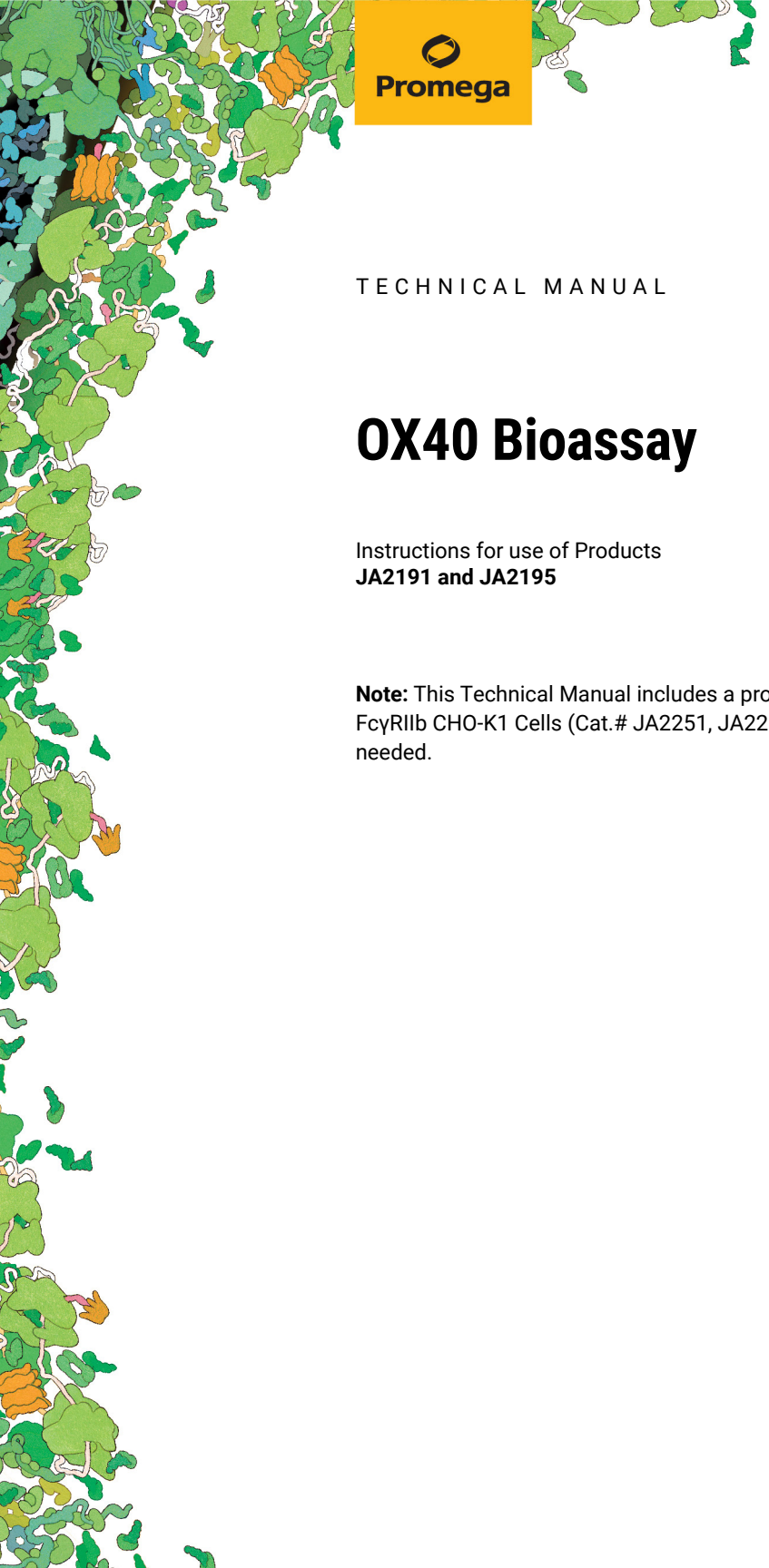

TECHNICAL MANUAL

# OX40 Bioassay

Instructions for use of Products
**JA2191 and JA2195**

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

Revised 5/26
TM581

Promega

# OX40 Bioassay

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**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[a–e] (Cat.# JA2191, JA2195), 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 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 OX40 Bioassay should be conducted with FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255) 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[e] and a standard luminometer such as the GloMax® Discover System.

