



TECHNICAL MANUAL

IL-23 Bioassay

Instructions for use of Products
JA2511 and JA2515

IL-23 Bioassay

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 Visit the website to verify that you are using the most current version of this Technical Manual.
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1. Description

Interleukin-23 (IL-23) was first described in 2000 when a computational screen identified a novel sequence (p19) that combined with the p40 subunit of IL-12 to create an active and composite cytokine. It is secreted by activated dendritic cells and possesses biological properties both distinct from and in common with IL-12 (1).

IL-23 is a member of the larger IL-12 cytokine family, which consists of IL-12, IL-23, IL-27, IL-35 and IL-39. This unique group of heterodimeric cytokines consists of an alpha chain subunit (p19, p28 or p35) and a beta chain subunit (p40 or Epstein-Barr virus induced gene 3), and signals through JAK/STAT pathways.

IL-23 binds and signals through a heterodimeric receptor complex. The p19 subunit binds to IL-23R and p40 subunit to IL-12R β 1. Similarly, the p35 and p40 subunits of IL-12 bind to IL-12R β 2 and IL-12 β 1, respectively.

The IL-23 receptor is found on natural killer cells, macrophages, memory T cells (Th17) and keratinocytes. In response to microbial pathogens and wound healing signals, IL-23 is secreted by activated dendritic cells and macrophages with subsequent neutrophil recruitment.

Upon IL-23 binding to Th17 cells, signaling begins with tyrosine kinase 2 (TYK2) recruitment to IL-12R β 1 and Janus kinase 2 (JAK2) recruitment to IL-23R. These kinases phosphorylate and activate signal transducer and activator of transcription 3 (STAT3), and to a lesser extent STAT4, STAT1 and STAT5 (2). Phosphorylated STAT3 complexes translocate to the nucleus, inducing expression of IL-17A, IL-23R and the transcription factor retinoid-related orphan receptor- γ t, thus stabilizing the Th17 phenotype (3).

Both IL-23 and IL-12 cytokines are involved in human T helper cell differentiation and survival. IL-12 binds and directly promotes the differentiation of naïve CD4+ T cells into T helper 1 cells. It is believed that IL-6, IL-1 β and TGF β are involved in the differentiation of human T helper 17 cells (Th17), while IL-23 is critical for their maturation, maintenance and pathogenicity (4–6).

Ustekinumab is a humanized antibody that targets the p40 cytokine subunit and prevents IL-12 and IL-23 binding to IL-12R β 1. It was FDA approved for the treatment of psoriasis in 2009, psoriatic arthritis in 2013 and most recently Crohn's disease in 2016 (7).

Recent research has indicated that IL-23 and the Th17 secretion of the pro-inflammatory cytokines IL-17, IL-22 and IL-21 are prominent contributors to the formation of psoriatic inflammation and plaques (8–10). By selectively targeting the IL-23p19 subunit, key therapeutic advantages may be achieved including preserving IL-12 Th1 pathogenic responses. Recently, several biologics targeting IL-23p19 have been approved for moderate-to-severe plaque psoriasis, including guselkumab, tildrakizumab and risankizumab.

The IL-23 Bioassay^(a-d) (Cat. # JA2511, JA2515) is a bioluminescent cell-based assay designed to measure IL-23 stimulation or inhibition. The IL-23 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-23 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-23 Bioassay, Propagation Model, Cat. # J3002).

The IL-23 Bioassay consists of human cells engineered to express the IL-23 receptor and a luciferase reporter driven by a response element (RE; Figure 1). When IL-23 binds to IL-23R, intracellular signals are transduced, resulting in luminescence. The bioluminescent signal is detected and quantified using Bio-Glo™ Luciferase Assay System^(d) (Cat.# G7940, G7941) and a standard luminometer, such as the GloMax® Discover System (see Related Products, Section 8).

