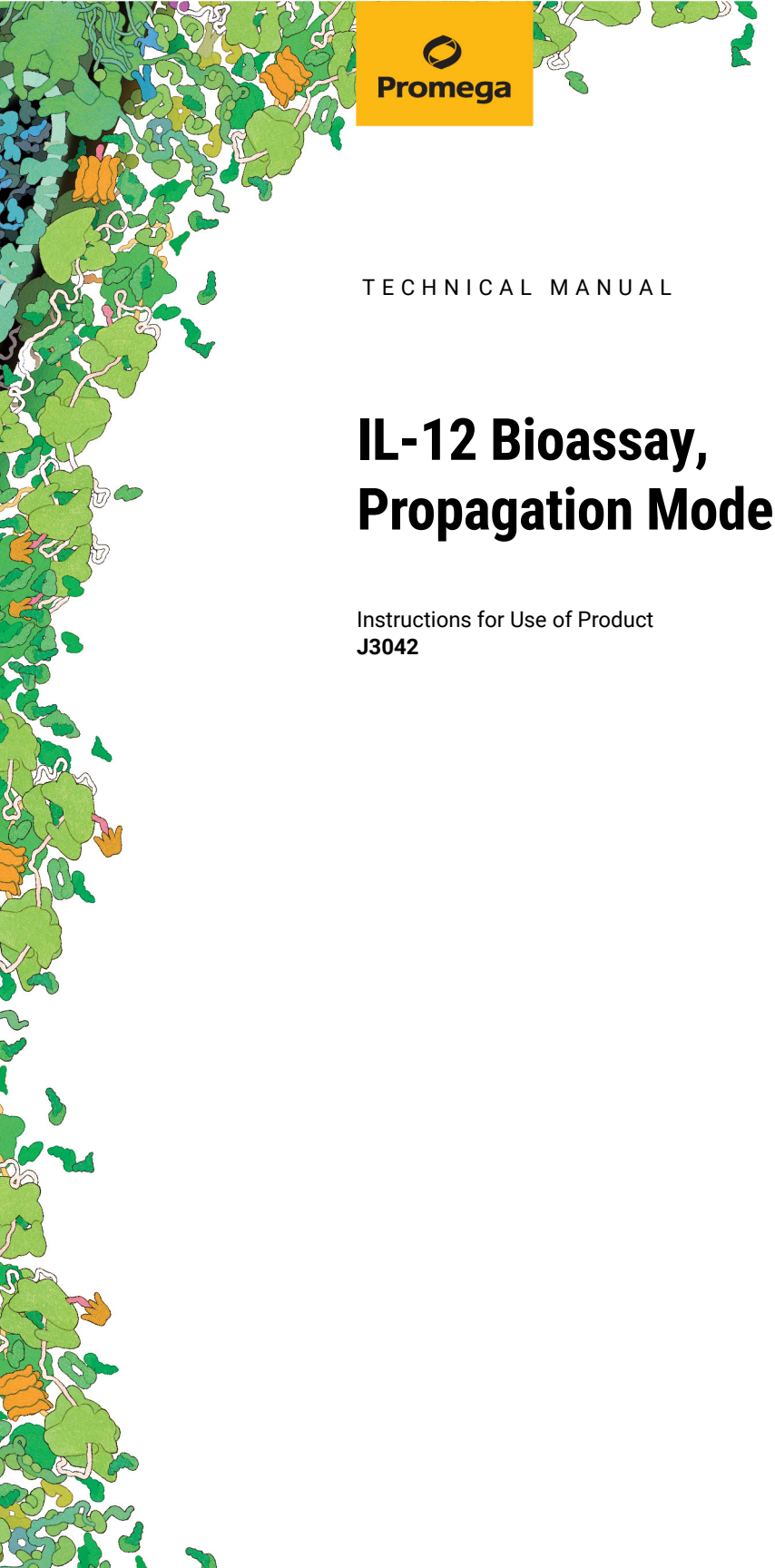

TECHNICAL MANUAL

IL-12 Bioassay, Propagation Model

Instructions for Use of Product
J3042

Revised 5/26
TM588

Promega

IL-12 Bioassay, Propagation Model

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

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1. Description

Interleukin-12 (IL-12) was first identified in the supernatant of Epstein-Barr virus-transformed B lymphoblasts in 1989 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 bacterial and microorganism stimulation of phagocytes and dendritic cells (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).

