TECHNICAL MANUAL

RANKL Bioassay

Instructions for Use of Products
JA2701 and JA2705

8/20
TM621

Promega

RANKL Bioassay

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.
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1. Description

Receptor activator of nuclear factor-κB (NF-κB; 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 NF-κB 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. In cells with excess RANKL or insufficient OPG, upregulated RANKL/RANK signaling leads to superfluous osteoclast formation and bone resorption, causing pathologic bone loss and destruction (3).

In osteosarcoma, in addition to cancer-induced bone destruction, RANKL is also involved in tumorigenesis and metastasis. RANKL inhibition significantly delays mammary tumor formation in carcinogen and hormone-induced breast cancer mouse models (4, 5). Denosumab is a human IgG2 monoclonal antibody that targets and inhibits RANKL. It is used to treat osteoporosis and hypercalcemia, as well as bone cancer and other cancer-associated bone disease. Denosumab blocks the RANK-RANKL interaction, inhibiting osteoclast formation, function and survival; thus, it decreases bone resorption and interrupts cancer-induced bone destruction (6).

The RANKL Bioassay[a–f] (Cat.# JA2701, JA2705) is a bioluminescent cell-based assay designed to measure RANK/RANKL pathway stimulation or inhibition. The RANKL Bioassay Cells are provided in a thaw-and-use format as cryopreserved cells that can be thawed, plated and used in an assay without the need for cell propagation. The RANKL Bioassay is also available in a Cell Propagation Model (CPM) format, which includes cryopreserved cells that can be thawed, propagated, and banked for long-term use (RANKL Bioassay, Propagation Model, Cat.# J3102).

The RANKL Bioassay is comprised of a human cell line engineered to express the RANK receptor and a luciferase reporter driven by a response element (RE). When RANKL binds the RANK receptor, transduced intracellular signals activate the RE, resulting in luminescence (Figure 1). The bioluminescent signal is detected and quantified using the Bio-Glo™ Luciferase Assay System[d] (Cat.# G7940, G7941) and a standard luminometer, such as the GloMax® Discover System (see Related Products, Section 8.B).

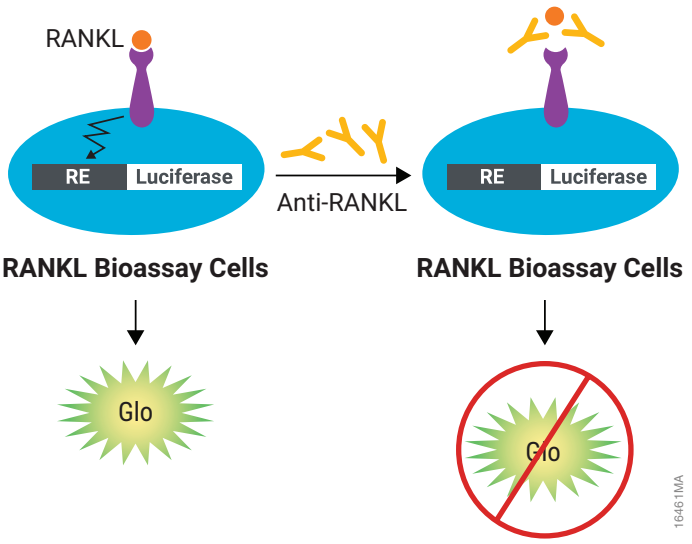


Figure 1. Representation of the RANKL Bioassay. The RANKL Bioassay consists of a genetically engineered cell line, RANKL Bioassay Cells. When RANKL binds to its receptor, receptor-mediated pathway signaling induces luminescence that can be detected upon the addition of Bio-Glo™ Reagent and quantified with a luminometer. Inhibition of RANKL binding by anti-RANKL antibody results in a decrease in luminescence.

