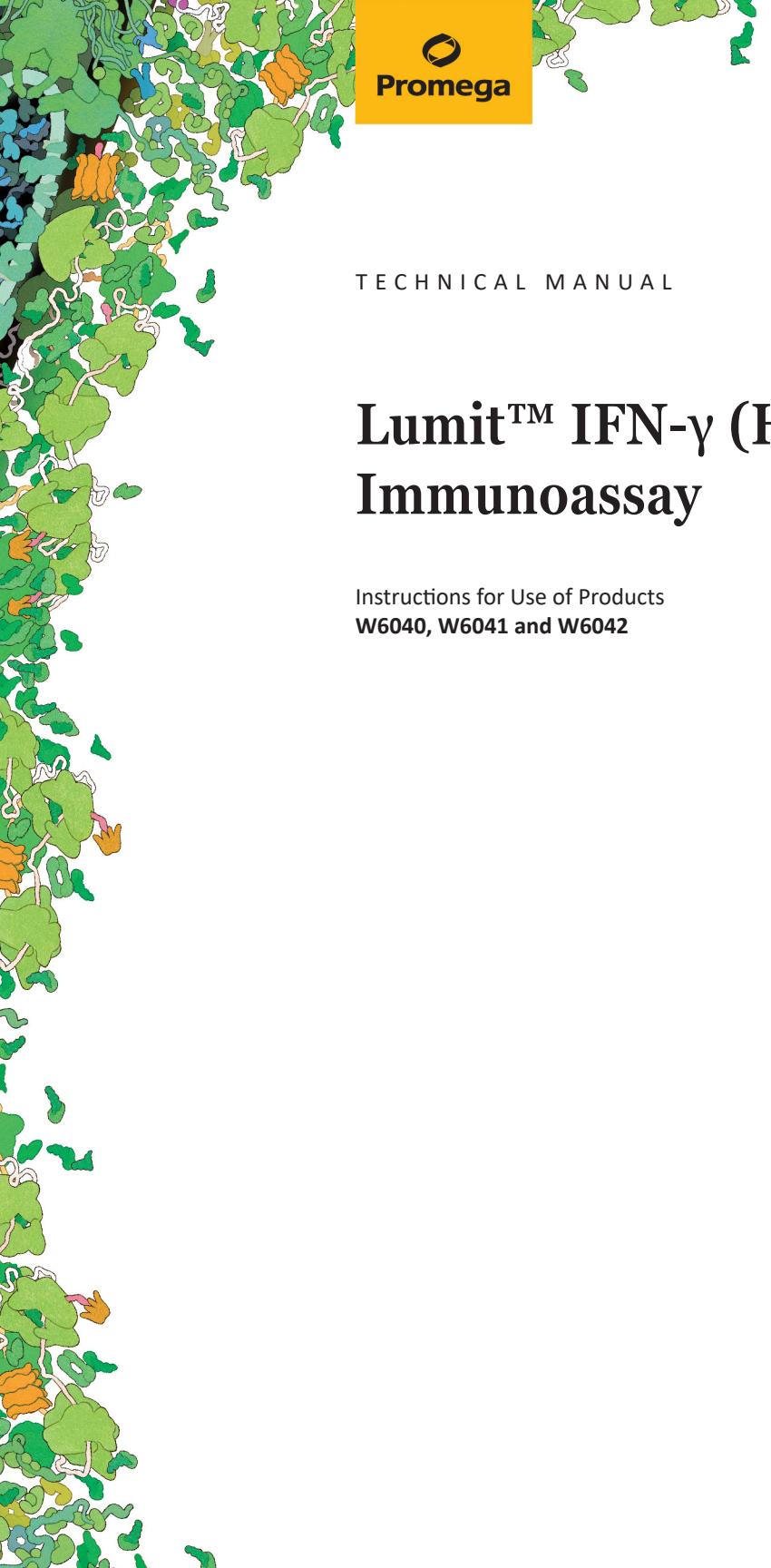

TECHNICAL MANUAL

# Lumit™ IFN-γ (Human) Immunoassay

Instructions for Use of Products
**W6040, W6041 and W6042**

3/22
TM686

Promega

# Lumit™ IFN-γ (Human) Immunoassay

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

## 1. Description

The Lumit™ IFN-γ (Human) Immunoassay[a,b] is a homogeneous, bioluminescent assay for detecting interferon gamma (IFN-γ) released from cells without the need for sample transfers or wash steps. Secreted by activated CD4+ and CD8+ T cells, γδ T cells, natural killer (NK) cells and antigen-presenting cells (e.g., macrophages), as well as other cells of the immune system, IFN-γ is a homodimer formed by the noncovalent association of two 17kDa polypeptide subunits (1). A critical player in the innate and adaptive immune system, IFN-γ has important antiviral and immunoregulatory properties, including complex effects on tumor biology (1,2) . While IFN-γ-based therapies continue to be of interest for cancer and other therapeutic applications (1), measuring the production of IL-2, IFN-γ and other cytokines in cell culture models is an important component of immunology research and related