NK, B and T cells are the primary IL-12 receptor-expressing cell types. 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 Jun 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 most recently Crohn's disease in 2016 (4).

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

The IL-12 Bioassay consists of a genetically engineered human cell line that expresses 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 Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941) and a standard luminometer, such as the GloMax® Discover System.

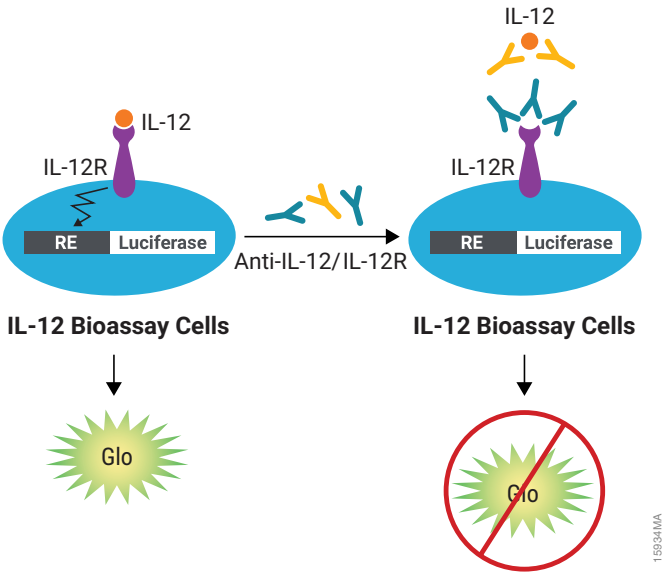
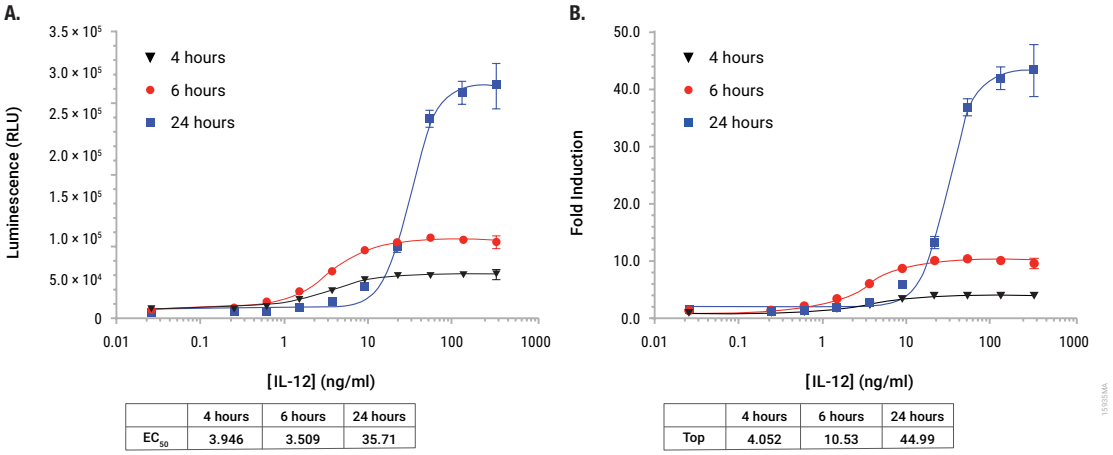


Figure 1. Representation of the IL-12 Bioassay. The IL-12 Bioassay consists of a genetically engineered cell line, IL-12 Bioassay Cells. When IL-12 binds to the IL-12 receptor (IL-12R), receptor-mediated pathway signaling induces luminescence that can be detected upon addition of Bio-Glo™ Reagent and 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 hours	6 hours	24 hours
EC_{50}	3.946	3.509	35.71

	4 hours	6 hours	24 hours
Top	4.052	10.53	44.99

Figure 2. The IL-12 Bioassay responds to recombinant IL-12. IL-12 Bioassay Cells were grown and prepared as described in this protocol and incubated with serial dilutions of recombinant IL-12. After a 4-, 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 cell propagation model (CPM) cells.

