TECHNICAL MANUAL

IL-12 Bioassay

Instructions for use of Products
JA2601 and JA2605

Revised 3/20
TM608

Promega

IL-12 Bioassay

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1. Description

Interleukin-12 (IL-12) was first identified in 1989 in the supernatant of B lymphocytes transformed with Epstein-Barr virus, and named "natural killer-stimulator factor" due to its ability to induce interferon (IFN)-γ and natural killer (NK) cell cytotoxicity when added to human peripheral blood lymphocytes (1). This pro-inflammatory, heterodimeric cytokine consists of covalently linked p40 and p35 subunits and is a member of the IL-12 cytokine family, which includes IL-12, IL-23, IL-27, IL-35 and IL-39. Both IL-12 and IL-23 are composite cytokines, sharing a common IL-12 p40 subunit and IL-12Rβ1 receptor. Cytokine specificity is derived from the unique IL-12 p35 subunit binding to IL-12Rβ2 and the IL-23p19 subunit binding to IL-23R. Both p35 and p40 genes need to be expressed within the same cell to produce the active heterodimer and subsequent IL-12 signaling.

IL-12 is secreted following stimulation of phagocytes and dendritic cells by bacteria and other microorganisms (2). Together with antigen presentation, IL-12 directs CD4+ T cells to differentiate into IFN-γ producing T helper 1 (Th1) cells and also induces lymphokine-activated killer cells and NK cells. These Th1 cells respond to intracellular pathogens (e.g., *Legionella pneumophila*, *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Salmonella spp*.) and promote delayed-type hypersensitivity and activation of macrophages (3).

The primary IL-12 receptor expressing cell types are NK, B and T cells. Signaling begins with tyrosine kinase 2 (TYK2) binding to the IL-12Rβ1 and Janus kinase 2 (JAK2) binding to IL-12Rβ2. These kinases phosphorylate and activate signal transducer and activator of transcription 4 (STAT4). The STAT4 complex translocates to the nucleus where, together with the Jun proto-oncogene (c-Jun), it binds to the IFN-γ promoter causing IFN-γ transcription and Th1 differentiation. Furthermore, IL-12-related production of IFN-γ triggers the production of C-X-C motif chemokine 10 (CXCL10), which in turn leads to an anti-angiogenic phenotype.

Ustekinumab is a humanized monoclonal antibody that targets IL-12/23 p40 and prevents IL-12 and IL-23 cytokine binding to IL-12Rβ1. It was approved by the FDA for the treatment of psoriasis in 2009, psoriatic arthritis in 2013 and Crohn's disease in 2016 (4).

The IL-12 Bioassay[a–e] (Cat.# JA2601, JA2605) is a bioluminescent cell-based assay designed to measure IL-12 stimulation or inhibition. The IL-12 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. The IL-12 Bioassay Cells are also available in a Cell Propagation Model (CPM; Cat.# J3042) format, as cryopreserved cells that can be thawed, propagated and banked for long-term use.

The IL-12 Bioassay consists of human cells engineered to express the IL-12 receptor and a luciferase reporter driven by a response element (RE). When IL-12 binds to IL-12R, it transduces intracellular signals resulting in luminescence (Figure 1). The bioluminescent signal is detected and quantified using the Bio-Glo™ Luciferase Assay System[e] (Cat.# G7940, G7941) and a standard luminometer, such as the GloMax® Discover System (see Related Products, Section 8).

