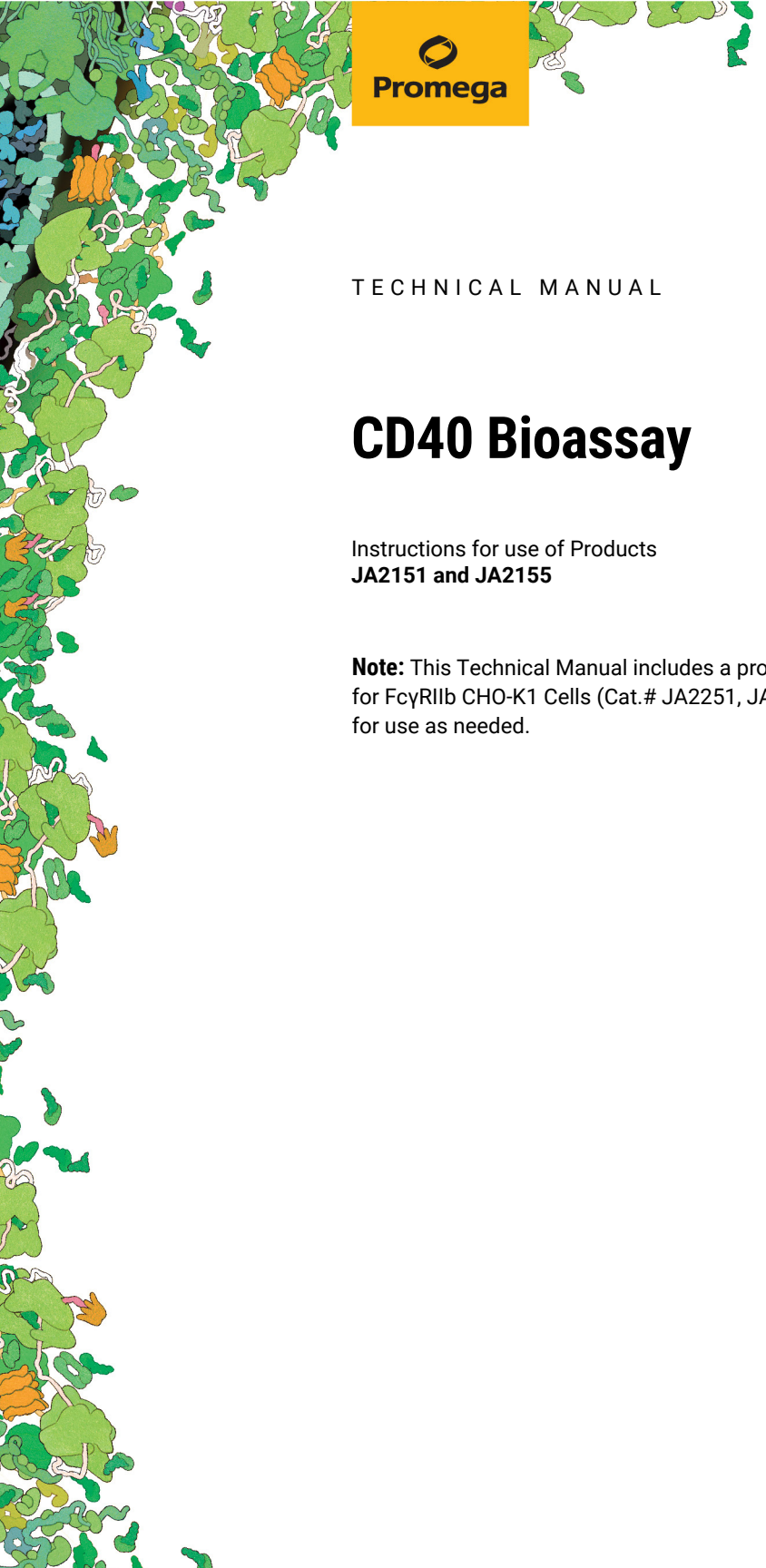

TECHNICAL MANUAL

CD40 Bioassay

Instructions for use of Products
JA2151 and JA2155

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

Revised 5/26
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CD40 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

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 agonist monoclonal antibodies has emerged as a novel strategy to enhance anti-tumor immune responses and promote immune-mediated tumor rejection (1,2).

