TECHNICAL MANUAL

NanoBRET™ Proteasomal Recruitment Assay

Instructions for Use of Products
ND2730

3/20
TM612

NanoBRET™ Proteasomal Recruitment Assay

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

Most eukaryotic proteins are degraded by recruiting and processing mediated by the 26S proteasome. The NanoBRET™ Proteasomal Recruitment Assay[a–i] is a live-cell assay that can monitor target proteins recruited to the 26S proteasome. This assay can also be used to study dynamic changes in 26S trafficking after cellular treatments, such as small molecules or pathway inducers, that would influence protein stabilization or degradation respectively.

This assay uses NanoBRET™ technology, a proximity-based method dependent upon energy transfer from a luminescent donor to a fluorescent acceptor that is measured using an instrument capable of reading dual-filtered luminescence (1). Because of the dynamics of protein stabilization or degradation, the optimal NanoBRET™ assay configuration is with the target protein as the luminescent donor and the 26S proteasomal component as the fluorescent acceptor. This means you can monitor target protein levels while simultaneously observing proteasomal recruitment, made possible due to the luminescent to fluorescent ratio in the NanoBRET™ assay. We identified an N-terminal HaloTag® fusion of PSMD3 (26S proteasome regulatory subunit 3) to be the optimal proteasomal protein for this general assay. This acceptor fusion can be labeled with the HaloTag® NanoBRET™ 618 Ligand to be a fluorescent acceptor. As the target will be variable, we offer a suite of tools for the user to generate the appropriate luminescent donor fusion, which can be either an ectopically expressed NanoLuc® fusion or an endogenous target tagged with HiBiT using CRISPR gene editing and complemented with LgBiT. The protocols here describe how to optimize the initial NanoBRET™ setup for either format, including donor tag placement, expression ratio for ectopic fusions and timing. Detecting the interaction requires the use of either the NanoBRET™ Nano-Glo® Detection System for endpoint detection, or the NanoBRET™ Nano-Glo® Kinetic Detection System for kinetic detection. This protocol also has optional cell viability analysis for multiplexing with the NanoBRET™ assay.

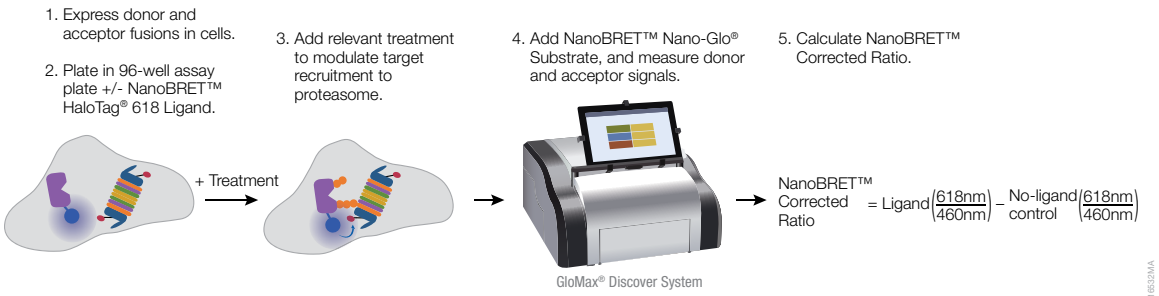

Figure 1. Overview of the five-step NanoBRET™ Proteasomal Recruitment Assay. First, the HaloTag®-PSMD3 acceptor fusion and NanoLuc® or HiBiT donor fusion are expressed in cells. Second, the cells are dispensed into 96-well plates, and samples designated experimental (with HaloTag® NanoBRET™ 618 Ligand) and control (without fluorescent ligand). Third, relevant treatments are added to induce recruitment modulation of the target to the proteasome. Fourth, the NanoBRET™ Nano-Glo® Substrate is added, and donor and acceptor signals are measured. Fifth, the corrected NanoBRET™ ratio is calculated.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT#
NanoBRET™ Proteasomal Recruitment Starter Kit	**1 each**	**ND2730**

The NanoBRET™ Nano-Glo® Detection System (NanoBRET™ NanoGlo® Substrate and HaloTag® NanoBRET™ 618 Ligand) is sufficient for approximately 200 assays performed in 96-well plates. Includes:

- HaloTag®-PSMD3 Fusion Vector
- pNLF1-N [CMV/Hygro] Vector
- pNLF1-C [CMV/Hygro] Vector
- HaloTag® Control Vector
- NanoLuc®-BRD4 FL Fusion Vector
- NanoBRET™ Nano-Glo® Detection System

Storage Conditions: Store all kit components at –30°C to –10°C. The HaloTag® NanoBRET™ 618 Ligand can be frozen and thawed up to 5 times.

Notes: Each starter kit contains vectors to create N- and C-terminal NanoLuc target protein fusions using standard cloning, the HaloTag®-PSMD3 Fusion Vector, positive control NanoLuc®-BRD4 FL Fusion Vector and negative control HaloTag® Control Vector. Individual components are available to purchase separately.