1. Description (continued)

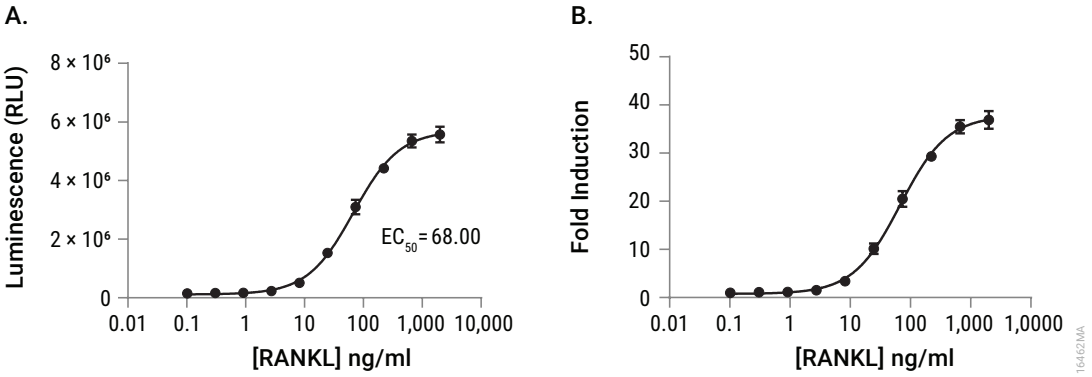


Figure 2. The RANKL Bioassay responds to recombinant RANKL. RANKL Bioassay Cells were grown and prepared as described in this protocol, and incubated with serial dilutions of recombinant RANKL. After a 6-hour incubation, Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. **Panel A** shows raw luminescence measurements. **Panel B** displays the calculated fold induction. Data were generated using thaw-and-use cells.

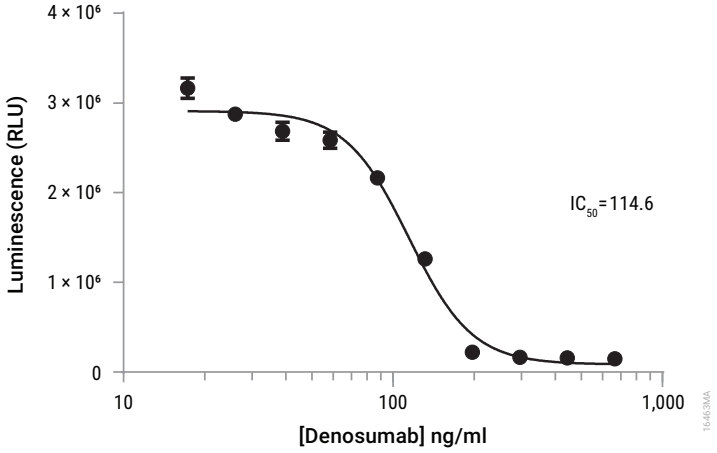


Figure 3. The RANKL Bioassay responds to denosumab. RANKL Bioassay Cells were grown and prepared as described in this protocol, and incubated with serial dilutions of denosumab (anti-RANKL) antibody and 100ng/ml RANKL. After a 6-hour incubation, Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

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

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	54.1
	75	79.9
	125	128.4
	150	152.7
Repeatability (% CV)	100% (Reference)	1.79
Intermediate Precision (% CV)		5.98
Linearity (r^2)		0.999
Linearity (y = mx + b)		y = 0.9819x – 5.5733

A 50–150% theoretical potency series of denosumab (anti-RANKL) was analyzed in triplicate in three independent experiments performed on three days by two analysts (for a total of six independent experiments). Bio-Glo™ Reagent was added, and luminescence was 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.

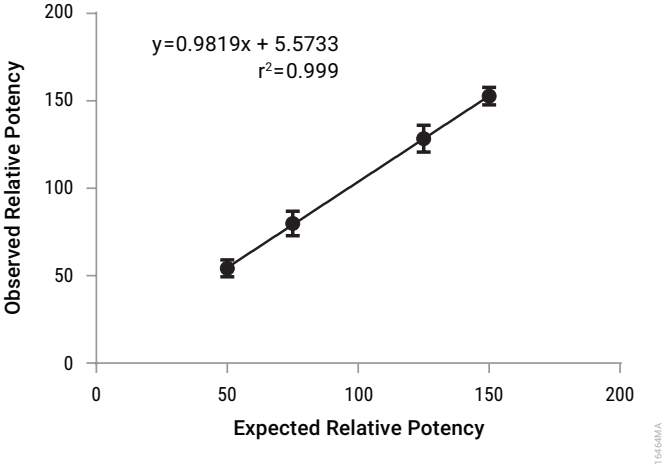
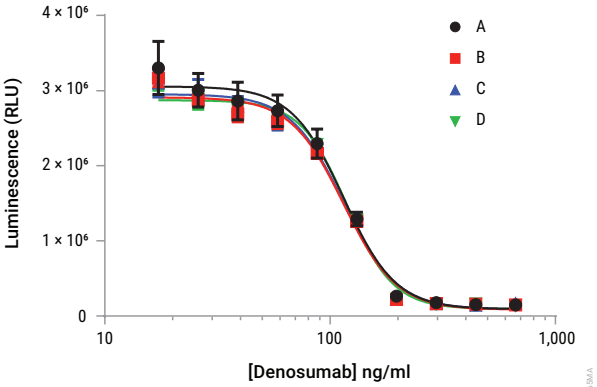


