TECHNICAL MANUAL

# HEK293 LgBiT Cell Line and LgBiT Expression Vector

Instructions for Use of Products
**N2672 and N2681**


4/20
TM620

Promega

# HEK293 LgBiT Cell Line and LgBiT Expression Vector

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

## 1. Description

The HEK293 LgBiT Cell Line and LgBiT Expression Vector are reagents for intracellular expression of the LgBiT protein that complements HiBiT, an 11-amino-acid peptide tag, to produce luminescence in live cells in the presence of substrate. The LgBiT reagents can be paired with HiBiT protein fusions that are expressed transiently, stably or endogenously inserted by CRISPR-Cas9, and used for endpoint or kinetic real-time monitoring of HiBiT protein levels. In addition, these products can be multiplexed with cell-health assays and bioluminescent resonance energy transfer (BRET)-based applications, including NanoBRET™ protein:protein and target engagement assays.

The HEK293 LgBiT Cell Line is a clonal cell line stably expressing the LgBiT protein under the control of a constitutive CMV promoter that maintains expression for at least 35 passages. For transient or stable expression of the LgBiT protein in other cell types, we recommend transfecting the LgBiT Expression Vector.

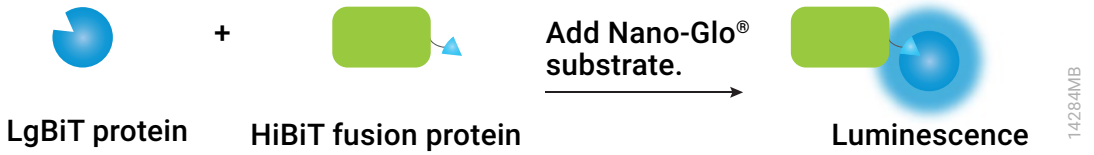


**Figure 1. The LgBiT protein complements HiBiT to produce luminescence in the presence of substrate.** When both the LgBiT protein and a HiBiT fusion protein are present in live cells, the high binding affinity between LgBiT and HiBiT results in spontaneous complementation, producing luminescence when substrate is added. The luminescent signal is directly proportional to intracellular protein level of the HiBiT fusion.

## 2. Product Components and Storage Conditions

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **HEK293 LgBiT Cell Line** | **1 each** | **N2672** |

Includes:

- 2 × 1ml HEK293 LgBiT Stable Cell Line

**Storage Conditions:** Upon arrival, immediately transfer cell vials to at or below –140°C (freezer or liquid nitrogen vapor phase) for long-term storage. Do not store cell vials submerged in liquid nitrogen. Do not store cell vials at –80°C as this will negatively affect cell viability and performance.


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| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **LgBiT Expression Vector** | **1 each** | **N2681** |

Includes:

- 20µg LgBiT Expression Vector

**Storage Conditions:** The LgBiT Expression Vector should be stored at –30°C to –10°C.

| Included Vector | Antibiotic Resistance Cassette |
|---|---|
| LgBiT Expression Vector | Kanamycin |

## Available Separately

### HiBiT Expression Vectors

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **pFC37K HiBiT CMV-neo Flexi® Vector** | **20µg** | **N2391** |
| **pFN38K HiBiT CMV-neo Flexi® Vector** | **20µg** | **N2401** |
| **pBiT3.1-N [CMV/HiBiT/Blast] Vector** | **20µg** | **N2361** |
| **pBiT3.1-C [CMV/HiBiT/Blast] Vector** | **20µg** | **N2371** |

### Luminescent Detection Systems

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **Nano-Glo® Live Cell Assay System** | **100 assays** | **N2011** |
| | **1,000 assays** | **N2012** |
| | **10,000 assays** | **N2013** |
| **Nano-Glo® Vivazine™ Live Cell Substrate** | **0.1ml** | **N2580** |
| | **1ml** | **N2581** |
| | **10ml** | **N2582** |
| **Nano-Glo® Endurazine™ Live Cell Substrate** | **0.1ml** | **N2570** |
| | **1ml** | **N2571** |
| | **10ml** | **N2572** |

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

### Cell Viability and Cell Toxicity Reagents

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| CellTox™ Green Cytotoxicity Assay | 10ml | G8741 |
| | 50ml | G8742 |
| | 100ml | G8743 |
| CellTiter-Glo® 2.0 Assay System | 10ml | G9241 |
| | 100ml | G9242 |
| | 500ml | G9243 |

## 3. Before You Begin

### 3.A. HiBiT Fusion Expression Format

HiBiT fusions can be expressed either exogenously as a HiBiT-target fusion or endogenously by inserting HiBiT into the target genomic locus using CRISPR-Cas9 gene editing. Both approaches result in a HiBiT fusion protein that will bind to LgBiT expressed from the HEK293 LgBiT Cell Line or by transfecting the LgBiT Expression Vector. Choose from MCS-based vectors and Flexi® Vectors to generate exogenous HiBiT target fusions at the N or C terminus.