drug discovery efforts. Indeed, IFN-γ release is a common measure of T cell activation in human peripheral blood mononuclear cell (PBMC) preparations, models reliant on use of purified CD4+ or CD8+ T cells, as well as in the characterization of cell therapies, including CAR-T cells (3).

The Lumit™ IFN-γ (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™ IFN-γ (Human) Immunoassay is based on NanoLuc® Binary Technology (NanoBiT®). NanoBiT is a luminescent structural complementation system designed for biomolecular interaction studies (4,5). 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 IFN-γ monoclonal antibodies covalently labeled with SmBiT or LgBiT. When the labeled antibodies recognize and bind to released IFN-γ, the complementary LgBiT and SmBiT molecules 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.

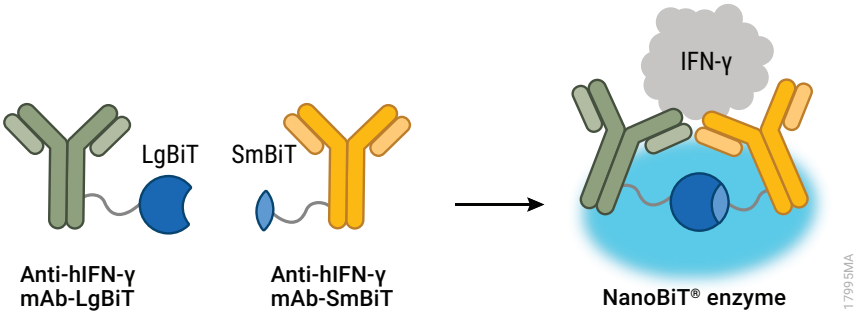


**Figure 1. Assay principle.** Primary monoclonal antibodies to human IFN-γ are labeled with SmBiT and LgBiT. In the presence of IFN-γ, SmBiT and LgBiT are brought into close proximity, forming the NanoBiT® enzyme. When Lumit™ Detection Reagent B is added, a bright luminescent signal is generated.