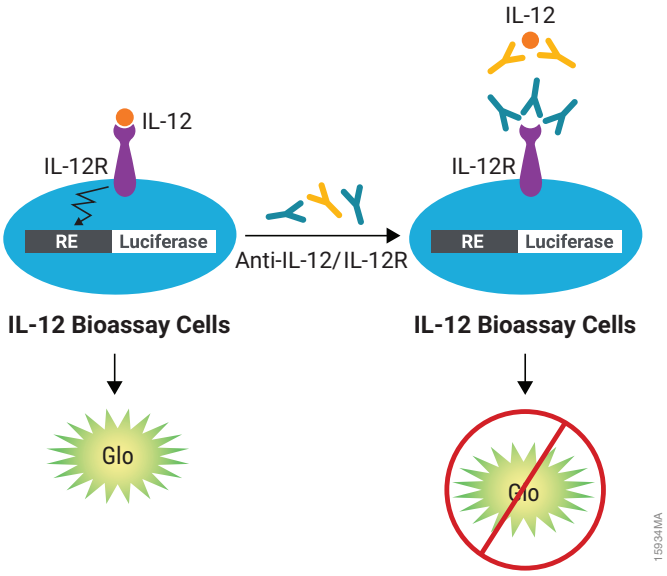
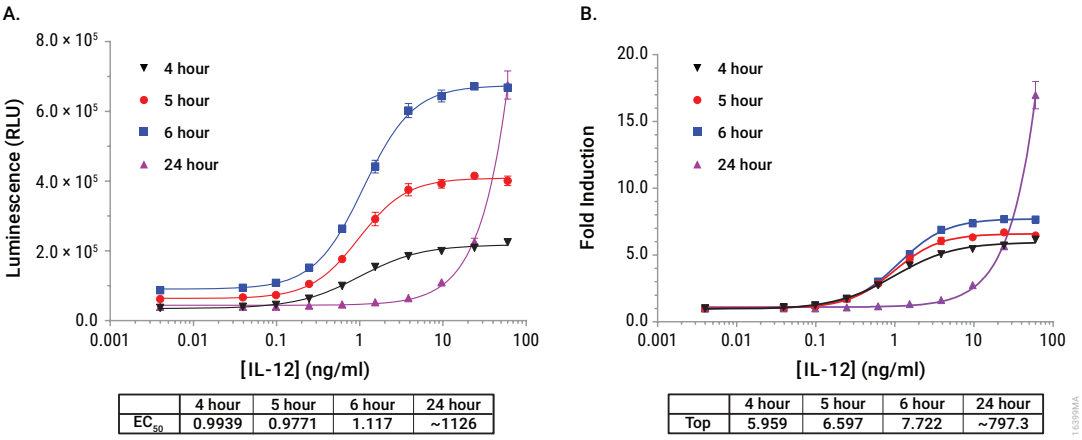


Figure 1. Representation of the IL-12 Bioassay. The IL-12 Bioassay consists of a genetically engineered cell line, IL-12 Bioassay Cells. When IL-12 binds to the IL-12 receptor (IL-12R), receptor-mediated pathway signaling induces luminescence that can be detected upon addition of Bio-Glo™ Reagent and quantified with a luminometer. Inhibition of IL-12 binding by anti-IL-12 or anti-IL-12R antibodies results in a decrease in luminescence.

1. Description (continued)



	4 hour	5 hour	6 hour	24 hour
EC_{50}	0.9939	0.9771	1.117	~1126

	4 hour	5 hour	6 hour	24 hour
Top	5.959	6.597	7.722	~797.3

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Figure 2. The IL-12 Bioassay responds to recombinant IL-12. IL-12 Bioassay Cells were prepared as described in this protocol and incubated with serial dilutions of recombinant IL-12. 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 4-parameter logistic (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-12 Bioassay Shows Precision, Accuracy and Linearity.

Parameter	Results	
Accuracy	% Expected Relative Potency	% Recovery
	50	101.9
	70	99.4
	140	98.7
	200	108.5
Repeatability (% CV)	100% (Reference)	3.99
Intermediate Precision (% CV)		9.6
Linearity (r^2)		0.994
Linearity (y = mx + b)		y = 1.096x – 7.15

A 50–200% theoretical potency series of ustekinumab (anti-IL-12) 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.