For information regarding HiBiT CRISPR endogenous tagging or available HiBiT CRISPR cell lines, including instructions for use as well as a list of existing HiBiT CRISPR pools or clones for purchase, visit: **www.promega.com/CRISPR/**

### 3.B. Luminescence Detection Format

To detect the luminescent HiBiT:LgBiT enzyme created with the HEK293 LgBiT Cell Line or LgBiT Expression Vector, use one of the nonlytic Nano-Glo® Live Cell detection systems. The Nano-Glo® Live Cell Assay System is limited to experiments ≤2 hours due to signal decay. The Nano-Glo® Endurazine™ and Vivazine™ Live Cell Substrates provide live-cell detection methods for several hours to days. The Vivazine™ substrate typically shows increased brightness but also an increased rate of signal decay compared to the Endurazine™ substrate. The Endurazine™ substrate will provide the maximum signal stability but lower initial signal intensity compared to other Nano-Glo® Live Cell Substrates.

### 3.C. Multiplexing with Cell Viability Assays

Live-cell luminescence depends on cell number and viability. If studying the modulation of HiBiT protein fusion levels, we recommend measuring cell viability to understand how differences in protein levels compare to changes in cell number or cell viability. Cell viability assays that can multiplex with HiBiT:LgBiT enzyme complementation in the same well include the fluorescent CellTox™ Green Cytotoxicity Assay and luminescent CellTiter-Glo® 2.0 Assay System.

### 3.D. LgBiT Vector Preparation

The amount of plasmid DNA provided with the LgBiT Expression Vector system is sufficient for a few initial testing experiments, but we recommend that the plasmid be archived and propagated as transfection-ready DNA. Follow standard conditions for transformation into *E. coli* for archiving and propagation, and for tissue-culture-grade DNA preparation. For the LgBiT Expression Vector, the LgBiT protein is constitutively expressed by a CMV promoter.

### 3.E. Instrumentation Information and Setup

**Note:** All experiments should be conducted using sterile, white, tissue culture-treated 96- or 384-well plates to minimize luminescence and fluorescence cross talk between wells.

#### Instrument Requirements for Measuring Luminescence

The HEK293 LgBiT Cell Line, LgBiT Expression Vector, and optional multiplexing with the CellTiter-Glo® 2.0 Assay System, require a plate-reading instrument capable of measuring luminescence (e.g., GloMax® Discover System). See Section 6.E for more information on multiplexing with the CellTiter-Glo® 2.0 Assay System.

#### Optional: Instrument Requirements for Optional Multiplexing with CellTox™ Green Cytotoxicity Assay

For multiplexing with the CellTox™ Green Cytotoxicity Assay, a multimodal plate reading instrument (e.g., GloMax® Discover System) capable of measuring luminescence as well as green fluorescence ($485–500nm_{Ex}/520–530nm_{Em}$) is required. See Section 6.D for more information on multiplexing with the CellTox™ Green Cytotoxicity Assay.

#### Optional: Instrument Requirements for Use in NanoBRET™ Target Engagement or Protein:Protein Interaction Assays

HiBiT fusion proteins expressed in combination with the LgBiT Protein are compatible for use as the luminescent donor in NanoBRET™ Target Engagement (TE) and Protein:Protein Interaction (PPI) Assays. To perform NanoBRET™ Assays, a luminometer capable of sequentially measuring dual wavelength windows is required. More information on NanoBRET™ TE and PPI assay design and specific instrumentation requirements for NanoBRET™ signal measurement can be found in the *NanoBRET™ Protein:Protein Interaction System Technical Manual* #TM439 or the relevant target engagement technical manual specific to the target of interest (e.g., *NanoBRET™ Target Engagement Intracellular Kinase Assay, Adherent Format, Technical Manual* #TM598)

## 4. Preparing the HEK293 LgBiT Cell Line

### Materials to be Supplied by User

(Composition of solutions are provided in Section 9.A.)