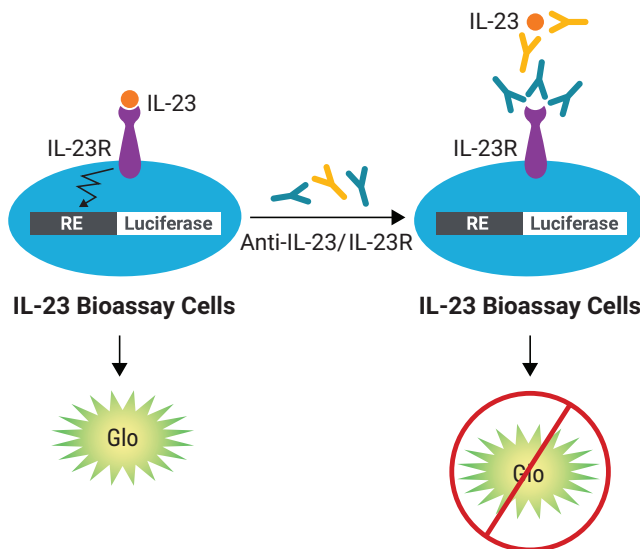


Figure 1. Representation of the IL-23 Bioassay. The IL-23 Bioassay consists of a genetically engineered cell line, IL-23 Bioassay Cells. When IL-23 binds to the IL-23 receptor (IL-23R), receptor-mediated pathway signaling induces luminescence that can be detected upon addition of Bio-Glo™ Reagent and quantified with a luminometer. Inhibition of IL-23 binding by anti-IL-23 or anti-IL-23R antibody results in a decrease in luminescence.

1. Description (continued)

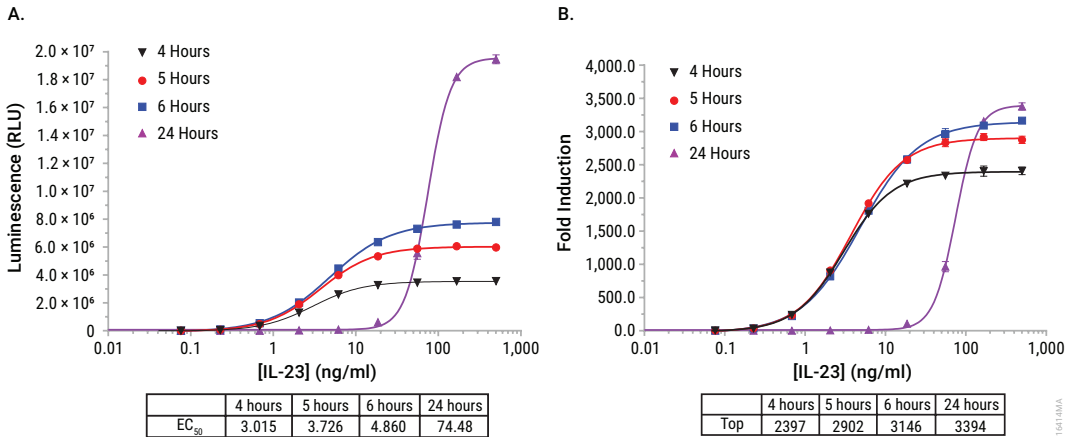


Figure 2. The IL-23 Bioassay responds to recombinant IL-23. IL-23 Bioassay Cells were prepared as described in this protocol and incubated with serial dilutions of recombinant IL-23. After a 4-, 5-, 6- or 24-hour incubation, Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. **Panel A** shows raw luminescence measurements. **Panel B** displays the calculated fold induction. Data were generated using thaw-and-use cells.

Table 1. The IL-23 Bioassay Shows Precision, Accuracy and Linearity.

Parameter	Results	
	% Expected Relative Potency	% Recovery
Accuracy	50	97.5
	70	102.5
	140	95
	200	91.9
Repeatability (% CV)	100% (Reference)	6.3
Intermediate Precision (% CV)		10.9
Linearity (r ²)		0.998
Linearity (y = mx + b)		y = 0.89x - 6.97

A 50–200% theoretical potency series of ustekinumab (anti-IL-23) 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.