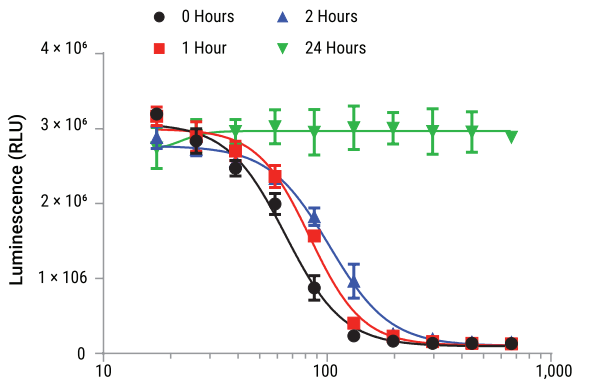
Figure 4. The RANKL Bioassay shows precision, accuracy, and linearity. A 50–150% theoretical potency series of RANKL was analyzed in triplicate in three independent experiments performed on three days by two analysts using the RANKL Bioassay (for a total of six independent experiments). Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Linearity and r^2 values were determined using GraphPad Prism® software. Data were generated using thaw-and-use cells.

1. Description (continued)



	A	B	C	D
IC_{50}	114.8	114.6	114.6	118.8

Figure 5. The RANKL Bioassay demonstrates repeatability. Four separate serial dilution series of denosumab were analyzed on four individual assay plates using the RANKL Bioassay. Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.



	0 Hours	1 Hour	2 Hours	24 Hours
IC_{50}	65.08	83.82	102.4	*

Figure 6. The RANKL Bioassay indicates stability. Denosumab was heated at 65°C for the indicated periods of time and used in the inhibition assay. Prolonged heating inactivated denosumab based on time heated, which is reflected by the RANKL assay. Data were generated using thaw-and-use cells. *Data for 24-hour treatment was not calculable.

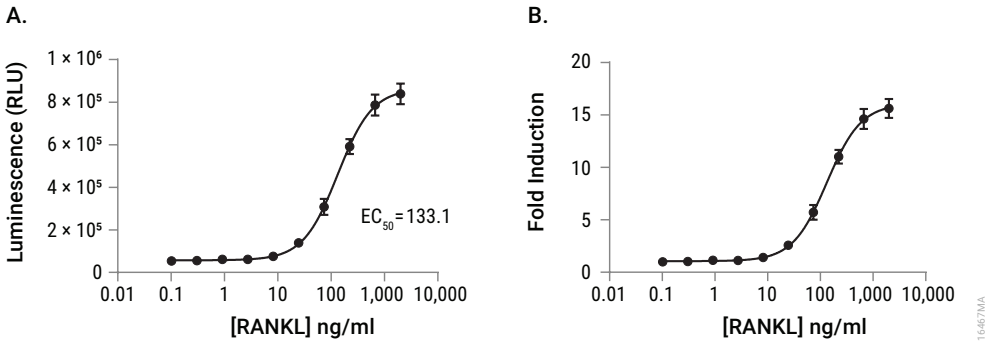


Figure 7. The RANKL Bioassay is amenable to 384-well plate format. The RANKL Bioassay was tested in 384-well format. RANKL Bioassay Cells were prepared and dispensed as 30µl/well. Serial threefold dilutions of recombinant human RANKL were prepared and added to cells after overnight incubation and removal of seeding media. After 6 hours of stimulation, Bio-Glo™ Reagent was added and luminescence was quantified using the GloMax® Discover System. Data were fitted to a four-parameter logistic curve using GraphPad Prism® software. **Panel A** shows raw luminescence measurements. **Panel B** displays the calculated fold induction. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
RANKL Bioassay	**1 each**	**JA2701**

