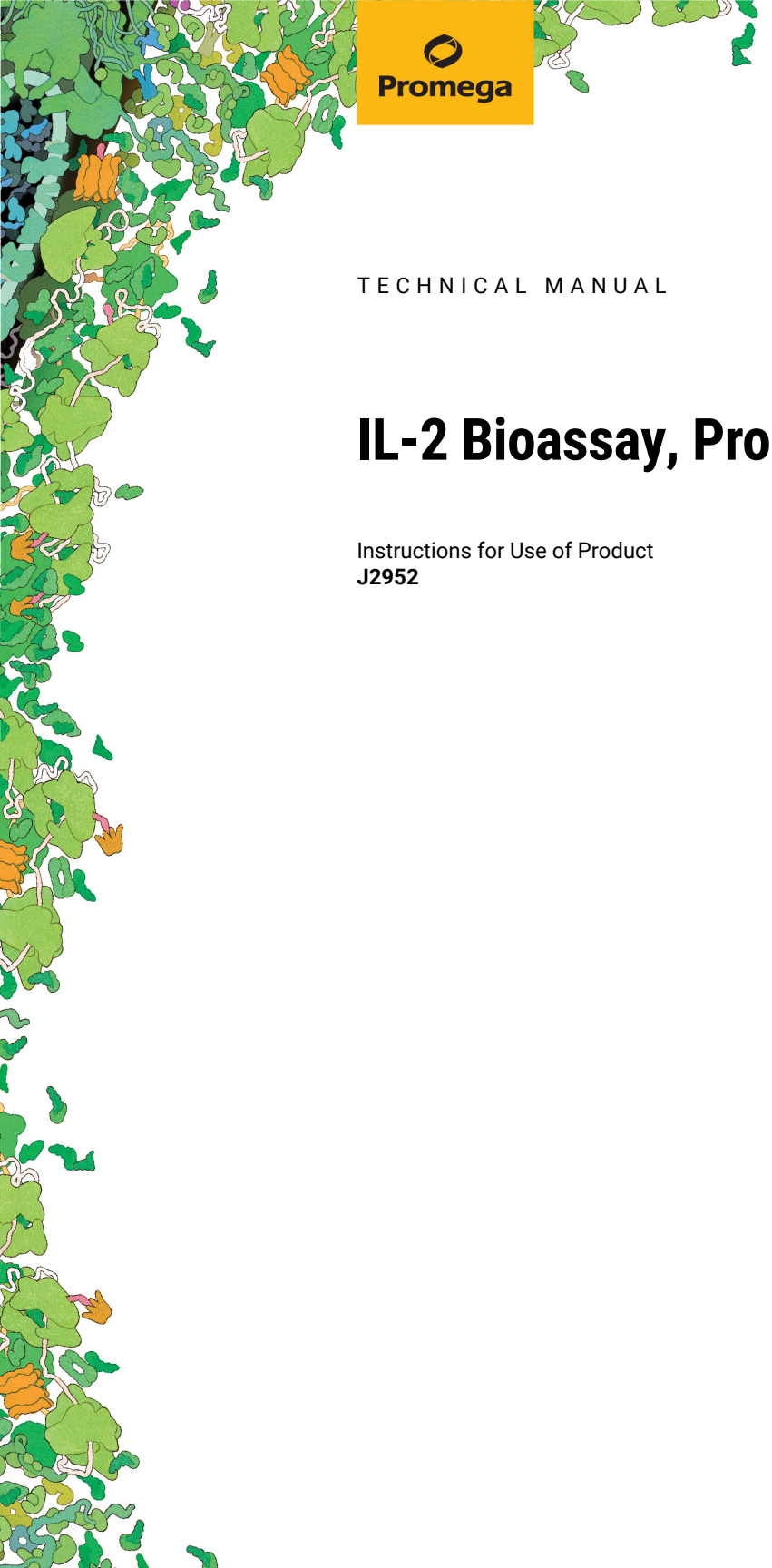


TECHNICAL MANUAL

IL-2 Bioassay, Propagation Model

Instructions for Use of Product
J2952

Revised 5/26
TM572

Promega

IL-2 Bioassay, Propagation Model

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

1. Description

Interleukin-2 (IL-2), originally described as "T cell growth factor" in 1976, is a small 15.5kDa monomer secreted by a variety of cell types including CD4+ and CD8+ T cells, natural killer (NK) cells and activated dendritic cells (1,2). IL-2 has pleiotropic effects on the immune system. It plays a critical role in the generation, maintenance and expansion of CD4+ regulatory T cells, promotes the cytotoxic activity of NK and CD8+ cells and governs homeostasis through the elimination of harmful autoreactive T cells via activation-induced cell death.

