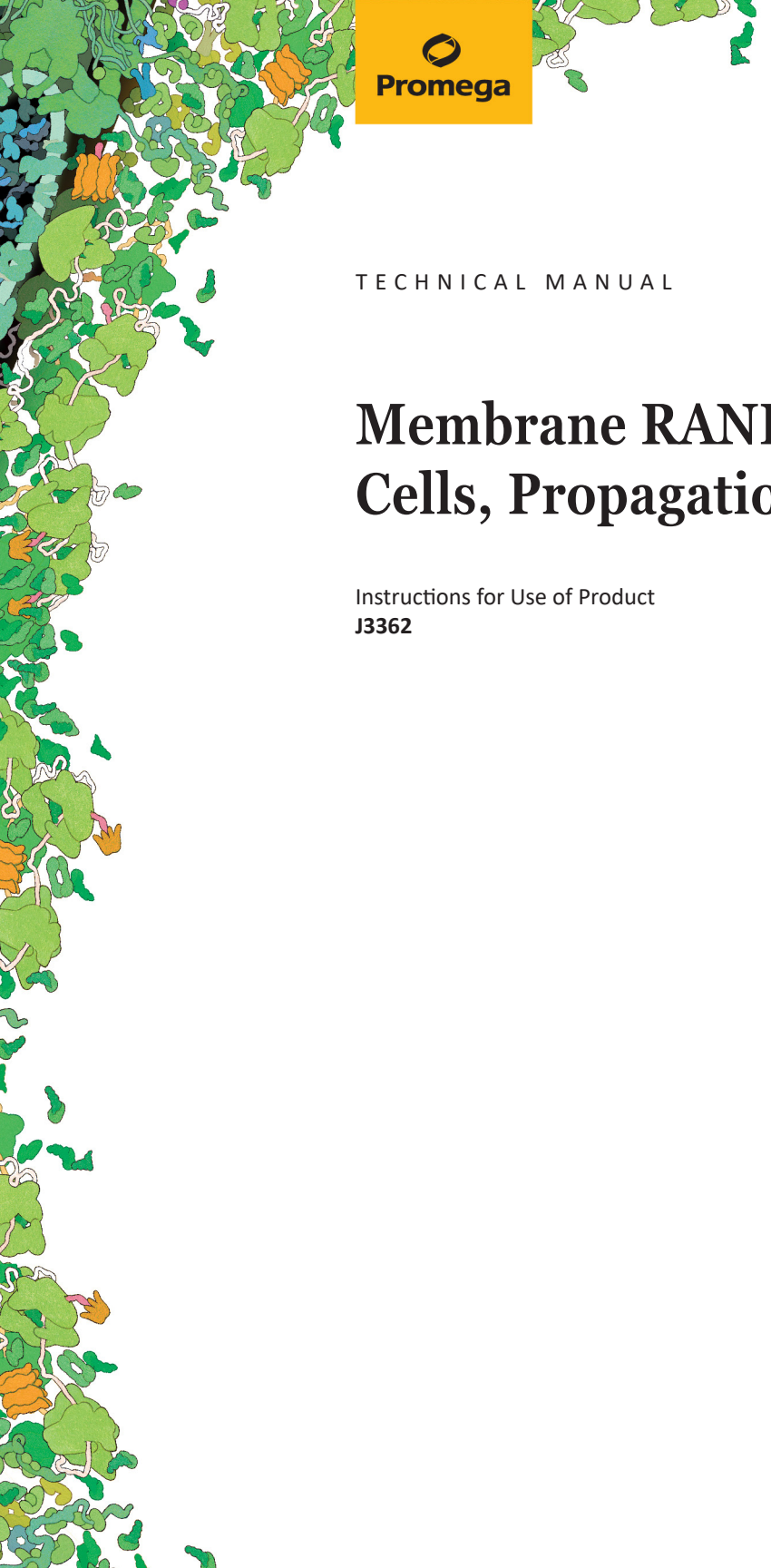

TECHNICAL MANUAL

Membrane RANKL Target Cells, Propagation Model

Instructions for Use of Product
J3362

11/21
TM671

Membrane RANKL Target Cells, Propagation Model

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

1. Description

Receptor activator of nuclear factor κ B (RANK/ TRANCE receptor/ TNFRSF11A) is a member of the tumor necrosis factor receptor (TNFR) family. Binding of its ligand RANKL to the receptor regulates osteoclast formation, activation and survival in bone modeling and remodeling, and several other pathologic conditions characterized by increased bone turnover (1).

The osteoclastogenesis signaling pathway is activated by osteoblasts producing RANKL, which binds to and activates the RANK receptor on osteoclast precursors. The adapter protein TRAF6 is recruited to the RANK receptor and activates NF-κB, which leads to its translocation to the nucleus. This increases the expression of c-FOS, which, together with NFATc1, increases the transcription of osteoclastogenic genes (2). Osteoprotegerin (OPG) binds to and inhibits RANKL. However, in cases of excess RANKL or insufficient OPG, excessive RANKL/RANK signaling leads to superfluous osteoclast formation and bone resorption and causes pathologic bone loss and destruction (3). In cases of osteosarcoma, in addition to cancer-induced bone destruction, RANKL has also been shown to be involved in tumorigenesis and metastasis. RANKL inhibition was shown to significantly delay mammary tumor formation in carcinogen and hormone-induced breast cancer mouse models (4,5). Denosumab is a fully human monoclonal antibody (IgG2) that binds RANKL and blocks RANK-RANKL interaction, which inhibits osteoclast formation, function and survival, thereby decreasing bone resorption and interrupting cancer-induced bone destruction (6).

RANKL is initially produced as a type II transmembrane protein (mRANKL), which is cleaved by proteases, resulting in soluble RANKL (7). Binding of RANKL-targeted antibodies to mRANKL-expressing cells may induce effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) to destroy the mRANKL-expressing cells (8). However, determining the ability of novel and biosimilar RANKL antibodies to induce cellular cytotoxicity is hampered by the lack of model cell lines naturally expressing mRANKL.

The Membrane RANKL Target Cells, Propagation Model[a,b] (Cat.# J3362) is a genetically engineered cell line stably expressing a cleavage-resistant form of mRANKL that enforces its surface expression. mRANKL Target Cells are provided in cell propagation model (CPM) format, which includes cryopreserved cells that can be thawed, propagated and banked for long-term use. They are designed to be used as target cells in assays that measure effector functions, such as ADCC and CDC, of anti-RANKL blockers. In addition, they also can be used to measure antibody binding affinity to mRANKL.

