

TECHNICAL MANUAL

Membrane RANKL Target Cells

Instructions for Use of Products
J3381 and J3385

Membrane RANKL Target Cells

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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- κ B, leading 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) that 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.

Membrane RANKL Target Cells^(a) (Cat. # J3381, J3385) are genetically-engineered cells stably expressing a cleavage-resistant form of mRANKL that enforces its surface expression. mRANKL Target Cells are provided in thaw-and-use format, which includes cryopreserved cells that are ready to be thawed for use as target cells in assays that measure the effector functions in ADCC and CDC for anti-RANKL blockers. In addition, they can be used to measure antibody binding affinity to mRANKL.

The Membrane RANKL Target Cells are also available in cell propagation model (CPM) format (Cat. # J3362), which includes cryopreserved cells that can be thawed, propagated and banked for long-term use.

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-TNF α 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-TNF α or anti-CD20 antibodies (Figure 4).

The ADCC Reporter Bioassay using mRANKL Target Cells is prequalified following International Council for Harmonisation 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).

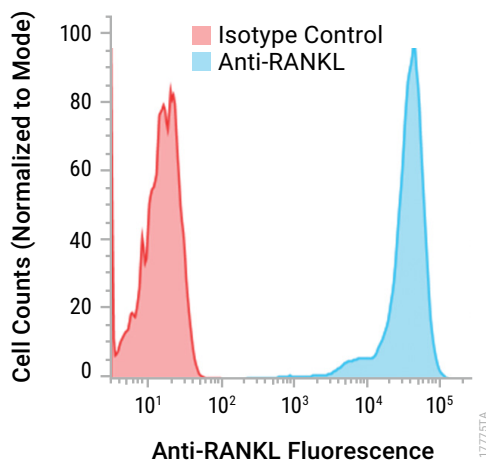


Figure 1. Surface expression of RANKL on mRANKL Target Cells. mRANKL Target Cells were labeled with PE-conjugated anti-RANKL or PE-conjugated isotype control antibodies. Cells were analyzed on a BD LSRFortessa™ X-20 flow cytometer. Data analysis was performed with FlowJo™ software.

1. Description (continued)

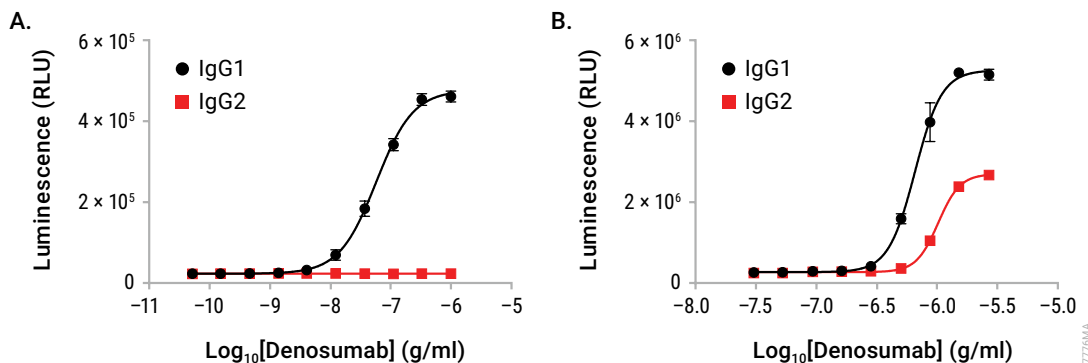


Figure 2. The ADCC Reporter Bioassay and CDC assay, performed with mRANKL Target Cells, reflects functional Fc activity of denosumab-IgG1 but not therapeutic denosumab (IgG2). **Panel A.** ADCC Effector Cells were cocultured with mRANKL Target Cells in the presence of serial titrations of antibodies, 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.