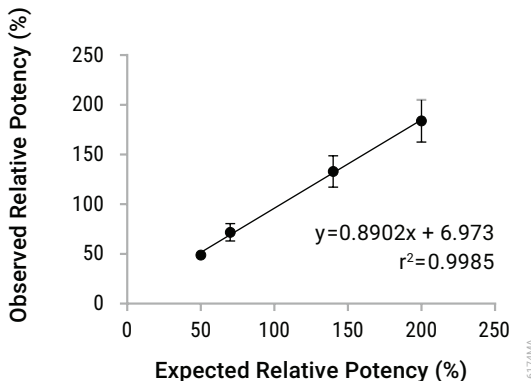


Figure 3. The IL-23 Bioassay shows precision, accuracy and linearity. A 50–200% theoretical potency series of ustekinumab was analyzed in triplicate in three independent experiments performed on three days by two analysts using the IL-23 Bioassay (for a total of six independent experiments). Bio-Glo™ Reagent was added and luminescence quantified using the GloMax® Discover System. Linearity and r² values were determined using GraphPad Prism® software. Data were generated using thaw-and-use cells.

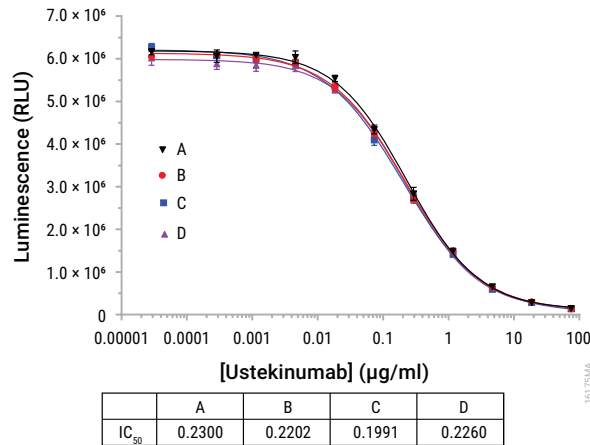


Figure 4. The IL-23 Bioassay demonstrates repeatability. Four separate dilution series of ustekinumab were analyzed on four individual assay plates using the IL-23 Bioassay. Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

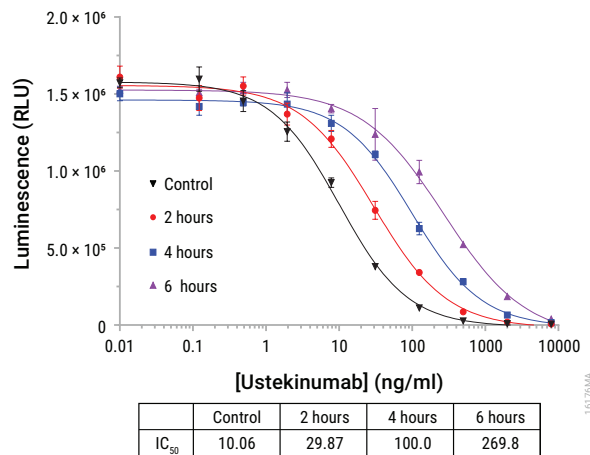


Figure 5. The IL-23 Bioassay indicates stability. Ustekinumab was heat treated at 65°C for 0–6 hours prior to use in the IL-23 Bioassay. Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells.