The OX40 Bioassay 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 1-day or 2-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 ligand or antibody samples; Figure 5), indicating potential for further development into a neutralizing antibody bioassay.

## 1. Description (continued)

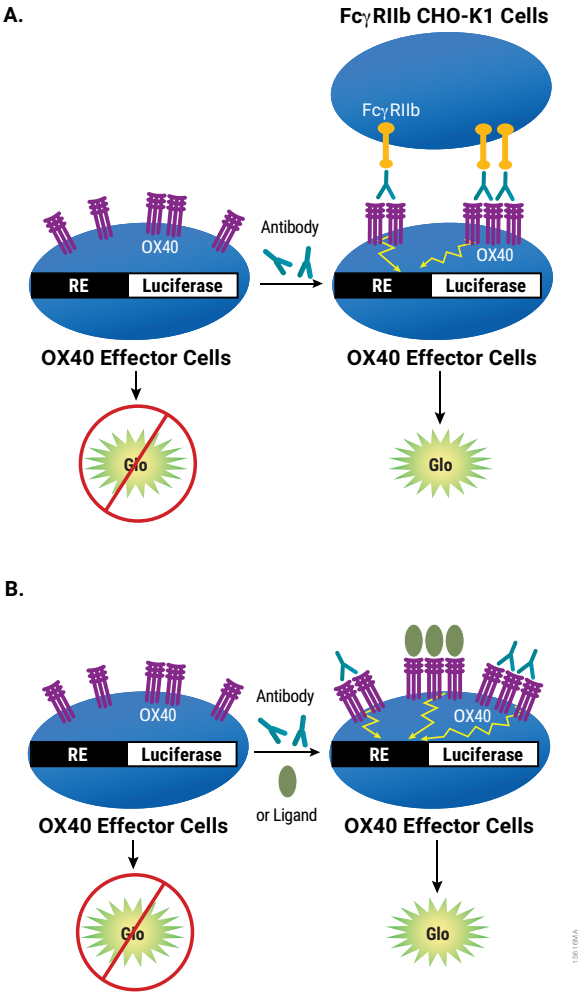


**Figure 1. Representation of the OX40 Bioassay. Panel A.** Assay with FcγRIIb-dependent agonist antibody. The bioassay consists of two engineered cell lines, OX40 Effector Cells and FcγRIIb CHO-K1 Cells. In the presence of FcγRIIb CHO-K1 Cells, the anti-OX40 antibody can be crosslinked, thereby inducing 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 the 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.

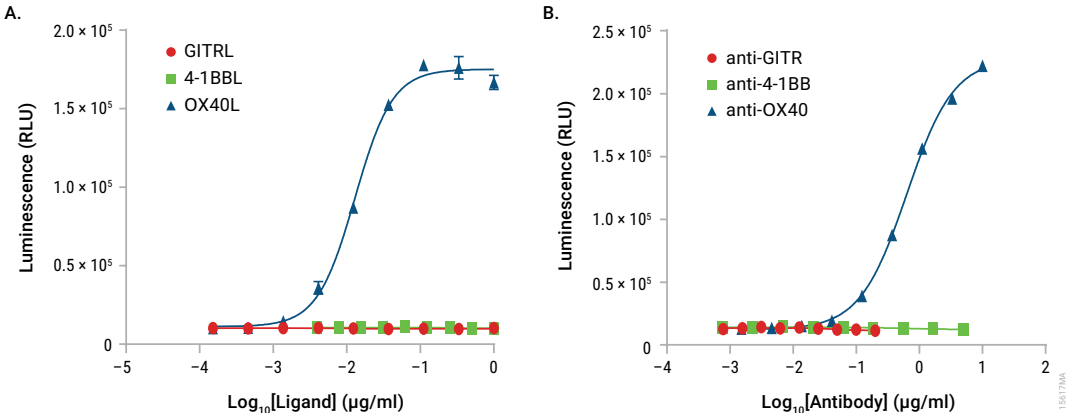


**Figure 2. The OX40 Bioassay reflects the mechanism of action (MOA) and shows specificity of biologics designed to activate OX40. Panel A.** OX40 Effector Cells were induced with a serial titration of ligands: GITRL crosslinked by anti-HA Ab; 4-1BBL crosslinked by anti-His Ab; or OX40L, as indicated. **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[e] 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)

