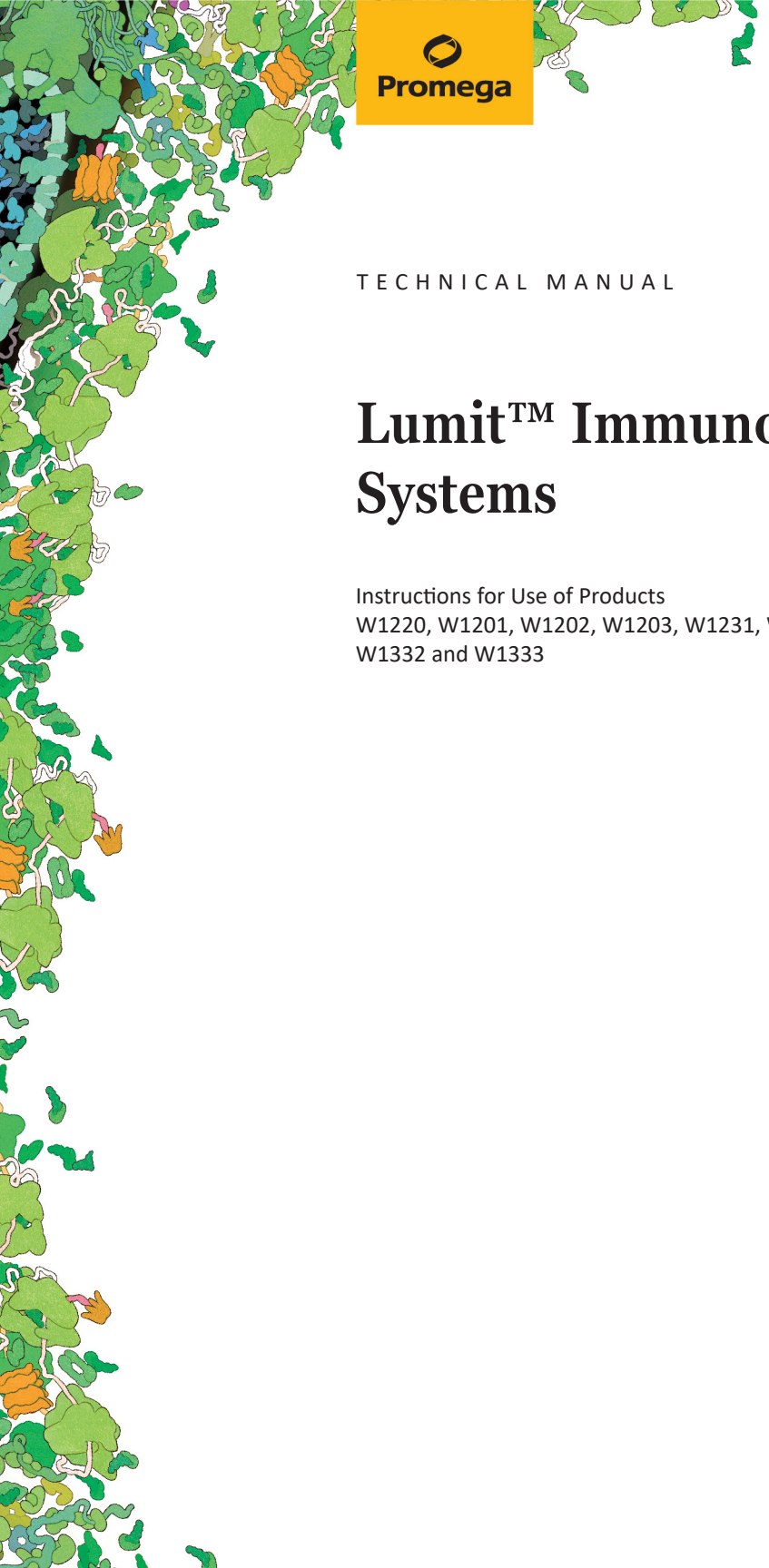


TECHNICAL MANUAL

Lumit™ Immunoassay Cellular Systems

Instructions for Use of Products
W1220, W1201, W1202, W1203, W1231, W1232, W1233, W1331, W1332 and W1333

Revised 4/21
TM613

Lumit™ Immunoassay Cellular Systems

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

Promega

1. Description

The Lumit™ Immunoassay Cellular System[b,c] is a homogeneous bioluminescent assay that measures levels of target proteins in cell lysates when used with the appropriate primary antibody pairs. It combines immunodetection and NanoLuc® Binary Technology (NanoBiT®). NanoBiT® is a structural complementation reporter ideal for protein:protein interaction (PPI) studies. The NanoBiT® System is composed of two subunits: Large BiT (LgBiT; 18kDa) and Small BiT (SmBiT; 11 amino acid peptide), that can be expressed as recombinant fusions or chemically conjugated to target proteins of interest. The LgBiT and SmBiT subunits have been optimized for stability and minimal self-association due to weak affinity (K_d = 190μM). When two proteins (one tagged with LgBiT, the other tagged with SmBiT) interact, the subunits are brought into proximity to form a functional enzyme that generates luminescence in the presence of its substrate (1).

In the Lumit™ Immunoassay Cellular System, NanoBiT® subunits are conjugated to a pair of secondary antibodies against two different species (anti-rabbit, anti-mouse; anti-goat is available separately). Seeded cells are lysed in multiwell plates using a NanoBiT® compatible lysis solution (Digitonin), and the target protein is detected by adding an antibody mix containing two primary antibodies against the target protein along with SmBiT- and LgBiT-conjugated secondary antibodies (e.g., Lumit™ Anti-Mouse Ab-LgBiT and Lumit™ Anti-Rabbit Ab-SmBiT). Binding of the primary/Lumit™ secondary antibody complexes to their corresponding epitopes brings NanoBiT® subunits into proximity to form an active NanoLuc® luciferase that generates light in proportion to the amount of target protein (Figure 1). When the primary antibody pair includes a phospho-specific antibody, the luminescence reflects the level of target protein phosphorylation (Figure 1, Panel A). On the other hand, to detect total protein level, the same concept is used except both primary antibodies recognize non-phosphorylated epitopes on the target protein (Figure 1, Panel A). The luminescent signal generated is determined using a luminometer as shown in Figure 2.

The Lumit™ Immunoassay Cellular System is solution-based, unlike standard immunodetection techniques such as ELISA or Western blot, the protocol does not include washing, liquid transfer or immobilization steps. Therefore, cells are lysed in the same well where antibody binding and luminescence generation occur (Figure 1B). The assay takes less than 2 hours to complete in a homogeneous “add-and-read” format. A key advantage of this approach is that it does not require cell engineering. Therefore, the phosphorylation or total amount of an intracellular protein may be detected at native levels in any cell type where it is expressed (2).

In addition, Lumit™ secondary antibodies can be adapted to detect any protein of interest, provided the appropriate primary antibodies (from mouse, rabbit or goat) are available. This assay can be performed in a single plate, with no need for medium removal or lysate transfer to new plate. The homogeneous format requires no washing steps, making the assay quicker than Western, ELISA or fluorescence-based methods. The assay works with a small number of cells and there is no IP step for phosphorylated proteins. Detection requires only a luminometer, and can be performed in 96- or 384-well plates. A Z´ factor >0.7 has been reported (3).