Included Vector	Antibiotic Resistance Cassette
HaloTag®-PSMD3 Fusion Vector	Kanamycin
HaloTag® Control Vector	Ampicillin
NanoLuc®-BRD4 FL Fusion Vector	Kanamycin

Available Separately

Detection Reagents

PRODUCT	SIZE	CAT#
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**
NanoBRET™ Nano-Glo® Substrate	**50µl**	**N1571**
	5 × 50µl	**N1572**
	2 × 1.25ml	**N1573**
HaloTag® NanoBRET™ 618 Ligand	**20µl**	**G9801**

2. Product Components and Storage Conditions (continued)

Vectors

PRODUCT	SIZE	CAT#
HaloTag®-PSMD3 Fusion Vector	20µg	N2701
pFN31A *Nluc* CMV-Hygro Flexi® Vector	20µg	N1311
pFN31K *Nluc* CMV-neo Flexi® Vector	20µg	N1321
pFC32A *Nluc* CMV-Hygro Flexi® Vector	20µg	N1331
pFC32K *Nluc* CMV-neo Flexi® Vector	20µg	N1341
pNLF1-N [CMV/Hygro] Vector	20µg	N1351
pNLF1-C [CMV/Hygro] Vector	20µg	N1361
NanoLuc®-BRD4 FL Fusion Vector	20µg	N1691
NanoBRET™ Positive Control	2 × 20µg	N1581
HaloTag® Control Vector	20µg	G6591

Transfection Reagents

PRODUCT	SIZE	CAT.#
FuGENE® HD Transfection Reagent	1ml	E2311
	5 × 1ml	E2312

3. Before You Begin

3.A. Assay Vector Preparation

The amount of each plasmid DNA provided with the system is sufficient for a few initial testing experiments, but we strongly advise that each 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 each vector, the fusion protein is constitutively expressed by a CMV promoter.

3.B. Instrument Information and Setup

To perform NanoBRET™ assays, a luminometer capable of sequentially measuring dual-wavelength windows is required. We recommend using a band pass (BP) filter for the donor signal and a long pass (LP) filter for the acceptor signal to maximize sensitivity. Filters outside of the recommended ranges will miss critical measurements and compromise data quality.

The NanoBRET™ 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. For example, a 450nm/BP80 will capture the 410–490nm range.

Note: A BP filter is preferred for the donor signal measurement to selectively capture the signal peak and avoid measuring any bleed-through into the acceptor peak. You can use a short pass (SP) filter that covers the 460nm area. However, this could result in an artificially large value for the donor signal and measuring the bleed-through into the acceptor peak, which could compress the ratio calculation and reduce the assay window.

The NanoBRET™ acceptor emission occurs at 618nm, to measure the acceptor signal we recommend a long pass filter starting at 600–610nm.

Instruments capable of dual luminescence measurements are either pre-equipped with a filter selection or the filters can be purchased and added separately. For instruments using mirrors select the luminescence mirror. An integration time of 0.2–1 second is typically sufficient. Ensure that the gain or PMT is optimized to capture the highest donor signal without reaching instrument saturation.

Consult with your instrument manufacturer to determine if the proper filters are pre-installed or what steps are needed to add filters to the luminometer. For example, a special holder or cube might be required for the filters to be mounted, and the shape and thickness may vary among instruments. We have experience with the following instruments and configurations:

1. The GloMax® Discover System (Cat.# GM3000) with pre-loaded filters donor 450nm/8nm BP and acceptor 600nm LP. Select the preloaded BRET:NanoBRET™ 618 protocol from the Protocol menu.
2. BMG Labtech CLARIOstar® with preloaded filters for donor 450nm/80nm BP and acceptor 610nm LP.
3. Thermo Varioskan® with filters obtained from Edmunds Optics, donor 450nm CWL, 25mm diameter, 80nm FWHM, Interference Filter and acceptor 1 inch diameter, RG-610 Long Pass Filter

Another popular instrument capable of measuring dual luminescence is the Perkin Elmer EnVision® and we recommend the following set-up:

- Mirror: Luminescence - Slot4
- Emission filter: Chroma Cat.# AT600LP - EmSlot4
- Second emission filter: Chroma Cat.# AT460/50m - EmSlot1
- Measurement height (mm): 6.5
- Measurement time (seconds): 1

3.C. NanoBRET™ Positive Control Vector

To ensure your instrument has been configured properly, we recommend testing with the NanoBRET™ Positive Control (Cat.# N1581; available separately). This vector is an artificial system that tethers together NanoLuc® and HaloTag® proteins, ensuring energy transfer. Because NanoLuc® luciferase is extremely bright and the energy transfer to the HaloTag® moiety is so efficient, the vector plasmid must be diluted with Transfection Carrier DNA to reduce its expression levels. Keep in mind that an actual protein pair is unlikely to show the same level of energy transfer efficiency and should not be compared to this artificial control. Representative data are show in Figure 2.

Note: If the NanoBRET Positive Control vector is to be used in the same plate as actual PPI partners, we recommend leaving an empty row of wells between PPI partners' sets and the NanoBRET™ Positive Control vector because the light from the control plasmid might cause cross-talk in adjoining wells.

