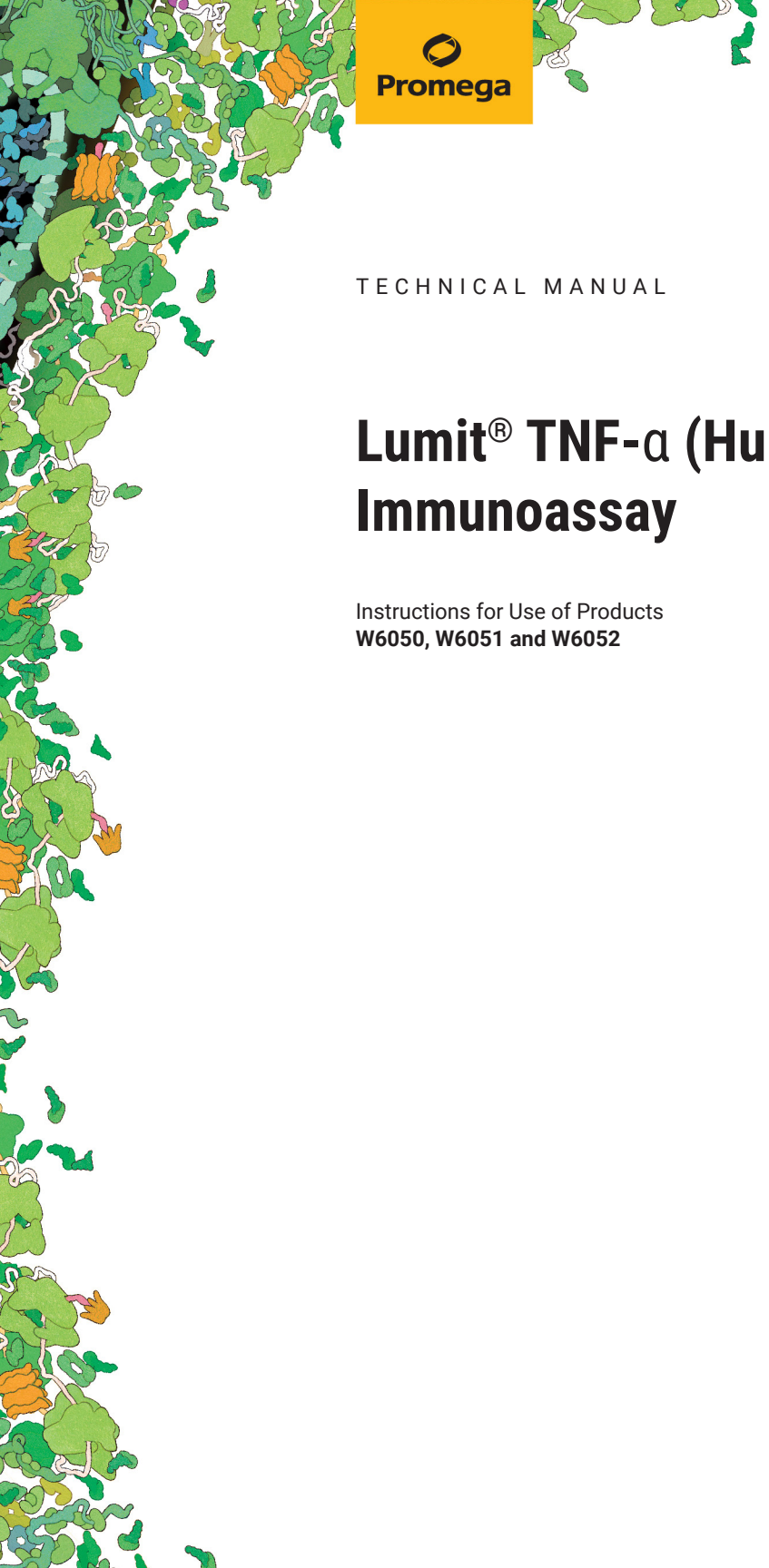

TECHNICAL MANUAL

Lumit® TNF-α (Human) Immunoassay

Instructions for Use of Products
W6050, W6051 and W6052

Revised 6/25
TM689

Lumit® TNF-α (Human) Immunoassay

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

1. Description

The Lumit® TNF-α (Human) Immunoassay[a,b] is a homogeneous, bioluminescent assay for detecting tumor necrosis factor alpha (TNF-α) released from cells without the need for sample transfers or wash steps. TNF-α is synthesized as a transmembrane protein and released as a bioactive, homotrimer comprised of three 17kDa monomers (1). Produced by both immune and nonimmune cells, TNF-α is a proinflammatory cytokine with important involvement in chronic inflammatory and autoimmune disorders (2), metabolic disease states such as diabetes and obesity (3) and in cancer biology (4,5). Indeed, inhibitors of TNF-α activity comprise an important class of therapeutic agents for treating inflammatory and autoimmune disorders and are of interest for additional applications (1,2,6). Hence, measuring TNF-α production in cell culture models is an important component of immunology research and various drug discovery and cell therapy efforts (7).

The Lumit® TNF-α (Human) Immunoassay has been developed for use with cell culture samples. Lumit® reagents can be dispensed directly into microplate wells containing cells and culture medium. Alternatively, medium from cell wells can be transferred to a separate plate for analysis. Assay performance with additional sample types must be determined by the user.

The Lumit® TNF-α (Human) Immunoassay is based on NanoLuc® Binary Technology (NanoBiT®). NanoBiT is a luminescent structural complementation system designed for biomolecular interaction studies (8,9). The system is composed of two subunits, Large BiT (LgBiT; 18kDa) and Small BiT (SmBiT; 11 amino acid peptide), that have been optimized for stability and minimal spontaneous association. In this assay, a sample is incubated with a pair of anti-human TNF-α monoclonal antibodies covalently labeled with SmBiT or LgBiT. When the labeled antibodies recognize and bind to released TNF-α, the complementary LgBiTs and SmBiTs are brought into proximity, thereby reconstituting the NanoBiT® enzyme and generating luminescence in the presence of the Lumit® substrate. Luminescence generated is directly proportional to the amount of analyte present in the sample.