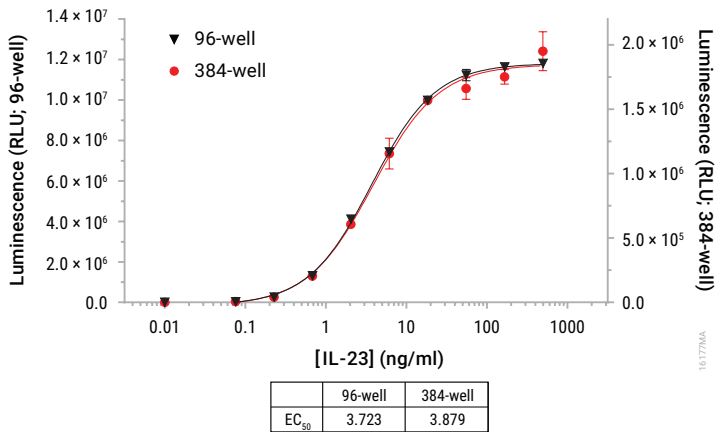


Figure 6. The IL-23 Bioassay is amenable to 384-well plate format. The IL-23 Bioassay was tested in 96- and 384-well formats. IL-23 Bioassay Cells were prepared and dispensed in 50 μ l (96-well) or 12.5 μ l (384-well) volumes. Serial threefold dilutions of recombinant human IL-23 were prepared and added to cells (25 μ l/well for 96-well format; 6.2 μ l/well for 384-well format). After 6 hours of stimulation, Bio-Glo™ Reagent was added (75 μ l/well for 96-well; 18.7 μ l/well for 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. (Costar® Cat.# 3570 384-well plates were used.)

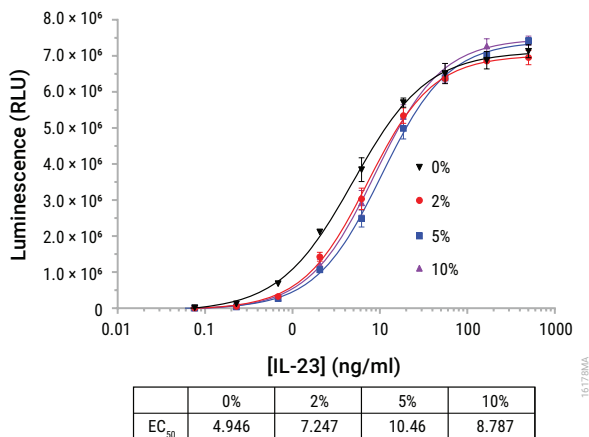


Figure 7. The IL-23 Bioassay tolerates up to 10% human serum. IL-23 Bioassay Cells were tested with a dose-response of recombinant IL-23 in the absence or presence of increasing concentrations of pooled normal human serum, resulting in final assay concentrations of 0–10% human serum. Bio-Glo™ Reagent 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.

