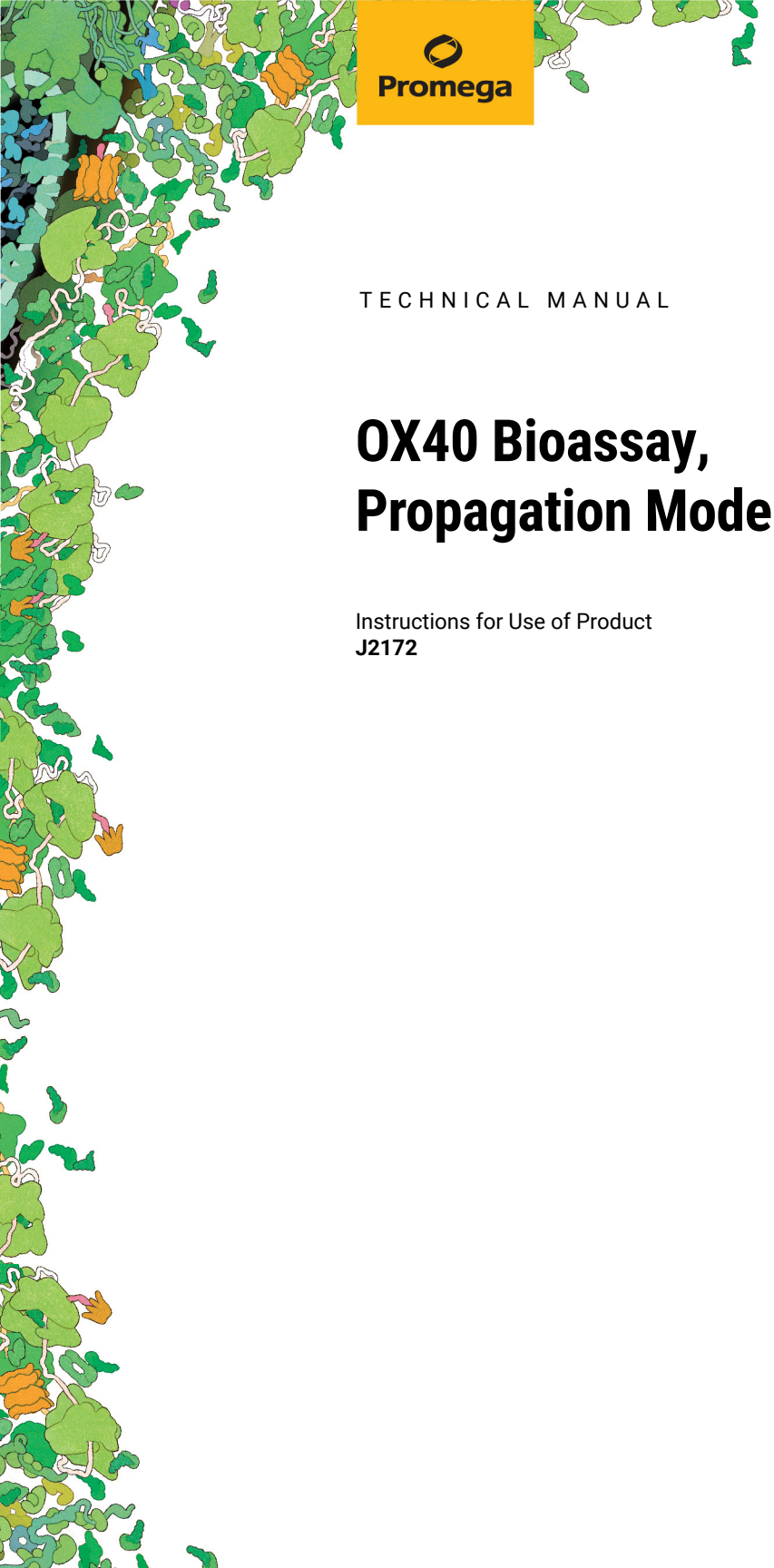

TECHNICAL MANUAL

OX40 Bioassay, Propagation Model

Instructions for Use of Product
J2172

Revised 5/26
TM580

Promega

OX40 Bioassay, Propagation Model

All technical literature is available at: www.promega.com/protocols/
Visit the website to verify that you are using the most current version of this Technical Manual.
Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

The human immune system is regulated by a complex network of inhibitory and stimulatory receptors that facilitate the elimination of pathogens, while maintaining tolerance to self-antigens. Stimulatory immune checkpoint receptors appear to have a significant role in cancer progression and autoimmune disease. Several costimulatory immune checkpoint receptors such as glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR), OX40, 4-1BB, 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).

OX40 (CD134/TNFRSF4), a member of the tumor necrosis factor (TNF) receptor superfamily, is a costimulatory receptor expressed primarily on activated T cells, and on neutrophils and natural killer (NK) cells to a lesser extent (4). When present on the cell surface, OX40 interacts with OX40 ligand (OX40L), and induces subsequent cell proliferation, survival and production of cytokines, particularly in T cells (5,6).

Current methods used to measure the activity of biologic drugs targeting OX40 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 quality controlled drug-development setting.

The OX40 Bioassay, Propagation Model[a–d] (Cat.# J2172), is a bioluminescent cell-based assay that overcomes the limitations of existing assays. It can be used to measure the potency and stability of ligands or agonist antibodies that can bind and activate OX40 (5,7). The assay consists of a genetically engineered Jurkat T cell line that expresses human OX40 and a luciferase reporter driven by a response element that can respond to OX40 ligand/agonist antibody stimulation. The OX40 Effector Cells are provided in Cell Propagation Model (CPM) format, which includes cryopreserved cells that can be thawed, propagated and banked for long-term use.

