγRIIb receptors on CHO-K1 cells. This crosslinking increases avidity and stabilizes the interaction of the antibody to the receptor, in certain cases. Based on the properties of the antibody tested, the Costimulatory Immune Checkpoint (i.e., OX40, CD40, GITR and 4-1BB) bioassays can be conducted in a single genetically engineered cell system or dual genetically engineered cell systems:

- FcγRIIb CHO-K1 Cells[a–d]: CHO-K1 cells engineered to express human FcγRIIb. The FcγRIIb CHO-K1 Cells are provided in Cell Propagation Model (CPM) format, which includes cryopreserved cells that can be thawed, propagated and banked for long-term use. These are not reporter cells, and function solely to crosslink anti-CD40, anti-OX40, anti-4-1BB or anti-GITR antibodies.

The Costimulatory Immune Checkpoint Bioassays (CD40, OX40, GITR or 4-1BB Bioassays), Propagation Model, reflect the mechanism of action (MOA) of biologics designed to activate CD40, OX40, GITR or 4-1BB, respectively. Specifically, CD40-, OX40-, GITR- or 4-1BB-mediated luminescence is detected following the addition of CD40, OX40, GITR or 4-1BB agonist antibodies or CD40, OX40, GITR or 4-1BB ligand, respectively. FcγRIIb CHO-K1 cells might be required to crosslink the antibodies but are not required for the ligands. It is recommended that while screening for agonist antibodies of costimulatory immune checkpoints, you should 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 (Figure 1).

The bioassays are prequalified according to ICH guidelines and show the precision, accuracy and linearity required for routine use in potency studies (Figures 2 and 3; Tables 1 and 2). The assay can be performed in a one-day or two-day time frame depending on antibody property. 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 (Figures 4 and 5). In addition, the bioassay can be used with up to 100% human serum (in antibody samples) with minimal impact on fold induction, indicating potential for further development into a neutralizing antibody bioassay.

