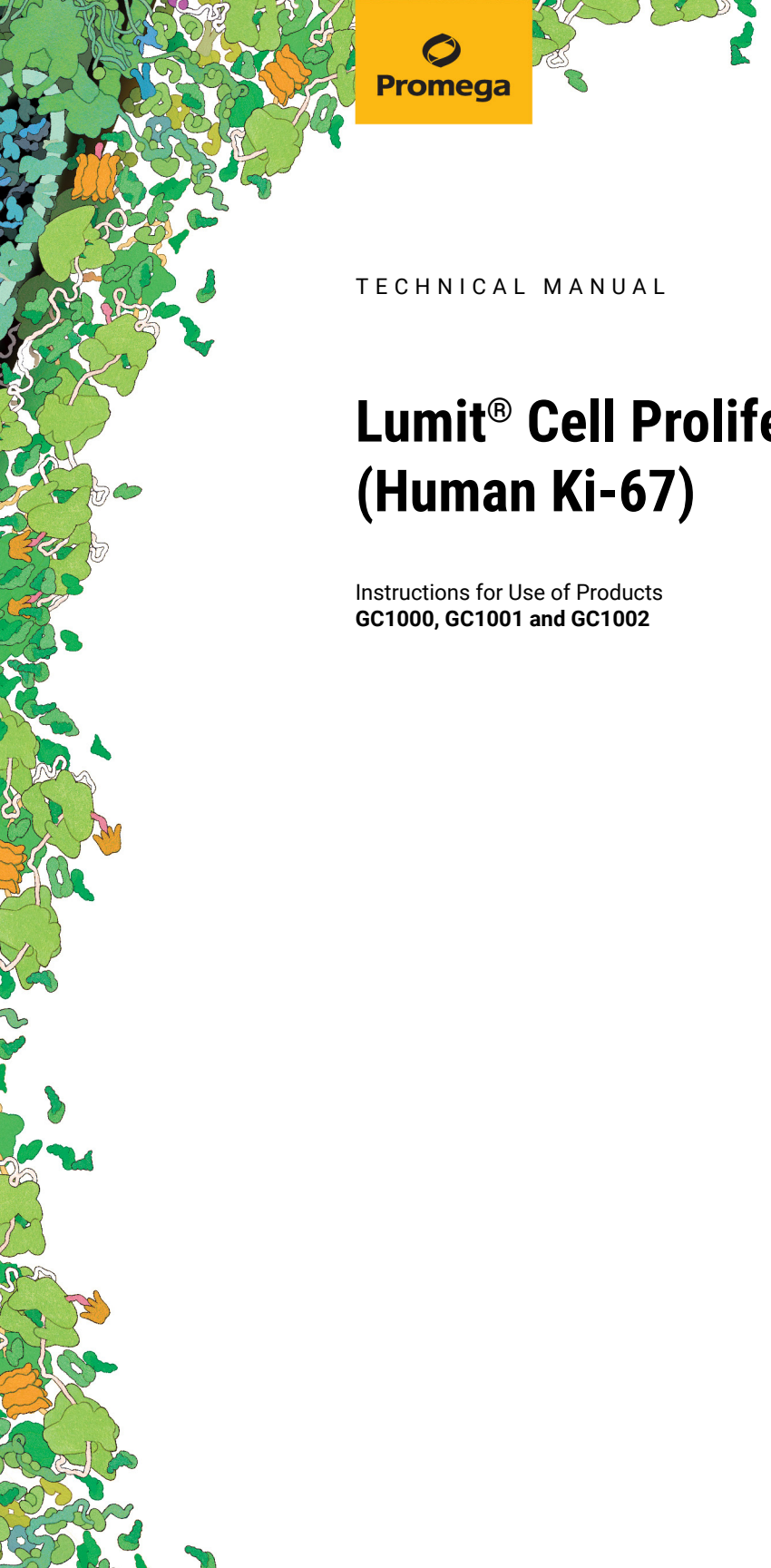

TECHNICAL MANUAL

Lumit® Cell Proliferation Assay (Human Ki-67)

Instructions for Use of Products
GC1000, GC1001 and GC1002

3/26
TM743

Lumit® Cell Proliferation Assay (Human Ki-67)

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® Cell Proliferation Assay (Human Ki-67)[a,b,c] is a homogeneous, bioluminescent assay for detecting relative levels of Ki-67 from cultured human cells without sample transfers or wash steps. Interpretation of Lumit® Cell Proliferation Assay results with respect to proliferative activity modulation can be enhanced by an understanding of whether a test agent induces cytotoxicity (cell death). The provided kit also includes CellTox™ Green Dye, enabling a same-well multiplex for cytotoxicity detection prior to addition of Lumit® Cell Proliferation Assay reagents.

Cell proliferation is the process of cell replication for increasing cell number. Ki-67 is a nuclear protein widely expressed in proliferating cells (G1, S, G2 and M cell cycle phases) but essentially undetectable in resting, nondividing cells (G0), whether they are quiescent, senescent or terminally differentiated. Because of this, Ki-67 is a widely used marker of proliferation, employed for such purposes as prognostic and predictive assessments on cancer biopsies, for the detection of proliferating T cells and for detection of antiproliferative compounds.

Common detection methods for Ki-67 include immunohistochemistry (IHC) on tissue sections, immunocytochemistry (ICC) of cultured cells, flow cytometry, Western blot and ELISA, all of which can be time-consuming, involve multiple steps and include sample transfer and/or washing steps. Likewise, other frequently used methods to monitor cell proliferation can be very time- and labor-intensive, such as DNA synthesis assays (e.g., EdU or BrdU incorporation), or provide limited response windows, such as metabolic assays for viable cell number or DNA content measurement.