The OX40 Bioassay should be conducted with FcγRIIb CHO-K1 Cells (Cat.# J2232) to test whether agonist antibodies activate OX40 in an FcγRIIb-dependent manner. FcγRIIb CHO-K1 cells may be required to crosslink agonist antibodies but are not required for testing ligands. It is recommended that, when screening for agonist antibodies of costimulatory immune checkpoints, you perform the assay both with and without FcγRIIb CHO-K1 cells to ascertain the need for these cells in enhancing the effect of the agonist antibodies raised against the costimulatory immune checkpoint targets.

Induction of the OX40 Effector Cells with an OX40 ligand or agonist antibody results in response element-mediated luminescence (Figure 1). The bioluminescent signal is quantified using the Bio-Glo™ Luciferase Assay System and a standard luminometer such as the GloMax® Discover System.

The OX40 Bioassay, Propagation Model, reflects the mechanism of action (MOA) of biologics designed to activate OX40. Specifically, OX40-mediated luminescence is detected following the addition of OX40 ligand and OX40 agonist antibodies (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, robust and compatible with both 96-well and 384-well plate formats used for antibody screening in early drug discovery (Figure 4). In addition, the bioassay can be used with up to 100% human serum (in antibody samples; Figure 5), indicating potential for further development into a neutralizing antibody bioassay.

1. Description (continued)

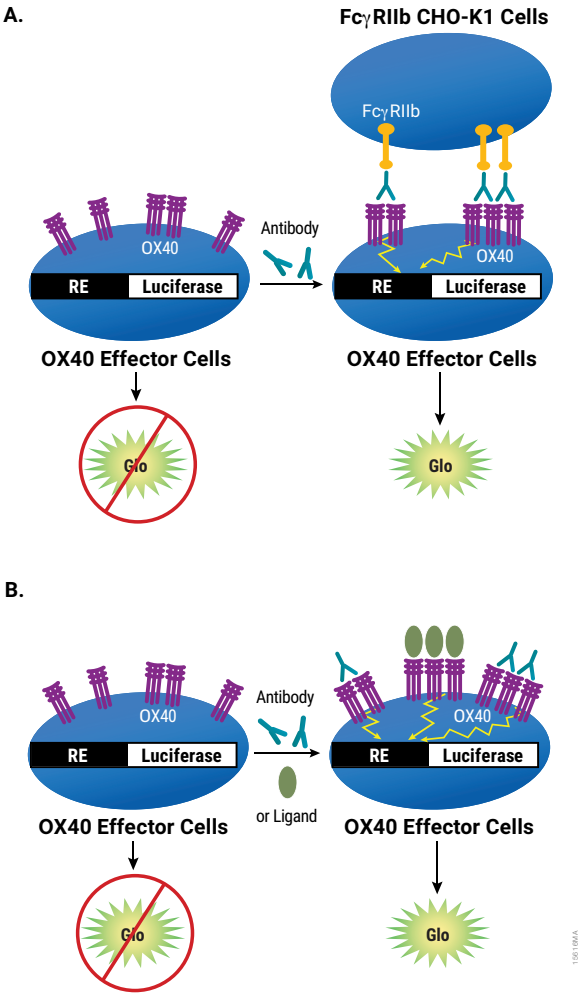

Figure 1. Representation of the OX40 Bioassay. Panel A. Assay with FcγRIIb-dependent agonist antibody. The bioassay consists of two engineered cell lines, OX40 Effector Cells and and FcγRIIb CHO-K1 Cells. In the presence of FcγRIIb CHO-K1 Cells, the anti-OX40 antibody can be crosslinked, thereby inducing OX40 pathway-activated luminescence. **Panel B.** Assay with FcγRIIb-independent agonist antibody or ligand. The bioassay consists of one engineered cell line, OX40 Effector Cells. In the absence of agonist antibody or OX40 ligand, the OX40 receptor is not activated and luminescence signal is low. The addition of agonist antibody or OX40 ligand induces the OX40 pathway-activated luminescence, which can be detected in a dose-dependent manner.

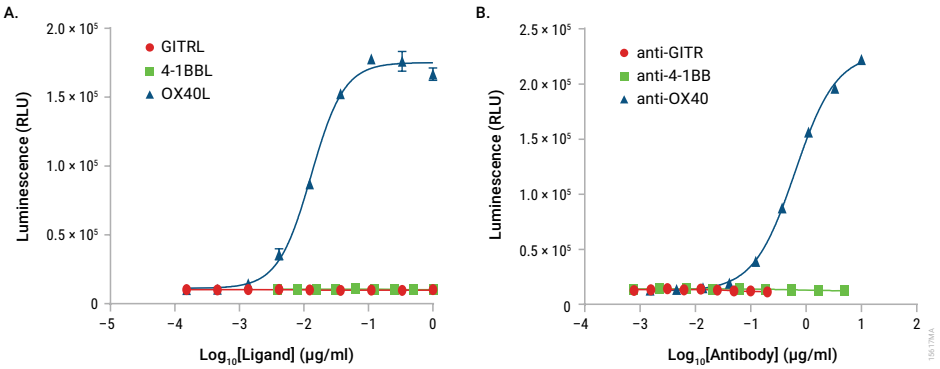

Figure 2. The OX40 Bioassay reflects the mechanism of action (MOA) and shows specificity of biologics designed to activate OX40. Panel A. OX40 Effector Cells were induced with a serial titration of ligands: GITRL crosslinked by anti-HA Ab; 4-1BBL crosslinked by anti-His Ab; or OX40L, as indicated. **Panel B.** OX40 Effector Cells were induced with a serial titration of anti-GITR antibody, anti-4-1BB antibody or anti-OX40 antibody, as indicated, in the presence of FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255). After a 5-hour induction, 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.

Table 1. The OX40 Bioassay Shows Precision, Accuracy and Linearity.

