TECHNICAL MANUAL

IL-6 Bioassay, Propagation Model

Instructions for use of Product
J2992

2/20
TM579

IL-6 Bioassay, Propagation Model

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1. Description

Interleukin-6 (IL-6, originally termed BCDF/BSF2) was first identified in 1983 alongside molecules with similar but distinct functional properties (IL-4/BCGF1/BSF1 and IL-5/BCGF2) (1,2). IL-6 is a small (~21kD) pleiotropic cytokine that is secreted by a wide range of cell types: immune (macrophages, dendritic, mast, B and T cells), hepatocytes, synovial fibroblasts, bone marrow, dermal fibroblasts and keratinocytes, mesangial, vascular endothelial and smooth muscle.

The IL-6 receptor (IL-6R) is composed of two subunits. IL-6Rα (CD126) is a single chain transmembrane protein that specifically binds the cytokine directly and is expressed on limited cell types including macrophages, neutrophils, CD4+ T cells, podocytes and hepatocytes. The second subunit is a large 130kDa signal transducing chain, glycoprotein 130 (gp130/CD130), that is expressed on most cell types.

IL-6 signals in three ways: classical signaling (membrane IL-6Rα and gp130), trans signaling (cytokine associated with soluble IL-6Rα) and trans presentation (dendritic IL-6/IL-6Rα complex presenting to a second cell expressing gp130) (3).

Downstream pathway signaling doesn't occur until the IL-6/IL-6Rα complex associates with gp130 and triggers a conformational change to activate tyrosine kinases JAK1, JAK2 and Tyk2, which in turn activate STAT3. Dimerized STAT3 translocates to the nucleus and activates transcription of a variety of genes, such as proinflammatory cytokines (IL-1β, IL-8), anti-apoptotic proteins (cyclin D1, MYC, Bcl-X) and immunosuppressive proteins (VEGF, IL-10, TGFβ) (4).

IL-6 is a member of the IL-6 cytokine family, which includes IL-11, leukemia inhibitory factor (LIF), oncostatin M, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and cardiotrophin-like cytokine (CLC). All of these cytokines signal through gp130 and the STAT3 pathway.

IL-6 is transiently secreted following tissue damage or stress (UV irradiation, reactive oxygen species, microbial, viral). It is also one of the cytokines released during bacterial sepsis (5). Furthermore, IL-6, along with transforming growth factor β (TGFβ), promotes differentiation of CD4+ T cells into Th17 cells and inhibits differentiation of regulatory T cells, thus playing a critical role in autoimmunity (6).

IL-6 has shown to be elevated in patients with rheumatoid arthritis (RA), Crohn's disease and Castleman's disease. Tocilizumab, the first successful IL-6R targeted therapy, blocks IL-6 signaling through both membrane and soluble forms of IL-6R. This biologic drug is approved for RA treatment in Japan, Europe and the USA (7). Additional IL-6 blocking therapeutic antibodies include siltuximab and sarilumab.

The IL-6 Bioassay, Propagation Model (Cat.# J2992) is a bioluminescent cell-based assay designed to measure IL-6 stimulation or inhibition. The IL-6 Bioassay Cells are provided in Cell Propagation Model (CPM) format, as cryopreserved cells that can be thawed, propagated and banked for long-term use (also offered in a thaw-and-use format; Cat.# JA2501, JA2505).

The IL-6 Bioassay consists of a human cell line engineered to express a luciferase reporter driven by a response element (RE). When IL-6 binds, the IL-6R transduces intracellular signals resulting in luminescence (Figure 1). The bioluminescent signal is detected and quantified using Bio-Glo™ Luciferase Assay System(e) (Cat.# G7940, G7941) and a standard luminometer, such as the GloMax® Discover System (see Related Products, Section 9.B).