The cell surface molecule CD40, expressed by B cells, dendritic cells and monocytes, is a member of the tumor necrosis factor receptor superfamily. CD40 ligand (CD154) is the primary ligand for CD40 and is expressed by activated T cells, which are critical regulators of cellular and humoral immunity. Signaling via CD40 triggers activation of antigen-presenting cells (APC). Agonist CD40 antibodies were found to mimic the signal of CD40 ligand and were capable of substituting for the function of CD4+ helper T cells in murine models of T cell-mediated immunity. Therefore, agonist CD40 antibodies can rescue the function of APC in tumor-bearing hosts and restore effective immune responses against tumor antigens. Subsequent data from multiple preclinical models has demonstrated synergistic enhancement from combining CD40 agonists with cytotoxics, especially chemotherapy (2,3).

Current methods used to measure the activity of biologic drugs targeting CD40 rely on primary human T cells and measurement of functional endpoints such as cell proliferation, cell surface marker expression, and interferon gamma (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 unqualified assay reagents. As a result, these assays are difficult to establish in a drug-development setting.

The CD40 Bioassay[a–e] (Cat.# JA2151, JA2155), 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 and other biologics that can bind and activate CD40 (4,5). The assay consists of a genetically engineered cell line that expresses human CD40 and a luciferase reporter driven by a response element that can respond to CD40 ligand/agonist antibody stimulation. The CD40 Effector Cells[a,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 CD40 Effector Cells are also provided in Cell Propagation Model (CPM) format (Cat.# J2132), as cryopreserved cells that can be thawed, propagated and banked for long-term use.

Based on the antibody properties to be tested, the CD40 Bioassay can be conducted in either single-cell systems or with FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255) to determine the agonist antibodies that can activate CD40 in a FcγR-dependent manner (6). Induction of the CD40 Effector Cells with CD40 ligand or an agonist CD40 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.

The CD40 Bioassay reflects the mechanism of action (MOA) of biologics designed to activate the CD40 receptor. Specifically, CD40-mediated luminescence is detected following the addition of CD40 agonist antibodies or CD40 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 1-day or 2-day time frame, depending on antibody properties. The bioassay workflow is simple and robust, and compatible with 96- 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 50% human serum (in antibody samples) with some loss in fold induction (Figure 5), indicating potential for further development in a neutralizing antibody bioassay.

1. Description (continued)

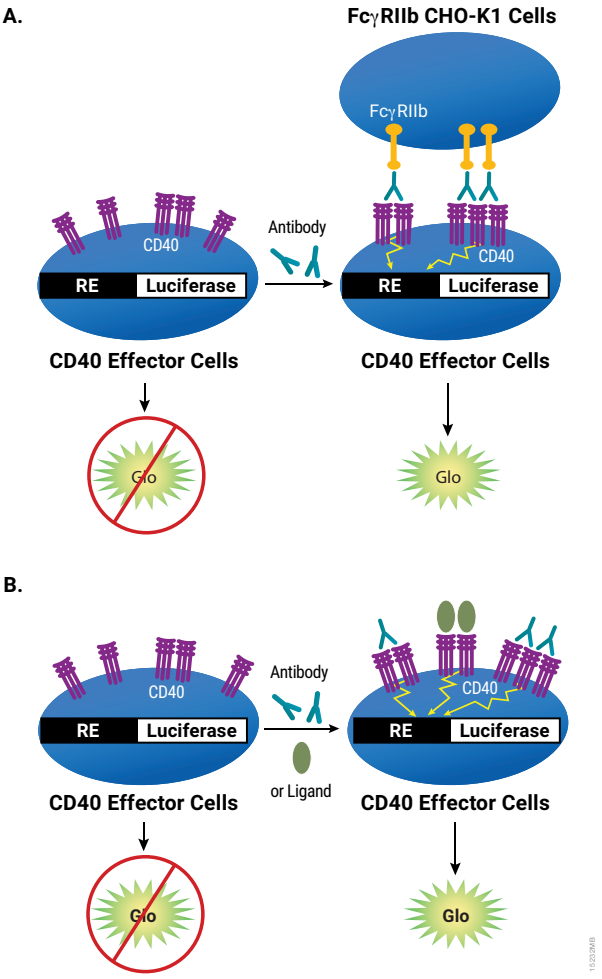

Figure 1. Representation of the CD40 Bioassay. Panel A. Assay with FcγRIIb-dependent antibody. The bioassay consists of two engineered cell lines, CD40 Effector Cells and FcγRIIb CHO-K1 Cells. In the presence of FcγRIIb CHO-K1 Cells, the anti-CD40 antibody is cross-linked, thereby inducing CD40 pathway-activated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