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mRANKL Target Cells express RANKL on the cell surface, as demonstrated by flow cytometry (Figure 1). Characterization and representative data were generated using a research-grade denosumab variant containing a human IgG1 Fc domain (denosumab-IgG1). In an ADCC Reporter Bioassay, ADCC activity is detected using denosumab-IgG1, but not with denosumab (IgG2 isotype; Figure 2). Furthermore, CDC function of denosumab-IgG1 is robust while CDC activity of denosumab (IgG2) is significantly reduced (Figure 2). The assay signal is specific to anti-RANKL antibodies in both ADCC and CDC assays. Using the ADCC Reporter Bioassay (Cat.# G7010), luminescence increases after adding anti-RANKL antibodies but not after adding anti-TNFα or anti-VEGF antibodies (Figure 3). In a CDC assay, mRANKL Target Cell death is detected following the addition of anti-RANKL antibodies but not after adding anti-TNFα or anti-CD20 antibodies (Figure 4). The ADCC Reporter Bioassay using mRANKL Target Cells is prequalified following International Council for Homogenization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines and shows the precision, accuracy and linearity required for routine use in potency and stability studies (Table 1 and Figure 5). The bioassay can be performed in a two-day timeframe, and the workflow is simple, robust and compatible with both 96- and 384-well plate formats used for antibody screening in early drug discovery (Figure 6).

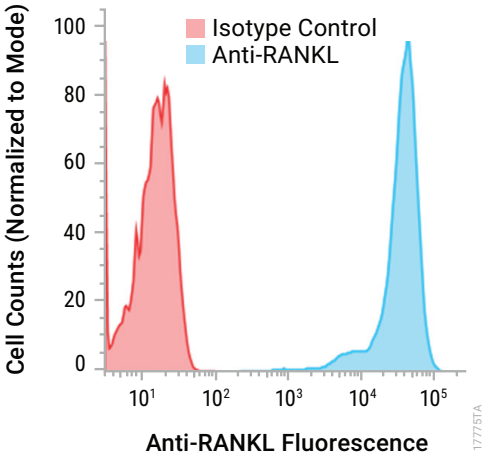

Figure 1. Surface expression of RANKL on mRANKL Target Cells. mRANKL Target Cells were labeled with PE-conjugated anti-RANKL or PE-conjugated isotype control antibodies. Cells were analyzed on a BD LSRFortessa™ X-20 flow cytometer. Data analysis was performed with FlowJo™ software.

1. Description (continued)

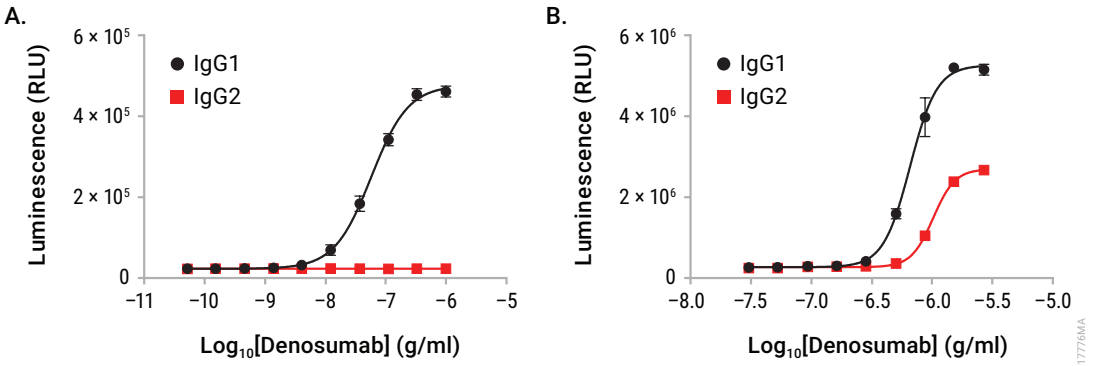

Figure 2. The ADCC Reporter Bioassay and CDC assay, performed with mRANKL Target Cells, reflects functional Fc activity of denosumab-IgG1 but not therapeutic denosumab (IgG2). Panel A. ADCC Effector Cells were cocultured with mRANKL Target Cells in the presence of serial titrations of antibodies, as indicated. After a 6-hour induction, Bio-Glo™ Reagent was added, and luminescence 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. **Panel B.** mRANKL Target Cells were incubated with 10% normal human serum complement and serial titrations of antibodies, as indicated. After a 6-hour incubation, CytoTox-Glo™ Reagent was added and luminescence 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.

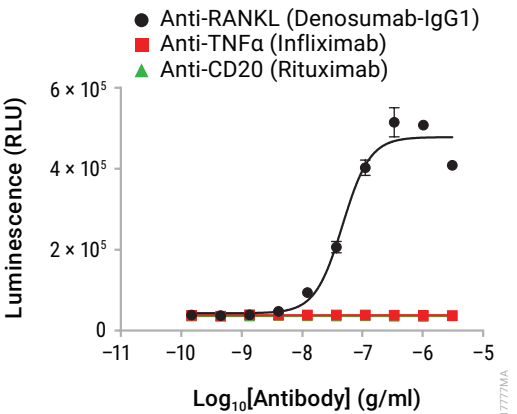

Figure 3. The ADCC Reporter Bioassay with mRANKL Target Cells reflects the mechanism of action (MOA) and shows specificity for antibodies that bind RANKL. ADCC Effector Cells were cocultured with mRANKL Target Cells in the presence of serial titrations of antibodies, as indicated. After a 6-hour induction, Bio-Glo™ Reagent was added and luminescence 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.

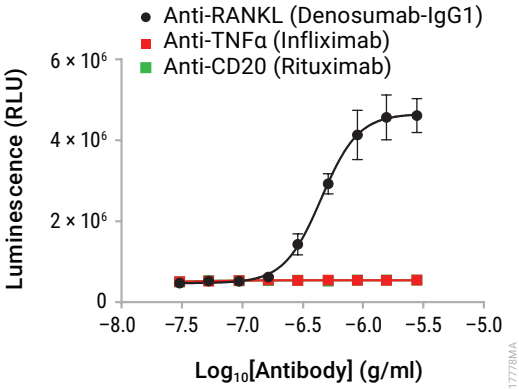

Figure 4. CDC assay with mRANKL Target Cells reflects the MOA and shows specificity for antibodies designed to bind RANKL. mRANKL Target Cells were incubated with 10% normal human serum complement in the presence of serial titrations of antibodies, as indicated. After a 6-hour incubation, CytoTox-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

Table 1. ADCC Reporter Bioassay using mRANKL Target Cells Shows Precision, Accuracy and Linearity.