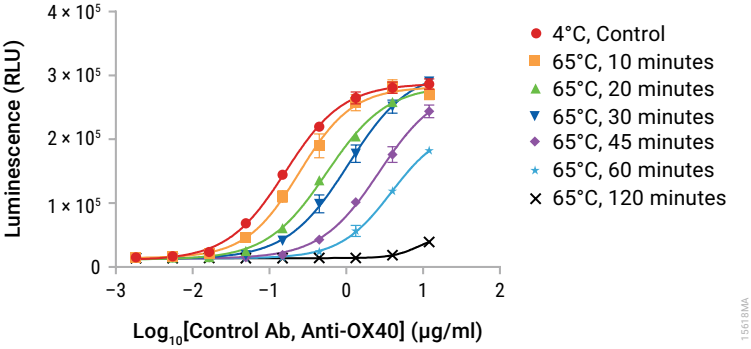


**Figure 3. The OX40 Bioassay is stability-indicating.** Samples of Control Ab, Anti-OX40 (Cat.# K1191), were maintained at 4°C (control) or heat-treated at 65°C for the indicated times, then analyzed using the OX40 Bioassay with FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255). After a 5-hour induction,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

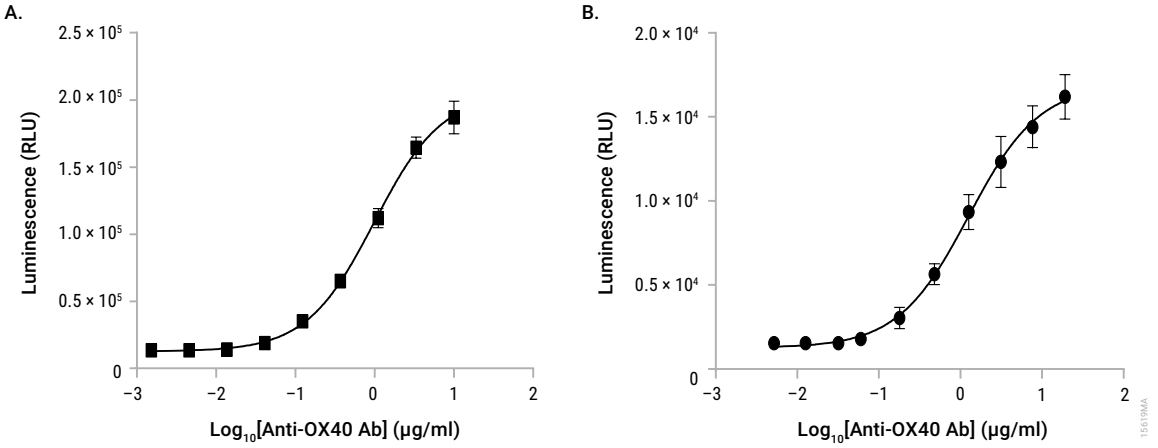


**Figure 4. The OX40 Bioassay is amenable to 384-well plate format and compatible with laboratory automation. Panel A.** The OX40 Bioassay was performed in 96-well plates as described in this technical manual using anti-OX40 antibody. **Panel B.** The OX40 Bioassay was performed in 384-well format using 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 $8 \times 10^3$ cells/10µl/well and incubated at 37°C for 5 hours. OX40 Effector Cells were then added to the plate at $1 \times 10^4$ 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)