3.C. NanoBRET™ Positive Control Vector (continued)

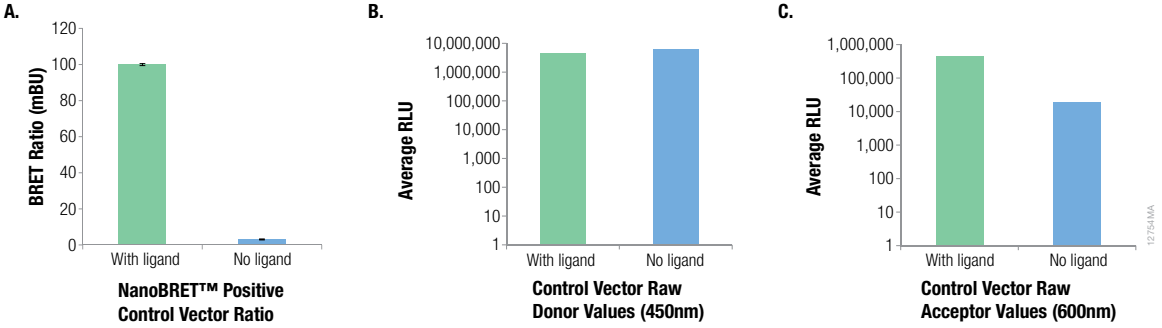

Figure 2. NanoBRET™ ratio and raw donor and acceptor measurements with the NanoBRET™ Positive Control vector. Panel A. Calculated NanoBRET™ ratio in experimental sample and no-ligand control. The no-ligand control represents the donor signal bleed-through into the acceptor channel of the NanoBRET™ ratio and should be subtracted from the experimental samples to obtain the corrected NanoBRET™ ratio. **Panel B.** Raw donor values in relative light units (RLU) measure instrument sensitivity. For most commonly used instruments, this value is typically 1,000,000 to 10,000,000 RLU for both sets of samples with or without ligand. **Panel C.** Raw acceptor values represent the energy transfer from donor to acceptor and should be higher in the experimental samples containing ligand while the no-ligand control samples represent bleed-through. Data generated using the GloMax® Discover System equipped with 450nm/8nm BP and 600nm LP filters.

4. Assay Design Parameters and Optimization

4.A. Configuring Fusion Tags

The NanoBRET™ system is a is a proximity-based assay that can detect protein interactions by measuring energy transfer from a bioluminescent protein donor to a fluorescent protein acceptor. In the NanoBRET™ Proteasomal Recruitment assay, we have optimized the tag placement of the HaloTag® acceptor fusion, provided as an N-terminal HaloTag®-PSMD3 fusion. To achieve an optimal NanoBRET™ signal, we recommend testing both N- and C-terminal tag placement for either the NanoLuc® or HiBiT donor, if development or functional physiology allow. To generate N- and C-terminal NanoLuc® fusions of the target protein, follow standard cloning procedures to introduce genes of interest into the NanoLuc® MCS-based fusion vectors or Flexi® Vectors as described in the individual vector protocols. For more information on generating HiBiT CRISPR insertions, see **www.promega.com** and purchase the supporting HEK293 LgBiT Stable Cell line (available by request through Custom Assay Services, CS1956D02).

4.B. Protein Expression Levels

Determining the protein expression level of the donor fusion relative to the acceptor fusion is important to maximize the NanoBRET™ signal and minimize background. In general, low levels of expression of the donor fusion are recommended. This optimization step is needed for ectopically-expressed NanoLuc® fusions, but not for endogenously tagged HiBiT fusions. To optimize donor expression level, we recommend performing donor dilution experiments, starting with an equal amount of donor DNA to acceptor DNA (1:1 ratio), and then diluting donor DNA to 1:10 and 1:100. Most frequently, we observe that a 1:100 donor-to-acceptor ratio is optimal for most NanoBRET™ pairs.

4.C. Test Compound Concentration and Treatment Time

The NanoBRET™ Proteasomal Recruitment Assay can be used to detect dynamic changes in relative recruitment of the target protein to the 26S proteasome. If using a compound to modulate target recruitment to the proteasome, we recommend testing a variety of concentrations and treatment times.

Note: We do not recommend using MG-132 proteasome inhibitor with this assay.

4.D. Controls

Two controls are described for the proteasomal recruitment assay. An unfused HaloTag® Control Vector is used as a negative control to assess interaction specificity. The positive control BRD4-NanoLuc® Fusion Vector can be used with a commercially available compound, dBET6 (2), a PROTAC compound which induces BRD4 trafficking to the proteasome and subsequent degradation. The HaloTag® Control Vector is designed for use when optimizing a new assay pair. The BRD4-NanoLuc® Fusion Vector can be used as an assay plate control during new assay optimization or during experimental testing of an optimized assay.

5. NanoBRET™ Proteasomal Recruitment Assay Protocol

Materials to be Supplied by User

- HEK293 or similar mammalian cells
- white, 96-well plate (Costar Cat.# 3917) or 384-well plate (Corning Cat.# 3570)
- tissue culture equipment and reagents (see Section 8.B, Composition of Buffers and Solutions)
- DPBS (Gibco Cat.# 14190)
- 0.05% Trypsin/EDTA (Gibco Cat.# 25300)
- FuGENE® HD Transfection Reagent (Cat.# E2311)
- DMEM (Gibco Cat.#11995)
- fetal bovine serum (Seradigm Cat.# 89510-194)
- Opti-MEM® I Reduced Serum Medium, no phenol red (Life Technologies Cat.# 11058-021)
- DMSO (Sigma Cat.# 2650)
- Nuclease-Free Water (Cat.# P1191)
- **Optional:** PSMD3/BRD4 control assay compound dBET6 (Selleckchem Cat.# S8762)
- user-defined test compounds
- luminometer capable of measuring dual-filtered luminescence (e.g., GloMax® Discover System Cat.# GM3000; see Section 3.B for more information)

5.A. Transfection Protocols for NanoBRET™ Proteasomal Recruitment Assay

The following transient transfection conditions are for mammalian HEK293 cells. Other cells lines may require optimization. If using a transfection reagent other than FuGENE® HD Transfection Reagent, follow the manufacturer's recommendations but keep the same relative ratio of donor-to-acceptor DNA.

