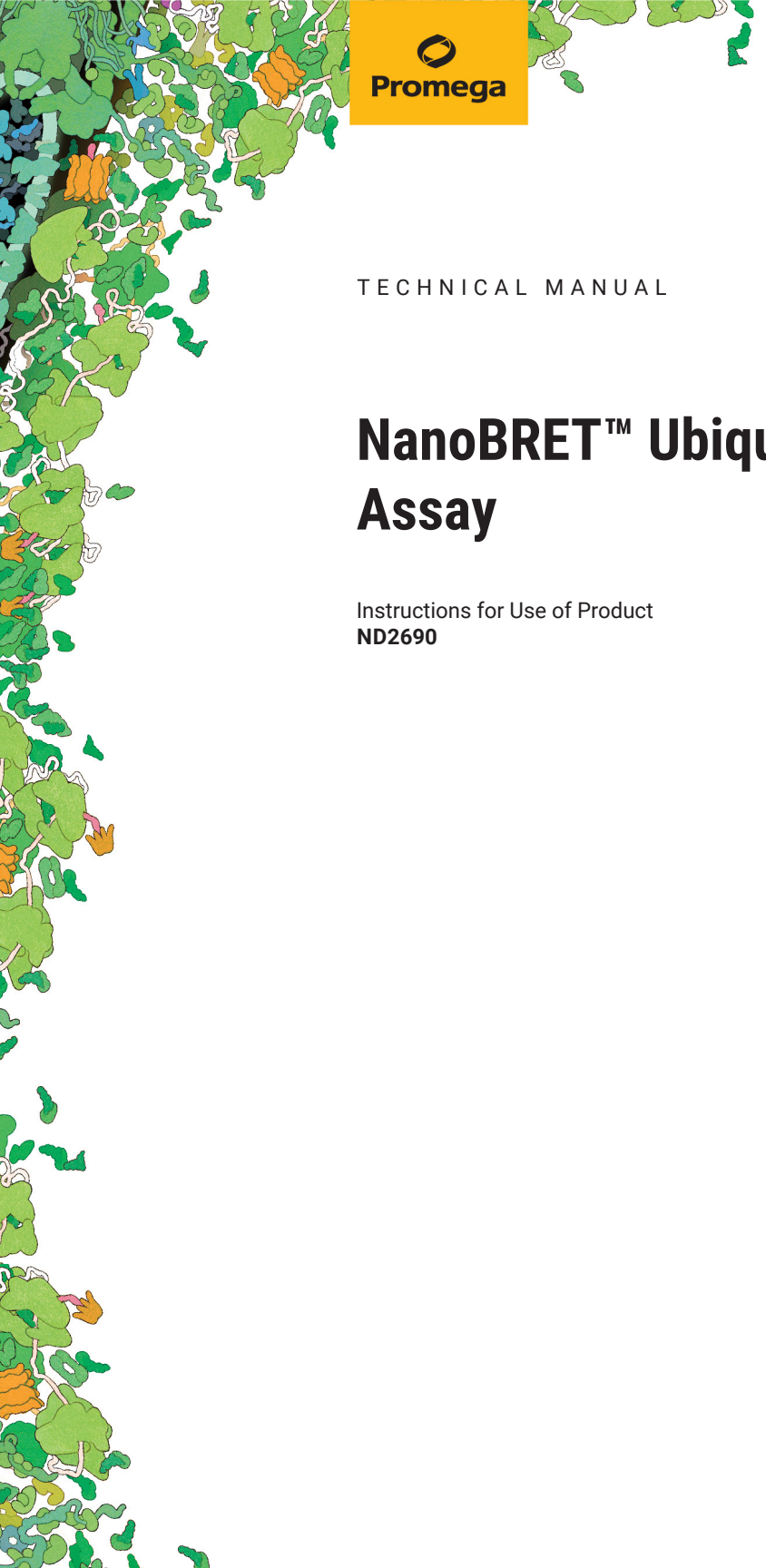

TECHNICAL MANUAL

NanoBRET™ Ubiquitination Assay

Instructions for Use of Product
ND2690

Revised 6/23
TM616

Promega

NanoBRET™ Ubiquitination 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

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1. Description

Proteins targeted for degradation via the ubiquitin proteasome system (UPS) require efficient ubiquitin conjugation prior to proteasome trafficking. Ubiquitination on any given target can vary in levels, mono- and poly-ubiquitination, and be mediated through a variety of amino acid linkages. The NanoBRET™ Ubiquitination Assay[a–g] is a live-cell ubiquitin assay, broadly measuring all types of ubiquitination on a target protein, both in terms of extent and linkage, but cannot discern amongst them. This assay can also be used to dynamically detect either an increase or a decrease in the relative level of target ubiquitination after cell treatments, including induction of signaling pathways or compound treatment (1).