Aldesleukin, a biologic based on human IL-2 with minor sequence modifications, is FDA approved for the treatment of both metastatic renal cell carcinoma (1992), and metastatic melanoma (1998) as a monotherapy (3). Its antitumor efficacy is achieved by increasing proliferation of NK cells, lymphokine-activated killer cells and other cytotoxic cells. Though initially promising, its current clinical usage is limited due to a variety of severe side effects at high dosages. Modern therapeutic strategies involving IL-2 that attempt to overcome the limitations of aldesleukin, include combination therapies (cell-based immunotherapy, chemotherapeutic agents, peptide vaccines and checkpoint inhibitors) and formats with sustained or targeted activity (immunocytokine, immunocomplex, PEGylation and protease activated; 4).

The IL-2 receptor (IL-2R) is a type 1 cytokine receptor consisting of 3 subunits: IL-2Rα (CD25), IL-2Rβ (CD122) and IL-2Rγ (CD132). 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. The IL-2 trimeric receptor form consists of all 3 subunits and has the highest affinity for IL-2 (K_d ~10^{-11}M). A heterodimeric form, containing only IL-2Rβ and γc, has medium affinity (K_d ~10^{-9}M), while the IL-2Rα alone has low affinity (K_d ~10^{-8}M) and lacks a cytoplasmic signaling domain (5). Various forms of the IL-2 receptors are located on T cells, B cells and NK cells. The highly expressed IL-2Rα subunit is responsible for the initial binding and conformation change of IL-2 prior to its association with the β and γ subunits and subsequent intracellular signaling.

When IL-2 is bound to either the dimeric or trimeric receptor forms, multiple signaling pathways are activated. JAK1 and JAK3 tyrosine kinases are recruited and activated at the receptor cytoplasmic domains. These kinases, in turn, activate the STAT, PI3-AKT and MAPK signaling pathways, which then mediate proliferation, survival, activation and differentiation in a multitude of immune cell types (3).

The IL-2 Bioassay, Propagation Model[a,b] (Cat.# J2952) is a bioluminescent cell-based assay designed to measure IL-2 stimulation or inhibition. The IL-2 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.# JA2201, JA2205).

A representation of the IL-2 Bioassay, Propagation Model, is shown in Figure 1.

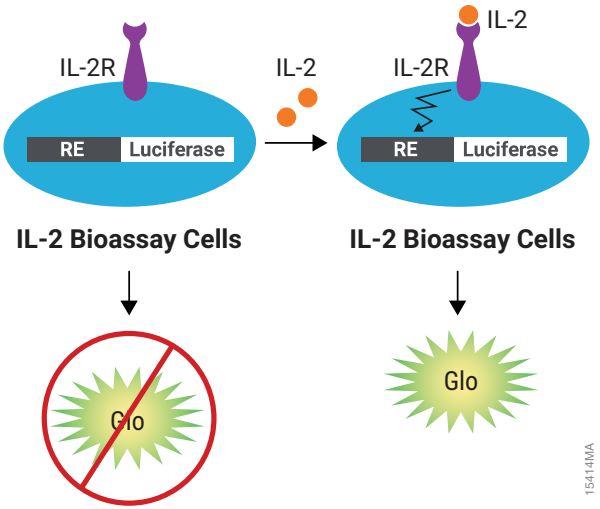
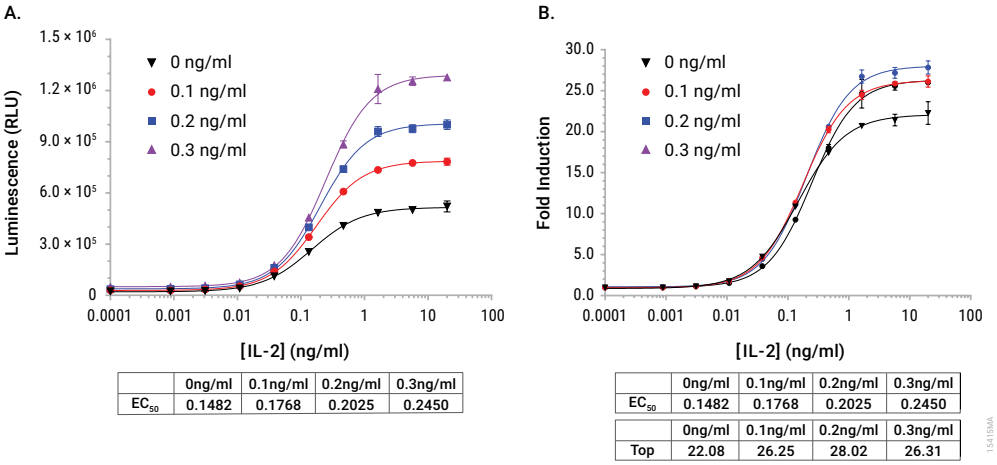