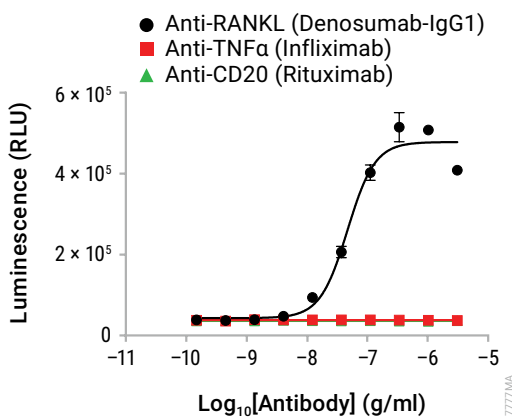


Figure 3. The ADCC Reporter Bioassay with mRANKL Target Cells reflects the mechanism of action (MOA) and shows specificity for antibodies that bind RANKL. ADCC Effector Cells were cocultured with mRANKL Target Cells in the presence of serial titrations of antibodies, as indicated. After a 6-hour induction,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

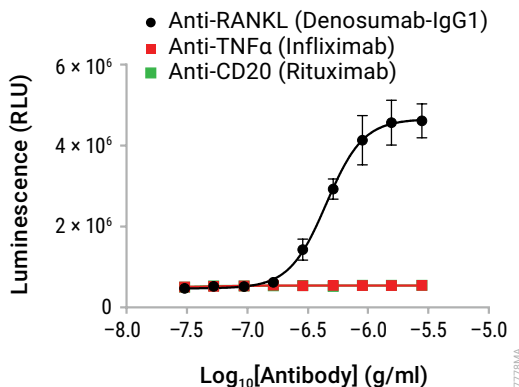


Figure 4. CDC assay with mRANKL Target Cells reflects the MOA and shows specificity for antibodies designed to bind RANKL. mRANKL Target Cells were incubated with 10% normal human serum complement in the presence of serial titrations of antibodies, as indicated. After a 6-hour incubation, CytoTox-Glo™ 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.		

1. Description (continued)

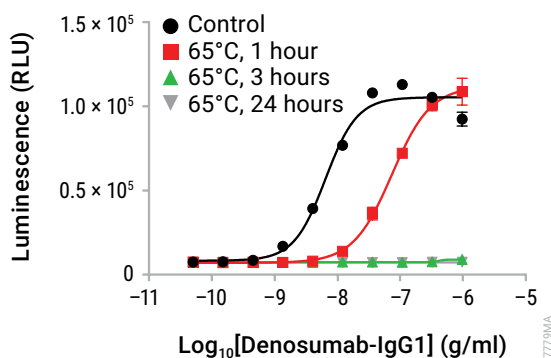


Figure 5. The ADCC Reporter Bioassay with mRANKL Target Cells is stability-indicating. Samples of anti-RANKL (denosumab-IgG1) were maintained at 4°C (control) or heat-treated at the indicated times and temperatures, then analyzed using the ADCC Reporter Bioassay with mRANKL Target Cells. Bio-Glo™ Reagent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

