

Detecting the Interaction of Target Proteins with E3 Ubiquitin Ligase Receptors, CRBN or VHL, using a NanoBRET™ Assay

Promega Corporation

Materials Required

- HEK293 or similar cultured mammalian cells; HaloTag®-VHL HEK293 stable cell line and HaloTag®-CRBN HEK293 stable cell line (optional) are available through **Custom Assay Services**
- 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)
- Nuclease-Free Water (Cat.# P1191)
- MG132 Proteasome Inhibitor (SelleckChem Cat.# S2619)
- FuGENE® HD Transfection Reagent (Cat.# E2311)
- NanoLuc®-fused target donor plasmid

Note: A collection of NanoLuc® target fusions 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.

- NanoBRET™ E3 Ligase Interaction Assays Kit (available through **Custom Assay Services**):
 - HaloTag® CRBN acceptor plasmid
 - HaloTag® VHL acceptor plasmid
 - NanoLuc® BRD4 donor plasmid (positive control)
 - HaloTag Negative Control Vector
- NanoBRET™ Nano-Glo® Detection System (Cat.# N1661)
- Nano-Glo® Vivazine™ Live Cell Substrate (Cat.# N2580)
- 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 a PROTAC-induced ternary complex between a target protein and the E3 ubiquitin ligase receptors, VHL or CRBN. The assays are based on NanoBRET™ technology, a proximity-based method dependent upon energy transfer from a luminescent donor to a fluorescent acceptor. In this assay, the target protein fused to NanoLuc® luciferase serves as the energy donor; and the N terminus of VHL or CRBN fused to HaloTag® protein serves as the energy acceptor. This protocol is based on either transient expression of both protein fusions, or stable expression of the energy acceptor in HEK293 cells with transient expression of the donor. We have found both approaches to be robust and reproducible methods for measuring the ternary complex, requiring minimal cell engineering.

However, there are alternative strategies for generating the target protein energy donor. Endogenous target proteins that have been 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 endogenously tagged proteins are discussed at the end of this protocol.

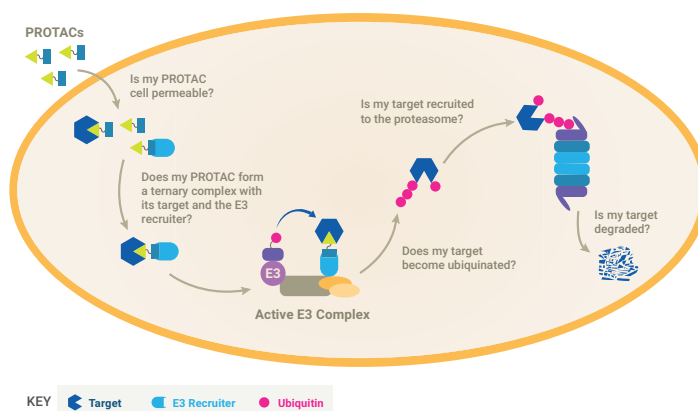


Figure 1. Overview of PROTAC-induced target protein degradation.

This protocol also includes three detection options: live cell endpoint measurement of ternary complex formation, extended live cell kinetic monitoring of ternary complex formation, and a lytic assay format for the assessment of potential non-permeable PROTACs. In addition, we describe how target protein levels can be measured using the NanoBRET™ assay results.

Instrument Requirements

To perform NanoBRET™ assays, an instrument capable of measuring dual-filtered luminescence values must be used. The NanoBRET™ bioluminescent donor emission occurs 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 emission occurs at 618nm. To measure the acceptor signal, we recommend a long pass filter starting at 600–610nm. For more information on instrument recommendations, please see the *NanoBRET™ Protein:Protein Interaction System Technical Manual #TM439*.