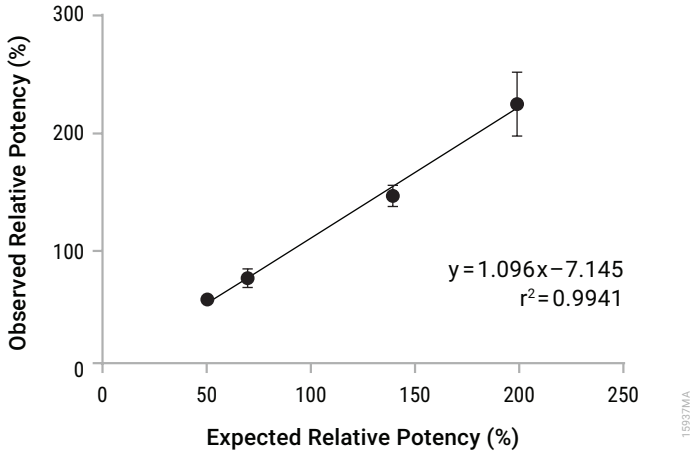
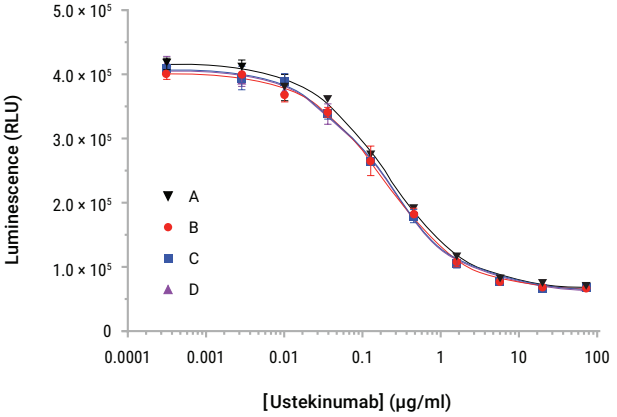
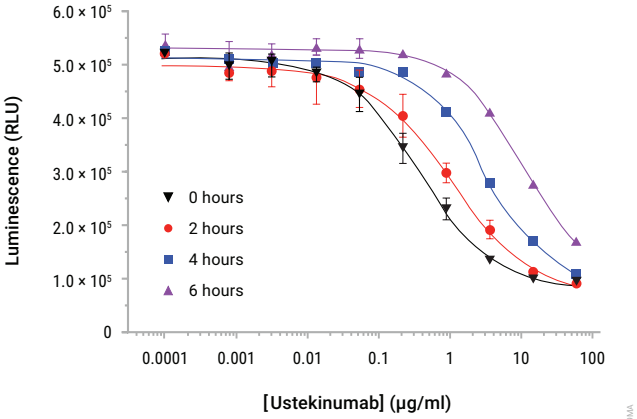


Figure 3. The IL-12 Bioassay shows precision, accuracy and linearity. A 50–200% theoretical potency series of ustekinumab was analyzed in triplicate in three independent experiments performed on three days by two analysts using the IL-12 Bioassay (for a total of six independent experiments). Bio-Glo™ 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}	0.1957	0.1896	0.1781	0.1847

Figure 4. The IL-12 Bioassay demonstrates repeatability. Four separate dilution series of ustekinumab (anti-IL-12) were analyzed on four individual assay plates using the IL-12 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. An EC_{90} concentration of IL-12 was used in this study. Data were generated using thaw-and-use cells.



	0 hours	2 hours	4 hours	6 hours
IC_{50}	0.3446	0.9580	2.728	8.999

Figure 5. The IL-12 Bioassay indicates stability. Ustekinumab was heat treated at 65°C for 0–6 hours prior to use in the IL-12 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. An EC_{90} concentration of IL-12 was used in this study. Data were generated using thaw-and-use cells.

