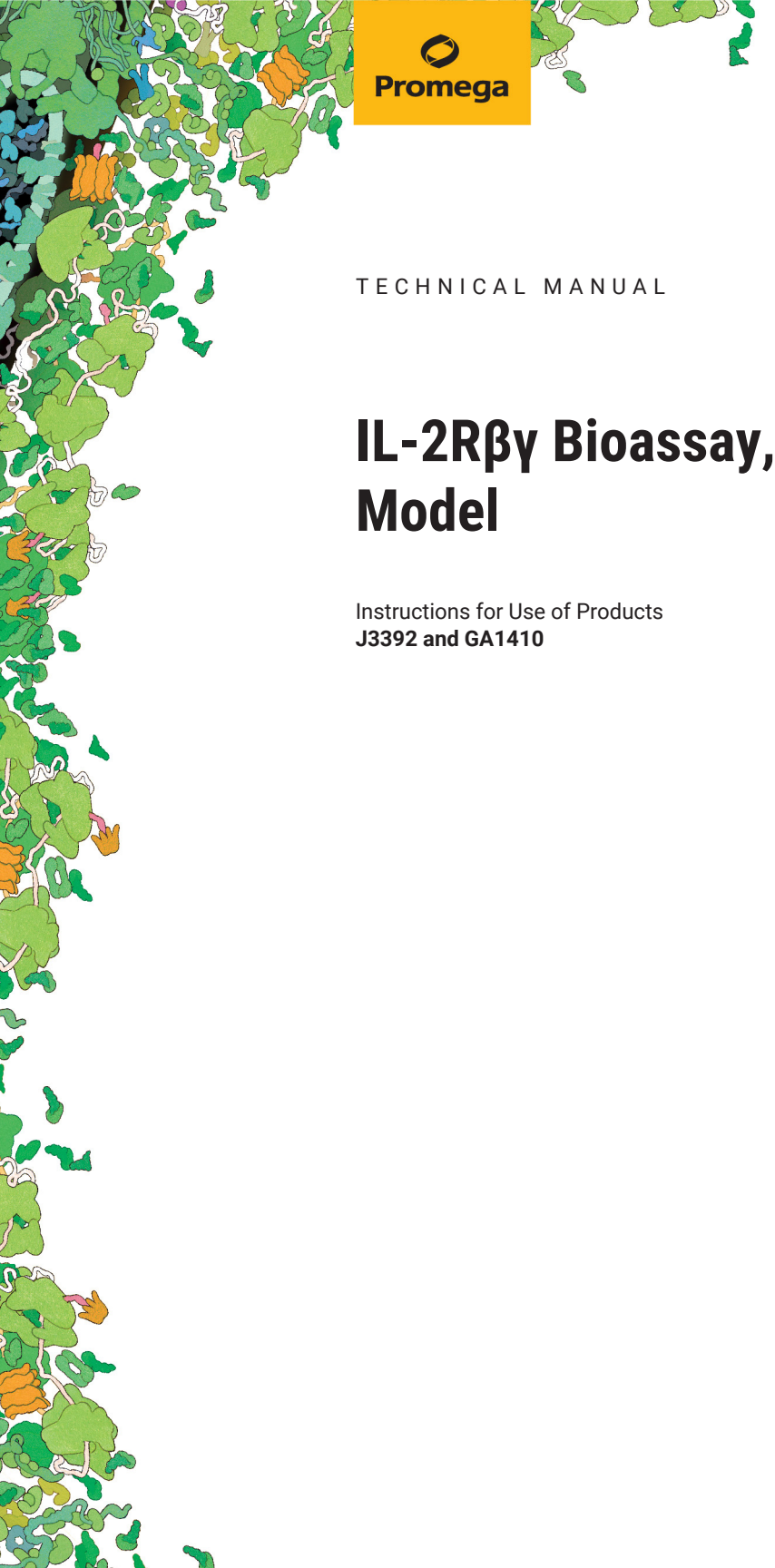


TECHNICAL MANUAL

IL-2Rβγ Bioassay, Propagation Model

Instructions for Use of Products
J3392 and GA1410

2/24
TM727

IL-2Rβγ Bioassay, 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

Promega

1. Description

The IL-2 receptor (IL-2R) is a type 1 cytokine receptor consisting of three subunits: IL-2Rα (CD25), IL-2/IL-15Rβ (CD122) and IL-2Rγ (CD132). The high-affinity (K_d ~10^{-11}M) trimeric receptor form consists of all three subunits and is found on regulatory T cells (Treg), activated CD4 and CD8 effector T cells and some natural killer (NK) cells, as well as various endothelial cell types. A medium-affinity (K_d ~10^{-9} M) heterodimeric receptor form, containing only IL-2Rβ and IL-2Rγ, is found on CD8+ effector memory T cells and most NK cells. IL-2Rγ is also known as the γ common chain (γc) and is found in receptors for IL-4, IL-7, IL-9, IL-15 and IL-21 (1).

High-dose recombinant IL-2 therapy (aldesleukin; trade name Proleukin) was approved in the 1990s for metastatic renal cell carcinoma and metastatic melanoma. Initially this therapy regime was thought to expand only patient effector T cells and NK cells. However, it was later discovered that high-dosage IL-2 promoted the expansion and development of immunosuppressive Treg cells, which are generally regarded as undesirable in the tumor microenvironment. In contrast, today low-dose regiments are being investigated for treatment of a variety of autoimmunity diseases, as Treg cells are involved in self-antigen tolerance. To avoid the pitfalls and side effects associated with high-dose IL-2 (short half-life, vascular leakage syndrome, hypotension and liver toxicity), other IL-2 and IL-15 molecules are in development that target the medium affinity receptor and the cell types that express it (2). For cancer therapeutic purposes, avoiding the high affinity receptor cell types (Treg and vascular and lung endothelial) and increasing the CD8-to-Treg ratio may enhance a positive and safer response.

