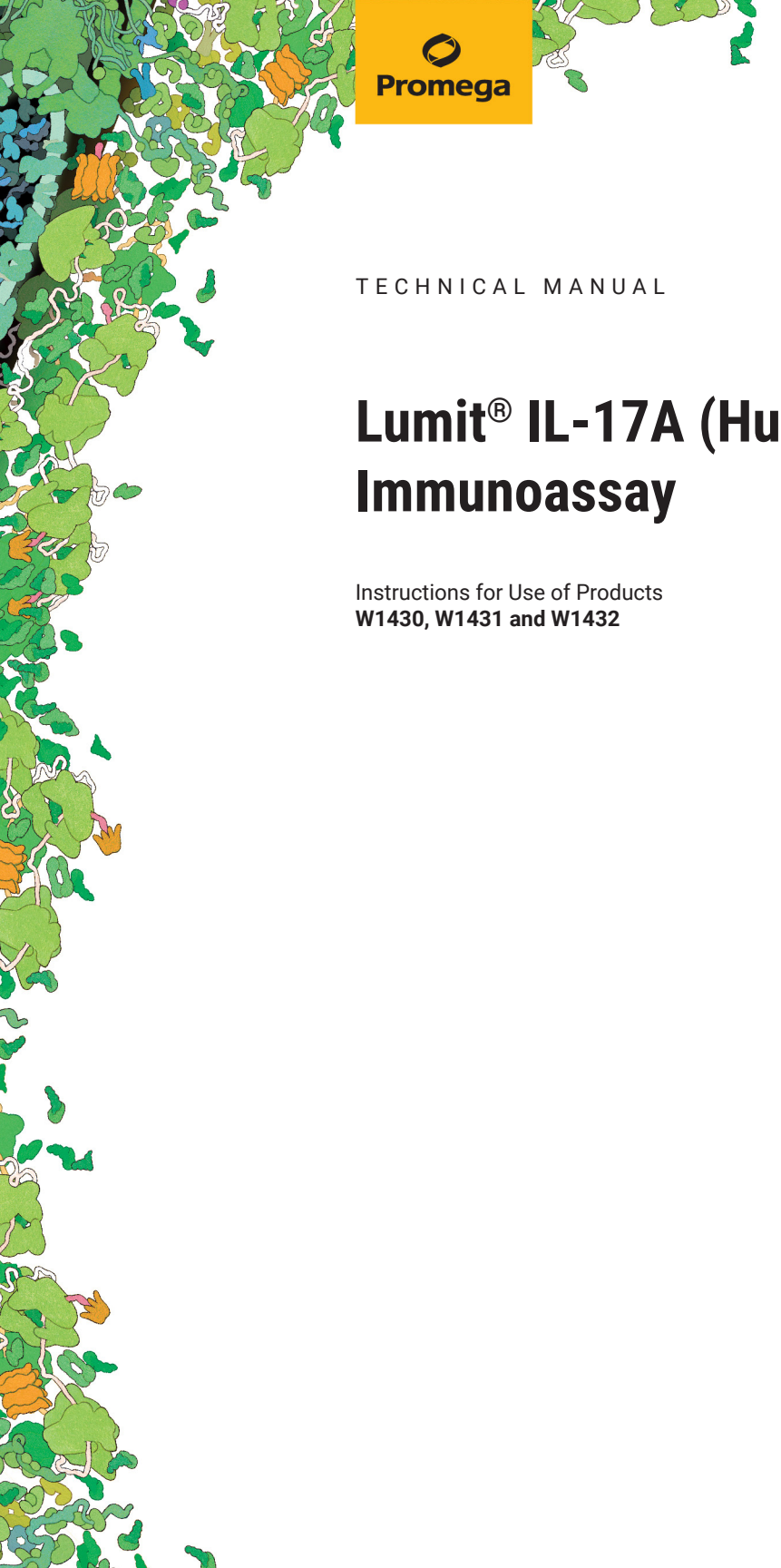


TECHNICAL MANUAL

Lumit® IL-17A (Human) Immunoassay

Instructions for Use of Products
W1430, W1431 and W1432

Revised 6/25
TM759

Promega

Lumit® IL-17A (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® IL-17A (Human) Immunoassay[a,b] is a homogeneous, bioluminescent assay for detecting interleukin-17A (IL-17A) released from cells without the need for sample transfers or wash steps. The assay is specific to IL-17A and does not detect IL-17B, IL-17D, IL-17E and IL-17F family members.