Figure 1. Representation of the IL-2 Bioassay, Propagation Model. The IL-2 Bioassay consists of a genetically engineered cell line, IL-2 Bioassay Cells. When IL-2 binds to its receptor (IL-2R), receptor-mediated pathway signaling induces luminescence that can be detected upon addition of Bio-Glo™ Reagent and quantified with a luminometer. In the absence of IL-2 no signaling occurs downstream of IL-2R and no luminescent signal is generated.

1. Description (continued)



	0ng/ml	0.1ng/ml	0.2ng/ml	0.3ng/ml
EC_{50}	0.1482	0.1768	0.2025	0.2450

	0ng/ml	0.1ng/ml	0.2ng/ml	0.3ng/ml
EC_{50}	0.1482	0.1768	0.2025	0.2450

	0ng/ml	0.1ng/ml	0.2ng/ml	0.3ng/ml
Top	22.08	26.25	28.02	26.31

Figure 2. The IL-2 Bioassay is responsive to recombinant IL-2. IL-2 Bioassay Cells were grown and prepared as described in this protocol and incubated with serial dilutions of recombinant IL-2. After a 6-hour incubation, Bio-Glo™ 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 CPM cells starved for 18 hours with 0–0.3ng/ml of IL-2. **Panel A** shows raw luminescence measurements. **Panel B** displays the calculated fold induction.

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

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	103.9
	70	99.8
	140	100.5
	200	101.6
Repeatability (% CV)	100% (Reference)	4.32
Intermediate Precision (% CV)		6.88
Linearity (r^2)		0.999
Linearity (y = mx + b)		y = 1.01x + 0.09

A 50–200% theoretical potency series of aldesleukin (therapeutic IL-2) 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 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.

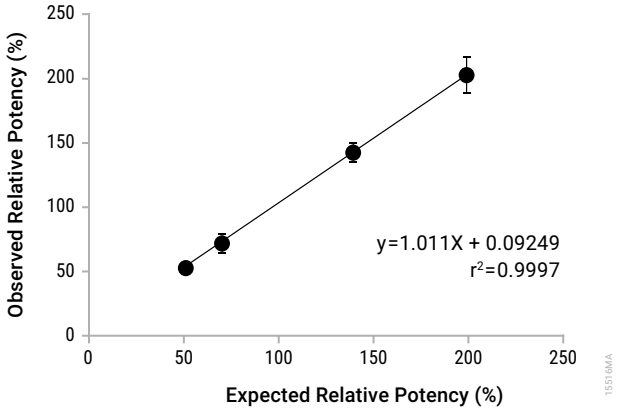


Figure 3. The IL-2 Bioassay shows precision, accuracy, and linearity. A 50–200% theoretical potency series of aldesleukin (therapeutic IL-2) was analyzed in triplicate in three independent experiments performed on three days by two analysts using the IL-2 Bioassay (for a total of six independent experiments). 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)

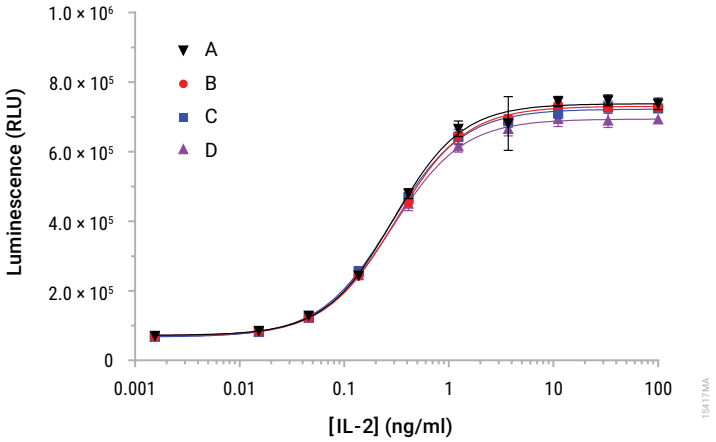


Figure 4. The IL-2 Bioassay demonstrates repeatability. Four separate serial dilution series of aldesleukin (therapeutic IL-2) were analyzed on four individual assay plates using the IL-2 Bioassay. Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. The EC_{50} values were 0.306, 0.309, 0.284 and 0.287ng/ml on the four assay plates. Data were generated using thaw-and-use cells.