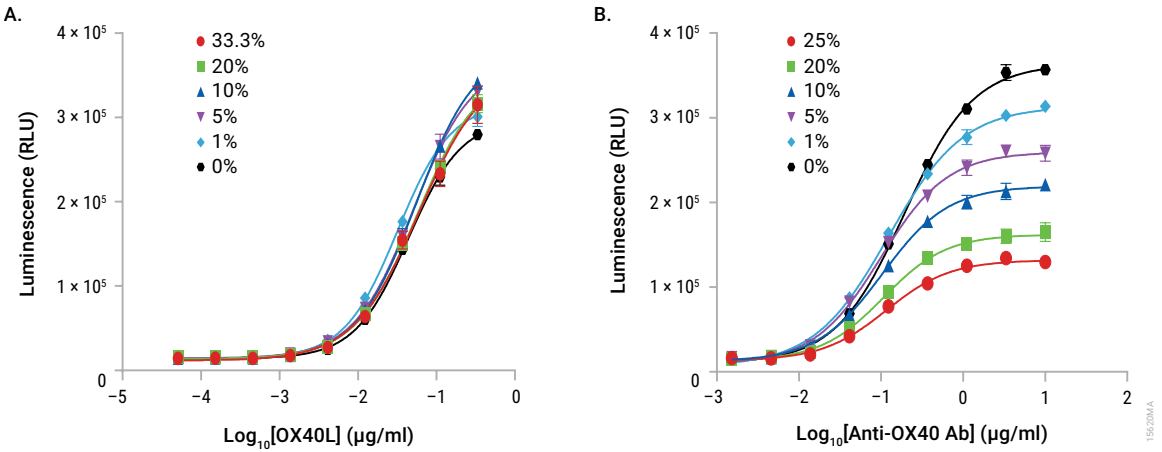


**Figure 5. The OX40 Bioassay is tolerant to human serum. Panel A.** OX40 ligand (OX40L, Biolegend Cat.# 555704) was analyzed in the presence of increasing concentrations of pooled normal human serum (0–100% in the ligand sample), resulting in final assay concentration of human serum (0–33.3%). **Panel B.** Anti-OX40 antibody was analyzed in the presence of FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255) and increasing concentrations of pooled normal human serum (0–100% in the antibody sample), resulting in final assay 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** | **1 each** | **JA2191** |

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 OX40 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.# |
|---|---|---|
| **OX40 Bioassay 5X** | **1 each** | **JA2195** |

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

- 5 vials OX40 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:** OX40 Bioassay components are shipped separately because of different temperature requirements. OX40 Effector Cells are shipped on dry ice. 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). 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 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 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) 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 8.A and 8.B, Representative Assay Results.

The OX40 Effector Cells are provided in frozen, thaw-and-use format and are ready to be used without any additional cell culture or propagation. When thawed and diluted as instructed, the cells will be at the appropriate concentration for the assay. The cells are sensitive, and care should be taken to follow cell thawing and plating procedures as described.

The OX40 Bioassay, produces a bioluminescent signal and should work with all major luminometers or luminescence plate readers for the detection of luminescence. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System. An integration time of 0.5 seconds/well was used for all readings.

### 3.A. Materials to Be Supplied By the User

- user-defined anti-OX40 antibodies or other biologics samples
- solid-white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917) for plating and reading luminescence
- sterile clear V-bottom 96-well plate with lid (e.g., Costar® Cat.# 3896 or Linbro Cat.# 76-223-05) for preparing antibody dilutions
- pipettes (single-channel and 12-channel; for best results use both manual and electronic pipettes as needed)
- sterile 15ml and 50ml conical tubes
- sterile 125ml media bottles (e.g., Fisher Scientific Cat.# 03-313-901)
- sterile reagent reservoirs (e.g., Costar®/Corning® Cat.# 4870)
- 37°C, 5% $CO_2$ incubator
- 37°C water bath
- plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent system)
- **optional:** solid-white, flat-bottom 384-well assay plates (e.g., Corning® Cat.# 3570)
- **optional:** FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255; We recommend this cell line when using this assay for the first time and/or testing an Ab that may be dependent on crosslinking FcγRIIb.)
- **optional:** Control Ab, Anti-OX40 (Cat.# K1191)
- **optional:** OX40 ligand (OX40L, Biolegend Cat.# 555704)

## 4. 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 (Cat.# JA2251, JA2255) and CPM format (Cat.# J2232; 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 in thaw-and-use format. Each test and reference antibody is run in triplicate, in a 10-point dilution series, in a single 96-well assay plate using the inner 60 wells. Other experimental 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, which is FcγRIIb-dependent and requires the use of FcγRIIb CHO-K1 Cells.

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

1. **Assay Buffer:** On the day before assay, prepare 70ml of assay buffer (95% RPMI 1640/5% FBS) in a 125ml media bottle. Thaw the FBS overnight at 4°C or in a 37°C water bath on the day of use. Add 3.5ml of FBS to 66.5ml of RPMI 1640 medium. Mix well and warm to 37°C before use. For reference, 70ml 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.