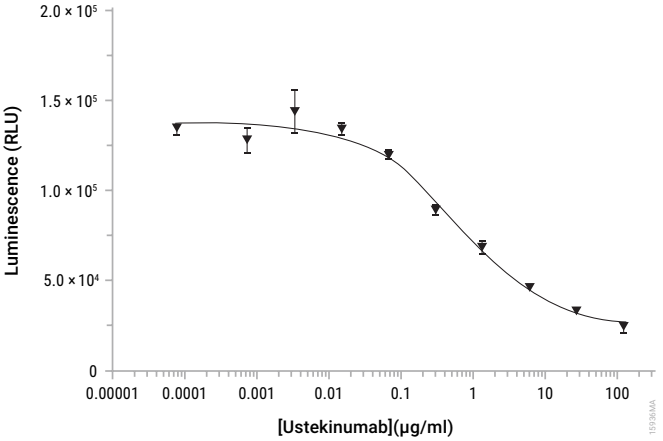


Figure 3. The IL-12 Bioassay responds to ustekinumab. IL-12 Bioassay Cells were grown and prepared as described in this protocol. Serial dilutions of ustekinumab (anti-IL-12 antibody) were combined with recombinant IL-12 (EC_{90} concentration) for 60 minutes. The antibody/IL-12 samples were then added to cells and the plate was further incubated for 5.5 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 CPM cells.

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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 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.

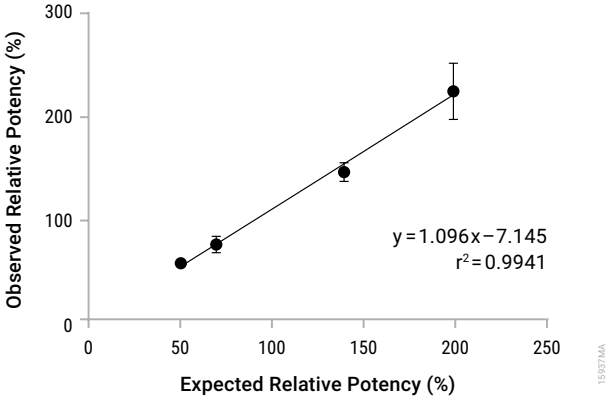
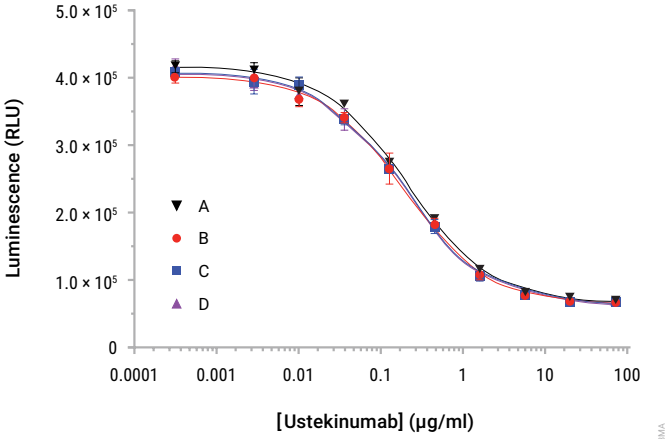
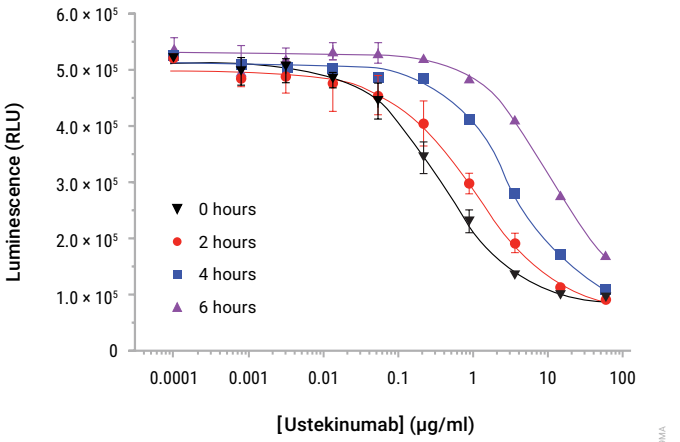


Figure 4. The IL-12 Bioassay shows precision, accuracy and linearity. A 50–200% theoretical potency series of ustekinumab was analyzed in triplicate in three independent experiments performed on three days by two analysts using the IL-12 Bioassay (for a total of six independent experiments). Bio-Glo™ 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 5. 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. 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 6. 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. Data were generated using thaw-and-use cells.