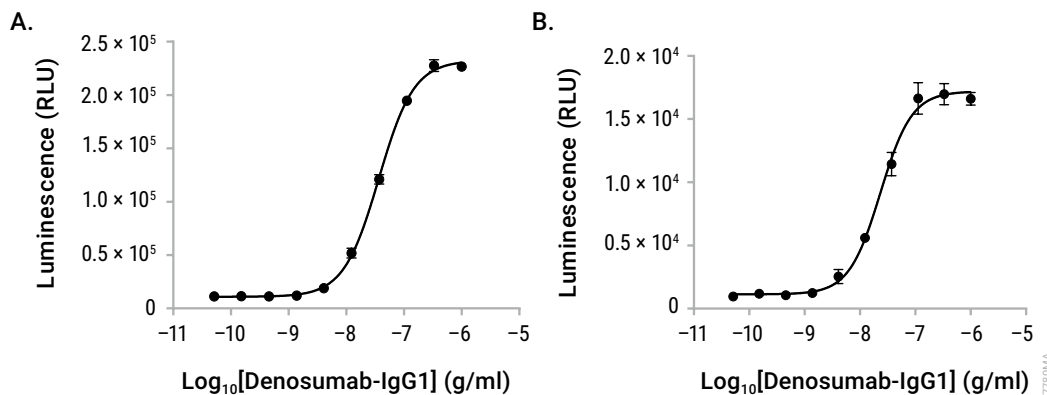


Figure 6. The ADCC Reporter Bioassay with mRANKL Target Cells is amenable to 384-well plate format. **Panel A.** The ADCC Reporter Bioassay with mRANKL Target Cells was performed in 96-well plates as described in this technical manual with a titration of anti-RANKL (denosumab-IgG1). **Panel B.** The 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 $2 \times 10^3/15\mu\text{l/well}$ 20 hours prior to the assay, in a 384-well white assay plate. On the day of the assay, $5\mu\text{l}$ of a threefold serial dilution of 5X concentrated denosumab-IgG1 was added to the wells, followed by $1.5 \times 10^4/5\mu\text{l/well}$ of ADCC Effector Cells. After a 6-hour induction at 37°C, 5% CO₂, $25\mu\text{l}$ 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 EC₅₀ 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	1 each	J3381

Not for Medical Diagnostic Use. Includes:

- 1 vial Membrane RANKL Target Cells (0.5ml per vial)

PRODUCT	SIZE	CAT.#
Membrane RANKL Target Cells 5X	1 each	J3385

Not for Medical Diagnostic Use. Includes:

- 5 vials Membrane RANKL Target Cells (0.5ml 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.

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

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 8.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.

mRANKL Target Cells are provided in frozen, thaw-and-use format and are ready for use 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. Do not overmix or overwarm the cell reagents.

The recommended cell plating densities, induction time and assay buffer components described in Sections 4 and 5 were established using denosumab-IgG1 (InvivoGen Cat.# hrankl-mab1) and ADCC Effector Cells (ADCC Reporter Bioassay) or in a CDC assay using the CytoTox-Glo™ Cytotoxicity Assay 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.

3. Before You Begin (continued)

The ADCC Reporter Bioassay and the CDC assay with CytoTox-Glo™ Cytotoxicity Assay 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 Detection System.

An integration time of 0.5 seconds/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.

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)
- **optional:** ADCC Reporter Bioassay (Cat.# G7102 G7010 or G7018; for ADCC Bioassay)
- **optional:** Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941; 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% CO₂ incubator
- 37°C water bath
- sensitive plate reader with glow luminescence measuring capability or luminometer (e.g., GloMax® Discover System, Cat.# GM3000, or equivalent instrument)

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

This assay protocol requires two engineered cell lines: ADCC Bioassay Effector Cells (Cat.# G7010, G7018) and Membrane RANKL Target Cells (Cat.# J3381, J3385). ADCC Bioassay Effector Cells and Membrane RANKL Target Cells are also available in Propagation Model format, Cat.# G7102 and Cat.# J3362, respectively.

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. 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 1µg/ml as a starting concentration (1X) and threefold serial dilution when testing denosumab-IgG1.

4.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.



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 3µg/ml of denosumab-IgG1 (dilu1, 3X final concentration) by adding 12µl of denosumab-IgG1 stock to 388µl of ADCC assay buffer.

4.A. Preparing ADCC Assay Reagents (continued)

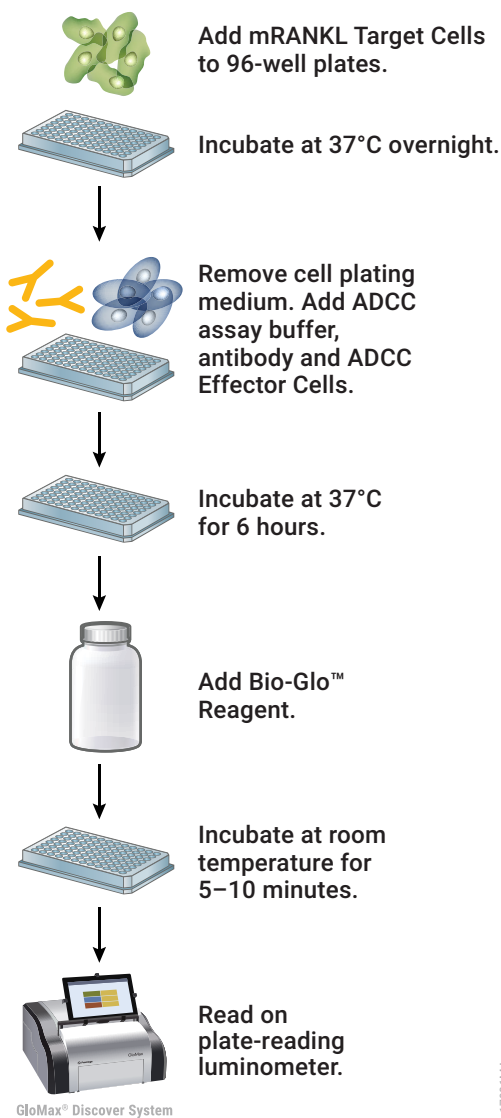


Figure 7. Schematic protocol for the ADCC Reporter Bioassay with mRANKL Target Cells.