Note: Follow the appropriate transfection protocol as DNA amounts required may differ for the assay control target versus a user-defined target. The four different transfection schemes are:

- Checking the instrument setup with the NanoBRET™ Positive Control
- Checking the NanoBRET™ Proteasomal Recruitment Assay performance with BRD4-NanoLuc® Fusion Vector and HaloTag®-PSMD3 OR HaloTag® Control Vector
- Assay optimization: User-generated NanoLuc® target fusion vectors transfected with HaloTag®-PSMD3 Fusion Vector
- Transfecting the HaloTag®-PSMD3 Fusion Vector into cells with an endogenously tagged HiBiT CRISPR fusion protein

Following the transfection step, all the remaining steps in the protocol remain identical regardless of type of transfection performed.

Transfection Conditions for the NanoBRET™ Positive Control to Check Instrument Setup (Optional)

1. Culture HEK293 cells appropriately prior to assay.
2. Remove medium from cell flask via 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 cell density to 4×10^5/ml in cell culture medium.
4. Plate 2ml of cells (800,000 cells) into each well of a six-well plate.
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 Transfection Carrier DNA + 0.002µg NanoBRET™ Positive Control Vector diluted 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. Dispense transfection mixture to wells with attached cells and express proteins for approximately 20 hours at 37°C, 5% CO_2.
10. Proceed to Section 5.B.

Transfection Conditions to Check the NanoBRET™ Proteasomal Recruitment Assay Performance

1. Culture HEK293 cells appropriately prior to assay.
2. Remove medium from cell flask via 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 cell density to 4×10^5/ml in cell culture medium.

4. Plate 2ml of cells (800,000 cells) into each well of a six-well plate.
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 HaloTag®-PSMD3 Fusion Vector or HaloTag® Control Vector + 0.02µg NanoLuc®-BRD4 Fusion Vector diluted 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. Dispense transfection mixture to wells with attached cells and express proteins for approximately 20 hours at 37°C, 5% CO_2.
10. Proceed to Section 5.B.

Transfection Conditions for Optimizing Donor Tag Placement and Donor-to-Acceptor Ratio

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) into a 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 DNA amounts according to the table below. Clone both N- and C-terminal NanoLuc® target protein fusions and test the fusion proteins to find the orientation that provides the best assay window.

Desired Ratio	HaloTag® Vector (Acceptor)	Amount of N- or C- terminal NanoLuc® Fusion Vector Diluted in Water (Donor)
1:1 (NanoLuc to HaloTag)	1µg PSMD3-HaloTag® Fusion Vector or HaloTag® Control Vector	1µg NanoLuc® Fusion Vector
1:10 (NanoLuc to HaloTag)	2µg PSMD3-HaloTag® Fusion Vector or HaloTag® Control Vector	0.2µg NanoLuc® Fusion Vector
1:100 (NanoLuc to HaloTag)		0.02µg NanoLuc® Fusion Vector

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 overnight (18–24 hours) at 37°C, 5% CO_2.
10. Proceed to Section 5.B.

5.A. Transfection Protocols for NanoBRET™ Proteasomal Recruitment Assay (continued)

Transfection Conditions for Endogenously Tagged HiBiT CRISPR Fusion Protein

Note: Cell lines which contain a HiBiT CRISPR fusion in a cell line stably expressing LgBiT (such as HEK293 LgBiT Stable Cells, Promega CAS# CS1956D02), do not require additional LgBiT expression. If the cell line being tested contains an endogenous HiBiT CRISPR fusion only, LgBiT must be expressed by transfecting the CMV LgBiT Vector (Promega CAS# CS1956B03).

1. Culture the cell line with endogenous target HiBiT knock-in appropriately prior to assay.
2. Remove medium from cell flask via aspiration, trypsinize, and allow cells to dissociate from the flask.
3. Neutralize trypsin using cell culture medium, count 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) 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 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. When ready to transfect, prepare a transfection mixture appropriate to your donor expression format, based on the table below:

Donor Expression Format	Desired Ratio	HaloTag® Vector	CMV LgBiT Vector
Endogenous HiBiT Fusion	1:1 (LgBiT to HaloTag)	1µg PSMD3-HaloTag® Fusion Vector or HaloTag® Control Vector	1µg LgBiT Vector
	1:10 (LgBiT to HaloTag)	2µg PSMD3-HaloTag® Fusion Vector or HaloTag® Control Vector	0.2µg LgBiT Vector
	1:100 (LgBiT to HaloTag)		0.02µg LgBiT Vector
HiBiT CRISPR Fusion Stably Expressing LgBiT	N/A	2µg PSMD3-HaloTag® Fusion Vector or HaloTag® Control Vector	N/A

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. Dispense transfection mixture to wells with attached cells and express proteins for approximately 20 hours at 37°C, 5% CO_2.
10. Proceed to Section 5.B.