Not for Medical Diagnostic Use. Each kit contains sufficient reagents for 120 assays using the inner 60 wells of two 96-well plates. Includes:

- 1 vial RANKL Bioassay Cells, 1.0×10^7 cells/ml (0.5ml per vial)
- 36ml RPMI 1640 Medium
- 4ml Fetal Bovine Serum
- 1 vial Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 10ml Bio-Glo™ Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
RANKL Bioassay 5X	**1 each**	**JA2705**

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

- 5 vials RANKL Bioassay Cells, 1.0×10^7 cells/ml (0.5ml per vial)
- 5 × 36ml RPMI 1640 Medium
- 5 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 5 × 10ml Bio-Glo™ Luciferase Assay Buffer

Note: RANKL Bioassay components are shipped separately because of differing temperature requirements. The RANKL Bioassay Cells are shipped on dry ice. The Bio-Glo™ Luciferase Assay System and Fetal Bovine Serum are shipped on dry ice separately from the cells. The RPMI 1640 Medium is shipped at ambient temperature.

Storage Conditions:

- Upon arrival, immediately transfer the cell vials to below –140°C (freezer or liquid nitrogen vapor phase) for long-term storage. Do not store cell vials submerged in liquid nitrogen. **Do not** store cell vials at –80°C because this will decrease cell viability and cell performance.
- Store Bio-Glo™ Luciferase Assay Substrate, Bio-Glo™ Luciferase Assay Buffer and Fetal Bovine Serum at –30°C to –10°C. Avoid multiple freeze-thaw cycles of the serum.
- For optimal performance, use reconstituted Bio-Glo™ Reagent on the day of preparation. However, once reconstituted, Bio-Glo™ Reagent can be stored at –20°C for up to 6 weeks.
- Store RPMI 1640 Medium at 4°C protected from light.

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 website such as the Certificate of Analysis.

The RANKL Bioassay is intended to be used with user-provided biologics designed to activate or inhibit the RANK/RANKL signaling pathway. The recommended cell plating density, induction time and assay buffer components described in Section 4 were established using research-grade recombinant human RANKL. You may need to adjust the parameters provided here and optimize assay conditions for other biologic samples. Data generated using these reagents are shown in Figure 2.

The RANKL Bioassay produces a bioluminescent signal and requires a sensitive luminometer or luminescence plate reader for the detection of luminescence. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System luminometer. An integration time of 0.5 second/well was used for all readings. The bioassay is compatible with most other plate-reading luminometers; however, relative luminescence unit readings may vary due to the sensitivity and settings of each instrument. The use of different instruments should not affect the measured relative potency of test samples.

Materials to Be Supplied by the User

(Composition of Buffers and Solutions is provided in Section 8.A.)

Reagents

- user-defined biologics samples
- **optional:** recombinant human RANKL (Biolegend Cat.# 591106)

Supplies and Equipment

- white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917)
- sterile clear 96-well plate with lid (e.g., Corning® Cat.# 3896 or Falcon Cat.# 353077) for preparing sample dilutions
- sterile 12-well reagent reservoir (e.g., Axygen Cat.# RES-MW12-LP-SI) for preparing sample dilutions
- pipettes (single-channel and 12-channel)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs
- humidified 37°C, 5% CO_2 incubator
- 37°C water bath
- plate reader that measures glow luminescence or luminometer (e.g., GloMax® Discover System)

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4. Stimulation Protocol

The RANKL Bioassay can be used to test RANKL and RANKL-blocking antibodies. This stimulation protocol illustrates the use of the RANKL Bioassay to examine two test samples against a reference sample in a single assay run. Each test and reference sample is run in triplicate, in a ten-point dilution series, in a single 96-well assay plate using the inner 60 wells (see Figure 9). Other experimental and plate layouts are possible but may require further optimization. The inhibition protocol (Section 5) illustrates the use of the RANKL Bioassay to block RANKL activity.

Notes:

1. When preparing test and reference samples, choose an appropriate starting concentration and dilution scheme to achieve a full dose-response curve with proper upper and lower asymptotes and sufficient points on the slope. For reference, we use 0–2µg/ml final concentration of recombinant human RANKL (Biolegend Cat.# 591106) as a sample range, with serial threefold dilutions to achieve full dose curves as a 10-point series. Concentration ranges and dilution schemes may need to be optimized for your samples.
2. When diluted as directed, each kit containing medium, serum and 1 vial of RANKL Bioassay Cells, is sufficient for 120 wells (two 96-well plates using inner-60 format). The thaw-and-use cells are for single use only and cannot be cultured or refrozen for second use. Please plan your experiments accordingly to optimize the use of the thaw-and-use cells.