Parameter	**Results**	
Accuracy	% Expected Relative Potency	% Recovery
	50	99.4
	70	100.8
	140	101.2
	200	109.9
Repeatability (% CV)	100% (Reference)	5.4
Intermediate Precision (% CV)		9.0
Linearity (r^2)		0.996
Linearity (y = mx + b)		y = 1.123x – 8.895
A 50–200% theoretical potency series of Control Ab, Anti-OX40, was analyzed in triplicate in three independent experiments performed on three days by two analysts. Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were analyzed and relative potencies were calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.		

1. Description (continued)

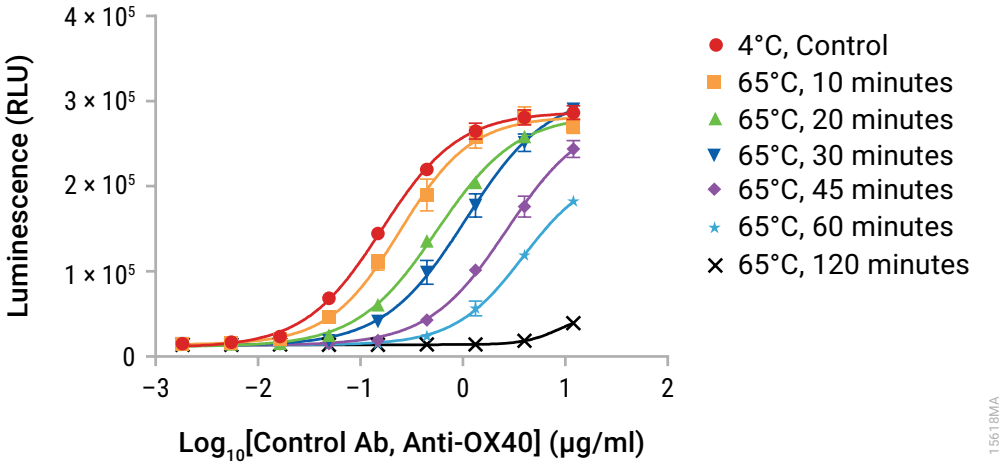

Figure 3. The OX40 Bioassay is stability-indicating. Samples of Control Ab, Anti-OX40 (Cat.# K1191), were maintained at 4°C (control) or heat-treated at 65°C for indicated times, and then analyzed using the OX40 Bioassay with FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255). After a 5-hour induction, Bio-Glo™ 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.

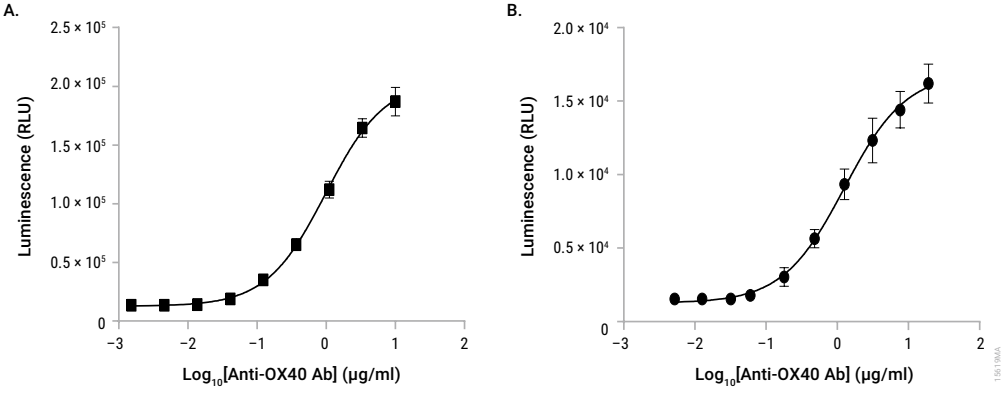

Figure 4. The OX40 Bioassay is amenable to 384-well plate format and compatible with laboratory automation. **Panel A.** The OX40 Bioassay was performed in 96-well plates as described in this technical manual using anti-OX40 antibody. **Panel B.** The OX40 Bioassay was performed in 384-well format using a Mantis® liquid handler to dispense the cells and Echo® Acoustic liquid handler for antibody handling. On the day before the assay, FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255) were plated at 8×10^3 cells/10µl/well and incubated at 37°C for 5 hours. OX40 Effector Cells were then added to the plate at 1.0×10^4 cells/10µl/well to co-incubate with FcγRIIb CHO-K1 Cells at 37°C overnight. On the day of the assay, anti-OX40 antibody was serially diluted and added to the plate at 0.2µl/well. After a 5-hour induction, 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 0.99µg/ml and 1.26µg/ml, and the fold inductions were 15 and 11 for 96-well and 384-well format, respectively. Data were generated using thaw-and-use cells.

