Kinetically Detecting and Quantitating PROTAC-Induced Degradation of Endogenous HiBiT-Tagged Target Proteins

Promega Corporation

Materials Required

- HEK293, LgBiT HEK293 stable cell line (available through **Custom Assay Services**) or similar cultured mammalian cells CRISPR-edited to knock-in HiBiT at the endogenous locus of the target protein
- LgBiT expression plasmid (available through **Custom Assay Services**; not needed if using LgBiT expression cell line)
- white, 96-well plate (Costar® Cat.# 3917) or 384-well plate (Corning® Cat.# 3570)
- tissue culture equipment and reagents
- DPBS (Invitrogen Cat.# 14190)
- 0.05% Trypsin/EDTA (Invitrogen Cat.# 25300)
- DMEM (Gibco Cat.#11995)
- Fetal bovine serum (Seradigm Cat.# 1500-050)
- Opti-MEM® I Reduced Serum Medium, no phenol red (Life Technologies 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) for extended kinetic monitoring
- CO_2-independent medium (Gibco Cat.# 18045-088) for extended kinetic monitoring in the absence of CO_2 control
- test PROTAC(s)

Media Composition

Cell Culture Medium

- 90% DMEM
- 10% FBS

Assay Medium

- 90% CO_2-independent medium or 90% DMEM if using a luminometer equipped with CO_2 control
- 10% FBS

Overview

Targeted protein degradation by PROTACs or other small molecule compounds is a powerful approach for studying the efficacy of removing a target protein from the cell. This protocol describes quantitative luminescent detection of target protein degradation kinetics in living cells that have been engineered using CRISPR-Cas9 to express HiBiT fused to a target protein. Detailed instructions for calculating and obtaining quantitative degradation parameters, rate, Dmax and DC_{50} are included.

Vector-based transient or stable expression of NanoLuc® or HiBiT protein fusions can also be used to monitor target protein levels. However, we recommend this approach only for *qualitative* degradation measurements. The calculated degradation rates, Dmax, DC_{50} values and recovery of ectopically expressed proteins differ greatly from those of endogenous proteins, which are regulated by native epigenetic and transcriptional mechanisms. If transient expression is desired, we recommend low expression levels either by using a thymidine kinase (TK) promoter, or diluting CMV plasmids in the transfection. For more information on HiBiT fusion protein expression levels, refer to the *Nano-Glo® HiBiT Lytic Detection System Technical Manual* #TM516.

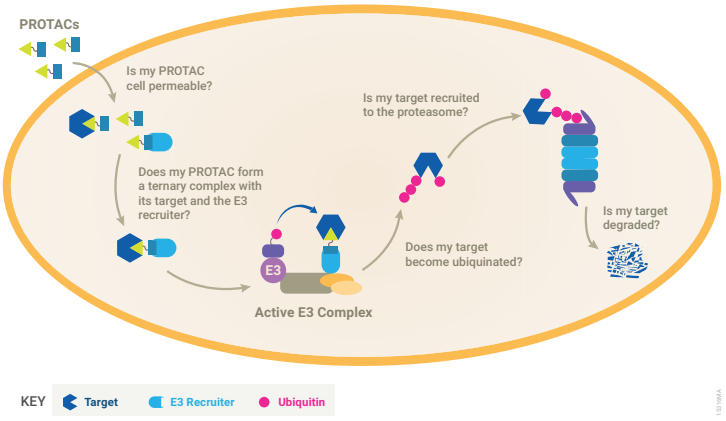

Figure 1. Overview of PROTAC-induced target protein degradation.

Protocol

Day 1: Transient Transfection of HEK293 Cells

Skip this section if using cells stably expressing LgBiT and proceed to Day 2.