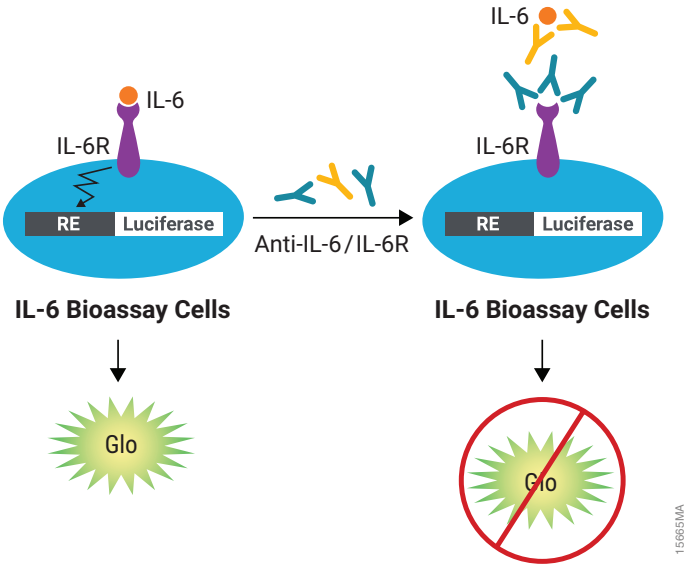
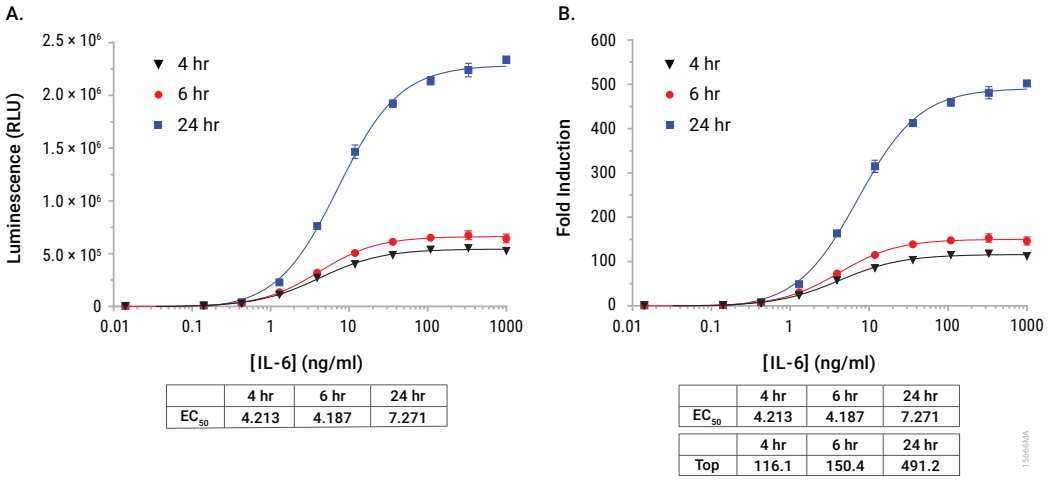


Figure 1. Representation of the IL-6 Bioassay. The IL-6 Bioassay consists of a genetically engineered cell line, IL-6 Bioassay Cells. When IL-6 binds to the IL-6 receptor (IL-6R), receptor-mediated pathway signaling induces luminescence that can be detected upon addition of Bio-Glo™ Reagent, and quantified with a luminometer. Inhibition of IL-6 binding by anti-IL-6 or anti-IL-6R antibody results in a decrease in luminescence.