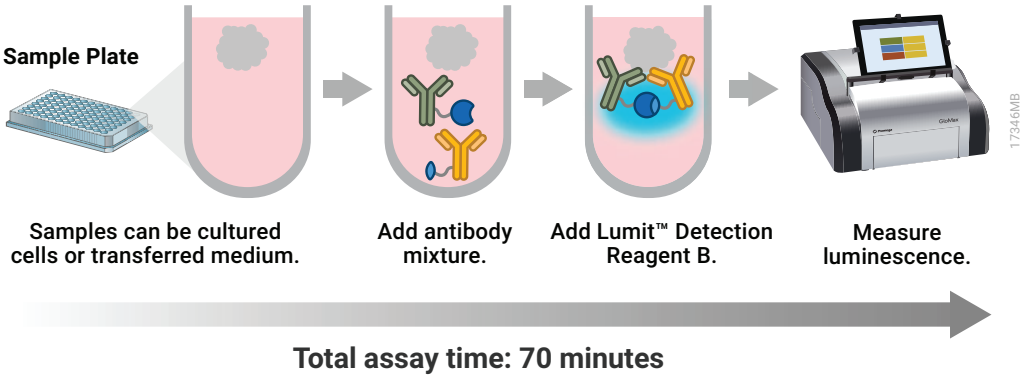


**Figure 2. Assay protocol.** The Lumit™ IFN-γ (Human) Immunoassay is performed directly on cells in culture or on medium transferred from the cell culture plate to a new assay plate. The Lumit™ Immunoassay protocol does not require wash steps and is complete in 70 minutes.

## 2. Product Components and Storage Conditions

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **Lumit™ IFN-γ (Human) Immunoassay** | **100 assays** | **W6040** |

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

- 30µl Anti-hIFN-γ mAb-SmBiT, 500X
- 30µl Anti-hIFN-γ mAb-LgBiT, 500X
- 25µl Human IFN-γ Standard
- 160µl Lumit™ Detection Substrate B
- 3.2ml Lumit™ Detection Buffer B

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **Lumit™ IFN-γ (Human) Immunoassay** | **1,000 assays** | **W6041** |

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

- 300µl Anti-hIFN-γ mAb-SmBiT, 500X
- 300µl Anti-hIFN-γ mAb-LgBiT, 500X
- 25µl Human IFN-γ Standard
- 1.25ml Lumit™ Detection Substrate B
- 25ml Lumit™ Detection Buffer B

## 2. Product Components and Storage Conditions (continued)

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **Lumit™ IFN-γ (Human) Immunoassay** | **5 × 100 assays** | **W6042** |

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

- 5 × 30µl Anti-hIFN-γ mAb-SmBiT, 500X
- 5 × 30µl Anti-hIFN-γ mAb-LgBiT, 500X
- 25µl Human IFN-γ 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 IFN-γ Standard at less than −65°C and all other components at −30°C to −10°C. After thawing, store Human IFN-γ Standard at +2°C to +10°C for up to 1 month. If storing the Human IFN-γ Standard for more than 1 month after thawing, dispense into aliquots and store at less than −65°C. After thawing, store the Anti-hIFN-γ mAb-SmBiT and Anti-hIFN-γ 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 IFN-γ. 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 course experiments 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 IFN-γ directly in cell culture wells. Add 20µl of a 5X antibody mixture to 80µl of cells or IFN-γ 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 IFN-γ in medium 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 IFN-γ Standard dilution series (Section 4.B or 5.B), the Anti-hIFN-γ 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 IFN-γ Standard dilutions, the Anti-hIFN-γ 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

| | **IFN-γ Standard Curve (pg/ml)*** | | **Test Samples** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| **A** | 10,000 | 10,000 | | | | | | | | | | |
| **B** | 3,000 | 3,000 | | | | | | | | | | |
| **C** | 900 | 900 | | | | | | | | | | |
| **D** | 270 | 270 | | | | | | | | | | |
| **E** | 81 | 81 | | | | | | | | | | |
| **F** | 24.3 | 24.3 | | | | | | | | | | |
| **G** | 7.29 | 7.29 | | | | | | | | | | |
| **H** | 0 | 0 | | | | | | | | | | |

***Note:** The indicated Human IFN-γ Standard dilution series listed above and described in the subsequent protocol directions 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 + 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)

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

This protocol describes how to detect IFN-γ released directly in assay wells containing cells and culture medium. For quantitation purposes, a standard curve is generated using an IFN-γ 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 determined empirically. 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, 6, 8 and 9.

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 IFN-γ.