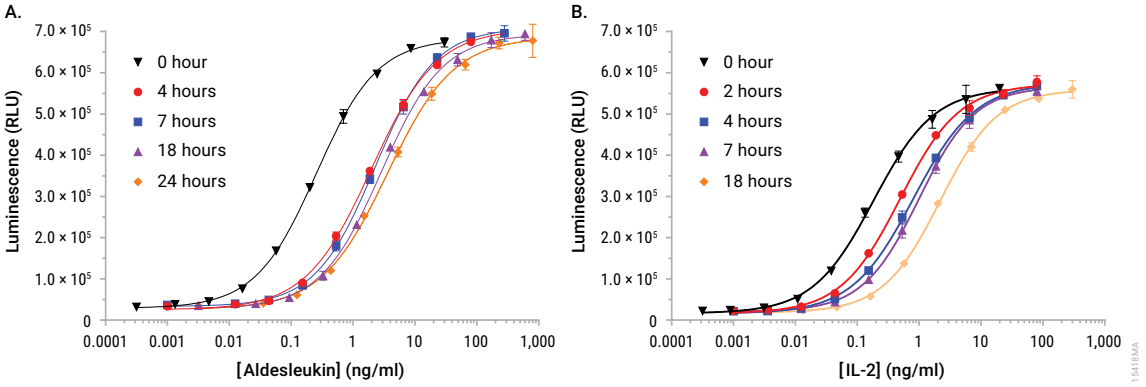


Figure 5. The IL-2 Bioassay indicates stability. Proleukin ® (aldesleukin) (**Panel A**, Cinigen, 1.1mg/ml undiluted) or a research grade equivalent (**Panel B**, Miltenyi Cat.# 130-097-742, 20µg/ml with BSA carrier) was heat stressed at 53°C hours prior to being tested in the IL-2 Bioassay. Bio-Glo™ 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.

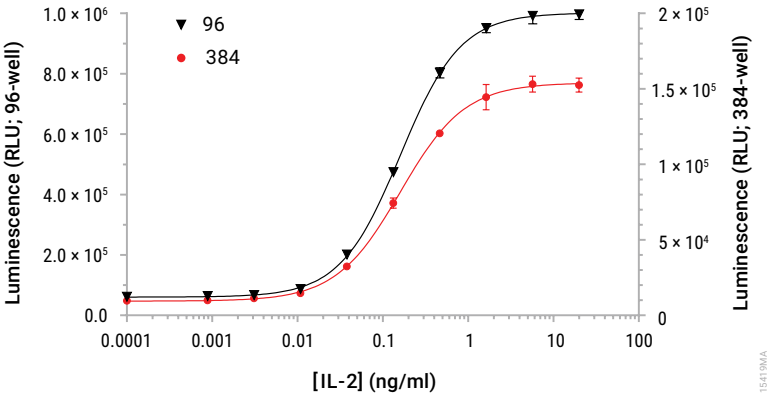
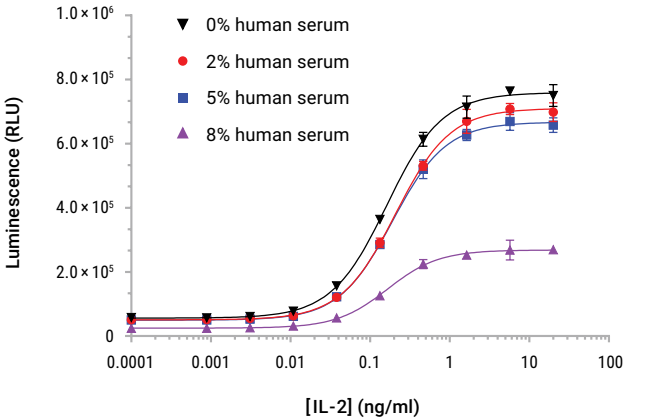