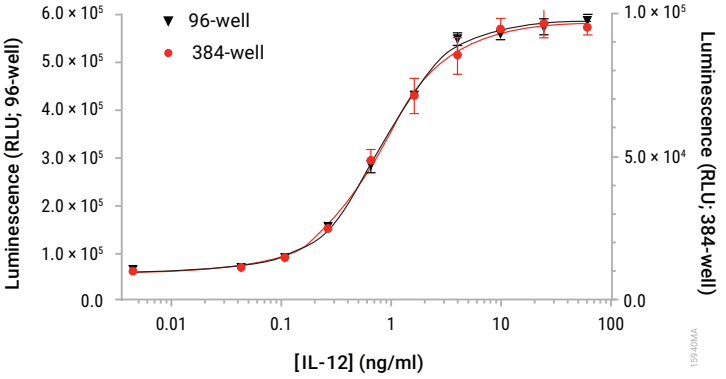
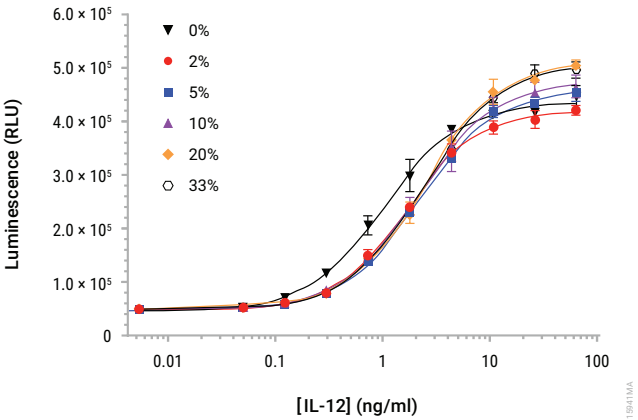
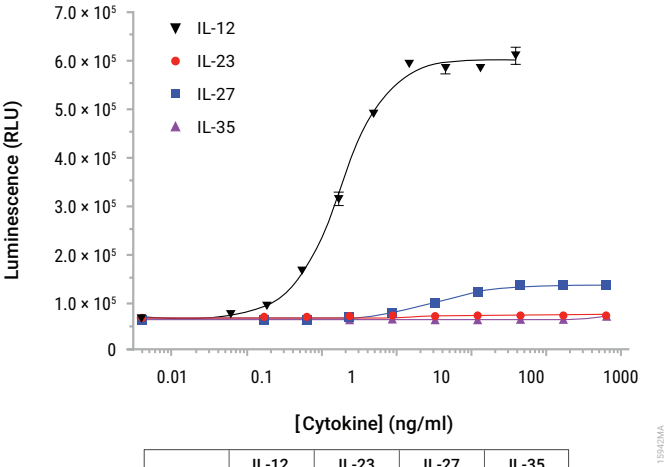


Figure 6. The IL-12 Bioassay is amenable to 384-well plate format. IL-12 Bioassay Cells were prepared and dispensed in 50µl (96-well) or 12.5µl (384-well) volumes. Serial 2.5-fold dilutions of recombinant human IL-12 were prepared and added to cells (25µl/well for 96-well plates; 6.2µl/well for 384-well plates). After 6 hours of stimulation, Bio-Glo™ Reagent was added (75µl/well for 96-well; 18.7µl/well for 384-well), and luminescence was quantified using the GloMax® Discover System. Data were fitted to a 4PL curve using GraphPad Prism® software. The IL-12 EC_{50} was 0.77ng/ml for 96-well plate and 0.75ng/ml for the 384-well plate. Data were generated using thaw-and-use cells.



	0%	2%	5%	10%	20%	33%
EC_{50}	0.8579	1.391	1.865	1.817	2.081	2.116

Figure 7. The IL-12 Bioassay tolerates human serum. IL-12 Bioassay Cells were tested with a dose-response of recombinant IL-12 in the absence or presence of increasing concentrations of pooled normal human serum, resulting in final assay concentrations of 0–33% 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.



	IL-12	IL-23	IL-27	IL-35
EC_{50}	0.6618	0.6037	7.780	~703.9

Figure 8. IL-12 Bioassay was tested for cytokine cross reactivity. IL-12 Bioassay Cells were tested using a panel of IL-12 family cytokines (IL-12, IL-23, IL-27 and IL-35). 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-12 Bioassay	**1 each**	**JA2601**

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-12 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-12 Bioassay 5X	**1 each**	**JA2605**

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-12 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-12 Bioassay components are shipped separately because of differing temperature requirements. The IL-12 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 –20°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 –20°C for up to 6 weeks.
- Store RPMI 1640 Medium at 4°C protected from light.