In contrast, the Lumit® Cell Proliferation Assay (Human Ki-67) provides a simple add-mix-measure format for detecting the dynamic and early changes in Ki-67 levels that accompany altered proliferative activity. In addition to the completely homogeneous assay format, the stable luminescent assay signal (half-life approximately 5 hours) makes the Lumit® Cell Proliferation Assay a robust solution for basic research and screening applications.

The Lumit® Cell Proliferation Assay (Human Ki-67) has been developed for use with human cell culture samples. Lumit® reagents can be dispensed directly into microplate wells containing cells and culture medium. Assay performance with sample types other than human cell culture models must be determined by the user.

The Lumit® Cell Proliferation Assay (Human Ki-67) 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 Ki-67 monoclonal antibodies covalently labeled with SmBiT or LgBiT. When the labeled antibodies recognize and bind to Ki-67, 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 (Ki-67) present in the sample.

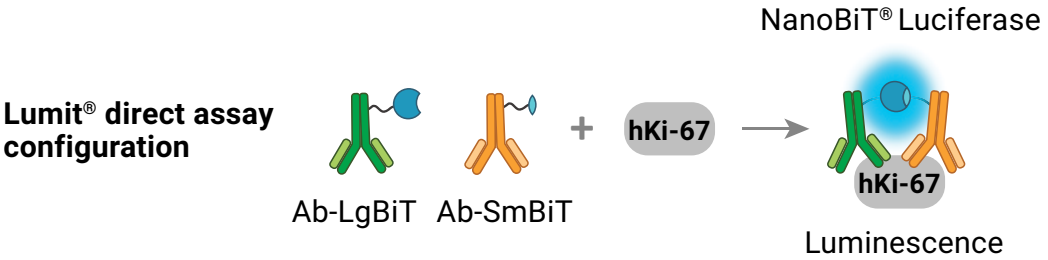

Figure 1. Assay principle. Primary monoclonal antibodies to human Ki-67 (hKi-67) are labeled with SmBiT and LgBiT. In the presence of hKi-67, SmBiT and LgBiT are brought into close proximity, forming the NanoBiT® enzyme. When assay detection reagent is added, a bright luminescent signal is generated.

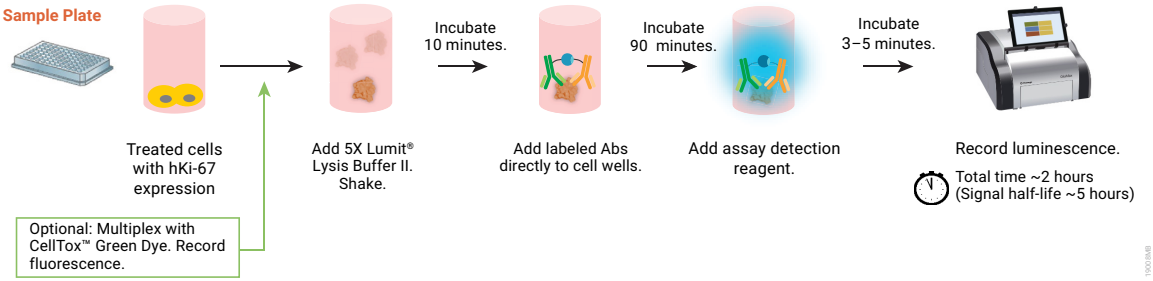

Figure 2. Assay protocol. Measurement of human Ki-67 levels using the Lumit® Cell Proliferation Assay (Human Ki-67) is performed directly on cells in culture with medium present (no need to remove). The Lumit® immunoassay protocol does not require wash steps and can be completed in approximately 2 hours. Antibody mixture consists of Anti-hKi-67 mAb SmBiT and Anti-hKi-67 mAb LgBiT. Twenty microliters of 5X Lumit® Lysis Buffer II is added to 80µl of treated cells in culture medium. Shake and incubate for 10 minutes. Following incubation, 100µl of 2X Lumit® Anti-hKi-67 mAb mixture is added and incubated for 90 minutes. Lastly, 50µl of assay detection reagent is added and luminescence is recorded. **Note:** CellTox™ Green Dye can be added to cells in culture to monitor cytotoxicity (cell death) after treatment with test agents but before addition of Lumit® Lysis Buffer II.

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2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Lumit® Cell Proliferation Assay (Human Ki-67)	**100 assays**	**GC1000**

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

- 60µl Anti-hKi-67 mAb SmBiT, 400X
- 60µl Anti-hKi-67 mAb LgBiT, 400X
- 1.3ml Lumit® Lysis Buffer II, 10X
- 1.8ml Lumit® Immunoassay Buffer C, 10X
- 600µl Ki-67 Assay Substrate
- 6ml Lumit® Detection Buffer B
- 25µl Human Ki-67 Protein (Partial) Positive Control
- 20µl CellTox™ Green Dye, 1000X

PRODUCT	SIZE	CAT.#
Lumit® Cell Proliferation Assay (Human Ki-67)	**1,000 assays**	**GC1001**

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

- 600µl Anti-hKi-67 mAb SmBiT, 400X
- 600µl Anti-hKi-67 mAb LgBiT, 400X
- 13ml Lumit® Lysis Buffer II, 10X
- 18ml Lumit® Immunoassay Buffer C, 10X
- 6ml Ki-67 Assay Substrate
- 60ml Lumit® Detection Buffer B
- 25µl Human Ki-67 Protein (Partial) Positive Control
- 200µl CellTox™ Green Dye, 1000X

PRODUCT	SIZE	CAT.#
Lumit® Cell Proliferation Assay (Human Ki-67)	**5 × 100 assays**	**GC1002**