IL-15 is a functionally related cytokine. However, unlike IL-2, it has no effect on Treg cells. IL-15 also has a trimeric receptor form, IL-15Rα (CD215), which contains a structural binding sushi domain at the N terminus, IL-2/IL-15Rβ (CD122) and IL-15Rγ (CD132). IL-15 can bind and signal effector cells in three ways. It predominantly signals in membrane form for a wide variety of cells that express both IL-15 and IL-15Rα, including monocyte, macrophage and dendritic cells (3). In this scenario, IL-15Rα chaperones IL-15 from the endoplasmic reticulum to the surface where it can trans-present to a variety of IL-15Rβγ-expressing cells (NK, CD8+ T, NKT and B cells; 4). A soluble form of the heterodimer receptor/cytokine complex can also be secreted for cytokine presentation to IL-15Rβγ-expressing effector cells (5). Finally, although monomeric IL-15 is rarely detected, it can bind directly to the IL-15Rβγ, but with lower affinity (6).

Multiple pathways can be activated by IL-2/IL-15 signaling. In lymphocytes, JAK/STAT signaling begins with JAK1 and JAK3 tyrosine kinase recruitment and activation at the receptor cytoplasmic domains. These kinases recruit and activate STAT3 and 5 with phosphorylated dimer/tetramer translocation to the nucleus for transcriptional activation of a variety of proteins, including Bcl-2, c-myc, c-fos and c-jun. In a second pathway, Shc adapter protein recruitment to the IL-2/IL-15Rβ subunit occurs with activation of Grb2. Grb2 is in the PI3K pathway and can ultimately phosphorylate Akt, or it can activate RAS-RAF and finally MAPK. These pathways affect cell proliferation, anti-apoptotic survival and cytotoxic effector functions.

The IL-2Rβγ Bioassay, Propagation Model[(a–c)] (Cat.# J3392) is a bioluminescent cell-based assay designed to measure human IL-2Rβγ receptor stimulation or inhibition. The IL-2Rβγ Bioassay Cells are provided in a Cell Propagation Model (CPM) format, which includes cryopreserved cells that can be thawed, propagated and banked for long-term use (also offered in a thaw-and-use format; Cat.# JA5101, JA5105). Cell banks for the IL-2Rβγ Bioassay, Propagation Model (Cat.# GA1410) are also available.

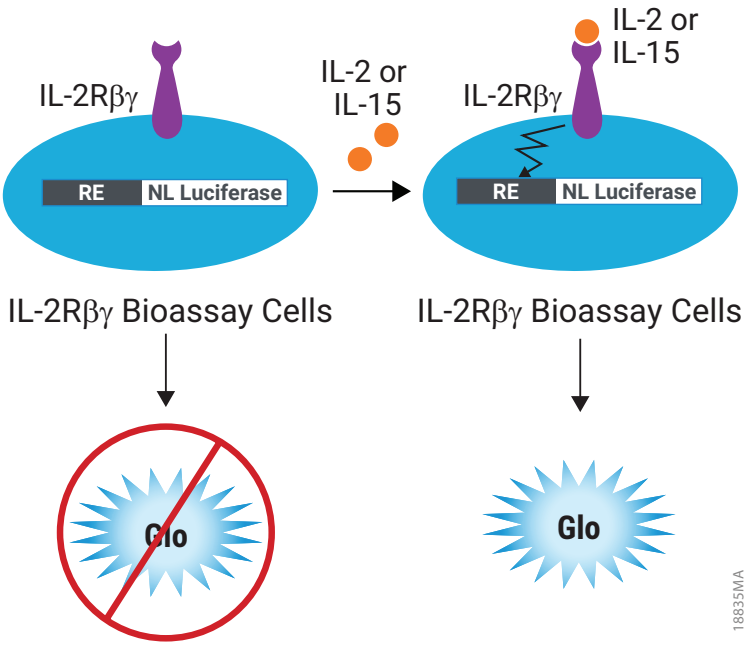
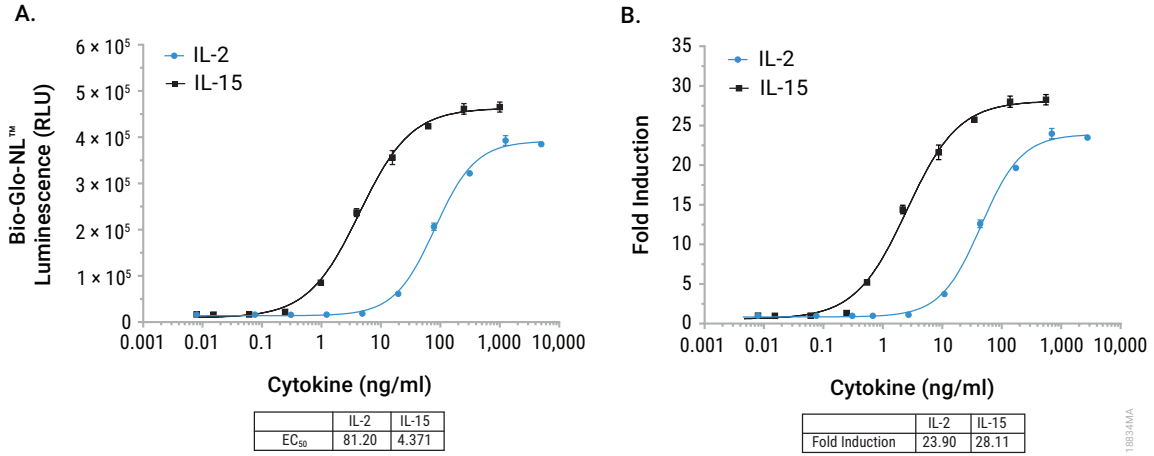


Figure 1. Representation of the IL-2Rβγ Bioassay. The IL-2Rβγ Bioassay consists of a genetically engineered cell line, IL-2Rβγ Bioassay Cells. When IL-2 or IL-15 bind to the medium affinity CD122/CD132 heterodimeric receptor complex (IL-2Rβγ), receptor-mediated pathway signaling induces luminescence that can be detected upon addition of Bio-Glo-NL™ Reagent (Cat.# J3081, J3082, J3083) and quantified with a luminometer. In the absence of cytokine, no signaling occurs downstream of the CD122/CD132 complex and a luminescent signal is not generated.