Media Composition

Cell Culture Medium

- 90% DMEM
- 10% 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 including both possible orientations of NanoLuc® luciferase fused to the N or C terminus of the target protein for maximal spatial proximity and energy transfer to the acceptor protein. For new targets, testing dilutions of donor DNA will ensure optimal saturation of donor molecules for the best possible assay window. We recommend testing donor dilutions of 1:1, 1:10, and 1:100. Specific recommended DNA amounts are included below. This protocol can be used with other cell types, but transfection conditions may need 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 ternary complex formation with CRBN can be induced by treatment with the PROTACs dBET1 or dBET6; and ternary complex formation with VHL can be induced by treatment with the PROTACs MZ1 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% CO₂.
6. Prepare a transfection mixture consisting of the DNA amounts listed in Table 1.

Note: If using a stable cell line expressing HaloTag®-VHL or HaloTag®-CRBN fusion proteins, transfections should consist of the same amounts of NanoLuc® fusion plasmid, but substitute promoterless carrier DNA for the HaloTag® plasmid amount.

Table 1. Transfection Mixtures

DNA Ratio (NanoLuc:HaloTag)	NanoLuc® Donor Plasmid*	HaloTag® Acceptor Plasmid
1:1	1µg NanoLuc® fusion	1µg VHL or CRBN vector
1:10	0.2µg NanoLuc® fusion	2µg VHL or CRBN vector
1:100	0.02µg NanoLuc® fusion	

*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, mix well and incubate at room temperature for 10 minutes.
9. Add transfection mixture to wells with attached cells and express overnight (18–24 hours) at 37°C, 5% CO₂.

Day 2: Replating Transfected Cells into Multiwell Plates and Adding HaloTag® NanoBRET™ 618 Ligand

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 assay medium (Opti-MEM® I Reduced Serum Medium, no phenol red + 4% FBS).
5. Count to estimate cell density and adjust density to 2 × 10⁵ 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% DMSO 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% CO₂ overnight (18–24 hours).

Day 3: NanoBRET™ Ternary Complex Assay

In live-cell mode, adding MG132 proteasome inhibitor prevents the proteasome-mediated degradation of BRD4 and enhances the NanoBRET™ assay windows with VHL and CRBN. While the interactions between BRD4 and the E3 substrate receptors are still observed in the absence of MG132, for the best assay window, we recommend preincubating with 10µM MG132 for up to 30 minutes prior to adding the PROTAC for either endpoint or kinetic live-cell assays.

Optional: To confirm target degradation by the PROTAC, a separate set of samples can be prepared without the MG132 inhibitor, and the luminescence (measured in relative light units [RLUs]) between the two sets of samples can be compared to determine the relative amount of degradation.

A. Live Mode—Endpoint

1. Prepare a 5X solution of MG132 proteasome inhibitor (50µM) in Opti-MEM® I Reduced Serum Medium, no phenol red, and add 25µl to each well of 96-well plate or 10µl for 384-well plate for a final concentration of 10µM.
2. Incubate plates at 37°C, 5% CO₂ for 30 minutes.
3. Prepare a 6X concentration of test PROTAC titration (starting at 60µM) in Opti-MEM® I Reduced Serum Medium, no phenol red, and add 25µl to each well of 96-well plate or 10µl for 384-well plate for a final concentration of 10µM at the highest point.
4. Incubate plates at 37°C, 5% CO₂ for 1–4 hours.
5. Prepare a 4X solution of NanoBRET™ Nano-Glo® Substrate in Opti-MEM® I Reduced Serum Medium, no phenol red. This is a 125-fold dilution of the stock reagent. For one 96-well plate, prepare a minimum of 5ml of medium + 20µl of stock reagent. For one 384-well plate, prepare a minimum of 8ml of medium + 32µl of stock reagent. For both multiwell formats, we recommend preparing at least 10% extra solution, especially if using automated dispensing.
Note: Use the 4X solution within 2 hours if stored at room temperature or within 4 hours if stored at 4°C.
6. Add 50µl of substrate for 96-well format or 20µl of substrate for 384-well format, and shake plate to mix for 30 seconds. We recommend using an electromagnetic mixer for the 384-well format.