- HEK293 or similar mammalian cells for use with LgBiT Expression Vector
- white, 96-well plate (Costar Cat.# 3917) or 384 well plate (Corning Cat.# 3570)
- tissue culture equipment and reagents
- DMEM (Gibco Cat.# 11995)
- Opti-MEM® I Reduced Serum Medium, no phenol red (Life Technologies Cat.# 11058-021)
- DPBS (Gibco Cat.# 14190)
- fetal bovine serum (Seradigm Cat.# 89510-194)
- 0.05% trypsin/EDTA (Gibco Cat.# 25300)
- hygromycin B solution (Gibco Cat.# 10-687-010)
- DMSO (Sigma Aldrich Cat.# D2650)
- HiBiT fusion vector of protein of interest (if using exogenous expression of HiBiT fusion)
- reagents and instrumentation for endogenous tagging of HiBiT to protein of interest via CRISPR-Cas9 (if using endogenous expression of HiBiT fusion)
- plate reader capable of measuring luminescence (e.g., GloMax® Discover System [Cat.# GM3000]; Section 3.E)
- FuGENE® HD Transfection Reagent (Cat.# E2311, E2312) for transfecting the LgBiT Expression Vector, HiBiT ectopic vectors or both
- Transfection Carrier DNA (Cat.# E4881, E4882) for transfecting the LgBiT Expression Vector, HiBiT ectopic vectors or both

### 4.A. Cell Thawing and Initial Cell Culture

1. Prepare 40ml of initial cell culture medium by adding 4ml of fetal bovine serum (FBS) to 36ml of DMEM. This initial cell culture medium (no hygromycin) will be used for culturing the cells immediately after thawing.
2. Transfer 9ml of prewarmed initial cell culture medium to a sterile 15ml conical tube.
3. Remove one vial of HEK293 LgBiT Stable Cells from storage at −140°C (or liquid $N_2$ vapor phase) and thaw in a 37°C water bath with gentle agitation (do not invert) until just thawed (typically 3 minutes).
4. Transfer all the cells (approximately 1ml) to the 15ml conical tube containing 9ml of prewarmed initial cell culture medium.
5. Centrifuge at 150 × *g* for 10 minutes at room temperature.
6. Carefully aspirate the medium and resuspend the cell pellet in 15ml of prewarmed cell culture medium (no antibiotics) and place the flask horizontally in a 37°C, 5% $CO_2$ incubator.
7. Incubate the cells for approximately 48–72 hours, or until 70–80% confluent, before passaging.

### 4.B. Cell Maintenance and Propagation

For cell maintenance and propagation starting from the next cell passage, use cell culture medium containing antibiotic (200µg/ml hygromycin). Once established in steady culture, cell viability is typically >95%. For best results, do not allow cells to become greater than 70–80% confluent prior to passaging. Seed cells at the recommended cell seeding densities (cells/cm$^2$) listed in the following table when passaging every 2, 3 or 4 days:

| Every 2 Days | Every 3 Days | Every 4 Days |
|---|---|---|
| $4.0–4.6 \times 10^4$ | $1.7–2.1 \times 10^4$ | $0.8–1.0 \times 10^4$ |

1. On the day of cell passage, aspirate the cell culture medium and wash the cells with 10ml of DPBS.
2. Add 2.5ml of 0.05% trypsin to each flask and incubate at room temperature or in a 37°C, 5% $CO_2$ incubator for 3–5 minutes or until the cells round up and detach from the bottom of the flask.
3. Neutralize the trypsin by adding 7.5ml of cell culture medium to each flask and transfer the cell suspension to a sterile 50ml conical centrifuge tube.
4. Centrifuge at 150 × *g* for 10 minutes at room temperature.
5. Carefully aspirate the medium and resuspend the cell pellet in 5–10ml of prewarmed cell culture medium by gentle mixing with a pipette to create a homogeneous cell suspension.
6. Count the cells and determine percent viability by Trypan blue staining. Calculate the cell numbers needed for replating based on desired cell seeding density per area and destination flask size and number.
7. Add an appropriate amount of cell culture medium with hygromycin to destination flasks to achieve the desired total culture volume per flask (be sure to account for the volume of cell suspension to be added). We suggest that you maintain a consistent ratio of culture volume to culture surface area (e.g., 15ml volume per T75 flask or 30ml volume per T150 flask).
8. Transfer the appropriate volume of cell suspension to new flasks to achieve the desired cell seeding density and total culture medium volume.
9. Place the flasks in a 37°C, 5% $CO_2$ incubator. Incubate the cells for approximately 48–96 hours (depending on plating density) before passaging again.

### 4.C. Cell Freezing and Banking

1. On the day of cell freezing, make new cell freezing medium and keep on ice.

   **Note:** Freezing medium is initial cell culture medium (no hygromycin) plus 5% DMSO.
2. Aspirate the cell culture medium and wash the cells with DPBS (10ml per T75 flask; 20ml per T150 flask).
3. Add 0.05% trypsin to each flask (2.5ml to each T75 flasks, 5ml to each T150 flasks) and incubate at room temperature or place in a 37°C, 5% $CO_2$ incubator for 3–5 minutes or until the cells round up and detach from the bottom of the flask.
4. Neutralize the trypsin by adding cell culture medium to each flask (7.5ml to each T75 flask; 15ml to each T150 flask). Gently mix the cells with a pipette to create a homogeneous cell suspension, then transfer the cell suspension to a sterile 50ml conical centrifuge tube.