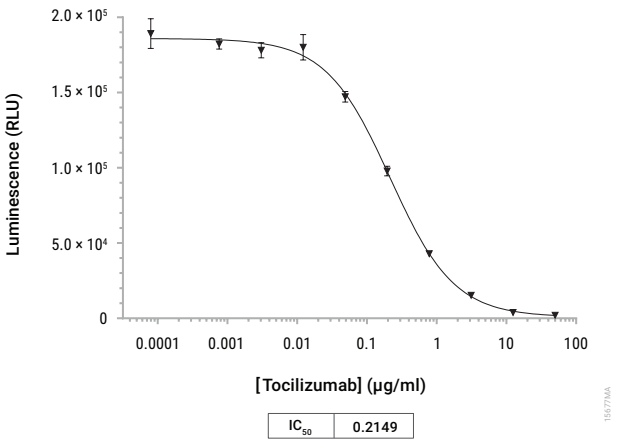
1. Description (continued)



	4 hr	6 hr	24 hr
EC_{50}	4.213	4.187	7.271

	4 hr	6 hr	24 hr
EC_{50}	4.213	4.187	7.271
Top	116.1	150.4	491.2

Figure 2. The IL-6 Bioassay responds to recombinant IL-6. IL-6 Bioassay Cells were grown and prepared as described in this protocol and incubated with serial dilutions of recombinant IL-6. After a 4-, 6- and 24-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. **Panel A** shows raw luminescence measurements. **Panel B** displays calculated fold induction. Data were generated using cell propagation model (CPM) cells.



IC_{50}	0.2149

Figure 3. The IL-6 Bioassay responds to tocilizumab. IL-6 Bioassay Cells were grown and prepared as described in this protocol, and incubated with serial dilutions of tocilizumab (anti-IL-6R) antibody for 20 minutes. IL-6 (EC_{90} concentration) was then added and plate was further incubated for 6 hours. 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 cell propagation model (CPM) cells.

Table 1. The IL-6 Bioassay Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	104
	75	101.3
	125	107
	150	106
Repeatability (% CV)	100% (Reference)	2.93
Intermediate Precision (% CV)		11.8
Linearity (r^2)		0.999
Linearity (y = mx + b)		y = 1.087x – 3.54

A 50–150% theoretical potency series of tocilizumab (anti-IL-6R) 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.

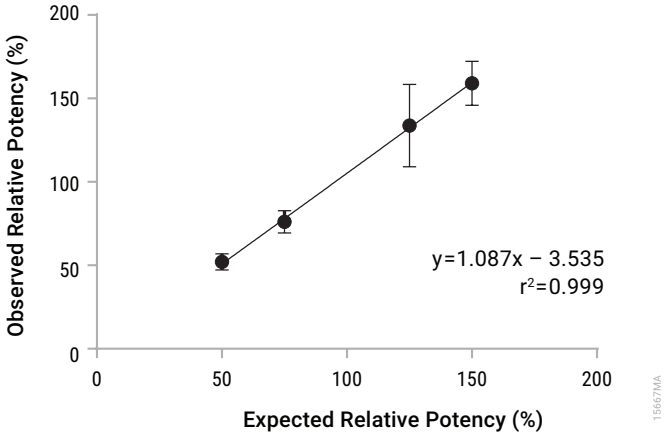
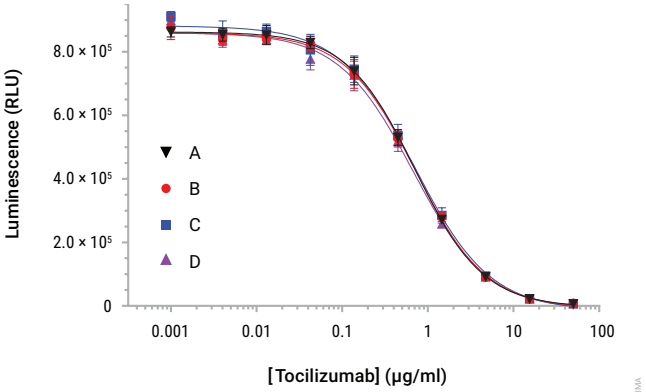
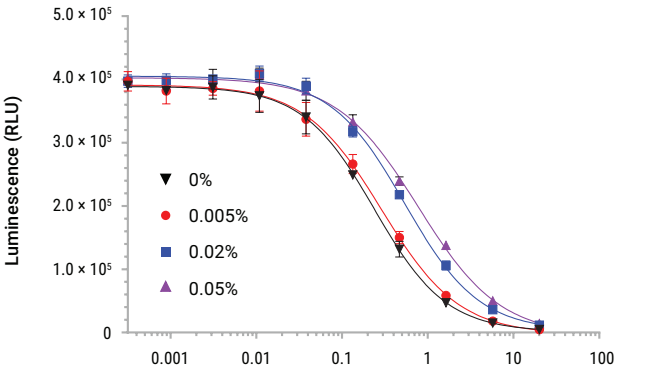