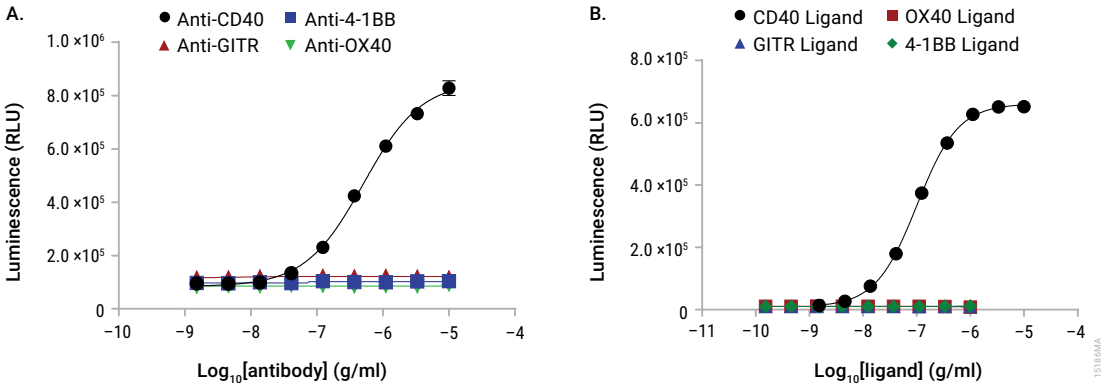

Figure 2. The CD40 Bioassay reflects the mechanism of action (MOA) and specificity of biologics designed to activate the CD40 receptor. Panel A. CD40 Effector Cells were induced with a serial titration of anti-CD40 antibody, anti-4-1BB antibody, anti-OX40 antibody or anti-GITR antibody, as indicated, in the presence of FcγRIIb CHO-K1 Cells. **Panel B.** CD40 Effector Cells were induced with a serial titration of CD40 Ligand, 4-1BB Ligand, OX40 Ligand or GITR Ligand, as indicated. For both panels, 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 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 (Cat.# K1181), was analyzed in triplicate in three independent experiments performed on three days by two analysts. 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.

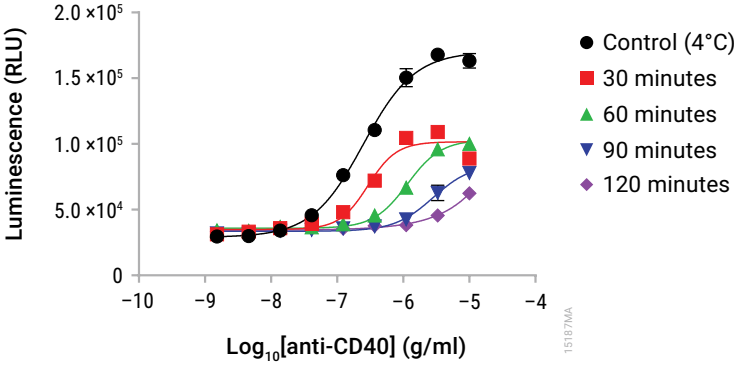

Figure 3. The CD40 Bioassay is stability-indicating. Samples of Control Ab, Anti-CD40 (Cat.# K1181), were maintained at 4°C (control) or heat-treated at 65°C for the indicated times, and then analyzed using the CD40 Bioassay with FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255). Bio-Glo™ Reagent was added, and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using the GraphPad Prism® software. Data were generated using thaw-and-use cells.