### 4.C. Cell Freezing and Banking (continued)

5. Count the cells and determine percent viability by Trypan blue staining.
6. Centrifuge at 150 × *g* for 10 minutes at room temperature.
7. Gently resuspend the cell pellet in ice-cold cell freezing medium to a final cell density of $2 \times 10^6$–$1 \times 10^7$ viable cells/ml. Combine the cell suspensions in a single tube and dispense 0.5–1ml aliquots into 1.2ml or 2.0ml cryovials.
8. Freeze the cells using a controlled-rate freezer (preferred) or with a freezing container or Styrofoam® rack in a –80°C freezer overnight. Transfer the vials to storage at –140°C or below (e.g., liquid $N_2$ vapor phase) for long-term storage. Do not store cells long-term at –80°C.

## 5. Establishing LgBiT Stable Cell Lines

The LgBiT Expression Vector expresses the LgBiT protein in a range of cell types and features LgBiT expressed under the control of the constitutive CMV promoter in mammalian cells. The vector also includes a hygromycin B resistance gene. Clonal cell lines stably expressing LgBiT can be generated using standard transfection and clonal line selection methods that are suitable for the cell type of interest. Mixed stable populations, although generally not considered optimal practice, may be suitable for some research purposes. The LgBiT Expression Vector can also be used to express the LgBiT protein in cell lines that contain an endogenously tagged HiBiT fusion.

The LgBiT Expression Vector features a kanamycin resistance gene for selection and propagation in bacterial cells. We recommend that the LgBiT Expression Vector be propagated and archived in *E. coli* upon receipt. DNA purification should be performed to produce transfection quality plasmid material, ensuring optimal transfection in mammalian cells.

We have transiently transfected HEK293 cells using the following protocol: First, dispense 800,000 cells into each well of a six-well plate and allow cells to adhere for ~4 hours. Next, add 2µg of LgBiT Expression Vector to 100µl of Opti-MEM® I Reduced Serum Medium, no phenol red, and mix well. Then add 6µl of FuGENE® HD Transfection Reagent, mix gently, and incubate for 10 minutes at room temperature. Slowly drop transfection mixture onto plated cells in medium, and gently rock plate to mix. Incubate cells at 37°C, 5% $CO_2$ for 24–48 hours to transfect and express LgBiT protein. These volumes can be scaled to larger formats by keeping a 3:1 ratio of reagent-to-nucleic acid (µl:µg). For additional information and transfection recommendations of other cells lines, visit: **www.promega.com/Resources/Tools/Transfection%20Assistant/**

## 6. Example Application: Targeted Protein Degradation

In this application, the target protein is fused to HiBiT using CRISPR-Cas9 gene editing and paired with the HEK293 LgBiT Stable Cell Line or LgBiT Expression Vector to characterize PROTAC function in live cells.

The HEK293 LgBiT Stable Cells and LgBiT Expression Vector can be used for quantitative luminescent detection of protein degradation kinetics in living cells that have been engineered using CRISPR-Cas9 to generate endogenously tagged HiBiT fusion target proteins. Exogenous expression of HiBiT protein fusions can also be used to monitor target protein levels, however, we recommend this approach only for qualitative assessment of degradation. The calculated degradation rates, $D_{max}$, $DC_{50}$ values and recovery of exogenously expressed proteins differ greatly from those of endogenous proteins, which are regulated by native epigenetic and transcriptional mechanisms.

**Note:** This protocol provides instructions for kinetic luminescence monitoring using a luminometer that is not equipped with $CO_2$ control. For luminometers which are equipped with $CO_2$ control, substitute an appropriate complete growth medium for the cell line being tested for $CO_2$-independent medium, and $CO_2$ should be set to 5%.