The Lumit® IL-17A (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.

The Lumit® IL-17A (Human) Immunoassay is based on NanoLuc® Binary Technology (NanoBiT). NanoBiT is a luminescent structural complementation system designed for biomolecular interaction studies (1,2). 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 IL-17A monoclonal antibodies covalently labeled with SmBiT or LgBiT. When the labeled antibodies recognize and bind to released IL-17A, the complementary LgBiT and SmBiT 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.

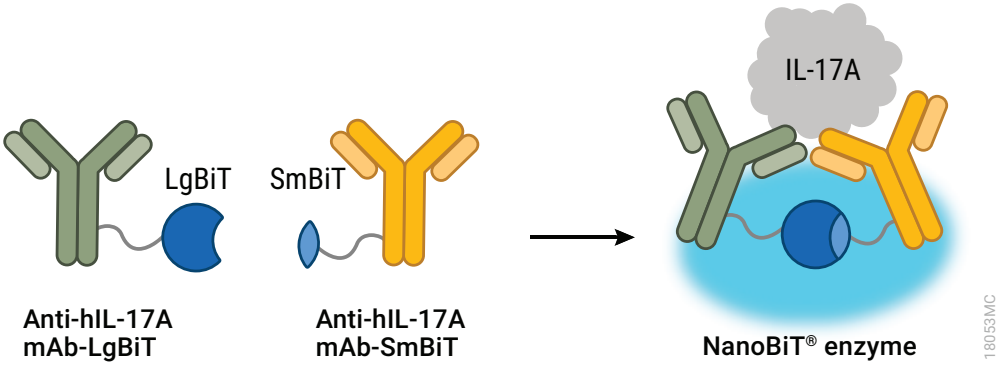


Figure 1. Assay principle. Primary monoclonal antibodies to human IL-17A are labeled with SmBiT and LgBiT. In the presence of IL-17A, SmBiT and LgBiT are brought into close proximity, forming the NanoBiT® enzyme. When Lumit® detection reagent B is added, a bright luminescent signal is generated.

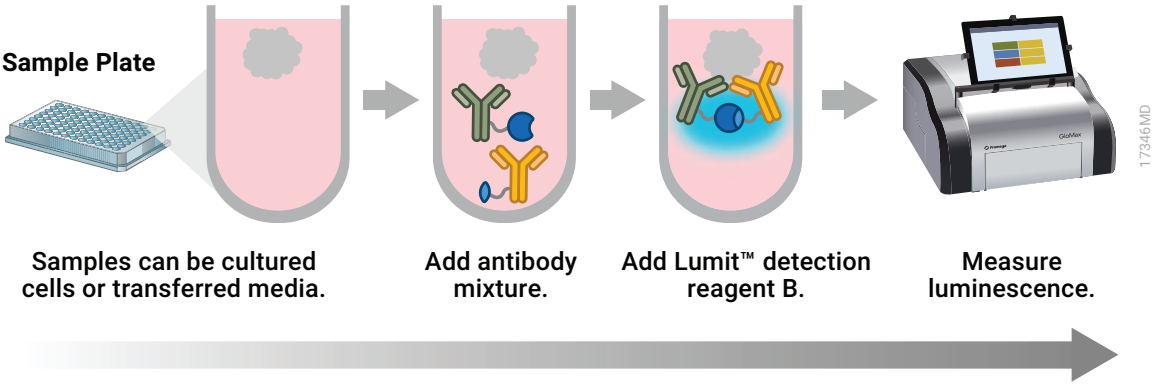


Figure 2. Assay protocol. The Lumit® IL-17A (Human) Immunoassay is performed directly on cells in culture or medium transferred from the cell culture plate to a new assay plate. The Lumit® Immunoassay protocol does not require wash steps and can be completed in 70 minutes. Antibody mixture consists of Anti-hIL-17A mAb-SmBiT and Anti-hIL-17A mAb-LgBiT.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Lumit® IL-17A (Human) Immunoassay	**100 assays**	**W1430**