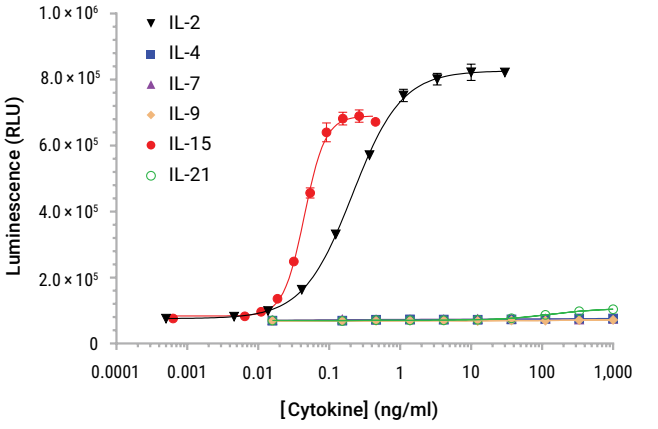
Figure 6. The IL-2 Bioassay is amenable to 384-well plate format. The IL-2 Bioassay was tested in 96- and 384-well formats. IL-2 Bioassay Cells were prepared and dispensed as 50µl (96-well) or 12.5µl (384-well) volumes. Serial 3.5-fold dilutions of recombinant IL-2 were prepared and added to cells (25µl/well for 96-well plates; 6.2µl/well for 384-well plates). After 6 hours of stimulation with recombinant IL-2, Bio-Glo™ Reagent was added (75µl/well for 96-well plates; 18.7µl/well for 384-well plates), 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. The IL-2 EC_{50} was 0.16ng/ml for both plate formats.

1. Description (continued)



	0%	2%	5%	8%
EC_{50}	0.1622	0.2085	0.1913	0.1659

Figure 7. The IL-2 Bioassay tolerates up to 5% human serum. IL-2 Bioassay Cells were tested with a dose-response of recombinant IL-2 in the absence or presence of increasing concentrations of pooled normal human serum, resulting in final assay concentrations of human serum (0–8%). Bio-Glo™ 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}	0.2118	0.04461

15421MA

Figure 8. IL-2 Bioassay cytokine specificity. IL-2 Bioassay Cells were tested with a panel of related type 1 cytokines (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21). Bio-Glo™ 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.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
IL-2 Bioassay, Propagation Model	**1 each**	**J2952**

Not for Medical Diagnostic Use. Includes:

- 2 vials IL-2 Bioassay Cells (CPM), 1.8 × 10^7 cells/ml (0.75ml per vial)

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

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.

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3. Before You Begin

Please read through the entire protocol to become familiar with the components and the assay procedure before beginning.

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

Cell thawing, propagation and banking should be performed exactly as described in Section 3.B. 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-2 Bioassay is intended to be used with user-provided biologics designed to activate or inhibit the IL-2 signaling pathway. The recommended cell plating density, induction time and assay buffer components described in Section 4 were established using research-grade recombinant human IL-2. 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 IL-2 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 seconds/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.

3.A. Materials to Be Supplied by the User

Composition of buffers and solutions is provided in Section 7.A.

Reagents

- Recombinant human IL-2 (e.g., PeproTech Cat.# 200-02 or Miltenyi Cat.# 130-097-742)
- User-defined biologics samples
- MEM nonessential amino acids (e.g., Corning® Cat.# 25-025-CI)
- DMSO (e.g., Sigma Cat.# D2650
- Iscove's DMEM (with glutamine) (e.g., Corning® Cat.# 10-016-CV)
- glutamine (e.g., Corning® Cat.# 25-005-CI)
- sodium pyruvate (e.g., Corning® Cat.# 25-000-CI)
- fetal bovine serum (e.g., HyClone Cat.# SH30070)
- hygromycin B (e.g., GIBCO® Cat.# 10687-010)
- 100mM acetic acid (sterile) (e.g., Sigma Cat.# A6283)
- D-PBS (e.g., Invitrogen Cat.# 14190144)
- bovine serum albumin, fraction V crystalline (e.g., Calbiochem Cat.# 12657)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941)

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
- 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)
- 37°C, 5% CO_2 humidified incubator
- 37°C water bath
- plate reader that measures glow luminescence or luminometer (e.g., GloMax® Discover System)

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3.B. Preparing IL-2 Bioassay Cells