1. Description (continued)

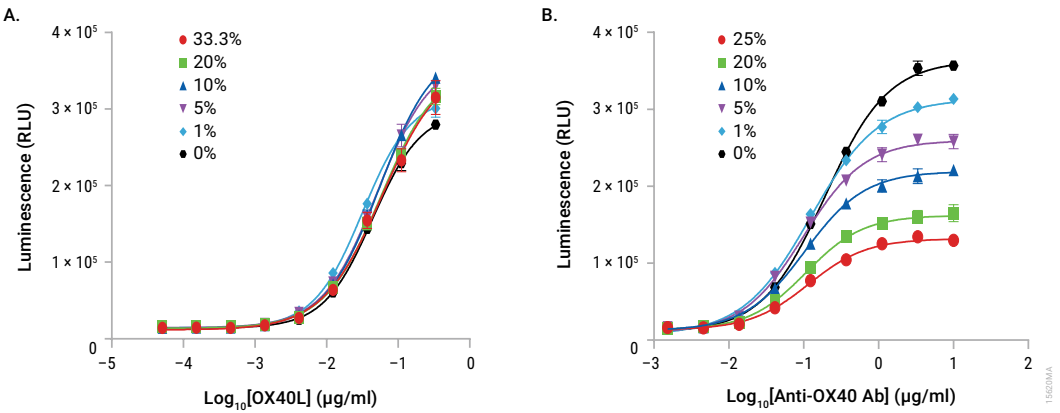

Figure 5. The OX40 Bioassay is tolerant to human serum. Panel A. OX40 ligand (OX40L, Biolegend Cat.# 555704) was analyzed in the presence of increasing concentrations of pooled normal human serum (0–100% in the ligand sample), resulting in final assay concentration of human serum (0–33.3%). **Panel B.** Control Ab, Anti-OX40 (Cat.#K1191), 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 (0–25%). After a 5-hour induction at 37°C, Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells. The OX40 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.#
OX40 Bioassay, Propagation Model	**1 each**	**J2172**

Not for Medical Diagnostic Use. Includes:

- 2 vials OX40 Effector Cells (CPM), 2.0 × 10^7 cells/ml (1.0ml per vial)

Note: Thaw and propagate one vial to create frozen cell banks before use in an assay. The second vial should be reserved for future use.

Storage Conditions: Upon arrival, immediately transfer the cell vials to below –140°C (freezer or liquid nitrogen vapor phase) for long-term storage. Do not store cell vials submerged in liquid nitrogen. **Do not** store cell vials at –80°C because this will decrease cell viability and cell performance.

3. Before You Begin

Please read through the entire protocol to become familiar with the components and the assay procedure before beginning.

Note the catalog number and lot number from the cell vial box label. This information can be used to download documents for the specified product from the website, such as Certificate of Analysis.

The OX40 Bioassay, Propagation Model, is intended to be used with user-provided antibodies or ligands designed to activate OX40. Control Ab, Anti-OX40 (Cat.# K1191), and FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255) and FcγRIIb CHO-K1 Cells, Propagation Model (Cat.# J2232), are available separately for use in assay optimization and routine quality control. We strongly recommend including OX40 Ligand or Control Ab, Anti-OX40, and FcγRIIb CHO-K1 Cells as a positive control in the first few assays to gain familiarity with the assay. Data generated using these reagents are shown in Figures 2–5 and Sections 9.A and 9.B, Representative Assay Results.

Cell thawing, propagation and banking should be performed exactly as described in Section 4. Cell seeding and propagation densities have been optimized to ensure stable cell growth, which is reflected in a steady cell doubling rate, to achieve optimal, consistent performance. An accurate, reliable and reproducible cell counting method is required for routine cell culturing and optimal bioassay performance. The recommended cell plating densities, induction time and assay buffer components described in Sections 5 and 6 were established using OX40 Ligand and Control Ab, Anti-OX40, respectively. You may need to adjust the parameters provided here and optimize assay conditions for your own antibody or biologic.

The OX40 Bioassay, Propagation Model, produces a bioluminescent signal and should work with all major luminometers or luminescence plate readers for the detection of luminescence. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System. An integration time of 0.5 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 9.C.)

Reagents

- user-defined anti-OX40 antibodies or other biologics samples
- RPMI 1640 Medium with L-glutamine and HEPES (e.g., Corning® Cat.# 10-041-CV or GIBCO® Cat.# 22400105)
- Ham's F-12 Medium with L-glutamine (e.g., GIBCO® Cat.# 11765062)
- fetal bovine serum (e.g., HyClone Cat.# SH30070.03 or GIBCO® Cat.# 16000044)
- hygromycin B (e.g., GIBCO® Cat.# 10687010)
- G418 sulfate solution (e.g., GIBCO® Cat.# 10131035)
- sodium pyruvate (e.g., GIBCO® Cat.# 11360070)
- MEM nonessential amino acids, 100X (e.g., GIBCO® Cat.# 11140050)
- DMSO (e.g., Sigma Cat.# D2650)
- DPBS (e.g., GIBCO® Cat.# 14190)
- Accutase® solution (e.g., Sigma Cat.# A6964)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941)
- **optional:** FcγRIIb CHO-K1 Cells (If using this assay for the first time and/or testing an Ab that might be dependent on crosslinking by FcγRIIb, this cell line is required: Cat.# JA2251, JA2255)
- **optional:** Control OX40 ligand (Biolegend, Cat.# 555704)
- **optional:** Control Ab, Anti-OX40 (Cat.# K1191)

Supplies and Equipment

- solid-white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917) for plating and reading luminescence
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896 or Linbro Cat.# 76-223-05) for preparing antibody dilutions
- pipettes (single-channel and 12-channel; for best results use both manual and electronic pipettes as needed)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Costar®/Corning® Cat.# 4870)
- 37°C, 5% CO_2 incubator
- 37°C water bath
- plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent system)

4. Preparing OX40 Effector Cells

4.A. Cell Thawing and Initial Cell Culture

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

1. Prepare 50ml of initial cell culture medium by adding 5ml of FBS, 0.5ml of 100X MEM nonessential amino acids (NEAA) and 0.5ml of 100mM sodium pyruvate to 44ml of RPMI 1640 medium prewarmed to 37°C. This initial cell culture medium will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of prewarmed initial cell culture medium to a 50ml conical tube.
3. Remove one vial of OX40 Effector Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed (typically 2–3 minutes).
4. Transfer all of the cells (approximately 1ml) to the 50ml conical tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 90 × *g* for 10 minutes.
6. Carefully aspirate the medium, and resuspend the cell pellet in 25ml of prewarmed initial cell culture medium.
7. Transfer the cell suspension to a T75 tissue culture flask, and place the flask horizontally in a 37°C, 5% CO_2 incubator.
8. Incubate for approximately 48 hours before passaging the cells.