- 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

- Prepare a 1X solution of Vivazine™ substrate in Opti-MEM® I Reduced Serum Medium, no phenol red, by diluting stock reagent 1:100.
- Aspirate medium from plate, and add 80µl (96-well) or 40µl (384-well) of Vivazine™ solution to each well.
- Incubate plate for 30–60 minutes at 37°C, 5% CO₂ to equilibrate the luminescence.
- Prepare a 10X solution of MG132 proteasome inhibitor (100µM) in Opti-MEM® I Reduced Serum Medium, no phenol red, and add 10µl to each well of a 96-well plate or 5µl for a 384-well plate for a final concentration of 10µM (final concentration after adding PROTAC).
- Incubate plates at 37°C, 5% CO₂ for up to 30 minutes while preparing PROTAC titration.
- Prepare a 10X concentration of test PROTAC titration (starting at 10µM) in Opti-MEM® I Reduced Serum Medium, no phenol red, 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.
- 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.

C. Lytic Mode

- Prepare a 5X solution consisting of 250µg/ml digitonin in Opti-MEM® I Reduced Serum Medium, no phenol red, for a final concentration of 50µg/ml. Prepare enough volume with which to use as the diluent for titration of test PROTAC.
- Prepare a 5X concentration of test PROTAC titration (starting at 5µM) in digitonin solution and add 25µl to each well of a 96-well plate or 10µl for a 384-well plate, for a final starting concentration of 1µM.
- Prepare a 6X concentration NanoBRET™ Nano-Glo® Substrate 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.

- Shake plate on an orbital shaker for 3 minutes. We recommend using an electromagnetic mixer for the 384-well format.
- Measure donor emission (460nm) and acceptor emission (618nm) using a NanoBRET™ PPI Assay-compatible luminometer (see Instrument Requirements). As different target:PROTAC:E3 ligase ternary complexes may differ in terms of kinetic formation, we recommend initially collecting measurements at multiple time points up to 2 hours to determine the optimal signal window.

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, subtract 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.

Figure 2. Corrected BRET ratio calculation.

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

Considerations for Alternative Energy Donor Strategies

The ternary complex formation assays can also be used with alternative strategies to tag the target protein, creating the energy donor. Endogenous target proteins can be tagged with NanoLuc[®] luciferase 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 Table 2. 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.

Table 2. Alternative Transfection Mixtures

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

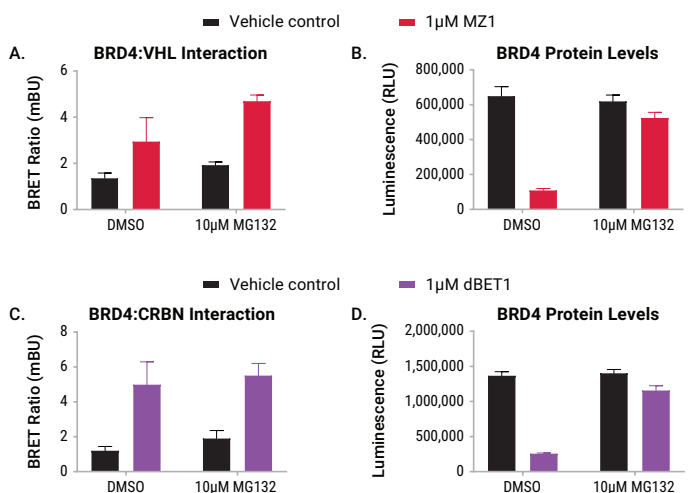


Figure 3. Measuring PROTAC-induced BRD4 VHL/CRBN ternary complex formation and BRD4 protein levels using live-cell endpoint detection. Cells were transfected with a 1:100 ratio of NanoLuc[®]-BRD4 donor plasmid to HaloTag[®]-VHL or HaloTag[®]-CRBN acceptor plasmid, plated as described and pretreated for 30 minutes with 10µM proteasome inhibitor MG132 or an equivalent amount of DMSO (vehicle control). **Panels A and B.** The BRD4/VHL assay cells were subsequently treated with 1µM MZ1 (VHL-based PROTAC) or an equivalent amount of DMSO for 1 hour. **Panels C and D.** The BRD4/CRBN assay cells were subsequently treated with 1µM dBET1 (Cereblon-based PROTAC) or an equivalent amount of DMSO for 1 hour. In all cases, an increase in BRET ratio was observed when adding the specific PROTAC molecule, which was further stabilized in the presence of the proteasome inhibitor MG132 (**Panels A and C**). Using the same set of data, BRD4 degradation was monitored by looking at the decrease in luminescence (as measured by RLUs) when treated with either PROTAC compound in the sets lacking proteasome inhibitor (vehicle control). As expected, adding the proteasome inhibitor in the MG132 sets greatly prevented or reduced degradation (**Panels B and D**).