5.B. Replating Transfected HEK293 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 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 2×10^5 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% 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 with endpoint detection: Dispense 40µl of each pool of the cells prepared in Step 6 in at least 3–4 wells.

   384-well format with kinetic detection: Dispense 36µl 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).
9. Proceed to Section 5.C.

5.C. Adding Test Compounds and Detecting Proteasomal Recruitment

Determine which detection protocol to follow. Choose between measuring the endpoint or assessing kinetic changes in live cells.

Live-Cell Endpoint Detection using the NanoBRET™ Nano-Glo® Detection System

1. Prepare a 5X concentration of test compound in Opti-MEM® I Reduced Serum Medium, no phenol red.

   96-well format: Add 25µl to each well for a final concentration of 1µM.

   384-well format: Add 10µl to each well for a final concentration of 1µM.

   Note: For the PSMD3/BRD4 control assay, treat with 1µM dBET6 for 2 hours.

5.C. Adding Test Compounds and Detecting Proteasomal Recruitment (continued)

2. Prepare a 5X concentration of DMSO to use as a negative control by adding an equivalent amount of DMSO to Opti-MEM® I Reduced Serum Medium, no phenol red.

   96-well format: Add 25µl to each negative control well.

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

3. Incubate plates at 37°C, 5% CO_2 for desired treatment time.

4. Prepare a 6X solution of NanoBRET™ Nano-Glo® Substrate in Opti-MEM® I Reduced Serum Medium, no phenol red. This is an 83.3-fold dilution of the stock reagent. For one 96-well plate, prepare a minimum of 5ml of medium + 60µl of stock reagent. For one 384-well plate, prepare a minimum of 8ml of medium + 96µl of stock reagent. For both multiwell formats, we recommend preparing at least 10% extra solution to account for dead volume, 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. Add substrate to cells and shake plate to mix for 30 seconds. (We recommend using an electromagnetic mixer for the 384-well format.)

   96-well format: Add 25µl of substrate.

   384-well format: Add 10µl of substrate.

5. Measure donor emission (460nm) and acceptor emission (618nm) within 10 minutes of substrate addition using a NanoBRET™ Assay-compatible luminometer (see instrument requirements in Section 3.B).

6. Proceed to Section 5.D for NanoBRET™ calculations.

Live-Cell Kinetic Detection using NanoBRET™ Nano-Glo® Kinetic Detection System

1. Prepare substrate as follows:

   96-well format: Prepare a 1X solution of Nano-Glo® Vivazine™ substrate (a 1:100 dilution of the stock reagent) in Opti-MEM® I Reduced Serum Medium, no phenol red + 4% FBS.

   384-well format: Prepare a 2X solution of Nano-Glo® Vivazine™ substrate (a 1:50 dilution of the stock reagent) in Opti-MEM® I Reduced Serum Medium, no phenol red + 4% FBS.

2. Add Vivazine™ solution to each well.

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

   384-well format: Dispense 36µl of 2X Vivazine™ solution to 36µl of cells.

3. Incubate plate for 30–60 minutes at 37°C, 5% CO_2 to equilibrate substrate luminescence.

4. Prepare a 10X concentration of test PROTAC in Opti-MEM® I Reduced Serum Medium, no phenol red.

   96-well format: Add 10µl to each well for a final concentration of 1µM.

   384-well format: Add 8µl to each well for a final concentration of 1µM.

   Note: For the PSMD3/BRD4 control assay, treat with 1µM dBET6.

5. Prepare a 10X concentration of DMSO to use as a negative control by adding an equivalent amount of DMSO to Opti-MEM® I Reduced Serum Medium, no phenol red.

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

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

6. Immediately collect kinetic measurements of donor emission (460nm) and acceptor emission (618nm) every 3–5 minutes up to 6 hours after adding test compound titration using a NanoBRET™ Assay-compatible luminometer (see instrument requirements in Section 3.B).

7. Proceed to Section 5.D for NanoBRET™ calculations.

5.D. NanoBRET™ Calculations

1. 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:

   $$\frac{618nm_{Em}}{460nm_{Em}} = \text{Raw NanoBRET™ Ratio} = BU$$

2 To convert raw NanoBRET™ units (typically decimal values) to milliBRET units (mBU; whole numbers), multiply each raw BRET value by 1,000.

   $$\frac{618nm_{Em}}{460nm_{Em}} = BU \times 1{,}000 = mBU$$

3. Determine the mean NanoBRET™ ratio for each set of samples: Experimental samples with HaloTag® NanoBRET™ 618 Ligand and no-ligand control samples. To factor in donor-contributed background or bleedthrough, subtract the no-ligandcontrol mean from the Experimental mean for the corrected NanoBRET™ ratio.