Additional protocols for many common cellular targets are available. See the application notes at:
www.promega.com/products/immunoassay-elisa/lumit-immunoassays/lumit-immunoassay-cellular-systems/

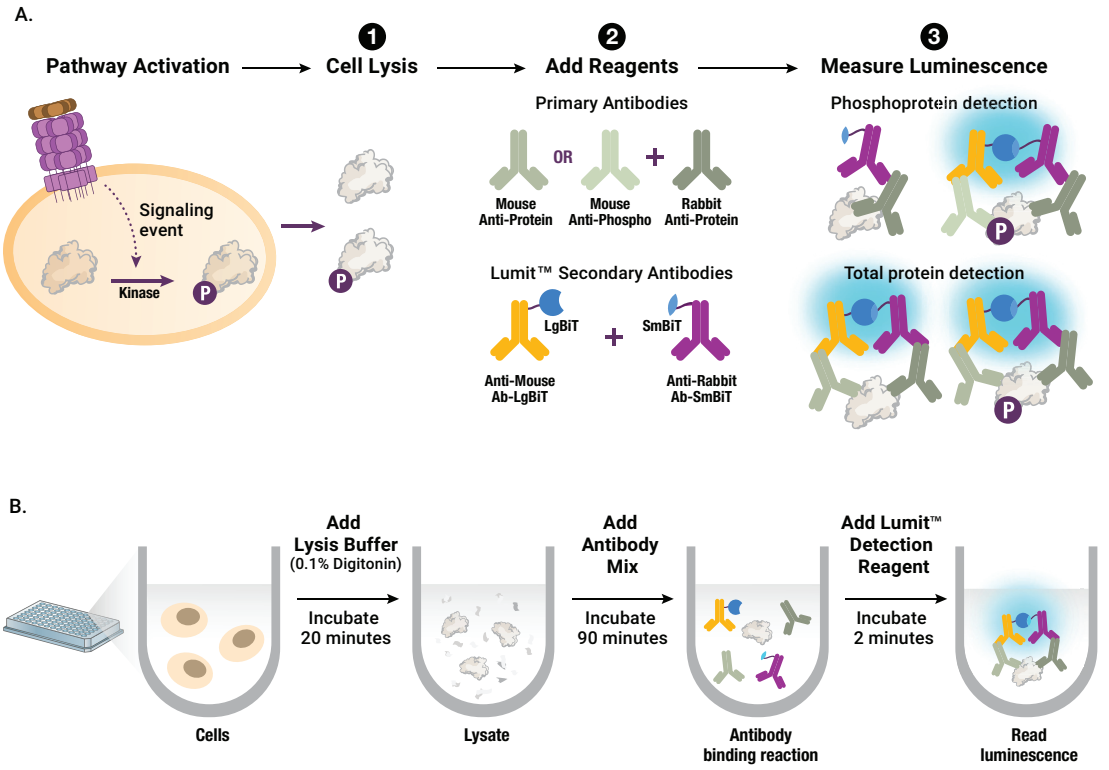


Figure 1. Principle of the Lumit™ Immunoassay Cellular System. Panel A. Phosphorylated or total target proteins in lysed cells after stimulation are recognized by each primary antibody pair. The Lumit™ secondary antibodies then recognize their cognate primary antibodies, bringing the NanoBiT® subunits into close proximity to form a functional enzyme that generates bright luminescence. **Panel B.** The Lumit™ Immunoassay is an endpoint homogeneous assay; cell lysis, antibody binding to the target and luminescence generation all happen in solution without transfer or wash steps.

1. Description (continued)

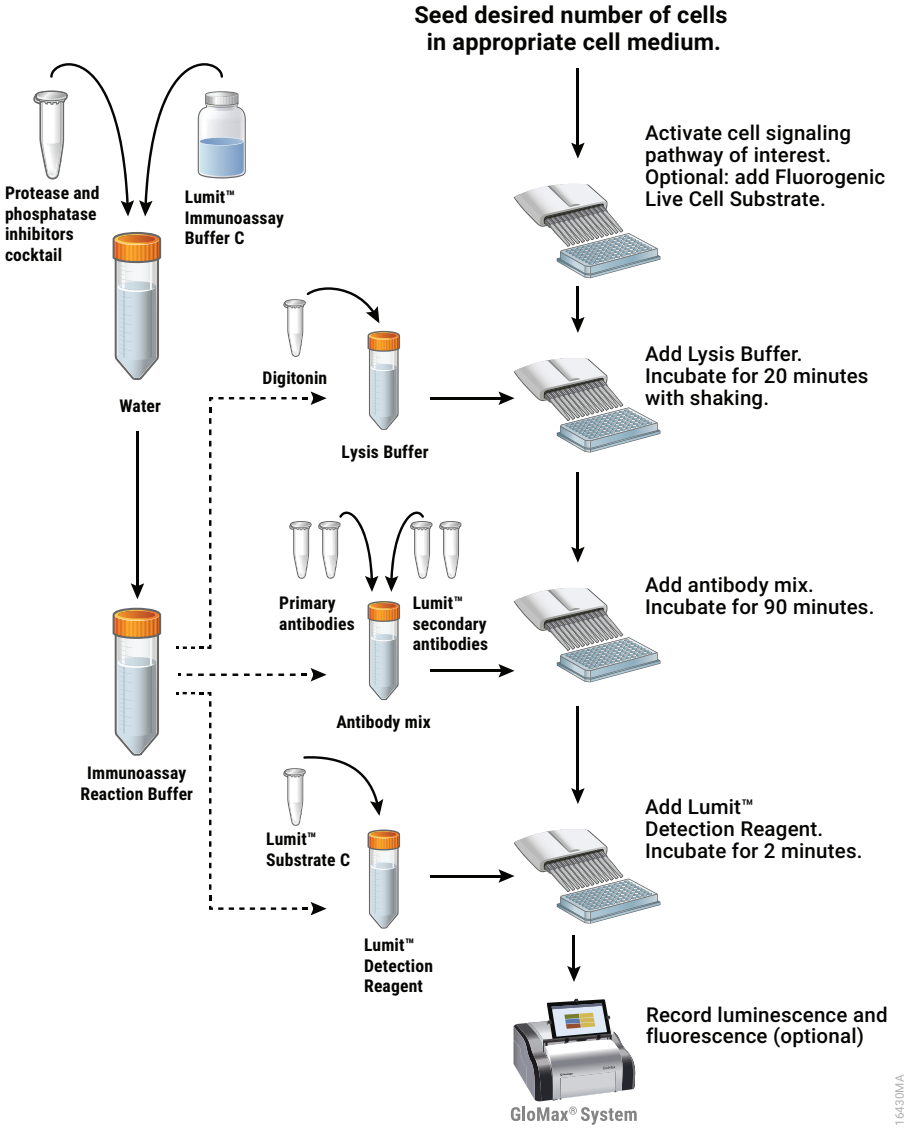


Figure 2. Lumit™ Immunoassay Cellular System protocol.

Signaling pathway activation leads to a multitude of cellular responses, including enzyme activity modulation, gene expression and protein translocation or degradation. Reversible protein phosphorylation is a common mechanism that transduces the signal from an upstream activation event to downstream cellular responses. The Lumit™ Immunoassay Cellular System can be applied to any signaling pathway marker protein or phosphoprotein in which primary antibodies from appropriate species are available (2). An example of detection using two markers of NF-κB pathway regulation is shown in Figure 3.

Notes:

1. The following Lumit™ secondary antibodies are currently offered: anti-rabbit, anti-mouse and anti-goat. Therefore, the Lumit™ Immunoassay Cellular System can be used with target-selective primary antibody pairs from mouse/rabbit, mouse/goat, or rabbit/goat.
2. Pairs of primary antibodies from the same species (e.g., mouse/mouse) are incompatible with the Lumit™ Immunoassay Cellular System.
3. The performance of the Lumit™ Immunoassay Cellular System may depend on the affinity of the primary antibodies against the target and the Lumit™ secondary antibodies set used. For example, when testing a rabbit/mouse antibody pair, we recommend using the Lumit™ Immunoassay Cellular System - Starter Kit (Cat.# W1220) as it contains both combinations of SmBiT- and LgBiT- conjugated secondary antibodies. This Kit will allow you to identify the Lumit™ secondary antibody combination that works best for your target protein.