   **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 IFN-γ Standard Dilution

Shortly before completing cell treatments, prepare IFN-γ 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 IFN-γ 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 10,000pg/ml human IFN-γ by diluting Human IFN-γ Standard (10µg/ml) 1:1,000 in prewarmed cell culture medium. For example, prepare 2,000µl of 10,000pg/ml human IFN-γ by adding 2µl of Human IFN-γ Standard stock to 1,998µl 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.

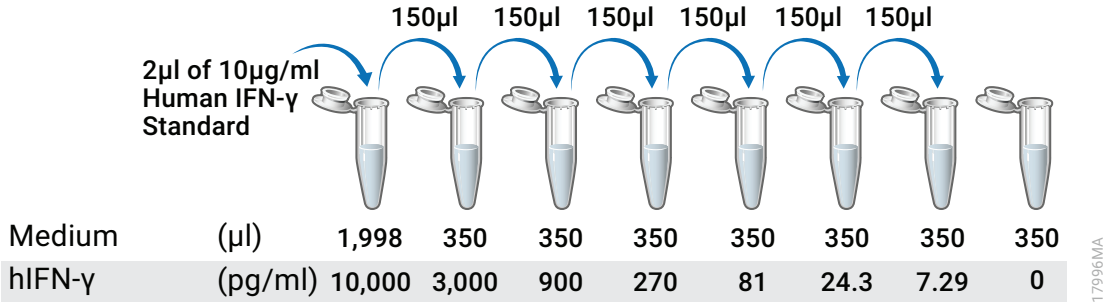


**Figure 3. Human IFN-γ dilution series.**

5. Prepare 3.33-fold serial dilutions of standard. Transfer 150µl from the 10,000pg/ml initial human IFN-γ 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 10,000pg/ml to 7.29pg/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 IFN-γ Standard (10µg/ml) can be stored at 4°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 IFN-γ Standard controls on each assay plate for normalization.

Promega

### 4.C. Adding 5X Anti-hIFN-γ Antibody Mixture to Assay Wells

**Note:** If using multiple cell models each requiring different culture medium, separate 5X Anti-hIFN-γ antibody mixtures must be generated in each medium used in the study.

1. Remove the Anti-hIFN-γ 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 buffer warming as necessary. If using the 25ml buffer in Cat.# W6041, you may need to initiate buffer warming further in advance of its use in Section 4.D.

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

3. Immediately prior to use, prepare a 5X antibody mixture by diluting both antibodies 1:100 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 | 2,352µl |
| Anti-hIFN-γ mAb-SmBiT | 24µl |
| Anti-hIFN-γ mAb-LgBiT | 24µl |

4. Add the 5X Anti-hIFN-γ 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-hIFN-γ antibody mixture to 80µl/well of cells or IFN-γ standard dilutions.

   **384-well plate:** Dispense 5µl/well of 5X Anti-hIFN-γ antibody mixture to 20µl/well of cells or IFN-γ 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-hIFN-γ 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™ IFN-γ (Human) Immunoassay (Cat.# W6041) 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.# W6041 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.

Promega

## 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 IFN-γ 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, 6, 8, and 9.

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-hIFN-γ antibody mixture (prepared in Section 4.C).

### 5.B. Preparing Human IFN-γ Standard Dilutions

Shortly before completing cell treatments, prepare IFN-γ standard 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 IFN-γ Standard (approximately 15 minutes) immediately before use.
2. Briefly centrifuge the tube before opening, then mix by pipetting.
3. Prepare an initial concentration of 10,000pg/ml human IFN-γ by diluting Human IFN-γ Standard (10µg/ml) 1:1,000 in prewarmed cell culture medium. For example, prepare 2,000µl of 10,000pg/ml human IFN-γ by adding 2µl of the Human IFN-γ Standard stock to 1,998µl 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 10,000pg/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 10,000pg/ml to 7.29pg/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 IFN-γ Standard (10µg/ml) can be stored at 4°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 IFN-γ Standard controls on each assay plate for normalization.