4.B. Cell Maintenance and Propagation

Note: For cell maintenance and propagation starting from the second cell passage, use the cell growth medium containing antibiotics, and monitor cell viability and doubling rate during propagation. The cell growth rate will stabilize by 7–10 days after thawing, at which time cell viability is typically >90%, and the average cell doubling rate is ~30 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 25 passages if passaging is performed on a Monday-Wednesday-Friday schedule.

1. On the day of cell passage, measure cell viability and density by Trypan blue staining.
2. Seed the cells at a density of 4×10^5 cells/ml if passaging every 2 days (e.g., Monday-Wednesday or Wednesday-Friday) or 2×10^5 cells/ml if passaging every 3 days (e.g., Friday-Monday). Always maintain the flasks in a horizontal position in the incubator.
3. Maintain the cell culture by adding fresh cell growth medium to the cell suspension in the original flask or by transferring the cells to a new flask while maintaining a consistent ratio of culture volume to flask surface area (e.g., 25ml volume per T75 flask or 50ml volume per T150 flask).
4. Place the flasks horizontally in a 37°C, 5% CO_2 incubator.

4.C. Cell Freezing and Banking

1. On the day of cell freezing, make fresh cell freezing medium and keep on ice.
2. Gently mix the cells with a pipette to create a homogenous cell suspension.
3. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of cell freezing medium needed based on desired cell freezing densities of 0.5×10^7–1×10^7 cells/ml.
4. Transfer the cell suspension to 50ml sterile conical tubes or larger sized centrifuge tubes, and centrifuge at 130 × *g*, 4°C for 10–15 minutes.
5. Carefully aspirate the supernatant; avoid disturbing the cell pellet.
6. Gently resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of 0.5×10^7–1×10^7 cells/ml. Combine the cell suspensions into a single tube and dispense into cryovials.
7. Freeze the cells using a controlled-rate freezer (preferred), or a Mr. Frosty® or a Styrofoam® rack in a –80°C freezer overnight. Transfer the vials to at or below –140°C for long-term storage.

5. Assay Protocol for FcγRIIb-Dependent Antibodies

This assay protocol requires two engineered cell lines: OX40 Effector Cells and FcγRIIb CHO-K1 Cells. The FcγRIIb CHO-K1 Cells are provided in Thaw-and-Use format (*OX40 Bioassay Technical Manual,* #TM581) and CPM format (see *FcγRIIb CHO-K1 Propagation Model Technical Manual*, #TM569, for details). Either cell format can be used in this assay.

The procedure below illustrates the use of the OX40 Bioassay to test two FcγRIIb-dependent antibody samples against a reference sample in a single assay run using the FcγRIIb CHO-K1 Cells CPM format. Each test and reference antibody is run in triplicate, in a 10-point dilution series, in a single 96-well assay plate using the inner 60 wells. Other experimental plate layouts are possible but may require further optimization.

Note: When preparing test and reference antibodies, choose an appropriate starting concentration and dilution scheme to achieve a complete dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 10µg/ml as a starting concentration (1X) and threefold serial dilution when testing Control Ab, Anti-OX40.

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

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

2. **Assay Buffer:** On the day of the assay, prepare an appropriate amount of assay buffer (95% RPMI 1640/5% 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, 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 5% FBS. This concentration of FBS works well for the Control Ab, Anti-OX40, that we tested. If assay performance is not ideal using 5% FBS assay buffer when testing your antibody of interest, try optimizing the FBS concentration from 0.5–10% in assay buffer.

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 (180µl each) and one reference antibody (400µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Notes:

   a. If you are using Control Ab, Anti-OX40 (Cat.# K1191), as a reference antibody in your assay, prepare a 400µl starting dilution with 30µg/ml of anti-OX40 antibody (dilu1, 3X final concentration) by adding 12µl of anti-OX40 stock (1,000µg/ml) to 388µl of assay buffer. Store the antibody starting dilution on ice until ready to use in the assay.

   b. To streamline assay setup, prepare antibody serial dilutions prior to harvesting and plating OX40 Effector Cells.

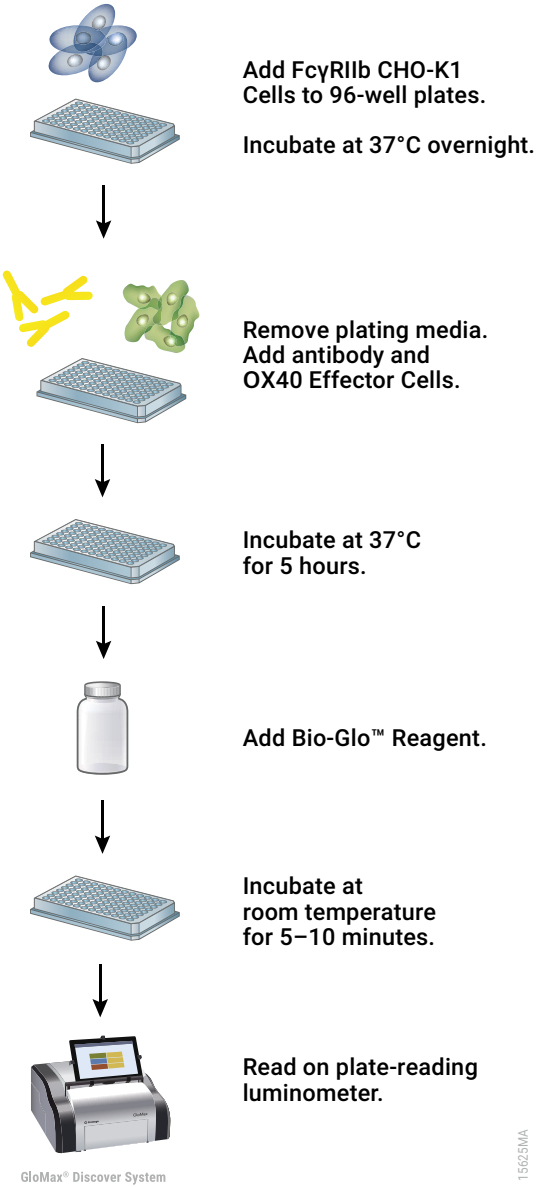

Figure 6. Schematic protocol for OX40 Bioassay, Propagation Model, with FcγRIIb-dependent antibody.

