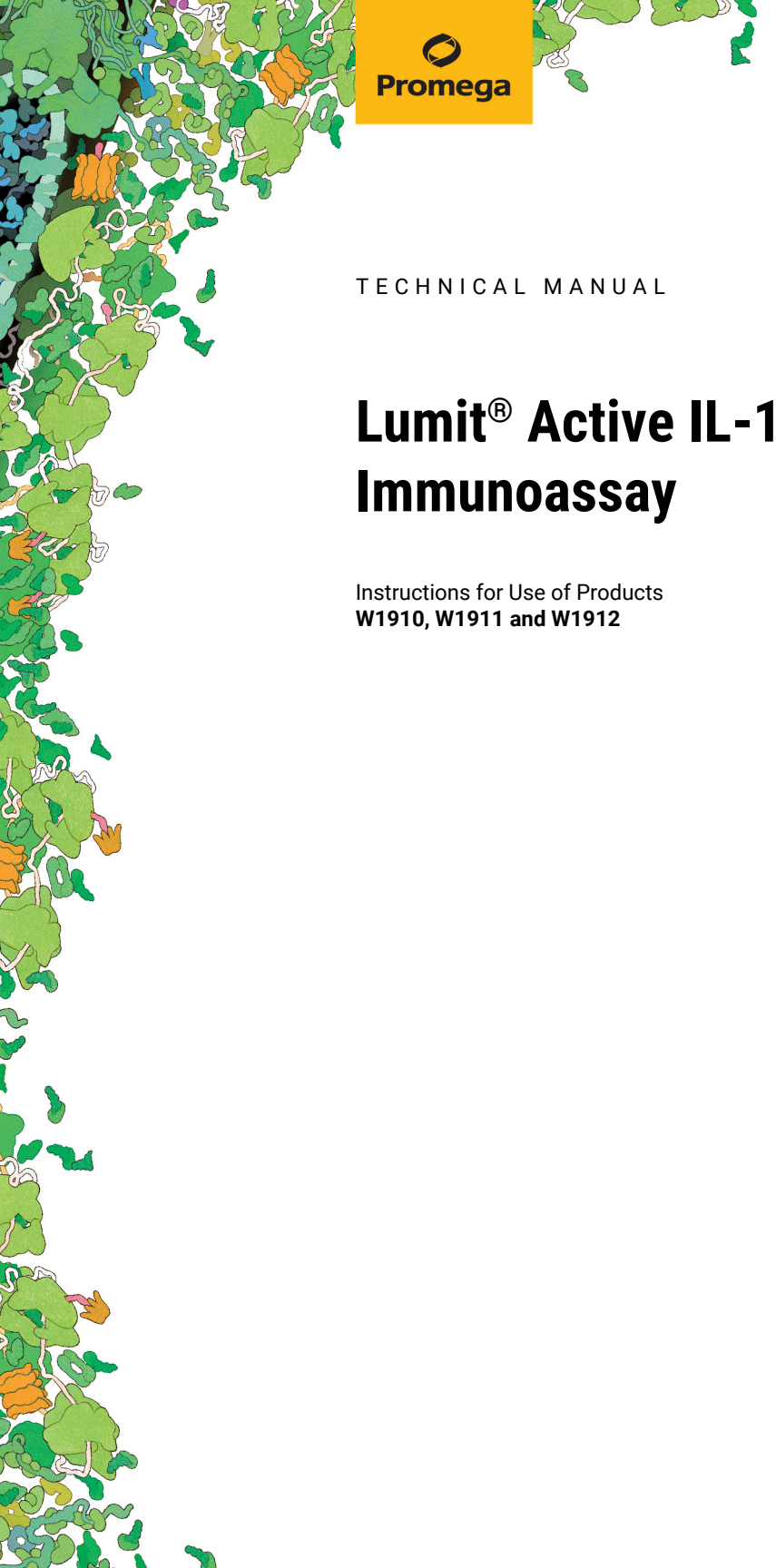

TECHNICAL MANUAL

Lumit® Active IL-18 (Human) Immunoassay

Instructions for Use of Products
W1910, W1911 and W1912

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Lumit® Active IL-18 (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® Active IL-18 immunoassay[a–d] is a homogeneous, bioluminescent assay for monitoring released active interleukin 18 (IL-18) directly from human cells without the need for sample transfers or wash steps.

IL-18 and IL-1β are critical to innate immunity as key proinflammatory cytokines that respond to microbial infection or tissue injury. IL-18 and IL-1β are processed and released following inflammasome oligomerization, an event triggered by pathogens or other damage-associated molecular patterns (DAMPs; 1,2). Both cytokines are synthesized as inactive precursors (proIL-18 and proIL-1β), requiring proteolytic processing for bioactivity. ProIL-18 is constitutively expressed, while proIL-1β typically requires a signal for upregulation. IL-1β has a more restricted expression pattern, primarily confined to myeloid cells, while IL-18 is widely expressed in myeloid cells as well as many epithelial and endothelial cells (3,4). Canonical inflammasomes recruit apoptosis-associated speck-like protein containing a CARD (ASC) and the protease caspase-1, which processes IL-18, IL-1β, and the pyroptosis effector gasdermin D (GSDMD; 5). Noncanonical inflammasomes form in response to lipopolysaccharide (LPS) from intracellular bacteria. Intracellular LPS binds caspase-4 directly and activated caspase-4 cleaves GSDMD causing pyroptosis (6). Recently human proIL-18 (but not mouse) has been shown to be efficiently cleaved by caspase-4, as well as caspase-1 (7). IL-18 is highly regulated by an inactivating binding protein, IL-18BP, with very high affinity for active IL-18 (3). To provide an assay selective for active IL-18, IL-18BP is used for detection in this Lumit® immunoassay because it primarily binds to active IL-18 (3).

The Lumit® Active IL-18 (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 sample types other than cell culture medium must be determined by the user.

Assay Principle

Lumit® Flex technology is a luminescent structural complementation system consisting of two small peptide tags (Peptide α and Peptide β) and a reagent-based polypeptide (Lumit® Flex Detection Protein). The peptide tags are attached to antibodies or proteins, resulting in subunit complementation after target binding and adding the polypeptide. In the Lumit® Active IL-18 immunoassay, human IL-18 binding protein (hIL-18BP) and anti-human IL-18 antibody are covalently labeled with Peptide β or Peptide α subunits of NanoLuc® luciferase, respectively. The stable hIL-18BP-Peptide β (hIL-18BP-β) is incubated with cells during treatment and binds to active IL-18 as it is released. Anti-hIL-18 mAb-Peptide α (Anti-hIL-18 mAb-α) is then added and binds to the active IL-18 already complexed with hIL-18BP-β, bringing the complementary α and β peptides into proximity with each other. The Lumit® Flex Detection Protein, added with the Lumit® substrate, reconstitutes the functional luciferase and generates luminescence in the presence of the substrate. Luminescence generated is directly proportional to the amount of active IL-18 in the sample.

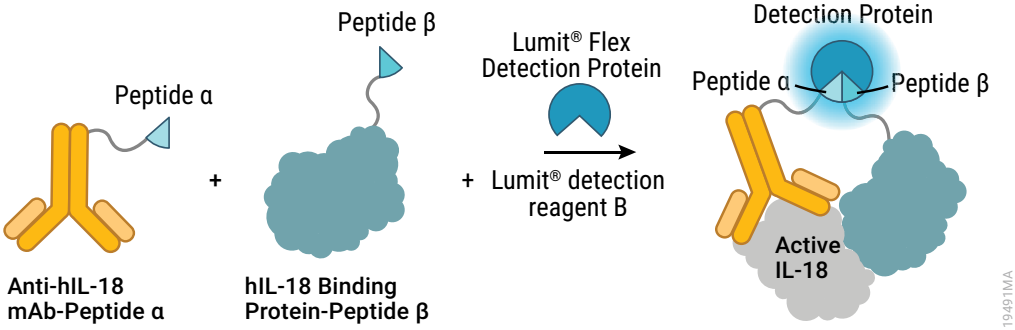

Figure 1. Assay principle. A primary monoclonal antibody directed toward human IL-18 and a human IL-18 binding protein are labeled with Peptide α and Peptide β, respectively. In the presence of active IL-18, Peptide α and Peptide β are brought into close proximity. When Lumit® Detection reagent B containing the Lumit® Flex Detection Protein is added, a functional luciferase is formed, and a bright luminescent signal is generated in the presence of Lumit® Substrate.