1. Description (continued)



	IL-2	IL-15
EC_{50}	81.20	4.371

	IL-2	IL-15
Fold Induction	23.90	28.11

Figure 2. The IL-2Rβγ Bioassay responds to recombinant IL-2 and IL-15. IL-2Rβγ Bioassay cells were prepared as described in this protocol and incubated with serial dilutions of recombinant IL-2 and IL-15. After a 6-hour incubation, Bio-Glo-NL™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. The IL-2 EC_{50} was 81ng/ml (**Panel A**), with a fold induction of approximately 24 (**Panel B**). The IL-15 EC_{50} was 4.4ng/ml with a fold induction of approximately 28. Data were generated using cells from culture. **Panel A** shows raw luminescence measurements. **Panel B** displays the calculated fold induction.

Table 1. The IL-2Rβγ Bioassay Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	90.3
	75	102.6
	125	105.7
	150	101.7
Repeatability (% CV)	100% (Reference)	3.6
Intermediate Precision (% CV)		8.4
Linearity (r^2)		0.996
Linearity (y = mx + b)		y = 1.08x–6.35

A 50–150% theoretical potency series of recombinant human IL-15 was analyzed in triplicate in three independent experiments performed on three days by two analysts (for a total of six independent experiments). Bio-Glo-NL™ 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)

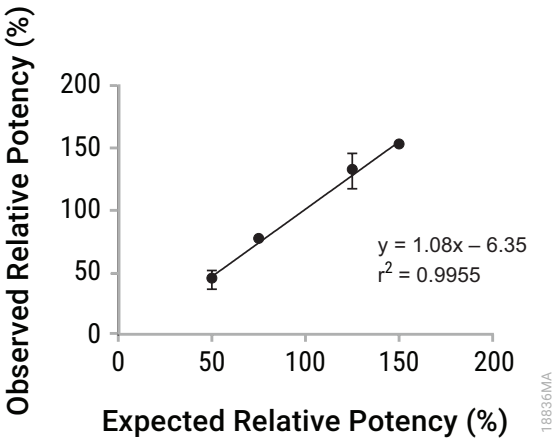
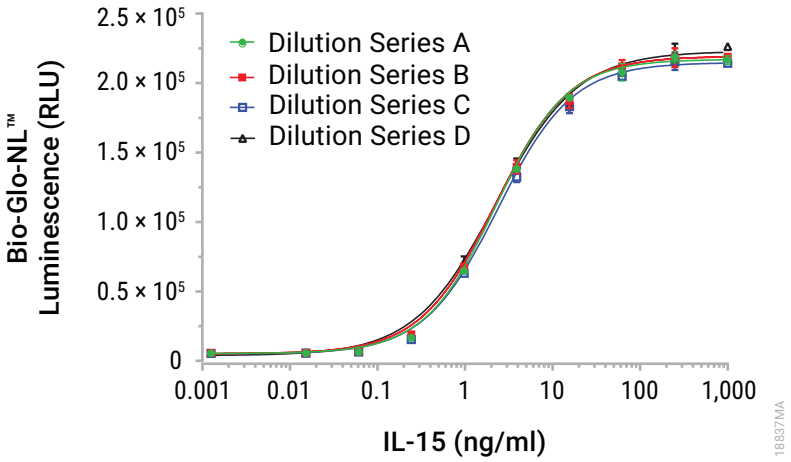


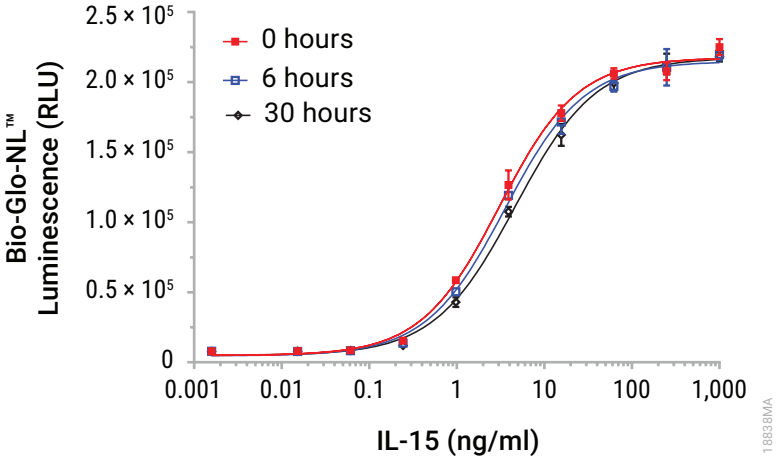
Figure 3. The IL-2Rβγ Bioassay shows precision, accuracy and linearity. A 50–150% theoretical potency series of recombinant human IL-15 was analyzed in triplicate in three independent experiments performed on three days by two analysts using the IL-2Rβγ Bioassay (for a total of six independent experiments). Bio-Glo-NL™ Reagent was added and luminescence 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.