1. Description (continued)

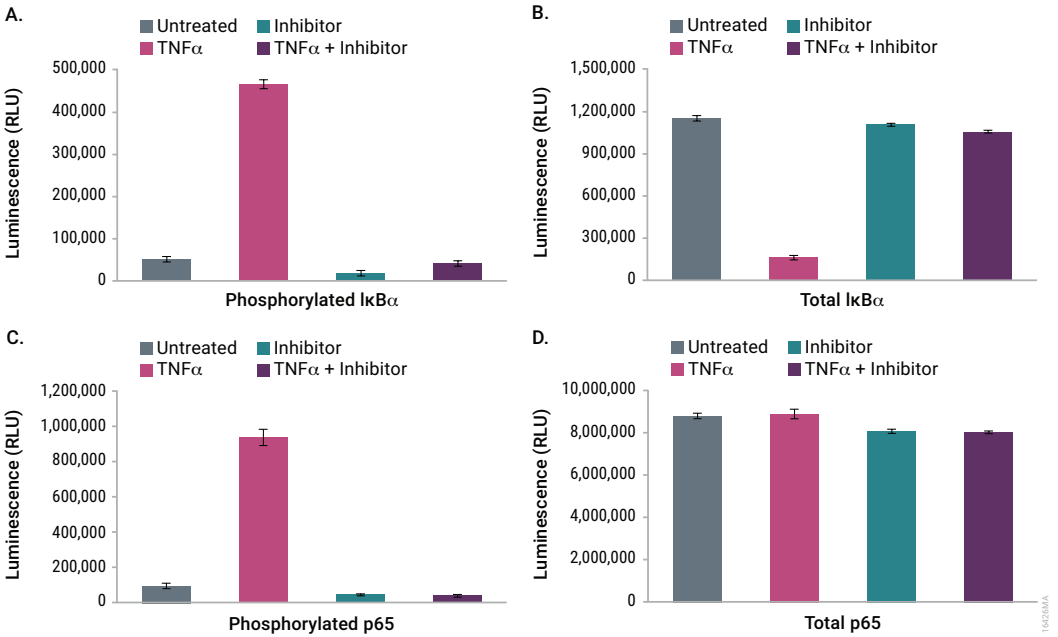


Figure 3. Detection of total and phosphorylated target proteins in NF-κB signaling pathway using the Lumit™ Immunoassay Cellular System. Seeded MCF-7 cells (50,000) were pretreated with an IKK complex specific inhibitor, IKK16 (10µM, 1 hour), and then treated with TNFα (20ng/ml) for 30 minutes (IκBα) or 10 minutes (p65). **Panel A.** Cells were pretreated with MG132 (20µM, 1 hour) and then phosphorylated IκBα (S32)was detected using the Lumit™ Immunoassay and 150ng/ml of the following antibodies: Mouse anti-IκBα (Cell Signaling Technology Cat.# 4814) and rabbit anti-phospho-IκBα (Cell Signaling Technology Cat.# 2859). **Panel B.** Total IκBα levels were measured using 150ng/ml of the following antibodies: Mouse anti-IκBα (Cell Signaling Technology Cat.# 4814) and rabbit anti-IκBα (Cell Signaling Technology Cat.# 4812). **Panel C.** Phosphorylated p65 (S536) levels were measured using 150ng/ml of the following antibodies: Mouse anti-phospho-p65 (Cell Signaling Technology Cat.# 13346), rabbit anti-p65 (Cell Signaling Technology Cat.# 8242). **Panel D.** Total p65 levels were measured using the Lumit™ Immunoassay and 150ng/ml of the following antibodies: Rabbit anti-p65 (Cell Signaling Technology Cat.# 8242) and mouse anti-p65 (Cell Signaling Technology Cat.# 6956).

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Lumit™ Immunoassay Cellular System - Starter Kit	**200 assays**	**W1220**

This system is sufficient for 200 assays (including 100 assays with the Anti-Mouse Ab-LgBiT/Anti-Rabbit Ab-SmBiT combination and 100 assays with the Anti-Mouse Ab-SmBiT/Anti-Rabbit Ab-LgBiT combination) if performed in 96-well plates using 50µl, 50µl and 25µl of cell lysate, antibody mix and Detection Reagent, respectively, per assay. This system can also be used in 384-well plates using 12.5µl:12.5µl:6.25µl for a total of 800 assays. Includes:

- 2 × 50µl Digitonin, 2%
- 2 × 1.5ml Lumit™ Immunoassay Buffer C, 10X
- 2 × 200µl Lumit™ Substrate C
- 30µl Lumit™ Anti-Mouse Ab-LgBiT
- 30µl Lumit™ Anti-Rabbit Ab-SmBiT
- 30µl Lumit™ Anti-Mouse Ab-SmBiT
- 30µl Lumit™ Anti-Rabbit Ab-LgBiT
- 1ml Antibody Dilution Buffer
- 10µl Fluorogenic Live Cell Substrate (GF-AFC Substrate), 100mM

PRODUCT	SIZE	CAT.#
Lumit™ Immunoassay Cellular System - Set 1	**100 assays**	**W1201**

This system is sufficient for 100 assays if performed in 96-well plates using 50µl, 50µl and 25µl of cell lysate, antibody mix and Detection Reagent, respectively, per assay. This system can also be used in 384-well plates using 12.5µl:12.5µl:6.25µl for a total of 400 assays. Includes:

- 50µl Digitonin, 2%
- 1.5ml Lumit™ Immunoassay Buffer C, 10X
- 200µl Lumit™ Substrate C
- 30µl Lumit™ Anti-Mouse Ab-LgBiT
- 30µl Lumit™ Anti-Rabbit Ab-SmBiT
- 1ml Antibody Dilution Buffer
- 10µl Fluorogenic Live Cell Substrate (GF-AFC Substrate), 100mM

2. Product Components and Storage Conditions (continued)

PRODUCT	SIZE	CAT.#
Lumit™ Immunoassay Cellular System - Set 1	**1,000 assays**	**W1202**

This system is sufficient for 1,000 assays if performed in 96-well plates using 50µl, 50µl and 25µl of cell lysate, antibody mix and Detection Reagent, respectively, per assay. This system can also be used in 384-well plates using 12.5µl:12.5µl:6.25µl for a total of 4,000 assays. Includes:

- 500µl Digitonin, 2%
- 15ml Lumit™ Immunoassay Buffer C, 10X
- 2 × 1ml Lumit™ Substrate C
- 300µl Lumit™ Anti-Mouse Ab-LgBiT
- 300µl Lumit™ Anti-Rabbit Ab-SmBiT
- 1ml Antibody Dilution Buffer
- 2 × 10µl Fluorogenic Live Cell Substrate (GF-AFC Substrate), 100mM

PRODUCT	SIZE	CAT.#
Lumit™ Immunoassay Cellular System - Set 1	**10,000 assays**	**W1203**

This system is sufficient for 10,000 assays if performed in 96-well plates using 50µl, 50µl and 25µl of cell lysate, antibody mix and Detection Reagent, respectively, per assay. This system can also be used in 384-well plates using 12.5µl:12.5µl:6.25µl for a total of 40,000 assays. **Note:** Cat.# W1203 is 10 × Cat.# W1202. Includes:

- 10 × 500µl Digitonin, 2%
- 10 × 15ml Lumit™ Immunoassay Buffer C, 10X
- 20 × 1ml Lumit™ Substrate C
- 10 × 300µl Lumit™ Anti-Mouse Ab-LgBiT
- 10 × 300µl Lumit™ Anti-Rabbit Ab-SmBiT
- 10 × 1ml Antibody Dilution Buffer
- 20 × 10µl Fluorogenic Live Cell Substrate (GF-AFC Substrate), 100mM