**Materials to Be Supplied By User**
(Composition of solutions are provided in Section 9.A.)

- HEK293 LgBiT Cell Line or similar cultured mammalian cells where CRIPSR-Cas9 was used to knock-in HiBiT at the endogenous locus of target protein
- LgBiT Expression Vector if not using a LgBiT-expressing stable cell line
- white, 96-well plate (Corning Cat.# 3917) or 384-well plate (Corning Cat.# 3570)
- tissue culture equipment and reagents
- DPBS (Gibco Cat.# 14190)
- 0.05% trypsin/EDTA (Gibco Cat.# 25300)
- DMEM (Gibco Cat.# 11995)
- fetal bovine serum (Seradigm Cat.# 89510-194)
- Opti-MEM® I Reduced Serum Medium, no phenol red (Gibco Cat.# 11058-021) if using LgBiT plasmid
- FuGENE® HD Transfection Reagent (Cat.# E2311) if using LgBiT plasmid
- Nano-Glo® Endurazine™ Live Cell Substrate (Cat.# N2570/1/2) for extended kinetic monitoring
- $CO_2$-independent medium (Gibco Cat.# 18045-088) for extended kinetic monitoring in a luminometer not equipped with $CO_2$ control
- test PROTAC(s)

### 6.A. Transiently Transfecting LgBiT Expression Vector

**Note:** If using a HiBiT CRISPR line developed with the HEK293 LgBiT Cell Line or other LgBiT-expressing cell line, skip to Section 6.B.

1. Culture CRISPR-Cas9 HiBiT knock-in HEK293 cells appropriately prior to assay.
2. Remove medium from cell flask by aspiration, wash cells with DPBS, trypsinize and allow cells to dissociate from the flask bottom.
3. Neutralize trypsin using cell culture medium, count cells to estimate density and resuspend to a final density of $4 \times 10^5$ cells/ml in cell culture medium.

### 6.A. Transiently Transfecting LgBiT Expression Vector (continued)

4. Plate 2ml of cells (800,000 cells) into each well of a six-well plate sufficient for the number of planned assays. After transfection and cell division, three wells of a six-well plate yield enough cells for assaying one 96-well plate. For larger scale experiments, transfect cells in T flasks or dishes, scaling the quantity of transfection materials accordingly.
5. Allow the cells to attach and recover for 4–6 hours at 37°C, 5% $CO_2$.
6. Prepare a transfection mixture consisting of 2µg LgBiT plasmid diluted in 100µl of Opti-MEM® I Reduced Serum Medium, no phenol red.

   **Notes:**

   1. The amount of LgBiT plasmid may need to be adjusted based on the expression level of the endogenous target protein, and the background LgBiT signal observed in the non-HiBiT-expressing parental cell line. For low-expressing targets, we recommend testing LgBiT plasmid dilutions of 10- to 100-fold in Transfection Carrier DNA (Cat.# E4881).
   2. Transfection reagents should be equilibrated to room temperature.
7. Add 6µl of FuGENE® HD Transfection Reagent, mix well and incubate at room temperature for 10 minutes.
8. Add transfection mixture to wells with attached cells, and express overnight (18–24 hours) at 37°C, 5% $CO_2$.
9. Proceed to Section 6.B.

### 6.B. Plating Cells into Multiwell Plates

1. Remove medium from cells by aspiration, wash cells with DPBS, dispense 0.05% trypsin-EDTA, and allow cells to dissociate from the flask bottom.
2. Neutralize trypsin using serum-containing cell culture medium, mix to collect and resuspend cells, and transfer cell suspension to a conical tube.
3. Spin cells down at 125 × *g* for 5 minutes. Discard cell culture medium and resuspend cells in an equal volume of cell culture medium.
4. Count to estimate cell density and adjust density to $2 \times 10^5$ cells/ml in assay medium. To cover an entire 96-well plate, you need at least 10ml of cells at this density. For a 384-well plate, you need approximately 16ml of cells at this density.
5. Plate cells for each experimental condition as follows:

   **96-well format:** Dispense 100µl of cells prepared in Step 4 into at least 3–4 wells.

   **384-well format:** Plate 36µl of cells prepared in Step 4 in at least 3–4 wells. **Note:** For the 384-well format, the assay is performed with a mixture of growth medium and $CO_2$-independent medium.
6. Incubate plates at 37°C, 5% $CO_2$ overnight (18–24 hours).
7. If performing kinetic luminescence detection without cytotoxicity multiplexing, proceed to Section 6.C. If multiplexing kinetic luminescence detection with the CellTox™ Green Cytotoxicity Assay, proceed to Section 6.D.

### 6.C. Assaying Degradation

1. Prepare substrate as follows:

   **96-well format:** Prepare a 1X solution of Nano-Glo® Endurazine™ Live Cell Substrate by diluting stock reagent 1:100 in $CO_2$-independent medium + 10% FBS.

   **384-well format:** Prepare a 2X solution of Nano-Glo® Endurazine™ Live Cell Substrate by diluting stock reagent 1:50 in $CO_2$-independent medium + 10% FBS.

