TECHNICAL MANUAL

IL-6 Bioassay

Instructions for Use of Products
JA2501 and JA2505

Revised 5/26
TM593

Promega

IL-6 Bioassay

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

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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 stressors, including UV irradiation, reactive oxygen species, and microbial and viral agents. 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 is 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 United States (7). Additional IL-6 blocking therapeutic antibodies include siltuximab and sarilumab.

The IL-6 Bioassay[a–d] (Cat.# JA2501, JA2505) is a bioluminescent cell-based assay designed to measure IL-6 stimulation or inhibition. The IL-6 Bioassay Cells are provided in a thaw-and-use format, as cryopreserved cells that can be thawed, plated and used in an assay without the need for cell propagation. IL-6 Bioassay Cells are also available in a cell propagation model (CPM) format, as cryopreserved cells that can be thawed, propagated and banked for long-term use (IL-6 Bioassay, Propagation Model, Cat.# J2992).

The IL-6 Bioassay consists of human cells engineered to express the IL-6 receptor and 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[c] (Cat.# G7940, G7941) and a standard luminometer, such as the GloMax® Discover System.

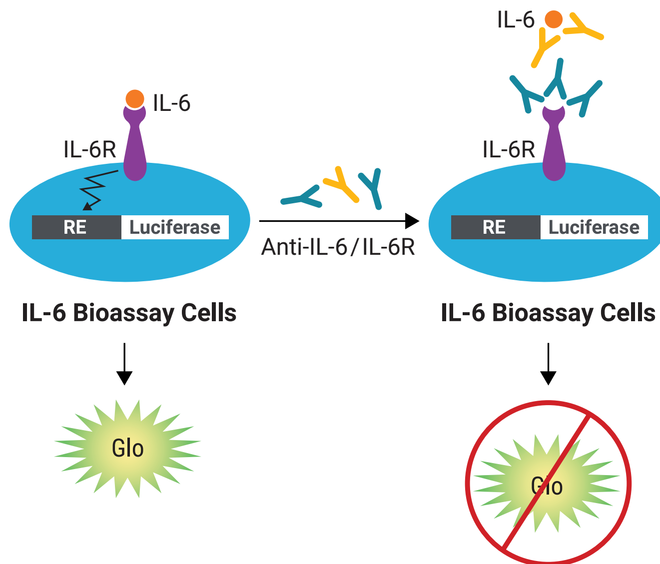
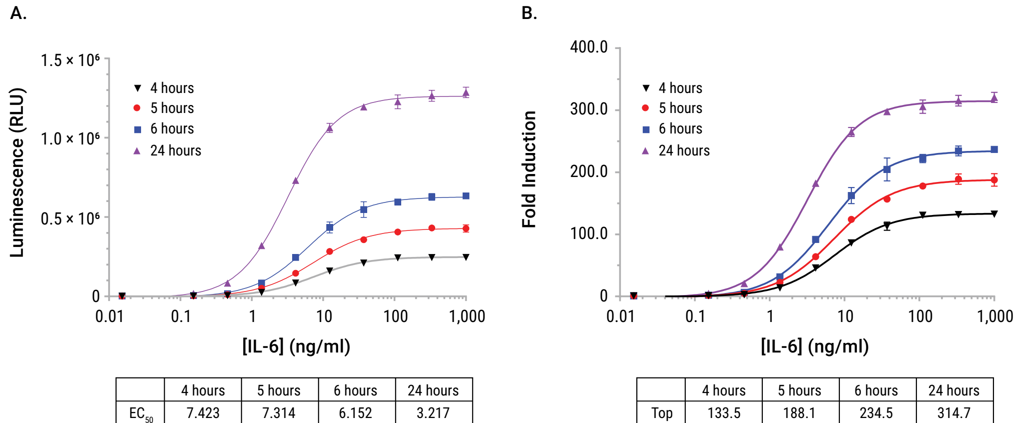


Figure 1. Representation of the IL-6 Bioassay. The IL-6 Bioassay consists of a genetically engineered cell line, IL-6 Bioassay Cells. When IL-6 binds to the IL-6 receptor (IL-6R), receptor-mediated pathway signaling induces luminescence that can be detected upon addition of Bio-Glo™ Reagent[(c)], and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