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

- 30µl Anti-hIL-17A mAb-SmBiT, 500X
- 30µl Anti-hIL-17A mAb-LgBiT, 500X
- 25µl Human IL-17A Standard
- 160µl Lumit® Detection Substrate B
- 3.2ml Lumit® Detection Buffer B

PRODUCT	SIZE	CAT.#
Lumit® IL-17A (Human) Immunoassay	**1,000 assays**	**W1431**

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

- 300µl Anti-hIL-17A mAb-SmBiT, 500X
- 300µl Anti-hIL-17A mAb-LgBiT, 500X
- 25µl Human IL-17A Standard
- 1.25ml Lumit® Detection Substrate B
- 25ml Lumit® Detection Buffer B

2. Product Components and Storage Conditions (continued)

PRODUCT	SIZE	CAT.#
Lumit® IL-17A (Human) Immunoassay	**5 × 100 assays**	**W1432**

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

- 5 × 30µl Anti-hIL-17A mAb-SmBiT, 500X
- 5 × 30µl Anti-hIL-17A mAb-LgBiT, 500X
- 25µl Human IL-17A 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 IL-17A Standard at less than –65°C and all other components at –30°C to –10°C. After thawing, Human IL-17A Standard can be stored for up to 1 month at +2°C to +10°C. If storing the Human IL-17A Standard for more than 1 month after thawing, dispense into aliquots and store at less than –65°C. After thawing, store the Anti-hIL-17A mAb-SmBiT and Anti-hIL-17A 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 IL-17A (hIL-17A). The direct protocol typically achieves higher sensitivity than the optional sample transfer protocol and requires less sample manipulation. The optional sample transfer protocol provides flexibility for same well sampling during treatment exposure time courses and split-sample analysis for assessment of multiple cytokine levels from the same sample.

Direct (No-Transfer) Protocol for Cultured Cells (Section 4): Measure human IL-17A directly in cell culture wells. Add 20µl of a 5X antibody mixture to 80µl of cells or IL-17A 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 IL-17A in medium samples transferred from treated cell wells. Transfer 50µl of culture medium from cell wells to a separate assay plate. Add 50µl of a 2X antibody mixture to 50µl of transferred sample or standard dilutions and incubate for 60 minutes. Following incubation, add 25µl of Lumit® detection reagent B and record luminescence.

Note: Assay volumes are scalable and can be adjusted based on sample sizes. The protocols in Sections 4 and 5 list common volumes for 96- and 384-well plates. Other volumes 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 (nonanalyte-mediated antibody pairing) and reduced assay sensitivity. When using serum-free, albumin-free medium, assay background may be reduced and sensitivity improved by preparing the assay antibody mixture 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 Human IL-17A Standard dilution series (Section 4.B or 5.B), the Anti-hIL-17A antibody mixture (Section 4.C or 5.C) and Lumit® detection reagent B (Section 4.D or 5.D) on the day of use. Do **not** reuse Human IL-17A Standard dilutions, the Anti-hIL-17A antibody mixture or the Lumit® detection reagent B. 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-17A Standard Curve (pg/ml)*		**Test Samples**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	25,000	25,000										
B	7,500	7,500										
C	2,250	2,250										
D	675	675										
E	203	203										
F	60.8	60.8										
G	18.2	18.2										
H	0	0										

***Note:** The indicated Human IL-17A 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® 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 IL-17A released directly in assay wells containing cells and culture medium. For quantitation purposes, a standard curve is generated using an IL-17A 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 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.