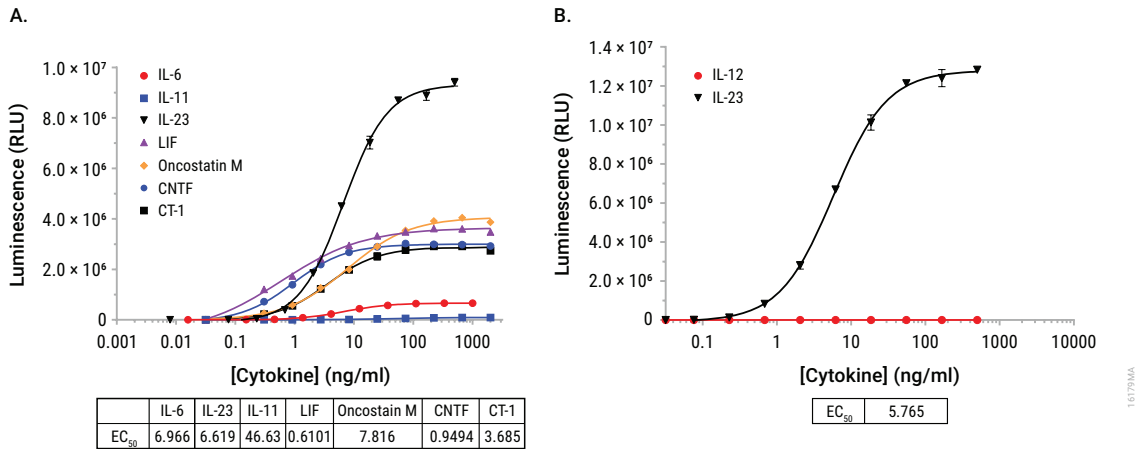


Figure 8. IL-23 Bioassay was tested for cytokine cross reactivity. Panel A. IL-23 Bioassay Cells were tested using a panel of IL-6 family cytokines (IL-6, IL-11, IL-23, LIF, Oncostatin M, CNTF and CT-1). Following a 6-hour treatment, Bio-Glo™ Reagent was added, and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. Data were generated using thaw-and-use cells. **Panel B** shows a lack of IL-23 Bioassay response to the structurally related cytokine IL-12.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT. #
IL-23 Bioassay	1 each	JA2511

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-23 Bioassay Cells (1.2×10^7 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-23 Bioassay 5X	1 each	JA2515

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-23 Bioassay Cells (1.2×10^7 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-23 Bioassay components are shipped separately because of differing temperature requirements. The IL-23 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 4°C protected from light.

3. Before You Begin

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

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-23 Bioassay is intended to be used with user-provided biologics designed to activate or inhibit the IL-23 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-23. 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-23 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.

Materials to Be Supplied by the User

Reagents

- user-defined biologics samples
- **optional:** recombinant human IL-23 (e.g., PeproTech Cat. # 200-23)

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% CO₂ incubator
- 37°C water bath
- plate reader that measures glow luminescence or luminometer (e.g., GloMax® Discover System)

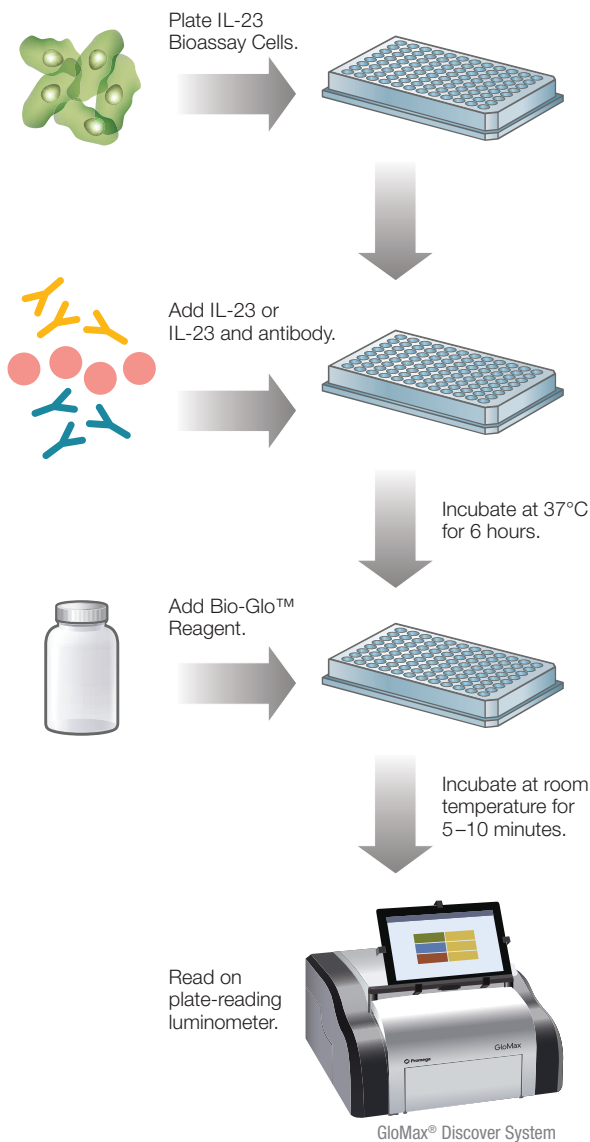


Figure 9. IL-23 Bioassay schematic protocol.