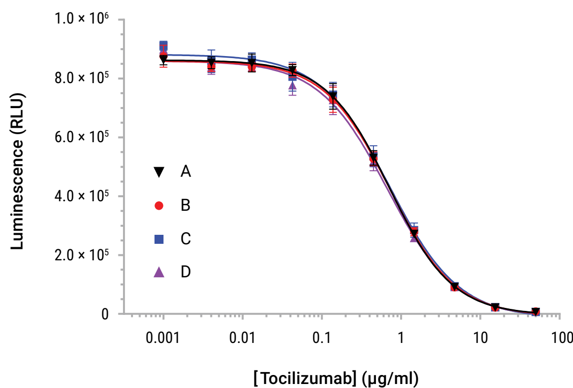
1. Description (continued)



	4 hours	5 hours	6 hours	24 hours
EC_{50}	7.423	7.314	6.152	3.217

	4 hours	5 hours	6 hours	24 hours
Top	133.5	188.1	234.5	314.7

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-, 5-, 6- or 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 thaw-and-use cells.



	A	B	C	D
IC_{50}	0.7095	0.7166	0.7125	0.6721

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Figure 3. 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. IL-6 Bioassay Cells were 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 the 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 thaw-and-use 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 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.

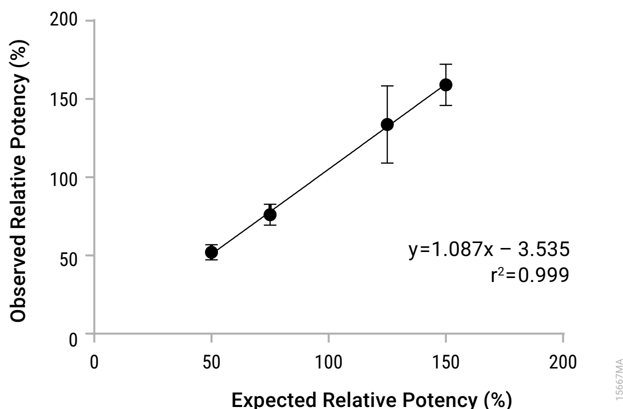
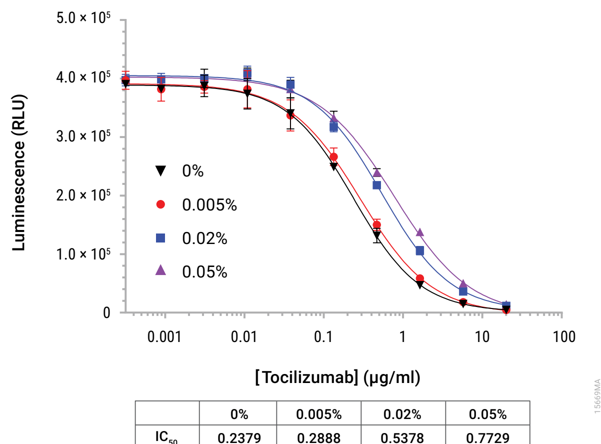


Figure 4. The IL-6 Bioassay shows precision, accuracy and linearity. A 50–150% theoretical potency series of tocilizumab was analyzed in triplicate in three independent experiments performed on three days by two analysts using the IL-6 Bioassay (for a total of six independent experiments). Bio-Glo™ Reagent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



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

Figure 5. 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.

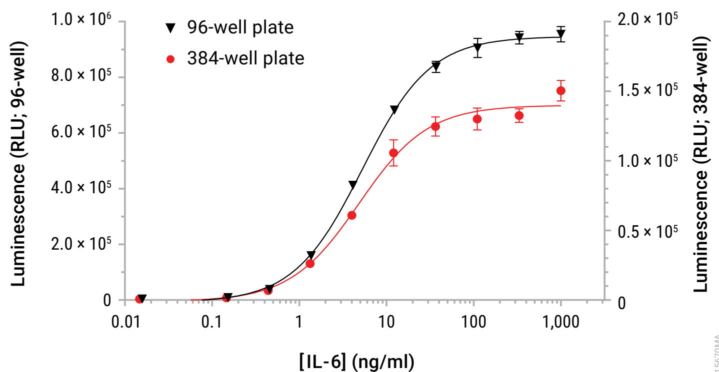
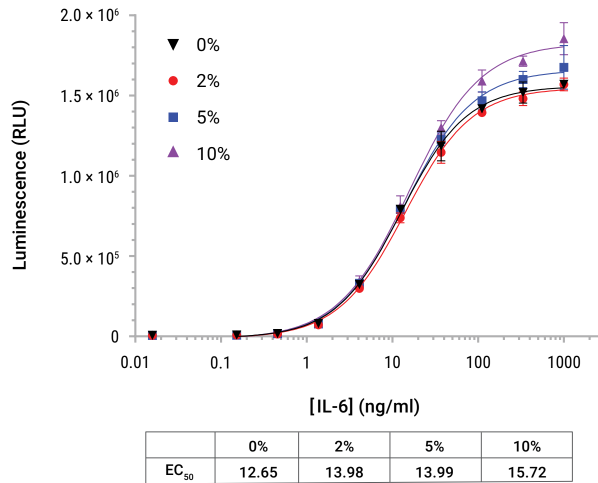
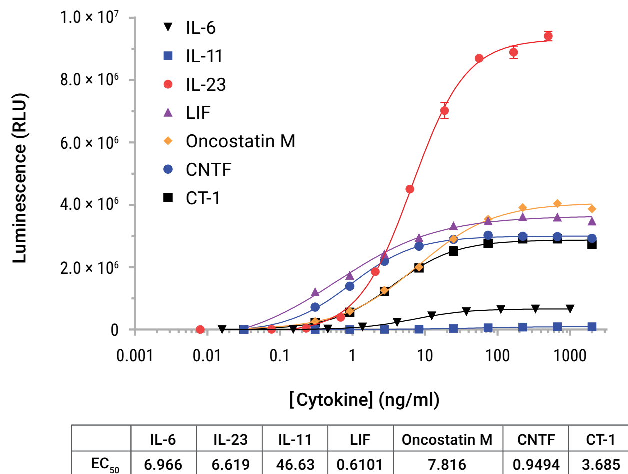