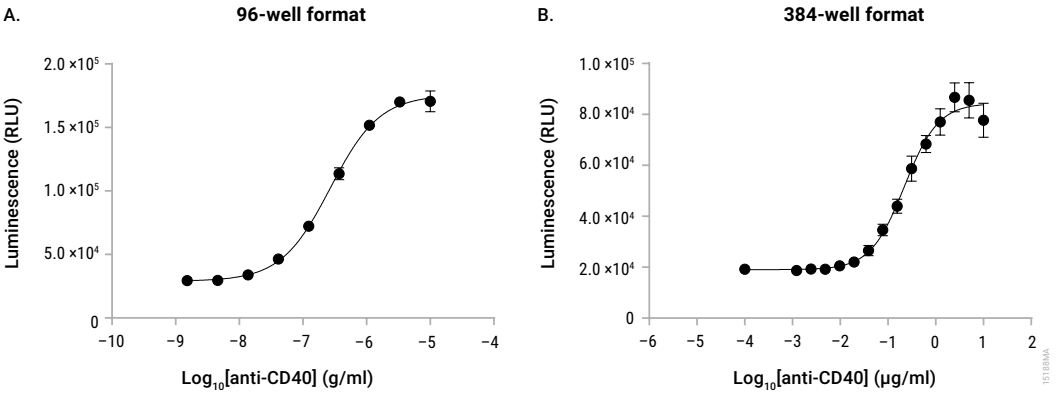

Figure 4. The CD40 Bioassay is amenable to 384-well plate format and compatible with laboratory automation. Panel A. The CD40 Bioassay was performed in 96-well plates as described in this technical manual using Control Ab, Anti-CD40 (Cat.# K1181). **Panel B.** The CD40 Bioassay was performed in 384-well format 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 assay, CD40 Effector Cells were plated at 8×10^3 cells/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/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- and 384-well format, respectively. Data were generated using thaw-and-use cells.

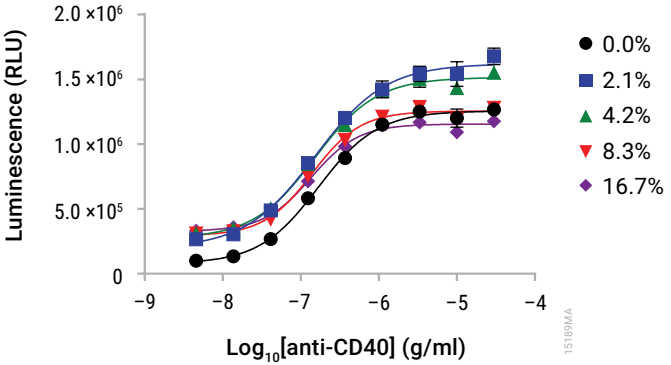

Figure 5. The CD40 Bioassay is tolerant to human serum. Control Ab, Anti-CD40 (Cat.# K1181) was analyzed in the presence of FcγRIIb CHO-K1 Cells and increasing concentrations of pooled normal human serum (0–50% in the antibody sample), resulting in final assay concentration of human serum (0–16.7%). 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 CD40 Bioassay is tolerant to serum in 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.#
CD40 Bioassay	**1 each**	**JA2151**

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 CD40 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.#
CD40 Bioassay 5X	**1 each**	**JA2155**

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

- 5 vials CD40 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 CD40 Bioassay components are shipped separately because of different temperature requirements. The CD40 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.

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 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 web site such as Certificate of Analysis.

The CD40 Bioassay, is intended to be used with user-provided ligands, antibodies or other biologics designed to activate or block CD40 receptor signal. Control Ab, Anti-CD40 (Cat.# K1181) and FcγRIIb CHO-K1 Cells (Cat.# JA2251) are available separately for use in assay optimization and routine quality control. We strongly recommend including Control Ab, Anti-CD40, as a positive control in the first few assays to gain familiarity with the assay. Data generated using these reagents are shown in Figures 3–5 and Sections 8.A (antibodies) and 8.B (ligands), Representative Assay Results.

The CD40 Bioassay produces a bioluminescent signal and is expected to be compatible with all standard luminometers or luminescence plate readers. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System. An integration time of 0.5 seconds/well was used for all readings.