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3. Before You Begin

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

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 web site, such as the Certificate of Analysis.

The IL-12 Bioassay is intended for use with user-provided biologics designed to activate or inhibit the IL-12 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-12. 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-12 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-12 (e.g., PeproTech IL-12 Cat.# 200-12)

Supplies and Equipment

- white, flat-bottom 96-well assay plates (e.g., Corning® Cat.# 3917)
- sterile, clear 96-well plates with lids (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
- plate reader that measures glow luminescence or luminometer (e.g., GloMax® Discover System, Cat.# GM3000)
- **optional:** clear 384-well plates with lids (e.g., Costar® Cat.# 3570)

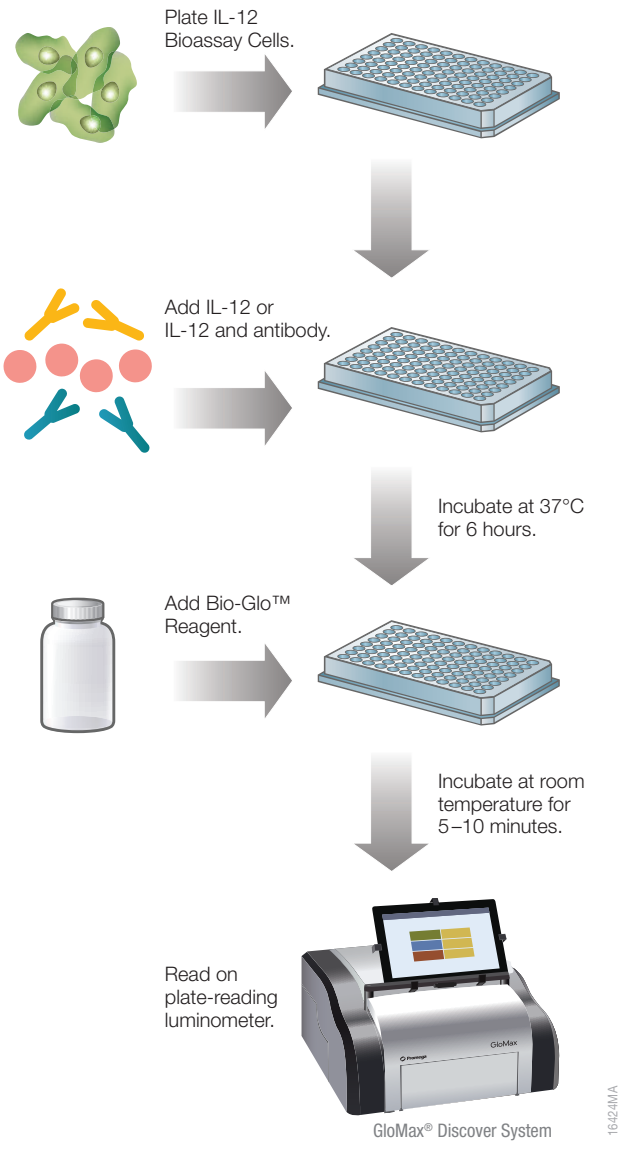

Figure 9. IL-12 Bioassay schematic protocol.

4. Stimulation Protocol

The IL-12 Bioassay can be used to test IL-12 cytokine and IL-12/IL-12R blocking antibodies. This stimulation protocol illustrates the use of the IL-12 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 10-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-12 activity.

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 0–60ng/ml of recombinant IL-12 (PeproTech Cat.# 200-12) as a sample range, with serial 2.5-fold dilutions to achieve full dose curves as a 10-point series. Concentration ranges and dilution schemes may need to be optimized for your samples.
2. When diluted as directed, each kit containing medium, serum, and 1 vial of IL-12 Bioassay Cells is sufficient for 120 wells (two 96-well plates using inner 60 wells). The thaw-and-use cells are for single use only and cannot be cultured or refrozen for a second use. Please plan your experiments accordingly to optimize 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 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-12 Bioassay Cells

The thaw-and-use IL-12 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 materials.