Figure 6. The IL-6 Bioassay is amenable to 384-well plate format. The IL-6 Bioassay was tested in 96- and 384-well formats by preparing and dispensing IL-6 Bioassay Cells in 50µl (96-well) or 12.5µl (384-well) volumes. Serial threefold dilutions of recombinant human IL-6 were prepared and added to cells (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 the 96-well plate and 4.9ng/ml for the 384-well plate. (Costar® Cat.# 3570 384-well plates were used.)



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

Figure 7. 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 8. 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 a 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	**1 each**	**JA2501**

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

- 1 vial IL-6 Bioassay Cells, 1.2 × 107 cells/ml (0.65ml per vial)
- 36ml RPMI 1640 Medium
- 4ml Fetal Bovine Serum
- 1 vial Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 10ml Bio-Glo™ Luciferase Assay Buffer

PRODUCT	SIZE	CAT.#
IL-6 Bioassay 5X	**1 each**	**JA2505**

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

- 5 vials IL-6 Bioassay Cells, 1.2 × 107 cells/ml (0.65ml per vial)
- 5 × 36ml RPMI 1640 Medium
- 5 × 4ml Fetal Bovine Serum
- 5 vials Bio-Glo™ Luciferase Assay Substrate (lyophilized)
- 5 × 10ml Bio-Glo™ Luciferase Assay Buffer

Note: IL-6 Bioassay components are shipped separately because of differing temperature requirements. The IL-6 Bioassay Cells are shipped on dry ice. The Bio-Glo™ Luciferase Assay System and Fetal Bovine Serum are shipped on dry ice separately from the cells. The RPMI 1640 Medium is shipped at ambient temperature.

Storage Conditions:

- Upon arrival, immediately transfer the cell vials to below –140°C (freezer or liquid nitrogen vapor phase) for long-term storage. Do not store cell vials submerged in liquid nitrogen. **Do not** store cell vials at –80°C because this will decrease cell viability and cell performance.
- Store Bio-Glo™ Luciferase Assay Substrate, Bio-Glo™ Luciferase Assay Buffer and Fetal Bovine Serum at –30°C to –10°C. Avoid multiple freeze-thaw cycles of the serum.
- For optimal performance, use reconstituted Bio-Glo™ Reagent on the day of preparation. However, once reconstituted, Bio-Glo™ Reagent can be stored at –30°C to –10°C for up to 6 weeks.
- Store RPMI 1640 Medium at +2°C to +10°C protected from light.

3. Before You Begin

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

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.

The IL-6 Bioassay is intended for use 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 4 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.