   Mean mBU experimental – Mean mBU no-ligand control = Mean corrected mBU

4. **Optional:** Z´ and Z factor calculations can be generated to gauge assay consistency. The Z´ factor estimates assay consistency by comparing the mean and standard deviation values of the experimental samples and the no-ligand control samples.

   $$Z' \text{ factor} = 1 - \left[\frac{(3X \text{ STDV experimental} + 3X \text{ STDV no-ligand control})}{(\text{Mean mBU experimental} - \text{Mean mBU no-ligand control})}\right]$$

In the presence of a degradation compound, a Z factor (different from a Z´ factor) takes into account both the assay variability and the difference between a treated sample and a vehicle control (delta). Use corrected mBU and STDV for these calculations. In general, an assay with a Z´ or Z value between 0.5–1 is considered to be robust with lower assay variability. Treated samples refer to samples treated with compound. In the following example, the calculation reflects an expected increase in proteasomal recruitment, as seen with the PSMD3/BRD4 assay. However, if calculating Z factor for an assay with an expected decrease, subtract the mean mBU for the treated samples from the mean mBU for the untreated samples to ensure a positive value in the denominator.

$$Z \text{ factor} = 1 - \left[\frac{(3X \text{ STDV untreated} + 3X \text{ STDV treated})}{(\text{Mean mBU treated} - \text{Mean mBU untreated})}\right]$$

6. Representative Data

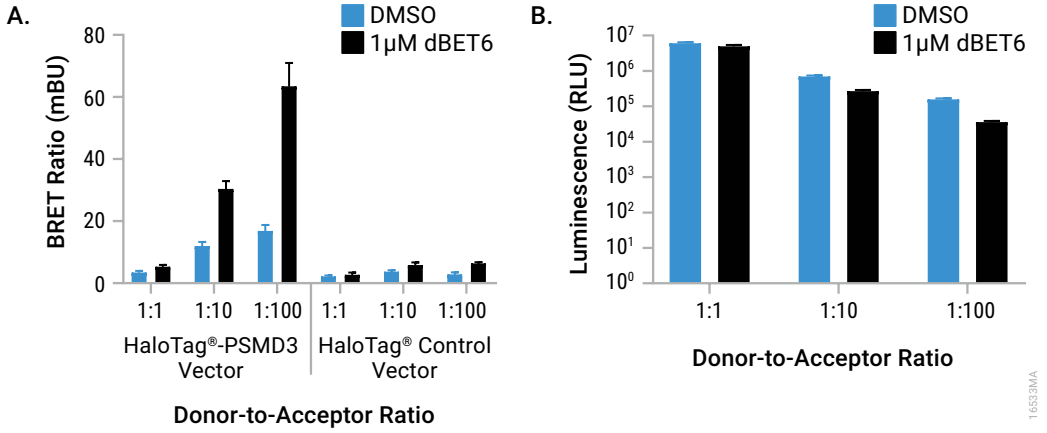

Figure 3. NanoBRET™ Proteasomal Recruitment Assay using the control HaloTag®-PSMD3 and NanoLuc®-BRD4 fusion proteins. Panel A. Donor-to-acceptor ratios of 1:1, 1:10 and 1:100 were tested with both the specific HaloTag®-PSMD3 Fusion Vector and HaloTag® Control Vector. Samples were treated with 1µM dBET6 PROTAC compound or DMSO control for 2 hours and NanoBRET™ signal was measured on a GloMax® Discover System instrument using the NanoBRET™ Nano-Glo® Detection System. A specific increase in the NanoBRET™ signal was observed for BRD4/PSMD3 fusion proteins when treated with dBET6 compared to the HaloTag® protein control. **Panel B**. NanoBRET™ luminescent donor values for NanoLuc®-BRD4 fusion protein at 1:1, 1:10 and 1:100 donor-to-acceptor ratios with and without 1µM dBET6 treatment. The dBET6 treatment resulted in an expected decrease in donor luminescence.

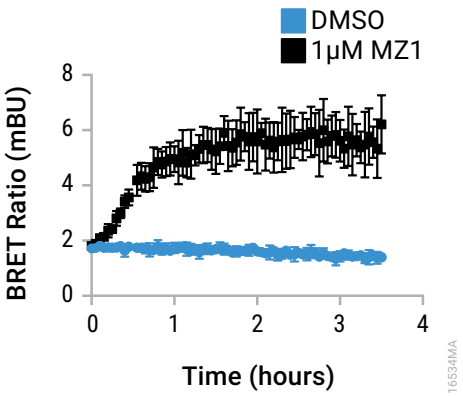

Figure 4. Measurements taken with NanoBRET™ Proteasomal Recruitment Assay in Kinetic Format. The HaloTag®-PSMD3 Fusion Vector was transiently transfected into cells stably expressing NanoLuc®-BRD4 fusion protein. Cells were treated with 1µM MZ1 or DMSO and NanoBRET™ signal was measured continuously for 3.5 hours using the NanoBRET™ Kinetic Detection System. Data were collected using a GloMax® Discover System.