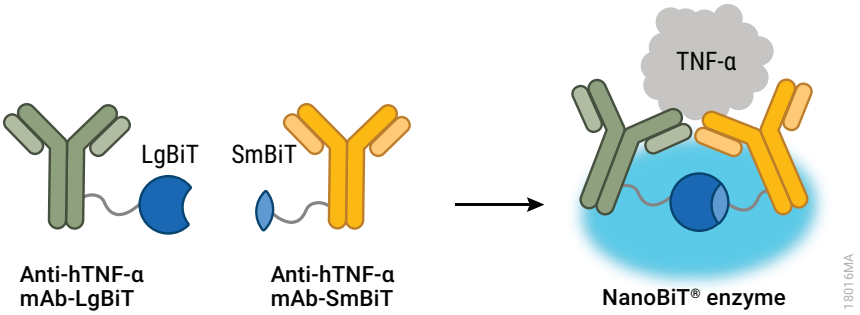

Figure 1. Assay principle. Primary monoclonal antibodies to human TNF-α are labeled with SmBiT and LgBiT. In the presence of TNF-α, SmBiT and LgBiT are brought into close proximity, forming the NanoBiT® enzyme. When Lumit® Detection Reagent B is added, a bright luminescent signal is generated.

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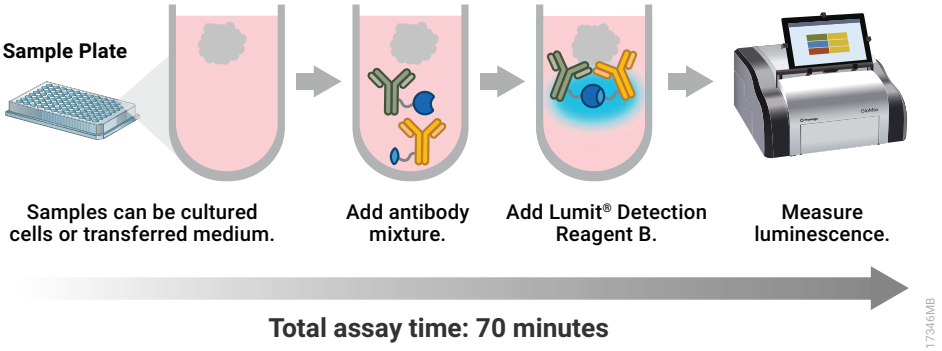

Figure 2. Assay protocol. The Lumit® TNF-α (Human) Immunoassay is performed directly on cells in culture or on medium transferred from a cell culture plate to a new assay plate. The Lumit® Immunoassay protocol does not require wash steps and is complete in 70 minutes.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Lumit® TNF-α (Human) Immunoassay	**100 assays**	**W6050**

Sufficient for 100 assays in 96-well plates; volumes can be adjusted for alternate plate sizes. Includes:

- 15µl Anti-hTNF-α mAb-SmBiT, 1000X
- 15µl Anti-hTNF-α mAb-LgBiT, 1000X
- 25µl Human TNF-α Standard
- 160µl Lumit® Detection Substrate B
- 3.2ml Lumit® Detection Buffer B

PRODUCT	SIZE	CAT.#
Lumit® TNF-α (Human) Immunoassay	**1,000 assays**	**W6051**

Sufficient for 1,000 assays in 96-well plates; volumes can be adjusted for alternate plate sizes. Includes:

- 150µl Anti-hTNF-α mAb-SmBiT, 1000X
- 150µl Anti-hTNF-α mAb-LgBiT, 1000X
- 25µl Human TNF-α Standard
- 1.25ml Lumit® Detection Substrate B
- 25ml Lumit® Detection Buffer B

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2. Product Components and Storage Conditions (continued)

PRODUCT	SIZE	CAT.#
Lumit® TNF-α (Human) Immunoassay	**5 × 100 assays**	**W6052**

Sufficient for 500 assays in 96-well plates; volumes can be adjusted for alternate plate sizes. Includes:

- 5 × 15µl Anti-hTNF-α mAb-SmBiT, 1000X
- 5 × 15µl Anti-hTNF-α mAb-LgBiT, 1000X
- 25µl Human TNF-α Standard
- 5 × 160µl Lumit® Detection Substrate B
- 5 × 3.2ml Lumit® Detection Buffer B

Storage Conditions: Store complete kit at less than –65°C upon receipt. Alternatively, store the Human TNF-α Standard at less than –65°C and all other components at –30°C to –10°C. After thawing, store Human TNF-α Standard at +2°C to +10°C for up to 1 month. If storing the Human TNF-α Standard for more than 1 month after thawing, dispense into aliquots and store at less than –65°C. After thawing, store the Anti-hTNF-α mAb-SmBiT and Anti-hTNF-α mAb-LgBiT at –30°C to –10°C. After thawing, store Lumit® Detection Buffer B at room temperature. Store Lumit® Detection Substrate B protected from light.

3. Before You Begin

There are two protocols for measuring human TNF-α. The direct protocol typically achieves higher sensitivity than the transfer protocol and requires less sample manipulation. The optional sample transfer protocol provides flexibility for same well sampling during treatment exposure time courses and split-sample analysis for assessment of multiple cytokine levels from the same sample.

Direct (No-Transfer) Protocol for Cultured Cells (Section 4): Measure human TNF-α directly in cell culture wells. Add 20µl of a 5X antibody mixture to 80µl of cells or TNF-α standard dilutions in culture medium and incubate for 60 minutes. Following incubation, add 25µl of Lumit® Detection Reagent B and record luminescence.

Optional Sample Transfer Protocol (Section 5): Measure human TNF-α in medium samples transferred from treated cell wells. Transfer 50µl of culture medium from cell wells to a separate assay plate. Add 50µl of a 2X antibody mixture to 50µl of transferred sample or standard dilutions and incubate for 60 minutes. Following incubation, add 25µl of Lumit® Detection Reagent B and record luminescence.

Note: Assay volumes are scalable and can be adjusted based on sample sizes. The protocols in Sections 4 and 5 list common volumes for 96- and 384-well plates. Other volumes may be used, maintaining the recommended antibody and detection reagent final concentrations. We recommend using standard tissue culture medium supplemented with 5-10% fetal bovine serum (FBS). Lesser concentrations of FBS may produce higher background (nonanalyte-mediated antibody pairing) and higher replicate variability. The use of medium without phenol red may increase assay sensitivity and reduce inner-filter effects from luminescence quenching.