### 5.C. Adding 2X Anti-hIFN-γ Antibody Mixture to Sample Wells

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

1. Remove the Anti-hIFN-γ 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.# W6041, you may need to initiate buffer thawing further in advance of its use in Section 5.D.

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

3. Immediately prior to use, prepare a 2X antibody mixture by diluting both antibodies 1:250 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 | 5,952µl |
| Anti-hIFN-γ mAb-SmBiT | 24µl |
| Anti-hIFN-γ mAb-LgBiT | 24µl |

4. Add the 2X Anti-hIFN-γ antibody mixture to transferred culture medium (samples) or IFN-γ 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-hIFN-γ antibody mixture to 50µl/well of medium or standard dilutions.

   **384-well plate:** Dispense 12.5µl/well of 2X Anti-hIFN-γ 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-hIFN-γ 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 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 into room temperature Lumit™ Detection Buffer B to ensure 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™ IFN-γ (Human) Immunoassay (Cat.# W6041) 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.# W6041 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, 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 (6).

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 release in various cell samples.

## 7. Representative Data

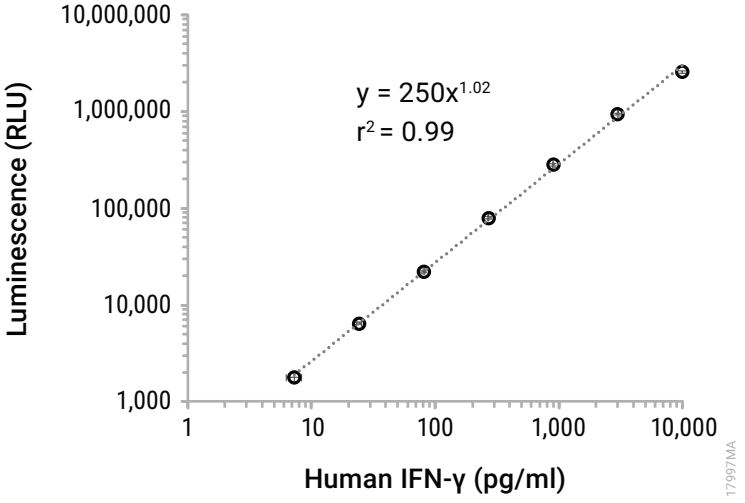


**Figure 4. Standard curve for the Lumit™ IFN-γ (Human) Immunoassay.** This is a representative standard curve and should not be used for calculation of unknowns. Generate a standard curve on each assay plate to interpolate the cytokine concentration in experimental samples. **Note:** The plotted luminescence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

## 7. Representative Data (continued)

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

| Assay Precision | | | |
|---|---|---|---|
| **Number of Replicates** | 40 | 40 | 40 |
| **Expected (pg/ml)** | 10,000 | 1,000 | 100 |
| **Mean (pg/ml)** | 9,630 | 991 | 101 |
| **Standard Deviation** | 241 | 32 | 3 |
| **Percent CV** | 2.5 | 3.2 | 3.1 |
| **Average Percent of Expected** | 96.3 | 99.1 | 100.9 |
| **Percent Range** | 86–100 | 92–110 | 93–112 |

**Note:** The minimal detectable dose (MDD), determined at two standard deviations above background, was 1.1 ± 0.1pg/ml of hIFN-γ.