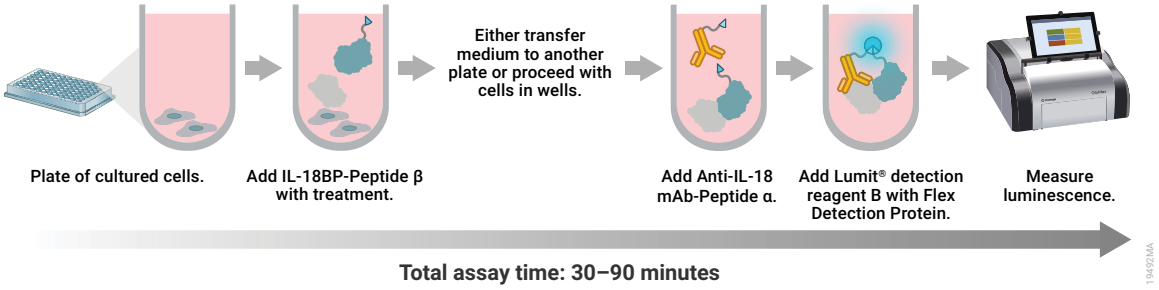

Figure 2. Assay workflow. The Lumit® Active IL-18 (Human) Immunoassay is performed directly on cells in culture or on medium transferred from the cell culture plate to a new assay plate. The Lumit® Immunoassay protocol does not require wash steps and is complete in 30–90 minutes after treatment.

Due to the instability of active IL-18 in culture medium at 37°C (see Section 8, Figure 4), the hIL-18BP-β must be added just prior to or with the cell treatment to capture and stabilize the released active IL-18.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Lumit® Active IL-18 (Human) Immunoassay	**100 assays**	**W1910**

Sufficient for 100 assays in 96-well plates; adjust assay volume for alternate plate sizes. Includes:

- 20µl hIL-18BP-Peptide β, 1,000X
- 20µl Anti-hIL-18 mAb-Peptide α, 1,000X
- 450µl Lumit® Flex Detection Protein
- 25µl Human Active IL-18 Standard
- 160µl Lumit® Detection Substrate B
- 3.2ml Lumit® Detection Buffer B

PRODUCT	SIZE	CAT.#
Lumit® Active IL-18 (Human) Immunoassay	**1,000 assays**	**W1911**

Sufficient for 1,000 assays in 96-well plates; adjust assay volume for alternate plate sizes. Includes:

- 200µl hIL-18BP-Peptide β, 1,000X
- 200µl Anti-hIL-18 mAb-Peptide α, 1,000X
- 4.5ml Lumit® Flex Detection Protein
- 25µl Human Active IL-18 Standard
- 1.25ml Lumit® Detection Substrate B
- 25ml Lumit® Detection Buffer B

PRODUCT	SIZE	CAT.#
Lumit® Active IL-18 (Human) Immunoassay	**5 × 100 assays**	**W1912**

Sufficient for 500 assays in 96-well plates; adjust assay volume for alternate plate sizes. Includes:

- 5 × 20µl hIL-18BP-Peptide β, 1,000X
- 5 × 20µl Anti-hIL-18 mAb-Peptide α, 1,000X
- 5 × 450µl Lumit® Flex Detection Protein
- 25µl Human Active IL-18 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. After the kit components have thawed, store as listed below:

Components	Storage after Thawing
Human Active IL-18 Standard	1 month at +2°C to +10°C. For longer term, dispense into aliquots and store at –30°C to –10°C
hIL-18BP-Peptide β and Anti-hIL-18 mAb-Peptide α	Up to 3 months at –30°C to –10°C. For longer term, dispense into aliquots and store at less than –65°C.
Lumit® Flex Detection Protein and Lumit® Detection Substrate B	–30°C to –10°C, protecting Lumit® Detection Substrate B from light
Lumit® Detection Buffer B	Room temperature (+15°C to +30°C)

2.A. Prepared Reagent Storage Conditions

Store remaining 10X hIL-18BP-β solution (Section 4.B) at 2–10°C until use when adding to standard curve wells.

3. Before You Begin

There are three protocols for measuring human IL-18 (hIL-18). The direct and alternative direct protocols typically achieve higher sensitivity than the optional sample transfer protocol and require less sample manipulation. The direct protocol is acceptable for all cell treatment durations, while the alternative direct protocol is suitable for treatments of 8 hours or less. The optional sample transfer protocol provides flexibility for same well sampling during treatment exposure time courses and split-sample analysis for assessing other cell measurements from the same sample.

Note: The Lumit® Active IL-18 (Human) Immunoassay cannot be multiplexed with any NanoBiT® complementation assays (e.g., Lumit® Immunoassays and Nano-Glo® HiBiT Lytic Detection System).

Direct (No-Transfer) Protocol (Section 4)

Standard protocol

With treatment, add 10X hIL-18BP-Peptide β.

↓

After treatment, add 10X Anti-hIL-18 mAb-Peptide α. Incubate 60 minutes.

↓

Add Lumit® detection reagent B with Lumit® Flex Detection Protein. Incubate 30 minutes.

↓

Measure luminescence.

Total assay time: 90 minutes

Optional Sample Transfer Protocol (Section 5)

Cells can be used in additional assays

With treatment, add 10X hIL-18BP-Peptide β.

↓

After treatment, transfer cell medium to a separate plate.

↓

Add 1X hIL-18BP-Peptide β and 2X Anti-hIL-18 mAb-Peptide α. Incubate 60 minutes.

↓

Add Lumit® detection reagent B with Lumit® Flex Detection Protein. Incubate 30 minutes.

↓

Measure luminescence.

Total assay time: 90 minutes

Alternative Direct Protocol (Section 6)

For treatments ≤8 hours

With treatment, add a 5X mixture of hIL-18BP-Peptide β and Anti-hIL-18 mAb-Peptide α.

↓

After treatment, add Lumit® detection reagent B with Lumit® Flex Detection Protein. Incubate 30 minutes.

↓

Measure luminescence.

Total assay time: 30 minutes

3. Before You Begin (continued)

Direct (No-Transfer) Protocol for Cultured Cells (Section 4): Measure human IL-18 directly in cell culture wells. Add 10µl of 10X hIL-18BP-β to 80µl of cells prior to or with treatment, as well as to assay wells with 80µl of Human IL-18 Standard dilutions prepared in culture medium for standard curve generation. After treatment, add 10µl of 10X Anti-hIL-18 mAb-α to cells and hIL-18 standard dilutions and incubate for 60 minutes. Following incubation, add 25µl of Lumit® detection reagent B with Lumit® Flex Detection Protein and record luminescence after 30 minutes.

Optional Sample Transfer Protocol (Section 5): Measure human IL-18 in medium samples transferred from treated cell wells. Add 10µl of 10X hIL-18BP-β to cells prior to or with treatment for a total volume of 100µl. After treatment, transfer 50µl of culture medium from cell wells to a separate assay plate. Add 50µl of a mixture of 1X hIL-18BP-β and 2X Anti-hIL-18 mAb-α to 50µl of transferred sample or standard dilutions and incubate for 60 minutes. Following incubation, add 25µl/well of Lumit® detection reagent B with Lumit® Flex Detection Protein included. Record luminescence in 30 minutes.

Alternative Direct Protocol (Section 6): This protocol option is a same-day treatment and read for cultured cells with fewer additions but is not suitable for overnight treatment times. For this alternative protocol, add 20µl of a 5X mixture of hIL-18BP-β and Anti-IL-18 mAb-α to 80µl of cells with treatment and 80µl of IL-18 Standard dilutions. After treatment, add 25µl of Lumit® detection reagent B with Lumit® Flex Detection Protein included. Record luminescence after 30 minutes. We **do not** recommend adding the Anti-IL-18 mAb-α to cell cultures for overnight or longer.

Note: Assay volumes are scalable and can be adjusted based on sample sizes. The protocols in Sections 4, 5 and 6 list common volumes for 96- and 384-well plates. Other volumes can be used as long as you maintain 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 and reduced assay sensitivity. When using serum-free, albumin-free medium, assay background may be reduced and sensitivity improved by preparing the hIL-18BP-Peptide β and Anti-hIL-18 mAb-Peptide α in medium supplemented with bovine serum albumin (BSA) to deliver 0.05% BSA final after addition to sample. (Using significantly higher albumin concentrations may further compromise assay performance.) Using medium without phenol red may increase assay sensitivity and reduce inner-filter effects from luminescence quenching.