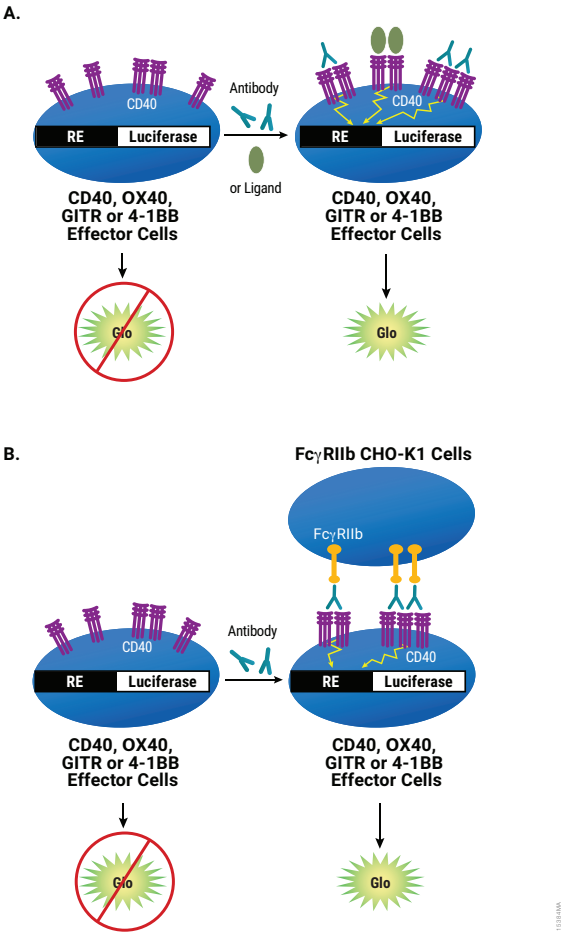

Figure 1. Representation of costimulatory bioassays (CD40, OX40, GITR or 4-1BB) with FcγRIIb-independent and FcγRIIb-dependent antibodies. Panel A. Assay with FcγRIIb-independent Antibody. The bioassay consists of one engineered cell line: CD40, OX40, GITR or 4-1BB Effector Cells. In the absence of agonist antibody or ligand, the CD40, OX40, GITR or 4-1BB receptor is not activated and luminescence signal is low. The addition of agonist antibody or ligand induces the CD40, OX40, GITR or 4-1BB pathway-activated luminescence, which can be detected in a dose-dependent manner by addition of Bio-Glo™ Reagent and quantitation with a luminometer. **Panel B.** Assay with FcγRIIb-dependent Antibody. The bioassay consists of two engineered cell lines: CD40, OX40, GITR or 4-1BB Effector Cells and FcγRIIb CHO-K1 Cells. In the presence of FcγRIIb CHO-K1 Cells, the anti-CD40, -OX40, -GITR or 4-1BB antibody is cross-linked, thereby inducing CD40, OX40, GITR or 4-1BB pathway-activated luminescence, which can be detected in a dose-dependent manner by addition of Bio-Glo™ Reagent and quantitation with a luminometer.

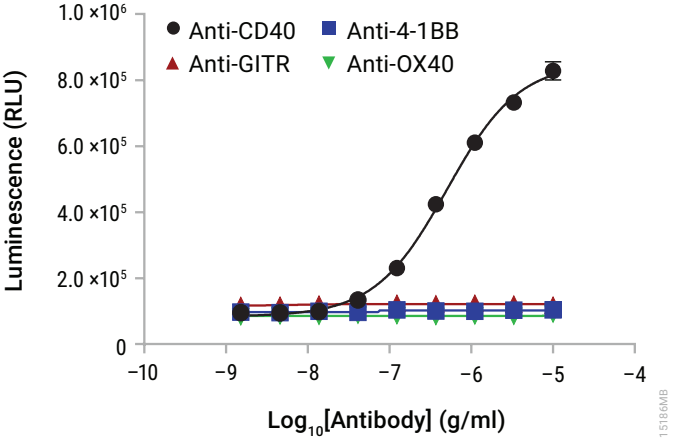

Figure 2. Using the CD40 Bioassay in the presence of FcγRIIb CHO-K1 Cells reflects the mechanism of action (MOA) and specificity of biologics designed to activate CD40 receptor. When incubated in the presence of FcγRIIb CHO-K1 Cells, CD40 Effector Cells were induced respectively with a serial titration of control antibodies: Anti-CD40, Anti-4-1BB, Anti-OX40 or Anti-GITR as indicated. 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.

Table 1. The CD40 Bioassay Shows Precision, Accuracy and Linearity.

Parameter	**Results**	
Accuracy	% Expected Relative Potency	% Recovery
	50	98.7
	70	99.8
	140	104.0
	200	100.8
Repeatability (% CV)	100% (Reference)	5.1
Intermediate Precision (% CV)		7.0
Linearity (r^2)		0.997
Linearity (y = mx + b)		y = 1.028X–0.0284

A 50–200% theoretical potency series of Control Ab, Anti-CD40, 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.

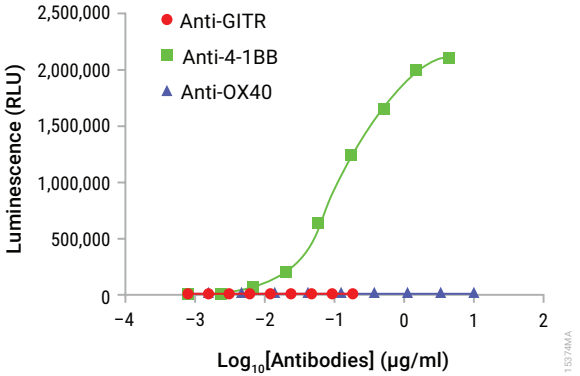

Figure 3. Using the 4-1BB Bioassay in the presence of FcγRIIb CHO-K1 Cells reflects the mechanism of action (MOA) and shows specificity of biologics designed to activate 4-1BB. When incubated in the presence of FcγRIIb CHO-K1 Cells, 4-1BB Effector Cells were induced respectively with a serial titration of control antibodies: Anti-4-1BB, Anti-OX40 or Anti-GITR as indicated. 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.