1. Remove one vial of IL-12 Bioassay Cells from storage at –140°C and transfer to the bench on dry ice.
2. Add 9.1ml 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 9.1ml 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. 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 2.5-fold 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 2.5-fold serial dilutions, you will need a minimum of 400µl of a reference sample at 3X the highest concentration in your dose-response curve. You will need a minimum of 200µl 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-12 stimulation using recombinant human IL-12 as your reference sample (PeproTech IL-12 Cat.# 200-12), we recommend starting with a 3X concentration of 180ng/ml and performing serial 2.5-fold dilutions. When using other reference sources of IL-12, the starting concentration may need to be adjusted.

Prepare serial dilutions on the day of the assay, after plating the IL-12 Bioassay Cells.

1. To a sterile clear 96-well plate, add 200µl of reference sample starting dilution (dilu1, 3X final concentration) to wells A11 and B11 (see Figure 11).
2. Add 200µl 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 these four rows, from column 10 to column 2.
4. Transfer 80µl of the sample starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
5. Repeat equivalent 2.5-fold serial dilutions across the columns from right to left until you reach column 3. Remove 80µl from column 3 so that all wells contain 120µl. Do not dilute into column 2.
6. Cover the plate with a lid and proceed to Section 4.E.

4.E. IL-12 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 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 to not create bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, C1 and D1 of each assay plate to determine 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 = $$\frac{\text{RLU (sample – background)}}{\text{RLU (no drug control – background)}}$$

   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 the IL-12 response using appropriate curve-fitting software (such as GraphPad Prism®).

5. Inhibition Protocol

The IL-12 Bioassay Cells can be used to measure inhibition of IL-12 signaling using a blocking antibody such as ustekinumab, which targets the p40 subunit of IL-12. A preliminary stimulation experiment with IL-12 is necessary to determine the EC_{90} concentration to be used for an inhibition assay. To facilitate workflow, a 5.5-hour incubation can be used instead of the 6 hours recommended in the stimulation protocol. The bioassay cells can be plated during the antibody + cytokine pre-incubation. This inhibition protocol can be modified for other blocking antibodies, depending on their mechanism of action.

5.A. Preparing Antibody Dilutions

1. Prepare serial dilutions of ustekinumab antibody in warm assay buffer as 6X final concentration. We recommend a final ustekinumab concentration of 0–60µg/ml using serial 3.5-fold dilutions.
2. To a sterile clear 96-well plate, add 105µl of **reference** antibody sample starting dilution (dilu1, 6X final concentration) to wells A11 and B11 (see Figure 11).
3. Add 105µl of **test** antibody samples 1 and 2 starting dilution (dilu1, 6X final concentration) to wells C11 and D11, respectively.
4. Add 75µl of assay buffer to the other wells in these four rows, from column 10 to column 2.
5. Transfer 30µl of the sample starting dilutions from column 11 into column 10. Mix well by pipetting. Avoid creating bubbles.
6. Repeat equivalent 3.5-fold serial dilutions across the columns from right to left until you reach column 3. Remove 30µl from column 3 so that all wells have a 75µl volume. Do not dilute into column 2.
7. Prepare IL-12 in warm assay buffer at 6X the predetermined EC_{90} response concentration.
8. Combine an equal volume (75µl) of IL-12 with each antibody dilution (75µl) . Mix by pipetting.

   Note: Each component is now at 3X its final concentration.
9. Incubate IL-12 and antibody samples for 60 minutes in a humidified 37°C, 5% CO_2 incubator.
10. Proceed to Section 5.B. and plate the bioassay cells.

5.B. Plating IL-12 Bioassay Cells

The thaw-and-use IL-12 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.