Figure 4. The IL-6 Bioassay shows precision, accuracy and linearity. A 50–150% theoretical potency series of tocilizumab was analyzed in triplicate in three independent experiments performed on three days by two analysts using the IL-6 Bioassay (for a total of six independent experiments). Bio-Glo™ Reagent 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
IC_{50}	0.7095	0.7166	0.7125	0.6721

Figure 5. The IL-6 Bioassay demonstrates repeatability. Four separate serial dilution series of tocilizumab were analyzed on four individual assay plates using the IL-6 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.



	0%	0.005%	0.02%	0.05%
IC_{50}	0.2379	0.2888	0.5378	0.7729

Figure 6. The IL-6 Bioassay indicates stability. Tocilizumab was treated with various concentrations of hydrogen peroxide (0–0.05%) for 18 hours at 26°C prior to use in the IL-6 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.

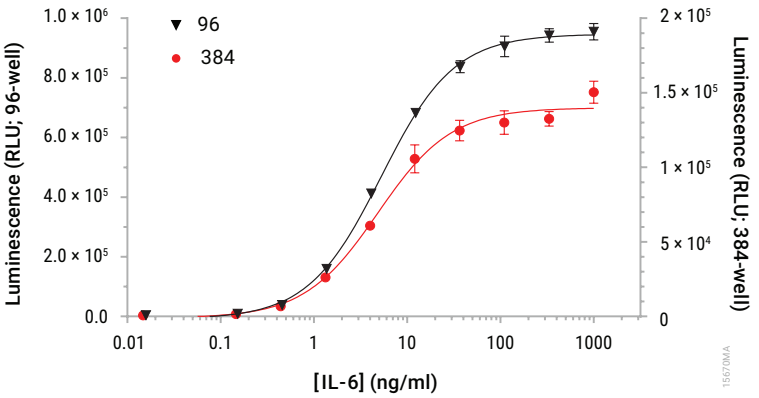
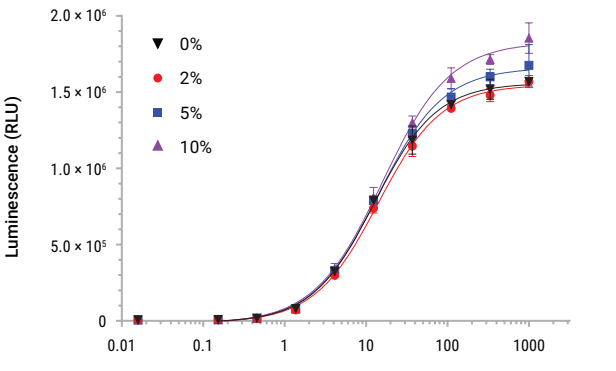
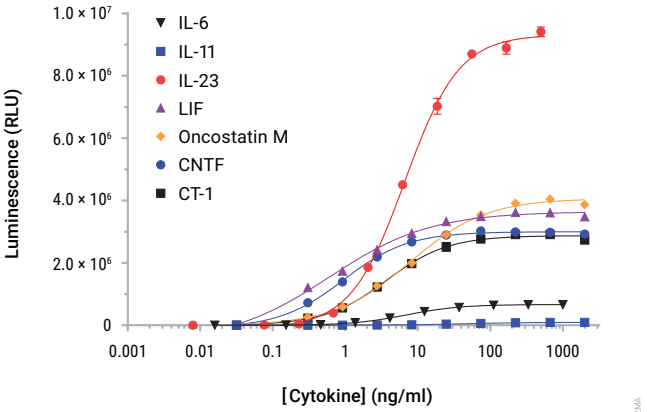