Table 2. 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. 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.		

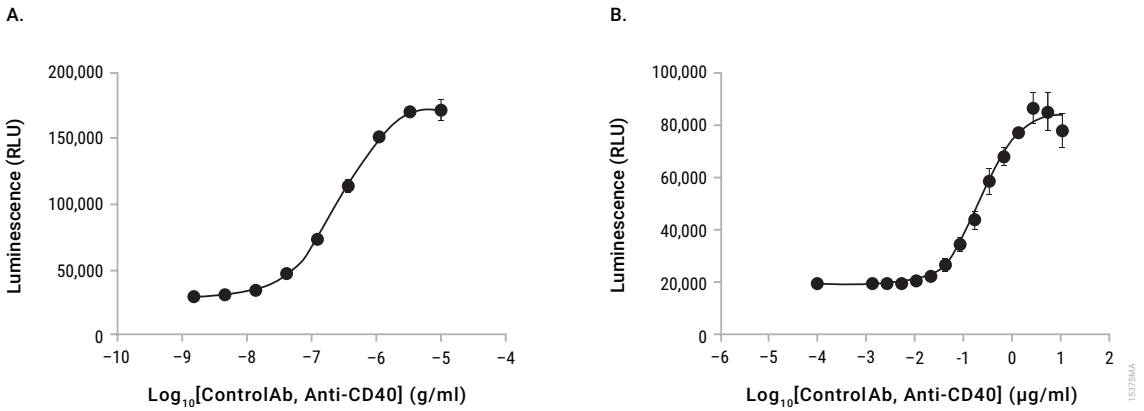

Figure 4. The assay is amenable to 384-well plate format and compatible with laboratory automation when using CD40 Effector Cells and FcγRIIb CHO-K1 Cells. The CD40 Bioassay was performed in the presence of FcγRIIb CHO-K1 cells using Control antibody, Anti-CD40, either in 96-well format (**Panel A**) or in 384-well format (**Panel B**) using a ThermoFisher Multidrop™ Combi nL Reagent Dispenser to dispense the cells and assay buffer and Echo® Acoustic liquid handler for antibody handling. On the day before the assay, CD40 effector cells were plated at 8×10^3 cells per 10µl/well. The next day, assay buffer was added to the plate at 5µl/well. The Control Ab, Anti-CD40, was dispensed in submicroliter volumes to the assay plate and serially titrated by direct dilution. Finally, FcγRIIb CHO-K1 cells were added at 10×10^3 cells per 5µl/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 0.27µg/ml and 0.23µg/ml, and the fold inductions were 6.3 and 4.7 for 96-well and 384-well format, respectively. Data were generated using thaw-and-use cells.