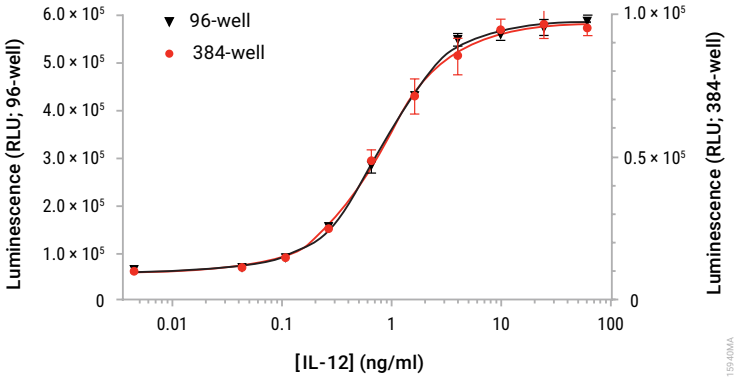
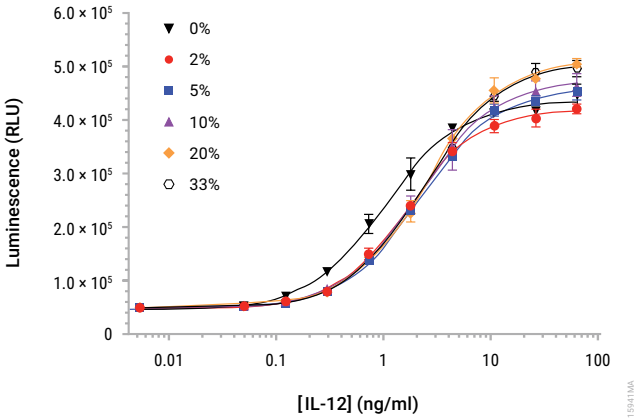
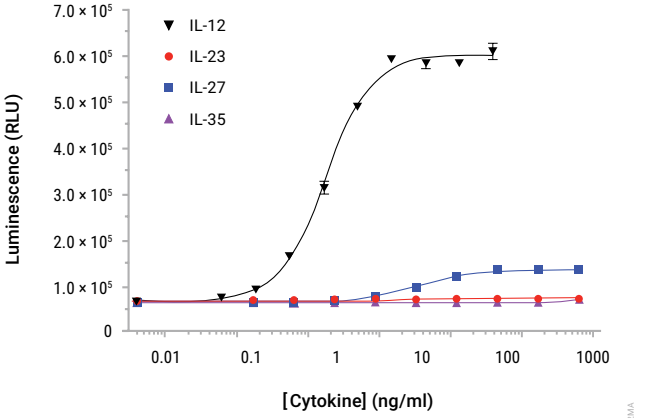


Figure 7. The IL-12 Bioassay is amenable to 384-well plate format. IL-12 Bioassay Cells were prepared and dispensed in 50µl (96-well) or 12.5µl (384-well) volumes. Serial 2.5-fold dilutions of recombinant human IL-12 were prepared and added to cells (25µl/well 96-well; 6.2µl/well 384-well). After 6 hours of stimulation, Bio-Glo™ Reagent was added (75µl/well 96-well; 18.7µl/well 384-well; Costar® Cat. #3570 384-well plates were used), 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 8. 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 9. 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, Propagation Model	**1 each**	**J3042**

Not for Medical Diagnostic Use. Includes:

- 2 vials IL-12 Bioassay Cells (1.2×10^7 cells/ml; 0.65ml per vial)

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

Storage Conditions: Upon arrival, immediately transfer the cell vials to below –140°C (freezer or liquid nitrogen vapor phase) for long-term storage. Do not store cell vials submerged in liquid nitrogen. **Do not** store cell vials at –80°C because this will decrease cell viability and cell performance.

3. Before You Begin

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

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

Cell thawing, propagation and banking should be performed exactly as described in Section 4. Cell seeding and propagation densities have been optimized to ensure stable cell growth, which is reflected in a steady cell doubling rate, to achieve optimal, consistent performance.