PRODUCT	SIZE	CAT.#
Lumit™ Immunoassay Cellular System - Set 2	**100 assays**	**W1331**

This system is sufficient for 100 assays if performed in 96-well plates using 50µl, 50µl and 25µl of cell lysate, antibody mix and Detection Reagent, respectively, per assay. This system can also be used in 384-well plates using 12.5µl:12.5µl:6.25µl for a total of 400 assays. **Note:** Cat.# W1331 is a combination of Cat.# W1231, W1041 and W1051. Includes:

- 50µl Digitonin, 2%
- 1.5ml Lumit™ Immunoassay Buffer C, 10X
- 200µl Lumit™ Substrate C
- 30µl Lumit™ Anti-Mouse Ab-SmBiT
- 30µl Lumit™ Anti-Rabbit Ab-LgBiT
- 1ml Antibody Dilution Buffer
- 10µl Fluorogenic Live Cell Substrate (GF-AFC Substrate), 100mM

PRODUCT	SIZE	CAT.#
Lumit™ Immunoassay Cellular System - Set 2	**1,000 assays**	**W1332**

This system is sufficient for 1,000 assays if performed in 96-well plates using 50µl, 50µl and 25µl of cell lysate, antibody mix and Detection Reagent, respectively, per assay. This system can also be used in 384-well plates using 12.5µl:12.5µl:6.25µl for a total of 4,000 assays. **Note:** Cat.# W1332 is a combination of Cat.# W1232, W1042 and W1052. Includes:

- 500µl Digitonin, 2%
- 15ml Lumit™ Immunoassay Buffer C, 10X
- 2 × 1ml Lumit™ Substrate C
- 300µl Lumit™ Anti-Mouse Ab-SmBiT
- 300µl Lumit™ Anti-Rabbit Ab-LgBiT
- 1ml Antibody Dilution Buffer
- 2 × 10µl Fluorogenic Live Cell Substrate (GF-AFC Substrate), 100mM

PRODUCT	SIZE	CAT.#
Lumit™ Immunoassay Cellular System - Set 2	**10,000 assays**	**W1333**

This system is sufficient for 10,000 assays if performed in 96-well plates using 50µl, 50µl and 25µl of cell lysate, antibody mix and Detection Reagent, respectively, per assay. This system can also be used in 384-well plates using 12.5µl:12.5µl:6.25µl for a total of 40,000 assays. **Note:** Cat.# W1333 is a combination of 10 × Cat.# W1232, 10 × Cat.# W1042 and 10 × Cat.# W1052. Includes:

- 10 × 500µl Digitonin, 2%
- 10 × 15ml Lumit™ Immunoassay Buffer C, 10X
- 20 × 1ml Lumit™ Substrate C
- 10 × 300µl Lumit™ Anti-Mouse Ab-SmBiT
- 10 × 300µl Lumit™ Anti-Rabbit Ab-LgBiT
- 10 × 1ml Antibody Dilution Buffer
- 20 × 10µl Fluorogenic Live Cell Substrate (GF-AFC Substrate), 100mM

PRODUCT	SIZE	CAT.#
Lumit™ Immunoassay Lysis and Detection Kit	**100 assays**	**W1231**

This system is sufficient for 100 assays if performed in 96-well plates using 50µl, 50µl and 25µl of cell lysate, antibody mix and Detection Reagent, respectively, per assay. This system can also be used in 384-well plates using 12.5µl:12.5µl:6.25µl for a total of 400 assays. Includes:

- 50µl Digitonin, 2%
- 1.5ml Lumit™ Immunoassay Buffer C, 10X
- 200µl Lumit™ Substrate C
- 1ml Antibody Dilution Buffer
- 10µl Fluorogenic Live Cell Substrate (GF-AFC Substrate), 100mM

2. Product Components and Storage Conditions (continued)

PRODUCT	SIZE	CAT.#
Lumit™ Immunoassay Lysis and Detection Kit	**1,000 assays**	**W1232**

This system is sufficient for 1,000 assays if performed in 96-well plates using 50µl, 50µl and 25µl of cell lysate, antibody mix, and Detection Reagent, respectively, per assay. This system can also be used in 384-well plates using 12.5µl:12.5µl:6.25µl for a total of 4,000 assays. Includes:

- 500µl Digitonin, 2%
- 15ml Lumit™ Immunoassay Buffer C, 10X
- 2 × 1ml Lumit™ Substrate C
- 1ml Antibody Dilution Buffer
- 2 × 10µl Fluorogenic Live Cell Substrate (GF-AFC Substrate), 100mM

PRODUCT	SIZE	CAT.#
Lumit™ Immunoassay Lysis and Detection Kit	**10,000 assays**	**W1233**

This system is sufficient for 10,000 assays if performed in 96-well plates using 50µl, 50µl and 25µl of cell lysate, antibody mix and Detection Reagent, respectively, per assay. This system can also be used in 384-well plates using 12.5µl:12.5µl:6.25µl for a total of 40,000 assays. **Note:** Cat.# W1233 is 10 × Cat.# W1232. Includes:

- 10 × 500µl Digitonin, 2%
- 10 × 15ml Lumit™ Immunoassay Buffer C, 10X
- 20 × 1ml Lumit™ Substrate C
- 10 × 1ml Antibody Dilution Buffer
- 20 × 10µl Fluorogenic Live Cell Substrate (GF-AFC Substrate), 100mM

Storage Conditions: Store all components at –30°C to –10°C. Before use, thaw all components completely at room temperature except the antibodies, which should be kept at –30°C to –10°C until use. Mix all components thoroughly before use. Store all components at –30°C to –10°C immediately after use. Lumit™ Substrate C and GF-AFC Substrate must be stored in the dark.

Notes:

1. Antibody Dilution Buffer is used to dilute primary antibodies to a working solution after an optimal assay concentration is identified (see Section 6).
2. GF-AFC Substrate is a cell viability substrate for cell number normalization. This is a nonlytic, single-reagent-addition fluorescence assay component that measures the relative number of viable cells in a population. It is based on measurement of a conserved and constitutive protease activity within live cells and therefore serves as a biomarker of cell viability. The substrate enters intact cells, where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. The live-cell protease becomes inactive upon cell lysis. After lysis, the accumulated fluorescence is measured at the end of the Lumit™ Immunoassay along with luminescence (Figure 4). Fluorescence can also be read before the lysis step to check for cell viability.
3. See Table 1, Section 4.A, for volume ratios for various plate formats.