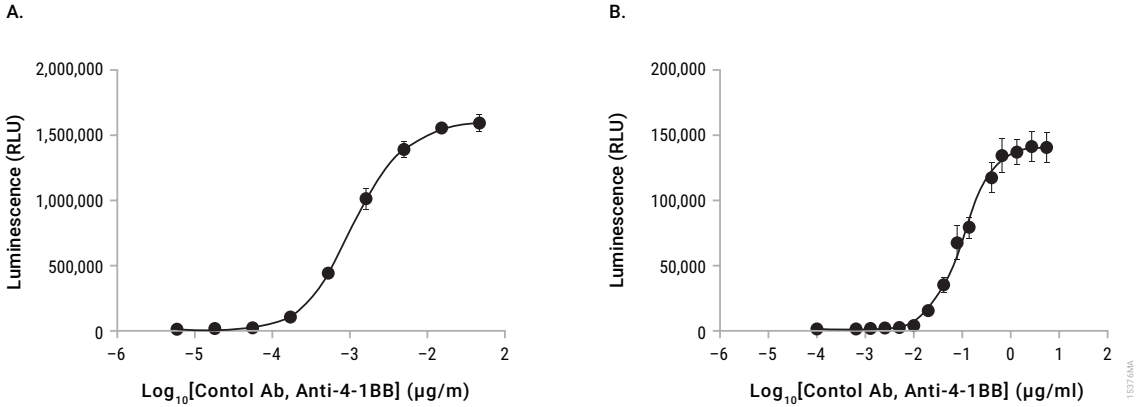

Figure 5. The assay is amenable to 384-well plate format and compatible with laboratory automation when using 4-1BB Effector Cells and FcγRIIb CHO-K1 Cells. The 4-1BB Bioassay was performed in the presence of FcγRIIb CHO-K1 cells using Control Ab, Anti-4-1BB, either in 96-well format (**Panel A**) or in 384-well format (**Panel B**) 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 were plated at 8×10^3 cells per 10µl/well. The next day, Control Ab, Anti-4-1BB, 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 per 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.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
FcγRIIb CHO-K1 Cells, Propagation Model	**1 each**	**J2232**

Not for Medical Diagnostic Use. Includes:

- 2 vials FcγRIIb CHO-K1 Cells (CPM), 12.8×10^6 cells/ml (1.0ml per vial)

Notes: 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 the Certificate of Analysis.

The FcγRIIb CHO-K1 Cells, Propagation Model, are intended for use with user-provided antibodies or other biologics designed to activate target receptors. They can be used in conjunction with CD40 Effector Cells or 4-1BB Effector Cells, as described above, to detect agonist antibodies that require FcγRIIb crosslinking in order to activate the target receptor. Data generated using these reagents are shown in Figures 2–5, above.

Cell thawing, propagation and banking should be performed exactly as described in Section 3. Cell seeding and propagation densities have been optimized to ensure stable cell growth, which is reflected in a steady cell doubling rate, to achieve optimal, consistent performance. An accurate, reliable, and reproducible cell counting method is required for routine cell culturing and optimal bioassay performance. The recommended cell plating densities, induction time and assay buffer components described in Section 4 were established using Control Ab, Anti-4-1BB. You may need to adjust the parameters provided here and optimize assay conditions for your own antibody or biologic, depending on the costimulatory bioassay of interest.

The CD40 and 4-1BB Bioassays produce a bioluminescent signal and require a sensitive luminometer or luminescence plate reader for the detection of luminescence. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System (see Section 7.B, Related Products). An integration time of 0.5 second/well was used for all readings.

3.A. Materials to Be Supplied by the User

(Composition of Buffers and Solutions is provided in Section 7.A.)

Reagents

- User-defined anti-4-1BB antibodies or other biologics
- Ham's F12 Medium with L-glutamine (e.g., GIBCO® Cat.# 11765-054)
- RPMI 1640 Medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO® Cat.# 22400-089)
- Fetal bovine serum (e.g., HyClone Cat.# SH300070.03 or GIBCO® Cat.# 16000044)
- Blasticidin S (e.g., Invitrogen Cat.# A11139-03)
- 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)

Supplies and Equipment

- white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917)
- T75 tissue culture flask (e.g., Corning® Cat.# CLS430641U)
- sterile, clear V-bottom 96-well plate with lid (e.g., Costar® Cat. Cat.# 3896 or Linbro Cat.# 76-223-05) for preparing antibody dilutions
- pipettes (single-channel and twelve-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)

3.B. Preparing FcγRIIb CHO-K1 Cells

Cell Thawing and Initial Cell Culture

1. Prepare 70ml of initial cell culture medium by adding 7ml of FBS to 63ml of Ham's F12 medium prewarmed to 37°C. This initial cell culture medium will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of pre-warmed initial cell culture medium to a 15ml conical tube.
3. Remove one vial of FcγRIIb CHO-K1 cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed (typically 2–3 minutes).
4. Transfer all of the cells (approximately 1ml) to the 15ml conical tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 180 × *g* for 5 minutes.
6. Carefully aspirate the medium, and resuspend the cell pellet in 60ml of pre-warmed initial cell culture medium.
7. Transfer the cell suspension to either four T75 tissue culture flasks (15ml each) or two T150 tissue culture flasks (30ml each), and place the flask horizontally in a 37°C, 5% CO_2 incubator.
8. Incubate for approximately 48 hours before passaging the cells.