4. Stimulation Protocol

The IL-23 Bioassay can be used to test IL-23 cytokine and IL-23/IL-23R blocking antibodies. This stimulation protocol illustrates the use of the IL-23 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-23 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 0–500ng/ml of recombinant human IL-23 (PeproTech Cat.# 200-23) as a sample range, with serial threefold dilutions to achieve full dose curves as ten-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-23 Bioassay Cells, is sufficient for 120 wells (two 96-well plates using inner 60 well format). These 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 in 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 40ml of assay buffer, add 4ml of FBS to 36ml 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-23 Bioassay Cells

The thaw-and-use IL-23 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-23 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 the vial.
4. Gently mix the cell suspension by pipeting, then transfer 0.65ml of the cells to the 15ml conical tube containing 10.4ml of assay buffer. Mix well by gently pipeting 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% CO₂ 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. Wells A2, B2, C2 and D2 contain 120µl of assay buffer without sample as a negative control.

4.D. Preparing Serial Dilutions

The instructions described here are for preparation of a single stock of threefold serial dilutions of a single sample for analysis in triplicate (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.

Serial dilutions should be prepared on the day of the assay, after plating the IL-23 Bioassay Cells.

Recommended starting concentration of reference samples

For IL-23 stimulation using recombinant human IL-23 as your reference sample (PeproTech IL-23 Cat.# 200-23), we recommend starting with a 3X concentration of 1,500ng/ml and performing serial threefold dilutions. When using other reference sources of IL-23, 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.
3. Add 120µl of assay buffer to the other wells in rows A, B, C and D, 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 volume of 120µl. Do not dilute into column 2.
6. Cover the plate with a lid and proceed to Section 4.E.

4.E. IL-23 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% CO₂ 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 EC₅₀ 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 Log₁₀[sample] and fold induction versus Log₁₀[sample]. Fit curves and determine the EC₅₀ value of the IL-23 response using appropriate curve fitting software (such as GraphPad Prism®).

5. Inhibition Protocol

IL-23 Bioassay Cells can be used to measure inhibition of IL-23 signaling using a blocking antibody such as ustekinumab, which targets the p40 subunit of IL-23. A preliminary stimulation experiment with IL-23 is necessary to determine the EC₈₀ concentration, which is used for an inhibition assay. To facilitate workflow, a 5-hour stimulation can be used instead of the 6 hours recommended in the stimulation protocol. The IL-23 Bioassay Cells can be plated during the antibody + cytokine pre-incubation. This inhibition assay uses an eleven-point dilution series due to the response seen with ustekinumab. This inhibition protocol can be modified for other blocking antibodies, depending on their mechanism of action.

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	dilu 10	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	Reference Sample
B		no drug	dilu 10	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	Reference Sample
C		no drug	dilu 10	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	Test Sample 1
D		no drug	dilu 10	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	Test Sample 2
E													
F													
G													
H													

Figure 12. Example plate layout showing reference and test sample eleven-point serial dilutions. Wells A2, B2, C2 and D2 contain 120µl of assay buffer without sample as a negative control.

5.A. Preparing Antibody Dilutions

1. Prepare serial dilutions of ustekinumab antibody in warm assay buffer as 6X final concentration. We recommend a final concentration of ustekinumab of 0–75µg/ml as serial fourfold dilutions, using an eleven-point dilution series.
2. To a sterile clear 96-well plate, add 80µl of reference antibody sample starting dilution (dilu1, 6X final concentration) to wells A12 and B12 (see Figure 12).
3. Add 80µl of test antibody samples 1 and 2 starting dilution (dilu1, 6X final concentration) to wells C12 and D12.
4. Add 60µl of assay buffer to the other wells in these four rows, from column 11 to column 2.
5. Transfer 20µl of the sample starting dilutions from column 12 into column 11. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent fourfold serial dilutions across the columns from right to left until you reach column 3. Remove 20µl from column 3 so that all wells have a volume of 60µl. Do not dilute into column 2.
7. Prepare IL-23 in warm assay buffer at 6X the predetermined EC₈₀ response concentration.
8. Combine an equal volume of IL-23 (60µl) with each antibody dilution (60µl). Mix by pipetting.
Note: Each component is now at 3X its final concentration.
9. Incubate IL-23 and antibody samples for 75 minutes in a humidified 37°C, 5% CO₂ incubator.
10. Proceed to Section 5.B. to plate the bioassay cells.