Available Separately

Lumit™ Secondary Antibodies[(a)]

PRODUCT	SIZE	CAT.#
Lumit™ Anti-Mouse Ab-LgBiT	30µl	W1021
Lumit™ Anti-Mouse Ab-LgBiT	300µl	W1022
Lumit™ Anti-Mouse Ab-SmBiT	30µl	W1051
Lumit™ Anti-Mouse Ab-SmBiT	300µl	W1052
Lumit™ Anti-Rabbit Ab-LgBiT	30µl	W1041
Lumit™ Anti-Rabbit Ab-LgBiT	300µl	W1042
Lumit™ Anti-Rabbit Ab-SmBiT	30µl	W1031
Lumit™ Anti-Rabbit Ab-SmBiT	300µl	W1032
Lumit™ Anti-Goat Ab-LgBiT	30µl	W1061
Lumit™ Anti-Goat Ab-LgBiT	300µl	W1062
Lumit™ Anti-Goat Ab-SmBiT	30µl	W1071
Lumit™ Anti-Goat Ab-SmBiT	300µl	W1072

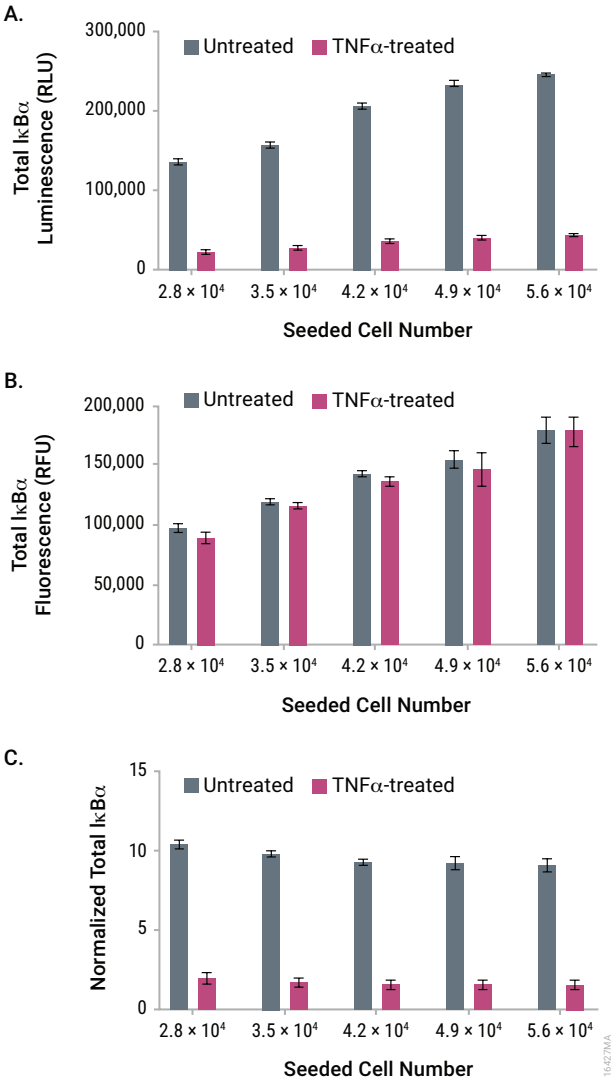


Figure 4. Normalization of luminescence data to cell number per well. Different cell numbers were treated with 50ng/ml TNFα for 30 minutes. GF-AFC Substrate was added to all cells 30 minutes before lysis. The Lumit™ Immunoassay was used to detect total IkBα level using 150ng/ml of the following antibodies: Mouse anti-IκBα (Cell Signaling Technology Cat.# 4814) and rabbit anti-IκBα (Cell Signaling Technology Cat.# 4812). Luminescence (**Panel A**) and fluorescence (**Panel B**) were read at the end of the experiment. **Panel C.** Normalized luminescence RLUs to fluorescence RFU values. Luminescence and fluorescence were recorded using the GloMax® Discover (Cat.# GM3000).

3. General Considerations

Lysis buffer: Commonly used detergents to lyse cells have a negative effect on NanoBiT® complementation. Digitonin is the most compatible detergent for the Lumit™ Immunoassay Cellular System. The optimized Digitonin treatment described in this manual effectively exposes intracellular targets to the Lumit™ Detection Reagent without affecting NanoBiT® complementation. We compared the protein level and phosphorylation using different cell disruption protocols, such as detergent-free sonication and other lysis buffers. We obtained similar results using detergent-free sonication and digitonin-based lysis. The latter was chosen because of its compatibility with high-throughput formats.

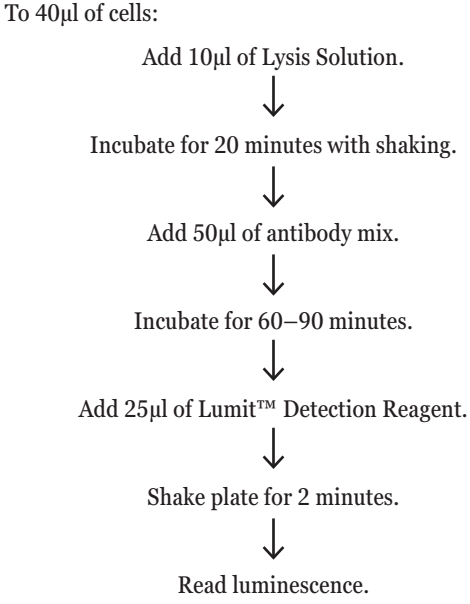
Antibodies: The Lumit™ Immunoassay Cellular System is a generic kit that requires the user to provide compatible primary antibodies. For broad target coverage, we offer multiple versions of the Lumit™ secondary antibodies in a kit with lysis and detection components or as stand-alone products. To find an optimal primary antibody pair for your target, use either 1) the Lumit™ Immunoassay Cellular System - Starter Kit (Cat.#W1220), when only testing rabbit/mouse antibody pairs. or 2) the Lumit™ Immunoassay Lysis and Detection Kit (Cat.# W1231) and any combination of the stand-alone Lumit™ Anti-Mouse, Anti-Rabbit or Anti-Goat labeled with LgBiT or SmBiT.

Plates and Instruments: We recommend using standard solid white multiwell plates suitable for luminescence measurements (e.g., Corning Cat.# 3570, 3693 and 3917). Luminescence can be recorded on a variety of plate readers although the relative light units will depend on the instrument. Assay well geometry and small dispensing volumes may affect mixing efficiency. Thus, poor assay homogeneity in individual wells may result in increased reaction noise, reduced signals or both.

4. Preparing for the Lumit™ Immunoassay Cellular System

General Protocol of the Lumit™ Immunoassay Cellular System (2:2:1 format)

Below is a quick reference protocol. For more details regarding cells and reagent preparation and detailed protocols, see Sections 4.A, 4.B and 5.



Materials to Be Supplied by the User

- a pair of primary antibodies against the target protein. Pairs can be made of antibodies from rabbit/mouse, mouse/goat or rabbit/goat
- solid white, multiwell plate (**Note:** Do not use black or clear plates). White plates with clear bottoms can also be used. However, when reading the luminescence signal, use white 3M paper to cover the bottom of the plate.
- multichannel pipette or automated pipetting station
- luminometer capable of reading multiwell plates (e.g., GloMax® Discover, Cat.# GM3000)
- plate shaker
- protease inhibitor cocktail or protease/phosphatase inhibitor cocktail (e.g., Halt™ Protease and Phosphatase Inhibitor Cocktail, 100X, Thermo Scientific Cat.# 78440)

4.A. Preparing Cells for the Lumit™ Immunoassay Cellular System

This protocol is designed for a 96-well plate using a 50µl:50µl:25µl ratio of cell lysate to antibody mix to Detection Reagent volumes. To perform the assay in a 384-well plate, reduce volumes fourfold. Other volumes may be used as described in Table 1, provided the 2:2:1 ratio of the assay component volumes is maintained.

Table 1. Assay Component Volumes for Various Plate Formats.

Plate Type	Cell Lysate[1] (µl)	Antibody Mix (µl)	Substrate (µl)
96-well plate	50	50	25
96-well plate (half volume)	25	25	12.5
384-well plate	12.5	12.5	6.25

[1]Cell lysate volume includes cell medium, treatment and lysis solution.

Adherent Cells

1. Maintain adherent cells in an appropriate growth medium. Split the cells at 70–90% confluency.
2. Count cells and seed them into plates in complete growth medium as follows:

   For 96-well plates, seed 50,000 cells per well.

   For 384-well plates, seed 10,000 cells per well.

   Note: To reach an optimal signal, cell number should be determined experimentally because the signal is dependent on the cell type and signaling pathway. Generally, an optimal signal change can be obtained in a range of 10,000–80,000 cells per well of a 96-well plate.
3. Incubate the plate at the appropriate cell culture conditions overnight.