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. When stored at ambient temperature, the reconstituted Bio-Glo™ Reagent will lose ~18% signal after 24 hours.

3. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 4X 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-OX40 (Cat.# K1191), as a reference antibody in your assay, prepare a 400µl starting dilution with 40µg/ml of anti-OX40 antibody (dilu1, 4X final concentration) by adding 16µl of anti-OX40 stock (1,000µg/ml) to 384µl of assay buffer. Store the antibody starting dilution on ice until ready to use in the assay.

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

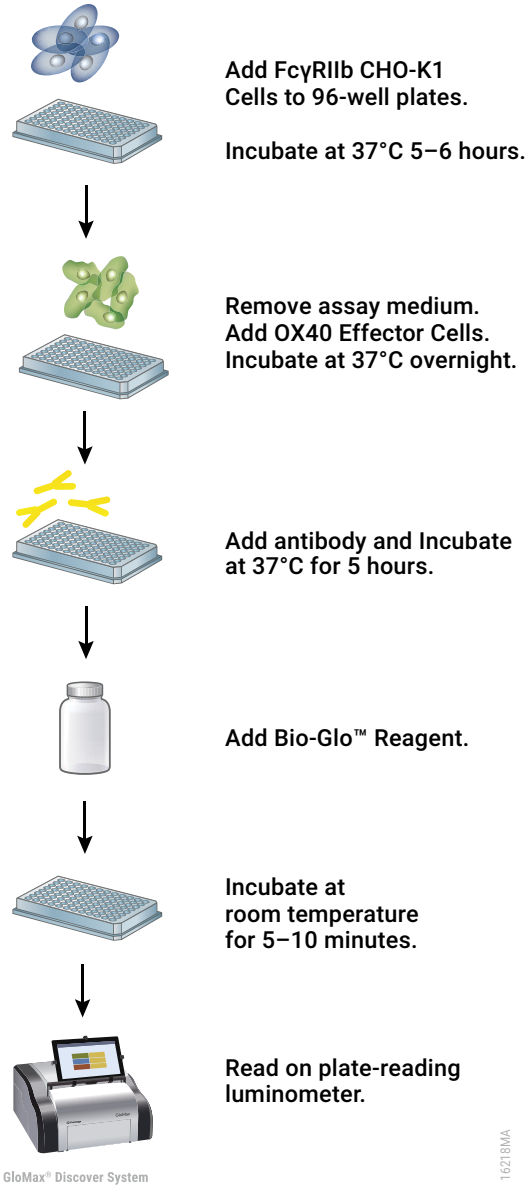


**Figure 6. Schematic protocol for OX40 Bioassay with FcγRIIb-dependent antibody.**

### 4.B. Plate Layout Design

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

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

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

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

The thaw-and-use FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255) are available separately. These cells are sensitive, and care should be taken to follow the cell thawing and plating procedures exactly as described. Do not overmix or overwarm the cell reagents. No additional cell culture or cell manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at one time.

**Note:** Perform the following steps using aseptic technique in a sterile cell culture hood early in the morning on the day before the assay.

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

1. On the day before the assay, prepare 70ml of fresh assay buffer (RPMI/5% FBS) as described in Section 4.A. Divide the assay buffer into three aliquots, one for FcγRIIb CHO-K1 preparation (30ml), one for OX40 Effector Cells preparation (30ml) and one for antibody serial dilution preparation (10ml). Store two aliquots (30ml and 10ml) at 4°C for later use.
2. Warm the assay buffer (30ml aliquot) in a 37°C water bath for 15 minutes then transfer 14.5ml of assay buffer to a 15ml conical tube.
3. Remove one vial of thaw-and-use FcγRIIb CHO-K1 Cells from –140°C storage and transfer to the bench on dry ice. Thaw the vial in a 37°C water bath until cells are just thawed (about 2 minutes). While thawing, gently agitate and visually inspect.
4. Gently mix the cell suspension in the vial by pipetting, and then transfer 0.5ml of cells to the tube containing 14.5ml of assay buffer. Mix well by gently inverting 1–2 times.
5. Transfer the cell suspension to a sterile reagent reservoir. Immediately, using a multichannel pipette, dispense 100µl of the cell suspension to each of the inner 60-wells of two 96-well, white, flat-bottom assay plates.
6. Add 100µl of assay buffer per well to the outside wells of the assay plates.
7. Cover the assay plates with a lid and incubate the cells in a 37°C, 5% $CO_2$ incubator for 5–6 hours.