Cell Thawing and Initial Cell Culture

IL-2 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 70ml of thaw medium (see Section 7.A) and prewarm to 37°C. This medium will be used for culturing the cells immediately after thawing.
2. Transfer 8ml of thaw medium into a 15ml conical tube.
3. Remove one vial of IL-2 Bioassay Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed (typically 2–3 minutes).
4. Spray vial with 70% ethanol and transfer to cell culture hood.
5. Transfer all of the cells (approximately 0.75ml) to the 15ml conical tube containing 8ml of prewarmed thaw medium.
6. Centrifuge at 150 × *g* for 5 minutes.
7. Carefully aspirate the medium and resuspend the cell pellet in 20ml of prewarmed thaw medium in a 50ml conical tube.
8. Count cells with Trypan blue and determine cell number and viability.
9. Adjust to 2.5×10^5 cells/ml with additional thaw medium (final cell suspension volume will be approximately 50ml) and transfer the cell suspension evenly into two T75 flasks. Place the flasks horizontally in a 37°C, 5% CO_2 humidified incubator and incubate for 2 days.

   Note: Observe cells immediately after placing in flask, and again several hours later. Significant cell death and debris will be observed within 2 hours; this is normal upon thawing.

Cell Maintenance and Propagation

Note: For cell maintenance and propagation starting from the second cell passage, use growth medium containing selection antibiotic (see Section 7.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 >95% and the average cell doubling rate is approximately 15 hours. Passage number should be recorded for each passage. Cells are expected to retain their functionality for up to 35 passages.

10. Pipet cells to create a single cell suspension.
11. Sample and count by Trypan blue exclusion.
12. Recommended density for passaging cells is as follows:
    a. For 2-day culture: 7×10^4 cells/ml
    b. For 3-day culture: 3×10^4 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 according to surface area of flask.
13. Place the flasks horizontally in a 37°C, 5% CO_2 humidified incubator.

    Note: Do not allow cells to exceed 1×10^6 cells/ml, as they will rapidly deplete medium nutrients, IL-2 and begin to lose viability.

Cell Freezing and Banking

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

14. On the day of cell freezing, prepare new cell freezing medium (see Section 7.A) and keep on ice.
15. 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 2×10^6–2×10^7 cells/ml.
16. Transfer cells to 50ml sterile conical tubes or larger sized centrifuge tubes, and centrifuge cells at 150 × *g* for 10–15 minutes.
17. Gently aspirate the supernatant, being careful not to disturb the cell pellet.
18. Carefully resuspend 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.
19. Freeze using a controlled-rate freezer (or use an insulated Mr. Frosty® or a Styrofoam® type of cell freezing container at –80°C overnight).
20. Transfer to –140°C or below for long-term storage.

4. Assay Protocol

The IL-2 Bioassay can be used to test IL-2 type biological samples such as recombinant human IL-2 and aldesleukin (therapeutic IL-2). This protocol illustrates the use of the IL-2 Bioassay to examine two test samples against a reference sample in a single assay run (Figure 9). 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 (Figure 10). Other experimental and plate layouts are possible but may require further optimization.

Notes:

1. To become fully responsive, the IL-2 Bioassay Cells require an IL-2 starvation period (0–0.3ng/ml of IL-2 for 18–20 hours). A range of concentrations can be used depending on desired fold of induction response, EC_{50} and luminescence (see Figure 2).
2. 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 0–20ng/ml of recombinant IL-2 (Peprotech Cat.# 200-02) as a sample range, with serial 3.5-fold dilutions to achieve full dose curves as ten-point series. Concentration ranges and dilution schemes may need to be optimized for your samples.
3. While maintaining the IL-2 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. Culture viability should be >95% prior to use in IL-2 Bioassay.