	A	B	C	D
EC_{50}	2.389	2.356	2.555	2.411

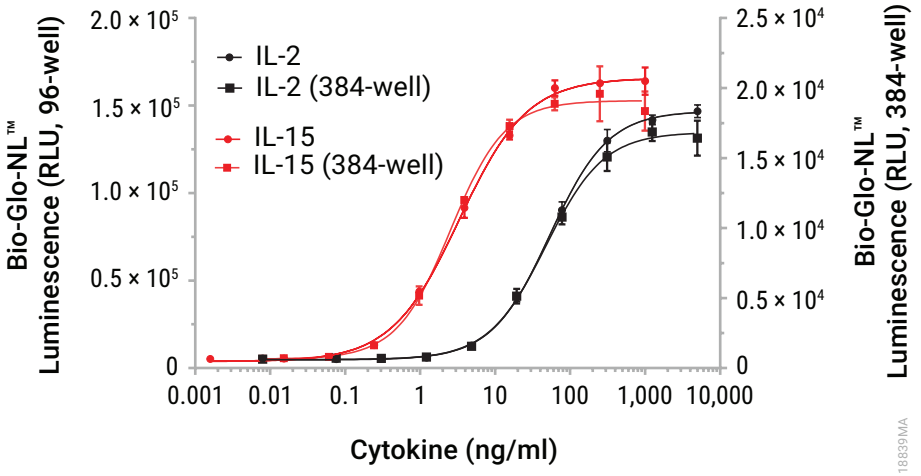
Figure 4. IL-2Rβγ Bioassay demonstrates repeatability. Four separate serial dilution series of recombinant human IL-15 (A, B, C and D) were analyzed on four individual assay plates using the IL-2Rβγ Bioassay. Bio-Glo-NL™ Reagent was added and luminescence quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. The EC_{50} values are shown for the four assay plates. Data were generated using thaw-and-use cells.

1. Description (continued)



Hours	0	6	30
EC_{50}	3.071	3.575	4.658

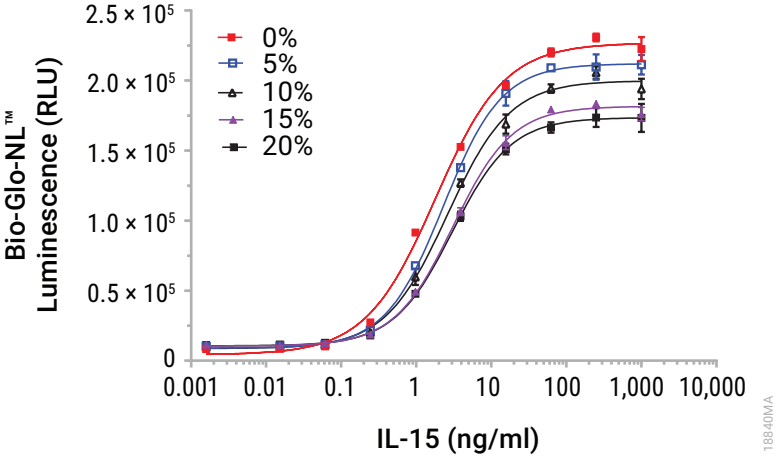
Figure 5. The IL-2Rβγ Bioassay indicates stability. Recombinant human IL-15 (15µg/ml with BSA carrier) was heat stressed at 53°C for 0–30 hours prior to being tested in the IL-2Rβγ Bioassay. Bio-Glo-NL™ Reagent was added and luminescence quantified using the GloMax® Discover System. Fold induction data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.



	IL-2	IL-15	IL-2 (384)	IL-15 (384)
EC_{50}	53.46	3.327	47.59	2.554

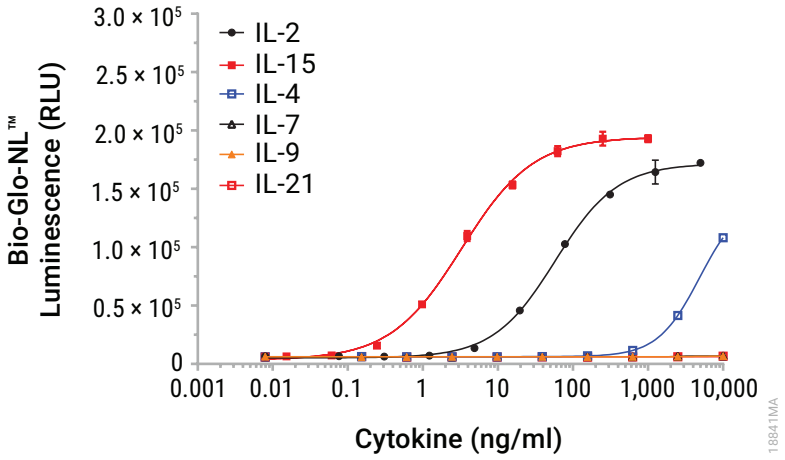
Figure 6. The IL-2Rβγ Bioassay is amenable to 384-well plate format. The IL-2Rβγ Bioassay was tested in 96- and 384-well formats. IL-2Rβγ Bioassay Cells were prepared and dispensed as 50µl (96-well) or 12.5µl (384-well) volumes. Serial fourfold dilutions of recombinant human IL-15 were prepared and added to cells (25µl/well in the 96-well format; 6.2µl/well in the 384-well format). After a 6-hour stimulation with recombinant IL-15, Bio-Glo-NL™ Reagent was added (75µl/well in the 96-well format; 18.7µl/well in the 384-well format) and luminescence quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells. The IL-2 EC_{50} was approximately 54 (96-well format) and 48ng/ml (384-well format) as shown in the table. The IL-15 EC_{50} was approximately 3.3ng/ml (96-well) and 2.6ng/ml (384-well).