Figure 7. The IL-6 Bioassay is amenable to 384-well plate format. The IL-6 Bioassay was tested in 96- and 384-well formats by preparing and dispensing IL-6 Bioassay Cells in 50µl (96-well) or 12.5µl (384-well) volumes. Serial threefold dilutions of recombinant human IL-6 were prepared and added to cells (25µl/well 96-well; 6.2µl/well 384-well). After 6 hours of stimulation, Bio-Glo™ Reagent was added (75µl/well 96-well; 18.7µl/well 384-well), 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-6 EC_{50} was 5.3ng/ml for 96-well plate, and 4.9ng/ml for the 384-well plate.



	0%	2%	5%	10%
EC_{50}	12.65	13.98	13.99	15.72

Figure 8. The IL-6 Bioassay tolerates up to 5% human serum. IL-6 Bioassay Cells were tested with a dose-response of recombinant IL-6 in the absence or presence of increasing concentrations of pooled normal human serum, resulting in final assay concentrations of human serum (0–10%). 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-6	IL-23	IL-11	LIF	Oncostatin M	CNTF	CT-1
EC_{50}	6.966	6.619	46.63	0.6101	7.816	0.9494	3.685

Figure 9. IL-6 Bioassay cytokine cross reactivity. IL-6 Bioassay Cells were tested using a panel of IL-6 family cytokines (IL-11, IL-23, LIF, Oncostatin M, CNTF and CT-1). Each of these proteins signals through the gp130 receptor. Following 6-hour treatment, 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-6 Bioassay, Propagation Model	**1 each**	**J2992**

Not for Medical Diagnostic Use. Includes:

- 2 vials IL-6 Bioassay Cells, 1.2 × 10^7 cells/ml (0.65ml 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.

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.

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-6 Bioassay is intended to be used with user-provided biologics designed to activate or inhibit the IL-6 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-6. 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-6 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.

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Materials to Be Supplied by the User

(Composition of buffers and solutions is provided in Section 9.A.)

Reagents

- user-defined biologics samples
- DMEM (high glucose with pyruvate; e.g., Invitrogen Cat.#11995-065)
- fetal bovine serum (e.g., HyClone Cat.# SH30070)
- hygromycin B (e.g., Invitrogen Cat.# 10687-010)
- G418 antibiotic (e.g., Invitrogen Cat.#10131-035)
- D-PBS (e.g., Invitrogen Cat.# 14190144)
- Accutase® or equivalent (e.g., Innovative Cell Technologies Cat.# AT104)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941)
- **optional:** recombinant Human IL-6 (e.g., PeproTech IL-6 Cat.# 200-06)

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

4. Preparing IL-6 Bioassay Cells

4.A. Cell Thawing and Initial Cell Culture

Note: IL-6 Bioassay Cells are grown as **adherent** cultures.

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

1. Prepare 60ml of thaw medium (see Section 9.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-6 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 0.65ml) 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 42ml of prewarmed thaw medium in a 50ml conical tube.
8. Count cells with Trypan blue and determine cell number and viability.
9. Transfer the cell suspension evenly into three 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

Note: For cell maintenance and propagation starting from the second cell passage, use growth medium containing selection antibiotic (see Section 9.A), and monitor cell viability and doubling rate during propagation. Cell growth rate will stabilize by approximately 7–10 days post-thaw. At this time, cell viability is typically >95% and the average cell doubling rate is approximately 21 hours. Passage number should be recorded for each passage. Cells are expected to retain their functionality for up to 30 passages.