1. Culture HEK293 cells appropriately prior to assay.
2. Remove medium from cell flask by aspiration, 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 4×10^5 cells/ml in cell culture medium.
4. Plate 2ml of cells (800,000 cells) per well into a six-well plate. 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.
   Note: The amount of LgBiT plasmid may need to be adjusted based on a) the expression level of the endogenous target protein, and b) the background LgBiT signal observed in the non-HiBiT-expressing parental cell line. For low expressing targets, we recommend testing dilution of LgBiT plasmid 10- to 100-fold in carrier DNA.
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.

Day 2: Replating Transfected HEK293 Cells into Multiwell Plates

1. For each well in a six-well plate, remove medium from cells and wash with 1ml of DPBS. Discard.
2. Add 0.5ml of 0.05% trypsin-EDTA and incubate at room temperature until cells lift from the well bottom.
3. Add 2ml of cell culture medium to neutralize trypsin, mix to collect and resuspend cells, and transfer cell suspension to a 15ml conical tube.
4. Spin cells down at 125 × *g* for 5 minutes. Discard cell culture medium and resuspend in an equal volume of fresh cell culture medium.
5. Count to estimate cell density and adjust density to 2×10^5 cells/ml in cell culture medium. To cover an entire 96-well plate, you need at least 10ml of cells at this concentration. For a 384-well plate, you need approximately 16ml of cells at this concentration.
6. Plate 100µl of cells into a 96-well or 40µl of cells into a 384-well plate.
8. Incubate plates at 37°C, 5% CO_2 overnight (18–24 hours).

Day 3: Degradation Assay

1. Prepare a 1X solution of Endurazine™ substrate in assay medium by diluting stock reagent 1:100.
2. Aspirate cell culture medium from plate and add 90µl (96-well) or 45µl (384-well) of Endurazine™ solution to each well.
3. Incubate plate for 2.5 hours at 37°C, 5% CO_2 to equilabrate the luminescence.
4. Prepare a 10X concentration of test PROTAC titration (starting at 10µM) in assay medium and add 10µl to each well of 96-well plate or 5µl for 384-well plate for a final concentration of 1µM at the highest point.
5. Collect kinetic measurements of luminescence in luminometer pre-equilibrated to 37°C.

Optional: Endpoint Multiplexing with the CellTiter-Glo® 2.0 Assay

Cell health can be measured with the ready-to-use CellTiter-Glo® 2.0 Assay (Cat.# G9241), a luminescent assay that quantitates the amount of ATP present, which indicates the presence of metabolically active cells.

1. Equilibrate CellTiter-Glo® 2.0 Reagent to room temperature.
2. Following degradation measurements, add 100µl of CellTiter-Glo® 2.0 Reagent per well of the plate, and mix on a plate shaker at 500–700rpm for 5 minutes.
3. Incubate the plate at room temperature for 30 minutes to allow cell lysis and quenching of HiBiT signal.
4. After the 30-minute incubation is complete, measure total luminescence on a luminometer. If using the GloMax® Discover Instrument, read the plate by selecting the CellTiter-Glo® protocol.

Kinetic Degradation Analysis

Note: If your luminometer lacks humidity control, measure luminescence with the lid on, if possible, but avoid conditions that promote condensation on the lid. If using a GloMax® Discover instrument, include a heating step at 37°C in your kinetic loop. Software update 3.1.0 adjusts the temperature of heating elements within the instrument to prevent condensation on the lid.

From the kinetic luminescence measurements collected from endogenously expressed proteins, several degradation parameters can be quantitated to characterize different PROTAC compounds or responses from different family members to the same PROTAC compound. These parameters include the degradation rate, degradation maximum (Dmax) and DC_{50}. To generate degradation profiles from which to calculate these parameters, the raw luminescence (measured in relative light units [RLUs]) for each PROTAC concentration must first be normalized to the replicate averaged "No PROTAC" condition at every time point to account for the steady decline in RLU due to substrate depletion. This results in a degradation curve that is expressed as Fractional RLU (Equation 1).

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

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. Excluding the first few data points from the fit may be helpful as there may be a brief lag before observing degradation.