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 (2). Due to ubiquitination potentially leading to protein loss, the optimal NanoBRET™ Ubiquitination Assay configuration is with the target protein as the luminescent donor and the ubiquitin protein as the fluorescent acceptor. This means you can monitor potential protein loss of the target protein while simultaneously assessing ubiquitination, made possible due to luminescent to fluorescent ratio in the NanoBRET™ assay. We provide an N-terminal HaloTag® fusion of the first repeat of UBB (polyubiquitin-B) with no modifications to the endogenous C-terminal sequence necessary to mediate conjugation. This fusion protein can be fluorescently labeled with the NanoBRET™ HaloTag® 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 endogenous target tagged with HiBiT using CRISPR gene editing and complemented with LgBiT. This protocol describes 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.

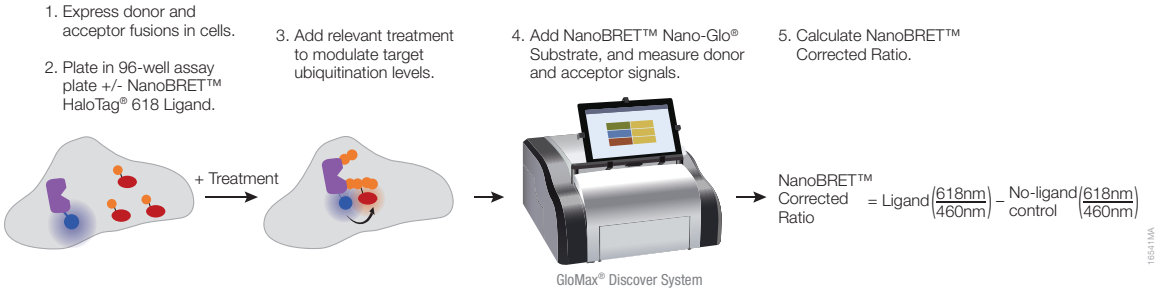

Figure 1. Overview of the five-step NanoBRET™ Ubiquitination Assay. First, the HaloTag®-Ubiquitin acceptor fusion and NanoLuc® or HiBiT donor fusion are expressed in cells. Second, the cells are replated into 96-well assay plates, and experimental samples are established, including the NanoBRET™ HaloTag® 618 Ligand (for experimental samples) and control samples (no fluorescent ligand). Third, relevant treatments are added to modulate (either an increase or a decrease) in target ubiquitination. Fourth, the NanoBRET™ Nano-Glo® Substrate is added, and donor and acceptor signals are measured on an instrument capable of measuring dual-filtered luminescence equipped with appropriate filters. Fifth, the corrected NanoBRET™ ratio is calculated by subtracting the NanoBRET™ ratios of the control (no fluorescent ligand) samples from the NanoBRET™ ratios of the experimental (NanoBRET™ HaloTag® 618 Ligand) samples.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
NanoBRET™ Ubiquitination Starter Kit	**1 each**	**ND2690**

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

- 20µg HaloTag®-Ubiquitin Fusion Vector
- 20µg pNLF1-N [CMV/Hygro] Vector
- 20µg pNLF1-C [CMV/Hygro] Vector
- 20µg HaloTag® Control Vector
- 20µg NanoLuc®-BRD4 FL Fusion Vector
- 20µl HaloTag® NanoBRET™ 618 Ligand
- 50µl NanoBRET™ Nano-Glo® Substrate

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.

Note: Each starter kit contains vectors to create N- and C-terminal NanoLuc® target protein fusions using standard cloning; the HaloTag®-Ubiquitin Fusion Vector, positive control NanoLuc®-BRD4 FL Fusion Vector and negative control HaloTag® Control Vector. Individual components are available to purchase separately. See Section 8.D, Related Products.

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

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.

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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 bleedthrough 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 bleedthrough 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:

- The GloMax® Discover System (Cat.# GM3000) with preloaded filters for donor 450nm/8nm BP and acceptor 600nm LP. Select the preloaded BRET:NanoBRET™ 618 protocoll from the Protocol menu.
- BMG Labtech CLARIOstar® with preloaded filters for donor 450nm/80nm BP and acceptor 610nm LP.
- 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 crosstalk in adjoining wells.

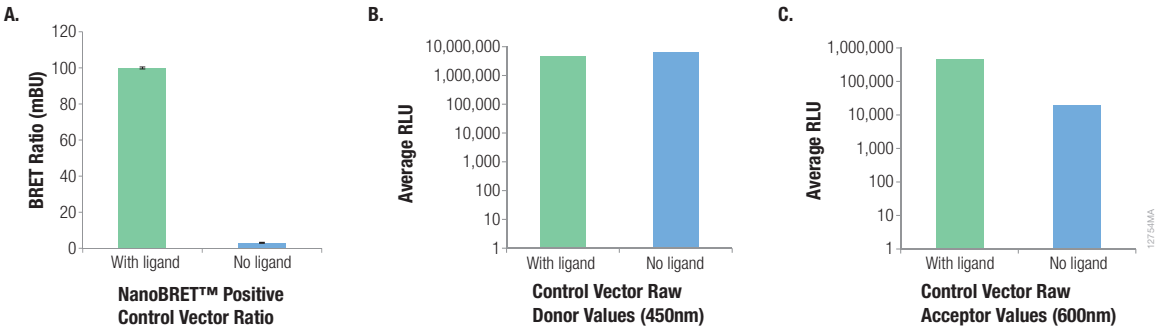

Figure 2. NanoBRET™ ratio and raw donor and acceptor measurements with the NanoBRET™ Positive Control vector. **Panel A.** Calculated NanoBRET™ ratio in experimental sample and no-ligand control. The no-ligand control represents the donor signal bleedthrough into the acceptor channel of the NanoBRET™ 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 bleedthrough. Data generated using the GloMax® Discover System equipped with 450nm/8nm BP and 600nm LP filters.