Reagent Preparation and Storage

Prepare the Human TNF-α Standard dilution series (Section 4.B or 5.B), the Anti-hTNF-α Antibody Mixture (Section 4.C or 5.C) and Lumit® Detection Reagent B (Section 4.C or 5.C) on the day of use. Do **not** reuse Human TNF-α Standard dilutions, the Anti-hTNF-α Antibody Mixture or the Lumit® Detection Reagent B.

Use personal protective equipment and follow your institution's safety guidelines and disposal requirements when working with biohazardous materials such as cells and cell culture reagents.

Plate Map

	TNF-α Standard Curve (pg/ml)*		**Test Samples**									
	1	2	3	4	5	6	7	8	9	10	11	12
A	25,000	25,000										
B	7,500	7,500										
C	2,250	2,250										
D	675	675										
E	203	203										
F	60.8	60.8										
G	18.2	18.2										
H	0	0										

***Note:** The indicated Human TNF-α Standard dilution series listed above and described in the subsequent protocol are only a recommended dilution series.

Materials to Be Supplied by the User

- cells (human-derived)
- culture medium (e.g., RPMI 1640; GIBCO® Cat.# 22400-089 and 10% heat-inactivated, fetal bovine serum; GIBCO® Cat.# A3840001)
- white, multiwell tissue culture plates (solid white or white with clear bottom) compatible with a luminometer (e.g., 96-well Corning® Cat.# 3917)
- multichannel pipette or automated pipetting station
- dilution tubes or multichamber, dilution reservoir (e.g., Dilux® D-1002)
- reagent reservoir trays (e.g., Midwest Scientific Cat.# RR25)
- plate shaker for mixing multiwell plates
- luminometer capable of reading multiwell plates (e.g., GloMax® Discover System, Cat.# GM3000)

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4. Direct (No-Transfer) Protocol for Cultured Cells

This protocol describes how to detect TNF-α released directly in assay wells containing cells and culture medium. For quantitation purposes, a standard curve is generated using an TNF-α standard diluted in culture medium.

4.A. Cell Plating and Treatment

1. Plate cells into a 96- or 384-well, white (or white with clear bottom), tissue culture plate. Leave columns 1 and 2 empty. Allow cells to attach if using adherent cells.

   Note: While the broad dynamic range of the assay offers considerable flexibility for cell number, the optimal number of cells dispensed per well for a specific cell model should be empirically determined. Ensure the maximum level of cytokine released does not exceed the linear range of the detection chemistry. Within that constraint, cell number can be increased to meet the detection requirements of low-level cytokine production. See example cell numbers used for human PBMC in Figures 5, 7 and 8.

2. Treat cells by adding a volume of test agent to each well such that the total volume is as follows:

   96-well plate: 80µl per well

   384-well plate: 20µl per well

   For example, if 60µl of cells are plated per well in a 96-well plate, add 20µl of 4X treatment agent in culture medium. Cells are typically treated for 6–24 hours or longer, depending on stimuli, to observe release of significant levels of TNF-α.

   Optional: If manually dispensing into the 384-well assay format, after various additions, briefly centrifuge the microplate (700–900rpm for approximately 10 seconds) then briefly mix with a plate shaker to ensure proper mixing.

4.B. Preparing Human TNF-α Standard Dilution

Shortly before completing cell treatments, prepare TNF-α dilutions in the identical culture medium used for cell samples.

Note: If using multiple cell models each requiring different culture medium, separate standard dilution series must be generated in each medium used in the study.

1. Thaw the Human TNF-α Standard (approximately 15 minutes at room temperature) immediately before use.
2. Briefly centrifuge the tube before opening, then mix by pipetting.
3. Prepare an initial concentration of 25,000pg/ml human TNF-α by diluting Human TNF-α Standard (10µg/ml) 1:400 in prewarmed cell culture medium. For example, prepare 800µl of 25,000pg/ml human TNF-α by adding 2µl of the Human TNF-α Standard stock to 798µl of culture medium (see Figure 3).
4. Set up seven tubes (or seven chambers in a dilution reservoir) with 350µl of culture medium in each.

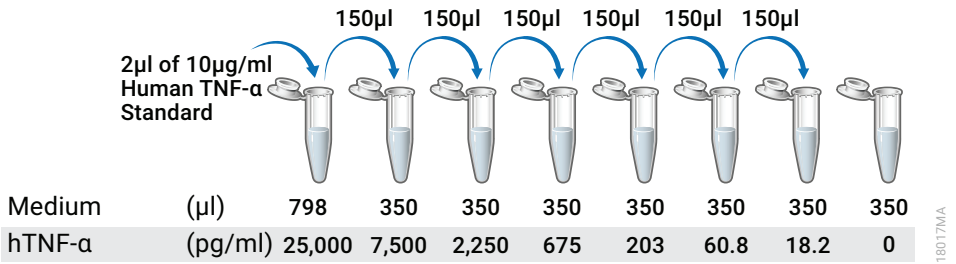

Figure 3. Human TNF-α dilution series.

5. Prepare 3.33-fold serial dilutions of standard. Transfer 150µl from the 25,000pg/ml initial human TNF-α dilution (Step 3) to 350µl of culture medium for the second dilution. Mix by pipetting and repeat five times to generate seven standard dilutions with a range of 25,000pg/ml to 18.2pg/ml. The last well or chamber should contain only culture medium as the background control.

   Note: Change pipette tips between each dilution step and use aerosol filter tips to avoid analyte carryover.

6. After the cell treatment is complete, add the standard dilutions and background control in duplicate to two columns in the assay plate (see the plate map in Section 3).

   96-well plate: Dispense 80µl per well.

   384-well plate: Dispense 20µl per well.

   Notes:

   a. Unused Human TNF-α Standard (10µg/ml) can be stored at +2°C to +10°C for 1 month. If storing the standard for more than 1 month, dispense into aliquots and store at less than –65°C. Avoid multiple freeze-thaw cycles.

   b. We recommend incorporating Human TNF-α Standard controls on each assay plate for normalization.

   c. Additional Lumit® Human TNF-α Standard (10µg/ml) is available for standalone purchase (Cat.# W137A-C).

