Detecting Ubiquitination of Target Proteins in Live Cells using a NanoBRET™ Assay

Promega Corporation

Materials Required

- HEK293 or similar cultured mammalian cells
- white, 96-well plates (Costar® Cat.# 3917) or 384-well plates (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)
- DMSO (Sigma-Aldrich® Cat.# 2650)
- Nuclease-Free Water (Cat.# P1191)
- FuGENE® HD Transfection Reagent (Cat.# E2311)
- NanoBRET™ Ubiquitin Assay Kit (contact Custom Assay Services at **CAS@promega.com** for information on obtaining this kit)
  - HaloTag® Ubb acceptor plasmid
  - NanoLuc® BRD4 donor plasmid (positive control)
  - HaloTag® Negative Control Vector
  - NanoBRET™ Nano-Glo® Detection System
- NanoLuc®-fused target donor plasmid
  Note: A collection of NanoLuc® target fusions consisting of kinases, epigenetic proteins, nuclear receptors and transcription factors are available on **promega.com**, or through **Custom Assay Services**. Alternatively, ORFs can be obtained through **Find My Gene** and subcloned into NanoLuc® fusion vectors using either **Flexi®** or **multiple cloning site (MCS)** compatible cloning strategies.
- Endpoint Assay: NanoBRET™ Nano-Glo® Detection System (Cat.# N1661)
- Kinetic-Live Mode Assay: Nano-Glo® Vivazine™ Live Cell Substrate (Cat.# N2580) for extended kinetic monitoring longer than 1 hour and HaloTag® NanoBRET™ 618 Ligand (Cat.# G9801)
- test PROTAC(s)
- Optional Positive Controls
  - BRD4 PROTACS: dBET1 (Cayman Chemical Cat.# 18044), MZ1 (Tocris Cat.# 6154), dBET6 (MedChemExpress Cat. # HY-112588), ARV-771 (MedChemExpress Cat.# HY-100972)

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 is designed for detecting and optimizing PROTAC-induced ubiquitination of a target protein. The assay is based on NanoBRET™ technology, a proximity-based method dependent upon energy transfer from a luminescent donor to a fluorescent acceptor.

The live-cell assay described here uses the target protein fused to NanoLuc® luciferase as the energy donor and an N-terminal fusion of HaloTag® protein to ubiquitin as the energy acceptor. The assay is based on ectopic expression of both fusions that are introduced into the cell using transient transfection. Ectopic expression of the protein fusion partners provides an accurate method for measuring target ubiquitination that requires minimal cellular engineering. However, there are alternative strategies for generating the target protein energy donor. Endogenous target proteins tagged with NanoLuc® luciferase or HiBiT using CRISPR-Cas9 are one option. Ectopic expression of HiBiT protein fusions is an additional alternative. Use of HiBiT-tagged proteins in this assay will also require intracellular expression of its complementation partner LgBiT. Further considerations for using alternate target protein tagging strategies are discussed at the end of this protocol.

Instrument Requirements

To perform NanoBRET™ assays, use an instrument capable of measuring dual-filtered luminescence values. The NanoBRET™ bioluminescent donor emits at 460nm. To measure the donor signal, we recommend a band pass (BP) filter that covers close to 460nm with a band pass range of 8–80nm. The NanoBRET™ acceptor emits at 618nm. To measure the acceptor signal, we recommend a long pass (LP) filter starting at 600–610nm. For more information on instrument recommendations please see the *NanoBRET™ Protein:Protein Interaction System Technical Manual* #TM439.

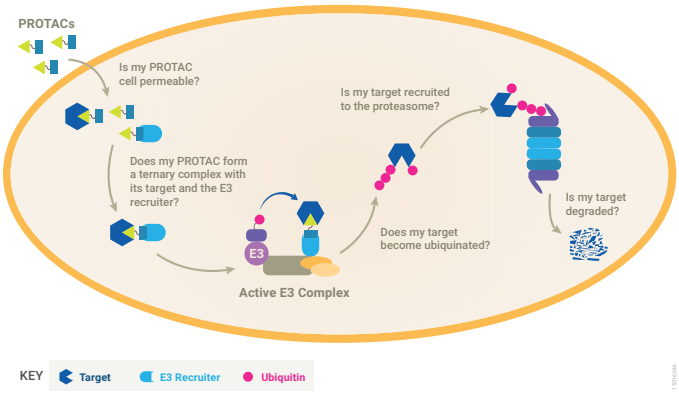

Figure 1. Key steps in PROTAC-mediated protein degradation. PROTACs permeate cells to engage target proteins with E3 ligase machinery, resulting in ubiquitination and proteasomal degradation of the target proteins.

Media Composition

Cell Culture Medium

- 90% DMEM
- 10% fetal bovine serum (FBS)

Assay Medium

- 96% Opti-MEM® I Reduced Serum Medium, no phenol red
- 4% FBS

Protocol

For testing any new target in this assay, we recommend fusing NanoLuc® luciferase in both orientations to the N or C terminus of the target protein to test for maximal spatial proximity and energy transfer to the acceptor protein. For new targets, testing dilutions of donor DNA relative to acceptor DNA will ensure optimal saturation of donor molecules that will result in the best possible assay window. We recommend testing donor-to-acceptor ratios of 1:1, 1:10 and 1:100. Specific recommended DNA amounts are included below. This protocol can be used for cell types other than HEK293, but the transfection conditions may require further optimization.