### 4.D. Preparing and Plating OX40 Effector Cells

**Note:** The thaw-and-use OX40 Effector Cells included in this kit are sensitive, and care should be taken to follow the cell thawing and plating procedures exactly as described. Do not overmix or overwarm the cell reagents. No additional cell culture or cell manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at one time.

1. Warm the assay buffer (30ml aliquot) in a 37°C water bath for 15 minutes and transfer 11.5ml of assay buffer to a 15ml conical tube.
2. Remove one vial of thaw-and-use OX40 Effector Cells from –140°C storage and transfer to the bench on dry ice. Thaw the vial in a 37°C water bath until cells are just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial.
3. Gently mix the cell suspension in the vial by pipetting, then transfer 0.5ml of cells to the 15ml conical tube containing 11.5ml of assay buffer. Mix well by gently inverting 1–2 times.
4. Remove the 96-well assay plates containing FcγRIIb CHO-K1 Cells from 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.
5. Transfer the OX40 Effector Cells prepared in Step 3 to a sterile reagent reservoir. Immediately, using a multichannel pipette, dispense 60µl of cell suspension to each of the inner 60-wells of the plates containing preplated FcγRIIb CHO-K1 Cells.
6. Add 80µl of assay buffer 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.

### 4.E. Preparing Antibody Serial Dilutions and Setting Up Assay

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 4X the highest antibody concentration in your dose-response curve. You will need 180µl of each test antibody at 4X 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-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, warm the assay buffer (10ml aliquot, described in Section 4.C, Step 1) in a 37°C water bath for 15 minutes.
2. To a sterile, clear V-bottom 96-well plate, add 180µl of reference antibody starting dilution (dilu1, 4X final concentration) to wells A11 and B11 (see Figure 8).
3. Add 180µl of test antibodies 1 and 2 starting dilution (dilu1, 4X 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. Remove the preplated FcγRIIb CHO-K1 and OX40 Effector Cells from the incubator. Using an electronic multichannel pipette, add 20µl of the appropriate antibody titration to the assay plates according to the plate layout in Figure 7.
8. Cover the plate with a lid and incubate in a 37°C, 5% $CO_2$ incubator for 5 hours.

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

### 4.E. Preparing Antibody Serial Dilutions and Setting Up Assay (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 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.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 80µl of Bio-Glo™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
3. Add 80µl of Bio-Glo™ Reagent to wells B1, C1 and D1 of each assay plate to measure the background signal.
4. Incubate at ambient temperature for 5–15 minutes.

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

### 4.G. Data Analysis

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

## 5. Assay Protocol for Ligand or FcγRIIb-Independent Antibodies

This assay protocol illustrates the use of the OX40 Bioassay to test two antibody samples against a reference sample in a single assay run. Each test and reference antibody is run in triplicate, in a 10-point dilution series, in a single 96-well assay plate using the inner 60 wells. Other experimental and plate layouts are possible but may require further optimization.

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

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

1. **Assay Buffer:** On the day before the assay, prepare 36ml of assay buffer (95% RPMI 1640/5% FBS) in a 50ml conical tube. Thaw the FBS overnight at 4°C, or in a 37°C water bath on the day of use. Add 1.8ml of FBS to 34.2ml of RPMI 1640 medium. Mix well and warm to 37°C before use. For reference, 36ml 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 OX40 ligand 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. When stored at ambient temperature, the reconstituted Bio-Glo™ Reagent will lose ~18% signal after 24 hours.

3. **Test and Reference Samples:** Using assay buffer as the diluent, prepare starting dilutions (dilu1, 4X final concentration) of two test antibodies (180µl each) and one reference ligand (400µl) in 1.5ml tubes. Store the tubes containing ligand or antibody starting dilutions appropriately before making antibody serial dilutions.

   **Note:** If you are using OX40 ligand (OX40L, Biolegend Cat.# 555704) as a reference ligand, prepare a 400µl starting dilution with 4µg/ml OX40L (dilu1, 4X final concentration) by adding 8µl of OX40L stock (200µg/ml) to 392µl of assay buffer. The final (1X) starting concentration is 1µg/ml OX40L. Store the ligand starting dilution on ice until ready to use in the assay.