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

- 5 × 60µl Anti-hKi-67 mAb SmBiT, 400X
- 5 × 60µl Anti-hKi-67 mAb LgBiT, 400X
- 5 × 1.3ml Lumit® Lysis Buffer II, 10X
- 5 × 1.8ml Lumit® Immunoassay Buffer C, 10X
- 5 × 600µl Ki-67 Assay Substrate
- 5 × 6ml Lumit® Detection Buffer B
- 25µl Human Ki-67 Protein (Partial) Positive Control
- 200µl CellTox™ Green Dye, 1000X

Storage Conditions: Upon receipt, store the complete kit at –30°C to –10°C. Store Ki-67 Assay Substrate and CellTox™ Green Dye protected from light. Thaw all frozen components and equilibrate to room temperature before use. (Antibodies and substrate are not frozen at –30°C to –10°C.) Gently mix each component before use. Store unused 10X Lumit® Lysis Buffer II reagent stock at +2°C to +10°C. Unused Lumit® Detection Buffer B can be refrozen, stored at +2°C to +10°C or, for convenience, stored at room temperature. Store all other unused components at –30°C to –10°C as soon as possible after use. Human Ki-67 Protein (Partial) Positive Control is stable for at least three freeze-thaw cycles.

! Do not store prepared reagents.

3. Before You Begin

The protocol describes the luminescent detection of relative human Ki-67 protein levels directly in assay wells containing both cells and culture medium (including up to 10% FBS) without the need for sample washes or transfer.

Materials to Be Supplied By the User

- cells (human-derived)
- culture medium appropriate for human-derived cell line (including up to 10% FBS)
- white, multiwell tissue culture plates (solid white or white with clear bottom) compatible with luminescent and fluorescent measurements (e.g., 96-well Corning® Cat.# 3917)
- multichannel pipette or automated pipetting station
- dilution tubes or multichamber, dilution reservoir (e.g., Dilux® D-1002)
- reagent reservoir trays (e.g., Midwest Scientific Cat.# RR25)
- plate shaker appropriate for multiwell plate format used in assay
- multimodal plate reader (luminometer and fluorometer) capable of reading multiwell plates (e.g., GloMax® Discover System, Cat.# GM3000)
- hemacytometer and Trypan blue (or alternative method) for viable cell counting prior to cell plating
- positive control mitogens or anti-proliferative compounds
- water (purified; Type I or Type II)
- **optional:** protease inhibitor cocktail (e.g., Thermo Scientific Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free [100X] Cat.# PI78441)

Note: The Lumit® Cell Proliferation Assay (Human Ki-67) will detect Ki-67 protein levels from human cells. It will not detect mouse Ki-67 protein and has not been evaluated for Ki-67 detection from other nonhuman species.

Assay Protocol for Cultured Cells (Section 4): Measure human Ki-67 directly in cell culture wells. Add 20µl of 5X Lumit® Lysis Buffer II to 80µl of cells in culture medium, shake, then incubate for 10 minutes at room temperature. Following incubation, add 100µl of 2X antibody mixture in 1X Lumit® Immunoassay Buffer C and incubate for 90 minutes. Then add 50µl of assay detection reagent (1:10 dilution of Ki-67 Assay Substrate in Lumit® Detection Buffer B) and record luminescence.

Optional CellTox™ Green Dye Multiplex (Recommended): Decreases in human Ki-67 protein in cell culture can be a secondary effect of compound cytotoxicity inducing cell death. Thus, the assay kit is provided with CellTox™ Green Dye for the (optional) detection of cytotoxicity. This cell impermeant, fluorogenic DNA dye can be multiplexed with the luminescent Lumit® Cell Proliferation Assay (Human Ki-67) to distinguish between compounds that are antiproliferative in the absence of cell death (reduced Ki-67 levels without cytotoxicity) or are antiproliferative due to induced cell death (reduced Ki-67 levels secondary to cytotoxicity). CellTox™ Green Dye can be added during cell plating (or, alternatively, at the time of cell treatment) to monitor cytotoxicity (cell death) following treatment with test agents, but fluorescence must be measured before adding Lumit® Lysis Buffer II.

3. Before You Begin (continued)

Note: Assay volumes are scalable and can be adjusted based on sample size. The protocol in Section 4 lists recommended volumes for 96- and 384-well plates. Other volumes can be used if you maintain the recommended final antibody and detection reagent concentrations. Robust assay performance with proliferating human cells is observed with different media types, including different fetal bovine serum (FBS) concentrations. However, when using different media (including different FBS concentrations) within a single study, we recommend confirming that assay performance is unaffected with respect to detection of cell-based Ki-67 levels. Signal obtained from Human Ki-67 Protein (Partial) Positive Control is optimal in the presence of 5–10% fetal bovine serum (FBS) and may be reduced at lower FBS concentrations, although the positive control signal will still be strong. Phenol red–free medium may enhance assay sensitivity by minimizing inner-filter effects and luminescence quenching.

Reagent Preparation and Storage

Prepare the 5X Lumit® Lysis Buffer II (Section 4.C), 2X Anti-hKi-67 antibody mixture (Section 4.D) and assay detection reagent (Section 4.E) on the day of use. Do not reuse the 5X Lumit® Lysis Buffer II, 2X Anti-hKi-67 antibody mixture or assay detection reagent. To avoid reagent contamination, use only fresh, aerosol filter tips or cotton-plugged, sterile pipettes when withdrawing volumes from reagent stock components. Store unused 10X Lumit® Lysis Buffer II reagent stock at +2°C to +10°C. Store all other unused components at –30°C to –10°C as soon as possible after use.

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.

Table 1. An Example 96-well Format Plate Map.

Test compound dilution series →											Controls	Controls
	1	2	3	4	5	6	7	8	9	10	11	12
A											Untreated (no compound) control replicates	No-cell (background) control replicates
B												
C												
D												
E											Cytotoxicity positive control replicates	**Optional:** Ki-67 Protein (Partial) Positive Control replicates
F												
G												
H												

Always include replicate wells for the necessary controls such as the no-cell control wells (with assay reagent added) for background luminescent signal determination and the untreated (no compound) control wells.