   Note: If necessary, the cells can be cultured in serum-free media after the cells are stably adherent on the plate. Determine the length of serum starvation based on the signaling pathway of interest.
4. Replace the medium with 35µl of fresh cell medium.
5. To stimulate and/or inhibit a pathway of interest, treat the cells with 5µl of cell medium containing an appropriate dose of an activator and/or inhibitor. For control wells, add 5µl of cell medium containing vehicle (e.g., DMSO).
6. Proceed immediately to the Lumit™ Immunoassay Cellular System protocol (Section 5).

   Notes:

   1. Volumes used in Steps 4 and 5 can be adjusted to accommodate different treatments, as long as the final volume before adding lysis solution is 40µl for 96-well and 10µl for 384-well plates.
   2. To measure cell viability and normalize data to viable cell number, add Fluorogenic Live Cell Substrate (GF-AFC Substrate) to the cell medium during treatment (Step 4 or 5) to a final concentration of 50µM in the 40µl volume. The GF-AFC Substrate can be incubated with cells 0.5–3 hours before lysis, and the signal is stable for up to 3 hours.

4.A. Preparing Cells for the Lumit™ Immunoassay Cellular System (continued)

Suspension Cells

1. Maintain suspension cells in an appropriate cell growth medium.
2. Count cells and seed them in fresh medium as follows:

   For 96-well plates, seed 100,000 cells in 35µl of fresh medium per well.

   For 384-well plates, seed 20,000 cells per well.

   Note: To reach an optimal signal, cell number should be determined experimentally because the signal is dependent on cell line and cell signaling pathway. Generally, an optimal signal change can be obtained in a range of 100,000–200,000 cells per well of a 96-well plate.
3. To stimulate and/or inhibit the pathway, treat the cells with 5µl of cell medium containing an appropriate dose of an activator and/or inhibitor. For control wells, add 5µl of cell medium containing vehicle (e.g., DMSO).
4. Proceed immediately to the Lumit™ Immunoassay Cellular System protocol (Section 5).

   Notes:

   1. Volumes used in Steps 2 and 3 can be adjusted to accommodate different treatments, as long as the final volume before adding lysis solution is 40µl for 96-well and 10µl for 384-well plates.
   2. To measure cell viability and normalize data to viable cell number, add Fluorogenic Live Cell Substrate (GF-AFC Substrate) to the cell medium during treatment (Step 2 or 3) to a final concentration of 50µM in the 40µl volume. The GF-AFC Substrate can be incubated with cells 0.5–3 hours before lysis, and the signal is stable for up to 3 hours.

4.B. Preparing Reagents for the Lumit™ Immunoassay Cellular System

Calculate the required volumes of Immunoassay Reaction Buffer and Lysis Solution. The assay follows the format of 2:2:1 volume ratios of cell lysate to antibody mix to Detection Reagent.

Note: The following instructions will prepare enough reagents for 10 assay points.

1. **Immunoassay Reaction Buffer:** To make 1.5ml, mix 150µl of Lumit™ Immunoassay Buffer C and appropriate volume of protease and phosphatase inhibitors cocktail with up to 1.35ml of water.
2. **Lysis Solution:** Prepare 100µl Lysis Solution (0.1%) by adding 5µl of Digitonin (2%) to 95µl of Immunoassay Reaction Buffer.
3. **Antibody Mix:** (To be prepared during lysis step in Section 5): For each batch of 10 assays, prepare 500µl of antibody mix by mixing 3µl of each four antibodies as shown in Table 2 with 488µl of Immunoassay Reaction Buffer.

Table 2. Example of Antibody Mix Preparation (in µl per 10 assays).

Component		Volume (µl)
Primary Antibodies	Anti-phospho-epitope antibody	3
	Anti-protein antibody	3
Lumit™ Secondary Antibodies	Lumit™ Anti-Mouse Antibody-LgBiT	3
	Lumit™ Anti-Rabbit Antibody-SmBiT	3
Immunoassay Reaction Buffer		488

Note: In most cases, 15ng of each primary antibody per well (150ng/ml final concentration after antibody addition) is optimal. However, we recommend empirically determining optimal primary antibody concentrations (see Section 6). Based on the optimal concentration, an antibody working stock solution can be prepared in Antibody Dilution Buffer and stored at –30°C to –10°C. For example, for a 15ng per assay well optimum, a 50µg/ml antibody stock solution can be prepared and stored for subsequent assays. According to Table 2, 3µl of the antibody stock solution is used to prepare antibody mix for 10 assays.

4. **Lumit™ Detection Reagent:** (To be prepared during the antibody binding step in Section 5): Equilibrate Lumit™ Substrate C to room temperature. For 10 assays, prepare 250µl of fresh Lumit™ Detection Reagent by mixing 20µl of Lumit™ Substrate C with 230µl of Immunoassay Reaction Buffer (1:12.5 dilution) just before use.

5. Lumit™ Immunoassay Cellular System Protocol

The Lumit™ Immunoassay Cellular System consists of three solution additions to cells after treatment (Figures 1 and 2). The following protocol does not require removing medium from cells after treatment.

Notes:

1. If your assay requires cell treatment in higher volume, remove cell medium after treatment. Then, resuspend cells in 40µl of Immunoassay Reaction Buffer and continue with lysis as described below.
2. Before performing the Lumit™ Immunoassay, prepare cells and reagents as described in Section 4. However, the antibody mix and the Lumit™ Detection Reagent should be prepared just before use.
3. We recommend using a set of cell samples without primary antibodies as a control to assess the reagent background level. To calculate net signal, subtract the reagent background values from the sample values.
4. Many cell types have been lysed successfully with the following protocol. You can optimize the lysis steps below for your specific cell type by either extending the incubation time (Step 2) or using smaller volume with higher digitonin concentration (Step 1). Make sure the concentration of digitonin is 0.01% final after adding antibody mix (Step 4).

Protocol

1. Add 10µl of Lysis Solution to each well containing 40µl cells.

   Note: Digitonin concentration is now 0.02%, which creates a homogeneous lysate where intracellular target epitopes are exposed for detection by the Lumit™ reagents.
2. Shake the plate vigorously at approximately 800rpm for 20 minutes.
3. Prepare antibody mix as described in Section 4.B.
4. Add 50µl of antibody mix to the lysed cells.
5. Shake the plate gently at approximately 400rpm for 2 minutes.
6. Incubate the plate at 23°C for 90 minutes.

   Note: A shorter incubation may be sufficient, but the optimal time should be empirically determined.
7. Prepare Lumit™ Detection Reagent as described in Section 4.B.
8. Add 25µl of Lumit™ Detection Reagent to the assay wells.
9. Shake the plate gently at approximately 400rpm for 2 minutes.
10. Measure the luminescence with a plate-reading luminometer.

    Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.
11. **Optional:** If GF-AFC Substrate was used, measure the resulting fluorescence using a fluorometer (380–400nm$_{Ex}$/505nm$_{Em}$) and normalize luminescent RLUs to the fluorescent RFU values.

6. Guidelines for Establishing the Lumit™ Immunoassay for a New Target

The Lumit™ Immunoassay Cellular System has been used successfully to detect both the phosphorylation and total levels of several signaling proteins in unmodified cells (2). For each validated target, we provide an application note containing representative data, the specific information about primary antibodies used and recommended concentration. The application notes are available, via the expandable table, on the Lumit™ Immunoassay Cellular System web page: **www.promega.com/products/immunoassay-elisa/lumit-immunoassays/lumit-immunoassay-cellular-systems/**

For validated targets, we recommend following the protocol above and antibody conditions described in the application notes. To develop a Lumit™ Immunoassay for a new target, the following sections describe how to select primary antibodies and perform optimization.