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.

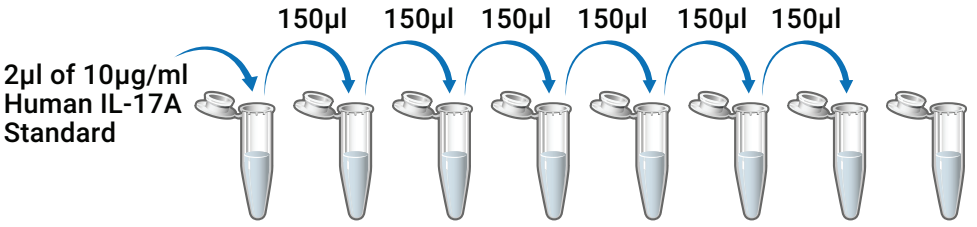
   384-well plate: 20µl per well.

   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 significant IL-17A release.

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

   Note: Assay sensitivity may be reduced in 384-well format compared to that in 96-well format.

4.B. Preparing Human IL-17A Standard Dilution



Medium	(µl)	798	350	350	350	350	350	350	350
hIL-17A	(pg/ml)	25,000	7,500	2,250	675	203	60.8	18.2	0

18054MC

Figure 3. Human IL-17A dilution series.

Shortly before completing cell treatments, prepare IL-17A 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 IL-17A Standard (approximately 15 minutes at room temperature) immediately before use.
2. Briefly centrifuge the tube before opening, then mix by pipetting.
3. Prepare an initial concentration of 25,000pg/ml human IL-17A by diluting Human IL-17A Standard (10µg/ml) 1:400 in prewarmed cell culture medium. For example, prepare 800µl of 25,000pg/ml human IL-17A by adding 2µl of the Human IL-17A Standard stock to 798µl of culture medium (see Figure 3).
4. Set up seven tubes (or seven chambers in a dilution reservoir) with 350µl of culture medium in each.
5. Prepare 3.33-fold serial dilutions of standard. Transfer 150µl from the 25,000pg/ml initial human IL-17A dilution (Step 3) to 350µl of culture medium for the second dilution. Mix by pipetting and repeat five times to generate seven standard dilutions with a range of 25,000pg/ml to 18.2pg/ml. The last well or chamber should contain only culture medium as the background control.

   Note: Change pipette tips between each dilution step and use aerosol filter tips to avoid analyte carryover.
6. After the cell treatment is complete, add the standard dilutions and background control in duplicate to two columns in the assay plate (see the plate map in Section 3).

   96-well plate: Dispense 80µl per well.

   384-well plate: Dispense 20µl per well.

   Notes:

   a. Unused Human IL-17A Standard (10µg/ml) can be stored at +2°C to +10°C for 1 month. If storing the standard for more than 1 month, dispense into aliquots and store at less than –65°C. Avoid multiple freeze-thaw cycles.

   b. We recommend incorporating Human IL-17A Standard controls on each assay plate for normalization.

   c. Additional Human IL-17A Standard (10µg/ml) is available for purchase (Cat.# W143A-C).

4.C. Adding 5X Anti-hIL-17A Antibody Mixture to Assay Wells

If using multiple cell models, each requiring a different culture medium, separate 5X Anti-hIL-17A antibody mixtures must be generated in each medium used in the study.

1. Remove the Anti-hIL-17A 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.# W1431, you may need to initiate buffer warming further in advance of its use in Section 4.D.

2. Briefly centrifuge the Anti-hIL-17A 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. Pipette 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-hIL-17A mAb-SmBiT	24µl
Anti-hIL-17A mAb-LgBiT	24µl

4. Add the 5X Anti-hIL-17A 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-hIL-17A antibody mixture to 80µl/well of cells or IL-17A standard dilutions.