3.A. Materials to Be Supplied by the User

Reagents

- Control Ab, Anti-CD40 (Cat.# K1181), available as a stand-alone reagent or user-defined anti-CD40 antibodies or other biologics
- **optional:** CD40 Ligand (R&D Systems, Cat.# 6420-CL/CF), as control

Supplies and Equipment

- white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917)
- 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., 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, Cat.# GM3000 or equivalent)

4. Assay Protocol for FcγRIIb-Dependent Antibodies

This assay protocol requires two engineered cell lines: CD40 Effector Cells and FcγRIIb CHO-K1 Cells. We provide FcγRIIb CHO-K1 Cells in Thaw and Use format (Cat.# JA2251, JA2255) and in Propagation Model format (Cat.# JA2232); see protocol for cell growth, thawing and banking. Either format may be used in this assay.

The procedure below illustrates the use of the CD40 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 threefold serial dilution when testing Control Ab, Anti-CD40.

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

1. **Cell Recovery Medium:** On the day before the assay, prepare 20ml of cell recovery medium (99% RPMI 1640/1% 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 0.2ml of FBS to 19.8ml of RPMI 1640 Medium. Mix well and warm to 37°C before use. Keep the rest of the FBS at 4°C for use on the day of assay.

2. **Assay Buffer:** On the day of the assay, prepare an appropriate amount of assay buffer (99.5% RPMI 1640/0.5% FBS). Mix well and warm to 37°C before use. For reference, 15ml 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 0.5% FBS. This concentration of FBS works well for the Control Ab, Anti-CD40, that we tested.

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

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

   If you are using a large (100ml) size of Bio-Glo™ Luciferase Assay System, dispense the reconstituted Bio-Glo™ Reagent into 10ml aliquots and store at –20°C for up to 6 weeks. Avoid repeated freeze-thaw cycles. On the day of the assay, thaw an appropriate amount of reconstituted Bio-Glo™ Reagent in a room temperature water bath for at least 1–2 hours before use. Bio-Glo™ Reagent can be stored at room temperature after reconstitution with ~18% loss in luminescence after 24 hours.

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

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 (360µl) 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-CD40 (Cat.# K1181), as a reference antibody in your assay, prepare 360µl of starting dilution with 30µg/ml anti-CD40 antibody (dilu1, 3X final concentration) by adding 10.8µl of anti-CD40 stock (1.0mg/ml) to 349.2µl of assay buffer. Store the antibody starting dilution on ice until ready to use in the assay.