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4.C. Adding 5X Anti-hTNF-α Antibody Mixture to Assay Wells

If using multiple cell models each requiring different culture medium, separate 5X Anti-hTNF-α antibody mixtures must be generated in each medium used in the study.

1. Remove the Anti-hTNF-α antibodies from storage immediately before use. Thaw if necessary.

   Note: Remove Lumit® Detection Buffer B from storage at the same time and equilibrate to room temperature if not already thawed. Use a water bath to accelerate thawing as necessary. If using the 25ml buffer in Cat.# W6051, you may need to initiate buffer thawing further in advance of its use in Section 4.D.

2. Briefly centrifuge the Anti-hTNF-α antibody tubes before opening, then mix by pipetting.

3. Immediately prior to use, prepare a 5X antibody mixture by diluting both antibodies 1:200 into a single volume of prewarmed culture medium. Pipet to mix the antibody solution. To assay a complete 96- or 384-well plate, including some reagent volume for pipetting loss, prepare the 5X antibody mixture as follows:

Reagent	Volume
culture medium	2,376µl
Anti-hTNF-α mAb-SmBiT	12µl
Anti-hTNF-α mAb-LgBiT	12µl

4. Add the 5X Anti-hTNF-α antibody mixture to wells containing cultured cells or standard dilutions, carefully avoiding cross contamination between wells by changing pipette tips if moving from high to low analyte levels.

   96-well plate: Dispense 20µl/well of 5X Anti-hTNF-α antibody mixture to 80µl/well of cells or TNF-α standard dilutions.

   384-well plate: Dispense 5µl/well of 5X Anti-hTNF-α antibody mixture to 20µl/well of cells or TNF-α standard dilutions.

5. Briefly mix with a plate shaker (e.g., 10 seconds at 250–350rpm).

6. Incubate for 45 minutes at 37°C in a humidified 5% CO_2 incubator.

4.D. Adding Lumit® Detection Reagent B to Assay Wells

While cells are incubating with the Anti-hTNF-α antibody mixture (Section 4.C), prepare the Lumit® Detection Reagent B.

1. Equilibrate the required volume of Lumit® Detection Buffer B to room temperature.
2. Remove the Lumit® Detection Substrate B from storage and mix. If Lumit® Detection Substrate B has collected in the cap or on the sides of the tube, briefly centrifuge.
3. Prepare a 1:20 dilution of Lumit® Detection Substrate B in room temperature Lumit® Detection Buffer B to create enough volume of Lumit® Detection Reagent B for the number of wells to be assayed. To assay a 96- or 384-well assay plate, including some reagent volume for pipetting loss, prepare 5X Lumit® Detection Reagent B as follows:

Reagent	Volume
Lumit® Detection Buffer B	3,040µl
Lumit® Detection Substrate B	160µl

Notes:

a. The 1,000 assay size Lumit® TNF-α (Human) Immunoassay (Cat.# W6051) contains 25ml of Lumit® Detection Buffer B and 1.25ml of Lumit® Detection Substrate B. There is sufficient overfill to prepare Lumit® Detection Reagent B for analyzing 5 or 10 plates at one time. If Cat.# W6051 is used for assaying 10 plates individually, mix 2,375µl of Lumit® Detection Buffer B + 125µl of Lumit® Detection Substrate B for each plate.

b. Once reconstituted, the Lumit® Detection Reagent B will lose 10% activity in approximately 3 hours at 20°C. At 4°C, the reconstituted reagent will lose 10% activity in approximately 7 hours.

4. After the incubation in Section 4.C, Step 6, equilibrate the assay plate with cells to room temperature for 15 minutes.
5. Add room temperature 5X Lumit® Detection Reagent B to each assay well of the plate.

   96-well plate: Dispense 25µl per well.

   384-well plate: Dispense 6.25µl per well.
6. Briefly mix with a plate shaker (e.g., 10 seconds at 300–500rpm).
7. Incubate at room temperature for 3–5 minutes.
8. Read luminescence.

   Note: Assay signal is stable with a half-life of approximately 2 hours, compatible with batch processing of multiple assay plates. We recommend incorporating standard controls on each assay plate for normalization.

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5. Optional Sample Transfer Protocol

This protocol describes transfer of sample medium from treated cell wells into a separate assay plate for subsequent cytokine detection, leaving the cells and remaining medium for additional uses. For quantitation purposes, a dilution series of Human TNF-α Standard prepared in culture medium is used to generate a standard curve.

5.A. Cell Plating and Treatment

1. Plate cells into a 96- or 384-well tissue culture plate. Leave columns 1 and 2 empty. Allow cells to attach if using adherent cells.

   Note: While the broad dynamic range of the assay offers considerable flexibility for cell number, the optimal number of cells dispensed per well for a specific cell model should be empirically determined. Ensure that the maximum level of cytokine released does not exceed the linear range of the detection chemistry. Within that constraint, cell number can be increased to meet the detection requirements of low-level cytokine production. See example cell numbers used for human PBMC in Figures 5, 7 and 8.

2. Treat cells by adding a volume of test agent to each well. The final treatment volume is flexible. Typical volumes are:

   96-well plate: 100–200µl per well.

   384-well plate: 25–50µl per well.

   Optional: If manually dispensing into the 384-well assay format, after various additions, briefly centrifuge the microplate (700–900rpm for approximately 10 seconds) then briefly mix with a plate shaker to ensure proper mixing.

3. After cell treatment is complete, transfer cell medium from each well to the corresponding wells of a separate white assay plate:

   96-well plate: Transfer 50µl per well.

   384-well plate: Transfer 12.5µl per well.

   Notes:

   a. If lower sample volumes are transferred, dilute with culture medium to 50µl for 96-well format and 12.5µl for 384-well format, subsequently accounting for sample dilution when calculating actual cytokine concentration released in cell wells.

   b. For enhanced 384-well assay sensitivity, if needed, you can transfer 20µl of sample (and standards) to assay wells, then add 5µl/well of 5X Anti-hTNF-α antibody mixture (prepared in Section 4.C).

5.B. Preparing Human TNF-α Standard Dilutions

Shortly before completing cell treatments, prepare TNF-α dilutions in the identical culture medium used for cell samples.