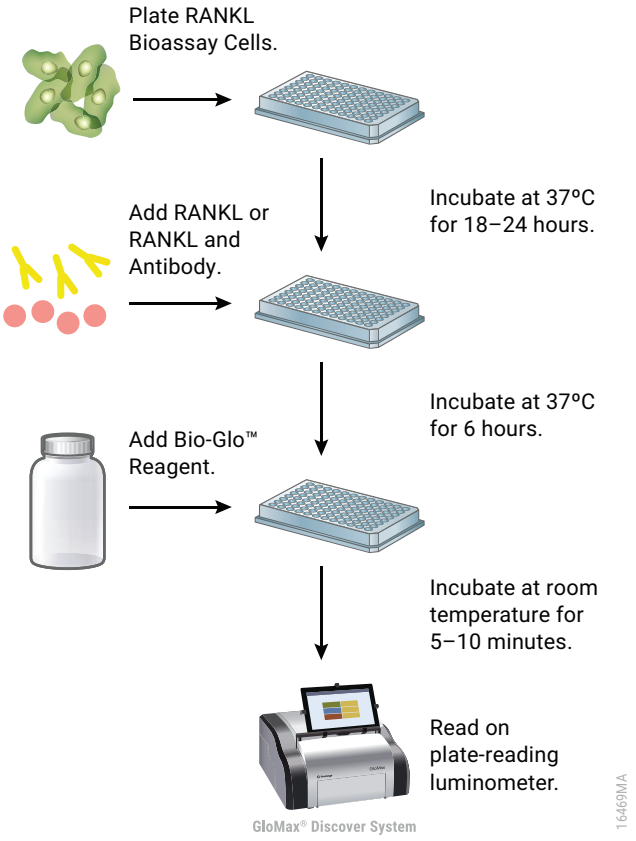

Figure 8. RANKL Bioassay schematic protocol.

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4.A. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 9 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference samples 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 drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Replicate 1
C	B	no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Replicate 1
D	B	no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Replicate 2
E	B	no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Replicate 2
F	B	no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Replicate 3
G	B	no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Replicate 3
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 9. Example plate layout. This suggested layout shows nonclustered locations for three replicates of each test and reference sample dilution series (dilu1–dilu9) and wells containing assay buffer (denoted by "B") alone.

4.B. Day One: Plating RANKL Bioassay Cells

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

1. Prepare 24ml of seeding medium by combining 23.76ml of RPMI with 240µl of FBS and warm to 37°C. Save the remaining RPMI at 4°C. You will need 12ml of RPMI on day 2.
2. Remove one vial of RANKL Bioassay Cells from storage at −140°C and transfer it to the bench on dry ice.
3. Add 15.6ml of prewarmed (37°C) seeding medium to a 50ml conical tube.
4. Warm the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect vial contents. Do not submerge the vial completely. Do not invert.
5. Gently mix the cell suspension by pipetting, then transfer 0.4ml of the cells to the 50ml conical tube containing 15.6ml of seeding medium. Mix well by gently pipetting or inverting five times.
6. Transfer the cell suspension to a sterile reagent reservoir.
7. Dispense 100µl/well (2.5×10^4 cells/well) using a multichannel pipette into the inner 60 wells of two solid white 96-well plates.
8. Transfer remaining 8.4ml of prepared seeding medium from Step 1 to a sterile reagent reservoir. Add 100µl/well of seeding medium to the outer 36 wells of each plate.
9. Incubate overnight (18–24 hours) at 37°C, 5% CO_2.

4.C. Day Two: Assay Day with Addition of Test and Reference Samples

Preparing Reagents for the Assay Day

1. **Bio-Glo™ Reagent:** For reference, 10ml of Bio-Glo™ Reagent is sufficient to assay 120 wells in a 96-well assay format. Thaw the Bio-Glo™ Luciferase Assay Buffer in a refrigerator 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 the Bio-Glo™ Luciferase Assay Buffer into the amber bottle containing the Bio-Glo™ Luciferase Assay Substrate and mix by inversion until the substrate is thoroughly dissolved. Equilibrate and store the reconstituted Bio-Glo™ Reagent at ambient temperature (22–25°C) protected from light before adding to assay plates.