7. 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	Causes and Comments
No NanoBRET™ ratio even with the NanoBRET™ Positive Control Assay	Improper instrument setup. • Make sure luminometer has the proper filters: 460nm/8–80nm BP for donor signal; 600–610nm LP for acceptor signal. • Make sure PMT or gain is set to detect donor signal without instrument saturation.
	Lack of expression of protein partners. Check expression of HiBiT or NanoLuc® fusions by luminescence or 460nm reading. Check expression of fluorescent HaloTag® fusion by cell-to-gel and band quantification in a fluoroimager. See the *HaloTag® MammalianPull-Down and Labeling Systems Technical Manual #*TM342 for more information.
	Donor and acceptor tags are not within proximity for energy transfer to occur. Test both N- and C-terminal tagging of HiBiT or NanoLuc® donor fusions.
	Lack of LgBiT protein expression when using HiBiT CRISPR cell lines. Check LgBiT expression by adding purified HiBiT Control Protein (Cat.# N3010) in the Nano-Glo® HiBiT Lytic Detection System (Cat.# N3030).
	Improper relative amounts of HaloTag® and NanoLuc® vectors. Follow the recommended ratios for transfecting HaloTag® and NanoLuc® vectors.
	Improper calculations. Divide the acceptor value by the donor value (618nm ÷ 460nm). Optionally multiply by 1,000 to convert to mBU. To account for background contribution, subtract the ratio of the no-ligand control from the ratio of the experimental samples.
Poor Z´ and Z factor values	High variability in numbers. • Ideally a robust assay has Z´ values of 0.5–1. Consider dispensing by automation to reduce variability. Z´ values could be lower in the 384 well format. • A Z factor value takes into account both the assay variability as well as the degree of effect of a modulator such as an inhibitor. A weak degradation compound will produce a small change (delta) between treated and untreated samples, resulting in a suboptimal Z factor value not due to the assay consistency.

7. Troubleshooting (continued)

Symptoms	Causes and Comments
Poor Z´ and Z factor values (continued)	NanoLuc® signal is close to the instrument limit of detection. The recommended amount of donor DNA in the control assay has been optimized for detection on most commonly used instruments. If the luminometer being used has lower sensitivity, increase the amount of NanoLuc® donor DNA. Do not exceed a 1:1 ratio between NanoLuc® donor and HaloTag® acceptor DNAs.
	Signal from a HiBiT CRISPR cell line is close to instrument limit of detection • Confirm that cell line being used is endogenously expressing HiBiT tagged protein. • Confirm that LgBiT protein is expressed within the cell by adding the HiBiT Control Protein (Cat.# N3010) in the Nano-Glo® HiBiT Lytic Detection System (Cat.# N3030).
Ratios and raw values are different from those shown on examples	The absolute raw values and ratios may vary among detection instruments. Confirm the proper biological response is observed such as the increase in BRD4 proteasomal recruitment with dBET6 treatment.
	The absolute raw values and ratios will vary among PPI systems. Absolute NanoBRET™ values are dependent on the proximity of the protein partners, the kD of the interaction, the relative occupancy with other interacting proteins, and the instrument set up.
Unable to express proteins	Suboptimal transfection conditions. Follow the recommended strategy for determining optimal relative amounts of HaloTag® and NanoLuc® fusion vectors.
Unable to detect signal modulation when using a known compound	Poor compound potency or permeability. Test a series of compound concentrations to determine optimal treatment concentration. For example, using 0.1–10µM of compound.
	Proteasomal recruitment is time dependent. Test multiple timepoints or perform the assay in kinetic format to determine optimal detection time.
	Donor expression is too high when using transient transfection. Reduce the relative level of donor expression or test target as a HiBiT CRISPR knock-in if possible.

Symptoms	Causes and Comments
Unable to detect signal modulation when using a known compound (continued)	Donor expression is too low. If endogeneously expressing a HiBiT fusion, test by transfecting a NanoLuc® fusion of the target protein. If using kinetic detection, first test and optimize tag orientation and protein expression levels in endpoint detection format.
	Compound concentration too low. Assay sensitivity can be decreased if compound treatment concentration is too low. Test higher concentrations of test compounds.
Can detect an increase in proteasomal recruitment but unable to measure degradation of HiBiT or NanoLuc® target.	Ectopic expression of NanoLuc® fusion donor. Reduce the relative level of NanoLuc® fusion expression because overexpression may mask degradation.
	Recruitment to the proteasome may not result in target degradation. This assay detects an increase in proximity of the target protein with the 26S proteasome. The target can be brought into proximity to the proteasome without degradation occurring.
Measure a decrease in NanoLuc® or HiBiT donor luminescence without an increase in proteasomal recruitment.	Poor cell health or compound toxicity. Ensure cells are still viable at the time of assay measurement by multiplexing with the CellTiter-Glo® 2.0 Assay.
Cannot detect NanoBRET™ ratio using other HaloTag® fluorescent ligands.	The optimal fluorescent ligand for HaloTag is the NanoBRET™ 618 Ligand. Do not use other HaloTag® fluorescent ligands. The signal will be reduced or absent.
Unequal amounts of detection reagents.	There is enough material provided for each of the detection reagent components for the number of assays. Because we recommend including a set of samples without HaloTag® NanoBRET™ 618 Ligand as a negative control, you may end up with extra ligand. The individual detection reagents are also available to purchase separately.
Discolored HaloTag® NanoBRET™ 618 Ligand.	The HaloTag® NanoBRET™ 618 Ligand is typically a hue of pink to red color but there might be instances where it appears a lighter hue or colorless. This is due to varying degrees of molecular closeness. In the close form the ligand is colorless. When added to medium, the ligand converts completely to the open usable form. To confirm chemical integrity, dilute 1µl of ligand in 1ml of Opti-MEM® I Reduced Serum Medium, no phenol red, and check fluorescence by exciting at 593nm±4nm and measuring the emission at 621nm±4nm. For the GloMax®-Multi Detection System, use the green channel (Ex: 525nm, Em: 580–640nm).