Parameter	**Results**	
Accuracy	% Expected Relative Potency	% Recovery
	50	46.0
	70	68.9
	100	100.3
	150	144.9
	200	214.7
Repeatability (% CV)	100% (Reference)	10.4
Intermediate Precision (% CV)		8.3
Linearity (r^2)		0.9928
Linearity (y = mx + b)		y = 1.093x – 9.838
A 50–200% simulated potency series of denosumab-IgG1 was analyzed in triplicate in three independent experiments performed on three days by two analysts using mRANKL Target Cells in the ADCC Reporter Bioassay. Bio-Glo™ Reagent was added, and luminescence quantified using the GloMax® Discover System. Data were analyzed and relative potencies calculated after parallelism determination using JMP® software. Data were generated using thaw-and-use cells.		

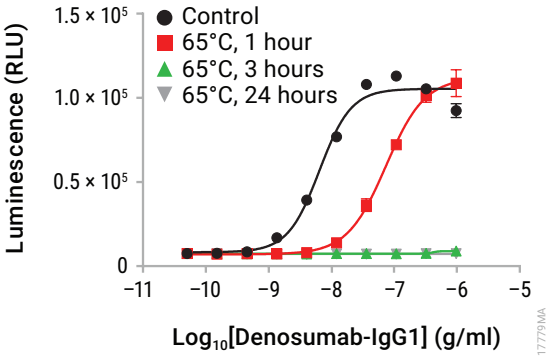

Figure 5. The ADCC Reporter Bioassay with mRANKL Target Cells is stability-indicating. Samples of anti-RANKL (denosumab-IgG1) were maintained at 4°C (control) or heat-treated at the indicated times and temperatures, then analyzed using the ADCC Reporter Bioassay with mRANKL Target Cells. Bio-Glo™ Reagent was added and luminescence 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.

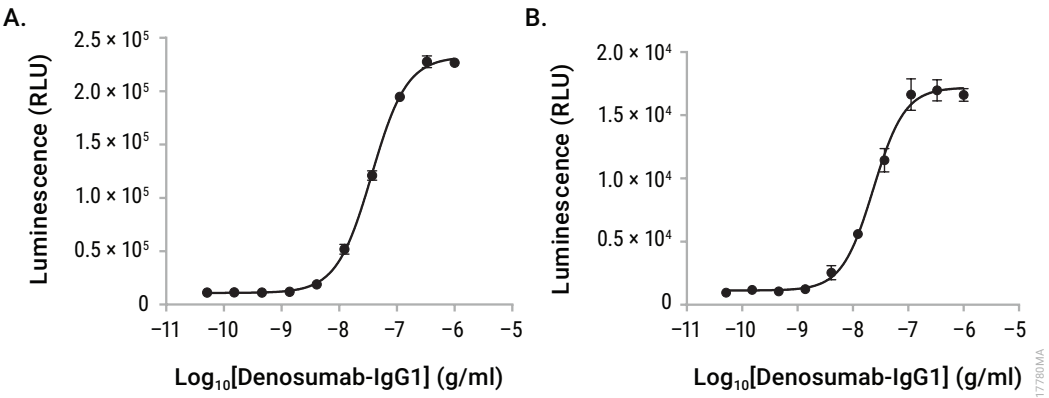

Figure 6. The ADCC Reporter Bioassay with mRANKL Target Cells is amenable to 384-well plate format. Panel A. The ADCC Reporter Bioassay with mRANKL Target Cells was performed in 96-well plates as described in this technical manual with a titration of anti-RANKL (denosumab-IgG1). **Panel B.** The ADCC Reporter Bioassay with mRANKL Target Cells was performed in 384-well plates as briefly described here. Thaw-and-use mRANKL Target Cells were thawed and plated at 2×10^3/15µl/well 20 hours prior to the assay, in a 384-well white assay plate. On the day of the assay, 5µl of a threefold serial dilution of 5X concentrated denosumab-IgG1 was added to the wells, followed by 1.5×10^4/5µl/well of ADCC Effector Cells. After a 6-hour induction at 37°C, 5% CO_2, 25µl of Bio-Glo™ Reagent was added per well and luminescence quantified using the GloMax® Discover System. Data were fitted to four-parameter logistic curves using GraphPad Prism® software. The EC_{50} values were 36.8 and 23.5ng/ml for 96- and 384-well plates, respectively. The fold induction was 20.6 and 18.0 for 96- and 384-well plates, respectively. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT. #
Membrane RANKL Target Cells, Propagation Model	**1 each**	**J3362**

Not for Medical Diagnostic Use. Includes:

- 2 vials Membrane RANKL Target Cells (CPM), 4×10^6 cells/ml (1.0ml per vial)

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.

Remove the product label from the box containing vials with cells or note the catalog number and lot number from the label. This information can be used to download documents for the specified product from the web site, such as the Certificate of Analysis.

Note: mRANKL Target Cells are intended for use with user-provided antibodies or other biologics designed to bind to RANKL. Data generated using denosumab-IgG1 are shown in Section 9.A, Representative Assay Results.

To measure ADCC activity, mRANKL Target Cells can be used in conjunction with the thaw-and-use cells in the ADCC Reporter Bioassay (Cat.# G7010, G7018) or ADCC Reporter Bioassay, Propagation Model (Cat.# G7102) to detect ADCC function of anti-RANKL antibodies. To measure CDC activity, we recommend using CytoTox-Glo™ Cytotoxicity Assay (Cat.# G9290) with normal human serum complement.

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 denosumab-IgG1 and ADCC Effector Cells (ADCC Reporter Bioassay) or in a CDC assay using CytoTox-Glo™ Reagent and complement-preserved human serum. You may need to adjust the parameters provided here and optimize assay conditions for your own assay readout and antibodies.

The ADCC Reporter Bioassay and the CDC assay with CytoTox-Glo™ Reagent produce a bioluminescent signal and require a sensitive luminescence plate reader. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System (see Section 9.C, Related Products). An integration time of 0.5 second/well was used for all readings. These bioassays are compatible with most other plate-reading luminometers, though relative luminescence unit (RLU) readings will vary with the sensitivity and settings of each instrument. If your luminometer or plate reader requires gain adjustment for luminescence, use the well with the highest antibody concentration.