As a positive control, NanoLuc®-BRD4 plasmid can be used in place of the donor DNA in the transfections at a 1:100 ratio of donor to acceptor, and BRD4 ubiquitination can be induced by treatment with BET family PROTACs dBET1, MZ1, dBET6 or ARV-771. As a negative control, unfused HaloTag® control plasmid can be used in place of the acceptor DNA in the transfections, maintaining the same donor to acceptor ratio by using the same amount of HaloTag® DNA as for the acceptor DNA.

Day 1: Transient Transfection of HEK293 Cells

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 DNA amounts according to the following table.

DNA Ratio (NanoLuc:HaloTag)	NanoLuc® donor plasmid*	HaloTag® acceptor plasmid
1:1	1µg vector	1µg Ubiquitin vector
1:10	0.2µg vector	2µg Ubiquitin vector
1:100	0.02µg vector	

*Dilutions made in water

7. Add 100µl of Opti-MEM® I Reduced Serum Medium, no phenol red, to the transfection mixture, and mix well.
8. Add 6µl of FuGENE® HD Transfection Reagent and incubate at room temperature for 10 minutes.
9. Add transfection mixture to wells with attached cells and express proteins for approximately 20 hours at 37°C, 5% CO_2.

Day 2: Replating Transfected HEK293 Cells into Multiwell Plates and Adding HaloTag® Ligand

1. For each well in a six-well plate, remove medium from cells and wash with 1ml of DPBS.
2. Add 0.5ml of 0.05% trypsin-EDTA and incubate at room temperature until cells lift from 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 assay medium.
5. Count to estimate cell density and adjust density to 2×10^5 cells/ml in assay 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. Divide cells into two pools and add HaloTag® NanoBRET™ 618 Ligand or DMSO vehicle as follows:
   Experimental samples (+ ligand): Add 1µl of 0.1mM HaloTag® NanoBRET™ 618 Ligand per milliliter of cells (100nM final concentration).
   No-acceptor controls (– ligand): Add 1µl of DMSO per milliliter of cells (0.1% final concentration).
7. Plate cells in the volumes indicated below:
   96-well format: Dispense 100µl of each pool of the cells prepared in Step 6 in at least 3–4 wells.
   384-well format: Dispense 40µl of each pool of the cells prepared in Step 6 in at least 3–4 wells.
8. Incubate plates at 37°C, 5% CO_2 overnight (18–24 hours).

Day 3: NanoBRET™ Live Cell Ubiquitination Assay

A. Live Mode—Endpoint

1. Prepare a 5X concentration of test PROTAC titration in Opti-MEM® I Reduced Serum Medium, no phenol red, and add 25µl to each well of a 96-well plate or 10µl for a 384-well plate for a final concentration of 1–10µM at the highest point.
2. Incubate plates at 37°C, 5% CO_2 for 1–4 hours.
3. Prepare a 6X solution of NanoBRET™ Nano-Glo® Substrate in Opti-MEM® I Reduced Serum Medium, no phenol red. This is an 83-fold dilution of the stock reagent. Add 25µl to each well of a 96-well plate or 10µl for a 384-well plate. For one 96-well plate, prepare a minimum of 2.5ml of medium + 30µl of stock reagent. For one 384-well plate, prepare a minimum of 4ml of medium + 48µl of stock reagent. For both multiwell formats, we recommend preparing at least 10% extra solution, especially if using automated dispensing.

   Note: Use the 6X solution within 2 hours if stored at room temperature or within 4 hours if stored at 4°C.
4. Shake plate to mix for 30 seconds. We recommend using an electromagnetic mixer for the 384-well format.
5. Measure donor emission (460nm) and acceptor emission (618nm) within 10 minutes of substrate addition using a NanoBRET™ PPI Assay-compatible luminometer (see Instrument Requirements).

B. Live Mode—Kinetic

1. Prepare a 1X solution of Vivazine™ substrate in Opti-MEM® I Reduced Serum Medium, no phenol red, by diluting stock reagent 1:100.
2. Aspirate medium from plate and add 90µl (96-well) or 45µl (384-well) Vivazine™ solution to each well.
3. Incubate plate for 60 minutes at 37°C, 5% CO_2 to equilibrate luminescence output.
4. Prepare a 10X stock of test PROTAC in Opti-MEM® I Reduced Serum Medium, no phenol red, for titration and add 10µl (96-well) or 5µl (384-well) to each well for a final concentration of 1–10µM at the highest point.
5. Collect kinetic measurements of donor emission (460nm) and acceptor emission (618nm) immediately after adding PROTAC titration using a NanoBRET™ PPI Assay-compatible luminometer (see Instrument Requirements) every 3–5 minutes.