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

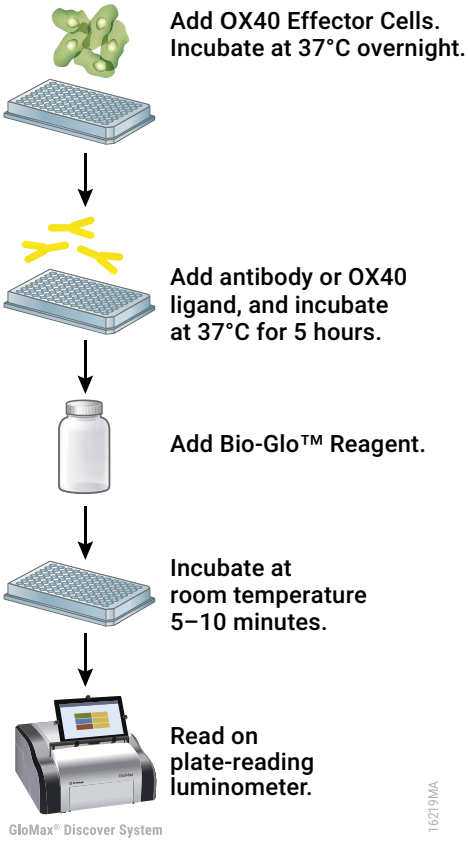


**Figure 9. Schematic protocol for OX40 Bioassay with OX40 ligand or FcγRIIb-independent antibody.**

### 5.B. Plate Layout Design

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

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

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

### 5.C. Preparing and Plating OX40 Effector Cells

**Note:** The thaw-and-use OX40 Effector Cells included in this kit are sensitive, and care should be taken to follow the cell thawing and plating procedures exactly as described. Do not overmix or overwarm the cell reagents. No additional cell culture or cell manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at one time.

1. On the day before the assay, prepare 36ml of assay buffer (95% RPMI 1640/5% FBS) as described in Section 5.A. Divide the assay buffer into two aliquots, one for OX40 Effector Cells preparation (26ml) and the other for antibody serial dilution preparation (10ml). Store the 10ml aliquot at 4°C for later use.
2. Warm the assay buffer (26ml aliquot) in a 37°C water bath for 15 minutes and transfer 11.5ml to a 15ml conical tube.
3. Remove one vial of thaw-and-use OX40 Effector Cells from –140°C storage and transfer to the bench on dry ice. Thaw the vial in a 37°C water bath until cells are just thawed (about 2 minutes). While thawing, gently agitate and visually inspect.
4. Gently mix the cell suspension in the vial by pipetting, then transfer 0.5ml of cells to the 15ml conical tube containing 11.5ml of assay buffer. Mix well by gently inverting 1–2 times.
5. Transfer the OX40 Effector Cell suspension to a sterile reagent reservoir. Immediately, using a multichannel pipette, dispense 60µl of cell suspension to each well of the inner 60 wells of two 96-well, white, flat-bottom assay plates.
6. Add 80µl of assay buffer 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.

### 5.D. Preparing Ligand or Antibody Serial Dilutions and Setting Up Assay

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 4X 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 4X 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 in Figure 11.

1. On the day of the assay, warm the 10ml assay buffer aliquot prepared in Section 5.C., Step 1.
2. To a sterile, clear V-bottom 96-well plate, add 180µl of reference ligand starting dilution (dilu1, 4X final concentration) to wells A11 and B11 (see Figure 11).
3. Add 180µl of test antibodies 1 and 2 starting dilution (dilu1, 4X 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. Remove the preplated OX40 Effector Cells from the incubator. Using an electronic multichannel pipette, add 20µl of the OX40L or appropriate antibody titration to the assay plates according to the plate layout in Figure 10.
8. Cover the plate with a lid and incubate in a 37°C, 5% $CO_2$ incubator for 5 hours.