Reagent Preparation and Storage

Prepare the hIL-18BP-β on treatment day. Prepare Human Active IL-18 standard curve (Section 4.C or 5.C), Anti-hIL-18 mAb-α and Lumit® detection reagent B + Lumit® Flex Detection Protein (Section 4.F or 5.F) on detection day. Do not reuse the diluted IL-18 standard curve, the diluted hIL-18BP-β, diluted Anti-hIL-18 mAb-α or the Lumit® detection reagent B + Lumit® Flex Detection Protein. To avoid reagent contamination, use only fresh, aerosol filter tips or cotton-plugged, sterile pipettes when withdrawing volumes from reagent stock components.

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

	IL-18 Standard Curve (pg/ml)*		Test Samples									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	5,714	5,714										
B	1,905	1,905										
C	635	635										
D	212	212										
E	70.5	70.5										
F	23.5	23.5										
G	7.8	7.8										
H	0	0										

***Note:** The indicated Human Active IL-18 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 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
- aerosol filter pipette tips
- dilution tubes or multichamber, dilution reservoir (e.g., Dilux® Cat.# 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 active IL-18 released directly in assay wells containing cells and culture medium. For quantitation purposes, a standard curve is generated using Human IL-18 Standard diluted in culture medium.

4.A. Cell Plating

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 cell number 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 THP-1 cells and PBMCs in Section 8, Representative Data, Figures 5–8.

4.B. Adding Human IL-18BP-Peptide β and Treatment to Cells

1. Remove the hIL-18BP-Peptide β, 1,000X, from storage immediately before use.
2. Gently tap the tube with a finger to ensure mixing, then briefly centrifuge before opening.
3. Dilute the hIL-18BP-Peptide β 1:100 in culture medium for a 10X working solution. Prepare sufficient volume for all sample and standard wells. To assay a complete 96- or 384-well plate, including some excess reagent volume, prepare 10X hIL-18BP-β as follows:

Reagent	Volume
culture medium	1,188µl
hIL-18BP-Peptide β, 1,000X	12µl

4. Add 10X hIL-18BP-β to the wells with plated cells, just prior to or with the cell treatment. (Reserve any remaining 10X IL-18BP-β solution for adding to standard curve wells, storing at 2–10°C until use.)

   96-well plate: Dispense 10µl of 10X hIL-18BP-β per well.

   384-well plate: Dispense 2.5µl of 10X hIL-18BP-β per well.

   After adding cells, hIL-18BP-β and test agent, the total volume should be:

   96-well plate: 90µl per well.

   384-well plate: 22.5µl per well.

   For example, if 70µl of cells are plated per well in a 96-well plate, add 10µl of 10X hIL-18BP-β followed by 10µl of 9X treatment agent in culture medium. The 10X hIL-18BP-β is stable and will be 1X after adding 10X Anti-hIL-18 mAb-α in Section 4.D. Cells can be treated overnight or for shorter periods (e.g., THP-1 cells can be primed with LPS overnight and then treated for 1 hour with nigericin to stimulate IL-18 release; in this model, the 10X hIL-18BP-β can be added with the LPS or alternatively, just prior to the nigericin treatment).

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

   Note: Assay sensitivity may be somewhat reduced in 384-well format as compared to that in 96-well format (see Section 8, Figure 5).

4.C. Preparing Human IL-18 Standard Dilution

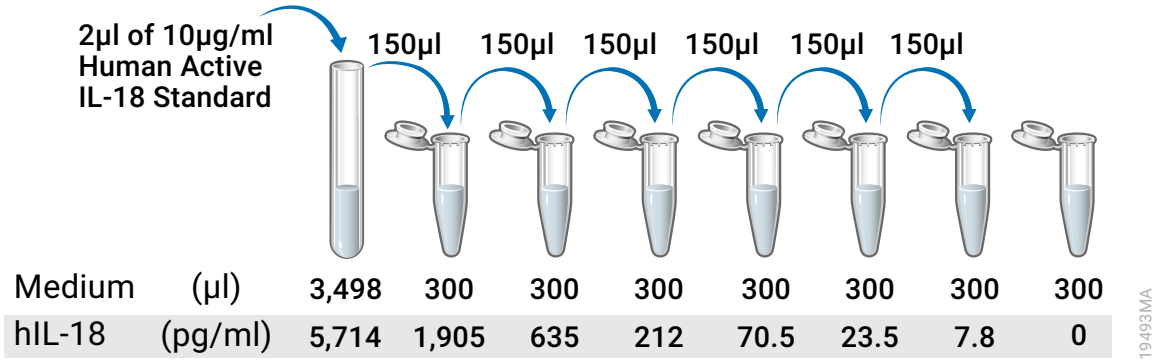

Figure 3. Human IL-18 dilution series.

Shortly before completing cell treatments, prepare IL-18 dilutions in the identical culture medium used for cell samples.

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

1. Thaw the Human Active IL-18 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 5,714pg/ml human IL-18 by diluting Human Active IL-18 Standard (10µg/ml) 1:1,750 in cell culture medium (typically HEPES-containing RPMI 1640 + 10% heat-inactivated FBS for human cells). For example, prepare 3,500µl of 5,714pg/ml human IL-18 by adding 2µl of the Human IL-18 Standard stock to 3,498µl of culture medium (see Figure 3).
4. Set up seven tubes (or seven chambers in a dilution reservoir) with 300µl of culture medium in each tube or chamber.
5. Prepare threefold serial dilutions of standard. Transfer 150µl from the 5,714pg/ml initial human IL-18 dilution (Step 3) to 300µl of culture medium for the second dilution. Mix and repeat this transfer five more times to generate seven standard dilutions with a range of 5,714pg/ml to 7.8pg/ml. The last well or chamber should contain only culture medium as the background control.

   Notes:

   a. Change pipette tips between each dilution step and use aerosol filter tips to avoid analyte carryover. The linear range of the assay is large, so carryover from high to low concentrations can compromise the standard curve.

   b. Due to the instability of active IL-18 in culture medium at 37°C, prepare the standard dilutions immediately before adding hIL-18BP-β.

4.C. Preparing Human IL-18 Standard Dilution (continued)

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.

7. Add 10X hIL-18BP-β (prepared in Section 4.B, Step 3) to wells with the standard dilutions and background control in the assay plate.

   96-well plate: Dispense 10µl per well.

   384-well plate: Dispense 2.5µl per well.

   Notes:

   a. Unused Human Active IL-18 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 –30 to –10°C. Avoid multiple freeze-thaw cycles.

   b. We recommend incorporating human IL-18 standard controls on each assay plate for normalization.

4.D. Adding 10X Anti-hIL-18 mAb-Peptide α to Assay Wells

If using multiple cell models, each requiring a different culture medium, separate 10X Anti-hIL-18 mAb-Peptide α antibody dilutions must be generated in each medium used in the study.

1. Remove the Anti-hIL-18 mAb-Peptide α, 1,000X, 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.# W1911, you may need to initiate buffer warming further in advance of its use in Section 4.D.

2. Gently tap the tube with a finger to ensure mixing, then briefly centrifuge before opening the Anti-hIL-18 mAb-α tube.

3. Immediately prior to use, prepare a 10X antibody solution by diluting the Anti-hIL-18 mAb-Peptide α, 1,000X, 1:100 into culture medium. Pipet to mix the antibody dilution. To assay a complete 96- or 384-well plate, including some excess reagent volume, prepare the 10X Anti-hIL-18 mAb-α solution as follows:

Reagent	Volume
culture medium	1,188µl
Anti-hIL-18 mAb-Peptide α, 1,000X	12µl

4. After treating the cells (Section 4.B, Step 4), add the 10X Anti-hIL-18 mAb-α solution to wells containing cultured cells and standard dilutions, carefully avoiding cross contamination between wells by changing pipette tips if moving from high to low analyte levels.

   96-well plate: Dispense 10µl/well of 10X Anti-hIL-18 mAb-α to 90µl/well of cells or IL-18 standard dilutions.

   384-well plate: Dispense 2.5µl/well of 10X Anti hIL-18 mAb-α to 22.5µl/well of cells or IL-18 standard dilutions

5. **Optional:** Briefly mix with a plate shaker (e.g., 10 seconds at 250–350rpm).
6. Incubate for 60 minutes at 37°C in a humidified 5% CO_2 incubator.

4.E. Adding Lumit® Detection Reagent B + Flex Detection Protein to Assay Wells

While cells are incubating with the hIL-18BP-β/Anti-hIL-18 mAb-α mixture (Section 4.D), prepare the Lumit® detection reagent B with Lumit® Flex Detection Protein.