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3.A. Materials to Be Supplied by the User

Reagents

- user-defined anti-RANKL antibodies or other biologics samples (e.g., denosumab-IgG1, InvivoGen Cat.# hrankl-mab1)
- Ham's F-12 Medium with L-glutamine (e.g., GIBCO™ Cat.# 11765062)
- fetal bovine serum (FBS; e.g., GIBCO™ Cat.# 35-015-CV or HyClone Cat.# SH30071.03)
- blasticidin (e.g., Invitrogen Cat.# A11139-03)
- DMSO (e.g., Sigma Cat.# D2650)
- Accutase® solution (e.g., Sigma Cat.# A6964)
- DPBS (e.g., GIBCO™ Cat.# 14190)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- **optional:** ADCC Bioassay Effector Cells, Propagation Model (Cat.# G7102)
- **optional:** super low IgG FBS (e.g., HyClone Cat.# SH30898; for ADCC Bioassay)
- **optional:** Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941; for ADCC Bioassay)
- **optional:** RPMI 1640 with L-glutamine and HEPES (GIBCO™ Cat.# 22400; for ADCC Bioassay)
- **optional:** normal human serum complement (Quidel Cat.# A112, A113; for CDC assay)
- **optional:** CytoTox-Glo™ Cytotoxicity Assay (Cat.# G9290; for CDC assay)

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) for preparing antibody dilutions
- sterile dilution reservoirs with lid (e.g., Dilux Cat.# D-1002) for higher volume 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
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System or equivalent instrument for ADCC Reporter Bioassay and CDC assay using the CytoTox-Glo™ Cytotoxicity Assay)

4. Preparing mRANKL Target Cells

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

4.A. Cell Thawing and Initial Cell Culture

1. Prepare 50ml of initial cell culture medium by adding 5ml of FBS to 45ml of Ham's F12 medium prewarmed to 37°C. This initial cell culture medium will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of prewarmed initial cell culture medium to a 50ml conical tube.
3. Remove one vial of mRANKL Target 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 150 × *g* for 10 minutes.
6. Carefully aspirate the medium and resuspend the cell pellet in 40ml of prewarmed initial cell culture medium.
7. Transfer the cell suspension to a T150 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

For cell maintenance and propagation starting from the second cell passage, use the cell growth medium containing antibiotics and monitor cell viability and doubling rate during propagation. The cell growth rate will stabilize by 7–10 days post-thaw, at which time cell viability is typically >95%, and the average cell doubling rate is ~24 hours. Passage number should be recorded for each passage. In our experience, cells maintain their functionality for up to 20 passages if passaging is performed on a Monday-Wednesday-Friday schedule.

1. On the day of cell passage, aspirate the cell culture medium and wash the cells with DPBS.
2. Add 2ml of Accutase® solution to each T75 flask (or 4ml of Accutase® for T150) and place in a 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.
3. Add 8ml of cell growth medium to each T75 flask (or 16ml for T150). Transfer the cell suspension to a sterile 15ml or 50ml conical tube.
4. Count the cells by Trypan blue staining. We suggest seeding the cells at a density of 4×10^4 cells/cm^2 if passaging every two days (e.g., Monday-Wednesday or Wednesday-Friday) or 2×10^4 cells/cm^2 if passaging every three days (e.g., Friday-Monday)
5. Add an appropriate amount of cell growth medium to a new flask.
6. Transfer the appropriate volume of cell suspension to achieve the desired cell seeding density per area.
7. Place the flasks horizontally in a humidified 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. Aspirate the cell culture medium and wash the cells with DPBS.
3. Add 2ml of Accutase® solution to each T75 flask and place in a 37°C, 5% CO_2 incubator for 5–7 minutes or until the cells round up and detach from the bottom of the flask.
4. Add 8ml of cell growth medium to each T75 flask. Transfer the cell suspension to a sterile 15ml or 50ml conical tube.
5. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of cell freezing medium needed based on desired cell freezing densities of 3×10^6–1.2×10^7 cells/ml.
6. Transfer the cell suspension to 50ml sterile conical tubes or larger-sized centrifuge tubes, and centrifuge at $150 \times g$, 4°C, for 10 minutes.
7. Carefully aspirate the supernatant and avoid disturbing the cell pellet.
8. Gently resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of 3×10^6–1.2×10^7 cells/ml. Combine the cell suspensions into a single tube and dispense into cryovials.
9. Freeze the cells using a controlled-rate freezer (preferred), or a Mr. Frosty® or a Styrofoam® rack in a –80°C freezer overnight. Transfer the vials to –140°C or below for long-term storage.

5. Assay Protocol for ADCC Reporter Bioassay with mRANKL Target Cells

This assay protocol requires two engineered cell lines: ADCC Bioassay Effector Cells, Propagation Model, and Membrane RANKL Target Cells, Propagation Model. Both cell lines are also available in thaw-and-use format (ADCC, Cat.# G7010; mRANKL, Cat.# J3381).

Note: Refer to the *ADCC Bioassay Effector Cells, Propagation Model Technical Manual* #TM385, for cell handling instructions for ADCC Bioassay Effector Cells.

The procedure below illustrates the use of the mRANKL Target Cells in the ADCC Reporter Bioassay to test two anti-RANKL antibody samples against a reference sample, in a single assay run using the mRANKL Target Cells, Propagation Model. Each test and reference antibody is run in triplicate, in a 10-point dilution series, in a single 96-well assay plate using the inner 60 wells. Other experimental and plate layouts are possible but may require further optimization.

Note: When preparing test and reference antibodies, choose an appropriate starting concentration and dilution scheme to achieve a complete dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 3µg/ml as a starting concentration (1X) and threefold serial dilution when testing denosumab-IgG1.

5.A. Preparing ADCC Assay Reagents

1. **mRANKL Target Cell Plating Medium:** On the day before the assay, prepare an appropriate amount of cell plating medium (90% Ham's F-12/10% 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 cell plating medium is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

2. **ADCC Assay Buffer:** On the day of the assay, prepare an appropriate amount of ADCC assay buffer (96% RPMI 1640/4% super low IgG FBS). Mix well and warm to 37°C before use. For reference, 30ml of ADCC 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 4% super low IgG FBS. This concentration and type of FBS works well for the anti-RANKL antibodies that we tested. If you experience assay performance issues when using ADCC assay buffer, we recommend testing different serum concentrations and types, in the range of 0.5–10%.

3. **Bio-Glo™ Reagent:** For reference, 10ml of Bio-Glo™ Reagent is sufficient to assay 120 wells in a 96-well 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 six weeks. Avoid repeated freeze-thaw cycles. On the day of the assay, thaw an appropriate amount of reconstituted Bio-Glo™ Reagent in a room temperature water bath for at least 1–2 hours before use. Approximate stability of Bio-Glo™ Reagent after reconstitution is an 18% loss of luminescence after 24 hours at ambient temperature.

   Note: The ADCC Reporter Bioassay is compatible only with the Bio-Glo™ Luciferase Assay System. **Do not** use the Bio-Glo-NL™ Luciferase Assay System with the ADCC Reporter Bioassay.