Note: If using multiple cell models, each requiring different culture medium, separate standard dilution series must be generated in each medium used.

1. Thaw the Human TNF-α Standard (approximately 15 minutes) immediately before use.
2. Briefly centrifuge the tube before opening, then mix by pipetting.
3. Prepare an initial concentration of 25,000pg/ml human TNF-α by diluting Human TNF-α Standard (10µg/ml) 1:400 in prewarmed cell culture medium. For example, prepare 800µl of 25,000pg/ml human TNF-α by adding 2µl of the Human TNF-α Standard stock to 798µl of culture medium (Figure 3).
4. Set up seven tubes (or seven chambers in a dilution reservoir) with 350µl of culture medium in each.
5. Proceed with 3.33-fold serial dilutions of standard. Transfer 150µl from the 25,000pg/ml stock to 350µl of culture medium for the second dilution. Mix and repeat five times to generate seven standard dilutions with a range of 25,000pg/ml to 18.2pg/ml. The last well or chamber should contain only culture medium as the background control.

   Note: Change pipette tips between each dilution step and use aerosol filter tips to avoid analyte carryover.
6. After transferring the culture medium from the treated cell wells to a separate assay plate, add the standard dilutions and background control in duplicate to two columns in the transfer plate (see the plate map in Section 3).

   96-well plate: Dispense 50µl per well.

   384-well plate: Dispense 12.5µl per well.

   Notes:

   a. Unused Human TNF-α Standard (10µg/ml) can be stored at +2°C to +10°C for 1 month. If storing the standard for more than 1 month, dispense into aliquots and store at less than –65°C. Avoid multiple freeze-thaw cycles.

   b. We recommend incorporating Human TNF-α Standard controls on each assay plate for normalization.

   c. Additional Lumit® Human TNF-α Standard (10µg/ml) is available for standalone purchase (Cat.# W137A-C).

5.C. Adding 2X Anti-hTNF-α Antibody Mixture to Sample Wells

If using multiple cell models each requiring different culture medium, separate 2X Anti-hTNF-α antibody mixtures must be generated in each medium used in the study.

1. Remove the Anti-hTNF-α antibodies from storage immediately before use. Thaw if necessary.

   Note: Remove Lumit® Detection Buffer B from storage at the same time and equilibrate to room temperature if not already thawed. Use a water bath to accelerate thawing as necessary. If using the 25ml buffer in Cat.# W6051, you may need to initiate buffer thawing further in advance of its use in Section 5.D.

2. Briefly centrifuge the Anti-hTNF-α antibody tubes before opening, then mix by pipetting.

3. Immediately prior to use, prepare a 2X antibody mixture by diluting both antibodies 1:500 into a single volume of prewarmed cell culture medium. Pipet to mix the antibody solution. To assay a complete 96- or 384-well plate, including some reagent volume for pipetting loss, prepare the 2X antibody mixture as follows:

Reagent	Volume
culture medium	5,976µl
Anti-hTNF-α mAb-SmBiT	12µl
Anti-hTNF-α mAb-LgBiT	12µl

4. Add the 2X Anti-hTNF-α antibody mixture to transferred culture medium (samples) or TNF-α standard dilutions, carefully avoiding cross contamination between wells by changing pipette tips if moving from high to low analyte levels.

   96-well plate: Dispense 50µl/well of 2X Anti-hTNF-α antibody mixture to 50µl/well of medium or standard dilutions.

   384-well plate: Dispense 12.5µl/well of 2X Anti-hTNF-α antibody mixture to 12.5µl/well of medium or standard dilutions.

5. Briefly mix with a plate shaker (e.g., 10 seconds at 250–350rpm).

6. Incubate for 60 minutes at room temperature.

   Note: To incubate at room temperature, a HEPES-containing medium will provide best results. Without HEPES, the buffering capacity outside of a CO_2 incubator is limited. The plates can also be incubated at 37°C in a CO_2 incubator if subsequently equilibrated to room temperature prior to addition of detection reagent.

5.D. Adding Lumit® Detection Reagent B to Sample Wells

While the samples and standard dilutions are incubating with the Anti-hTNF-α antibody mixture (Section 5.C), prepare the Lumit® Detection Reagent B.

1. Equilibrate the required volume of Lumit® Detection Buffer B to room temperature.
2. Remove the Lumit® Detection Substrate B from storage and mix. If the Lumit® Detection Substrate B has collected in the cap or on the sides of the tube, briefly centrifuge.
3. Prepare a 1:20 dilution of Lumit® Detection Substrate B into room temperature Lumit® Detection Buffer B to create enough volume of Lumit® Detection Reagent B for the number of wells to be assayed. For a 96- or 384-well assay plate, including some excess reagent volume, prepare 5X Lumit® Detection Reagent B as follows:

Reagent	Volume
Lumit® Detection Buffer B	3,040µl
Lumit® Detection Substrate B	160µl

Notes:

a. The 1,000 assay size of Lumit® TNF-α (Human) Immunoassay (Cat.# W6051) contains 25ml of Lumit® Detection Buffer B and 1.25ml of Lumit® Detection Substrate B. There is sufficient overfill to prepare Lumit® Detection Reagent B for analyzing 5 or 10 plates at once. If Cat.# W6051 is used for assaying 10 plates individually, mix 2,375µl of Lumit® Detection Buffer B + 125µl of Lumit® Detection Substrate B for each plate.

b. Once reconstituted, the Lumit® Detection Reagent B loses 10% activity in approximately 3 hours at 20°C. At 4°C, the reconstituted reagent loses 10% activity in approximately 7 hours.

4. After the incubation in Section 5.C, Step 6 is complete, add room temperature Lumit® Detection Reagent B to each assay well of the plate:

   96-well plate: Dispense 25µl per well.

   384-well plate: Dispense 6.25µl per well.

5. Briefly mix with a plate shaker (e.g., 10 seconds at 300–500rpm).
6. Incubate at room temperature for 3–5 minutes.
7. Read luminescence.

   Note: Assay signal is stable with a half-life of approximately 2 hours, compatible with batch processing of multiple assay plates.