   384-well plate: Dispense 5µl/well of 5X Anti-hIL-17A antibody mixture to 20µl/well of cells or IL-17A 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.

   Notes:

   a. This incubation step can be performed at room temperature and ambient atmosphere if the effect on cell responses and cell health is not a concern.

   b. 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 adding detection reagent.

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

While cells are incubating with the Anti-hIL-17A 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® IL-17A (Human) Immunoassay (Cat.# W1431) 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 for analyzing 5 or 10 plates at one time. If Cat.# W1431 is used to assay 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 +2°C to +10°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.

   Notes:

   a. Assay signal is stable with a half-life of approximately 2 hours, compatible with batch processing of multiple assay plates. We recommend incorporating the Human IL-17A 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 IL-17A 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 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.

2. Treat cells by adding a volume of test agent to each well. The final treatment volume is flexible, and can be adjusted to accommodate intended sample analyses, including split-sample analysis for multiple cytokines. Typical volumes are:

   96-well plate: 100–200µl per well

   384-well plate: 25–50µl per well

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

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. Be sure to account for sample dilution when calculating actual cytokine concentration released in cell wells.

   b. Assay sensitivity may be reduced in 384-well format compared to that in 96-well format.

   c. 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-hIL-17A antibody mixture (see Section 4.C for preparing the more concentrated Anti-hIL-17A antibody mixture).

5.B. Preparing Human IL-17A Standard Dilutions

Shortly before completing cell treatments, prepare IL-17A 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 IL-17A Standard (approximately 15 minutes at room temperature) immediately before use.
2. Briefly centrifuge the tube before opening, then mix by pipetting.
3. Prepare an initial concentration of 25,000pg/ml human IL-17A by diluting the Human IL-17A Standard (10µg/ml) 1:400 in prewarmed cell culture medium. For example, prepare 800µl of 25,000pg/ml human IL-17A by adding 2µl of the Human IL-17A Standard stock to 798µl of culture medium (Figure 3).
4. Set up seven tubes (or seven chambers in a dilution reservoir) with 350µl of culture medium in each.
5. Proceed with 3.33-fold serial dilutions of standard. Transfer 150µl from the 25,000pg/ml stock to 350µl of culture medium for the second dilution. Mix and repeat five times to generate seven standard dilutions with a range of 25,000pg/ml to 18.2pg/ml. The last well or chamber should contain only culture medium as the background control.

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

   96-well plate: Dispense 50µl per well.

   384-well plate: Dispense 12.5µl per well.

   Notes:

   a. Unused Human IL-17A Standard (10µg/ml) can be stored at +2°C to +10°C for 1 month. If storing the standard for more than 1 month, dispense into aliquots and store at less than –65°C. Avoid multiple freeze-thaw cycles.

   b. We recommend incorporating Human IL-17A Standard controls on each assay plate for normalization.

   c. Additional Human IL-17A Standard (10µg/ml) is available for purchase (Cat.# W143A-C).

5.C. Adding 2X Anti-hIL-17A Antibody Mixture to Sample Wells

If using multiple cell models, each requiring a different culture medium, separate 2X Anti-hIL-17A antibody mixtures must be generated in each medium used in the study.

1. Remove the Anti-hIL-17A 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.# W1431, you may need to initiate buffer warming further in advance of its use in Section 5.D.

2. Briefly centrifuge the Anti-hIL-17A 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. Pipette 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-hIL-17A mAb-SmBiT	24µl
Anti-hIL-17A mAb-LgBiT	24µl

4. Add the 2X Anti-hIL-17A antibody mixture to transferred culture medium (samples) or IL-17A 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-hIL-17A antibody mixture to 50µl/well of medium or standard dilutions.

   384-well plate: Dispense 12.5µl/well of 2X Anti-hIL-17A 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 adding detection reagent.

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

While the samples and standard dilutions are incubating with the Anti-hIL-17A antibody mixture (Section 5.C), prepare the Lumit® detection reagent B.