5.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 7 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibody to generate two ten-point dose-response curves for each plate.

Recommended Plate Layout Design													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)
B	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
C	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
D	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ab
G	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 7. Example plate layout showing nonclustered sample locations of test antibody and reference antibody dilution series, and wells containing assay buffer (denoted by "B") alone.

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

While maintaining the FcγRIIb CHO-K1 Cells (Cat.# J2232), follow the cell seeding density recommendations in the *FcγRIIb CHO-K1 Cells, Propagation Model, Technical Manual*, #TM569. Changes in cell culture volume or seeding density will affect cell growth rate and assay performance. Do not allow the cells to grow to 100% confluence. Only use the cells in the assay after the cell doubling rate has stabilized during propagation.

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

1. We recommend passaging the FcγRIIb CHO-K1 Cells two days before plating for the assay, as described in the *FcγRIIb CHO-K1 Cells, Propagation Model, Technical Manual,* #TM569, to ensure optimal and consistent assay performance.
2. On the day before performing the assay, prepare new FcγRIIb CHO-K1 cell plating medium (Ham's F-12/10% FBS) for the FcγRIIb CHO-K1 Cells.
3. Aspirate the cell culture medium from the FcγRIIb CHO-K1 Cells and wash with DPBS.

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

4. Add 2ml of Accutase® solution to each T75 flask, and place the flask in a 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.
5. Add 8ml of FcγRIIb CHO-K1 cell plating medium to the flask. Transfer the cell suspension to a 50ml (or larger) conical centrifuge tube.
6. Gently mix and count the FcγRIIb CHO-K1 Cells by Trypan blue staining.
7. Centrifuge at 230 × *g* for 5 minutes.
8. Gently resuspend the cell pellet in cell plating medium to achieve a concentration of 4×10^5 viable cells/ml.
9. Transfer the suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 100µl of the cell suspension to each of the inner 60 wells of a 96-well white flat-bottom assay plate. The final cell number in each well should be 4×10^4 cells/well.
10. Add 100µl of cell plating medium to each of the outside wells of the assay plates.
11. Place lids on the assay plates and incubate in a 37°C, 5% CO_2 incubator overnight (18–22 hours).

5.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparation of a single stock of 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, OX40, 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 5.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 plate with a lid and keep at ambient temperature (22–25°C) while preparing the OX40 Effector Cells.

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

Recommended Plate Layout for Antibody Dilutions Prepared from a Single Antibody Stock													
	1	2	3	4	5	6	7	8	9	10	11	12	
A		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ab
B		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ab
C													
D													
E		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 1
F													
G		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 2
H													

Figure 8. Example plate layout showing antibody serial dilutions.

5.E. Preparing OX40 Effector Cells

While maintaining the OX40 Effector Cells, it is important to follow the recommended cell seeding density. Changes in cell culture volume or seeding density will affect cell growth rate and assay performance. Only use the cells in the assay after the cell doubling rate has stabilized during propagation.

1. Passage the cells two or three days before performing the assay as described in Section 4.B. To ensure optimal and consistent assay performance, maintain the cell density, upon harvest, in the range of 0.8–1.5 × 10^6 cells/ml and cell viability greater than 90%.
2. Count the OX40 Effector Cells by Trypan blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of OX40 Effector Cells from the culture vessel to a 50ml conical tube or larger sized centrifuge tube.
4. Collect the cells at 130 × *g* for 10 minutes at ambient temperature, and resuspend the pellet in assay buffer to generate the targeted cell density of 1.0 × 10^6 cells/ml.
5. You will need at least 8ml of OX40 Effector Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.

5.F. Adding Antibody Samples and OX40 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 OX40 Effector Cells prepared in Section 5.E to a sterile reagent reservoir. Using a multichannel pipette, dispense 50µl (0.5×10^5 cells) of OX40 Effector Cells into the wells containing antibody.
5. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 5 hours.

5.G. Adding Bio-Glo™ Reagent

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

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

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

5.H. Data Analysis

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

6. Assay Protocol for Ligand or FcγRIIb-Independent Antibodies

This assay protocol illustrates the use of the OX40 Bioassay, Propagation Model, to test two antibody samples against a reference sample in a single assay run. Each test and reference antibody is run in triplicate, in a ten-point dilution series, in a single 96-well assay plate using the inner 60 wells. Other experimental and plate layouts are possible but may require further optimization.

Note: When preparing test antibodies or ligand, choose an appropriate starting concentration and dilution scheme to achieve a full dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 0.333µg/ml as a starting concentration (1X) and threefold dilution when testing OX40 Ligand (see Section 3).

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

1. **Assay Buffer:** On the day of the assay, prepare an appropriate amount of assay buffer (95% RPMI 1640/5% 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, 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 5% FBS. This concentration of FBS works well with the crosslinked Control Ligand, OX40L, that we tested.

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

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

   If you are using a large (100ml) size of Bio-Glo™ Luciferase Assay System, dispense the reconstituted Bio-Glo™ Reagent into 10ml aliquots and store at –20°C for up to 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.