8. Appendix

8.A. Multiplexing with the CellTiter-Glo® 2.0 Assay

In some cases, you may want to determine the cell viability or compound toxicity or both plus perform the NanoBRET™ assay. Multiplexing with another assay will give you more data from a single well. Assess cell health using the ready-to-use CellTiter-Glo® 2.0 Assay, a luminescent assay that quantitates the amount of ATP present, which indicates the presence of metabolically active cells. Figure 5 shows example data.

1. Equilibrate CellTiter-Glo® 2.0 reagent to room temperature.
2. Following NanoBRET™ measurements, add an equal volume of CellTiter-Glo® 2.0 Reagent per well and mix on a plate shaker at 500–700 rpm for 5 minutes. For example, add 125µl of CellTiter-Glo® 2.0 Reagent to a 96-well plate.

   Note: Total well volume in a 96-well plate should not exceed 250µl after adding Cell Titer-Glo® 2.0 Reagent. If multiplexing the CellTiter-Glo® 2.0 Assay with the NanoBRET™ assay where fusion proteins were transiently transfected, add a higher concentration of compound and Nano-Glo® substrate to maintain the 125µl well volume for the NanoBRET™ assay.
3. Incubate the plate at room temperature for 30 minutes to lyse cells and quench the NanoLuc® signal.
4. After the 30-minute incubation is complete, measure total luminescence on a luminometer. If using the GloMax® Discover System, select the CellTiter-Glo® protocol.
5. If determining compound toxicity, compare the luminescence (RLU) of vehicle-containing samples versus compound-containing samples. Keep in mind that even if some toxicity is observed, the NanoBRET™ ratio is only derived from the live cells in the NanoBRET™ assay.

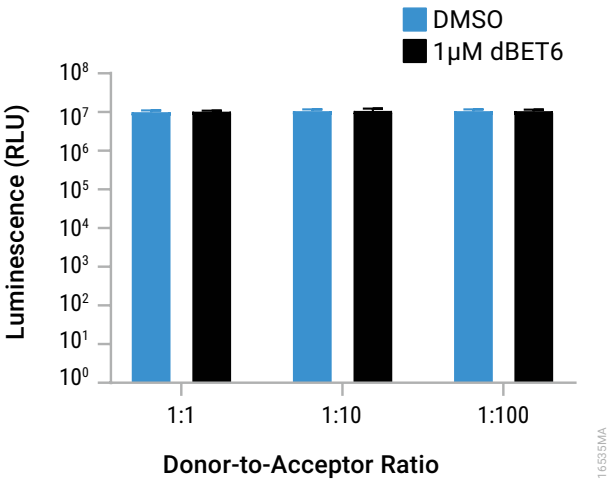

Figure 5. Example data for multiplexing the NanoBRET™ Proteasomal Recruitment Assay with the CellTiter-Glo® 2.0 Assay. Experimental samples for the NanoBRET™ Proteasomal Recruitment Assay as described in Figure 3 were multiplexed the CellTiter-Glo® 2.0 Assay to measure cell viability. There was no effect on cell viability observed with dBET6 treatment.

8.B. Composition of Buffers and Solutions

Cell Culture Medium

90% DMEM (Gibco Cat.#11995)

10% fetal bovine serum (Seradigm Cat.# 89510-194)

Assay Medium

96% Opti-MEM® I Reduced Serum Medium, no phenol red (Life Technologies Cat.# 11058-021)

4% fetal bovine serum (Seradigm Cat.# 89510-194)

8.C. References

1. Machleidt, T. *et al*. (2015) NanoBRET—A novel BRET platform for the analysis of protein-protein interactions. *ACS Chem. Biol.* **10**, 1797–1804.
2. Winter, G.E. *et al*. (2017) BET bromodomain proteins function as master transcription elongation factors independent of CDK9 recruitment. *Mol. Cell* **67**, 5–18.

8.D. Related Products

Product	Size	Cat.#
NanoBRET™ VHL Ternary Complex Starter Kit	1 each	ND2700
NanoBRET™ CRBN Ternary Complex Starter Kit	1 each	ND2720
NanoBRET™ Ubiquitination Starter Kit	1 each	ND2690
CellTiter-Glo® 2.0 Cell Viability Assay	10ml	G9241
	100ml	G9242
	500ml	G9243
NanoBRET™ PPI Control Pair (p53, MDM2)	1 each	N1641

Multimode Detection Instrument

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

Intracellular LgBiT Expression (Custom Reagents)

Product	Size	Cat.#
CMV LgBiT Vector	20µg	CS1956B03
HEK293 LgBiT Stable Cell Line	2 × 1ml	CS1956D02

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[e]U.S. Pat. No. 8,809,529, European Pat. No. 2635582, and other patents and patents pending.

[f]U.S. Pat. Nos. 7,425,436, 7,935,803, 8,466,269, 8,420,367, 8,742,086, 8,748,148, 9,416,353, 9,593,316 and other patents and patents pending.

[g]U.S. Pat. Nos. 10,067,149 and 10,024,862 and other patents and patents pending.

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