   If you are using a large (100ml) size of Bio-Glo™ Luciferase Assay System, dispense the reconstituted Bio-Glo™ Reagent into 10ml aliquots and store at −20°C for up to 6 weeks. Avoid repeated freeze-thaw cycles. On the day of the assay, thaw the 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 18% loss of luminescence after 24 hours at ambient temperature and 12% loss of luminescence after 5 days at 4°C.
2. **Assay Buffer:** Use RPMI 1640 without serum as assay buffer. Warm to 37°C prior to use.
3. **Test and Reference Samples:** Prepare starting dilutions (denoted as dilu1) of test and reference samples (see Figure 10). Using assay buffer as the diluent, prepare a minimum of 800µl of referemce sample starting dilution and a minimum of 400µl of each test sample starting dilution, in 1.5ml tubes. Store the tubes containing starting dilutions appropriately before making serial dilutions.

4.D. Preparing Serial Dilutions

The instructions described here are for preparation of a single stock of threefold serial dilutions of a single sample for analysis in triplicate (250µl of each dilution provides a sufficient volume). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples.

Note: For RANKL stimulation using recombinant human RANKL as your reference sample (Biolegend Cat.# 591106), we recommend starting with a 1X concentration of 2µg/ml and performing serial threefold dilutions. When using other reference sources of RANKL, the starting concentration may need to be adjusted. To perform the dilutions as described here, you will need a total of four dilution reservoirs, two for the test samples and two for the reference samples.

1. Add 375µl of each sample starting dilution (dilu1) to column 11 of a sterile 12-well dilution reservoir (see Figure 10).
2. Add 250µl of assay buffer to the other wells, from column 10 to column 2.
3. Transfer 125µl of the sample starting dilution from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
4. Repeat equivalent threefold serial dilutions across the columns from right to left until you reach column 3. Remove 125µl from column 3 so that all wells have a 250µl volume. Do not dilute into column 2.
5. Create the same dilution series for the remaining samples.

Recommended Reservoir Layout for Sample Dilutions

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

Figure 10. Example reservoir layout showing sample serial dilutions. Well 2 contains 250µl of assay buffer without sample.

4.E. RANKL Stimulation Assay

1. Invert the assay plate above a sink to remove the medium. Then, place the inverted plate on paper towels for 5–10 seconds to drain any remaining medium. Alternatively, use a multichannel pipette or aspirator to remove seeding media (~100µl) from each well.
2. Dispense 75µl of each sample to the preplated cells according to the plate layout in Figure 9.
3. Cover each assay plate with a lid and incubate in a humidified 37°C, 5% CO_2 incubator for 6 hours.
4. After the 6-hour incubation is complete, proceed to Section 4.F.

4.F. Adding Bio-Glo™ Reagent

Note: Prepare Bio-Glo™ Reagent as described in Section 4.C. Bio-Glo™ Reagent should be at ambient temperature (22–25°C) when added to assay plates.

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

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

4.G. Data Analysis

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

   Note: When calculating fold induction, if the no-drug control sample RLU is at least 100X the plate background RLU, there is no need to subtract plate background from sample RLU.
3. Graph data as RLU versus $\log_{10}$[sample] and fold induction versus $\log_{10}$[sample]. Fit curves and determine the EC_{50} value of RANKL response using appropriate curve fitting software (such as GraphPad Prism®).

5. Inhibition Protocol

The RANKL Bioassay Cells can be used to measure inhibition of RANK/RANKL signaling using a blocking antibody such as denosumab, which targets RANKL. A preliminary stimulation experiment with RANKL is necessary to determine the EC_{80} value, which is used during an inhibition assay. The protocol may be modified for other blocking antibodies, depending on their mechanism of action.