4. **Test and Reference Samples:** Using ADCC assay buffer as the diluent, prepare starting dilutions (dilu1, 3X final concentration) of two test antibodies (200µl 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 denosumab-IgG1 (100µg/ml stock) as a reference antibody in your assay, prepare a 400µl starting dilution of 9µg/ml of denosumab-IgG1 (dilu1, 3X final concentration) by adding 36µl of denosumab-IgG1 stock to 364µl of ADCC assay buffer.

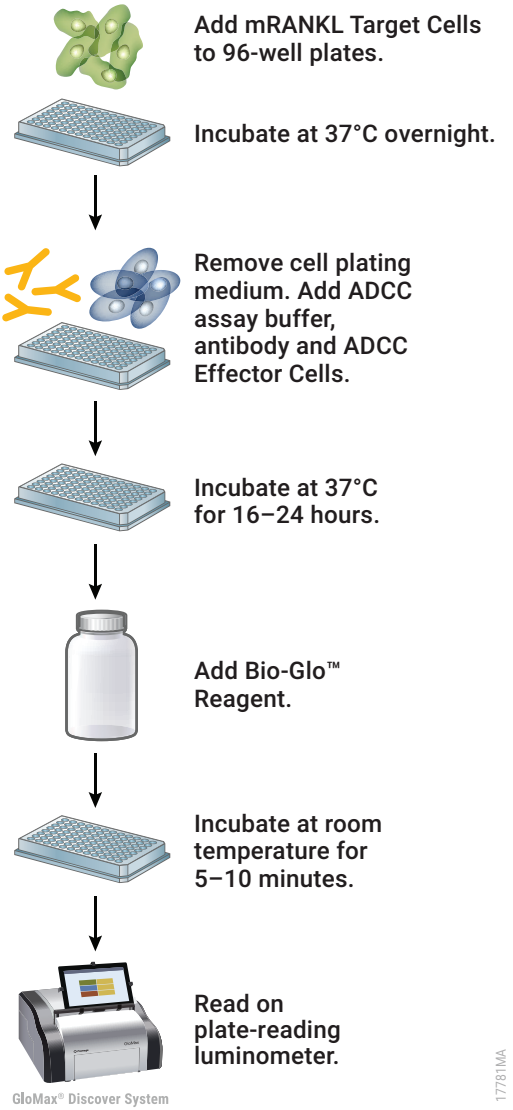

Figure 7. Schematic protocol for the ADCC Reporter Bioassay with mRANKL Target Cells, Propagation Model.

5.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 8 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibody to generate two 10-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 8. Example plate layout showing non-clustered sample locations of test antibody and reference antibody dilution series and wells containing ADCC assay buffer ("B") alone.

5.C. Preparing and Plating mRANKL Target Cells

While maintaining the mRANKL Target Cells, follow the recommended cell seeding density (refer to Section 4 for culture instructions). 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 mRANKL Target Cells two days before plating for the assay (as described in Section 4) to ensure optimal and consistent assay performance.
2. On the day before performing the assay, prepare mRANKL Target Cell plating medium (Ham's F-12/10% FBS).
3. Aspirate the cell culture medium from the mRANKL Target Cells and wash with DPBS.
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 mRANKL Target Cell plating medium to the flask. Transfer the cell suspension to a 50ml (or larger) conical centrifuge tube.
6. Gently mix and count the mRANKL Target Cells by Trypan blue staining.
7. Centrifuge at 150 × *g* for 10 minutes.
8. Gently resuspend the cell pellet in cell plating medium to achieve a concentration of 1×10^5 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 should be 1×10^4 cells/well.
10. Add 100µl of mRANKL Target 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 preparing a single stock of threefold serial dilutions of a single antibody for analysis in triplicate (100µl of each 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 a total of 400µl of reference antibody at 3X the highest antibody concentration in your dose-response curve. You will need 200µl 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: The instructions below use denosumab-IgG1; follow these instructions to prepare threefold serial dilutions. A threefold serial dilution for test antibodies is also listed as an example below.

1. On the day of the assay, prepare ADCC assay buffer as described in Section 5.A.
2. To a sterile clear V-bottom 96-well plate, add 200µl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 9).
3. Add 200µl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and G11, respectively (see Figure 9).
4. Add 120µl of ADCC 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.

   Note: Wells A2, B2, E2 and G2 contain 120µl of ADCC assay buffer without antibody as a negative control.
7. Cover the antibody dilution plate with a lid and keep at ambient temperature (22–25°C) while preparing the ADCC Effector Cells.

Recommended Plate Layout for Antibody Dilutions Prepared from a Single Antibody Stock.													
	1	2	3	4	5	6	7	8	9	10	11	12	
A		no 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 9. Example plate layout showing antibody serial dilutions.

5.E. Preparing ADCC Effector Cells

While maintaining the ADCC 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 and cell viability is greater than 95%.

1. Passage the cells 2 days before performing the assay.
2. Count the ADCC Effector Cells by Trypan blue staining and calculate the cell density and viability.
3. Transfer an appropriate amount of ADCC Effector Cells from the culture vessel to a 50ml conical or larger-sized centrifuge tube.
4. Pellet the cells at 130 × *g* for 10 minutes at ambient temperature and resuspend the pellet in ADCC assay buffer at 70% of the full volume needed to generate the targeted final cell density of 3×10^6 cells/ml, giving a final density of 7.5×10^4 cells/25µl/well.
5. Count the cells again and adjust the volume of ADCC assay buffer to achieve a final cell density of 3×10^6 cells/ml. You will need at least 3ml of ADCC Effector Cells to fill 120 assay wells, or the inner 60 wells of two assay plates.

5.F. Adding ADCC Assay Reagents and Cells to Plates

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

1. Remove the 96-well assay plates containing mRANKL Target Cells from the incubator. Invert the assay plate to remove the medium. Then, place the inverted plate on paper towels for 5–10 seconds to drain any remaining medium. Alternatively, remove 95µl of medium from each of the wells using a manual multichannel pipette.
2. Using a multichannel pipette, add 25µl of ADCC assay buffer to the inner 60 wells of both 96-well assay plates.
3. Using a multichannel pipette, add 25µl of the appropriate antibody dilution (Figure 9) to the assay plates according to the plate layout in Figure 8.
4. Mix the ADCC Effector Cells by inverting the tube, then transfer the suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 25µl of the cell suspension to each of the inner 60 wells of the assay plates. Gently swirl the assay plates to ensure mixing of the ADCC Effector Cells and antibody.
5. Add 75µl ADCC assay buffer to each of the outside wells of the assay plates.
6. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 16–24 hours.