6.A. Primary Antibody Selection

Sensitivity and specificity of the Lumit™ Immunoassay depend on the quality of primary antibodies used. It is important to select best-performing antibodies to take full advantage of the Lumit™ Immunoassay Cellular System. A good starting point is to select two to three rabbit/mouse, mouse/goat or rabbit/goat antibody pairs and use the corresponding Lumit™ secondary antibodies with the Lumit™ Immunoassay Lysis and Detection Kit. When only testing a rabbit/mouse antibody pair, we recommend using the Lumit™ Immunoassay Cellular System - Starter Kit (Cat.# W1220) as it contains both combinations of SmBiT- and LgBiT-conjugated secondary antibodies. Thus, it will allow you to identify the Lumit™ secondary antibody combination that works best for your target protein.

6.B. Selection of Optimal Primary Antibody Pair in Checkerboard Experiments

To select antibody pairs, we recommend performing a checkerboard experiment with various antibody pairs at a single concentration (e.g., 15ng/well) against the target protein in cells. Include cells treated to provide high- and low-end signals. Using this approach, select an antibody pair that provides the highest luminescent signal with the largest signal-to-background ratio. In a second checkerboard, titrate each antibody of the selected pair for optimal concentration. Verify selectivity by demonstrating an authentic biological response to inputs that change the target protein levels.

Note: The cells can be nontreated or treated with an activator or inhibitor that will change the target protein level. Sometimes, a treatment that will modulate target protein level in cells is not known; therefore, upper and lower signals in the checkerboard experiments cannot be achieved. In this case, we recommend using a purified recombinant version of the target protein if available.

Single Antibody Concentration Checkerboard Selection Experiment

In the example shown in Figure 5, three rabbit and three mouse antibodies against a target protein were used to create a nine-pair checkerboard. Each antibody was used at 150ng/ml final concentration after antibody addition (15ng/well). A modified Lumit™ Immunoassay protocol which includes the checkerboard scheme is described here.

6.B. Selection of Optimal Primary Antibody Pair in Checkerboard Experiments (continued)

1. Prepare 4ml of Immunoassay Reaction Buffer and 400µl of Lysis Solution as described in Section 4.B.
2. Prepare 40µl of cells per well in a 96-well plate as described in Section 4.A following the checkerboard scheme in Figure 5, Panel A.

   Note: If a recombinant version of the target protein is used instead of cells, prepare 800µl of protein at 1nM concentration in Immunoassay Reaction Buffer. Then add 40µl of Immunoassay Reaction Buffer in the wells marked "Nontreated" and 40µl of target protein solution in the wells marked "Treated".
3. Prepare 200µl of each primary antibody by diluting 200ng antibody in the appropriate volume of Immunoassay Reaction Buffer.
4. Prepare 800µl of Lumit™ secondary antibody mix by adding 12µl of Lumit™ Anti-Mouse Antibody-LgBiT and 12µl of Lumit™ Anti-Rabbit Antibody-SmBiT to 776µl of Immunoassay Reaction Buffer.
5. Add 10µl of Lysis Solution to all wells.
6. Shake the plate vigorously at approximately 800rpm for 20 minutes.
7. Add 15µl of each rabbit primary antibody to the corresponding wells as shown in Figure 5, Panel A.
8. Add 15µl of each mouse primary antibody to the corresponding wells as shown in Figure 5, Panel A.
9. Add 20µl of Lumit™ secondary antibody mix to all wells.
10. Shake the plate gently at approximately 400rpm for 2 minutes.
11. Incubate the plate at 23°C for 90 minutes.
12. Prepare 1ml of Lumit™ Detection Reagent as described in Section 4.B.
13. Add 25µl of Lumit™ Detection Reagent to all wells.
14. Shake the plate gently at around 400rpm for 2 minutes.
15. Measure the luminescence with a plate-reading luminometer.

    Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.
16. Use absolute luminescence readings, as well as signal-to-background ratios, to select an antibody combination for subsequent assays as described in Figure 5, Panel B.

In this experiment, the optimal antibody pair provides a balance between best luminescent signal and signal-to-background ratio.

Note: If no antibody pair is selected in this experiment, repeat the checkerboard selection using other available primary antibodies, increase the amount of antibody per well, or use the other combination of the Lumit™ Antibodies (e.g., Lumit™ Anti-Mouse Antibody-SmBiT and Lumit™ Anti-Rabbit Antibody-LgBiT). In some instances, the latter combination may perform better.

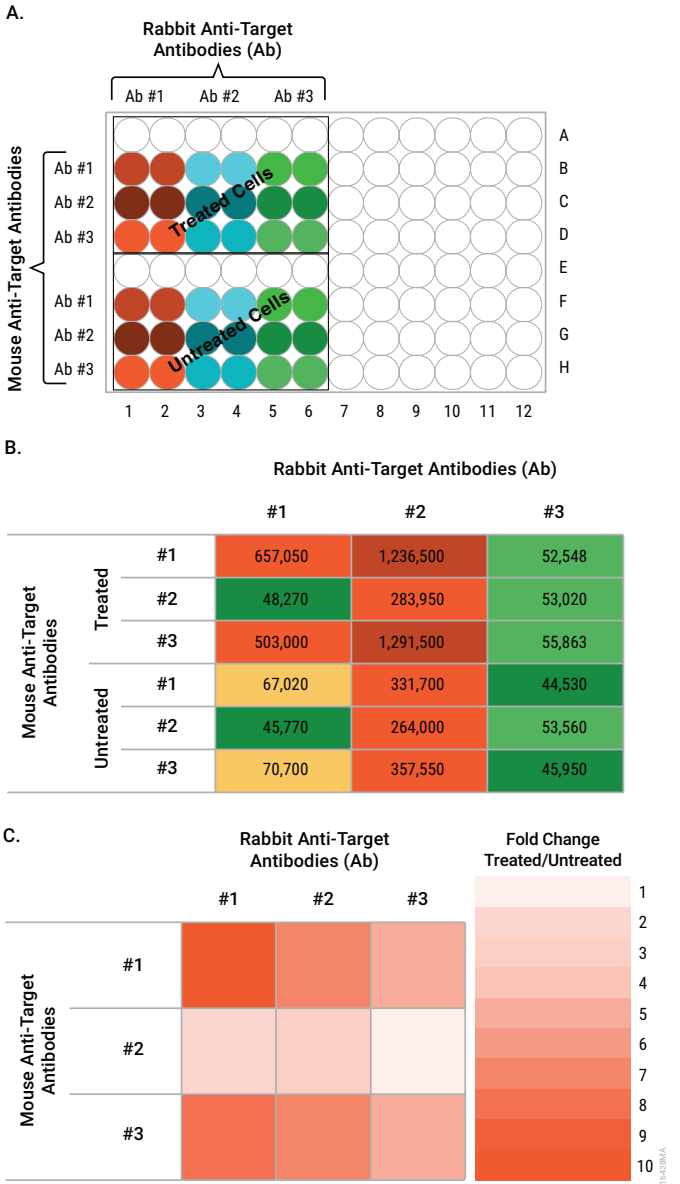


Figure 5. Single antibody concentration checkerboard selection experiment. Panel A. Checkerboard experiment design using three rabbit and three mouse antibodies designated #1, #2 and #3. **Panel B.** Example of average luminescence values obtained using a single-dose antibody (150ng/ml) for target protein detection. **Panel C.** Fold changes (Treated/Untreated) calculated from Panel B and represented in a heat map to determine the best antibody pair.