5.A. Preparing Antibody Dilutions (continued)

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	dilu 10	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	Reference Replicate 1
C	B	no drug	dilu 10	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	Test Replicate 1
D	B	no drug	dilu 10	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	Reference Replicate 2
E	B	no drug	dilu 10	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	Test Replicate 2
F	B	no drug	dilu 10	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	Reference Replicate 3
G	B	no drug	dilu 10	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	Test Replicate 3
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 13. Example plate layout. This suggested layout shows nonclustered locations for three replicates of each test and reference sample dilution series (dilu1–dilu10) and wells containing assay buffer alone (denoted by “B”).

5.B. Plating IL-23 Bioassay Cells

The thaw-and-use IL-23 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.

For the inhibition protocol described here, use the plate layout illustrated in Figure 13 as a guide. The protocol describes serial replicate dilutions ($n = 3$) of test and reference samples to generate two eleven-point dose-response curves for each plate.

1. Remove one vial of IL-23 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 the vial.
4. Gently mix the cell suspension by pipeting, 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 $50\mu\text{l}$ of the cell suspension to each of the inner 66 wells (B2–G12) of two 96-well assay plates. Optimal results depend on gently keeping the cells evenly resuspended during the plating process.
7. Add $75\mu\text{l}$ /well of warm assay buffer to the outer 30 wells of each plate.
8. Cover each assay plate with a lid and incubate at 37°C in a 5% CO_2 incubator for the remainder of the antibody + cytokine pre-incubation.

5.C. IL-23 Inhibition Assay

1. After the antibody + cytokine pre-incubation is complete, use a multichannel pipette to dispense $25\mu\text{l}$ of each antibody dilution sample to the $50\mu\text{l}$ of preplated cells.
2. Incubate for 5 hours (as done for preliminary EC_{80} concentration determination) in a humidified, 37°C , 5% CO_2 incubator.
3. After the 5-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. Due to the eleven-point dilution curve and 66 wells per plate being used for assay, 70µl/well of Bio-Glo™ Reagent is added to 75µl/well sample in the plate.

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 70µl of Bio-Glo™ Reagent to the inner 66 wells of the assay plate, taking care not to create bubbles.
3. Add 70µl 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 IC₅₀ 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 Log₁₀[sample] and fold induction versus Log₁₀[sample]. Fit curves and determine the IC₅₀ value of the IL-23 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)	<p>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.</p> <hr/> <p>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.</p> <hr/> <p>Low cell viability can lead to low luminescence readout and variability in assay performance.</p> <hr/> <p>Low activity of Bio-Glo™ Reagent leads to low RLU. Store and handle the Bio-Glo™ Reagent according to the instructions.</p>
Assay performance is variable	<p>Ensure that incubation times are consistent between assays.</p> <hr/> <p>Ensure that the Preparing and Plating protocols are strictly followed.</p> <hr/> <p>Ensure that IL-23 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.</p> <hr/> <p>IL-23 lot-to-lot activity differences may be observed. Consult cytokine provider for details.</p>
Weak assay response (low fold induction)	<p>IL-23 frozen single-use aliquot has lost biological activity. Follow manufacturer's recommendation for storage and stability.</p> <hr/> <p>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.</p>