1. On the day of cell passage, visualize cells under microscope and estimate confluency.
2. Remove growth medium.
3. Add 2ml of Accutase® to each T75 flask and rock flask several times to mix and coat cell surface.
4. Incubate at room temperature until cells begin to lift off (approximately 3–5 minutes).
5. Add 8ml of prewarmed growth medium and triturate cells to create a single cell suspension.
6. Sample and count by Trypan blue exclusion.
7. Add fresh growth medium and transfer cells to a new flask. Mix gently.
8. Recommended density for passaging cells is as follows:
   a. For 2-day culture: 2.6×10^4 cells/cm^2
   b. For 3-day culture: 1×10^4 cells/cm^2

   Note: We recommend using the following media volumes for routine cell propagation: 14ml for a T75 flask, 28ml for a T150 flask and 42ml for a T225 flask. Scale according to the surface area of the flask.
9. Place the flasks horizontally in a humidified 37°C, 5% CO_2 incubator.

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 9.A) and keep on ice.
2. Harvest cells as described in Steps 10–18 above.
3. 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.
4. Transfer cells to 50ml sterile conical tubes or larger sized centrifuge tubes, and centrifuge cells at 150 × *g* for 10–15 minutes.
5. Gently aspirate the supernatant, being careful not to disturb the cell pellet.
6. 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.
7. Freeze using a controlled-rate freezer (or use an insulated Mr. Frosty® or a Styrofoam® type of cell freezing container at –80°C overnight).
8. Transfer to –140°C or below for long-term storage.

5. Stimulation Protocol

The IL-6 Bioassay can be used to test IL-6 and IL-6 blocking antibodies. This protocol illustrates the use of the IL-6 Bioassay to examine two test samples against a reference sample in a single assay run (Figure 10). 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 11). Other experimental and plate layouts are possible but may require further optimization.

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 0–1µg/ml of recombinant IL-6 (Peprotech Cat.# 200-06) as a sample range, with serial threefold dilutions to achieve full dose curves as 10-point series. Concentration ranges and dilution schemes may need to be optimized for your samples.
2. While maintaining the IL-6 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 for use in the IL-6 Bioassay.

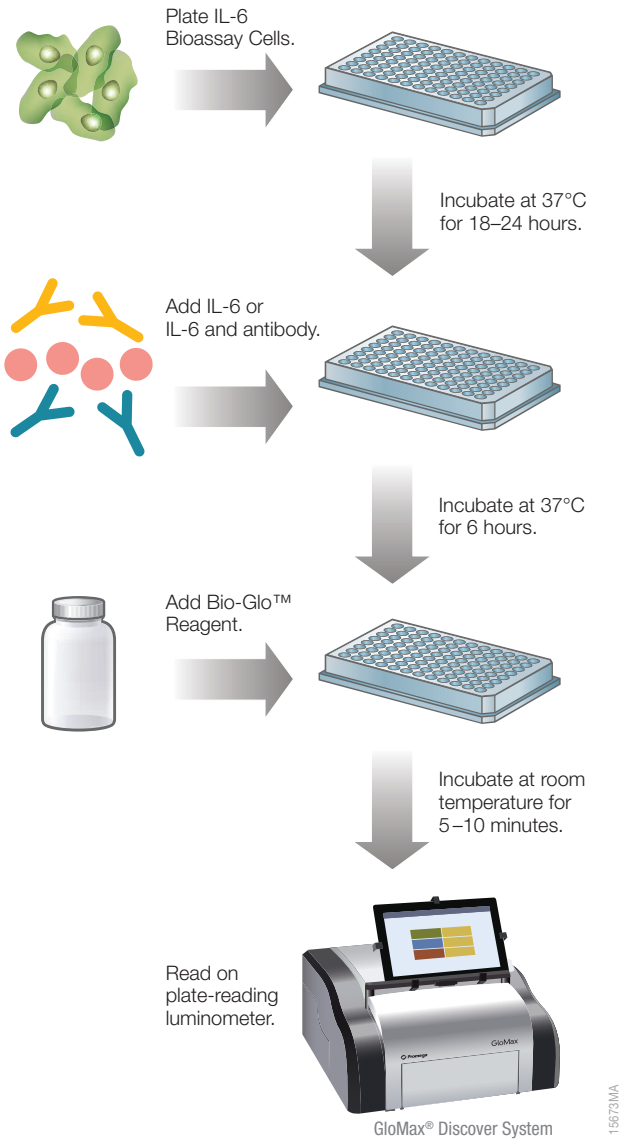

Figure 10. IL-6 Bioassay schematic protocol.