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

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

Notes:

a. The 1,000-assay size of Lumit® IL-17A (Human) Immunoassay (Cat.# W1431) 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 for analyzing 5 or 10 plates at once. If Cat.# W1431 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 +2°C to +10°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.

   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. 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 (3).

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 7, Figure 4) and subsequently used for interpolation of the concentration of cytokine release in various cell samples.

7. Representative Data

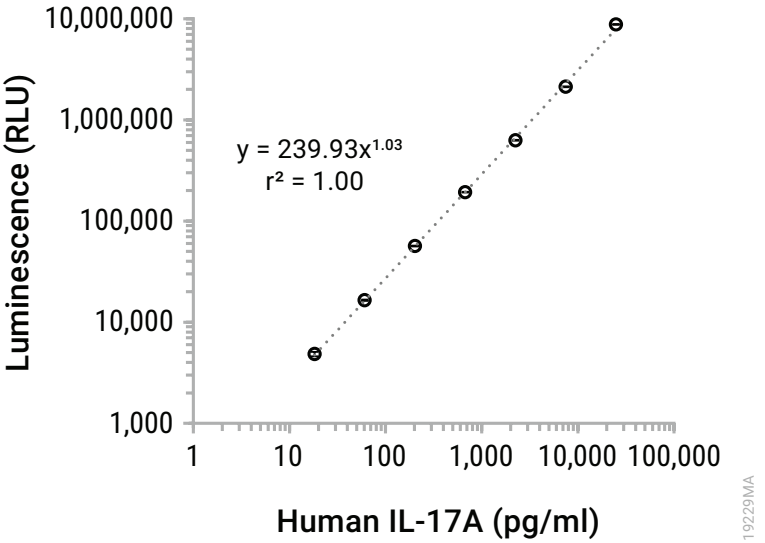


Figure 4. Standard curve for the Lumit® IL-17A (Human) Immunoassay. This is a representative standard curve and should not be used for calculation of unknowns. Generate a standard curve on each assay plate to interpolate the concentration of cytokine in experimental samples. **Note:** The plotted luminescence values were determined by subtracting background RLU.

Table 1. Intra-Assay Precision. Three samples of known concentrations of human IL-17A 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-17A quantities in each well by the various methods using GraphPad® Prism software.

	Cubic spline fit			Four parameter logistic (4PL) fit		
n	20	20	20	20	20	20
Expected (pg/ml)	20,000	2,000	200	20,000	2,000	200
Mean (pg/ml)	21,745	2,012	219	20,599	2,102	221
Standard Deviation	601	148	16	609	151	15
Percent CV	2.9	7.4	7.2	3.0	7.5	6.7
Average Percent of Expected	103.7	100.6	109.7	103.0	100.6	110.5
Percent Range	95–110	95–108	93–123	97–108	89–111	98–122

Note: The minimal detectable dose (MDD), determined at two standard deviations above background, was 1.4pg/ml.