7. References

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8. Related Products

Fc Effector Bioassays

Product	Size	Cat.#
ADCC Reporter Bioassay, Complete Kit (Raji)*	1 each	G7015
ADCC Reporter Bioassay, Core Kit*	1 each	G7010
ADCC Reporter Bioassay, F Variant, Core Kit**	1 each	G9790
ADCC Reporter Bioassay, Target Kit (Raji)*	1 each	G7016
Fc γ R11a-H ADCP Reporter Bioassay, Complete Kit**	1 each	G9901
Fc γ R11a-H ADCP Reporter Bioassay, Core Kit**	1 each	G9991
Mouse Fc γ RIV ADCC Bioassay, Complete Kit	1 each	M1201
Mouse Fc γ RIV ADCC Bioassay, Core Kit	1 each	M1211
Membrane TNF α Target Cells**	1 each	J3331
Membrane VEGF Target Cells**	1 each	J3351

*For Research Use Only. Not for use in diagnostic procedures.

**Not for Medical Diagnostic Use.

Additional kit formats are available.

Fc Effector Immunoassay

Product	Size	Cat.#
Lumit [®] FcRn Binding Immunoassay	100 assays	W1151

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

Immune Checkpoint Bioassays

Product	Size	Cat.#
4-1BB Bioassay	1 each	JA2351
CD28 Bioassay	1 each	JA6701
CD28 Blockade Bioassay	1 each	JA6101
CD40 Bioassay	1 each	JA2151
CTLA-4 Blockade Bioassay	1 each	JA3001
GITR Bioassay	1 each	JA2291
ICOS Bioassay	1 each	JA6801
ICOS Blockade Bioassay	1 each	JA6001
LAG-3/MHCII Blockade Bioassay	1 each	JA1111
OX40 Bioassay	1 each	JA2191

8. Related Products (continued)

Immune Checkpoint Bioassays (continued)

Product	Size	Cat.#
PD-1/PD-L1 Blockade Bioassay	1 each	J1250
PD-1+TIGIT Combination Bioassay	1 each	J2211
PD-L1 Negative Cells	1 each	J1191
TIGIT/CD155 Blockade Bioassay	1 each	J2201

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

Cytokine and Growth Factor Bioassays

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

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

Control Antibodies and Proteins

Product	Size	Cat.#
Control Ab, Anti-4-1BB	50µg	K1161
Control Ab, Anti-CD20	5µg	GA1130
Control Ab, Anti-OX40	50µg	K1191
Control Ab, Anti-CD40	50µg	K1181
Control Ab, Anti-CTLA-4	100µg	JA1020
Control Ab, Anti-LAG-3	100µg	K1150
Control Ab, Anti-PD-1	100µg	J1201
Control Ab, Anti-TIGIT	100µg	J2051
Control Ab, Anti-TIM-3	100µg	K1210
Recombinant VEGF ligand	10µg	J2371

Detection Reagents

Product	Size	Cat. #
Bio-Glo™ Luciferase Assay System	10ml	G7941
	100ml	G7940
Bio-Glo-NL™ Luciferase Assay System	10ml	J3081
	100ml	J3082
	1,000ml	J3083

Not for Medical Diagnostic Use.

Luminometers

Product	Size	Cat. #
GloMax® Navigator System	1 each	GM2000
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500

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Note: Additional Fc Effector, Immune Checkpoint, T Cell Activation and Cytokine, Macrophage and Target Cell Killing Bioassays are available. To view and order Promega Bioassay products visit:

www.promega.com/products/reporter-bioassays/ or email: EarlyAccess@promega.com. For information on custom bioassay development and services visit the Promega Tailored R&D Solutions website:

www.promega.com/custom-solutions/tailored-solutions/

9. Summary of Changes

The following changes were made to the 7/25 revision of this document:

1. Revised text about the label in Section 3.
2. Updated the fonts.
3. Made miscellaneous text edits, including removing one patent statement and updating another patent statement.
4. Updated Lumit trademark to registered.



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