3.A. Materials to Be Supplied by the User

Reagents

- user-defined biologics samples
- **optional:** recombinant human IL-6 (e.g., PeproTech 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)
- 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
- luminometer (e.g., GloMax® Discover System) or plate reader that measures glow luminescence

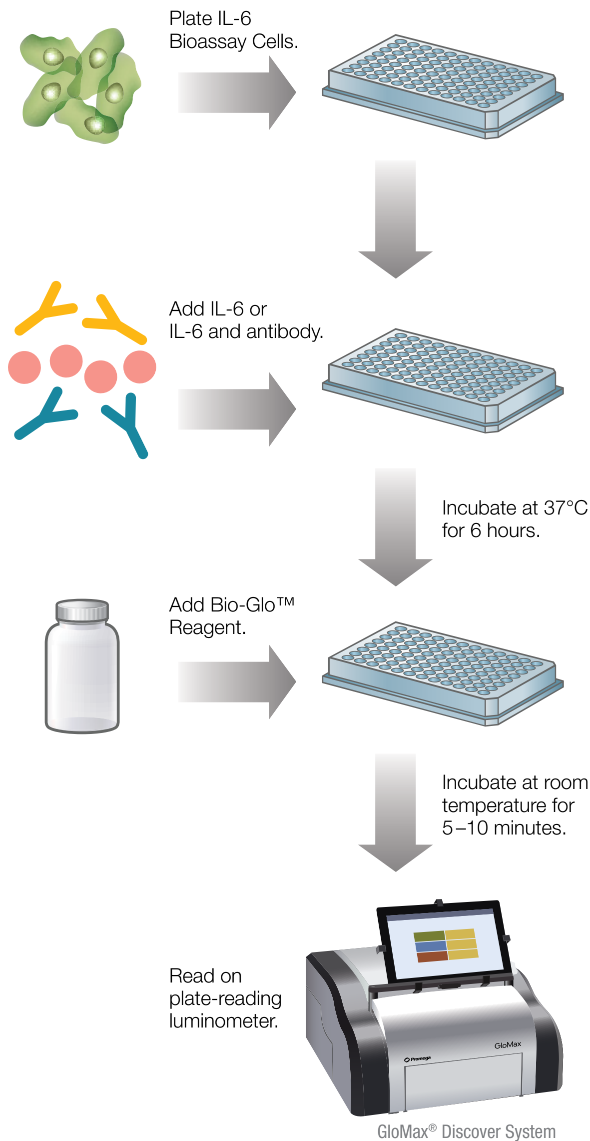


Figure 9. IL-6 Bioassay schematic protocol.

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4. Stimulation Protocol

The IL-6 Bioassay can be used to test IL-6 cytokine and IL-6/IL-6R blocking antibodies. This stimulation protocol illustrates the use of the IL-6 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 (Figure 10). Other experimental and plate layouts are possible but may require further optimization. The inhibition protocol (Section 5) illustrates the use of the bioassay to examine blockade of IL-6 activity.

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 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.

b. When diluted as directed, each kit containing medium, serum, and 1 vial of IL-6 Bioassay Cells is sufficient for 120 wells (two 96-well plates using inner-60 format). The thaw-and-use cells are for single use only and cannot be cultured or refrozen for second use. Please plan your experiments accordingly to optimize the use of the thaw-and-use cells.

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 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 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 alone (denoted by "B").

4.B. Preparing Reagents for the Assay

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.

   After reconstitution the Bio-Glo™ Reagent can be stored at room temperature with ~18% loss of luminescence after 24 hours or at 4°C with ~12% loss of luminescence after 5 days.

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 4ml of FBS to 46ml of RPMI 1640 medium to yield 90% RPMI 1640 medium/10% FBS. Mix well and warm to 37°C prior to use. For reference, 40ml of assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 plate wells.

4.C. Plating IL-6 Bioassay Cells

The thaw-and-use IL-6 Bioassay Cells included in this kit are sensitive; carefully follow the cell thawing and plating procedures exactly as described. Do not overmix or overwarm the cell reagents. No additional cell culture or manipulation is required. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at a time.

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

1. Remove one vial of IL-6 Bioassay Cells from storage at –140°C and transfer to the bench on dry ice.
2. Add 10.4ml of prewarmed (37°C) assay buffer to a 15ml conical tube.
3. Warm the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect. Try not to submerge the vial completely. Do not invert.
4. Gently mix the cell suspension by pipetting, then transfer 0.65ml of the cells to the 15ml conical tube containing 10.4ml of assay buffer. Mix well by gently pipetting or inverting 5 times.
5. Transfer the cell suspension to a sterile reagent reservoir.
6. Using a multichannel pipette, immediately dispense 50µl of the cell suspension to each of the inner 60 wells of two 96-well assay plates. Optimal results depend on gently keeping the cells evenly resuspended during the plating process.
7. Add 75µl/well of warm assay buffer to the outer 36 wells of each plate.
8. Cover each assay plate with a lid and incubate at 37°C in a 5% CO_2 incubator while preparing samples and dilutions.

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 120µl of assay buffer without sample as a negative control.

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4.D. Preparing Serial Dilutions

Note: Serial dilutions should be prepared after plating the IL-6 Bioassay Cells, on the day of assay.