1. Equilibrate the required volume of Lumit® Detection Buffer B to room temperature.
2. Remove the Lumit® Detection Substrate B and Lumit® Flex Detection Protein from storage and mix. If substrate or detection protein has collected in the cap or on the sides of the tube, briefly centrifuge.
3. Prepare a 5X mixture of Lumit® detection reagent B + detection protein by diluting the Lumit® Detection Substrate B 20-fold and the Lumit® Flex Detection Protein 7-fold into the Lumit® Detection Buffer B. To assay a 96- or 384-well assay plate, including some reagent volume for pipetting loss, prepare as follows:

Reagent	Volume
Lumit® Detection Buffer B	2,260µl
Lumit® Detection Protein	400µl
Lumit® Detection Substrate B	140µl

Notes:

a. The 1,000 assay size Lumit® Human IL-18 Immunoassay (Cat.# W1911) contains 25ml of Detection Buffer B and 1.25ml of Detection Substrate B. There is sufficient volume to prepare Lumit® detection reagent B + detection protein for analyzing 5 or 10 plates at one time. If Cat.# W1911 is used to assay 10 plates individually, mix 2,018µl of Lumit® Detection Buffer B + 357µl of Lumit® Flex Detection Protein + 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.D, Step 6, equilibrate the assay plate with cells to room temperature for 15 minutes.
5. Add room temperature 5X Lumit® detection reagent B + detection protein 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 30 minutes.

4.E. Adding Lumit® Detection Reagent B + Flex Detection Protein to Assay Wells (continued)

8. Measure luminescence.

   Notes:

   a. Assay signal is stable with a half-life of approximately 2 hours after reaching peak signal at approximately 30–40 minutes, compatible with batch processing of multiple assay plates. We recommend incorporating the Human Active IL-18 Standard controls on each assay plate for normalization.

   b. All standard luminescence plate readers are suitable for this assay. An integration time of 0.25–1 second per well should be suitable. The gain settings on some instruments might require optimizing to achieve sensitivity and dynamic range. Consult the instrument manual. The GloMax® Discover System (Cat.# GM3000) provides a pre-installed "Lumit Immunoassay" protocol under the 'Luminescence Protocols' tab with a 0.5 second integration time.

5. Optional Sample Transfer Protocol

This protocol describes sample medium transfer 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 Active IL-18 Standard prepared in culture medium is used to generate a standard curve.

Note: The Lumit® Active IL-18 (Human) Immunoassay cannot be multiplexed with any NanoBiT® complementation assays (e.g., Lumit® Immunoassays and Nano-Glo® HiBiT Lytic Detection System).

5.A. Cell Plating

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 dispensed cell number per well for a specific cell model should be empirically determined. Ensure that the maximum cytokine level released does not exceed the linear range of the detection chemistry. Within that constraint, cell number can be increased to meet the detection requirements in cases of low-level cytokine production. See example cell numbers used for THP-1 cells and PBMCs in Section 8, Figures 5–8.

5.B. Adding Human IL-18BP-Peptide β and Treatment to Cells

1. Remove the hIL-18BP-Peptide β, 1,000X, from storage immediately before use.
2. Gently tap the tube with a finger to ensure mixing, then briefly centrifuge before opening.
3. Prepare a 10X working solution by diluting the hIL-18BP-Peptide β 1:100 in culture medium. Prepare sufficient volume for all sample and standard wells. To assay a complete 96- or 384-well plate, including some excess reagent volume, prepare the 10X hIL-18BP-β as follows:

Reagent	Volume
culture medium	990µl
hIL-18BP-Peptide β, 1,000X	10µl

4. Add 10X hIL-18BP-β working solution to the wells with plated cells, just prior to or with the cell treatment, to achieve a 1X final concentration in the well.

   96-well plate: Dispense 10µl of 10X hIL-18BP-β per well.

   384-well plate: Dispense 2.5µl of 10X hIL-18BP-β per well.

   After adding cells, hIL-18BP-β and test agent, the total volume should be:

   96-well plate: 100µl per well

   384-well plate: 25µl per well

   For example, if 80µl of cells are plated per well in a 96-well plate, add 10µl of 10X hIL-18BP-β followed by 10µl of 10X treatment agent in culture medium. The 10X hIL-18BP-β working solution is very stable. Cells can be treated overnight or for shorter periods (e.g., THP-1 cells can be primed with LPS overnight and then treated for 1 hour with nigericin to stimulate release of IL-18; in this model, the 10X hIL-18BP-β can be added with the LPS or alternatively, just prior to the nigericin treatment).

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

   Note: Assay sensitivity may be somewhat reduced in 384-well format as compared to that in 96-well format (see Section 8, Figure 5).

6. Once the treatment incubation time is complete, transfer the sample cell supernatants to an assay plate.

   96-well plate: Transfer 50µl per well.

   384-well plate: Transfer 12.5µl per well.

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5.C. Preparing Human IL-18 Standard Dilutions

Shortly before completing cell treatments, prepare IL-18 dilutions in the identical culture medium used for cell samples.

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

1. Thaw the Human Active IL-18 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 5,714pg/ml human IL-18 by diluting Human Active IL-18 Standard (10µg/ml) 1:1,750 in cell culture medium (typically HEPES-containing RPMI 1640 + 10% heat-inactivated FBS for human cells). For example, prepare 3,500µl of 5,714pg/ml human IL-18 by adding 2µl of the Human IL-18 Standard stock to 3,498µl of culture medium (see Figure 3).
4. Set up seven tubes (or seven chambers in a dilution reservoir) with 300µl of culture medium in each tube or chamber.
5. Prepare threefold serial dilutions of standard. Transfer 150µl from the 5,714pg/ml initial human IL-18 dilution (Step 3) to 300µl of culture medium for the second dilution. Mix and repeat this transfer five more times to generate seven standard dilutions with a range of 5,714pg/ml to 7.8pg/ml. The last well or chamber should contain only culture medium as the background control. See Section 4.C for an illustration of dilution scheme.

   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 Active IL-18 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 –30°C to –10°C. Avoid multiple freeze-thaw cycles.

   b. We recommend incorporating Human Active IL-18 Standard controls on each assay plate for normalization.

5.D. Adding 2X Anti-hIL-18 mAb-Peptide α/hIL-18BP-Peptide β Mixture to IL-18 Standard Dilutions

If using multiple cell models, each requiring a different culture medium, separate 2X Anti-hIL-18 mAb-Peptide α/hIL-18BP-Peptide β mixtures must be generated in each medium used in the study.

1. Remove the Anti-hIL-18 mAb-Peptide α, 1,000X, and hIL-18BP-Peptide β, 1,000X, 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.# W1911, you may need to initiate buffer warming further in advance of its use in Section 5.E.

2. Gently tap the tube with a finger to ensure mixing before opening the Anti-hIL-18 mAb-α tube, and briefly centrifuge the hIL-18BP-β and Anti-hIL-18 mAb-α tubes before opening.

3. Immediately prior to use, prepare a 2X mixture by diluting the hIL-18BP-Peptide β and Anti-hIL-18 mAb-α 1:500 into a single volume of prewarmed cell culture medium. Pipet to mix the solution. To prepare sufficient volume for a standard curve, prepare the as follows:

Reagent	Volume
culture medium	996µl
Anti-hIL-18 mAb-Peptide α, 1,000X	2µl
hIL-18BP- Peptide β, 1,000X	2µl

4. Add 2X mixture to each standard dilution in duplicate to achieve 1X final concentration.

   96-well plate: Dispense 50µl of 2X Anti-hIL-18 mAb-α/hIL-18BP-β mixture per well.

   384-well plate: Dispense 12.5µl of 2X Anti-hIL-18 mAb-α/hIL-18BP-β mixture per well.

5.E. Adding 2X Anti-hIL-18 mAb-α/1X hIL-18BP-β Mixture to Sample Wells

1. Immediately prior to use, prepare a **2X** Anti-hIL-18 mAb-α/**1X** hIL-18BP-β mixture by diluting Anti-hIL-18 mAb-α 1:500 and hIL-18BP-β 1:1,000 into a single volume of culture medium. Pipet to mix the antibody solution. To assay a complete 96- or 384-well plate, including some excess reagent volume, prepare the 2X Anti-hIL-18 mAb-α/1X hIL-18BP-β mixture as follows:

Reagent	Volume
culture medium	5,982µl
Anti-hIL-18 mAb-Peptide α, 1,000X	12µl
hIL-18BP- Peptide β, 1,000X	6µl

2. Add the 2X Anti-hIL-18 mAb-α/1X hIL-18BP-β mixture to sample wells with transferred culture medium, carefully avoiding cross contamination between wells. Change pipette tips between rows to avoid cross contamination.