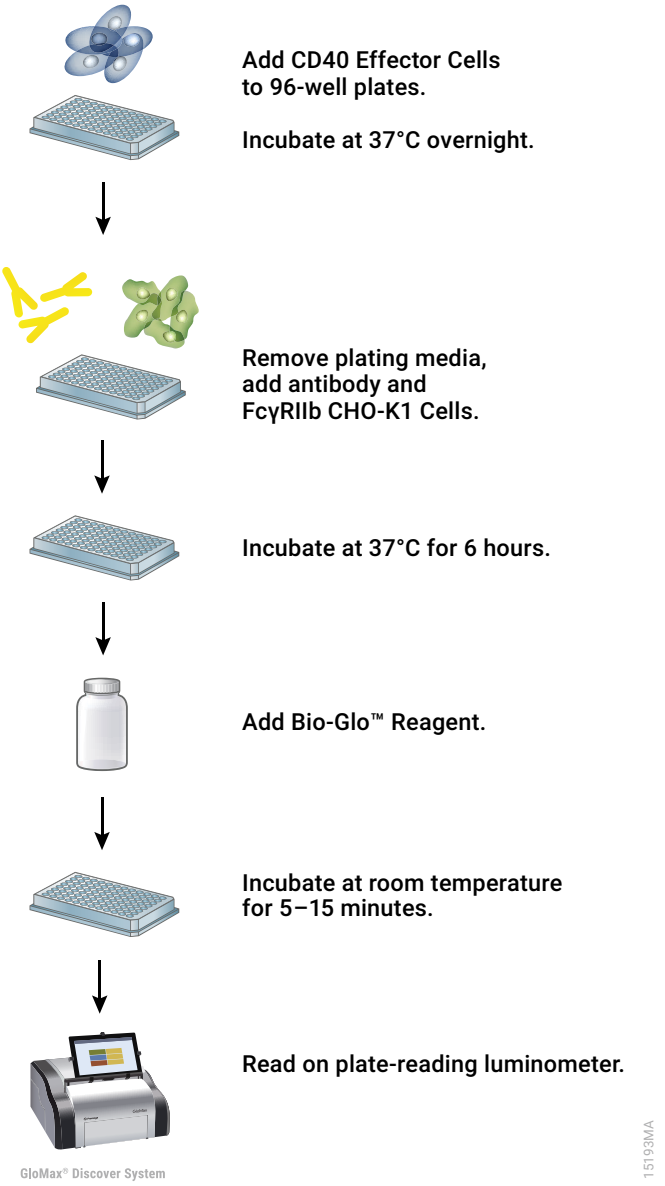

Figure 6. Schematic protocol for FcγRIIb-dependent CD40 Bioassay.

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

4.C. Preparing and Plating CD40 Effector Cells

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 20ml of fresh cell recovery medium (99% RPMI 1640/1% FBS).
2. Remove one vial of CD40 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 (approximately 2 minutes). While thawing, gently agitate and visually inspect.
3. Transfer the vial to a sterile hood, and gently resuspend the cell pellet by pipetting.
4. Transfer 0.3ml of cells from the vial to a 15ml conical tube containing 14.7ml of cell plating media.
5. Transfer the CD40 Effector Cells to a sterile reagent reservoir. Using a multichannel pipette, dispense 100µl (1.5×10^4 cells) of CD40 Effector Cells into the inner 60 wells of the assay plates.
6. Add 75µl of cell recovery medium to the outside wells of the 96-well assay plates.
7. Cover the assay plates with plate lids 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 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 360µl 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-CD40 (Cat.# K1181), 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 the assay, prepare an appropriate amount of assay buffer as described in Section 4.A.
2. To a sterile, clear V-bottom 96-well plate, add 180µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 11).
3. Add 180µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and G11, respectively (see Figure 11).
4. Add 120µl of assay buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 60µl of the 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 CD40 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 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 8. Example plate layout showing antibody serial dilutions. Wells A2, B2, E2 and G2 contain 120µl of assay buffer without antibody as a negative control.

4.E. Preparing FcγRIIb CHO-K1 Cells

1. Remove one vial of FcγRIIb CHO-K1 Thaw and Use 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 (approximately 2 minutes). While thawing, gently agitate and visually inspect.
2. Gently resuspend the cell pellet by pipetting up and down.
3. Transfer 0.5ml of cells from the vial to a 15ml conical tube containing 7.5ml of assay medium.

4.F. Adding Antibody Samples and FcγRIIb CHO-K1 Cells to Assay Plates

1. Remove the 96-well assay plates containing CD40 Effector Cells from the incubator. Using a manual multichannel pipette, carefully remove all medium from the inner 60 wells of each plate.
2. Using an electronic multichannel pipette, add 25µl of the appropriate antibody titration to the assay plates according to the plate layout in Figure 8.
3. Transfer the FcγRIIb CHO-K1 Cells prepared in Section 4.E to a sterile reagent reservoir. Using a multichannel pipette, dispense 50µl (4.0×10^4 cells) of FcγRIIb CHO-K1 Cells into the wells containing antibody or ligand.
4. 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. $$\text{Calculate fold induction} = \frac{\text{RLU (induced–background)}}{\text{RLU (no antibody control–background)}}$$
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 CD40 Bioassay to test two ligand or 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 and reference 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 10µg/ml as a starting concentration (1X) and threefold dilution when testing a CD40 ligand.

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

1. **Cell Recovery Medium:** On the day before the assay, prepare 20ml of cell recovery medium (99% RPMI 1640/1% 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 0.2ml of FBS to 19.8ml 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 (99.5% RPMI 1640/0.5% 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, 15ml 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 0.5% FBS. This concentration of FBS works well for the recommended CD40 ligand (CD40L).
3. **Bio-Glo™ Reagent:** For reference, 10ml of Bio-Glo™ Reagent is sufficient to assay 120 wells in a 96-well assay format. Thaw the Bio-Glo™ Luciferase Assay Buffer at 4°C overnight or in a room temperature water bath on the day of assay. Equilibrate the Bio-Glo™ Luciferase Assay Buffer to ambient temperature, protected from light.