- Cells treated with mitogenic or anti-proliferative compounds may require a long incubation period, thus evaporation can be more pronounced in edge wells. For long incubation periods, blank wells (medium only) around the plate edges can help minimize evaporation of the inner wells and help mitigate edge effects. For shorter incubation periods, blank wells may not be necessary and the full 96-well plate can be used.
- When multiplexing the Lumit® Cell Proliferation Assay with CellTox™ Green Dye for cytotoxicity determinations (recommended), we recommend including replicate cell wells treated with a reference agent known to induce a high level of cell death in the cell model as a cytotoxicity positive control.

4. Assay Protocol for Cultured Cells

This protocol describes the luminescent detection of relative human Ki-67 protein levels directly in assay wells containing cells and culture medium (including up to 10% FBS). This protocol also describes the fluorescent detection of cytotoxicity using the CellTox™ Green Dye, which can be multiplexed with luminescent human Ki-67 detection. Include the necessary controls described in Section 3 and refer to the example plate map (Table 1; 96-well format) when designing a study. The example plate map is an illustrative example and can be changed, provided that all the proper controls are included.

4.A. Cell Plating and Treatment

1. Plate cells with medium into a 96- or 384-well white (or white with clear bottom) tissue culture plate as follows:

   96-well plate: 40µl/well.

   384-well plate: 10µl/well.

   Allow cells to attach if using adherent cells (typically, approximately 4—6 hours). Be sure to include a set of control wells with medium only (no-cell control wells) for background determination.

   Note: While the broad dynamic range of the assay offers considerable flexibility for cell number, the optimal number of cells dispensed per well for a specific cell model should be empirically determined. Ensure the maximum hKi-67 level achieved under the employed conditions does not exceed the linear range of the detection chemistry for the cell model. Within that constraint, cell number can be increased to meet the detection requirements of low-level hKi-67 expression. See Figure 3 in Section 6.A (Appendix, Supporting Data) for an example of assay signal linearity versus cell number.

   Recommended: For same-well cytotoxicity assessment, dilute the 1,000X CellTox™ Green Dye 500-fold to 2X concentration into cells with medium before cell plating, or alternatively into medium with vehicle and test agents at treatment time. The final concentration of CellTox™ Green Dye during cell treatment should be 1X**.**

   Note: If the luminescent Lumit® Cell Proliferation Assay is multiplexed with the CellTox™ Green Cytotoxicity Assay, the no-cell control wells (background control) should also include the cell-impermeant CellTox™ Green Dye at the same dilution factor as wells containing cells.

4.A. Cell Plating and Treatment (continued)

2. Treat cells by adding a volume of 2X test agent or vehicle control to each well (including the no-cell control wells) such that the total volume is as follows:

   96-well plate: 80µl/well.

   384-well plate: 20µl/well.

   For example, if 40µl of cells are plated per well in a 96-well plate, add 40µl of 2X treatment agent in culture medium. Cells are typically treated for 24–72 hours, depending on test agent, to observe relative changes in hKi-67 levels. Include a set of replicate control wells for untreated (no compound, vehicle only) cells.

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

   Notes:

   a. Assay sensitivity may be reduced in 384-well format as compared to sensitivity in 96-well format.

   b. If multiplexing with the CellTox™ Green Dye for cytotoxicity determination, include a known cytotoxic agent in replicate wells as a positive control.

3. Incubate cells for the desired treatment time in a humidified 37°C, 5% CO_2 tissue culture incubator.

4. If multiplexing the luminescent Lumit® Cell Proliferation Assay with the CellTox™ Green Dye to determine cytotoxicity, briefly shake the plate, then read fluorescence (RFU) using a standard "green" filter set ($485nm_{Ex}/520nm_{Em}$).

   Note: Fluorescence readings should be taken after cell treatments but **before** adding the 5X Lumit® Lysis Buffer II (Section 4.C).

4.B. Optional: Preparing the Human Ki-67 Protein (Partial) Positive Control

Shortly before completing the desired cell treatment time, prepare a dilution of Human Ki-67 Protein (Partial) Positive Control for use as a positive control.

1. After the cell treatment is complete and CellTox™ Green fluorescence is measured (optional), prepare a 1/500 dilution of the Human Ki-67 Protein (Partial) Positive Control in the identical culture medium used for cell samples.

2. Add 80µl of the Human Ki-67 Protein (Partial) Positive Control dilution to the empty wells reserved for this positive control (see example plate map in Section 3).

   Note: The Ki-67 positive control at the prescribed dilution described above can be incorporated on at least one assay plate for a positive control to demonstrate successful implementation of the assay chemistry. **However, using this positive control as a quantitative standard for determining cell-based, native Ki-67 protein concentrations has not been verified.**

4.C. Adding 5X Lumit® Lysis Buffer II Mixture to Assay Wells

Shortly before completing the desired cell treatment time, thaw the Lumit® Lysis Buffer II, 10X, and equilibrate to room temperature. In addition, thaw the Lumit® Immunoassay Buffer C, 10X, to room temperature for use in Section 4.D.

1. Immediately prior to use, prepare a 5X Lumit® Lysis Buffer II mixture by diluting the Lumit® Lysis Buffer II, 10X, stock into a single volume of water. We recommend including a protease inhibitor cocktail mix in the dilution. Pipet to mix the solution. For example, to assay a complete 96- or 384-well plate, including some reagent volume for pipetting loss, prepare 2,600µl of 5X Lumit® Lysis Buffer II mix as follows:

Reagent	Volume
Water	1,170µl
Lumit® Lysis Buffer II, 10X	1,300µl
Halt™ Protease Inhibitor Cocktail (100X)	130µl

Notes:

a. Scale volumes up or down as appropriate to accommodate the desired number of assay wells. Include some excess reagent volume for pipetting loss.

b. We recommend using protease inhibitor cocktail. If it is not used, replace the protease inhibitor cocktail volume with water.