1. Description (continued)



	0%	5%	10%	15%	20%
EC_{50}	1.796	2.307	2.610	3.100	3.029

Figure 7. Human serum impact on the IL-2Rβγ Bioassay. IL-2Rβγ Bioassay Cells were tested with a dose-response of recombinant IL-15 in the absence or presence of increasing concentrations of pooled normal human serum, resulting in 0–20% human serum final assay concentrations. Bio-Glo-NL™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.



	IL-2	IL-15
EC_{50}	58.37	3.304

Figure 8. IL-2Rβγ Bioassay cytokine specificity. IL-2Rβγ Bioassay cells were tested with a panel of related type 1 cytokines (IL-2, IL-15, IL-4, IL-7, IL-9 and IL-21). Bio-Glo-NL™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Some IL-4 response was detectable at high concentrations, but no signal was generated by IL-7, IL-9 or IL-21 in the assay. We could not calculate EC_{50} values for these related cytokines over the range of concentrations tested. Data were generated using thaw-and-use cells.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
IL-2Rβγ Bioassay, Propagation Model	**1 each**	**J3392**

Not for Medical Diagnostic Use. Includes:

- 2 vials IL-2Rβγ Bioassay Cells (CPM), 1.6 × 10^7 cells/ml (1ml per vial)

Note: Thaw and propagate one vial to create frozen cell banks for use in an assay. Reserve the second vial for future use.

PRODUCT	SIZE	CAT.#
IL-2Rβγ Bioassay, Cell Bank	**1 each**	**GA1410**

Not for Medical Diagnostic Use. Includes:

- 50 vials IL-2Rβγ Bioassay Cells (CPM), 1.6 × 10^7 cells/ml (1ml 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, such as Certificate of Analysis, from the web site.

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.

The IL-2Rβγ Bioassay is intended for use with user-provided biologics designed to activate or inhibit the IL-2Rβγ signaling pathway. The recommended cell plating density, induction time and assay buffer components described in Section 5 were established using research-grade recombinant human IL-2 and IL-15. You may need to adjust the parameters provided here and optimize assay conditions for other biologic samples. Data generated using these reagents is shown in Figure 2.

The IL-2Rβγ Bioassay produces a bioluminescent signal and requires a sensitive luminometer or multimode plate reader for detecting luminescence. Bioassay development and performance data included in this Technical Manual were generated using the GloMax® Discover System instrument. 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 (RLU) 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.

3.A. Materials to Be Supplied By the User

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

Reagents

- Recombinant human IL-15 (e.g., PeproTech Cat.# 200-15) or Human IL-2 (e.g., PeproTech Cat.# 200-02 or Miltenyi Cat.# 130-097-742)
- user-defined biologics samples
- RPMI 1640 medium (e.g., ATCC Cat.# 30-2001 or GIBCO™ Cat.# A10491-01)
- fetal bovine serum (FBS; e.g., Avantor Seradigm Cat.# 89510-194 or HyClone Cat.# SH30070)
- hygromycin B (e.g., GIBCO™ Cat.# 10687-010)
- blasticidin S (e.g., GIBCO™ Cat.# A1113903 or Invivogen Cat.# ant-bl)
- DMSO (Sigma Cat.# 2650)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- Bio-Glo-NL™ Luciferase Assay System (Cat.# J3081, J3082, J3083)

Note: The IL-2Rβγ Bioassay Cells, Propagation Model, uses the Bio-Glo-NL™ Luciferase Assay System (Cat.# J3081, J3082, J3083) for detection. **Do not** use the Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941).

Supplies and Equipment

- white, flat-bottom 96-well assay plate (e.g., Corning® Cat.# 3917)
- sterile clear 96-well plate with lid (e.g., Corning® Cat.# 3370 or Falcon Cat.# 353077) for preparing sample dilutions
- pipettes (single-channel and 12-channel)
- T75 tissue culture flask (e.g., Corning® Cat.# 430641U)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning® Cat.# 4870)
- humidified 37°C, 5% CO_2 incubator
- 37°C water bath
- plate reader that measures glow luminescence (e.g., GloMax® Discover System)

4. Preparing IL-2Rβγ Bioassay Cells

4.A. Cell Thawing and Initial Cell Culture

IL-2Rβγ Bioassay Cells are grown in suspension culture.

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

1. Prepare 75ml of thaw medium (see Section 8.A) and prewarm to 37°C. This medium will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of thaw medium into a 50ml conical tube.

4.A. Cell Thawing and Initial Cell Culture (continued)

3. Remove one vial of IL-2Rβγ Bioassay Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (do not invert cell vial) until just thawed (typically 2–3 minutes).
4. Spray the vial with 70% ethanol and transfer to cell culture hood.
5. Transfer all of the cells (approximately 1ml) to the 50ml conical tube containing 9ml of prewarmed thaw medium.
6. Centrifuge at 120 × *g* for 5 minutes.
7. Carefully aspirate the medium and resuspend the cell pellet in 25ml of prewarmed thaw medium.
8. Count cells by Trypan blue exclusion and determine cell number and viability.
9. Adjust to 3×10^5 cells/ml with additional thaw medium (final cell suspension volume will be approximately 60ml) and transfer the cell suspension evenly into two T75 flasks. Place the flasks horizontally in a humidified 37°C, 5% CO_2 incubator and incubate for 2 days.

4.B. Cell Maintenance and Propagation

For cell maintenance and propagation starting from the second cell passage, use growth medium containing selection antibiotic (see Section 8.A), and monitor cell viability and doubling rate during propagation. The cell growth rate will stabilize by approximately 7–10 days post-thaw. At this time, the cell viability is typically >90% and the average cell doubling rate is approximately 24 hours. Passage number should be recorded for each passage. Cells are expected to retain most of their functionality for up to 25 passages.