The instructions described here are for preparation of a single stock of threefold serial dilutions of a single sample for analysis in triplicate (120µl 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 360µl of a reference sample at 3X the highest concentration in your dose-response curve. You will need a minimum of 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.

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 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 120µl of assay buffer to the other wells in these four rows, from column 10 to column 2.
4. Transfer 60µl of the sample starting dilutions from column 11 to 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 60µl from column 3 so that all wells have a 120µl volume. Do not dilute into column 2.
6. Cover the plate with a lid and proceed to Section 4.E.

4.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 10.
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 complete, 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 assay plate 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 plate, taking care not to create bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, C1 and D1 of the assay plate to measure the background signal.
4. Incubate at ambient temperature for 5–10 minutes.

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

4.G. Data Analysis

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

   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®).

5. 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 and blocks IL-6R. A preliminary stimulation experiment with IL-6 is necessary to determine the EC_{90} concentration, which is used during an inhibition assay. The protocol may be modified for other blocking antibodies, depending on their mechanism of action.

5.A. Plating IL-6 Bioassay Cells

The thaw-and-use IL-6 Bioassay Cells included in this kit are sensitive; carefully follow the cell thawing and plating procedures exactly as described. Do not overmix or overwarm the cell reagents. No additional cell culture or manipulation is required. We recommend that you thaw and dilute a maximum of two vials of thaw-and-use cells at one time.

1. Remove one vial of IL-6 Bioassay Cells from storage at –140°C and transfer to the bench on dry ice.
2. Add 4.9ml of prewarmed (37°C) assay buffer to a 15ml conical tube.
3. Warm the cells in a 37°C water bath until just thawed (about 2 minutes). While thawing, gently agitate and visually inspect. Try not to submerge the vial completely. Do not invert.
4. Gently mix the cell suspension by pipetting, then transfer 0.65ml of the cells to the 15ml conical tube containing 4.9ml of assay buffer. Mix well by gently pipetting or inverting 5 times.
5. Transfer the cell suspension to a sterile reagent reservoir.
6. Using a multichannel pipette, immediately dispense 25µl of the cell suspension to each of the inner 60 wells of two 96-well assay plates. Optimal results depend on gently keeping the cells evenly resuspended during the plating process.
7. Add 75µl/well of prewarmed assay buffer to the outer 36 wells of each plate.
8. Cover each assay plate with a lid and place in a 37°C, 5% CO_2 incubator.

5.B. Preparing Serial Dilutions

1. Prepare serial dilutions of tocilizumab antibody in warm assay buffer as 3X final concentration. We recommend a final concentration of tocilizumab of 0–50µg/ml as serial 3.25-fold dilutions.
2. To a sterile clear 96-well plate, add 200µl of **reference** antibody sample starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 11).
3. Add 200µl of **test** antibody samples 1 and 2 starting dilution (dilu1, 3X final concentration) to wells C11 and D11, respectively.
4. Add 135µl of assay buffer to the other wells in these four rows, from column 10 to column 2.
5. Transfer 60µl of the sample starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent 3.25-fold serial dilutions across the columns from right to left until you reach column 3. Remove 60µl from column 3 so that all wells have a 135µl volume. Do not dilute into column 2.

5.C. IL-6 Inhibition Assay

1. Using a multichannel pipette, dispense 25µl of each antibody dilution sample to the 25µl of preplated cells.
2. Incubate for 20 minutes in a humidified 37°C, 5% CO_2 incubator to allow tocilizumab antibody to bind to IL-6R.
3. Prepare IL-6 at 3X the predetermined EC_{90} IL-6 response concentration in warm assay buffer.
4. Remove the assay plate from the incubator and add 25µl/well of IL-6 (3X EC_{90}) to wells. The final sample volume is now 75µl/well (25µl of cells + 25µl of antibody + 25µl of IL-6).
5. Incubate for 6 hours (as done for preliminary EC_{90} concentration determination) in a humidified 37°C, 5% CO_2 incubator.
6. After the 6-hour incubation is complete, proceed to Section 5.D.

5.D. 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 plate 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 plate, taking care not to create bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, C1 and D1 of the 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.

5.E. 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 IC_{50} value of IL-6 response using appropriate curve fitting software (such as GraphPad Prism® software).

6. Troubleshooting

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

Symptoms	Causes and Comments
Low luminescence measurements (RLU readout)	Choose 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 that the Preparing and Plating protocol is strictly followed.
	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.

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7. References

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8. Summary of Changes

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

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

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