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

   Notes:

   a. If you are using OX40L (control OX40 ligand/TNFSF4, Biolegend, Cat.# 555704) as a reference ligand, prepare a 400µl starting dilution with 1µg/ml of OX40L by adding 2µl of OX40L, stock (200µg/ml) to 398µl of assay buffer. The final (1X) starting concentration is 0.333µg/ml of OX40L. Store the ligand starting dilution on ice until ready to use in the assay.

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

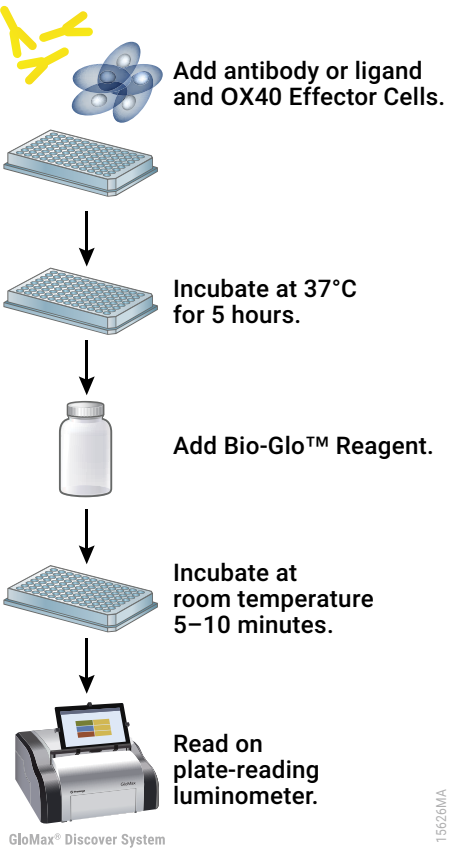

Figure 9. Schematic protocol for OX40 Bioassay, Propagation Model, with OX40 Ligand or FcγRIIb-independent antibody.

6.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 10 as a guide. The protocol describes serial replicate dilutions (n = 3) of test antibody and reference ligand to generate two ten-point dose-response curves for each plate.

Recommended Plate Layout Design													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)
B	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ligand
C	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
D	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ligand
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Ligand
G	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Ab
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 10. Example plate layout showing nonclustered sample locations of test antibody and reference ligand dilution series and wells containing assay buffer (denoted by "B") alone.

6.C. Preparing Ligand or Antibody Serial Dilutions

The instructions described here are for preparation of a single stock of threefold serial dilutions of a ligand for analysis in triplicate (120µl of each ligand dilution provides a sufficient volume for analysis in triplicate). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples. To prepare threefold serial dilutions for ligand, you will need 400µl of reference ligand at 3X the highest concentration in your dose response curve. To prepare threefold serial dilutions for test antibodies, you will need 180µl of each test antibody at 3X the highest antibody concentration in each of the test antibody dose-response curves. For other dilution schemes, adjust the volumes accordingly.

Note: If you are using OX40L (see Section 3) as a control in the assay, use the following instructions to prepare threefold serial dilutions. A threefold serial dilution for test antibodies is shown as an example on the next page.

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

1. On the day of assay, prepare an appropriate amount of assay buffer as described in Section 6.A.
2. To a sterile, clear V-bottom 96-well plate, add 180µl of reference ligand starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 11).
3. Add 180µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and G11, respectively (Figure 11).
4. Add 120µl of assay buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 60µl of the ligand or antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent threefold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.
7. Cover the plate with a lid and keep at ambient temperature (22–25°C) while preparing the OX40 Effector Cells.

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

Recommended Plate Layout for Antibody Dilutions Prepared from a Single Antibody Stock

	1	2	3	4	5	6	7	8	9	10	11	12	
A		no ligand	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ligand
B		no ligand	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Ligand
C													
D													
E		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 1
F													
G		no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Ab 2
H													

Figure 11. Example plate layout showing antibody serial dilutions.

6.D. Preparing OX40 Effector Cells

While maintaining the OX40 Effector Cells, it is important to follow the recommended cell seeding density. Changes in cell culture volume or seeding density will affect cell growth rate and assay performance. Only use the cells in the assay after the cell doubling rate has stabilized during propagation.

1. Passage the cells 2 days or 3 days before performing the assay, as described in Section 4.B. To ensure optimal and consistent assay performance, maintain the cell density, upon harvest, in the range of 0.8–1.5 × 10^6 cells/ml and cell viability at greater than 90%.
2. Count the OX40 Effector Cells by Trypan blue staining, and calculate the cell density and viability.
3. Transfer an appropriate amount of OX40 Effector Cells from the culture vessel to a 50ml conical tube or larger sized centrifuge tube.
4. Collect the cells at 130 × *g* for 10 minutes at ambient temperature, and resuspend the pellet in assay buffer to generate the targeted cell density of 1.0 × 10^6 cells/ml.
5. You will need at least 8ml of OX40 Effector Cells to fill 120 assay wells, using the inner 60 wells of two assay plates.

6.E. Adding Ligand or Antibody Samples and OX40 Effector Cells to Assay Plates

1. Using an electronic multichannel pipette, add 25µl of the appropriate antibody or ligand titration to the assay plates according to the plate layout in Figure 10.
2. Add 75µl of assay buffer to the outside wells of the 96-well assay plates.
3. Transfer the OX40 Effector Cells prepared in Section 6.D to a sterile reagent reservoir. Using a multichannel pipette, dispense 50µl (0.5 × 10^5 cells) of OX40 Effector Cells into the wells containing antibody or ligand.
4. Cover each assay plate with a lid and incubate in a 37°C, 5% CO_2 incubator for 5 hours.