Cell Maintenance and Propagation

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

1. On the day of cell passage, aspirate the cell culture medium and wash the cells with DPBS.
2. Add 3ml of Accutase solution to each T75 flask and place in a 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.

3. Add 7ml of cell growth medium to each T75 flask. Transfer the cell suspension to a sterile 15ml or 50ml conical tube.
4. Count the cells by Trypan blue staining. We suggest seeding the cells at a density of 4×10^4 cells/cm^2 if passaging every two days (e.g., Monday-Wednesday or Wednesday-Friday) or 2×10^4 cells/cm^2 if passaging every three days (e.g., Friday-Monday).
5. Add an appropriate amount of cell growth media to a new flask.
6. Transfer the appropriate volume of cell suspension to achieve the desired cell seeding density per area.
7. Place the flasks horizontally in a 37°C, 5% CO_2 incubator.

Cell Freezing and Banking

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

4. Example Assay Protocol for FcγRIIb-Dependent Antibodies Using 4-1BB Effector Cells

This assay protocol requires two engineered cell lines: 4-1BB Effector Cells and FcγRIIb CHO-K1 Cells. The FcγRIIb CHO-K1cells are provided in CPM format and Thaw-and-Use format, and either format can be used in this assay. Refer to the respective Costimulatory Bioassay protocols when using Thaw-and-Use cells.

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. 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 and reference antibodies, 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 10µg/ml as a starting concentration (1X) and 3-fold serial dilution when testing Control Antibody, Anti-4-1BB.

4.A. Preparing Assay Buffer, BioGlo™ Reagent and Antibody Samples

1. **FcγRIIb CHO-K1 Cell Recovery Medium:** On the day before the assay, prepare 40ml of Cell Recovery 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 4 ml 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-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 the 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 (22–25°C) water bath for at least 1–2 hours before use, protected from light. Approximate stability of Bio-Glo™ Reagent after reconstitution is an 18% loss of luminescence after 24 hours at ambient temperature.
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 400µl starting dilution with 30µg/ml Anti-4-1BB antibody (dilu1, 3X final concentration) by adding 12µl of anti-4-1BB, stock (1000 µg/ml) to 388µl of assay buffer. Store the antibody starting dilution on ice until ready to use in the assay.

Tip: To streamline assay setup, prepare antibody serial dilutions prior to harvesting and plating 4-1BB Effector cells.