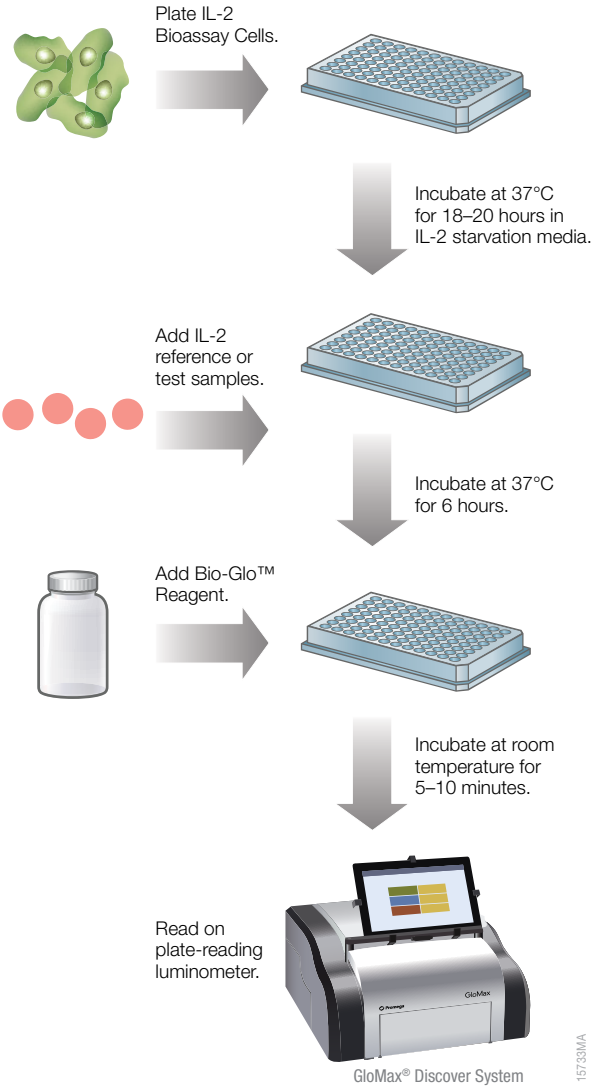

Figure 9. IL-2 Bioassay schematic protocol.

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4.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."

4.B. Day 1: Preparing and Plating IL-2 Bioassay Cells with IL-2 Starvation

1. Prepare an IL-2 working aliquot as described in Section 7.A.
2. Prepare 50ml of assay buffer as described in Section 7.A and warm to 37°C before use.
3. Use actively growing, healthy cells, harvested during a routine 2- or 3-day passage, pipet cells, sample and count by Trypan blue exclusion.
4. 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 2.4×10^6 cells are required (4×10^4 cells/well × 60 wells).
5. Place cells into 50ml centrifuge tubes and centrifuge at 150 × *g* for 10–15 minutes.
6. Remove supernatant. Carefully wash cell pellet with an equal volume of warm assay buffer.
7. Centrifuge cells at 150 × *g* for 10–15 minutes. Remove supernatant.
8. Suspend cells in assay buffer to an estimated 2×10^6 cells/ml and count by Trypan blue exclusion.
9. Adjust to 8×10^5 cells/ml using additional assay buffer.
10. Using the working aliquot of IL-2 (10–15µg/ml), prepare an intermediate dilution (100ng/ml) of IL-2 in assay buffer.
11. To the Bioassay cell suspension from Step 9, add a volume of diluted IL-2 to bring the final IL-2 concentration to 0–0.3ng/ml. Mix thoroughly. (See Figure 2 for example data regarding IL-2 starve concentration and response).

    Note: Activity of IL-2 varies by vendor. Appropriate starvation concentration may need to be adjusted depending on specific IL-2 used.
12. Dispense 50µl/well (4×10^4 cells/well) using the inner 60-wells of two 96-well plates. Add 75µl/well of assay buffer to the outer 36 wells.
13. Incubate 18–20 hours at 37°C, 5% CO_2.

    Note: This completes the low-dose IL-2 starvation required to generate IL-2 responsive cells.

4.C. Day 2: 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 of the Bio-Glo™ Luciferase Assay Buffer into the amber bottle containing the Bio-Glo™ Luciferase Assay Substrate and mix by inversion until the Substrate is thoroughly dissolved. Equilibrate and store the reconstituted Bio-Glo™ Reagent at ambient temperature (22–25°C) protected from light before adding to assay plates.

   If you are using a large (100ml) size of Bio-Glo™ Luciferase Assay System, dispense the reconstituted Bio-Glo™ Reagent into 10ml aliquots and store at –20°C for up to 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:** 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 supplemented Iscove's DMEM medium (IDMEM) to yield 90% IDMEM/ 10% FBS (see Section 7.A). Mix well and warm to 37°C prior to use. For reference, 30ml of assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 plate wells.

3. **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 350µl of reference sample starting dilution and 175µl 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

Serial dilutions should be prepared on the day of assay.

The instructions described here are for preparation of a single stock of 3.5-fold serial dilutions of a single sample for analysis in triplicate (125µl 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 3.5-fold serial dilutions, you will need a total of 350µl of a reference sample at 3X the highest concentration in your dose-response curve. You will need 175µl 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.