2. Add the 5X Lumit® Lysis Buffer II mixture to all assay wells including cultured cells (test wells or untreated controls), no-cell control wells (background controls), Ki-67 Protein (Partial) Positive Control wells and cytotoxicity positive control wells. Carefully avoid cross contamination between wells.

   96-well plate: Dispense 20µl/well of 5X Lumit® Lysis Buffer II mixture.

   384-well plate: Dispense 5µl/well of 5X Lumit® Lysis Buffer II mixture.

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

3. Mix assay plate as follows:

   96-well plate: Mix on a plate shaker for 10–15 seconds at 800rpm.

   384-well plate: Mix on a plate shaker for 2 minutes at 800rpm. A higher shaking speed may be required for adequate mixing of the 384-well format, depending on the plate shaker model used.

4. Incubate the assay plate for 10 minutes at room temperature.

   Note: For difficult-to-lyse samples, such as very large 3D spheroids, you may need to increase the shaking and incubation times during the lysis step for maximal Ki-67 recovery and detection.

4.D. Adding 2X Anti-hKi-67 Antibody Mixture to Assay Wells

The Lumit® Immunoassay Buffer C, 10X, should be thawed at room temperature as instructed in Section 4.C. At this time, the Lumit® Detection Buffer B should be thawed and equilibrated to room temperature for use in Section 4.E.

1. Remove the Anti-hKi-67 antibodies from storage immediately before use. Thaw if necessary.
2. Briefly centrifuge the Anti-hKi-67 antibody tubes before opening, then mix by gentle pipetting.
3. Immediately prior to use, prepare a 2X antibody mixture by diluting both 400X antibody stocks 1:200 into a single volume of 1X Lumit® Immunoassay Buffer C with additional use of water. Pipet to mix the antibody solution. For example, to assay a complete 96- or 384-well plate, including some reagent volume for pipetting loss, prepare 12,000µl of 2X antibody mixture as follows:

Reagent	Volume
Water	10,680µl
Lumit® Immunoassay Buffer C, 10X	1,200µl
Anti-hKi-67 mAb SmBiT, 400X	60µl
Anti-hKi-67 mAb LgBiT, 400X	60µl

Note: Scale volumes up or down as appropriate to accommodate the desired number of assay wells. Include some excess reagent volume for pipetting loss.

4. Add the 2X Anti-hKi-67 antibody mixture to all assay wells, including cultured cells (test wells or untreated controls), no-cell control wells (background controls), Human Ki-67 Protein (Partial) Positive Control wells and cytotoxicity positive control wells. Carefully avoid cross contamination between wells.

   96-well plate: Dispense 100µl/well of 2X Anti-hKi-67 antibody mixture.

   384-well plate: Dispense 25µl/well of 2X Anti-hKi-67 antibody mixture.

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

5. Mix assay plates as follows:

   96-well assay plate: Mix briefly on a plate shaker (e.g., 10–15 seconds at 500rpm).

   384-well assay plate: Mix briefly on a plate shaker (e.g., 10–15 seconds at 800rpm).

6. Incubate the assay plate for 90 minutes at room temperature.

4.E. Adding Assay Detection Reagent to Assay Wells

While test samples and controls are being incubated with the Anti-hKi-67 Antibody Mixture (Section 4.D, Step 6), prepare the assay detection reagent.

Note: The Lumit® Detection Buffer B should be thawed and equilibrated to room temperature as instructed in Section 4.D.

1. Remove the Ki-67 Assay Substrate from storage and mix. If Ki-67 Assay Substrate has collected in the cap or on the sides of the tube, briefly centrifuge.
2. Prepare a 1:10 dilution of Ki-67 Assay Substrate in Lumit® Detection Buffer B to create enough assay detection reagent for the number of wells to be assayed. Pipet to mix the solution. For example, to assay a complete 96- or 384-well assay plate, including some reagent volume for pipetting loss, prepare 6,000µl of assay detection reagent as follows:

Reagent	Volume
Lumit® Detection Buffer B	5,400µl
Ki-67 Assay Substrate	600µl

Notes:

a. Scale volumes up or down as appropriate to accommodate the desired number of assay wells. Include some excess reagent volume for pipetting loss.

b. Once reconstituted, the assay detection reagent will lose 10% activity in approximately 4 hours at 20°C.

3. Add the assay detection reagent to all assay wells including cultured cells (test wells or untreated controls), no-cell control wells (background controls), Ki-67 Protein (Partial) Positive Control wells and cytotoxicity positive control wells. Carefully avoid cross contamination between wells.

   96-well plate: Dispense 50µl/well of assay detection reagent.

   384-well plate: Dispense 12.5µl/well of assay detection reagent.

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

4. Briefly mix the assay plate with shaking:

   96-well plate: Mix briefly on a plate shaker (e.g., 10–15 seconds at 500rpm).

   384-well plate: Mix briefly on a plate shaker (e.g., 10–15 seconds at 800rpm).

5. Incubate at room temperature for 3–5 minutes.

4.E. Adding Assay Detection Reagent to Assay Wells (continued)

6. Measure luminescence.

   Notes:

   a. Assay signal is stable with a half-life of approximately 5 hours, compatible with batch processing of multiple assay plates.

   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 instrument 'Luminescence Protocols' tab with a 0.5-second integration time.