   96-well plate: Dispense 50µl/well of 2X Anti-hIL-18 mAb-α/1X hIL-18BP-β mixture.

   384-well plate: Dispense 12.5µl/well of 2X Anti-hIL-18 mAb-α/1X hIL-18BP-β mixture.

5.E. Adding 2X Anti-hIL-18 mAb-α/1X hIL-18BP-β Mixture to Sample Wells (continued)

3. **Optional:** Briefly mix with a plate shaker (e.g., 10 seconds at 250–350rpm).
4. Incubate for 60 minutes at 37°C in a 5% CO_2 humidified incubator.

   Note: The hIL-18BP-β binds to active hIL-18 more efficiently at 37°C. Therefore, the transferred culture medium and IL-18 standard dilutions must be incubated at 37°C.

5.F. Adding Lumit® Detection Reagent B + Detection Protein to Assay Wells

While the transferred culture medium plate is incubating with the Anti-hIL-18 mAb-α/hIL-18BP-β mixture (Section 5.D), prepare the Lumit® detection reagent B + detection protein.

1. Equilibrate the required volume of Lumit® Detection Buffer B to room temperature.
2. Remove the Lumit® Detection Substrate B and Flex Detection Protein from storage and mix. If the Lumit® Detection Substrate B or Flex Detection Protein has collected in the cap or on the sides of the tube, briefly centrifuge.
3. Prepare a 5X Lumit® detection reagent B + Detection Protein by diluting the Lumit® Detection Substrate B 20-fold and Lumit® Flex Detection Protein 7-fold into Lumit Detection Buffer B. To assay a 96- or 384-well assay plate, including some reagent volume for pipetting loss, prepare as follows:

Reagent	Volume
Lumit® Detection Buffer B	2,260µl
Lumit® Flex Detection Protein	400µl
Lumit® Detection Substrate B	140µl

Notes:

a. The 1,000-assay size of Lumit® Active IL-18 (Human) Immunoassay (Cat.# W1911) contains 25ml of Lumit® Detection Buffer B and 1.25ml of Lumit® Detection Substrate B. There is sufficient volume to prepare Lumit® detection reagent B + detection protein for analyzing 5 or 10 plates at once. If Cat.# W1911 is used for assaying 10 plates individually, mix 2,018µl of Lumit® Detection Buffer B + 357µl of Lumit® Flex Detection Protein + 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 2–10°C, the reconstituted reagent loses 10% activity in approximately 7 hours.

4. Equilibrate assay plate with cells to room temperature for 5–10 minutes.
5. Add room temperature Lumit® detection reagent B + detection protein 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 30 minutes.

7. Measure luminescence.

Notes:

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

b. All standard plate readers capable of reading luminescence are suitable for this assay. An integration time of 0.25–1 second per well should be suitable. Some instruments might require optimizing the gain settings to achieve sensitivity and dynamic range. Consult the instrument manual for instrument settings. The GloMax® Discover System (Cat.# GM3000) provides a pre-installed "Lumit Immunoassay" protocol under the 'Luminescence Protocols' tab with a 0.5 second integration time.

6. Alternative Direct Protocol for Cultured Cells: Simultaneous Addition of hIL-18BP-β, Anti-hIL-18 mAb-α and Treatment to Cells

This protocol is appropriate for use when treatment duration lasts ≤8 hours. We do not recommend overnight incubation with Anti-hIL-18 mAb-Peptide α.

6.A. Cell Plating

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 dispensed cell number per well for a specific cell model should be empirically determined. Cell numbers can be increased to meet the detection requirements of low-level cytokine production. See example cell numbers used for THP-1 cells and PBMCs in Section 8, Representative Data, Figures 2–5.

6.B. Adding Human IL-18BP-Peptide β, Anti-hIL-18 mAb-Peptide α and Treatment to Cells

Just before treating cells, make a 5X mixture of hIL-18BP-Peptide β and Anti-hIL-18 mAb-Peptide α.

1. Remove the hIL-18BP-Peptide β, 1,000X, and Anti-hIL-18 mAb-Peptide α, 1,000X from storage immediately before use.

   Note: Remove Lumit® Buffer B from –30°C to –10°C at the same time and equilibrate to room temperature if not already thawed.

2. Gently tap to mix and briefly centrifuge the hIL-18BP-β and Anti-hIL-18 mAb-α tubes before opening.

3. Immediately prior to use, prepare a 5X mixture by diluting the hIL-18BP-β and Anti-hIL-18 mAb-α 1:200 into a single volume of culture medium. Pipet to mix the solution. To assay a complete 96- or 384-well plate, including some excess reagent volume, prepare the 5X hIL-18BP-β/Anti-hIL-18 mAb-α mixture as follows:

Reagent	Volume
culture medium	2,376µl
Anti-hIL-18 mAb-Peptide α, 1,000X	12µl
hIL-18BP- Peptide β, 1,000X	12µl

6. Add 5X hIL-18BP-β/Anti-hIL-18 mAb-α and treatment to wells with plated cells to achieve a 1X final concentration in the well. (Reserve any remaining 5X hIL-18BP-β/Anti-hIL-18 mAb-α solution for adding to standard curve wells, storing at 2–10°C until use.)

   96-well plate: Dispense 20µl of 5X hIL-18BP-β/Anti-hIL-18 mAb-α per well.

   384-well plate: Dispense 5µl of 5X hIL-18BP-β/Anti-hIL-18 mAb-α per well.

   After adding cells, 5X hIL-18BP-β/Anti-hIL-18 mAb-α and test agent, the total volume should be:

   96-well plate: 100µl per well.

   384-well plate: 25µl per well.

   For example, if 70µl of cells are plated per well in a 96-well plate, add 20µl of 5X hIL-18BP-β/Anti-hIL-18 mAb-α followed by 10µl of 10X treatment agent in culture medium.

6.C. Preparing Human IL-18 Standard Dilution

Shortly before completing cell treatments, prepare IL-18 standard dilutions.

1. Thaw the Human Active IL-18 Standard immediately before use.
2. Briefly centrifuge the tube before opening, then mix by pipetting.
3. Prepare an initial concentration of 5,714pg/ml human IL-18 by diluting Human Active IL-18 Standard (10µg/ml) 1:1,750 in cell culture medium (typically HEPES-containing RPMI 1640 + 10% heat-inactivated FBS for human cells). For example, prepare 3,500µl of 5,714pg/ml human IL-18 by adding 2µl of the Human IL-18 Standard stock to 3,498µl of culture medium (see Figure 3).
4. Set up seven tubes (or seven chambers in a dilution reservoir) with 300µl of culture medium in each tube or chamber.
5. Prepare threefold serial dilutions of standard. Transfer 150µl from the 5,714pg/ml initial human IL-18 dilution (Step 3) to 300µl of culture medium for the second dilution. Mix and repeat this transfer five more times to generate seven standard dilutions with a range of 5,714pg/ml to 7.8pg/ml. The last well or chamber should contain only culture medium as the background control. See Section 4.C for illustration of dilution scheme.

   Notes:

   a. Change pipette tips between each dilution step and use aerosol filter tips to avoid analyte carryover. The linear range of the assay is large, so carryover from high to low concentrations can compromise the standard curve.

   b. Due to the instability of active IL-18 at 37°C in culture medium, prepare the standard dilutions immediately before adding the hIL-18BP-β.
6. During or just after the cell treatment, add the standard dilutions and background control in duplicate to two columns in the assay plate (see the plate map in Section 3, Before You Begin).

   96-well plate: Dispense 80µl per well.

   384-well plate: Dispense 20µl per well.
7. Immediately add reserved 5X hIL-18BP-β/Anti-IL-18 mAb-α.(see Section 6.B, Step 7) to the IL-18 standard dilutions to achieve a 1X final concentration, carefully avoiding cross contamination between wells. Change pipette tips between columns to avoid cross contamination.

   96-well plate: Dispense 20µl per well.

   384-well plate: Dispense 5µl per well.

   Notes:

   a. Unused Human Active IL-18 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 –30°C to –10°C. Avoid multiple freeze-thaw cycles.

   b. We recommend incorporating human IL-18 standard controls on each assay plate for normalization.