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

   If you are using a large (100ml) size of Bio-Glo™ Luciferase Assay System, dispense the reconstituted Bio-Glo™ Reagent into 10ml aliquots and store at –20°C for up to 6 weeks. Avoid repeated freeze-thaw cycles. On the day of the assay, thaw an appropriate amount of reconstituted Bio-Glo™ Reagent in a room temperature water bath for at least 1–2 hours before use. The Bio-Glo Reagent can be stored at room temperature after reconstitution with ~18% loss in luminescence after 24 hours.
4. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 3X final concentration) of two test antibodies or ligands (180µl each) and one reference ligand or reference antibody (360µl) in 1.5ml tubes. Store the tubes containing ligand or antibody starting dilutions appropriately before making antibody serial dilutions.

   Note: If you are using a CD40 Ligand (CD40L) as a reference ligand in your assay, prepare 360µl of starting dilution with 30µg/ml CD40L (dilu1, 3X final concentration). Store the antibody starting dilution on ice until ready to use in the assay.

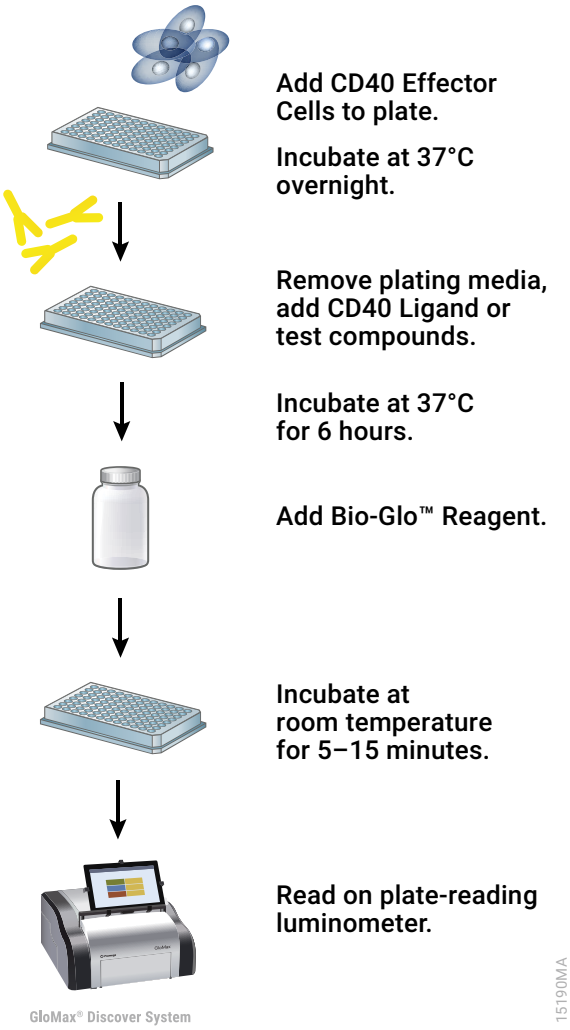

Figure 9. Schematic protocol for CD40 ligand or FcγRIIb-independent CD40 Bioassay.

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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 and reference antibodies 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 10. Example plate layout showing non-clustered sample locations of test and reference antibody dilution series and wells containing assay buffer (denoted by "B") alone.

5.C. Preparing and Plating CD40 Effector Cells

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

1. On the day before performing the assay, prepare 20ml of fresh cell recovery medium (99% RPMI 1640/1% FBS).
2. Remove one vial of CD40 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 (approximately 2 minutes). While thawing, gently agitate and visually inspect.
3. Transfer vial to a sterile hood, and gently resuspend the cell pellet by pipetting.
4. Transfer 0.3ml of cells from the vial to a separate 15ml conical tube containing 14.7ml of cell recovery medium.
5. Transfer the CD40 Effector Cells to a sterile reagent reservoir. Using a multichannel pipette, dispense 100µl (1.5×10^4 cells) of CD40 Effector Cells into the inner 60 wells of the assay plates.
6. Add 75µl of cell recovery medium to the outside wells of the 96-well assay plates.
7. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator overnight (18–22 hours).

5.D. 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 CD40 ligand, you will need 360µl 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: Prepare ligand or antibody on the day of the assay. If you are using Control Ligand, CD40L, as a control in the assay, follow the instructions below to prepare threefold serial dilutions.