2. Add Endurazine™ solution to each well.

   **96-well format:** Aspirate medium and dispense 90µl of 1X Endurazine™ solution.

   **384-well format:** Dispense 36µl of 2X Endurazine™ solution onto 36µl of cells. Do **not** aspirate cell medium.

3. Incubate plate for 2.5 hours at 37°C, 5% $CO_2$ to equilibrate luminescence.

4. Prepare a 10X concentration of test PROTAC titration (starting at 10µM) in $CO_2$-independent medium + 10% FBS.

   **96-well format:** Add 10µl to each well for a final concentration of 1µM at the highest point.

   **384-well format:** Add 8µl to each well for a final concentration of 1µM at the highest point.

5. Prepare a 10X concentration of DMSO to use as a negative control by adding an equivalent amount of DMSO to $CO_2$-independent medium + 10% FBS.

   **96-well format:** Add 10µl to each negative control well.

   **384-well format:** Add 8µl to each negative control well.

6. Collect kinetic measurements in a luminometer equilibrated to 37°C.

7. If multiplexing with the CellTiter-Glo® 2.0 Assay for endpoint cell viability testing, proceed to Section 6.E.

8. Proceed to Section 6.F for Kinetic Degradation Analysis.

### 6.D. Kinetic Multiplexing of the Degradation Assay with the CellTox™ Green Cytotoxicity Assay

Cell health and test PROTAC toxicity can be measured kinetically with the use of the CellTox™ Green Cytotoxicity Assay. The cytotoxicity assay can be multiplexed in the same well as the degradation protocol described. The CellTox™ Green Assay measures membrane integrity with a cyanine dye that enters and binds the DNA of compromised cells, producing a fluorescent signal.

1. Thaw the CellTox™ Green Dye, 1,000X, in a 37°C water bath.

2. Prepare substrate with CellTox™ Green Dye as follows:

   **96-well format:** Prepare a 1X solution by diluting Nano-Glo® Endurazine™ Live Cell Substrate stock reagent 1:100 and CellTox™ Green Dye 1:1,000 in $CO_2$-independent medium + 10% FBS.

   **384-well format:** Prepare a 2X solution by diluting Nano-Glo® Endurazine™ Live Cell Substrate stock reagent 1:50, and CellTox™ Green Dye 1:500 in $CO_2$-independent medium + 10% FBS.

### 6.D. Kinetic Multiplexing of the Degradation Assay with the CellTox™ Green Cytotoxicity Assay (continued)

3. Add Endurazine™ and CellTox™ Green solution to each well.

   **96-well format:** Aspirate medium and dispense 90µl of 1X Endurazine™ + CellTox™ Green solution.

   **384-well format:** Dispense 36µl of 2X Endurazine™ + CellTox™ Green solution to 36µl of cells. Do **not** aspirate cell medium.

4. Incubate plate for 2.5 hours at 37°C, 5% $CO_2$ to equilibrate luminescence.

5. **Recommended cytotoxicity positive control:** Add 2µl lysis solution to control wells, incubate for 15–20 minutes before measuring fluorescence.

6. Prepare a 10X concentration of test PROTAC titration (starting at 10µM) in $CO_2$-independent medium + 10% FBS.

   **96-well format:** Add 10µl to each well for a final concentration of 1µM at the highest point.

   **384-well format:** Add 8µl to each well for a final concentration of 1µM at the highest point.

7. Prepare a 10X concentration of DMSO to use as a negative control by adding an equivalent amount of DMSO to $CO_2$-independent medium + 10% FBS.

   **96-well format:** Add 10µl to each negative control well.

   **384-well format:** Add 8µl to each negative control well.

8. Collect kinetic measurements of luminescence and fluorescence (485–500$nm_{Ex}$/520–530$nm_{Em}$) in a luminometer equilibrated to 37°C.

9. Proceed to Section 6.F for Kinetic Degradation Analysis.

### 6.E. Endpoint Cell Viability Multiplexing with the CellTiter-Glo® 2.0 Assay

Cell health can be measured with the ready-to-use luminescent CellTiter-Glo® 2.0 Assay that quantitates the amount of ATP present, demonstrating the presence of metabolically active cells.

1. Equilibrate the CellTiter-Glo® 2.0 Reagent to room temperature.

2. Following degradation measurements, add CellTiter-Glo® 2.0 Reagent.

   **96-well format:** Add 100µl of CellTiter-Glo® 2.0 Reagent to each well of the plate.

   **384-well format:** Add 50µl of CellTiter-Glo® 2.0 Reagent to each well of the plate.

3. Mix on a plate shaker at 500–700rpm for 5 minutes.

4. Incubate the plate at room temperature for 30 minutes to lyse cells and quench the HiBiT signal.

5. After completing the 30-minute incubation, measure luminescence on a luminometer. If using the GloMax® Discover Instrument, read the signal by selecting the CellTiter-Glo® protocol.