   Note: The 16–24 hour assay time was optimized using denosumab-IgG1. We recommend optimizing assay time (5–24 hours) with your own antibody or other biologic samples.

5.G. Adding Bio-Glo™ Reagent

Note: The ADCC Reporter Bioassay is compatible only with the Bio-Glo™ Luciferase Assay System. **Do not** use the Bio-Glo-NL™ Luciferase Assay System with the ADCC Reporter Bioassay.

Bio-Glo™ Reagent should be at ambient temperature when added to assay plates.

1. Remove assay plates from the 37°C incubator and equilibrate to ambient temperature (22°C–25°C) on the bench for 15 minutes.
2. Add 75µl of Bio-Glo™ Reagent to the inner 60 wells of both assay plates using a manual multichannel pipette. Avoid creating any bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, D1 and F1 in each assay plate to determine plate background.
4. Incubate at ambient temperature for 5–20 minutes.
5. Measure luminescence using a plate reader with glow-type luminescence reading capabilities.

5.H. Data Analysis

1. Determine the plate background by calculating the average RLU from wells B1, D1 and F1.
2. Calculate Fold Induction:

$$\text{Fold Induction} = \frac{\text{RLU (induced} - \text{background)}}{\text{RLU (no antibody control} - \text{background)}}$$

3. Graph data as RLU versus Log_{10}[antibody] and fold induction versus Log_{10}[antibody]. Fit curves and determine the EC_{50} value of antibody response using appropriate curve fitting software (such as GraphPad Prism® software).

6. Assay Protocol for CDC Assay using mRANKL Target Cells

The procedure below illustrates the use of the mRANKL Target Cells in a CDC assay to test two anti-RANKL antibody samples against a reference sample, in a single assay run using the Membrane RANKL Target Cells, Propagation Model format. Each test and reference antibody is run in triplicate, in a 10-point dilution series, in a single 96-well assay plate. Other experimental and plate layouts are possible but may require further optimization.

Note: When preparing test and reference antibodies, choose an appropriate starting concentration and dilution scheme to achieve a complete dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 2µg/ml as a starting concentration (1X) and 1.75-fold serial dilution when testing denosumab-IgG1.

6.A. Preparing CDC Assay Reagents

1. **mRANKL Target Cell Plating Medium/CDC Assay Buffer:** On the day before the assay, prepare an appropriate amount of cell plating medium/CDC assay buffer (90% Ham's F-12/10% 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, 50ml of cell plating medium/CDC assay buffer is typically sufficient for 132 wells in a 96-well assay format (Figure 11). After plating mRANKL Target Cells, remaining CDC assay buffer can be stored at 4°C overnight for use on the day of the assay.

2. **CytoTox-Glo™ Reagent:** Prepare an appropriate amount of CytoTox-Glo™ Reagent on the day of the assay. Thaw the CytoTox-Glo™ Assay Buffer in a room-temperature water bath, and equilibrate to ambient temperature, protected from light. Thirty minutes prior to the end of assay time, transfer 5ml of buffer into one amber bottle containing the AAF-Glo™ Substrate and mix by inversion, until the substrate is thoroughly dissolved. For reference, 10ml of CytoTox-Glo™ Reagent is sufficient for 132 assay wells in a 96-well assay plate.

   For optimal results, use freshly prepared CytoTox-Glo™ Reagent. Use within 12 hours if stored at room temperature. CytoTox-Glo™ Reagent can be stored at 4°C for up to 7 days with no appreciable loss in performance. CytoTox-Glo™ Reagent can be stored in single-use aliquots for up to 4 months at −70°C. Freezing and thawing will damage the reagent and should be avoided.

3. **Normal Human Serum Complement:** Follow manufacturer's instructions for storage, preparation and handling.

   Note: The recommended assay conditions contain 10% normal human serum complement. This concentration and type of complement works well for the anti-RANKL antibodies we tested. If you experience assay performance issues when using normal human serum complement, we recommend testing concentrations in the range of 5–20%.

4. **Test and Reference Samples:** Using CDC assay buffer as the diluent, prepare stock starting dilutions (dilu1, 1.5X final concentration) of two test antibodies (400µl each) and one reference antibody (800µl) in 1.5ml tubes. Store the tubes containing antibody starting dilutions appropriately before making antibody serial dilutions.

   Note: If you are using denosumab-IgG1 (100µg/ml stock) as a reference antibody in your assay, prepare an 800µl starting dilution of 3µg/ml of denosumab-IgG1 (dilu1, 1.5X final concentration) by adding 24µl of denosumab-IgG1 stock to 776µl of CDC assay buffer.

6.A. Preparing CDC Assay Reagents (continued)

5. **Digitonin:** Prior to completion of assay, prepare 1mg/ml digitonin in CytoTox-Glo™ Assay Buffer from 20mg/ml stock provided in the CytoTox-Glo™ Cytotoxicity Assay (Cat.# G9290) as follows: Combine 5µl of digitonin stock with 95µl of CytoTox-Glo™ Assay Buffer for 1mg/ml digitonin (10X).

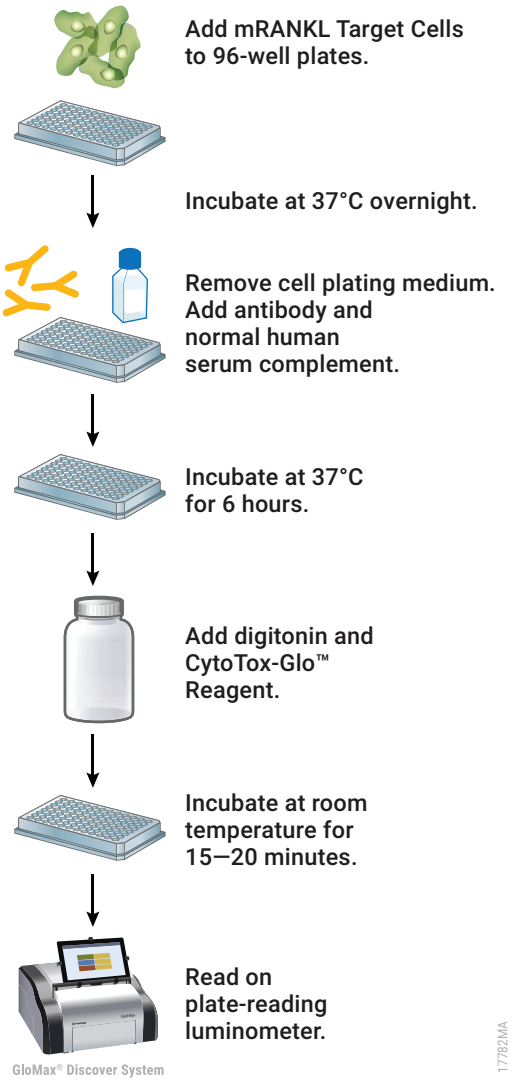

Figure 10. Schematic protocol for the CDC assay with Membrane RANKL Target Cells, Propagation Model.