6. Calculating Results

Create a standard curve for the known cytokine concentrations using software (e.g., GraphPad® Prism) capable of nonlinear regression analysis or cubic spline curve fitting.

Subsequently, interpolate the concentration of cytokine in various cell samples. The broad dynamic range of the Lumit® standard curve closely approaches linearity and is well-suited for second- or third-order polynomial regression curve fitting, as well as cubic spline curve fitting. Four-parameter logistic (4PL) curve fitting is also commonly used, but may not be ideal since the broad, linear dynamic range for the Lumit® standard curve is not well-suited for sigmoidal curve fitting (10).

Alternatively, while somewhat less accurate, a Log-Log plot of average RLU (background-subtracted) vs. cytokine standard concentrations can be fit with the Power trendline in Microsoft Excel® (see Section 7) and subsequently used for interpolation of the concentration of cytokine released in various cell samples.

7. Representative Data

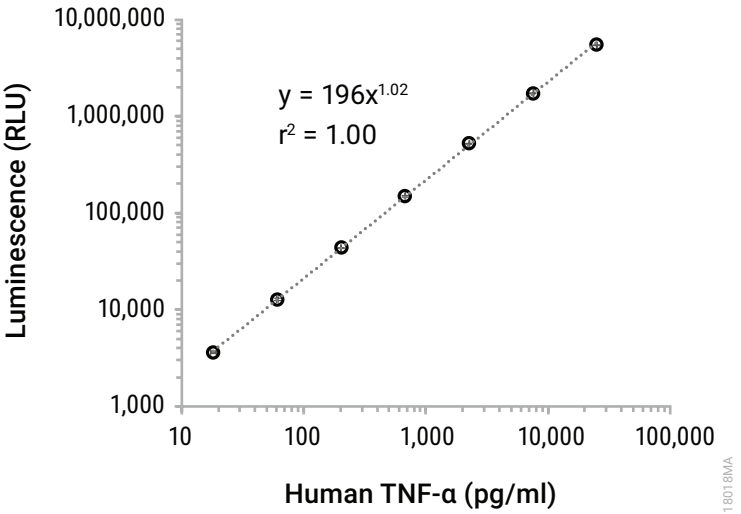

Figure 4. Standard curve for the Lumit® TNF-α (Human) Immunoassay. This is a representative standard curve and should not be used for calculation of unknowns. Generate a standard curve on each assay plate to interpolate the cytokine concentration in experimental samples. **Note:** The plotted luminescence values were determined by subtracting background RLU.

Table 1. Assay Precision. Three samples of known concentrations of human TNF-α were tested 20 times on two plates to assess assay precision. A standard curve was used on each assay plate to interpolate the TNF-α quantities in each well by using cubic spline fitting with GraphPad® Prism software.

Assay Precision			
Number of Replicates	40	40	40
Expected (pg/ml)	20,000	2,000	200
Mean (pg/ml)	19,359	2,036	209
Standard Deviation	759	62	5
Percent CV	3.9	3.1	2.4
Average Percent of Expected	96.8	101.8	104.7
Percent Range	86–108	95–112	97–112

Note: The minimal detectable dose (MDD), determined at two standard deviations above background, was determined to be 2.0 ± 0.4pg/ml of hTNF-α.

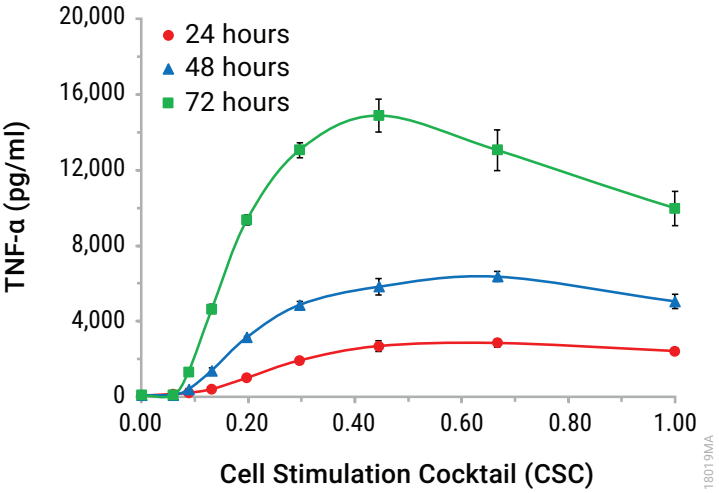

Figure 5. Lumit® detection of TNF-α released from human peripheral blood mononuclear cells (PBMC). PBMC pooled from four donors (BioIVT) were plated in RPMI 1640 + 10% heat- inactivated FBS at 100,000 cells/well in 96-well plates. Cells were treated with a titration of Cell Stimulation Cocktail (CSC; Invitrogen Cat.# 00-4970-93) for 24, 48 and 72 hours. 1X CSC is a mixture of 81nM phorbol 12-myristate 13-acetate (PMA) and 1.34µM ionomycin. Subsequently, the 5X Anti-hTNF-α antibody mixture was dispensed to the cell wells and incubated for 1 hour before Lumit® Detection Reagent B was added. Luminescence readings were analyzed using cubic spline interpolation (GraphPad® Prism 8) against the standard curve to determine levels of TNF-α released. Replicates: n = 3.

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7. Representative Data (continued)

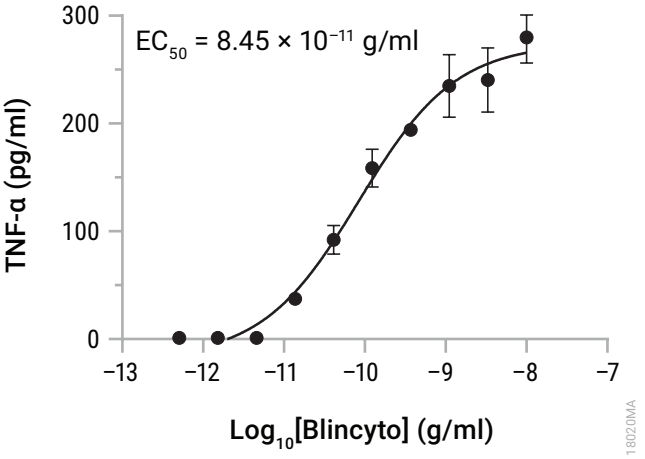

Figure 6. Simple detection of TNF-α release from a complex culture model in response to a biological agent. Purified CD8+ T cells were plated with target Raji B cells and treated with increasing concentrations of the bispecific T cell engager Blincyto®, a therapeutic monoclonal antibody that interacts with CD3 on effector T cells and CD19 on target cells. Consequent to effector cell activation and target cell killing, T cells release immunomodulatory cytokines, including TNF-α. Direct addition of Lumit® TNF-α (Human) Immunoassay reagents to this complex culture model enabled simple and rapid determination of cytokine release in response to drug treatment.