1. On the day of cell passage, observe cells under a light microscope and estimate confluency.
2. Triturate cells to create a single cell suspension.
3. Sample and count cells by Trypan blue exclusion.
4. Recommended density for passaging cells is as follows:
   a. For 2-day culture: 3×10^5 cells/ml
   b. For 3-day culture: 1.5×10^5 cells/ml

   Note: We recommend using the following media volumes for routine cell propagation: 25ml for a T75 flask, 50ml for a T150 flask and 75ml for a T225 flask. Scale accordingly for other flask surface areas.
5. Place the flasks horizontally in a humidified 37°C, 5% CO_2 incubator.

Do not allow cells to exceed 1.6×10^6 cells/ml, as they will rapidly deplete medium nutrients and begin to lose viability.

4.C. Cell Freezing and Banking

Note: We recommend making master and working cell banks at the earliest possible passage.

1. On the day of cell freezing, prepare new cell freezing medium (see Section 8.A) and keep on ice.
2. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of freezing medium needed based on desired cell freezing density. We recommend a freezing density range of 2×10^6–1×10^7 cells/ml.
3. Transfer cells to 50ml sterile conical tubes or larger-sized centrifuge tubes, and centrifuge at 120 × *g* for 10–15 minutes.
4. Gently aspirate the supernatant, being careful not to disturb the cell pellet.
5. Carefully resuspend the cell pellet in ice-cold freezing medium to desired final cell density. Combine the cell suspensions into a single tube and dispense 1ml into cryovials.
6. Freeze using a controlled-rate freezer (or use an insulated Mr. Frosty® or a Styrofoam® type of cell freezing container at –80°C overnight).
7. Transfer to –140°C or below for long-term storage.

5. Assay Protocol

The IL-2Rβγ Bioassay can be used to test IL-2- and IL-15-type biological samples that are known to bind and stimulate the IL-2Rβγ receptor. Recombinant human IL-2 or IL-15 can be used. This protocol illustrates the use of the IL-2Rβγ 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. Other experimental and plate layouts are possible but may require further optimization.

Notes:

a. 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 0–1µg/ml of recombinant human IL-15 (PeproTech Cat.# 200-15) or 0–5µg/ml of recombinant human IL-2 (PeproTech Cat.# 200-02) as a sample range with serial fourfold dilutions to achieve full dose curves as ten-point series. Concentration ranges and dilution schemes may need to be optimized for your samples.

b. While maintaining the IL-2Rβγ Bioassay Cells in culture, follow the recommended cell seeding density during routine propagation. Changes in cell culture volume or seeding density could affect subsequent assay performance. Only use cells in this assay after the doubling rate has stabilized during propagation. Use actively growing, healthy cells harvested as part of a routine 2- or 3-day passage; cell viability should be >90% prior to use in the assay.

5. Assay Protocol (continued)

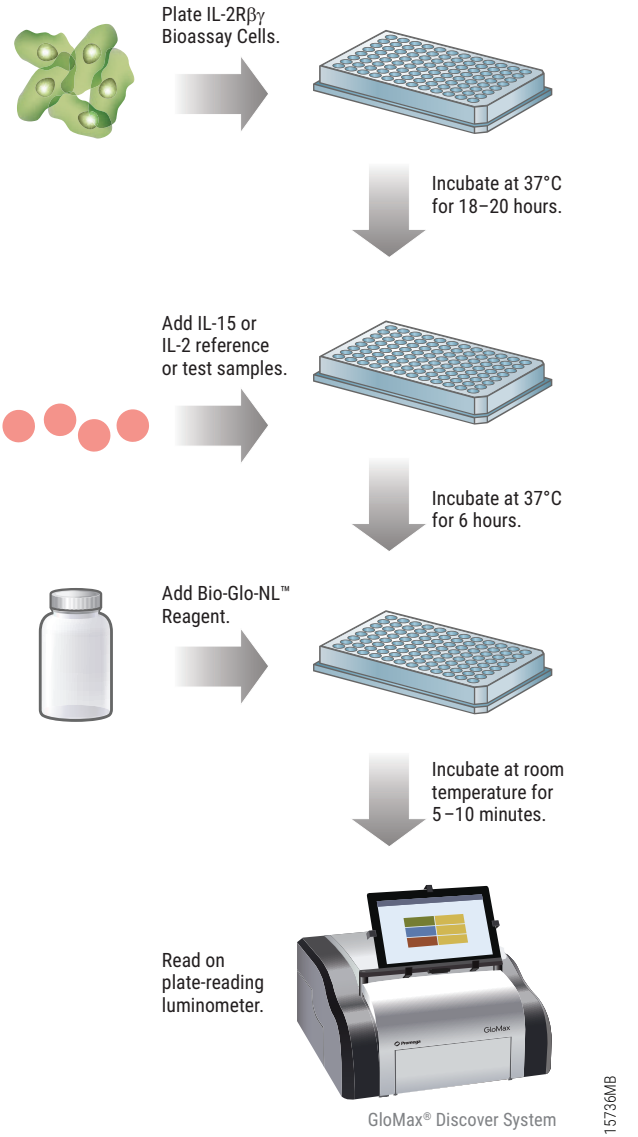


Figure 9. IL-2Rβγ Bioassay schematic protocol.

5.A. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 10 as a guide. The protocol describes serial replicate dilutions (n = 3) of test 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 10. 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.