6.D. Adding Lumit® Detection Reagent B + Detection Protein to Assay Wells

While cells are incubating with the hIL-18BP-β/Anti-hIL-18 mAb-α mixture and treatment (the Adding Human IL-18BP-Peptide β, Anti-hIL-18 mAb-Peptide α and Treatment to Cells section), prepare the Lumit® detection reagent B.

1. Equilibrate the required volume of Lumit® Detection Buffer B to ambient temperature.
2. Remove the Lumit® Substrate B and Lumit® Flex Detection Protein from storage, and mix. If the Lumit® Substrate B or Flex Detection Protein has collected in the cap or on the sides of the tube, briefly centrifuge.
3. Prepare a 5X mixture of Lumit® detection reagent B + detection protein by diluting the Lumit® Detection Substrate B 20-fold and the Lumit® Flex Detection Protein 7-fold into the Lumit Detection Buffer B. To assay a 96- or 384-well assay plate, including some reagent volume for pipetting loss, prepare as follows:

Reagent	Volume
Lumit® Detection Buffer B	2,260µl
Lumit® Flex Detection Protein	400µl
Lumit® Detection Substrate B	140µl

Notes:

a. The 1,000 assay size Lumit® Active Human IL-18 Immunoassay (Cat.# W1911) contains 25ml of Detection Buffer B and 1.25ml of Detection Substrate B. There is sufficient volume to prepare Lumit® detection reagent B + detection protein for analyzing 5 or 10 plates at once. If Cat.# W1911 is used for assaying 10 plates individually, mix 2,018µl of Lumit® Detection Buffer B + 357µl of Lumit® Flex Detection Protein + 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 2–10°C, the reconstituted reagent will lose 10% activity in approximately 7 hours.

4. Equilibrate assay plate with cells to room temperature for 5–10 minutes.
5. Add room temperature 5X Lumit® detection reagent B + detection protein 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 300–500 rpm).
7. Incubate 30 minutes at room temperature.
8. Read luminescence.

   Note: Assay signal has a half-life of approximately 2 hours after reaching peak signal at approximately 30–40 minutes, compatible with batch processing of multiple assay plates. We recommend incorporating standard controls on each assay plate for normalization.

7. 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 cytokine concentration 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 relative light units (RLU; background-subtracted) vs. cytokine standard concentrations can be fit with the Power trendline in Microsoft Excel® (see Section 8, Figure 4) and subsequently used for interpolation of the concentration of cytokine release in various cell samples.

8. Representative Data

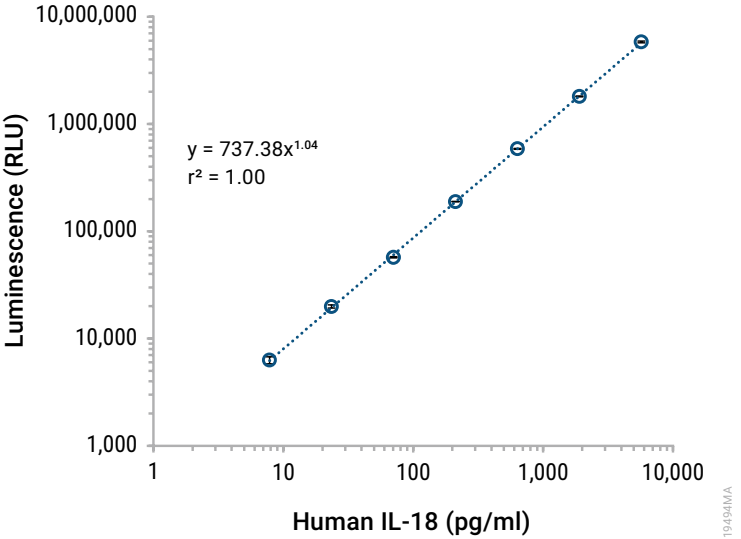

Figure 4. Standard curve for the Lumit® Active IL-18 (Human) Immunoassay. This is a representative standard curve and should not be used for calculation of unknowns. Generate a standard curve on each assay plate to interpolate the concentration of cytokine in experimental samples. **Note:** The plotted luminescence values were determined by subtracting background RLU.

Table 1. Intra-Assay Precision. Three samples of known concentrations of human IL-18 were tested with 20 replicates on one plate to assess intra-assay precision. A standard curve was used on the assay plate to interpolate the IL-18 quantities in each well by the various methods using GraphPad® Prism software.

	Third order polynomial (cubic) fit			Cubic spline fit		
Number of Replicates	20	20	20	20	20	20
Expected (pg/ml)	50	500	5,000	50	500	5,000
Mean (pg/ml)	50	492	5,153	52	487	5,139
Standard Deviation	1.3	4.6	54.0	1.5	4.7	55.0
Percent CV	2.57	0.94	1.05	2.91	0.96	1.07
Average Percent of Expected	99.1	98.3	103.1	103.5	97.4	102.8
Percent Range	94.9–103.8	96.4–99.5	100.7–105.3	98.6–109.2	95.5–98.6	100.4–105.1

Note: The minimal detectable dose (MDD), determined at two standard deviations above background, was 1.6pg/ml.

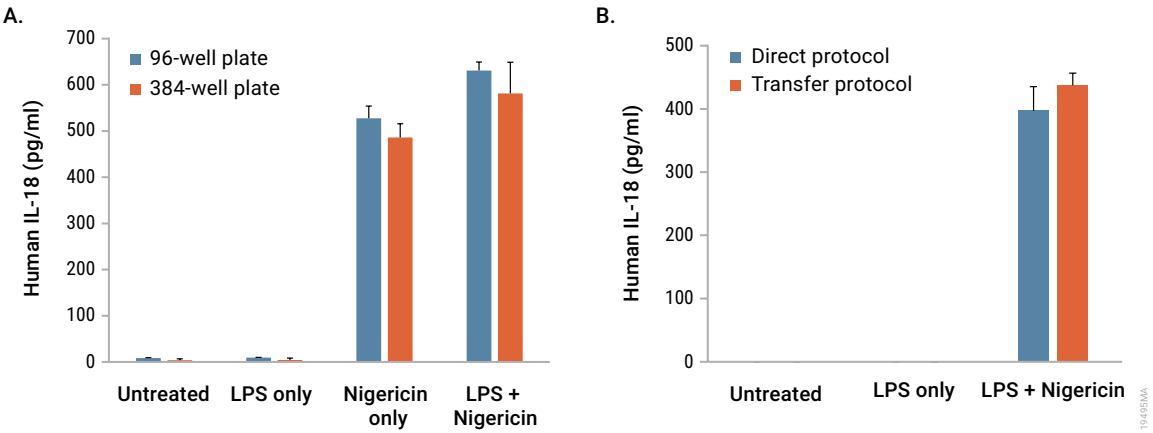

Figure 5. Active IL-18 released from THP-1 cells. Panel A. THP-1 cells (ATCC) were plated into 96-well or 384-well tissue culture plates at 50,000 or 12,500 cells/well, respectively, and were either LPS-primed overnight with 500ng/ml LPS or left untreated. The next day, hIL-18BP-Peptide β was added, followed by nigericin (10µM final) to activate the canonical inflammasome. After treating with nigericin for 1 hour, cells were incubated with the Anti-hIL-18-mAb-α for 1 hour. After equilibrating the plate to room temperature, Lumit® detection reagent B + detection protein was added. After 30 minutes, luminescence was recorded on a GloMax® Discover System (Cat.# GM3000). **Panel B.** THP-1 cells were plated into 96-well plates and LPS-primed overnight. The next day the hIL-18BP-β was added followed by nigericin (10µM final concentration). After a 1-hour nigericin treatment, half the culture medium was transferred to a separate plate. Both the transferred medium and cells with remaining medium were incubated with the anti-IL-18 mAb-α for 1 hour at 37°C/5% CO_2. After equilibrating the plate to room temperature, Lumit® detection reagent B + detection protein was added. After 30 minutes, luminescence was recorded on a GloMax® Discover System. IL-18 levels were interpolated from a standard curve using GraphPad® Prism software. Nigericin alone can activate the NLRP3 inflammasome in THP-1 cells (11,12) and LPS-priming slightly increases the release of active IL-18 triggered by nigericin. The transferred medium or the cells with the remaining half medium give the same concentration of released IL-18.