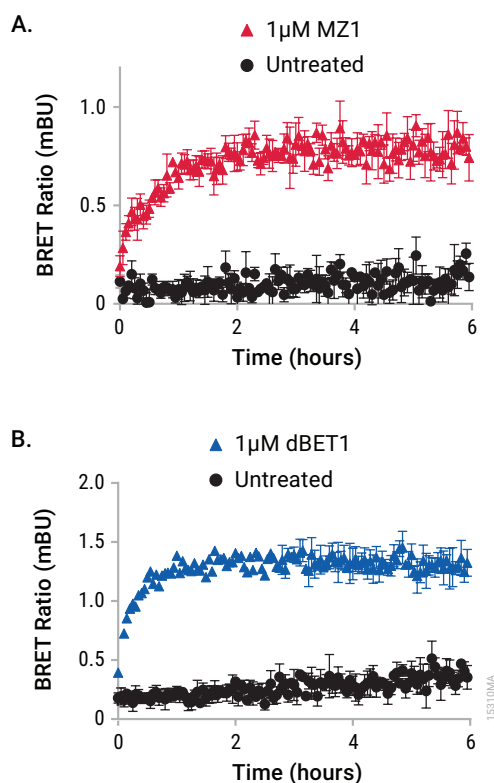


Figure 4. Kinetically monitoring BRD4/VHL and BRD4/CRBN ternary complex formation following PROTAC treatment. HEK293 cells stably expressing LgBiT were engineered using CRISPR-Cas9 to express endogenous HiBiT-BRD4, plated in six-well plates and transfected with 2μg of either HaloTag®-VHL or HaloTag®-CRBN acceptor plasmid. The following day, cells were replated as described in the presence of HaloTag® NanoBRET™ 618 Ligand. Medium was replaced with medium containing Vivazine™ substrate for 60 minutes, and then pretreated for 30 minutes with 10μM proteasome inhibitor MG132. After pretreatment, 1μM MZ1 was added to BRD4/VHL assay (**Panel A**), and 1μM dBET1 was added to BRD4/CRBN assay (**Panel B**), and NanoBRET™ measurements were collected every 3 minutes for 6 hours on a BMG LABTECH CLARIOstar® microplate reader.

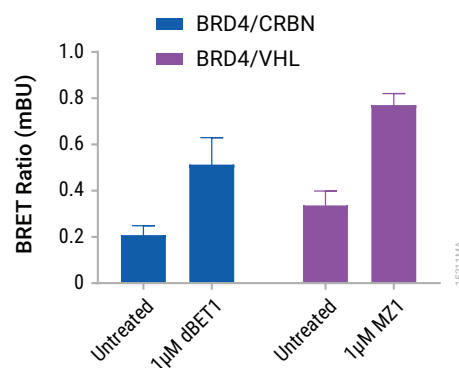


Figure 5. Measuring PROTAC-induced BRD4 VHL/CRBN ternary complex formation using lytic assay. HEK293 cells stably expressing LgBiT were engineered using CRISPR-Cas9 to express endogenous HiBiT-BRD4, plated in six-well plates and transfected with 2μg of either HaloTag®-VHL or HaloTag®-CRBN acceptor plasmid. The following day, cells were replated as described in the presence of HaloTag® NanoBRET™ 618 Ligand. Cells were permeabilized with 50μg/ml digitonin after adding either 1μM dBET1 or 1μM MZ1 PROTAC, and NanoBRET™ measurements were collected on a GloMax® Discover System.