4.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.

4.C. Plating mRANKL Target Cells

Thaw-and-use mRANKL Target 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 manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

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

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

1. On the day before performing the assay, prepare mRANKL Target Cell Plating Medium (90% Ham's F-12/10% FBS) as described in Section 4.A.

4.C. Plating mRANKL Target Cells (continued)

2. Transfer 19.5ml of cell plating medium to a 50ml conical tube.
3. Remove one vial of mRANKL Target Cells from storage at -140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial. Do not invert.
4. Gently mix the cell suspension by pipetting, then transfer 0.5ml cells to the tube containing 19.5ml of cell plating medium. Mix well by gently inverting the tube 1–2 times. Transfer the suspension to a sterile reagent reservoir. Using a multichannel pipette, immediately dispense 100 μl of the cell suspension to each of the inner 60 wells of a 96-well white flat-bottom assay plate.
5. Add 100 μl of mRANKL Target Cell plating medium to each of the outside wells of the assay plates.
6. Place lids on the assay plates and incubate in a 37°C , 5% CO_2 incubator overnight (18–22 hours).

4.D. Preparing Antibody Serial Dilutions

The instructions described here are for preparing a single stock of threefold serial dilutions of a single antibody for analysis in triplicate (100 μl 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 400 μl of reference antibody at 3X the highest antibody concentration in your dose-response curve. You will need 200 μl 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 4.A.
2. To a sterile clear V-bottom 96-well plate, add 200 μl of reference antibody starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 9).
3. Add 200 μl of test antibodies 1 and 2 starting dilution (dilu1, 3X final concentration) to wells E11 and G11, respectively (see Figure 9).
4. Add 120 μl of ADCC assay buffer to other wells in these four rows, from column 10 to column 2.
5. Transfer 60 μl 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 120 μl of ADCC assay buffer without antibody as a negative control.

7. Cover the antibody dilution plate with a lid and keep at ambient temperature ($22\text{--}25^{\circ}\text{C}$).

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.

4.E. Adding Antibody to mRANKL Target Cells in Assay Plates

1. Take the 96-well assay plate containing mRANKL Target Cells out of 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. Add 75µl of ADCC assay buffer to each of the outside wells of the assay plate.
5. Cover the assay plates with lids and keep the plates on the bench before adding ADCC Bioassay Effector cells at the next step.

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

The thaw-and-use ADCC Effector Cells in the ADCC Reporter Bioassay (Cat. # G7010) 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 manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

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

1. Label a sterile 15ml conical tube "Effector Cells". Add 3.6ml of prewarmed (37°C) ADCC assay buffer to the 15ml conical tube.
2. Remove one vial of ADCC Effector Cells from storage at -140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial. Do not invert the vial.
3. Gently mix the cell suspension by pipetting, then transfer 630µl cells to the 15ml conical tube containing 3.6ml of ADCC assay buffer. Mix well by gently inverting the tube.
4. 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.
5. Cover the assay plates with lids and incubate in a 37°C, 5% CO₂ incubator for 6 hours.

Note: The 6-hour assay time was optimized using denosumab-IgG1. We recommend optimizing assay time (5–24 hours) with your own antibody or other biologic samples.

4.G. Adding Bio-Glo™ Reagent

! 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.

Note: 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 (22–25°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.

4.H. Data Analysis

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

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

3. Graph data as RLU versus $\text{Log}_{10}[\text{antibody}]$ and fold induction versus $\text{Log}_{10}[\text{antibody}]$. 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 CDC Assay using mRANKL Target Cells

The procedure below illustrates the use of the mRANKL Target Cells in a CDC assay (using CytoTox-Glo™ Cytotoxicity Assay, Cat. # G9290) to test two anti-RANKL antibody samples against a reference sample in a single assay run using the Membrane RANKL Target Cells. 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 2.67 µg/ml as a starting concentration (1X) and 1.75-fold serial dilution when testing denosumab-IgG1.