NanoBRET™ Calculations

Divide the acceptor emission value (e.g., 618nm) by the donor emission value (e.g., 460nm) for each sample to generate raw NanoBRET™ ratio values. To remove background BRET due to donor bleedthrough in the acceptor channel, substract the BRET ratio calculated in the absence of HaloTag® NanoBRET™ 618 Ligand (average of no ligand control samples) from the BRET ratio calculated for each sample in the presence of ligand. Convert raw BRET units to milliBRET units (mBU) by multiplying each raw BRET value by 1,000.

$$\left[\left(\frac{618_{Em}}{460_{Em}}\right)_{Ligand} - \left(\frac{618_{Em}}{460_{Em}}\right)_{No\ Ligand}\right] \times 1{,}000 = \text{Corrected BRET ratio in mBU}$$

1530BMA

Figure 2. Corrected BRET ratio calculation.

Considerations for Alternate Energy Donor Strategies

The live-cell ubiquination assay can also be used with alternate strategies to tag the target protein, creating the energy donor. Endogenous target proteins can be tagged with NanoLuc® using CRISPR/Cas9. Alternatively, target proteins can be fused to the HiBiT tag and expressed either ectopically or through endogenous tagging. If using one of these alternative tagging strategies to create the energy donor, the transfection mixture for Day 1 should be modified according to the following table. If using HiBiT-fused target protein as the donor, LgBiT plasmid (CAS Part# CS1956B03) will also need to be included in the transfection mixture unless the cells stably express LgBiT protein.

Donor Expression Format	DNA Ratio	HaloTag® Plasmid	NanoLuc®, HiBiT or LgBiT Amounts*
	HiBiT:LgBiT:HaloTag		
Transient HiBiT fusion to target	1:1:2	1µg Ubiquitin vector	0.5µg HiBiT vector 0.5µg LgBiT vector
	1:1:10	2µg Ubiquitin vector	0.2µg HiBiT vector 0.2µg LgBiT vector
	1:1:100		0.02µg HiBiT vector 0.02µg LgBiT vector
	LgBiT:HaloTag		
Endogenous HiBiT fusion	1:1	1µg Ubiquitin vector	1µg LgBiT vector
	1:10	2µg Ubiquitin vector	0.2µg LgBiT vector
	1:100		0.02µg LgBiT vector
Endogenous NanoLuc® fusion OR Endogenous HiBiT fusion w/ stable LgBiT expression	N/A	2µg Ubiquitin vector	N/A

*Dilutions made in water

Representative Data

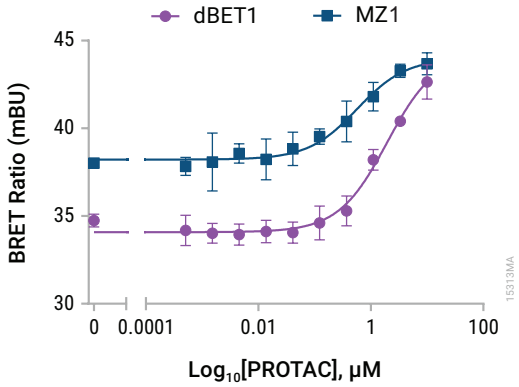

Figure 3. BRD4 ubiquitination following PROTAC treatment using a live-cell endpoint assay. HEK293 cells were transfected with NanoLuc®-BRD4 and HaloTag®-Ubiquitin plasmids at a 1:100 donor:acceptor ratio, plated at the density described in the presence of HaloTag® NanoBRET™ 618 Ligand, and treated with a serial dilution of 10µM dBET1 or MZ1 PROTAC compounds for 1 hour. NanoBRET™ Nano-Glo® substrate was added and BRET measurements collected on a GloMax® Discover. For both PROTACs, a dose-dependent increase in BRET ratio was observed. Error bars represent standard deviation, n = 3.

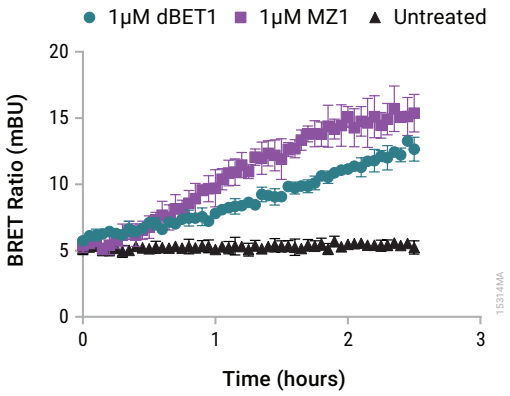

Figure 4. Kinetic monitoring of BRD4 ubiquitination. HEK293 cells stably expressing LgBiT were engineered using CRISPR-Cas9 to express endogenous HiBiT-tagged BRD4, plated in six-well plates and transfected with 2µg of HaloTag®-Ubiquitin acceptor plasmid. The following day, cells were replated at the density described in the presence of HaloTag® NanoBRET™ 618 Ligand. Medium was replaced with medium containing Nano-Glo® Vivazine™ substrate for 1 hour, and then treated with 1µM MZ1 or 1µM dBET1. NanoBRET™ measurements were collected every 3 minutes for 2.5 hours on a BMG Labtech CLARIOstar® microplate reader. Error bars represent standard deviation, n = 4.