5.A. RANKL Inhibition Assay

1. Prepare and plate RANKL Bioassay Cells as described in Sections 4.A and 4.B (see Figure 9). Incubate overnight in a humidified 37°C, 5% CO_2 incubator.
2. In a separate clear 96-well plate, prepare serial dilutions of denosumab in prewarmed assay buffer to a 2X final concentration. We recommend a final concentration of denosumab of 0–660ng/ml as serial 10-point 1.5-fold dilutions. Prepare at least 125µl of denosumab solution for each sample.
3. Prepare a RANKL solution at 2X the concentration of the predetermined EC_{80} response value in prewarmed assay buffer. Prepare at least 125µl of RANKL solution for each sample.
4. Combine an equal volume of RANKL solution with each antibody dilution. Mix by pipetting.

   Note: Each component is now at 1X its final concentration.
5. Remove the 96-well assay plates containing the overnight preplated RANKL Bioassay Cells from the incubator.
6. Invert the assay plate above a sink to remove the medium. Then, place the inverted plate on paper towels for 5–10 seconds to drain any remaining medium. Alternatively, use a multichannel pipette or aspirator to remove all the seeding media (~100µl) from each well of the assay plate.
7. Add 75µl of RANKL and denosumab dilutions from the dilution plate in Step 4 into each replicate well of the assay plate.
8. Cover each assay plate with a lid and incubate in a humidified 37°C, 5% CO_2 incubator for 6 hours.
9. After the 6-hour incubation is over, proceed to Section 5.B.

5.B. Adding Bio-Glo™ Reagent

Note: Prepare Bio-Glo™ Reagent as described in Section 4.C. Bio-Glo™ Reagent should be at ambient temperature (22–25°C) when added to assay plates.

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

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

5.C. Data Analysis

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

   Note: When calculating fold induction, if the no-drug control sample RLU is at least 100X the plate background RLU, there is no need to subtract plate background from sample RLU.
3. Graph data as RLU versus Log_{10}[sample] and fold induction versus Log_{10}[sample]. Fit curves and determine the IC_{50} value of RANKL inhibition 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** E-mail: **techserv@promega.com**

Symptoms	Possible Causes and Comments
Low luminescence measurements (RLU readout)	Choose an instrument designed for plate-reading luminescence detection. Instruments designed primarily for fluorescence detection are not recommended. Luminometers measure and report luminescence as relative values, and actual RLU numbers will vary between instruments.
	Insufficient cells per well can lead to low RLU. Handle and plate the cells according to the instructions to ensure a sufficient number of viable cells per well.
	Low cell viability can lead to low luminescence readout and variability in assay performance.
	Low activity of Bio-Glo™ Reagent leads to low RLU. Store and handle the Bio-Glo™ Reagent according to the instructions.
Assay performance is variable	Ensure that incubation times are consistent between assays.
	Ensure the plating protocol (see Section 4.B) is strictly followed.
	Cells must be treated the same way prior to each assay. Variability in cell growth rates and preculture plating densities will result in variable assay results.
	Ensure RANKL is prepared and stored properly with carrier protein. Follow manufacturer's protocol for initial rehydration of cytokines. Single use frozen aliquots are recommended for each assay.
	RANKL lot-to-lot activity differences may be observed. Consult cytokine provider for details.
Weak assay response (low fold induction)	RANKL frozen single-use aliquot has lost biological activity. Follow manufacturer's recommendation for storage and stability.
	If untreated control RLU is less than 100X above plate reader background RLU, subtract plate reader background RLU from all samples prior to calculating fold induction.

7. References

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2. Walsh, MC. *et al.* (2014) Biology of the RANKL–RANK–OPG system in immunity, bone, and beyond. *Fron. Immunol.* **5(511)**, 1–11.
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8. Appendix

8.A. Composition of Buffers and Solutions

seeding medium

99%	RPMI 1640
1%	fetal bovine serum

assay buffer

100%	RPMI 1640

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8.B. Related Products

Cytokine and Growth Factor Bioassays

Product	Size	Cat.#
RANKL Bioassay, Propagation Model	1 each	J3102
VEGF Bioassay	1 each	GA2001
VEGF Bioassay 5X	1 each	GA2005
VEGF Bioassay, Cell Propagation Model	1 each	GA1082
Recombinant VEGF	10µg	J2371
IL-2 Bioassay	1 each	JA2201
IL-2 Bioassay 5X	1 each	JA2205
IL-2 Bioassay, Propagation Model	1 each	J2952
IL-6 Bioassay	1 each	JA2501
IL-6 Bioassay 5X	1 each	JA2505
IL-6 Bioassay, Propagation Model	1 each	J2992
IL-12 Bioassay	1 each	JA2601
IL-12 Bioassay 5X	1 each	JA2605
IL-12 Bioassay, Propagation Model	1 each	J3042
IL-15 Bioassay	1 each	JA2011
IL-15 Bioassay 5X	1 each	JA2015
IL-15 Bioassay, Propagation Model	1 each	J2962
IL-23 Bioassay	1 each	JA2511
IL-23 Bioassay 5X	1 each	JA2515
IL-23 Bioassay, Propagation Model	1 each	J3002

Not for Medical Diagnostic Use.