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

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| 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 reference ligand and antibody serial dilutions.**

### 5.E. Adding Bio-Glo™ Reagent

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

1. Remove the assay plates from the incubator and equilibrate to ambient temperature for 10–15 minutes.
2. Using a manual multichannel pipette, add 80µl of Bio-Glo™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
3. Add 80µl of Bio-Glo™ Reagent to wells B1, C1 and D1 of each assay plate to measure the background signal.
4. Incubate at ambient temperature for 5–15 minutes.

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

### 5.F. Data Analysis

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

## 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 FcγRIIb CHO-K1 Cells since your Ab of your interest may depend on crosslinking by FcγRIIb. In the case of ligands, crosslinking by an antibody may be necessary. |
| Variability in assay performance | Inappropriate cell handling during cell thawing, including long water bath incubation times, can cause low assay performance and high assay variation. |
| Weak assay response (low fold induction) | Low activity of Bio-Glo™ Reagent leads to low RLU. Store and handle the Bio-Glo™ Reagent according to the instructions. |
| | Optimize the concentration range of your test sample(s) to achieve a full dose response with complete upper and lower asymptotes. The $EC_{50}$ value obtained in the OX40 Bioassay may vary from the $EC_{50}$ value obtained using other methods such as primary T cell-based assays. |
| | Determine if the antibody used is dependent on crosslinking by testing in the presence of FcγRIIb Cells. |
| | If untreated control RLU is less than 100-fold above plate reader background RLU, subtract plate background RLU from all samples before calculating fold induction. |

## 7. References


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## 8. Appendix

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

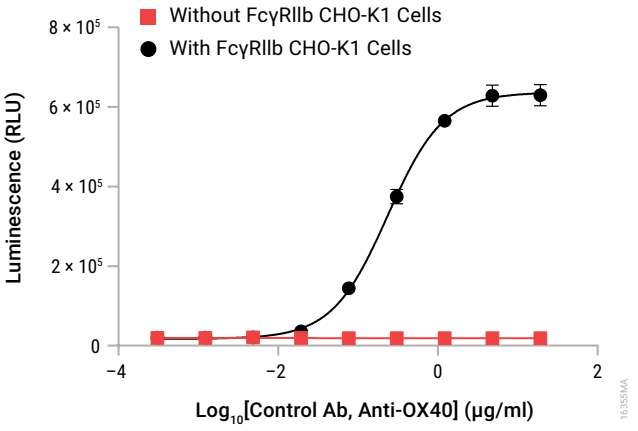


**Figure 12. The OX40 Bioassay measures the activity of Control Antibody, Anti-OX40.** Thaw-and-use FcγRIIb CHO-K1 Cells (Cat.# JA2251, JA2255) and OX40 Effector Cells (Cat.# JA2191, JA2195) were thawed and plated as instructed, and incubated overnight. The following day, a titration of Control Ab, Anti-OX40, (Cat.# K1191; Section 4.C) was added. 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 ~0.24µg/ml, and the fold induction was ~30.

### 8.B. Representative Assay Results with OX40 Ligand

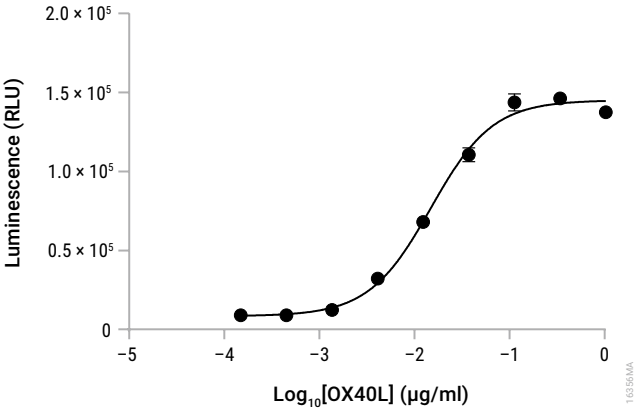


**Figure 13. The OX40 Bioassay measures the activity of OX40 Ligand.** Thaw-and-use OX40 Effector Cells were plated overnight. The following day, cells were incubated with various concentrations of OX40 ligand (Section 5.C). After 5 hours of induction at 37°C, Bio-Glo™ Reagent was added and luminescence was determined using a GloMax® Discover System. Four-parameter logistic curve analysis was performed with GraphPad Prism® software. The $EC_{50}$ response determined was ~0.015µg/ml, and the fold induction was ~15.2.

## 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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[d]Patent Pending.

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