5.B. Day 1: Preparing and Plating IL-2Rβγ Bioassay Cells

1. Prepare 50ml of assay buffer as described in Section 8.A and warm to 37°C before use.
2. Use actively growing, healthy cells, harvested during a routine 2- or 3-day passage. Triturate cells, sample and count by Trypan blue exclusion.
3. Based on the number of samples and plates, estimate the number of cells required and include 50–100% extra to account for loss during centrifugations. For each assay plate, a minimum of 1.8×10^6 cells will be required (3×10^4 cells/well × 60 wells).
4. Add cells to 50ml tubes and centrifuge at 120 × *g* for 10 minutes.
5. Remove supernatant.

6. Suspend cells in assay buffer to an estimated 2×10^6 cells/ml and count by Trypan blue exclusion.
7. Adjust to 6×10^5 cells/ml using additional assay buffer.
8. Dispense 50µl/well (3×10^4 cells/well) using the inner 60-wells of two 96-well plates. Add 75µl/well of assay buffer to the outer 36-wells.
9. Incubate 18–20 hours at 37°C, 5% CO_2.

Note: This bioassay can also be performed in a one-day format, where the cells are plated and stimulated within one day. Slightly lower fold induction may be observed.

5.C. Day 2 (Assay Day): Preparing Reagents, Test and Reference Samples

Prepare the following reagents before starting the assay.

Bio-Glo-NL™ Luciferase Assay Reagent: For reference, 10ml of Bio-Glo-NL™ Reagent is sufficient to assay 120 wells in a 96-well assay format. Store Bio-Glo-NL™ Luciferase Assay Substrate at –30°C to –10°C. Thaw the Bio-Glo-NL™ Luciferase Assay Buffer at room temperature (do not exceed 25°C) during the 6-hour assay induction period. We recommend preparing the reconstituted Bio-Glo-NL™ Reagent immediately before use. Do not store the reconstituted reagent. Once reconstituted, Bio-Glo-NL™ Reagent will lose 10% activity in approximately 8 hours at room temperature. For instructions on use of the Bio-Glo-NL™ Luciferase Assay System, please refer to the *Bio-Glo-NL™ Luciferase Assay System Quick Protoco*l, #FB227.

Note: The IL-2Rβγ Bioassay Cells, Propagation Model, are compatible only with Bio-Glo-NL™ Luciferase Assay Reagent. **Do not** use Bio-Glo™ Luciferase Assay Reagent with the IL-2Rβγ Bioassay.

Assay Buffer: Ensure that an appropriate amount of assay buffer is prepared for the assay. Thaw the fetal bovine serum (FBS) overnight at 4°C, or in a 37°C water bath, taking care not to overheat it. To make 50ml of assay buffer, add 5ml of FBS to 45ml of RPMI 1640 medium to yield 90% RPMI 1640/10% FBS (see Section 8.A). Mix well and warm to 37°C prior to use. For reference, 50ml of assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 wells.

Test and Reference Samples: Prepare starting dilutions (denoted as dilu1, 3X final concentration) of test and reference samples (see Figures 10 and 11). Using assay buffer as the diluent, prepare 360µl of reference sample starting dilution and 180µl of each test sample starting dilution in 1.5ml tubes. Store the tubes containing starting dilutions appropriately before making serial dilutions.

5.D. Preparing Serial Dilutions

Prepare serial dilutions on the day of the assay.

The instructions described here are for preparing a single stock of fourfold serial dilutions of a single sample for analysis in triplicate (135µl 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 fourfold serial dilutions, you will need a total of 360µl of a reference sample at 3X the highest concentration in your dose-response curve. You will need 180µl of each test sample at 3X the highest concentration in each of the test sample dose-response curves. For other dilution schemes, adjust the volumes accordingly.

Note: For IL-15 stimulation using recombinant human IL-15 as your reference sample (PeproTech IL-15, Cat.# 200-15), we recommend starting with a 3X concentration of 3µg/ml and performing serial fourfold dilutions. When using other reference sources of IL-15, the starting concentration may need to be adjusted.

1. To a sterile clear 96-well plate, add 180µl of reference sample starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 11).
2. Add 180µl of test samples 1 and 2 starting dilution (dilu1, 3X final concentration) to wells C11 and D11, respectively.
3. Add 135µl of assay buffer to other wells in these four rows, from column 10 to column 2.
4. Transfer 45µl of the sample starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
5. Repeat equivalent fourfold serial dilutions across the columns from right to left until you reach column 3. Remove 45µl from column 3 so all wells have 135µl volume. Do not dilute into column 2.
6. Cover the plate with a lid and set aside.

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

Figure 11. Example plate layout showing reference and test sample serial dilutions. Wells A2, B2, C2 and D2 contain 135µl of assay buffer without sample as a negative control.

5.E. IL-15 Stimulation Assay

1. Using a multichannel pipette, dispense 25µl of each sample to the 50µl of preplated cells according to the plate layout in Figure 10.
2. Cover each assay plate with a lid and incubate in a humidified 37°C, 5% CO_2 incubator for 6 hours.
3. After a 6-hour incubation, proceed to Section 5.F.

5.F. Preparing and Adding Bio-Glo-NL™ Reagent

We recommend preparing the Bio-Glo-NL™ Luciferase Assay Reagent immediately before use. Ensure that the Bio-Glo-NL™ Luciferase Assay Buffer is equilibrated to room temperature (do not exceed 25°C) before reconstituting the reagent. Do not store the reconstituted reagent. Once reconstituted, Bio-Glo-NL™ Reagent will lose 10% activity in approximately 8 hours at room temperature.