1. Remove one vial of IL-12 Bioassay Cells from storage at −140°C and transfer to the bench on dry ice.
2. Add 9.1ml 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 pipetting, then transfer 0.65ml of the cells to the 15ml conical tube containing 9.1ml of assay buffer. Mix well by gently pipetting or inverting five 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. To optimize results, gently keep 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 for the reminder of the antibody + cytokine pre-incubation.

5.C. IL-12 Inhibition Assay

1. Using a multichannel pipette, dispense 25µl of each antibody dilution sample into wells containing 50µl of preplated cells.
2. Incubate for 5.5 hours (as done for preliminary EC_{90} concentration determination) in a humidified 37°C, 5% CO_2 incubator.
3. After the 5.5-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.

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 to not create bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, C1 and D1 of each assay plate to determine 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-12 inhibition 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** 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 that the Preparing and Plating protocol is strictly followed.
	Ensure that IL-12 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-12 lot-to-lot activity differences may be observed. Consult cytokine provider for details.
Weak assay response (low fold induction)	IL-12 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

1. Kobayashi, M. *et. al.* (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* **170**, 827–45.
2. Ma, X. and Trinchieri, G. (2001) Regulation of interleukin-12 production in antigen-presenting cells. *Adv. Immunol.* **79**, 55–92.
3. Trinchieri, G. (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* **3**, 133–46.
4. Benson, J.M. *et. al.* (2011) Therapeutic targeting of the IL-12/23 pathways: generation and characterization of ustekinumab. *Nat. Biotechnol.* **29(7)** 615–24.

8. 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-6 Bioassay, Propagation Model	1 each	J2992
IL-12 Bioassay, Propagation Model	1 each	J3042
IL-15 Bioassay	1 each	JA2011
IL-15 Bioassay 5X	1 each	JA2015
IL-15 Bioassay, Propagation Model	1 each	J2962
IL-23 Bioassay	1 each	JA2511
IL-23 Bioassay 5X	1 each	JA2515
IL-23 Bioassay, Propagation Model	1 each	J3002

Not for Medical Diagnostic Use.

Immune Checkpoint Bioassays

Product	Size	Cat.#
CD40 Bioassay	1 each	JA2151
CD40 Bioassay 5X	1 each	JA2155
CD40 Bioassay, Propagation Model	1 each	J2132
FcγRIIb CHO-K1 Cells	1 each	JA2251
FcγRIIb CHO-K1 Cells 5X	1 each	JA2255
FcγRIIb CHO-K1 Cells, Propagation Model	1 each	J2232
GITR Bioassay	1 each	JA2291
GITR Bioassay 5X	1 each	JA2295
GITR Bioassay, Propagation Model	1 each	J2272
LAG-3/MHCII Blockade Bioassay	1 each	JA1111
LAG-3/MHCII Blockade Bioassay 5X	1 each	JA1115
LAG-3/MHCII Blockade Bioassay, Propagation Model	1 each	JA1112
Control Ab, Anti-LAG-3	100µg	K1150
TCR Activating Antigen Stock Solution	500µl	K1201
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
CTLA-4 Blockade Bioassay	1 each	JA3001
CTLA-4 Blockade Bioassay 5X	1 each	JA3005
Control Antibody, Anti-CTLA-4	100µg	JA1020
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
Control Ab, Anti-CD40	50µg	K1181

Note: Additional Bioassays are available from Promega Custom Assay Services. To view and order products see Early Access Bioassays at:
www.promega.com/applications/biologics-drug-discovery/functional-bioassays/target-pathway-assays/
or email: **CAS@promega.com**

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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.

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.

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

For Research Use Only. Not For Use in Diagnostic Procedures.

9. Summary of Changes

The following change was made to the 3/20 revision of TM608:

In Section 2. Product Components and Storage Conditions, the amount of RPMI 1640 Medium was changed to 36ml.

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[b] Cells were obtained under license from AdVec Inc.

[c] U.S. Pat. No. 8,008,006 and European Pat. No. 1341808.

[d] Patents Pending.

[e] U.S. Pat. No. 10,077,244 and other patents.

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