### 6.F. Kinetic Degradation Analysis

The luminescent kinetic measurements collected from endogenously expressed proteins provide several degradation parameters that can be used to characterize different PROTAC compounds or responses from different family members to the same PROTAC compound. These parameters include the degradation rate, degradation maximum ($D_{max}$), and $DC_{50}$. To generate degradation profiles from which to calculate these parameters, the relative luminescent units (RLUs) for each PROTAC concentration must first be normalized to the replicate averaged No PROTAC condition at every time point to account for changes in free furimazine concentration over time. This results in a degradation curve that is expressed as "Fractional RLU" (Equation 1).

**Equation 1:** $$\text{Fractional RLU} = \frac{RLU_{PROTAC}}{RLU_{No\ PROTAC}}$$

From the degradation profiles, a single-component exponential decay model (Equation 2) can be fit to the initial degradation portion of each curve to the point where the data reaches a plateau. Consider excluding the first few data points from the fit as there may be a brief lag before observed degradation.

**Equation 2:** $y = (y_0 - \text{Plateau})e^{-\lambda t} + \text{Plateau}$

Using Equation 2, the parameter $\lambda$ represents the degradation rate constant, and the Plateau represents the lowest amount of protein remaining. $D_{max}$ is expressed as the maximum fractional amount of degraded protein and is calculated as 1 − Plateau. Plotting $D_{max}$ for each concentration of PROTAC yields a degradation potency curve and $DC_{50}$ value that accounts for any difference in time that each concentration takes to reach its $D_{max}$.

## 7. Representative Data for Kinetic Degradation

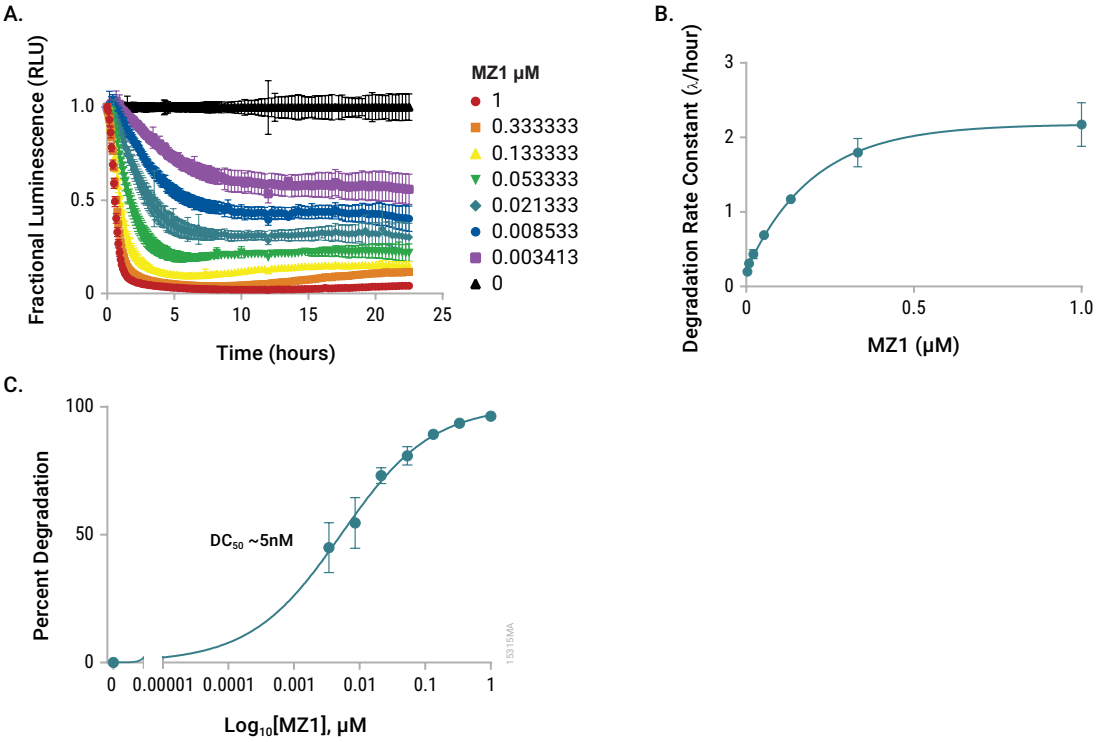


**Figure 2. Endogenous HiBiT-BRD4 kinetic degradation assay.** HEK293 cells stably expressing LgBiT were engineered using CRISPR-Cas9 to express endogenous HiBiT-BRD4 and plated as described. Medium was replaced with $CO_2$-independent medium containing Nano-Glo® Endurazine™ substrate for 2.5 hours before adding a titration of 1µM MZ1 PROTAC (3). Kinetic luminescence measurements of degradation at each PROTAC concentration (**Panel A**) were collected on a GloMax® Discover, and calculations for degradation rate (**Panel B**), and $D_{max}$ (**Panel C**) were made according to Section 6.F.