5.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, the 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.

5.A. Preparing CDC Assay Reagents (continued)

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 4µg/ml of denosumab-IgG1 (dilu1, 1.5X final concentration) by adding 32µl of denosumab-IgG1 stock to 768µl of CDC assay buffer.

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).

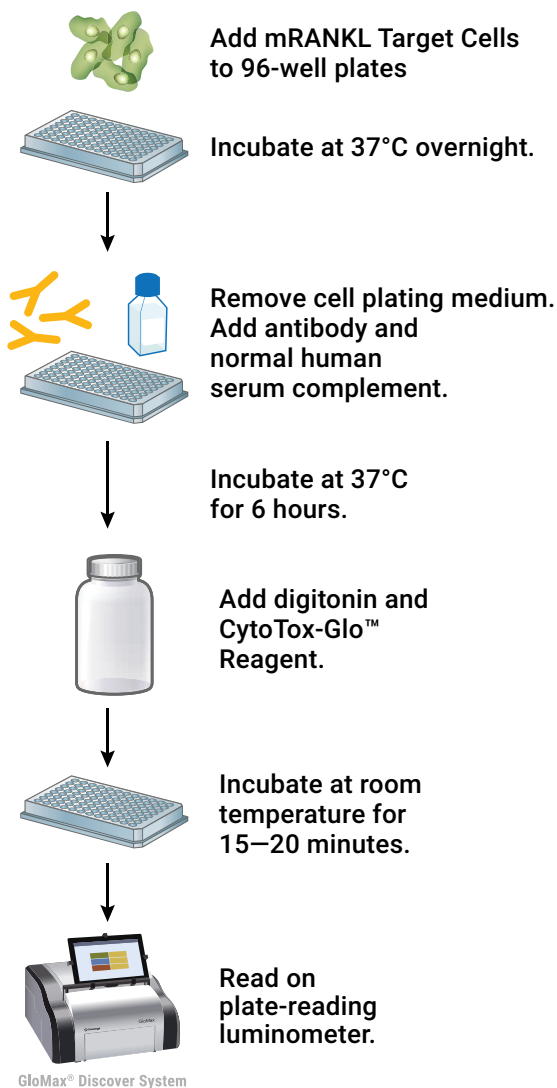


Figure 10. Schematic protocol for the CDC assay with Membrane RANKL Target Cells.

5.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").

5.C. Plating mRANKL Target Cells

Thaw-and-use mRANKL Target 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 manipulation is required or recommended. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at any one time.

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

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

1. On the day before the assay, prepare 50ml of mRANKL Target Cell plating medium by combining 45ml of Ham's F-12 with 5ml of FBS to yield 90% Ham's F-12/10% FBS. Mix well and warm to 37°C prior to use.
2. Transfer 19.5ml of mRANKL Target Cell plating medium to a 50ml conical tube.

3. Remove one vial of mRANKL Target Cells from storage at -140°C and transfer to the bench on dry ice. Thaw the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect the vial. Do not invert.
4. Gently mix the cell suspension by pipetting, then transfer 0.5ml cells to the tube containing 19.5ml of cell plating medium. Mix well by gently inverting the tube 1–2 times. Transfer the suspension to a sterile reagent reservoir.
5. Using a multichannel pipette, immediately dispense 100 μl of the cell suspension B2 through G12 of a 96-well white flat-bottom assay plate (Figure 10).
6. Add 100 μl of mRANKL Target Cell plating medium to each of the empty wells of the assay plates (Figure 11).
7. Place lids on the assay plates and incubate in a 37°C , 5% CO_2 incubator overnight

5.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 (200 μl 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 800 μl of reference antibody at 1.5X the highest antibody concentration in your dose-response curve. You will need 400 μl 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 5.A.
2. To a sterile 12-well reservoir labeled reference, add 800 μl 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 400 μl of Test antibodies 1 and 2 starting dilution (dilu1, 1.5X final concentration) to well 11 (Figure 12).
4. For reference antibody, add 340 μl of CDC assay buffer to other wells, 2 through 10 and 12 (well 1 is empty).
5. For test antibodies 1 and 2, add 170 μl of CDC assay buffer to other wells, 2 through 10 and 12 (well 1 is empty).
6. Transfer 453 μl 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 340 μl of CDC assay buffer without antibody as a negative control. Well 12 contains 340 μl of CDC assay buffer without antibody and will be used for digitonin addition at end of the assay.