5.A. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 11 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference samples to generate two ten-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 11. 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 One: Preparing and Plating IL-6 Bioassay Cells

1. Prepare 50ml of assay buffer as described in Section 9.A and warm to 37°C before use.
2. Aspirate growth medium.
3. Add 2ml of Accutase® to a T75 flask and rock the flask several times to mix and coat the cell surface.
4. Incubate at room temperature until cells begin to lift off (approximately 3–5 minutes).
5. Add 8ml of assay buffer and triturate cells to create a single cell suspension.
6. Sample and count by Trypan blue exclusion.
7. 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 1.5×10^6 cells are required (2.5×10^4 cells/well × 60 wells).

8. Place cells into 50ml centrifuge tubes and centrifuge at 150 × *g* for 5–10 minutes.

9. Remove supernatant. Resuspend cells in assay buffer to an estimated 2×10^6 cells/ml and count again by Trypan blue exclusion.

10. Adjust to 5×10^5 cells/ml using additional assay buffer.

11. Dispense 50µl/well (2.5×10^4 cells/well) using a multichannel pipette into the inner 60-wells of two solid white 96-well plates. Add 75µl/well of assay buffer to outer 36 wells.

12. Incubate 18–24 hours at 37°C, 5% CO_2.

5.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 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 DMEM medium to yield 90% DMEM/10% FBS (see Section 9.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 11 and 12). Using assay buffer as the diluent, prepare a minimum of 390µl of reference sample starting dilution and a minimum of 195µl of each test sample starting dilution in 1.5ml tubes. Store the tubes containing starting dilutions appropriately before making serial dilutions.

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5.D. Preparing Serial Dilutions

Note: Serial dilutions should be prepared on the day of the assay.

The instructions described here are for preparation of a single stock of threefold serial dilutions of a single sample for analysis in triplicate (130µl of each dilution provides a sufficient volume for analysis in triplicate). Alternatively, you can prepare three independent stocks of serial dilutions to generate triplicate samples. To prepare threefold serial dilutions, you will need a minimum of 390µl of a reference sample at 3X the highest concentration in your dose-response curve. You will need a minimum of 195µl 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-6 stimulation using recombinant human IL-6 as your reference sample (PeproTech IL-6 Cat.# 200-06), we recommend starting with a 3X concentration of 3µg/ml and performing serial threefold dilutions. When using other reference sources of IL-6, the starting concentration may need to be adjusted.

1. To a sterile clear 96-well plate, add 195µl of reference sample starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 11).
2. Add 195µl of test samples 1 and 2 starting dilution (dilu1, 3X final concentration) to wells C11 and D11, respectively.
3. Add 130µl of assay buffer to other wells in these four rows, from column 10 to column 2.
4. Transfer 65µl of the sample starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
5. Repeat equivalent threefold serial dilutions across the columns from right to left until you reach column 3. Remove 65µl from column 3 so that all wells have a volume of 130µl. 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 130µl of assay buffer without sample as a negative control.

5.E. IL-6 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 11.
2. Cover each assay plate with a lid and incubate in a humidified 37°C, 5% CO_2 incubator for 6 hours.

   Note: Other induction times can be used. See Figure 2.
3. After the 6-hour incubation is completed, proceed to Section 5.F.

5.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 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.

5.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 IL-6 response using appropriate curve fitting software (such as GraphPad Prism®).