1. On the day of the assay, prepare an appropriate amount of assay buffer as described in Section 5.A.
2. To a sterile, clear V-bottom 96-well plate, add 180µl of reference ligand 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 the other wells in these four rows, from column 10 to column 2.
5. Transfer 60µl of the ligand or antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent threefold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.
7. Cover the assay plate with a lid and keep at ambient temperature (22–25°C) while preparing the CD40 Effector Cell plates.

5.D. Preparing Ligand or Antibody Serial Dilutions (continued)

Recommended Plate Layout for Antibody Dilutions Prepared from a Single Antibody Stock													
	1	2	3	4	5	6	7	8	9	10	11	12	
A		no 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. Wells A2, B2, E2 and G2 contain 120µl of assay buffer without antibody as a negative control.

5.E. Adding Ligand or Antibody Samples to Assay Plates

1. Remove assay plates from the incubator and carefully remove all cell recovery medium from the inner 60 wells of each plate.
2. Using an electronic multichannel pipette, add 25µl of the appropriate antibody or ligand titration to the assay plates according to the plate layout in Figure 11.
3. Add additional 50µl of assay buffer to all assay wells for a final volume of 75µl per well.
4. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 6 hours.

5.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.

5.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).

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 use the FcγRIIb cells since your Ab of your interest may be dependent on crosslinking by FcγRIIb. In the case of ligands, crosslinking by an antibody may be necessary.
Variability in assay performance	Low activity of Bio-Glo™ Reagent leads to low RLU. Store and handle the Bio-Glo™ Reagent according to the instructions.
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 may vary from the EC_{50} value obtained using other methods.
	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

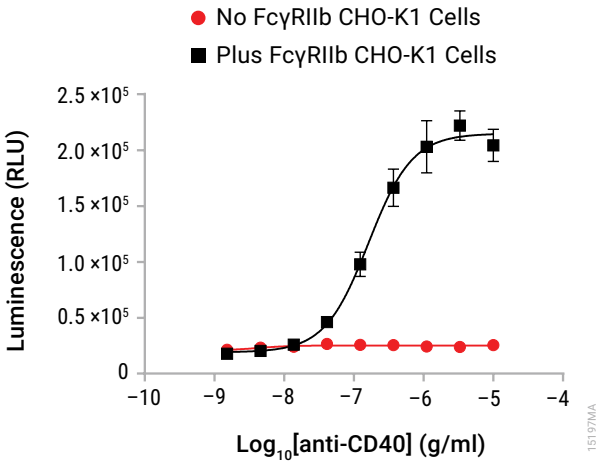

Figure 12. The CD40 Bioassay measures the activity of Control Ab, Anti-CD40. The day before the assay, CD40 Effector Cells were plated and allowed to attach overnight. On the assay day, a titration of Control Ab, Anti-CD40 (Cat.# K1181), was added followed by the addition of FcγRIIb CHO-K1 Cells. After a 6-hour 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 0.163µg/ml, and the fold induction was 11.7.

8.B. Representative Assay Results with CD40 Ligand

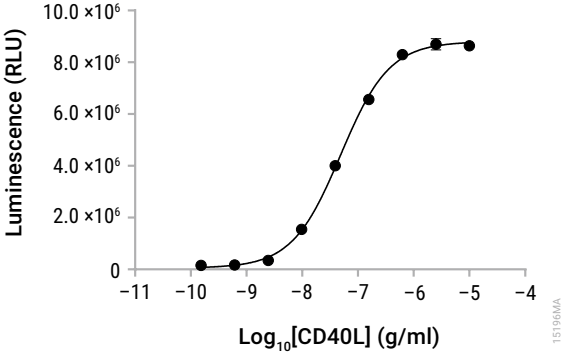

Figure 13. The CD40 Bioassay measures the activity of CD40 ligand. The day before the assay, CD40 Effector Cells were plated in a 96-well plate at 25,000 cells/well. On the assay day, a serial dilution of CD40L was added to assay plates. After a 6-hour induction at 37°C, Bio-Glo™ Luciferase Assay Reagent 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 49ng/ml, and the fold induction was 63-fold.

9. Summary of Changes

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

1. Removed section 8.C, Related Products.
2. Made minor text and formatting edits.

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