## 8. Troubleshooting

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

| Symptoms | Possible Causes and Comments |
|---|---|
| Low or no luminescence measured | HiBiT fusion is not expressed. If performing CRISPR-Cas9 gene editing to introduce the HiBiT tag, be sure that the target is expressed in the cell line being used. |
| | Low cell viability can lead to low luminescence readout. When testing compound treatment, multiplex with CellTiter-Glo® 2.0 Cell Viability Assay or CellTox™ Green Cell Toxicity Assay to assess the effect of test compounds on cell viability. |
| | Insufficient cell number per well can lead to low luminescence. Handle and plate cells as instructed to ensure sufficient number of viable cells per well. |
| | To confirm LgBiT expression in the HEK293 LgBiT Stable Cell Line or in cells transfected with the LgBiT Expression Vector, measure luminescence using the Nano-Glo® HiBiT Lytic Detection System (Cat.# N3030) with the HiBiT Control Protein (Cat.# N3010) in place of the LgBiT Protein. See the *Nano-Glo® HiBiT Lytic Detection System Technical Manual* #TM516 for more information. Alternatively, perform a Western blot using the Anti-LgBiT Monoclonal Antibody (Cat.# N7100). |
| Cells do not grow after thawing | Be sure to follow all instructions on thawing and propagating cells with proper cell density upon receipt. |
| No decrease in RLU when treating with a known degradation compound | Overexpression of a target protein can mask degradation. Reduce the relative level of HiBiT fusion expression if using exogenous expression, or test target as a HiBiT CRISPR fusion if possible. |

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## 9. Appendix

### 9.A. Composition of Buffers and Solutions

**Initial Cell Culture Medium**

| | |
|---|---|
| 90% | DMEM |
| 10% | fetal bovine serum |

**Cell Culture Medium**

| | |
|---|---|
| 90% | DMEM |
| 10% | fetal bovine serum |
| 200µg/ml | hygromycin B Solution |

**Cell Freeze Medium**

| | |
|---|---|
| 85% | DMEM |
| 10% | fetal bovine serum |
| 5% | DMSO |

### 9.B. References


1. Riching, K.M. *et al*. (2018) Quantitative live-cell kinetic degradation and mechanistic profiling of PROTAC mode of action. *ACS Chem. Biol.* **13**, 2758–70.
2. Schwinn, M.K. *et al*. (2017) CRISPR-mediated tagging of endogenous proteins with a luminescent peptide. *ACS Chem. Biol.* **13**, 467–74.
3. Zengerle, M., Chan, K.H. and Ciulli, A. (2015) Selective small molecule induced degradation of the BET bromodomain protein BRD4. *ACS Chem. Biol.* **10**, 1770–7.

## 9.C. Related Products

| Product | Size | Cat.# |
|---|---|---|
| NanoBRET™ VHL Ternary Complex Starter Kit | 1 each | ND2700 |
| NanoBRET™ CRBN Ternary Complex Starter Kit | 1 each | ND2720 |
| NanoBRET™ Proteasomal Recruitment Starter Kit | 1 each | ND2730 |
| NanoBRET™ Ubiquitination Starter Kit | 1 each | ND2690 |
| NanoBRET™ Nano-Glo® Detection System | 200 assays | N1661 |
| | 1,000 assays | N1662 |
| | 10,000 assays | N1663 |
| NanoBRET™ Nano-Glo® Kinetic Detection System | 200 assays | N2583 |
| | 1,000 assays | N2584 |
| | 10,000 assays | N2585 |
| Nano-Glo® HiBiT Lytic Detection System | 10ml | N3030 |
| | 100ml | N3040 |
| | 10 × 100ml | N3050 |
| NanoBRET™ TE Intracellular HDAC Assays | 100 assays | N2080 |
| | 1,000 assays | N2081 |
| NanoBRET™ TE Intracellular BET BRD Assays | 100 assays | N2130 |
| | 1,000 assays | N2131 |
| NanoBRET™ TE Intracellular Kinase Assay, K-4 | 100 assays | N2520 |
| | 1,000 assays | N2521 |
| NanoBRET™ TE Intracellular Kinase Assay, K-5 | 100 assays | N2500 |
| | 1,000 assays | N2501 |
| HiBiT Control Protein | 100µl | N3010 |
| Anti-LgBiT Monoclonal Antibody | 100µg | N7100 |

### Multimode Detection Instrument

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

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