6.B. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 11 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference antibody to generate two 10-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	D	Reference Ab
C	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Test Ab
D	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Reference Ab
E	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Test Ab
F	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Reference Ab
G	B	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	D	Test Ab
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 11. Example plate layout showing nonclustered sample locations of test antibody and reference antibody dilution series and wells containing digitonin (“D”) or CDC assay buffer (“B”).

6.C. Preparing and Plating mRANKL Target Cells

While maintaining the mRANKL Target Cells, follow the recommended cell seeding density (refer to Section 4 for culture instructions). 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 mRANKL Target Cells two days before plating for the assay (see Section 4) to ensure optimal and consistent assay performance.
2. On the day before the assay, prepare an appropriate amount of mRANKL Target Cell plating medium (90% Ham’s F-12/10% FBS).
3. Aspirate the cell culture medium from the mRANKL Target Cells and wash with DPBS.
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 mRANKL Target Cell plating medium to the flask. Transfer the cell suspension to a 50ml (or larger) conical centrifuge tube.
6. Gently mix and count the mRANKL Target Cells by Trypan blue staining.
7. Centrifuge at 150 × *g* for 10 minutes.
8. Gently resuspend the cell pellet in cell plating medium to achieve a concentration of 1×10^5 viable cells/ml.
9. Transfer the cell suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 100µl of the cell suspension to wells B2 through G12 of a 96-well white flat-bottom assay plate (Figure 11). The final cell number in each well should be 1×10^4 cells/well.
10. Add 100µl of cell plating medium to each of the empty wells of the assay plates (Figure 11).
11. Place lids on the assay plates and incubate in a 37°C, 5% CO_2 incubator overnight (18–24 hours).

6.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparation of single stocks of 1.75-fold serial dilutions of a single antibody for analysis in triplicate (200µl of each 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 1.75-fold serial dilutions, you will need 800µl of reference antibody at 1.5X the highest antibody concentration in your dose-response curve. You will need 400µl of each test antibody at 1.5X the highest antibody concentration in each of the test antibody dose-response curves. For other dilution schemes, adjust the volumes accordingly.

Note: The instructions below use denosumab-IgG1; follow the instructions below to prepare 1.75-fold serial dilutions. A 1.75-fold serial dilution for test antibodies is listed as an example below as well.

1. On the day of the assay, warm CDC assay buffer prepared the day before to 37°C. Otherwise, prepare an appropriate amount of CDC assay buffer as described in Section 6.A.
2. To a sterile 12-well reservoir labeled reference, add 800µl of appropriate reference antibody starting dilution (dilu1, 1.5X final concentration) to well 11 (Figure 12).
3. To two additional sterile 12-well reservoirs labeled test 1 and test 2, add 400µl of Test antibodies 1 and 2 starting dilution (dilu1, 1.5X final concentration) to well 11 (Figure 12).
4. For reference antibody, add 340µl of CDC assay buffer to other wells, 2 through 10 and 12 (well 1 is empty).
5. For test antibodies 1 and 2, add 170µl of CDC assay buffer to other wells, 2 through 10 and 12 (well 1 is empty).
6. Transfer 453µl of the reference antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
7. Repeat equivalent reference antibody 1.75-fold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.

   Note: Well 2 contains 340µl of CDC assay buffer without antibody as a negative control. Well 12 contains 340µl of CDC assay buffer without antibody and will be used for digitonin addition at end of the assay.
8. Transfer 227µl of the test 1 and 2 antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.

9. Repeat equivalent test 1 and 2 antibody 1.75-fold serial dilutions across the columns from right to left through column 3. Do not dilute into column 2.

   Note: Well 2 contains 170µl of CDC assay buffer without antibody, as a negative control. Well 12 contains 170µl of CDC assay buffer without antibody and will be used for digitonin addition at end of assay. Cover the antibody dilution reservoirs with lids and keep at ambient temperature (22–25°C) while preparing the normal human serum complement.

Recommended 12-well Reservoir Layouts for Antibody Dilutions Prepared from a Single Antibody Stock.

1	2	3	4	5	6	7	8	9	10	11	12	
	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	no Ab	Reference Ab

1	2	3	4	5	6	7	8	9	10	11	12	
	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	no Ab	Test Ab 1

1	2	3	4	5	6	7	8	9	10	11	12	
	no Ab	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	no Ab	Test Ab 2

Figure 12. Example 12-well reservoir layouts showing antibody serial dilutions.

6.E. Preparing Normal Human Serum Complement

Note: Normal human serum complement is heat labile and care must be taken to ensure it is fully intact when used in the assay. Follow manufacturer's instructions for storage and handling.

1. Dilute the normal human serum complement with CDC assay buffer, to achieve a 30% solution. Once diluted in the assay plate, this gives a 10% final concentration. You will need at least 4ml of 30% complement to fill 120 assay wells, or 60 wells of two assay plates.

6.F. Adding Antibody and Complement to CDC Assay Plates

1. Remove the 96-well assay plates containing mRANKL Target Cells from the incubator. Invert the assay plate to remove the medium. Then place the inverted plate on paper towels for 5–10 seconds to drain any remaining medium. Alternatively, remove 95µl of medium from each of the wells using a manual multichannel pipette.
2. Using a multichannel pipette, add 50µl of appropriate antibody dilution to the assay plates according to the plate layout in Figure 11.
3. Using a multichannel pipette, add 25µl of the 30% normal human serum complement to each of the inner 60 wells of the assay plates (see Figure 11).
4. Using a multichannel pipette, add 25µl of CDC assay buffer to wells B12–G12 (digitonin wells).
5. Add 75µl of CDC assay buffer to each of the empty outside wells of the assay plates.

6. Cover the assay plates with lids and incubate in a 37°C, 5% CO_2 incubator for 6 hours.

   Note: The 6-hour assay time was optimized using denosumab-IgG1. We recommend optimizing assay time (3–24 hours) with your own antibody or other biologic samples.