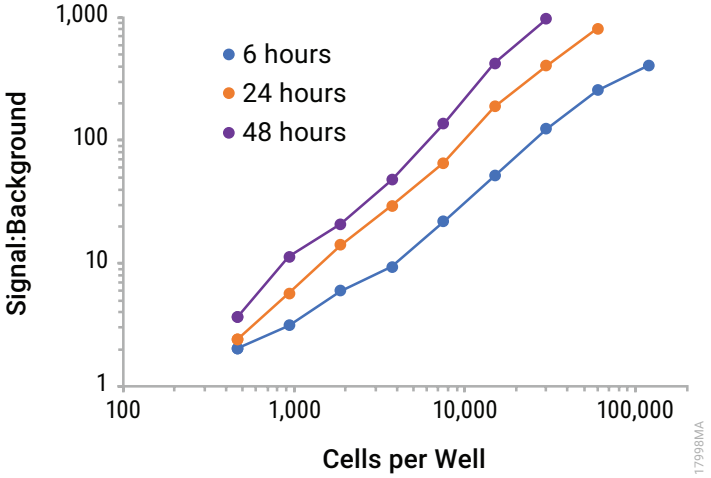


**Figure 5. Optimization of cell number for Lumit™ IFN-γ (Human) Immunoassay performance.** Human peripheral blood mononuclear cells (PBMC) pooled from four donors (BioIVT) were plated in RPMI 1640 + 10% heat-inactivated FBS at increasing numbers of cells/well in 96-well plates and treated with 1X Cell Stimulation Cocktail (CSC; Invitrogen Cat.# 00-4970-93) for 6, 24 and 48 hours. 1X CSC is a mixture of 81nM phorbol 12-myristate 13-acetate (PMA) and 1.34µM ionomycin. Luminescence measurements obtained by direct reagent addition (Section 4) to stimulated and untreated cell wells were used to calculate signal-to-background values. Stimulated IFN-γ release was reliably observed over a wide range of cell numbers, even as few as 1,000 cells/well.

## 7. Representative Data (continued)

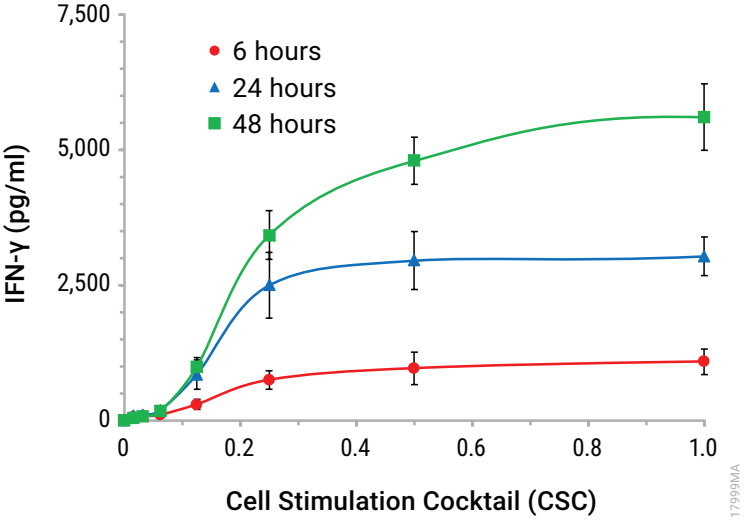


**Figure 6. Lumit™ detection of IFN-γ released from human peripheral blood mononuclear cells (PBMC).** PBMC pooled from four donors (BioIVT) were plated in RPMI 1640 + 10% heat-inactivated FBS at 10,000 cells/well in 96-well plates. Cells were treated with a titration of Cell Stimulation Cocktail (CSC) for 6, 24, or 48 hours. Subsequently, the 5X Anti-hIFN-γ antibody mixture was dispensed to the cell wells and plates incubated for 1 hour before adding Lumit™ Detection Reagent B. Luminescence readings were analyzed using cubic spline interpolation (GraphPad® Prism 8) against the standard curve to determine levels of IFN-γ released. Replicates: n = 3.