5. Calculating Results

To properly analyze data from the Lumit® Cell Proliferation Assay (Human Ki-67) and CellTox™ Green Cytotoxicity Assay (see Technical Manual #TM365), carefully follow these steps:

5.A. CellTox™ Green Cytotoxicity Assay (Fluorescence)

1. Measure fluorescence in all replicate wells, including no-cell (background) control wells.
2. Calculate the average fluorescence for each condition.
3. Subtract the average background fluorescence from all sample wells (untreated, treated and cytotoxicity control).
4. Plot the background-subtracted values to assess cytotoxicity (increased fluorescence reflects higher cell death).

5.B. Lumit® Cell Proliferation Assay (Luminescence)

1. Measure luminescence in all replicate wells, including no-cell (background) control wells.
2. Calculate the average luminescence for each condition.
3. Subtract the average background luminescence from all sample wells (untreated, treated, Ki-67 Protein [Partial] Positive Control and cytotoxicity control).
4. Plot the background-subtracted values to reflect changes in relative Ki-67 protein levels.

5.C. Optional Normalization

Perform normalization if Ki-67 is present in untreated controls.

1. Normalize background-subtracted luminescence values from treated wells to the average untreated control.
2. Express results as a percentage of the untreated control for comparison.

6. Appendix

6.A. Representative Data

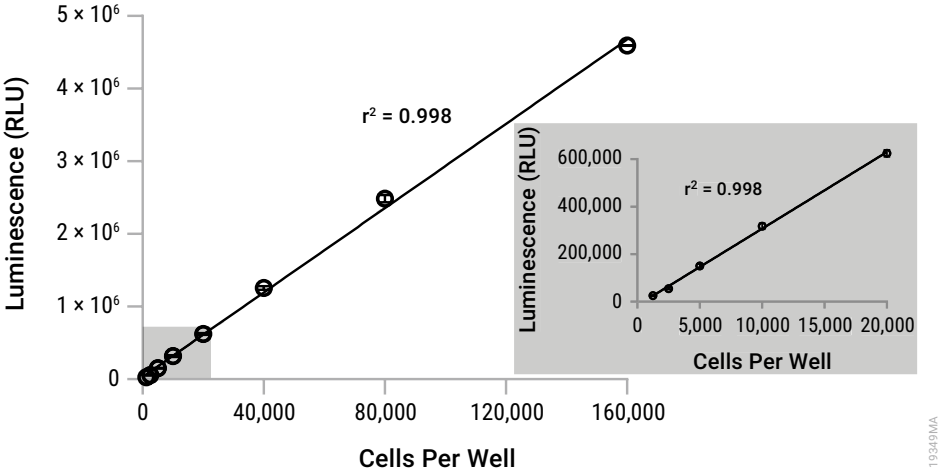

Figure 3. Luminescence is proportional to Ki-67 levels. The Lumit® Cell Proliferation Assay (Human Ki-67) was applied to increasing numbers of proliferating Jurkat cells in 96-well format, resulting in proportional increases in assay signal. These results demonstrate the effective detection of human Ki-67 protein levels over a wide range of cell number.
Note: The plotted luminescence values were determined by subtracting background RLU.

6.A. Representative Data (continued)

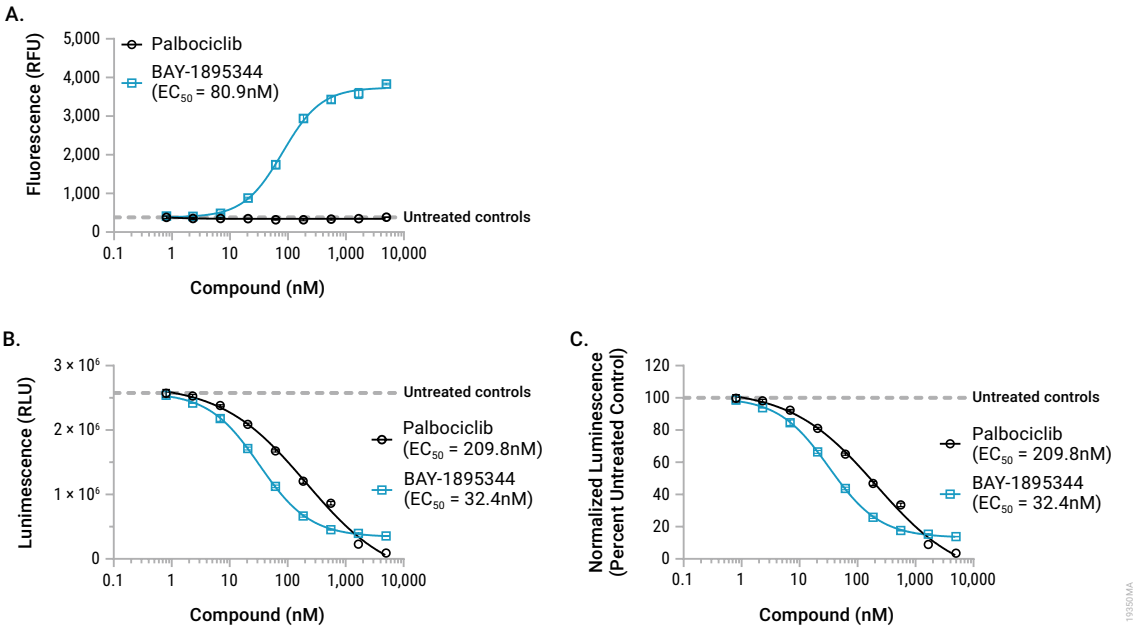

Figure 4. Multiplex application of CellTox™ Green Cytotoxicity Assay and Lumit® Cell Proliferation Assay to control and drug-treated Jurkat cells in suspension (96-well format; 20,000/well; 48-hour treatments). Panel A. Compared to untreated Jurkat cells, palbociclib demonstrates no cytotoxicity at 48 hours, as determined with the CellTox™ Green Cytotoxicity Assay. In contrast, BAY-1895344 demonstrates significant dose-dependent cell death. **Note:** The plotted fluorescence values were determined by subtracting background RFU. **Panel B.** In the same assay wells, the subsequent application of the Lumit® Cell Proliferation Assay demonstrates a dose-dependent decrease in Ki-67 levels in response to both palbociclib and BAY-1895344 treatment. **Note:** The plotted luminescence values were determined by subtracting background RLU. **Panel C.** The luminescence data from Panel B was reanalyzed and graphed as normalized luminescence (percent of untreated controls). Taken together, these results demonstrate the antiproliferative effects of palbociclib without apparent cytotoxicity, while the antiproliferative effects of BAY-1895344 in this model include the provocation of overt cell death.