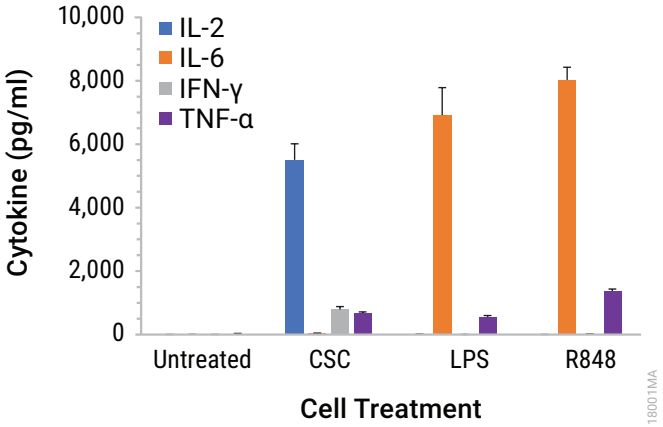

Figure 7. Detecting multiple cytokines by split-sample analysis via the optional sample transfer protocol. Human PBMC were plated in a 96-well plate at 50,000 cells/well in RPMI 1640 + 10% heat-inactivated FBS. Cell wells were treated in quadruplicate in a 100µl final volume for 24 hours with either 1X Cell Stimulation Cocktail (CSC), 2EU/ml lipopolysaccharide (LPS; InvivoGen Cat.# tlrl-3pelps) or 8µg/ml R848 (InvivoGen Cat.# tlrl-r848). Subsequently, from each cell well, four 20µl sample aliquots were transferred into a white, 384-well assay plate. These split-sample aliquots were individually assayed with different Lumit® cytokine immunoassays to determine IL-2, IL-6, IFN-γ and TNF-α levels previously released into the treated cell wells. After adding 5X anti-cytokine antibody mixture (5µl) for each assay and incubating for 1 hour at room temperature, 6.25µl of Lumit® Detection Reagent B was added. Luminescence readings from assay wells were analyzed using cubic spline interpolation (GraphPad® Prism 9) against respective standard curves to determine the levels of individual cytokines. Given the nonlytic nature of the assay chemistry, this simple approach for profiling cytokine release can be applied equally to adherent or suspension cell models.

8. 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
No signal from treated cells	Make sure that human cells are used with the Lumit® TNF-α (Human) Immunoassay. The Lumit® TNF-α (Human) Immunoassay will not effectively detect TNF-α from other species. In addition to the Human TNF-α Standard, consider using treated human PBMC as a positive control.
Human TNF-α standard curve is not linear	The 3.33-fold dilutions should be carefully created without carryover from a higher concentration. We recommend changing aerosol filter pipette tips after each dilution step to prevent carryover. The sensitivity of the assay and the broad linear range (>3 logs) means that any carryover will disrupt the linear range. Also, make sure that no TNF-α contaminates the background control.
The relative light units (RLU) for the standard curve are low and/or variable	Warm culture medium, Human TNF-α Standard and Lumit® Detection Buffer B to room temperature before use. There may be some variation in RLU due to culture conditions, temperature, etc., but as long as the standard curve is run on the same plate as the test samples under the same conditions, released TNF-α can be accurately quantitated.

9. Appendix

9.A. Assessing Cross-Reactivity of Lumit® Cytokine Immunoassays

Table 2. Testing Immunoassay Cross-Reactivity. The specificity of Lumit® cytokine immunoassays were assessed against a panel of their respective recombinant standards. Each Lumit® cytokine immunoassay was applied to 10ng/ml of the indicated cytokine standards in triplicate assay wells, in addition to wells containing only medium for background determination. Background luminescence was subtracted prior to normalization of average assay signals with each cytokine to that of the intended target of the Lumit® cytokine immunoassay. For the Lumit® TNF-α (Human) Immunoassay, signals obtained from the tested cytokines were less than or equal to 0.03% of the signal from human TNF-α.

	Lumit® Cytokine Immunoassay (Percent Assay Control)						
Standard (10ng/ml)	**hIL-1β**	**hIL-2**	**hIL-4**	**hIL-6**	**hIL-10**	**hIFN-γ**	**hTNF-α**
hIL-1β	100	0.01	0.02	0	0	0.02	0
hIL-2	0	100	0.02	0	0	0.01	0
hIL-4	0.06	0.01	100	0	0.02	0.01	0
hIL-6	0.07	0.02	0.06	100	0.02	0.03	0
hIL-10	0.18	0.03	0.03	0.03	100	0.04	0
hIFN-γ	0.10	0.03	0.03	0.03	0.02	100	0.03
hTNF-α	0.01	0.01	0.02	0.02	0.01	0.03	100