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

In Equation 2, the parameter ƛ represents the degradation rate constant, and the Plateau represents the maximal amount of protein loss. Dmax is expressed as the maximum fractional amount of degraded protein, and is calculated as 1–Plateau. Plotting Dmax 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 Dmax.

Representative Data

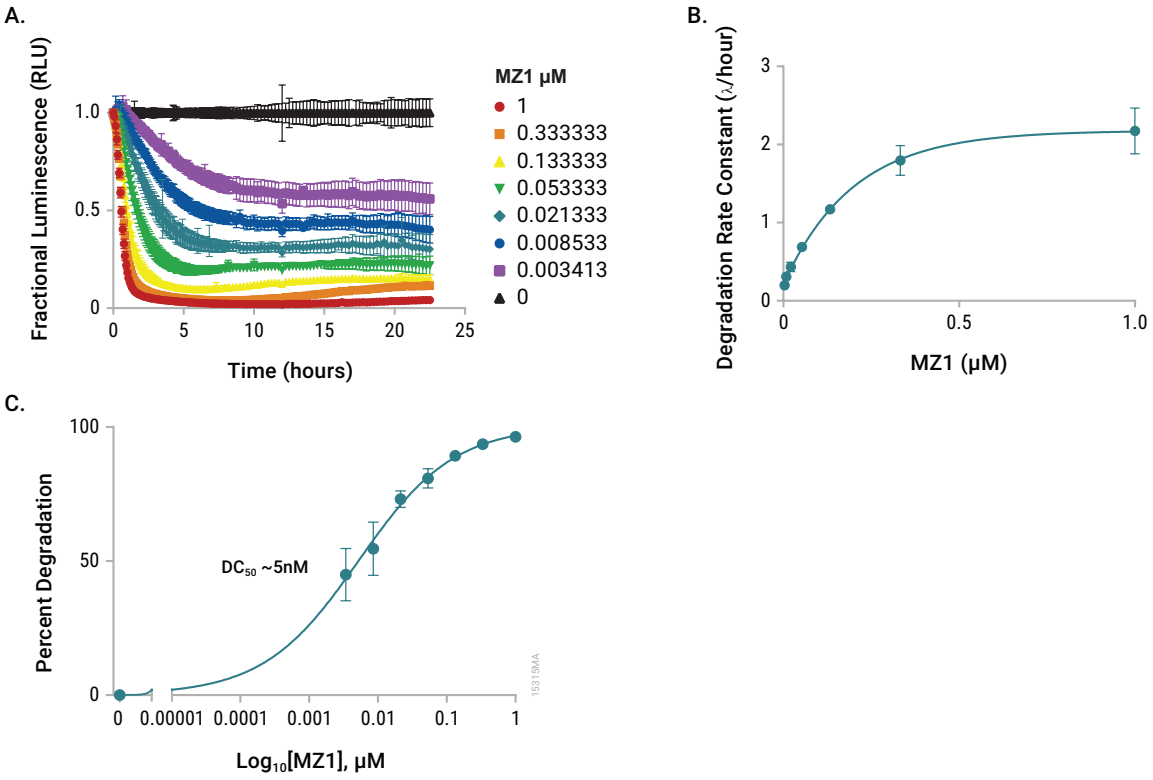

Figure 2. Degradation kinetics of endogenous HiBiT-BRD4 following PROTAC treatment. HEK293 cells stably expressing LgBiT were engineered using CRISPR-Cas9 to express endogenous HiBiT-BRD4 and plated as described. Medium was replaced with CO_2-independent medium containing Nano-Glo® Endurazine™ substrate for 2.5 hours, before adding a titration of 1µM MZ1 PROTAC. Kinetic luminescence measurements of degradation at each PROTAC concentration (**Panel A**) were collected on a GloMax® Discover instrument, and calculations for degradation rate (**Panel B**) and Dmax (**Panel C**) were made according to the previous section on Kinetic Degradation Analysis.