1. Remove the Bio-Glo-NL™ Luciferase Assay Substrate from –30°C to –10°C storage. Briefly centrifuge the tubes if the substrate has collected in the cap or on the sides of the tubes.
2. Prepare the desired amount of reconstituted Bio-Glo-NL™ Reagent by combining one volume of substrate with 50 volumes of buffer. For example, if the experiment requires 10ml of reagent, add 200µl of substrate to 10ml of buffer. Ten milliliters (10ml) of Bio-Glo-NL™ Reagent is sufficient for 120 wells (two assay plates, using the inner 60 wells of each plate).
3. Remove the assay plates from the incubator, remove the plate lid, and equilibrate to ambient temperature for 15 minutes.
4. Using a multichannel pipette, add 75µl of Bio-Glo-NL™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
5. Add 75µl of Bio-Glo-NL™ Reagent to wells B1, C1 and D1 of each assay plate to measure the background signal.
6. 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 EC_{50} value and fold induction.
7. Measure luminescence using a luminometer or luminescence plate reader.

5.G. Data Analysis

1. Measure plate background by calculating the average relative light units (RLU) from wells B1, C1 and D1.
2. Calculate fold induction:

$$\text{Fold induction} = \frac{\text{RLU (sample–background)}}{\text{RLU (no drug control–background)}}$$

   Note: When calculating fold induction, if the no drug control sample RLU values are at least 100X the plate background RLU values, 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 response using appropriate curve fitting software (such as GraphPad Prism®).

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	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 values will vary between instruments. 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 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-NL™ Reagent leads to low RLU. Store and handle the Bio-Glo-NL™ Reagent according to the instructions.
	Ensure that you are using Bio-Glo-NL™ Reagent, which is designed for NanoLuc® Luciferase reporter bioassays. IL-2Rβγ Bioassay Cells are not compatible with Bio-Glo™ Reagent, which is designed for firefly luciferase reporter bioassays.
Variability in assay performance	Ensure that incubation times are consistent between assays.
	Ensure that the preculture protocol is strictly followed for either a 2- or 3-day incubation period.
	Cells must be treated the same way prior to the assay for each assay. Variability in cell growth rates and preculture plating densities will result in variable assay results.
	Ensure cells are counted in a consistent manner.
Weak assay response (low fold induction)	Ensure starting cell viability of plated cells is >90%.
	If untreated control RLU is less than 100-fold above plate reader background RLU, subtract plate reader background RLU from all samples prior to calculating fold induction.

7. References

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3. Castillo, E.F. and Schluns, K.S. (2012) Regulating the immune system via IL-15 transpresentation. Cytokine **59**, 479–90.
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5. Bergamaschi, C. *et al.* (2012) Circulating IL-15 exists as heterodimeric complex with soluble IL-15Rα in human and mouse serum. *Blood* **120**, e1–8.
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8. Appendix

8.A. Composition of Buffers and Solutions

thaw medium

90% RPMI 1640
10% fetal bovine serum

Prepare and use within 10 days. Store at 4°C.

growth medium

90% RPMI 1640
4µg/ml blasticidin
550µg/ml hygromycin B
10% fetal bovine serum

Prepare and use within 10 days. Store at 4°C.

freezing medium

70% RPMI 1640
20% fetal bovine serum
10% DMSO

Prepare and keep at 4°C during use.

assay buffer

90% RPMI 1640
10% fetal bovine serum

Prepare and use within 5 days. Store at 4°C.

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8.B. 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 RANKL Target Cells**	1 each	J3381

*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 and sizes are available.

Macrophage-Directed Bioassays

Product	Size	Cat.#
SIRPα/CD47 Blockade Bioassay	1 each	JA6011
SIRPα/CD47 Blockade Bioassay, Fc-Dependent	1 each	JA4801
TLR Bioassay	1 each	JA9011
ADCP Reporter Bioassay (THP-1)	1 each	JA9411

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

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8.B. Related Products (continued)

T Cell Activation Bioassays

Product	Size	Cat.#
T Cell Activation Bioassay (IL-2)	1 each	J1651
T Cell Activation Bioassay (NFAT)	1 each	J1621
T Cell Activation Bioassay (TCRαβ-KO, CD4+)	1 each	GA1172
T Cell Activation Bioassay (TCRαβ-KO, CD8+)	1 each	GA1162
T Cell Activation Bioassay (TCRαβ-KO, CD4+, CD8+)	1 each	GA1182

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

Cytokine and Growth Factor Bioassays

Product	Size	Cat.#
IL-2 Bioassay	1 each	JA2201
IL-6 Bioassay	1 each	JA2501
IL-12 Bioassay	1 each	JA2601
IL-15 Bioassay	1 each	JA2011
IL-23 Bioassay	1 each	JA2511
RANKL Bioassay	1 each	JA2701
VEGF Bioassay	1 each	GA2001

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-CD40	50µg	K1181
Control Ab, Anti-CTLA-4	100µg	JA1020
Control Ab, Anti-LAG-3	100µg	K1150
Control Ab, Anti-OX40	50µg	K1191
Control Ab, Anti-PD-1	100µg	J1201
Control Ab, Anti-SIRPα	50µg	K1251
Control Ab, Anti-TIGIT	100µg	J2051
Control Ab, Anti-TIM-3	100µg	K1210
Recombinant VEGF ligand	10µg	J2371

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, Macrophage and Target Cell Killing Bioassays are available. To view and order Promega Bioassay products visit: **www.promega.com/products/reporter-bioassays/** or e-mail: **EarlyAccess@promega.com.** For information on custom bioassay development and services visit the Promega Tailored R&D Solutions web site: **www.promega.com/custom-solutions/tailored-solutions/**

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[b]U.S. Pat. Nos. 8,557,970, 8,669,103, 9,777,311, 9,840,730, 9,951,373, 10,233,485, 10,633,690, 10,774,364, 10,844,422, 11,365,436, 11,661,623, 11,667,950; European Pat. Nos. 2456864, 2635595, 2990478, 3181687, 3409764; Japanese Pat. Nos. 6038649, 6155424, 6227615, 6374420, 6539689; and other patents and patents pending.

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