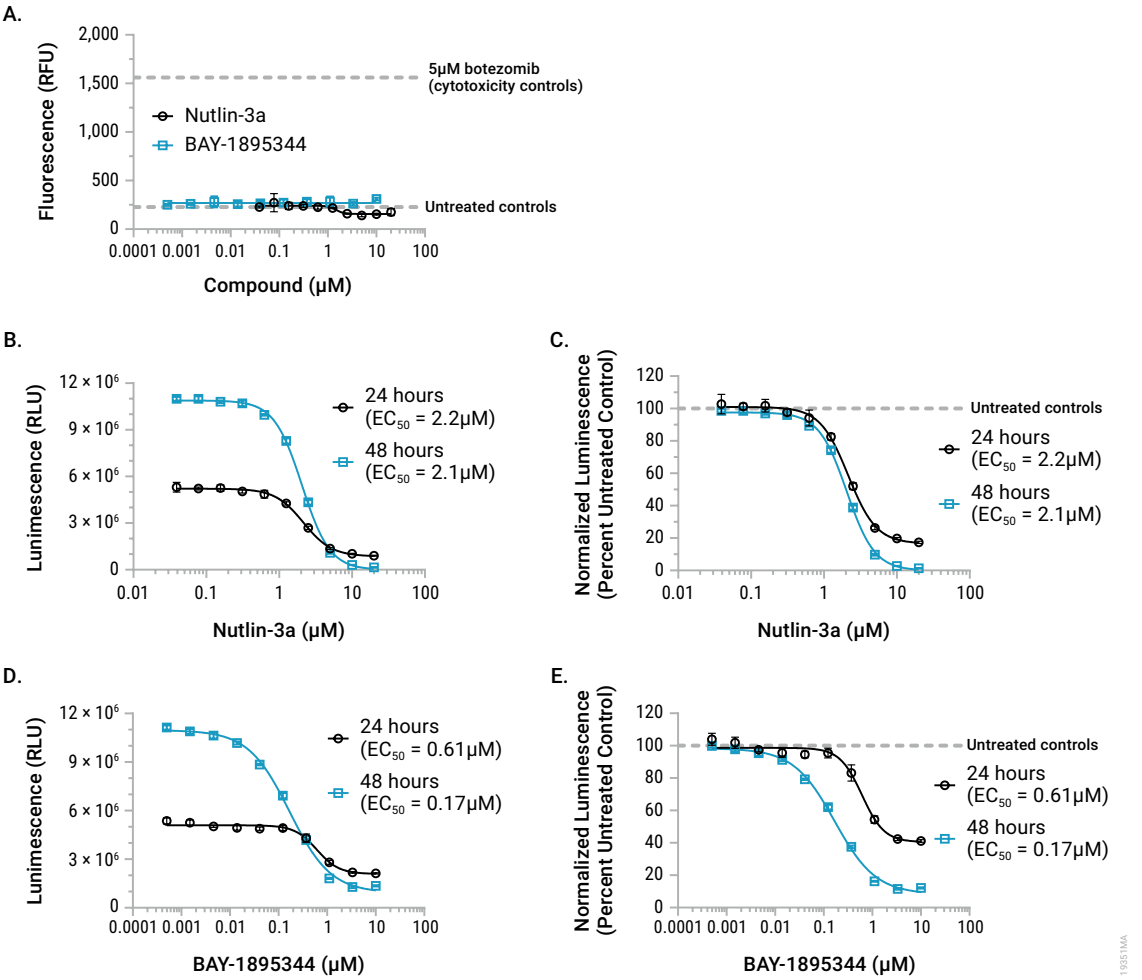

Figure 5. Multiplex application of CellTox™ Green Cytotoxicity Assay and Lumit® Cell Proliferation Assay to control and drug-treated HCT116 monolayer cells (96-well format; 4,000/well; 24- and 48-hour treatments). Panel A. Compared to untreated HCT116 cells, nutlin-3a and BAY-1895344 demonstrate no cytotoxicity at 48 hours, as determined with the CellTox™ Green Cytotoxicity Assay. The fluorescence signal elicited with the cytotoxicity positive control (5µM bortezomib) is indicated for comparison. **Note:** The plotted fluorescence values were determined by subtracting background RFU. **Panels B and D.** In the same assay wells, the subsequent application of the Lumit® Cell Proliferation Assay demonstrates a dose-dependent decrease in Ki-67 levels in response to treatment with either nutlin-3a (**Panel B**) or BAY-1895344 (**Panel D**). Note: The plotted luminescence values were determined by subtracting background RLU. **Panels C and E**. The luminescence data from **Panels B** and **D**, respectively, were reanalyzed and graphed as normalized luminescence (percent of untreated controls). Taken together, these results demonstrate antiproliferative effects without cell death for both nutlin-3a and BAY-1895344 in this cell model. In addition, while antiproliferative efficacy is increased with treatment time for both agents, a potency increase with time is only observed for BAY-1895344.