The IL-12 Bioassay is intended to be used 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 Sections 5 and 6 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.

3.A. Materials to Be Supplied by the User

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

Reagents

- user-defined biologics samples
- DMEM high glucose with pyruvate (e.g., GIBCO® Cat.#11995-065)
- fetal bovine serum (e.g., HyClone™ Cat.# SH3007002)
- blasticidin (e.g., GIBCO® Cat.# A11139-03)
- puromycin (e.g., GIBCO® Cat.# A11138-03)
- D-PBS (e.g., GIBCO® Cat.# 14190144)
- Accutase® or equivalent (e.g., Innovative Cell Technologies Cat.# AT104)
- DMSO (e.g., Sigma Cat.# D2650)
- Trypan blue solution (e.g., Sigma Cat.# T8154)
- Bio-Glo™ Luciferase Assay System (Cat.# G7940, G7941)
- **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 plate with lid (e.g., Corning® Cat.# 3896 or Falcon Cat.# 353077) for preparing sample dilutions
- pipettes (single-channel and 12-channel)
- T75 tissue culture flask (e.g., Corning® Cat.# 430641U)
- sterile 15ml and 50ml conical tubes
- sterile reagent reservoirs (e.g., Corning® Cat.# 4870)
- humidified 37°C, 5% CO_2 incubator
- 37°C water bath
- plate reader that measures glow luminescence or luminometer (e.g., GloMax® Discover System, Cat.# GM3000)
- **optional:** T225 tissue culture flask (e.g., Corning® Cat.# 431082)

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4. Preparing IL-12 Bioassay Cells

4.A. Cell Thawing and Initial Cell Culture

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

Note: IL-12 Bioassay Cells are grown as **adherent** cultures.

1. Prepare 65ml of thaw medium (see Section 9.A) and prewarm to 37°C. This medium will be used for culturing the cells immediately after thawing.
2. Transfer 8ml of thaw medium into a 15ml conical tube.
3. Remove one vial of IL-12 Bioassay Cells from storage at –140°C and thaw in a 37°C water bath with gentle agitation (no inversion) until just thawed (typically 2–3 minutes).
4. Spray vial with 70% ethanol and transfer to cell culture hood.
5. Transfer all of the cells (approximately 0.65ml) to the 15ml conical tube containing 8ml of prewarmed thaw medium.
6. Centrifuge at 150 × *g* for 5 minutes.
7. Carefully aspirate the medium and resuspend the cell pellet in 42ml of prewarmed thaw medium in a 50ml conical tube.
8. Count cells with Trypan blue and determine cell number and viability.
9. Transfer the cell suspension evenly into **three** T75 flasks (or one T225 flask). Place the flasks horizontally in a humidified 37°C, 5% CO_2 incubator and incubate for **two days**.

4.B. Cell Maintenance and Propagation

Note: For cell maintenance and propagation starting from the second cell passage, use growth medium containing selection antibiotic (see Section 9.A), and monitor cell viability and doubling rate during propagation. Cell growth rate will stabilize by approximately 7–10 days after thawing. At this time, cell viability is typically >90% and the average cell doubling rate is approximately 24 hours. Passage number should be recorded for each passage. Cells are expected to retain their functionality for up to 26 passages.

1. On the day of cell passage, visualize cells under microscope and estimate confluency.
2. To harvest cells for passaging, aspirate medium and gently rinse cell monolayer with D-PBS, being careful not to disrupt the cell monolayer. Carefully aspirate D-PBS.
3. Add 2ml of Accutase® to each T75 flask and rock flask several times to mix and coat the cell surface.
4. Incubate at room temperature until cells begin to lift off (approximately 3–5 minutes).
5. Once cells detach, add 8ml of prewarmed growth medium to the Accutase® and pipet to gently break up cell clumps and make a single cell suspension.
6. Sample and count by Trypan blue exclusion.
7. Add newly prepared growth medium and transfer cells to new flask. Mix gently.