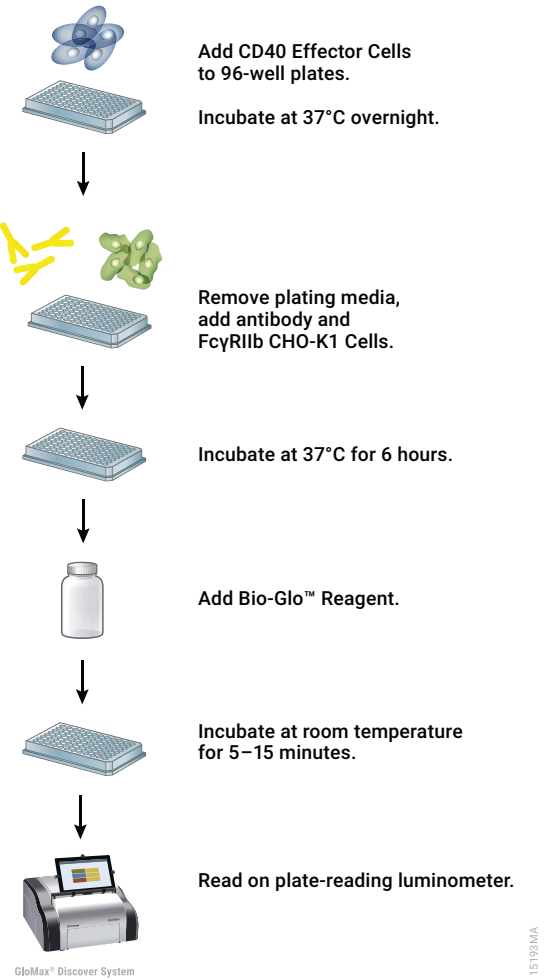

Figure 6. Costimulatory Bioassay (CD40, OX40, 4-1BB or GITR) schematic protocol for FcγRIIb-dependent antibody. The addition of Effector Cells and FcgRIIb CHO-K1 cells may differ in order for certain bioassays. Refer to the appropriate Bioassay CPM Technical Literature for specific instructions.

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 non-clustered sample locations of test and reference antibody dilution series and wells containing assay buffer alone (denoted by "B").

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

While maintaining the FcγRIIb CHO-K1 Cells (Cat.# J2232), follow the recommended cell seeding density. 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, as described above, to ensure optimal and consistent assay performance.
2. On the day before performing the assay, prepare cell recovery 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.

4. Add 3ml 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 7ml of FcγRIIb CHO-K1 cell recovery 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 10 minutes.
8. Gently resuspend the cell pellet in cell recovery 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 recovery 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).

4.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparation of a single stock of 3-fold 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 3-fold 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 was 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. 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).
3. Add 180µl 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 other wells in these four rows, from column 10 to column 2.
5. Transfer 60µl of the 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 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 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 4-1BB Effector Cells

While maintaining the 4-1BB 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. For more information on cell seeding density, please refer to the *4-1BB Bioassay, Cell Propagation Model Technical Manual* #TM551.

Note: Passaging cells less than 40 hours before the assay is not recommended for this specific assay.

1. Passage the cells two days or three days before performing the assay 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 at greater than 90%.
2. Count the 4-1BB Effector Cells by Trypan blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of 4-1BB 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 × 10^6 cells/ml.
5. You will need at least 8ml of 4-1BB Effector Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.

4.F. Adding Antibody Samples and 4-1BB Effector Cells to Assay Plates

1. Take the 96-well assay plates containing FcγRIIb CHO-K1 Cells out of 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 4-1BB Effector Cells prepared in Section 4.E to a sterile reagent reservoir. Using a multichannel pipette, dispense 50µl (0.5×10^5 cells) of 4-1BB Effector Cells into the wells containing antibody or ligand.
5. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 6 hours.

4.G. 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 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.H. Data Analysis

1. Determine the plate background by calculating the average RLU from wells B1, C1 and D1.
2. $$\text{Calculate fold induction} = \frac{\text{RLU (induced}-\text{background)}}{\text{RLU (no antibody control}-\text{background)}}$$
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. Troubleshooting

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

Symptoms	Causes and Comments
Low luminescence measurements (RLU readout)	Choose an instrument designed for plate-reading luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual RLU numbers will vary between instruments. Some older models of luminometers with low sensitivity should be avoided. We recommend using a high gain setting if you are using a luminometer with an adjustable gain.
	Insufficient cells per well can lead to low RLU. Handle and plate the 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.
	We recommend using Accutase for dissociation of FcγRIIb CHO-K1 cells prior to plating for Bioassays. Use of other dissociation methods (e.g., Trypsin) may result in lower RLU values due to loss of FcγRIIb receptors expressed on the CHO-K1 cell surface.
Variability in assay performance	Assay performance can be affected by variations in cell growth conditions including plating and harvest density, centrifuge times and speeds, and freezing and/or DMSO exposure times during cell banking.
	Poor cell viability and variations in doubling time may affect assay performance. Ensure consistent cell growth by handling the cells exactly according to the instructions. Avoid one-day cell passages whenever possible.
	Ensure you are using high-quality cell culture reagents (especially serum) and plastic ware for maintaining cells in culture.
	Ensure accurate and consistent cell counting methods.