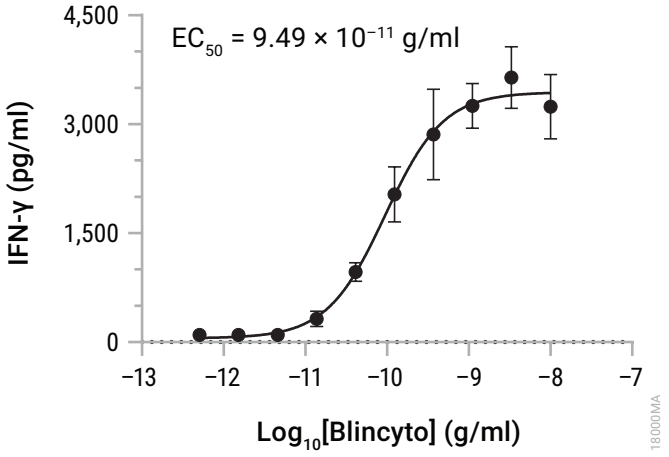


**Figure 7. Simple detection of IFN-γ release from a complex culture model in response to a biological agent.** Purified CD8+ T cells were plated with target Raji B cells and treated with increasing concentrations of the bispecific T cell engager Blincyto®, a therapeutic monoclonal antibody that interacts 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 IFN-γ. Direct addition of Lumit™ IFN-γ (Human) Immunoassay reagents to this complex culture model enabled simple and rapid determination of cytokine release in response to drug treatment.

## 7. Representative Data (continued)

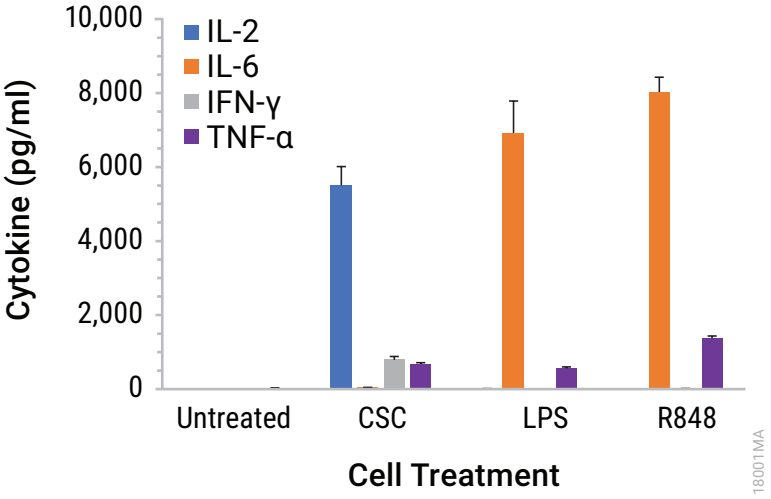


**Figure 8. Multiple cytokine detection by split-sample analysis via optional sample transfer protocol.** Human PBMC were plated in a 96-well plate at 50,000 cells/well in RPMI 1640 + 10% heat-inactivated FBS. Cell wells were treated in quadruplicate in a 100µl final volume for 24 hours with either 1X Cell Stimulation Cocktail (CSC; Invitrogen Cat.# 00-4970-93), 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**. E-mail: **techserv@promega.com**

| Symptoms | Causes and Comments |
|---|---|
| No signal from treated cells | Make sure that human cells are used with the Lumit™ IFN-γ (Human) Immunoassay. The Lumit™ IFN-γ (Human) Immunoassay will not effectively detect IFN-γ from other species. In addition to the Human IFN-γ Standard, consider using treated human PBMC as a positive control. |
| Human IFN-γ 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 IFN-γ contaminates the background control. |
| The relative light units (RLU) for the standard curve are low and/or variable | Warm culture medium, Human IFN-γ 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 IFN-γ can be accurately quantitated. |

Promega

## 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™ IFN-γ (Human) Immunoassay, signals obtained from the tested cytokines were less than or equal to 0.04% of the signal from human IFN-γ.

| | 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™ IFN-γ (Human) Immunoassay