8. Transfer 227 μl of the test 1 and 2 antibody starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.

5.D. Preparing Antibody Serial Dilutions (continued)

- 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 the end of the 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.

5.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.

- 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.

5.F. Adding Antibody and Complement to Assay Plates

- 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.
- Using a multichannel pipette, add 50µl of appropriate antibody dilution to the assay plates according to the plate layout in Figure 11.
- 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).
- 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% CO₂ 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.

5.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 5.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 5.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.

5.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:

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

4. Calculate percent specific lysis:

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

5. Graph data as RLU versus Log₁₀[antibody], fold induction versus Log₁₀[antibody] and percent specific lysis versus Log₁₀[antibody]. Fit curves and determine the EC₅₀ 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 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 EC₅₀ values obtained in the ADCC Reporter Bioassay or CDC assay with mRANKL Target Cells may vary from the values 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.
Variability in assay performance	Ensure that incubation times are consistent between assays. Ensure that the plating protocols are strictly followed.

7. References

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8. Appendix

8.A. Representative Assay Results

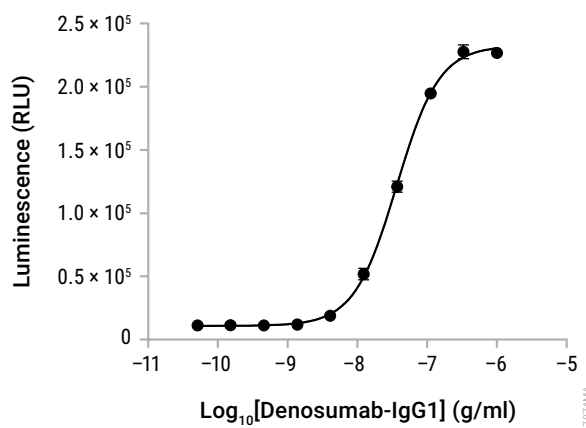


Figure 13. The ADCC Reporter Bioassay with mRANKL Target Cells measures the activity of the anti-RANKL antibody denosumab-IgG1. mRANKL Target Cells were added to a 96-well assay plate 18 hours prior to the assay. On the day of the assay, ADCC Effector Cells and a titration of denosumab-IgG1 were added. 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 EC₅₀ was 37ng/ml for denosumab-IgG1 and fold induction was 20.5.

8.A. Representative Assay Results (continued)

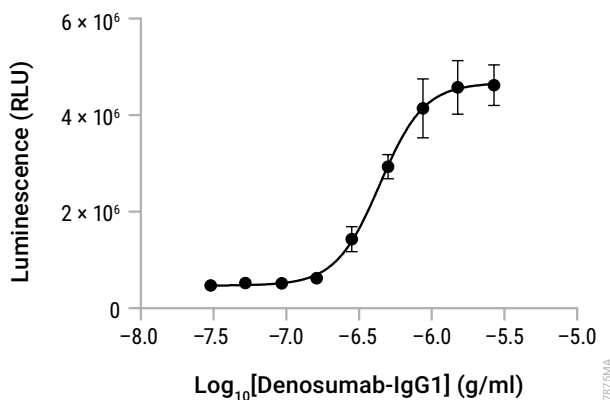


Figure 14. A CDC assay measures the activity of the anti-RANKL antibody denosumab-IgG1. mRANKL Target Cells were added to a 96-well assay plate 18 hours prior to the assay. On the day of assay, 10% normal human serum complement and a titration of denosumab-IgG1 were added. After a 6-hour induction at 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 EC₅₀ was 441ng/ml, the fold induction was 9.9 and the percent specific lysis was 57%.

9. Summary of Changes

The following changes were made to the 7/26 revision of this document:

1. Removed Section 8.B, Related Products.
2. Made minor text edits.

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