6.A. Representative Data (continued)

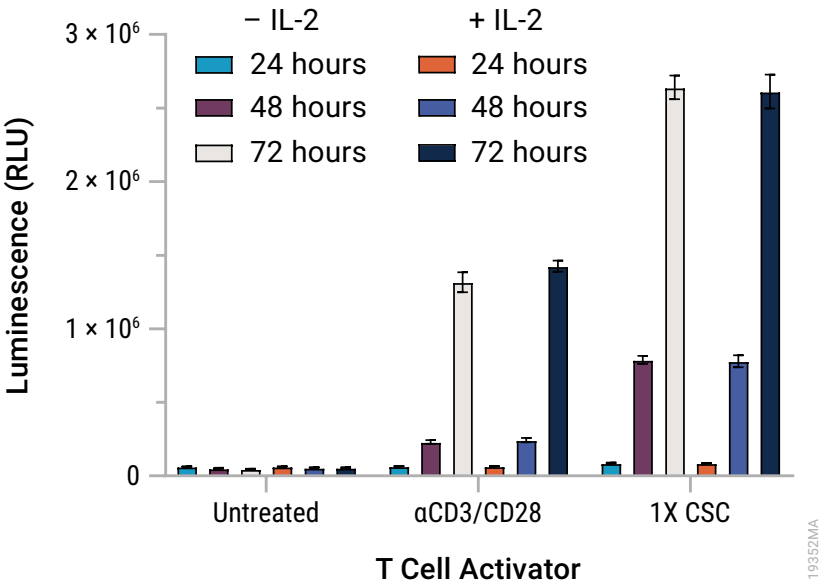

Figure 6. The Lumit® Cell Proliferation Assay (Human Ki-67) application to untreated (nonproliferating) and treated human CD8+ T-cells (96-well format; 80,000/well; 24-, 48- and 72-hour treatments). Application of the Lumit® Cell Proliferation Assay demonstrates a time-dependent increase in Ki-67 levels in response to prior treatment of human CD8+ T cells with the T-cell activators αCD3/CD28 or CSC (Cell Stimulation Cocktail). **Note:** The plotted luminescence values were determined by subtracting background RLU. Of note, the maximum increase in cell number in response to mitogen treatment for up to 72 hours was less than 34% (data not shown). Given that significant T-cell expansion typically occurs >3 days after T-cell activation, upregulation of Ki-67 levels is a relatively early indicator of T-cell proliferation.

6.B. Additional Considerations

Cell models and standard operating procedures: The Lumit® Cell Proliferation Assay (Human Ki-67) is a sensitive, homogeneous lytic assay intended to measure experimental levels of Ki-67 expression. Care should be taken to validate a model (cell number and exposure period) whereby untreated cells maintain optimal viability over the time course. Special attention should be taken to gently passage sensitive cell types that are susceptible to mechanical or enzymatic dispersion (adherent cell lines). For these cell lines, we recommend an acclimation period of at least 4–6 hours after passage. Failure to optimize cell health parameters may lead to decreases in Ki-67 levels in untreated wells.

Temperature: The NanoBiT® enzymatic activity and the reconstituted NanoBiT® Luciferase is temperature dependent. Strive to keep the assay plate equilibrated to room temperature during the application of the Lumit® Cell Proliferation Assay reagents, especially at the time of assay detection reagent addition and subsequent recording of assay luminescence. Failure to do so will negatively affect replicate variability.

Spectral interferences: Test or control compounds with visible color can absorb light in the visible spectrum and quench luminescence in a dose-dependent manner. Other compounds that are DNA intercalators or minor groove binders can interfere with DNA binding dyes such as the CellTox™ Green Dye. Also, test or control compounds that are antiproliferative or provoke proliferation will cause differential cell growth between treated and untreated wells, which in turn, can influence phenol-red containing medium. In some cases, this can result in variations in pH in control vs. treated cell wells and a concomitant effect on phenol red color intensity. In extreme cases, this "inner-filter" variation may alter observed luminescence intensity when compared to untreated controls.

6.C. 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
Greatly reduced assay sensitivity when applied to human serum samples	Several factors within human serum samples can interfere with the homogeneous Lumit® Cell Proliferation Assay performance and, thereby, significantly reduce assay sensitivity. For this reason, we do not recommend use of the Lumit® Cell Proliferation Assay on human serum samples.
Experimental RLU are not identical to the numbers in this Technical Manual	Bioluminescent signal intensity (e.g., 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 luminescent signal over background for assayed cell wells is low	There may be variations in luminescent signal due to culture conditions, temperature, etc. Confirm that the assay is being performed on human cells. Nonproliferating cells will have very low Ki-67 expression. Highly proliferating human cell lines (e.g., HeLa, HCT116, Jurkat) or Human Ki-67 Protein (Partial) Positive Control can serve as positive controls for Ki-67 luminescent detection. If little-to-no specific signal is observed, make sure the assay procedure in Section 4 was performed correctly. For difficult-to-lyse samples, such as very large 3D spheroids, increased shake and incubation times during the lysis step may be required to achieve maximal Ki-67 recovery and detection. Cell lysis and Ki-67 exposure for immunoassay detection may be incomplete due to improper handling of the Lumit® Lysis Buffer II, 10X, stock reagent. Be sure to equilibrate the stock reagent to room temperature and gently mix before use. After use, do not refreeze unused Lumit® Lysis Buffer II, 10X, storing at +2°C to +10°C, avoiding multiple freeze-thaw cycles.

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6.D. 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.

[a]U.S. Pat. Nos. 9,797,889; 9,797,890; 10,107,800; 11,493,504; European Pat. No. 2970412; Japanese Pat. No. 7280842; 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. Nos. 8,598,198 and 9,458,499; European Pat. No. 2408863; and Japanese Pat. No. 5746135.