6.B. Selection of Optimal Primary Antibody Pair in Checkerboard Experiments (continued)

Antibody Titration Checkerboard Selection Experiment

In the example shown in Figure 6, rabbit primary antibody #1 and mouse primary antibody #1 were titrated to determine the optimal concentration of each. Each antibody will be used at 75ng/ml, 150ng/ml and 300ng/ml final concentration after antibody addition (7.5ng, 15ng and 30ng per well). A modified Lumit™ Immunoassay protocol that includes the checkerboard scheme is described here.

1. Prepare 7ml of Immunoassay Reaction Buffer and 700µl of Lysis Solution as described in Section 4.
2. Prepare 40µl of cells per well in a 96-well plate as described in Section 4 following the checkerboard scheme in Figure 6, Panel A.

   Note: If a recombinant version of the target protein is to be used instead of cells, add 40µl of Immunoassay Reaction Buffer in the wells marked "Untreated" and 40µl of 1nM target protein solution in the wells marked "Treated".
3. Prepare 300µl of each primary antibody dilution by mixing an appropriate volume of Immunoassay Reaction Buffer with 150ng, 300ng or 600ng antibody for the 75ng/ml, 150ng/ml or 300ng/ml concentrations, respectively.
4. Prepare 1,400µl of Lumit™ antibody mix by adding 21µl of Lumit™ Anti-Mouse Antibody-LgBiT and 21µl of Lumit™ Anti-Rabbit Antibody-SmBiT to 1,358µl of Immunoassay Reaction Buffer.
5. Add 10µl of Lysis Solution to all wells.
6. Shake the plate vigorously at approximately 800rpm for 20 minutes.
7. Add 15µl of each rabbit primary antibody dilution to the corresponding wells as shown in Figure 6, Panel A.
8. Add 15µl of each mouse primary antibody dilution to the corresponding wells as shown in Figure 6, Panel A.
9. Add 15µl of Immunoassay Reaction Buffer to the "0ng antibody" control wells (rows D and H, columns 7 and 8) as shown in Figure 6, Panel A.
10. Add 20µl of Lumit™ antibody mix to all wells.
11. Shake the plate gently at approximately 400rpm for 2 minutes.
12. Incubate the plate at 23°C for 90 minutes.
13. Prepare 1.8ml of Lumit™ Detection Reagent as described in Section 3.B.
14. Add 25µl of Lumit™ Detection Reagent to all wells.
15. Shake the plate gently at approximately 400rpm for 2 minutes.
16. Measure the luminescence with a plate-reading luminometer.

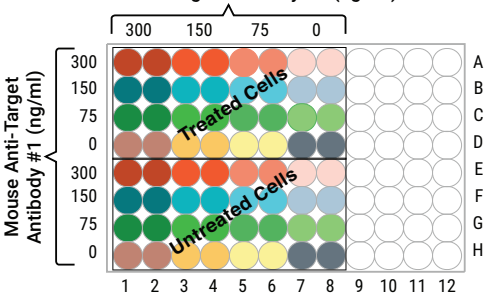
    Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

17. Subtract the background value of "no primary antibodies" control (wells D7, D8, H7 and H8; Figure 6, Panel A) from the corresponding experimental samples. Use the normalized luminescence values as well as signal-to-background ratio to select an antibody concentration combination for subsequent assays as described in Figure 6, Panel C and 6, Panel D.

    Note: In this experiment, the optimal antibody concentrations provides a balance between best luminescent signal and signal-to-background ratio.

18. Make primary antibody working stock solutions at the chosen concentration in the Antibody Dilution Buffer and store at –30°C to –10°C. For example, if the optimal amount is found to be 150ng/ml, a 50µg/ml concentrated primary antibody working stock solution can be made and stored for subsequent assays.

A.



B.

Rabbit Anti-Target Antibody #1 (ng/ml)

Mouse Anti-Target Antibody #1 (ng/ml)		300	150	75	0
Treated	300	492,425	429,850	330,300	39,270
	150	707,025	666,250	494,575	41,700
	75	648,205	548,664	541,500	43,850
	0	47,000	49,090	48,090	44,970
Untreated	300	75,751	69,255	69,970	40,430
	150	74,061	65,046	60,865	43,400
	75	71,818	66,101	65,039	44,000
	0	48,700	51,990	50,830	45,890

C.

Rabbit Anti-Target Antibody #1 (ng/ml)

Mouse Anti-Target Antibody #1 (ng/ml)		300	150	75
Treated	300	447,455	384,880	285,330
	150	662,055	621,280	449,605
	75	603,235	503,694	496,530
Untreated	300	29,861	23,365	24,080
	150	28,171	19,156	14,975
	75	25,928	20,211	19,149

D.

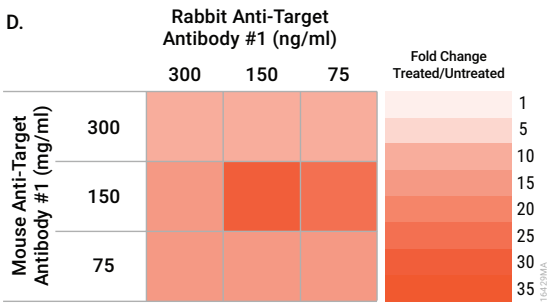


Figure 6. Antibody titration checkerboard selection experiment. Panel A. Checkerboard experiment design using three dilutions of one rabbit and one mouse antibody selected from the single-dose checkerboard experiment. **Panel B.** Example of average luminescence values obtained using combinations of three dilutions of the rabbit and mouse antibodies for target protein detection. **Panel C.** Net luminescence values after subtraction of the "no primary antibodies" control. **Panel D.** Fold changes (Treated/Untreated) calculated from Panel C and represented in a heat map to determine the best antibody concentration for subsequent experiments.

7. 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. *et al.* (2020) A homogeneous bioluminescent immunoassay approach to probe cellular signaling pathway regulation. *Commun. Biol.* **3**, 8.

3. Zegzouti, H. *et al.* (2020) A versatile bioluminescent immunoassay approach to probe cellular signaling pathway regulation. Poster presented at the 2020 Annual Conference of the Society for Laboratory Automation and Screening (SLAS), available online at **www.promega.com/resources/**

8. Related Products

Kinase Assays

Product	Size	Cat.#
ADP-Glo™ Kinase Assay	400 assays	V6930
NanoBRET™ TE Intracellular Kinase Assay, K-3	100 assays	N2600
NanoBRET™ TE Intracellular Kinase Assay, K-4	100 assays	N2520
NanoBRET™ TE Intracellular Kinase Assay, K-5	100 assays	N2500
NanoBRET™ TE Intracellular Kinase Assay, K-8	100 assays	N2620
NanoBRET™ TE Intracellular Kinase Assay, K-9	100 assays	N2630
NanoBRET™ TE Intracellular Kinase Assay, K-10	100 assays	N2640
NanoBRET™ TE Intracellular Kinase Assay, K-11	100 assays	N2650

Instrument

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000

9. Summary of Changes

The following changes were made to the 4/21 revision of this Technical Manual:

1. The url for the product web page was updated in Section 6 and added to Section 1.
2. General text and figure edits were made.
3. The cover image was updated.

[a]All of the stand-alone antibodies have a Limited Use Label License associated with them: BY USE OF THIS PRODUCT, RESEARCHER AGREES TO BE BOUND BY THE TERMS OF THIS LIMITED USE LABEL LICENSE. If researcher is not willing to accept the terms of this label license, and the product is unused, Promega will accept return of the unused product and provide researcher with a full refund.

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(i) use Lumit™-branded luminescent assay reagents (LARs) for all determinations of luminescence activity of this product and its derivatives; or

(ii) contact Promega to obtain a license for use of the product and its derivatives with LARs not manufactured by Promega.

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

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

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