8. Maintain cells at 15%–95% confluency. Do not allow cells to become 100% confluent prior to passaging as this may impact performance in subsequent passages. Recommended seeding density for passaging cells is as follows:

   a. For two-day culture: 2.7×10^4 cells/cm^2

   b. For three-day culture: 1.35×10^4 cells/cm^2

   Note: We recommend using the following media volumes for routine cell propagation: 14ml for a T75 flask, 28ml for a T150 flask and 42ml for a T225 flask. Scale according to surface area of flask.

9. Place the flasks horizontally in a humidified 37°C, 5% CO_2 incubator.

   Note: It is important to maintain a consistent passaging regimen to obtain consistent assay results.

4.C. Cell Freezing and Banking

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

1. On the day of cell freezing, prepare new cell freezing medium (see Section 9.A) and store on ice.
2. Remove a sample for cell counting by Trypan blue staining. Calculate the volume of freezing medium needed based on desired cell freezing density. We recommend a freezing density range of 1×10^6–1.5×10^7 cells/ml.
3. Transfer cells to 50ml sterile conical tubes or larger sized centrifuge tubes, and centrifuge cells at 150 × *g* for 10–15 minutes.
4. Gently aspirate the supernatant, being careful not to disturb the cell pellet.
5. Carefully resuspend cell pellet in ice-cold freezing medium to the desired final cell density. Combine the cell suspensions into a single tube and dispense 1ml into cryovials.
6. Freeze using a controlled-rate freezer (or use an insulated Mr. Frosty® or a Styrofoam® type of cell freezing container at –80°C overnight).
7. Transfer to –140°C or below for long-term storage.

5. Assay Protocol

The IL-12 Bioassay can be used in two different formats: IL-12 stimulation (described in Section 5.E.) and inhibition (described in Section 6.). This 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 ten-point dilution series, in a single 96-well assay plate using the inner 60 wells (Figure 11). Other experimental plate layouts are possible but may require further optimization.

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 0–400ng/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 ten-point series. Concentration ranges and dilution schemes may need to be optimized for your samples.

b. While maintaining IL-12 Bioassay Cells in culture, follow the recommended cell seeding density during routine propagation. Changes in cell culture volume or seeding density could affect subsequent assay performance. Only use cells in this assay after the doubling rate has stabilized during propagation. Use actively growing, healthy cells harvested as part of a routine two- or three-day passage. Culture viability should be >90% prior to use in the IL-12 Bioassay.

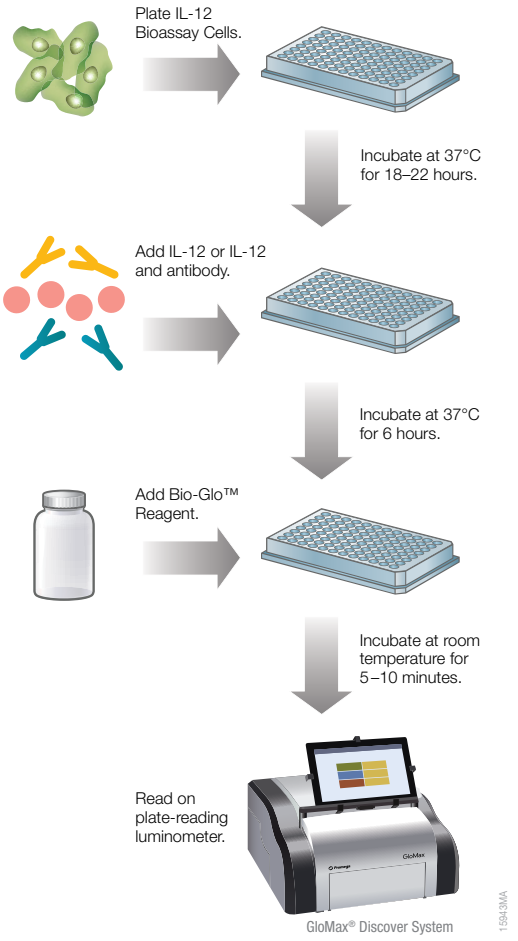

Figure 10. IL-12 Bioassay schematic protocol.

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5.A. Plate Layout Design

For the protocol described here, use the plate layout illustrated in Figure 11 as a guide. The protocol describes serial replicate dilutions (n = 3) of test and reference samples to generate two ten-point dose-response curves for each plate.