Notes on recommended starting concentration of reference samples:

For IL-2 stimulation using recombinant human IL-2 as your reference sample (PeproTech IL-2 Cat.# 200-02), we recommend starting with a 3X concentration of 60ng/ml and performing serial 3.5-fold dilutions. When using other reference sources of IL-2, the starting concentration may need to be adjusted.

1. To a sterile clear 96-well plate, add 175µl of reference sample starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 11).
2. Add 175µl of test samples 1 and 2 starting dilution (dilu1, 3X final concentration) to wells C11 and D11, respectively.
3. Add 125µl of assay buffer to other wells in these four rows, from column 10 to column 2.
4. Transfer 50µl of the sample starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
5. Repeat equivalent 3.5-fold serial dilutions across the columns from right to left until you reach column 3. Remove 50µl from column 3 so all wells have 125µl 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. Note: Wells A2, B2, C2 and D2 contain 125µl of assay buffer without sample as a negative control.

4.E. IL-2 Stimulation Assay

1. Using a multichannel pipette, dispense 25µl of each sample to the 50µl of preplated/prestarved cells according to the plate layout in Figure 10.
2. Cover each assay plate with a lid and incubate in a 37°C, 5% CO_2 humidified incubator for 6 hours.
3. After the 6-hour incubation is over, proceed to Section 4.F.

4.F. Adding Bio-Glo™ Reagent

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

1. Remove the assay plates from the incubator, remove the plate lid and equilibrate 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 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. 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 IL-2 response using appropriate curve fitting software (such as GraphPad Prism®).

5. 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	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.
	Starve cells with a higher concentration of IL-2 (see Figure 2).
	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 preculture protocol is strictly followed for either 2-day or 3-day incubation period.
	Cells must be treated the same way prior to assay for each assay. Variability in cell growth rates and preculture plating densities will result in variable assay results.
Weak assay response (low fold induction)	Ensure starting cell viability of plated cells is >95% prior to starvation.
	Ensure cells are washed with Assay Buffer without IL-2 prior to starvation.
	Ensure that the assay incubation period is 6 hours and not overnight. Overnight assay incubation does not work for this assay.
	Ensure that preculture protocol is followed exactly, and that cells are cells are doubling approximately every 16–18 hours.
	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.

6. References

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4. Boyman, O. and Arenas-Ramirez, N. (2019) Development of a novel class of interleukin-2 immunotherapies for metastatic cancer. *Swiss Med. Wkly*. **149**, w14697.
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7. Appendix

7.A. Composition of Buffers and Solutions

thaw medium

87%	Iscove's DMEM (with glutamine)
1%	MEM nonessential amino acids
2mM	glutamine
1mM	sodium pyruvate
40ng/ml	IL-2 (recombinant human)
10%	fetal bovine serum

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

freeze medium

67%	Iscove's DMEM (with glutamine)
1%	MEM nonessential amino acids
2mM	glutamine
1mM	sodium pyruvate
20ng/ml	IL-2 (recombinant human)
20%	fetal bovine serum
10%	DMSO

Prepare fresh and keep at 4°C during use.

growth medium

87%	Iscove's DMEM (with glutamine)
1%	MEM nonessential amino acids
2mM	glutamine
1mM	sodium pyruvate
20ng/ml	IL-2 (recombinant human)
400µg/ml	hygromycin B
10%	fetal bovine serum

Prepare and use IL-2 supplemented medium within 5 days. Store at 4°C.

assay buffer

87%	Iscove's DMEM (with glutamine)
1%	MEM nonessential amino acids
2mM	glutamine
1mM	sodium pyruvate
10%	FBS

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

7.A. Composition of Buffers and Solutions (continued)

recombinant IL-2

100µg/ml	lyophilized IL-2
100mM	acetic acid (sterile)
10mg/ml	bovine serum albumin

Reconstitute lyophilized IL-2 to 100µg/ml using sterile acetic acid (or manufacturer's recommended buffer). Dilute IL-2 to 10–15µg/ml with filter-sterilized D-PBS containing 10mg/ml bovine serum albumin. Prepare aliquots and store at –80°C where they are stable for a minimum of 6 months. Thawed aliquots are stable at 4°C for 1 week. Once thawed, do not refreeze aliquots.

8. Summary of Changes

The following changes were made to the 5/26 revision of this document:

1. Removed Section 7.B, Related Products.
2. Made minor text edits and formatting changes.

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