6. Inhibition Protocol

The IL-6 Bioassay Cells can be used to measure inhibition of IL-6 signaling using a blocking antibody such as tocilizumab, which targets the IL-6 receptor (IL-6R). A preliminary stimulation experiment with IL-6 is necessary to determine the EC_{90} concentration, which is used during an inhibition assay. This protocol may be modified for other blocking antibodies, depending on their mechanism of action.

6.A. IL-6 Inhibition Assay

1. Prepare and plate IL-6 Bioassay Cells as described in Section 5.B. Incubate overnight in a humidified, 37°C, 5% CO_2 incubator.
2. In a separate sterile clear 96-well plate, prepare serial dilutions of tocilizumab antibody in warm assay buffer as 2X final concentration. We recommend a final concentration of 0–40µg/ml tocilizumab as serial threefold dilutions.
3. Remove the 96-well assay plates containing the overnight preplated IL-6 Bioassay Cells from the incubator. 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 manual multichannel pipette to carefully remove 50µl of medium from each of the wells.
4. Immediately add 35µl/well of antibody dilution series samples to emptied wells containing cells.
5. Incubate for 20 minutes in a humidified 37°C, 5% CO_2 incubator to allow antibody to bind to the IL-6R.
6. Prepare IL-6 at 2X the predetermined EC_{90} IL-6 response concentration in warm assay buffer.
7. Remove the assay plate from the incubator and add 35µl to each well. Final sample volume is now 70µl/well (35µl of antibody + 35µl of IL-6).
8. Incubate for 4–6 hours (as was done for preliminary EC_{90} concentration determination) in a humidified 37°C, 5% CO_2 incubator.
9. Proceed to Section 6.B.

6.B. 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 70µl of Bio-Glo™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
3. Add 70µl 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 IC_{50} value and fold induction.
5. Measure luminescence using a luminometer or luminescence plate reader.

6.C. Data Analysis

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

   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 IL-6 inhibition response using appropriate curve fitting software (such as GraphPad Prism® software).

7. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	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 Preparing and Plating 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.
	Ensure that IL-6 is prepared and stored properly with carrier protein. Follow manufacturer's protocol for initial rehydration of cytokine. Single use frozen aliquots are recommended for each assay.
	IL-6 lot-to-lot activity differences may be observed. Consult cytokine provider for details.
Weak assay response (low fold induction)	IL-6 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.

8. References

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3. Garbers, C. *et al.* (2018) Interleukin-6: designing specific therapeutics for a complex cytokine. *Nat. Rev. Drug Disc.* **17,** 395–412.
4. Vainer, N. *et al.* (2018) Systematic literature review of IL-6 as a biomarker or treatment target in patients with gastric, bile duct, pancreatic and colorectal cancer. *Oncotarget* **9(51)**, 29820–41.
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9. Appendix

9.A. Composition of Buffers and Solutions

thaw medium

90% DMEM (high glucose with pyruvate)
10% fetal bovine serum

Prepare and use within 2 weeks, stored at 4°C.

freeze medium

85% DMEM (high glucose with pyruvate)
10% fetal bovine serum
5% DMSO

Prepare immediately before use. Maintain at 4°C during use.

growth medium

90% DMEM (high glucose with pyruvate)
10% fetal bovine serum
600µg/ml G418 antibiotic
200µg/ml hygromycin B

Prepare and use within 2 weeks, stored at 4°C.

assay buffer

90% DMEM (high glucose with pyruvate)
10% FBS

Prepare and use within 2 weeks, stored at 4°C.

9.B. Related Products

Cytokine and Growth Factor Bioassays

Product	Size	Cat.#
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-15 Bioassay	1 each	JA2011
IL-15 Bioassay 5X	1 each	JA2015
IL-15 Bioassay, Propagation Model	1 each	J2962

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.

**Not for Medical Diagnostic Use.

Additional kit formats are available.

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9.B. Related Products (continued)

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
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-L1 Negative Cells	1 each	J1191
Control Ab, Anti-PD-1	100µg	J1201
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
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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