Recommended Plate Layout Design													
	1	2	3	4	5	6	7	8	9	10	11	12	
A	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)
B	B	no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Replicate 1
C	B	no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Replicate 1
D	B	no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Replicate 2
E	B	no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Replicate 2
F	B	no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Reference Replicate 3
G	B	no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1	B	Test Replicate 3
H	B	B	B	B	B	B	B	B	B	B	B	B	Assay Buffer (B)

Figure 11. Example plate layout. This suggested layout shows nonclustered locations for three replicates of each test and reference sample dilution series (dilu1–dilu9) and wells containing assay buffer (denoted by "B") alone.

5.B. Day One: Preparing and Plating IL-12 Bioassay Cells

1. Prepare 50ml of assay buffer as described in Section 9.A and warm to 37°C before use.
2. Aspirate medium and gently rinse cell monolayer with D-PBS, being careful not to disrupt the cell monolayer. Carefully aspirate D-PBS.
3. Add 2ml of Accutase® to each T75 flask and rock the flask several times to mix and coat the cell surface.
4. Incubate at room temperature until cells begin to lift off (approximately 3–5 minutes).
5. Once cells detach, add 8ml of prewarmed growth medium to the Accutase® and pipet to gently break up cell clumps making a single cell suspension.
6. Sample and count by Trypan blue exclusion.
7. Based on the number of samples and plates, estimate the number of cells required and include 50–100% extra to account for loss during centrifugations. For each assay plate, a minimum of 2.4×10^6 cells are required (4×10^4 cells/well × 60 wells).
8. Place cells into a 50ml centrifuge tubes and centrifuge at 150 × *g* for 5–10 minutes.
9. Remove supernatant. Resuspend cells in assay buffer to an estimated 1.5×10^6 cells/ml and count again by Trypan blue exclusion.
10. Adjust to 8×10^5 cells/ml using additional assay buffer.
11. Dispense 50µl/well (4×10^4 cells/well) using a multichannel pipette into the inner 60 wells of two solid white 96-well plates. Add 75µl/well of assay buffer to outer 36 wells.
12. Incubate 18–22 hours at 37°C, 5% CO_2.

5.C. Day Two: Assay Day with Addition of Test and Reference Samples

Preparing Reagents for the Assay Day

1. **Bio-Glo™ Reagent:** For reference, 10ml of Bio-Glo™ Reagent is sufficient to assay 120 wells in a 96-well assay format. Thaw the Bio-Glo™ Luciferase Assay Buffer in a refrigerator overnight or in a room-temperature water bath on the day of assay. Equilibrate the Bio-Glo™ Luciferase Assay Buffer to ambient temperature, protected from light. Transfer all of the Bio-Glo™ Luciferase Assay Buffer into the amber bottle containing the Bio-Glo™ Luciferase Assay Substrate and mix by inversion until the Substrate is thoroughly dissolved. Equilibrate and store the reconstituted Bio-Glo™ Reagent at ambient temperature (22–25°C) protected from light before adding to assay plates.

   If you are using a large (100ml) size of Bio-Glo™ Luciferase Assay System, dispense the reconstituted Bio-Glo™ Reagent into 10ml aliquots and store at –20°C for up to 6 weeks. Avoid repeated freeze-thaw cycles. On the day of the assay, thaw the appropriate amount of reconstituted Bio-Glo™ Reagent in a room temperature water bath for at least 1–2 hours before use. Approximate stability of Bio-Glo™ Reagent after reconstitution is 18% loss of luminescence after 24 hours at ambient temperature and 12% loss of luminescence after 5 days at 4°C.

2. **Assay Buffer:** Ensure that an appropriate amount of assay buffer is prepared. Thaw the fetal bovine serum (FBS) overnight at 4°C, or in a 37°C water bath, taking care not to overheat it. To make 50ml of assay buffer, add 5ml of FBS to 45ml of DMEM to yield 90% DMEM/10% FBS (see Section 9.A). Mix well and warm to 37°C prior to use. For reference, 50ml of assay buffer is typically sufficient for 120 wells in a 96-well assay format using the inner 60 plate wells.

3. **Test and Reference Samples:** Prepare starting dilutions (denoted as dilu1, 3X final concentration) of test and reference samples (see Figures 11 and 12). Using assay buffer as the diluent, prepare a minimum of 400µl of reference sample starting dilution and a minimum of 200µl of each test sample starting dilution in 1.5ml tubes. Store the tubes containing starting dilutions appropriately before making serial dilutions.