6.F. Adding Bio-Glo™ Reagent

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

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

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

6.G. Data Analysis

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

7. Troubleshooting

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

Symptoms	Causes and Comments
Low luminescence measurements (RLU readout)	Choose an instrument designed for plate-reading luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual RLU numbers will vary between instruments.
	Insufficient cells per well can lead to low RLU. Handle and plate cells according to the instructions to ensure a sufficient number of viable cells per well.
	Low activity of Bio-Glo™ Reagent leads to low RLU. Store and handle the Bio-Glo™ Reagent according to the instructions.
	If performing the assay for the first time, we recommend that you 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.
Variability in assay performance	Variations in cell growth conditions including cell plating and harvest density, cell viability and cell doubling time. Ensure consistent cell growth by handling the cells exactly according to the instructions.
	Inappropriate cell handling during cell harvest, including long centrifuge times and high centrifuge speeds, may cause low assay performance and high assay variation.
	Inappropriate cell counting methods may lead to variation in cell numbers in culture and assays and cause high assay variation. Ensure accurate and consistent cell counting methods.

7. Troubleshooting (continued)

Symptoms	Causes and Comments
Weak assay response (low fold induction)	Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The EC_{50} value obtained in the OX40 Bioassay may vary from the EC_{50} value obtained using other methods such as primary T cell-based assays.
	The assay is sensitive to the concentration of FBS in assay buffer. Optimize the FBS concentration from 0.5%-10% in assay buffer if assay performance is not ideal.
	Determine if the antibody used is dependent on crosslinking for performance by testing in the presence of FcγRIIb cells.
	If untreated control RLU is less than 100-fold above plate reader background RLU, subtract plate background RLU from all samples before calculating fold induction.

8. References

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9. Appendix

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

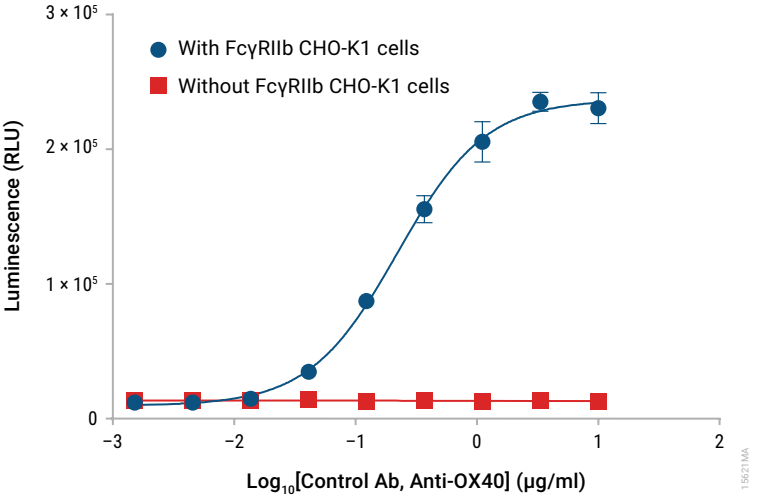

Figure 12. The OX40 Bioassay measures the activity of Control Antibody, Anti-OX40. FcγRIIb CHO-K1 Cells were plated overnight. The following day, a titration of Control Ab, Anti-OX40, (Section 5.D) was added followed by OX40 Effector Cells. After 5 hours of induction at 37°C, Bio-Glo™ Reagent was added, and luminescence was determined using a GloMax® Discover System. Four-parameter logistic curve analysis was performed with GraphPad Prism® software. The EC_{50} response determined was ~0.22µg/ml, and the fold induction was ~19.2.

9.B. Representative Assay Results with OX40 Ligand

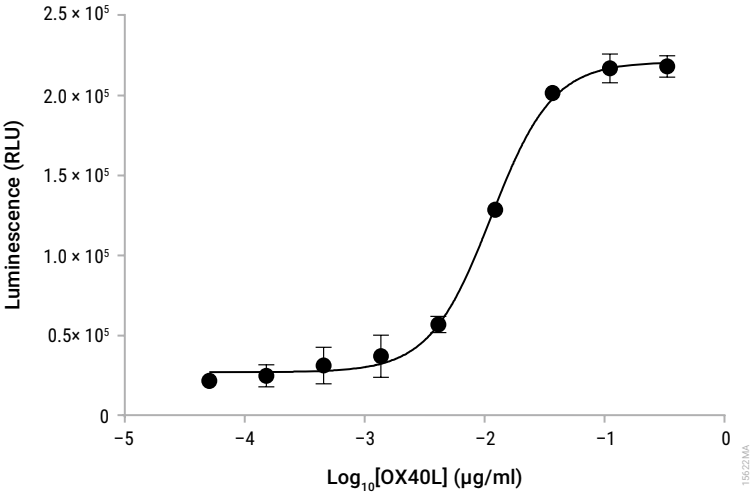

Figure 13. The OX40 Bioassay measures the activity of OX40 Ligand. On the assay day, OX40 Effector Cells were plated in a 96-well plate at 50,000 cells/well. Cells were incubated with various concentrations of OX40L (Section 6.C). After a 5-hour induction at 37°C, Bio-Glo™ Reagent was added, and luminescence was determined using a GloMax® Discover System. Four-parameter logistic curve analysis was performed with GraphPad Prism® software. The EC_{50} response determined was ~0.011µg/ml, and the fold induction was ~10.1.

9.C. Composition of Buffers and Solutions

initial cell culture medium for OX40 Effector Cells

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

cell growth medium for OX40 Effector Cells

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

cell freezing medium for OX40 Effector Cells

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

cell recovery medium for FcγRIIb CHO-K1 Cells

90%	Ham's F12
10%	FBS

assay buffer

95%	RPMI 1640 with L-glutamine and HEPES
5%	FBS

10. Summary of Changes

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

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

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