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 CD40 Bioassay or 4-1BB Bioassay may vary from the EC_{50} obtained using other methods.
	If untreated control RLU is less than 100-fold above plate reader background RLU, subtract plate reader background RLU from all samples prior to calculating fold induction.

6. References

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7. Appendix

7.A. Composition of Buffers and Solutions

initial cell culture medium for FCγRIIb CHO-K1 Cells

90% Ham's F12 with L-glutamine
10% FBS

cell growth medium for FCγRIIb CHO-K1 Cells

90% Ham's F12 with L-glutamine
10% FBS
10µg/ml Blasticidin S

cell freezing medium for FCγRIIb CHO-K1 Cells

85% Ham's F12 with L-glutamine
10% FBS
5% DMSO

cell recovery medium for FCγRIIb CHO-K1 Cells

90% Ham's F12 with L-glutamine
10% FBS

assay buffer

99% RPMI 1640 with L-glutamine
1% FBS

7.B. Related Products

Fc Effector Bioassays

Product	Size	Cat.#
ADCC Reporter Bioassay, Complete Kit (Raji)*	1 each	G7015
ADCC Reporter Bioassay, Core Kit*	1 each	G7010
ADCC Reporter Bioassay, F Variant, Core Kit**	1 each	G9790
ADCC Reporter Bioassay, Target Kit (Raji)*	1 each	G7016
FcγRIIa-H ADCP Reporter Bioassay, Complete Kit**	1 each	G9901
FcγRIIa-H ADCP Reporter Bioassay, Core Kit**	1 each	G9991
Mouse FcγRIV ADCC Bioassay, Complete Kit	1 each	M1201
Mouse FcγRIV ADCC Bioassay, Core Kit	1 each	M1211
Membrane TNFα Target Cells**	1 each	J3331
Membrane VEGF Target Cells**	1 each	J3351
Membrane RANKL Target Cells**	1 each	J3381

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

Fc Effector Immunoassay

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

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

Immune Checkpoint Bioassays

Product	Size	Cat.#
4-1BB Bioassay	1 each	JA2351
CD28 Bioassay	1 each	JA6701
CD28 Blockade Bioassay	1 each	JA6101
CD40 Bioassay	1 each	JA2151
CTLA-4 Blockade Bioassay	1 each	JA3001
GITR Bioassay	1 each	JA2291
ICOS Bioassay	1 each	JA6801
ICOS Blockade Bioassay	1 each	JA6001
LAG-3/MHCII Blockade Bioassay	1 each	JA1111
OX40 Bioassay	1 each	JA2191
PD-1/PD-L1 Blockade Bioassay	1 each	J1250
PD-1+TIGIT Combination Bioassay	1 each	J2211
PD-L1 Negative Cells	1 each	J1191
TIGIT/CD155 Blockade Bioassay	1 each	J2201

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T Cell Activation Bioassays

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

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7.B. Related Products (continued)

Cytokine and Growth Factor Bioassays

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

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

Control Antibodies and Proteins

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

Detection Reagent

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

Not for Medical Diagnostic Use.

Luminometers

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

For Research Use Only. Not for use in diagnostic procedures.

Note: Additional Fc Effector, Immune Checkpoint, T Cell Activation, Cytokine, Macrophage, Primary Cell and Target Cell Killing Bioassays are available. To view and order Promega Bioassay products visit: **www.promega.com/products/reporter-bioassays/** or email: **EarlyAccess@promega.com**. For information on custom bioassay development and services visit the Promega Tailored R&D Solutions website: **www.promega.com/custom-solutions/tailored-solutions/**

8. Summary of Changes

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

1. Updated fonts and made miscellaneous text edits.
2. Revised text about the label in Section 3.
3. Corrected the assay buffer composition in Section 7.A.
4. Removed an expired patent statement, added a patent statement and updated another patent statement.
5. Updated two third party trademarks.
6. Updated the Lumit trademark.

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