8. Representative Data (continued)

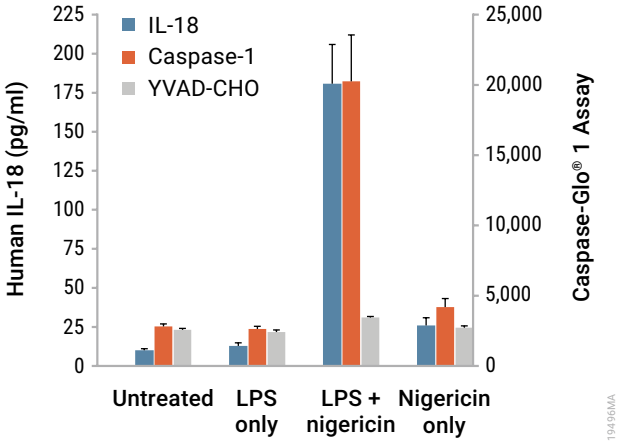

Figure 6. Active IL-18 release multiplexed with caspase-1 activity assay from inflammasome-activated PBMC. Peripheral blood mononuclear cells (PBMC) pooled from four donors (BioIVT) were plated in a 96-well-plate at 150,000 cells/well and primed with 500ng/ml LPS for 2.5 hours with hIL-18BP-β added followed by nigericin treatment (10µM final concentration) for 1.5 hours. Half of the medium was transferred to a new 96-well plate for a Caspase-Glo® 1 Inflammasome Assay (Cat.# G9951) following the protocol described in Technical Manual #TM456, using the YVAD-CHO caspase-1 inhibitor to confirm caspase-1 activity. hIL-18 mAb-α was added to the remaining cells and medium and incubated for 1 hour at 37°C/5% CO_2. The Lumit® detection reagent B + detection protein was added, and luminescence was recorded after 30 minutes on a GloMax® Discover System (Cat.# GM3000).

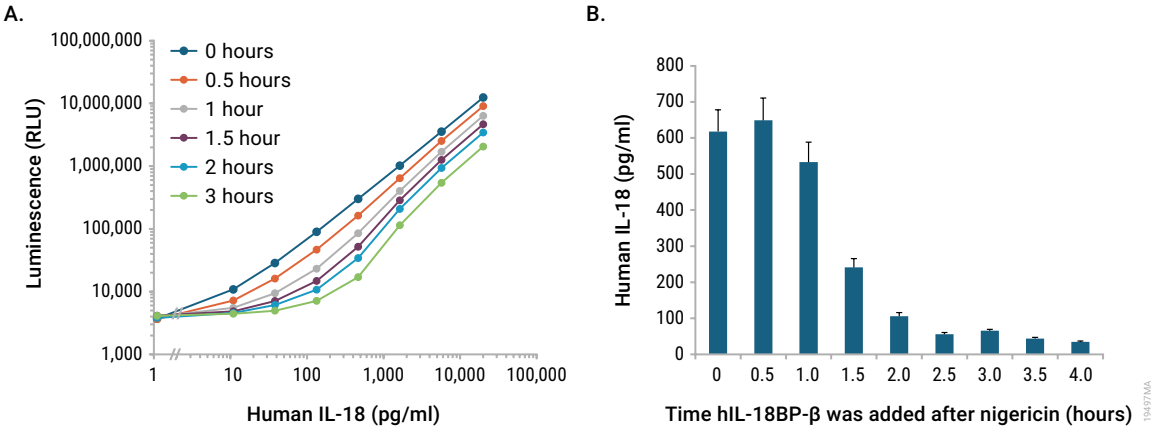

Figure 7. Instability of active IL-18 in 37°C culture medium. Panel A. Dilutions of recombinant active IL-18 in culture medium to generate standard curves were incubated at 37°C for various times before adding the hIL-18BP-β. Anti-hIL-18 mAb-α was then added to all the IL-18 dilutions and incubated for 1 hour at 37°C. After equilibrating the plate to room temperature, the Lumit® detection reagent B + detection protein was added. Luminescence was recorded after 30 minutes on a GloMax® Discover System (Cat.# GM3000). The instability of IL-18 in culture medium is temperature dependent, with greater instability at higher temperatures. **Panel B.** THP-1 cells were LPS-primed overnight and then nigericin treated for 4 hours with hIL-18BP-β added either before the nigericin treatment (time 0) or at various times after adding nigericin. The IL-18-mAb-α was added after 4 hours and incubated for 1 hour at 37°C/5% CO_2. After bringing the plate to room temperature, Lumit® detection reagent B + detection protein was added, and luminescence was recorded after 30 minutes on a GloMax® Discover System. The signal from released IL-18 is lost after 2.5 hours at 37°C in culture medium, demonstrating the need for capturing active IL-18 upon release with the hIL-18BP-β. Once bound, the IL-18/IL-18BP complex is stable.

8. Representative Data (continued)

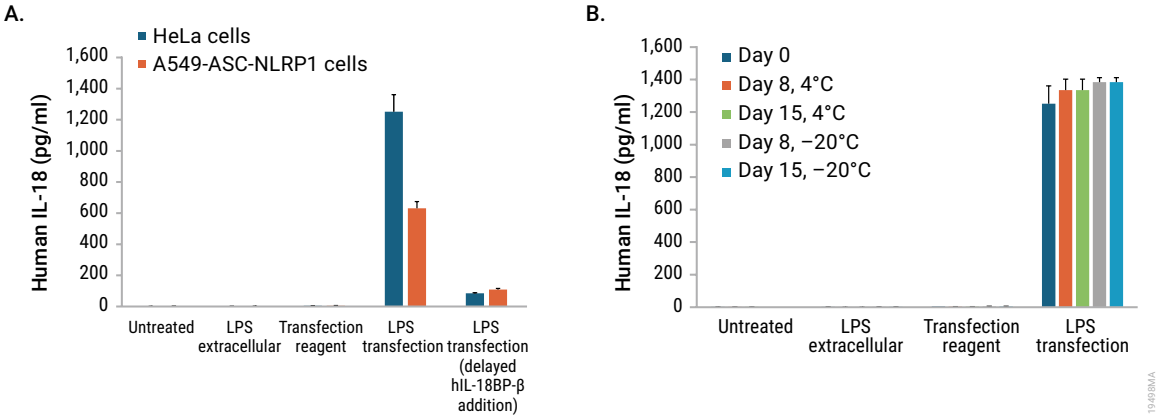

Figure 8. Noncanonical NLRP3 inflammasome activation and stability of active IL-18 bound to hIL-18BP-β. Panel A. HeLa cells (ATCC) and A549-ASC-NLRP1 cells (InvivoGen; both plated at 2 × 10^5 cells/ml) were transfected using FuGENE® HD Transfection Reagent (Cat.# E2311) with LPS in 12-well plates to stimulate the noncanonical inflammasome. HeLa and A549-ASC-NLRP1 cells were primed with IFN-γ (10ng/ml) in culture medium overnight. The next day, the FuGENE® HD Transfection Reagent was prepared with or without LPS (40µg/ml) in OptiMEM® I Reduced Serum Medium. The DMEM/10% FBS culture medium was removed from the cells and OptiMEM® medium was added followed by FuGENE® HD Transfection Reagent with or without LPS. Cells were incubated for 30 minutes before removing the transfection reagent. Cells were washed with culture medium followed by adding the hIL-18BP-β in DMEM/10% FBS and incubating for 4 hours at 37°C/5% CO_2. Supernatants were removed, centrifuged at 3,800 × *g* for 10 minutes and plated into 96-well plates. Anti-hIL-18 mAb-α was added and incubated for 1 hour at 37°C/5% CO_2. After equilibrating the plate to room temperature, the Lumit® detection reagent B + detection protein was added, and luminescence was recorded after 30 minutes on a GloMax® Discover System (Cat.# GM3000). **Panel B.** HeLa cells were transfected using FuGENE® HD Transfection Reagent with LPS as in Panel A, but the supernatants were collected and stored at 4°C or –20°C for the number of days noted. The supernatants were thawed and dispensed into 96-well plates and incubated with Anti-hIL-18 mAb-α for 1 hour at 37°C/5% CO_2. After equilibrating the plate to room temperature, the Lumit® detection reagent B + detection protein was added, and luminescence was recorded after 30 minutes on a GloMax® Discover System. The IL-18/hIL-18BP-β complex is stable and can be stored at 4°C or –20°C for later testing.