5.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:

- 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 1,200ng/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.
- Prepare and store IL-12 according to the manufacturer's instructions. Single-use frozen aliquots are recommended for each assay.

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, respectively.
3. Add 120µl of assay buffer to 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 set aside.

Recommended Plate Layout for Sample Dilutions Prepared from a Single Sample Stock													
	1	2	3	4	5	6	7	8	9	10	11	12	
A		no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Sample
B		no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Reference Sample
C		no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Sample 1
D		no drug	dilu9	dilu8	dilu7	dilu6	dilu5	dilu4	dilu3	dilu2	dilu1		Test Sample 2
E													
F													
G													
H													

Figure 12. 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.

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

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

5.F. Adding Bio-Glo™ Reagent

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

1. Remove the assay plates from the incubator, remove the plate lid and equilibrate to ambient temperature for 10–15 minutes.
2. Using a multichannel pipette, add 75µl of Bio-Glo™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, C1 and D1 of each assay plate to 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.

5.G. Data Analysis

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

   Note: When calculating fold induction, if the no-drug control sample RLU is at least 100X the plate background RLU, there is no need to subtract plate background from sample RLU.
3. Graph data as RLU versus Log_{10}[sample] and fold induction versus Log_{10}[sample]. Fit curves and determine the EC_{50} value of the IL-12 response using appropriate curve fitting software (such as GraphPad Prism®).

6. 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, which is used during an inhibition assay. To facilitate workflow, a 5.5-hour stimulation can be used instead of the 6 hours recommended in the stimulation protocol. This inhibition protocol can be modified for other blocking antibodies, depending on their mechanism of action.

6.A. IL-12 Inhibition Assay

1. Prepare and plate IL-12 Bioassay Cells as described in Section 5.B. Incubate overnight in a humidified 37°C, 5% CO_2 incubator.
2. In a separate sterile clear 96-well plate, prepare serial dilutions of ustekinumab in warm assay buffer as 6X final concentration. We recommend a final concentration of 0–125µg/ml ustekinumab as serial 4.5-fold dilutions.
3. Prepare IL-12 in warm assay buffer at 6X the predetermined EC_{90} response concentration.
4. Combine an equal volume of IL-12 with each antibody dilution. Mix by pipetting.

   Note: Each component is now at 3X its final concentration.
5. Incubate IL-12 and antibody samples for 60 minutes in a humidified 37°C, 5% CO_2 incubator.
6. Remove the 96-well assay plates containing the overnight preplated IL-12 Bioassay Cells from the incubator.
7. Add 25µl/well of IL-12 and antibody samples to cells. Final volume is now 75µl/well.
8. Incubate for 5.5 hours in a humidified 37°C, 5% CO_2 incubator.
9. After the incubation, proceed to Section 6.B.

6.B. Adding Bio-Glo™ Reagent

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

1. Remove the assay plates from the incubator, remove the plate lid and equilibrate to ambient temperature for 10–15 minutes.
2. Using a multichannel pipette, add 75µl of Bio-Glo™ Reagent to the inner 60 wells of the assay plates, taking care not to create bubbles.
3. Add 75µl of Bio-Glo™ Reagent to wells B1, C1 and D1 of each assay plate to measure the background signal.
4. Incubate at ambient temperature for 5–10 minutes.

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

6.C. Data Analysis

1. Measure plate background by calculating the average RLU from wells B1, C1 and D1.
2. 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).

7. 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 for either 2-day or 3-day incubation period.
	Cells must be treated the same way prior to each assay. Variability in cell growth rates and preculture plating densities can effect assay results.
	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.

8. 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.

9. Appendix

9.A. Composition of Buffers and Solutions

thaw medium

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

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

freeze medium

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

Prepare immmediately before use and maintain at 4°C.

growth medium

90% DMEM (high glucose with pyruvate)
10% fetal bovine serum
4µg/ml blasticidin
0.35µg/ml puromycin

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

assay buffer

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

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

10. Summary of Changes

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

1. Removed Section 9.B, Related Products.
2. Made minor text and formatting edits.

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