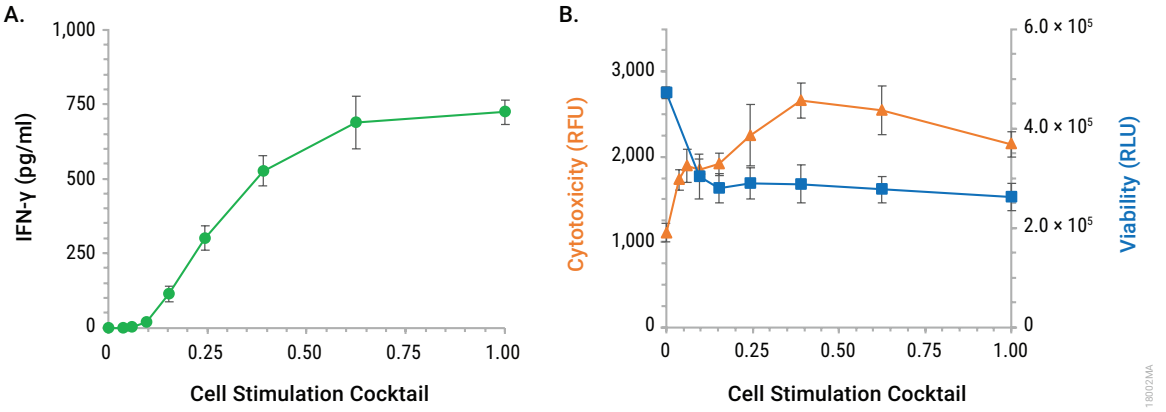


**Figure 9. Same-well determination of IFN-γ release, cytotoxicity and viability for treated human PBMC.** Human PBMC were plated in RPMI 1640 + 10% heat-inactivated FBS at 20,000 cells/well in an all-white 96-well plate and treated at a total volume of 70µl with increasing concentrations of Cell Stimulation Cocktail (CSC) for 24 hours. Following treatment, 10µl of 10X CellTox™ Green Reagent (prepared in Assay Buffer as described in the *CellTox™ Green Cytotoxicity Assay Technical Manual* #TM375) and 20µl of 5X Anti-hIFN-γ 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 IFN-γ 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 the luminescence signal (RLU) was measured. Luminescence reflects cell viability. **Panel A.** Dose-dependent release of human IFN-γ was observed in response to CSC treatment. **Panel B.** Some degree of increased cytotoxicity and decreased cell viability was observed in response to 24-hour treatment with increasing CSC. **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 4,770 RFU while viability signals (shown as luminescence using the CellTiter-Glo® Viability Assay, Cat.# G7570) were reduced by more than 94% relative to untreated cells (data not shown).

Promega

## 9.C. References


1. Castro, F. *et al.* (2018) Interferon-gamma at the crossroads of tumor immune surveillance or evasion. *Front. Immunol.* **9**, 847.
2. Schroder, K. *et al.* (2004) Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* **75(2)**,163–89.
3. Teng, R. *et al.* (2019) Chimeric antigen receptor-modified T cells repressed solid tumors and their relapse in an established patient-derived colon carcinoma xenograft model. *J. Immunother.* **42**, 33–42.
4. Dixon, A.S. *et al.* (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11**, 400–8.
5. Hwang, B.B. *et al.* (2020) A homogeneous bioluminescent immunoassay to probe cellular signaling pathway regulation. *Commun. Biol.* **3**, 8.
6. Herman, R.A., Scherer, P.N. and Shan, G. (2008) Evaluation of logistic and polynomial models for fitting sandwich-ELISA calibration curves. *J. Immunol. Methods* **339**, 245–58.


## 9.D. Related Products

### Lumit™ Immunoassays

| Product | Size | Cat.# |
|---|---|---|
| Lumit™ FcRn Binding Immunoassay | 100 assays | W1151 |
| Lumit™ HMGB1 (Human/Mouse) Immunoassay | 100 assays | W6110 |
| Lumit™ Human IL-1β Immunoassay | 100 assays | W6010 |
| 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 |
| Lumit™ TNF-α (Human) Immunoassay | 100 assays | W6050 |

Additional sizes available.

### Lumit™ Immunoassay Reagents

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

* Additional sizes available.

## 9.D. Related Products (continued)

### 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.

[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, and other patents and patents pending.