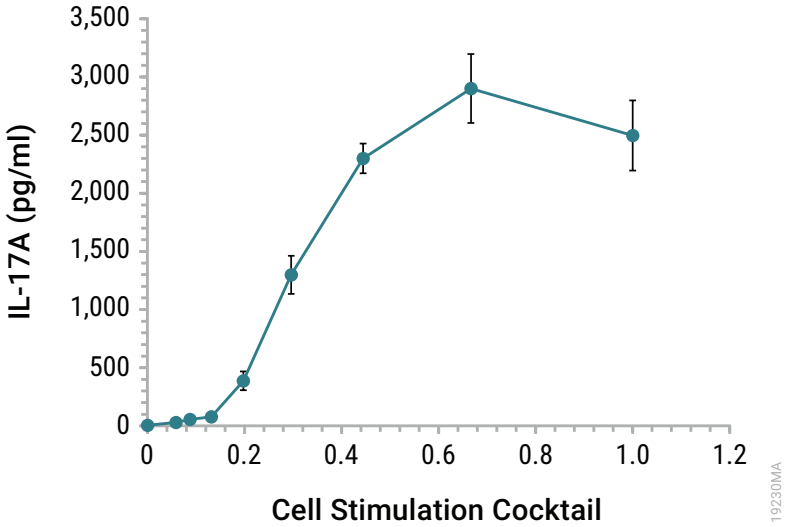


Figure 5. Lumit® detection of IL-17A released from human peripheral blood mononuclear cells (PBMC). PBMC pooled from four donors (BioIVT) were plated in 96-well plate format at 100,000 cells/well in RPMI 1640 + 10% heat-inactivated FBS. Cells were treated in quadruplicate for 48 hours with increasing concentrations of Cell Stimulation Cocktail (CSC; Invitrogen Cat.# 00-4970-93). 1X CSC is a mixture of 81nM phorbol-12-myristate 13-acetate (PMA) and 1.34µM ionomycin. Following treatment, the 5X Anti-hIL-17A antibody mixture was dispensed into the cell wells and incubated for 1 hour before Lumit® detection reagent B was added. Luminescence readings for unknowns were interpolated against a standard curve using four-parameter logistic (4PL) fitting (GraphPad® Prism) to determine levels of IL-17A released.

8. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. Email: **techserv@promega.com**

Symptoms	Causes and Comments
No signal from treated cells	Use only human cells with the Lumit® IL-17A (Human) Immunoassay. The Lumit® IL-17A (Human) Immunoassay will not effectively detect IL-17A from other species. In addition to the Human IL-17A Standard, consider using treated human PBMC as a positive control.
Human IL-17A 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 assay sensitivity and the broad linear range (>3 logs) means that any carryover will disrupt the linear range. Also, make sure that no IL-17A contaminates the background control.
The relative light units (RLU) for the standard are low and/or variable	Warm culture medium, Human IL-17A 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-17A can be accurately quantitated.
High assay background and reduced signal-to-background ratio when using medium with low or no FBS (or albumin)	Using medium with less than 5% FBS can produce higher background (nonanalyte-mediated antibody pairing) and reduce assay sensitivity. When using serum-free, albumin-free medium, you can reduce assay background and improve sensitivity by preparing the assay antibody mixture in medium supplemented with BSA to deliver 0.05% BSA final after addition to sample. (**Note:** Using significantly higher albumin concentrations than indicated can further compromise assay performance.)
Greatly reduced assay sensitivity when applied to human serum samples	Several factors within human serum samples can interfere with the homogeneous Lumit® IL-17A (Human) Immunoassay performance and, thereby, significantly reduce assay sensitivity. For this reason, we do not recommend use of the Lumit® IL-17A (Human) Immunoassay on human serum samples.

Symptoms	Causes and Comments
Experimental RLUs are not identical to the numbers in this Technical Manual	Bioluminescent signal intensity (i.e., absolute RLU) will vary between laboratories due to several factors, such as specific experimental conditions (buffers, volumes), as well as plates and plate readers. Establish the assay performance in your lab with the sample buffer, plates and instruments you will be using. The Human IL-17A Standard provided in the kit can be used to generate a standard curve that will help establish the parameters expected in your lab.

9. Appendix

9.A. References

1. Dixon, A.S. *et al*. (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol*. **11**, 400–8.
2. Hwang, B.B. *et al*. (2020) A homogeneous bioluminescent immunoassay to probe cellular signaling pathway regulation. *Commun. Biol.* **3**, 8.
3. 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.

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9.B. Related Products

Lumit® Cytokine Immunoassays and Supporting Reagents

Product	Size	Cat.#
Lumit® Human IL-17A Standard	25µl	W143A-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-10 (Human) Immunoassay	100 assays	W6070
Lumit® TNF-α (Human) Immunoassay	100 assays	W6050

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.

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.

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9.B. Related Products (continued)

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	JE9400
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.

10. Summary of Changes

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

1. Removed an expired patent statement.
2. Made miscellaneous text edits.

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

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