6.G. Adding Digitonin and CytoTox-Glo™ Reagent

1. During the 6-hour incubation time, reconstitute the CytoTox-Glo™ Reagent according to the instructions in Section 6.A.
2. At the end of the 6-hour incubation, remove the assay plates from the incubator and immediately add 8µl of 1mg/ml digitonin (Section 6.A) to wells B12–G12, per the plate layout in Figure 11.
3. Gently shake plate briefly to mix the digitonin in the well.
4. Allow plate to equilibrate to ambient temperature (22–25°C) for 15–20 minutes.
5. Add 40µl/well of CytoTox-Glo™ Reagent (ambient temperature) to all wells containing mRANKL Target Cells (wells B2–G12).
6. Add 40µl/well of CytoTox-Glo™ Reagent to wells B1, D1 and F1 to determine plate background.
7. Incubate at ambient temperature for 15 minutes.
8. Measure luminescence using a plate reader with glow-type luminescence reading capabilities.

6.H. Data Analysis

1. Determine the plate background by calculating the average RLU from wells B1, D1 and F1.
2. Determine the maximum killing by calculating the average RLU from wells B12–G12.
3. Calculate fold induction:

$$\text{Fold Induction} = \frac{\text{RLU (antibody} - \text{background)}}{\text{RLU (no antibody control} - \text{background)}}$$

4. Calculate percent specific lysis:

$$\text{Percent Specific Lysis} = \frac{\text{RLU (antibody} - \text{background)}}{\text{RLU (digitonin} - \text{background)}} \times 100$$

5. Graph data as RLU versus Log_{10}[antibody], fold induction versus Log_{10}[antibody] and percent specific lysis 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**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
Low luminescence measurements (RLU readout)	Choose a sensitive instrument designed for luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual numbers will vary between instruments.
	Some models of luminometers with low sensitivity should be avoided. If using a reader with an adjustable gain, we recommend a high gain setting.
	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 or CytoTox-Glo™ Reagent leads to low RLU. Store and handle the reagents according to the instructions.
Weak assay response (low fold induction)	Optimize the concentration range of your test sample(s) to achieve a complete dose response with complete upper and lower asymptotes. The EC_{50} values obtained in the ADCC Reporter Bioassay or CDC assay with mRANKL Target Cells may vary from the EC_{50} value obtained using other methods.
	Optimize the ADCC Reporter Bioassay incubation time within a range of 5–24 hours or the CDC assay within a range of 3–24 hours, and choose the incubation time that gives optimal response.
	Optimize the super low IgG FBS concentration from 0.5–10% in ADCC assay buffer if ADCC Reporter Bioassay performance is not ideal.
	Optimize the normal human serum complement from 5–20% if CDC assay performance is not ideal. Complement must be kept on ice until ready to dilute.

7. Troubleshooting (continued)

Symptoms	Causes and Comments
Variability in assay performance	Variations in cell growth conditions including cell plating, harvest density, cell viability and cell doubling time can cause low assay performance and high assay variation. Use high-quality cell culture reagents (especially serum) and plasticware for maintaining cells in culture. 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 can cause poor assay performance and high assay variation. Centrifuge the cells exactly according to the instructions.
	Inappropriate cell freezing/DMSO exposure can cause poor assay performance and high assay variation. Freeze the cells exactly according to the instructions.
	Inappropriate cell counting methods can lead to variation in cell numbers in culture and assays and cause high assay variation. Ensure consistent and accurate cell counting methods.

8. References

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9. Appendix

9.A. Representative Assay Results

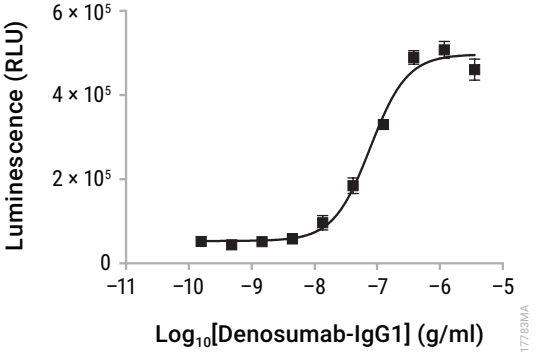

Figure 13. The ADCC Reporter Bioassay with mRANKL Target Cells measures the activity of the anti-RANKL antibody denosumab-IgG1. mRANKL Target Cells were added to a 96-well assay plate 18 hours prior to the assay. On the day of the assay, ADCC Effector Cells and a titration of denosumab-IgG1 were added. After a 24-hour induction at 37°C, Bio-Glo™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. The EC_{50} was 69ng/ml for denosumab-IgG1 and fold induction was 10.2.

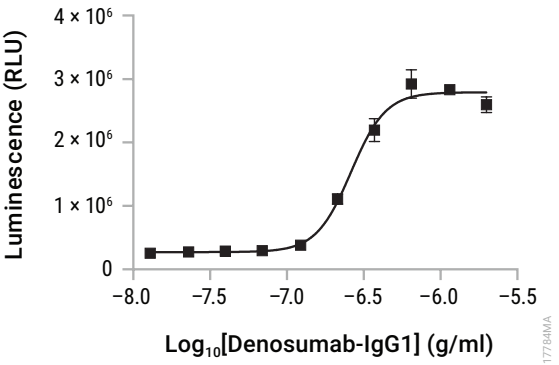

Figure 14. A CDC assay measures the activity of the anti-RANKL antibody denosumab-IgG1. mRANKL Target Cells were added to a 96-well assay plate 18 hours prior to the assay. On the day of assay, 10% normal human serum complement and a titration of denosumab-IgG1 were added. After a 6-hour induction at 37°C, CytoTox-Glo™ Reagent was added and luminescence measured using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. For denosumab-IgG1, the EC_{50} was 261ng/ml, the fold induction was 11.0 and the percent specific lysis was 53%.

9.B. Composition of Buffers and Solutions

initial cell culture medium for mRANKL Target Cells

90%	Ham's F-12
10%	FBS

cell growth medium for mRANKL Target Cells

90%	Ham's F-12
10%	FBS
10µg/ml	blasticidin

cell plating medium for mRANKL Target Cells

90%	Ham's F-12
10%	FBS

cell freezing medium for mRANKL Target Cells

85%	Ham's F-12
10%	FBS
5%	DMSO

ADCC Assay Buffer

96%	RPMI 1640 with L-glutamine and HEPES
4%	Super Low IgG FBS

CDC Assay Buffer

90%	Ham's F-12
10%	FBS

9.C. Related Products

Fc Effector Bioassays

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

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

Fc Effector Immunoassay

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

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

Immune Checkpoint Bioassays

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

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

Control Antibodies and Proteins

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

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9.C. Related Products (continued)

Detection Reagents

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

Not for Medical Diagnostic Use.

Luminometers

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

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

Note: Additional Fc Effector, Immune Checkpoint, T Cell Activation and Cytokine Bioassays are available through Promega Elite Access. To view and order Promega Bioassay products visit: **www.promega.com/products/reporter-bioassays/** or e-mail: **eliteaccess@promega.com**

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