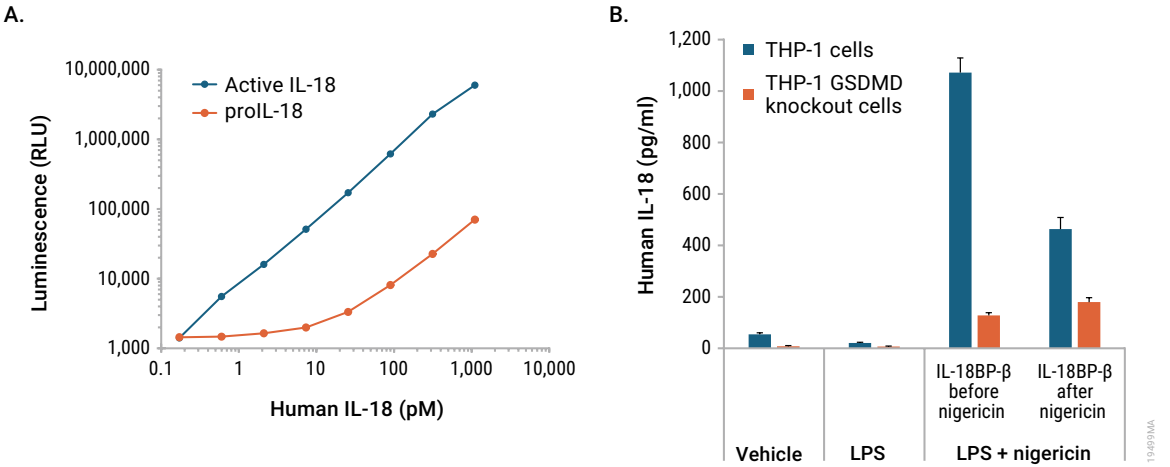

Figure 9. Cross-reactivity of hIL-18BP with proIL-18 and confirming detection of active hIL-18. Panel A. Dilutions of IL-18 and pro-IL-18 (with GST tag, Sino Biological) at equimolar concentrations were incubated with a hIL-18BP-β/Anti-hIL-18 mAb-α mixture for 1 hour at 37°C/5% CO_2. After equilibrating the plate to room temperature, the Lumit® detection reagent B + Flex Detection Protein was added, and luminescence was recorded after 30 minutes on a GloMax® Discover System (Cat.# GM3000). While IL-18BP is not reported to bind to proIL-18, we detected a 1–2% cross-reactivity of hIL-18BP with recombinant proIL-18. **Panel B.** To confirm detection of active IL-18 and not cross-reactivity with proIL-18, the hIL-18BP-Peptide β can be added before or after treating cells, within the same day. The instability of active IL-18 in culture medium at 37°C will confirm active IL-18 with a decrease in signal when hIL-18BP-β is added after treatment. THP-1 GSDMD knockout cells (InvivoGen) undergo a delayed pyroptosis that is caspase-3 and GSDME mediated (13). Caspase-3 cleaves proIL-18 into an inactive form of IL-18 (6). THP-1 and THP-1 (GSDMD$^{-/-}$) were plated into 96-well plates (50,000 cells/well) and either primed with 500ng/ml LPS overnight or left untreated. The next day, hIL-18BP-β was added, followed by nigericin (10µM final concentration) to activate the canonical inflammasome. The THP-1 cells were treated with nigericin for 1 hour and the THP-1 (GSDMD$^{-/-}$) cells were treated for 5.5 hours to induce delayed GSDME-mediated pyroptosis. A significant amount of IL-18 was released from THP-1 cells, and the amount decreased if the hIL-18BP-β was not added until after treatment, as expected. Tenfold less IL-18 was detected from the THP-1 (GSDMD$^{-/-}$) cells, but this amount did not decrease with delayed hIL-18BP-β addition. ProIL-18 is more stable than active IL-18 in culture medium and abundant in many cell types; thus, low levels may be detected from dead cells. If the treatment time is within a day, adding hIL-18BP-β before and after treatment will distinguish active IL-18 from proIL-18. The minimal signal from THP-1 (GSDMD$^{-/-}$) cells is proIL-18, since the signal does not decrease after 5.5 hours.

8. Representative Data (continued)

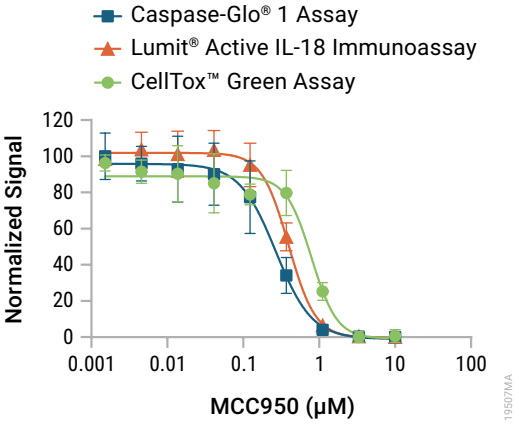

Figure 10. Monitoring caspase-1 activity, active IL-18 release and cell death from the same cells. The NLRP3 inflammasome inhibitor, MCC950 (also called CRID3), inhibits caspase-1 activity, IL-18 release and pyroptosis (14). THP-1 cells were dispensed into 96-well plates at 50,000 cells/well in RPMI/10% FBS and primed with 500ng/ml LPS overnight. The next day a dose range of MCC950 was added, followed by hIL-18BP-β and nigericin (10µM final concentration) for a total of 150µl/well. After a 1-hour nigericin treatment, 50µl of culture medium was transferred for Caspase-Glo® 1 Inflammasome Assay (Cat.# G9951) and 50µl of culture medium was transferred for analysis with the Lumit® Active hIL-18 (Human) Immunoassay. The remaining cells were monitored for cell death with CellTox™ Green Cytotoxicity Assay (Cat.# G8741). MCC950 inhibited caspase-1 activity, IL-18 release and pyroptosis. The IC_{50} for MCC950 is higher in FBS-containing medium (15).

9. 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	Use only human cells with the Lumit® Active IL-18 (Human) Immunoassay. The Lumit® Active IL-18 (Human) Immunoassay will not effectively detect IL-18 from other species. In addition to the Human Active IL-18 Standard, consider using treated human THP-1 cells as a positive control.
Human IL-18 standard curve is not linear	The threefold 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 assay sensitivity and the broad linear range (>3 logs) means that any carryover will disrupt the linear range. Also, make sure that no IL-18 contaminates the background control.
The relative light units (RLU) for the standard are low and/or variable	Warm culture medium, Human IL-Active IL-18 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 IL-18 can be accurately quantitated.
No luminescence detected	Make sure the Lumit® Flex Detection Protein is added to the Lumit® detection reagent B.

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10. Appendix

10.A. References

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10.B. Related Products

Lumit® Cytokine Immunoassays and Supporting Reagents

Product	Size	Cat.#
Lumit® Human Active IL-18 Standard	25µl	W191A-C

Additional cytokine standards available.

Product	Size	Cat.#
Lumit® HMGB1 (Human/Mouse) Immunoassay	100 assays	W6110
Lumit® IFN-β (Human) Immunoassay	100 assays	W1810
Lumit® IFN-γ (Human) Immunoassay	100 assays	W6040
Lumit® IL-1β (Human) Immunoassay	100 assays	W6010
Lumit® IL-1β (Mouse) Immunoassay	100 assays	W7010
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-8/CXCL8 (Human) Immunoassay	100 assays	W1460
Lumit® IL-10 (Human) Immunoassay	100 assays	W6070
Lumit® IL-12 p70 (Human) Immunoassay	100 assays	W1850
Lumit® IL-17A (Human) Immunoassay	100 assays	W1430
Lumit® TNF-α (Human) Immunoassay	100 assays	W6050
Lumit® VEGF-A (Human) Immunoassay	100 assays	W1880

Additional sizes available.

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.

Inflammation Assays

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

Additional sizes available.

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10.C. Related Products (continued)

Cell Viability Assays

Product	Size	Cat.#
CellTiter-Glo® 2.0 Cell Viability Assay	10ml	G9241
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.

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.

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
Glucose Uptake-Glo™ Assay	5ml	J1341
Glycogen-Glo™ Assay	5ml	J5051
Pyruvate-Glo™ Assay	5ml	J4051
Malate-Glo™ Assay	5ml	JE9100
Glutamine/Glutamate-Glo™ Assay	5ml	J8021
Glycerol-Glo™ Assay	5ml	J3150
Triglyceride-Glo™ Assay	5ml	J3160
Cholesterol/Cholesterol Ester-Glo™ Assay	5ml	J3190
BHB-Glo™ (Ketone Body) Assay	5ml	JE9500
BCAA-Glo™ Assay	5ml	JE9300

Additional sizes available.

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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, and other patents and patents pending.

[c]U.S. Pat. No. 11,493,504; European Pat. Nos. 3783011 and 2970412; and Japanese Pat. No. 7280842.

[d]Patents Pending.

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