9.B. Multiplexing Assays with Lumit® TNF-α (Human) Immunoassay

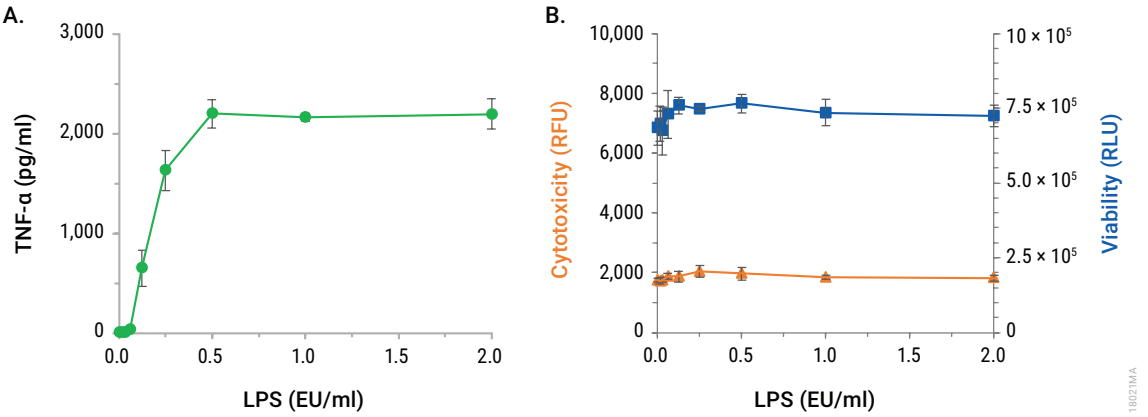

Figure 8. Same-well determination of TNF-α release, cytotoxicity and viability for treated human PBMC. Human PBMC were plated in RPMI 1640 + 10% heat-inactivated FBS at 30,000 cells/well in an all-white 96-well plate and treated at a total volume of 70µl with increasing concentrations of lipopolysaccharide (LPS) for 36 hours. Following treatment, 10µl of 10X CellTox™ Green Reagent (prepared in Assay Buffer as described in *CellTox™ Green Cytotoxicity Assay Technical Manual*, #TM375) and 20µl of 5X Anti-hTNF-α antibody mixture (Section 4) were added to each well. The assay plate was incubated for 45 minutes at 37°C in a humidified 5% CO_2 incubator prior to measuring fluorescence (RFU) indicating cytotoxicity. The assay plate was allowed to equilibrate to room temperature for 15 minutes before adding 25µl of Lumit® Detection Reagent B to assess human TNF-α release. Lastly, 100µl of the lytic CellTiter-Glo® Reagent was added to each well (as described in the *CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin* #TB288) and the plate was shaken for 2 minutes at 350rpm, then incubated for 10 minutes before measuring the luminescence signal (RLU). Luminescence reflects cell viability. **Panel A.** Dose-dependent release of human TNF-α was observed in response to LPS treatment. **Panel B.** Minimal impact on signals for cytotoxicity and cell viability assays were observed in response to 36-hour treatment with increasing LPS concentrations. **Note:** Maximal cell death provoked by adding a reference cytotoxic agent (50µg/ml digitonin) produced fluorescence readings (using the CellTox™ Green Cytotoxicity Assay, Cat.# G8741) of more than 16,500 RFU while viability signals (shown as luminescence using the CellTiter-Glo® Viability Assay, Cat.# G7570) were reduced by 97% relative to untreated cells (data not shown).

9.C. References

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8. Dixon, A.S. *et al.* (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11**, 400–8.
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9.D. Related Products

Lumit® Immunoassays

Product	Size	Cat.#
Lumit® Human TNF-α Standard	25µl	W137A-C
Lumit® FcRn Binding Immunoassay	100 assays	W1151
Lumit® HMGB1 (Human/Mouse) Immunoassay	100 assays	W6110
Lumit® Human IL-1β Immunoassay	100 assays	W6010
Lumit® IFN-γ (Human) Immunoassay	100 assays	W6040
Lumit® IL-2 (Human) Immunoassay	100 assays	W6020
Lumit® IL-4 (Human) Immunoassay	100 assays	W6060
Lumit® IL-6 (Human) Immunoassay	100 assays	W6030
Lumit® IL-10 (Human) Immunoassay	100 assays	W6070
Lumit® Mouse IL-1β Immunoassay	100 assays	W7010

Additional sizes available.

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9.D. Related Products (continued)

Lumit® Immunoassay Reagents

Product	Size	Cat.#
Lumit® Immunoassay Labeling Kit	1 each	VB2500
Lumit® Detection Reagent B*	100 assays	VB4050
Lumit® Immunoassay Cellular Systems–Starter Kit	200 assays	W1220

*Additional sizes available.

Inflammation Assays

Product	Size	Cat.#
Caspase-Glo® 1 Inflammasome Assay	10ml	G9951
RealTime-Glo™ Extracellular ATP Assay	200 assays	GA5010

Additional sizes available.

Cell Viability Assays

Product	Size	Cat.#
CellTiter-Glo® 2.0 Cell Viability Assay	10ml	G9241
CellTiter-Glo® Cell Viability Assay	10ml	G7570
RealTime-Glo™ MT Cell Viability Assay	100 reactions	G9711
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080

Additional sizes available.

Cytotoxicity Assays

Product	Size	Cat.#
LDH-Glo™ Cytotoxicity Assay	10ml	J2380
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
CellTox™ Green Cytotoxicity Assay	10ml	G8741

Additional sizes available.

Energy Metabolism and Oxidative Stress Assays

Product	Size	Cat.#
NAD/NADH-Glo™ Assay	10ml	G9071
NADP/NADPH-Glo™ Assay	10ml	G9081
ROS-Glo™ H_2O_2 Assay	10ml	G8820
GSH/GSSG-Glo™ Assay	10ml	V6611
Lactate-Glo™ Assay	5ml	J5021
Glucose-Glo™ Assay	5ml	J6021
Glutamine/Glutamate-Glo™ Assay	5ml	J8021
Glycerol-Glo™ Assay	5ml	J3150
Triglyceride-Glo™ Assay	5ml	J3160
Cholesterol/Cholesterol Ester-Glo™ Assay	5ml	J3190

Additional sizes available.

Apoptosis Assays

Product	Size	Cat.#
Caspase-Glo® 3/7 Assay System	2.5ml	G8090
Caspase-Glo® 8 Assay System	2.5ml	G8200
Caspase-Glo® 9 Assay System	2.5ml	G8210
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	100 assays	JA1011

Additional sizes available.

10. Summary of Changes

The following changes were made to the 6/25 revision of this document:

1. Updated the fonts.
2. Added Lumit® Human TNF-α Standard to Sections 4.B, 5.B and 9.D.
3. Updated the Lumit trademark and a third party trademark attribution.
4. Updated one patent statement.

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[a]U.S. Pat. Nos. 9,797,889; 9,797,890; 10,107,800; 10,648,971; and other patents and patents pending.

[b]U.S. Pat. No. 8,809,529, European Pat. No. 2635582, Japanese Pat. No. 5889910 and other patents and patents pending.

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