T Cell Activation Bioassays

Product	Size	Cat.#
T Cell Activation Bioassay (NFAT)	1 each	J1621
T Cell Activation Bioassay (NFAT) 5X	1 each	J1625
T Cell Activation Bioassay (IL-2)	1 each	J1651
T Cell Activation Bioassay (IL-2) 5X	1 each	J1655

Not for Medical Diagnostic Use.

Fc Effector Bioassays

Product	Size	Cat.#
ADCC Reporter Bioassay, Complete Kit (Raji)*	1 each	G7015
ADCC Reporter Bioassay, Target Kit (Raji)*	1 each	G7016
ADCC Reporter Bioassay, Core Kit*	1 each	G7010
ADCC Reporter Bioassay, F Variant, Core Kit**	1 each	G9790
FcγRIIa-H ADCP Reporter Bioassay, Complete Kit**	1 each	G9901
FcγRIIa-H ADCP Reporter Bioassay, Core Kit**	1 each	G9991

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

Immune Checkpoint Bioassays

Product	Size	Cat.#
CD40 Bioassay	1 each	JA2151
CD40 Bioassay 5X	1 each	JA2155
CD40 Bioassay, Propagation Model	1 each	J2132
Control Ab, Anti-CD40	50µg	K1181
CTLA-4 Blockade Bioassay	1 each	JA3001
CTLA-4 Blockade Bioassay 5X	1 each	JA3005
CTLA-4 Blockade Bioassay, Propagation Model	1 each	JA1400
Control Antibody, Anti-CTLA-4	100µg	JA1020
LAG-3/MCHII Blockade Bioassay	1 each	JA1111
LAG-3/MHCII Blockade Bioassay 5X	1 each	JA1115
LAG-3/MHCII Blockade Bioassay, Propagation Model	1 each	JA1112
TCR Activating Antigen Stock Solution	500µl	K1201
Control Ab, Anti-LAG-3	100µg	K1150
PD-1/PD-L1 Blockade Bioassay	1 each	J1250
PD-1/PD-L1 Blockade Bioassay 5X	1 each	J1255
PD-1 Blockade Bioassay, Propagation Model	1 each	J1252
PD-L1 Negative Cells	1 each	J1191
Control Ab, Anti-PD-1	100µg	J1201

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Immune Checkpoint Bioassays (continued)

Product	Size	Cat.#
OX40 Bioassay	1 each	JA2191
OX40 Bioassay 5X	1 each	JA2195
OX40 Bioassay, Propagation Model	1 each	J2172
Control Antibody, Anti-OX40	50µg	K1191
TIGIT Negative Cells	1 each	J1921
PD-1+TIGIT Combination Bioassay	1 each	J2211
PD-1+TIGIT Combination Bioassay 5X	1 each	J2215
Control Ab, Anti-TIGIT	100µg	J2051
TIM-3 Bioassay	1 each	JA2211
TIM-3 Bioassay 5X	1 each	JA2215
TIM-3 Bioassay, Propagation Model	1 each	JA2222
4-1BB Bioassay	1 each	JA2351
4-1BB Bioassay 5X	1 each	JA2355
4-1BB Bioassay, Propagation Model	1 each	J2332
Control Ab, Anti-4-1BB	50µg	K1161

Note: Additional Bioassays are available from Promega Custom Assay Services. To view and order products from Custom Assay Services see Early Access Bioassays at:
www.promega.com/applications/biologics-drug-discovery/functional-bioassays/target-pathway-assays/
or email: **CAS@promega.com**

Detection Reagents

Product	Size	Cat.#
Bio-Glo™ Luciferase Assay System	10ml	G7941
	100ml	G7940

Not for Medical Diagnostic Use.

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000

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