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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™ Ubiquitination assay, we have optimized the tag placement of the HaloTag® acceptor fusion, provided as an N-terminal HaloTag®-Ubiquitin fusion. To achieve an optimal NanoBRET signal, we recommend testing both N- and C-terminal tag placement on 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 dilute 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™ Ubiquitination Assay can be used to detect both increases and decreases in relative target ubiquitination. If using a compound to modulate ubiquitination levels, 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 ubiquitination 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 (3), a PROTAC compound that 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™ Ubiquitination 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.# 14190-144)
- FuGENE® HD Transfection Reagent (Cat.# E2311)
- DMEM (GIBCO™ Cat.#11995-065)
- 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)
- 0.05% Trypsin/EDTA (GIBCO™ Cat.# 25300-054)
- ubiquitin/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™ Ubiquitination 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 manufacturers 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™ Ubiquitination Assay performance with NanoLuc®-BRD4 FL Fusion Vector and HaloTag®-Ubiquitin **or** HaloTag® Control Vector
- Assay optimization: User-generated NanoLuc® target fusion vectors transfected with HaloTag®-Ubiquitin Fusion Vector
- Transfecting the HaloTag®-Ubiquitin 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.

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

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™ Ubiquitination 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 1:100 transfection mixture consisting of 2µg of HaloTag®-Ubiquitin Fusion Vector OR HaloTag® Control Vector + 0.02µg of 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 HaloTag®-Ubiquitin Fusion Vector or HaloTag® Control Vector	1µg NanoLuc® Fusion Vector
1:10 (NanoLuc to HaloTag)	2µg HaloTag®-Ubiquitin 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™ Ubiquitination 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 via the CMV LgBiT Vector (Promega CAS# CS1956B03) by addition to the transfection mixture.

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:HaloTag)	1µg HaloTag®-Ubiquitin Fusion Vector or HaloTag® Control Vector	1µg LgBiT vector
	1:10 (LgBiT:HaloTag)	2µg HaloTag®-Ubiquitin Fusion Vector or HaloTag® Control Vector	0.2µg LgBiT vector
	1:100 (LgBiT:HaloTag)		0.02µg LgBiT vector
HiBiT CRISPR Fusion Stably Expressing LgBiT	N/A	2µg HaloTag®-Ubiquitin 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 2.2×10^5 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 90µl of each pool of the cells prepared in Step 6 in at least 3–4 wells.

   384-well format with endpoint detection: Dispense 36µl 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 Ubiquitination

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

Note regarding Dose-Response Curves (DRC): If compounds are to be tested at a range of concentrations, perform serial dilutions in diluent containing the same amount of solvent as the highest concentration. For example, if the highest 10X concentration contains 1% DMSO, subsequent dilutions should be done in assay medium containing 1% DMSO to keep the final concentration at 0.1% DMSO for all samples. For a vehicle or zero control, add DMSO-containing medium without compound. The NanoBRET™ assay has been tested at up to 0.5% final DMSO concentration with no consequence. Higher DMSO concentrations may be tolerated in the assay.

5.C. Adding Test Compounds and Detecting Ubiquitination (continued)

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

1. Prepare a 10X concentration of test compound in Opti-MEM™ I Reduced Serum Medium, no phenol red (e.g., 10µM for a 1µM final concentration). 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 of test compound or DMSO solution to each well.

   384-well format: Add 4µl of test compound or DMSO solution to each well.

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

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

3. Prepare a 5X solution of NanoBRET™ Nano-Glo® Substrate in Opti-MEM™ I Reduced Serum Medium, no phenol red. This is an 100-fold dilution of the stock reagent. For one 96-well plate, prepare a minimum of 2.5ml of medium + 25µl of stock reagent. For one 384-well plate, prepare a minimum of 4ml of medium + 40µl of stock reagent. Substrate volume in the kit allows for the preparation of 4% extra detection solution.

   Note: Use the 5X 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 compound in Opti-MEM™ I Reduced Serum Medium, no phenol red (e.g., 10µM for a 1µM final concentration). 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 of test compound or DMSO solution to each well.

   384-well format: Add 8µl of test compound or DMSO solution to each well.

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

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

6. 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.

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

5.D. NanoBRET™ Calculations (continued)

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 of 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 ubiquitination, as seen with the ubiquitin/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 for the denominator.

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

6. Representative Data

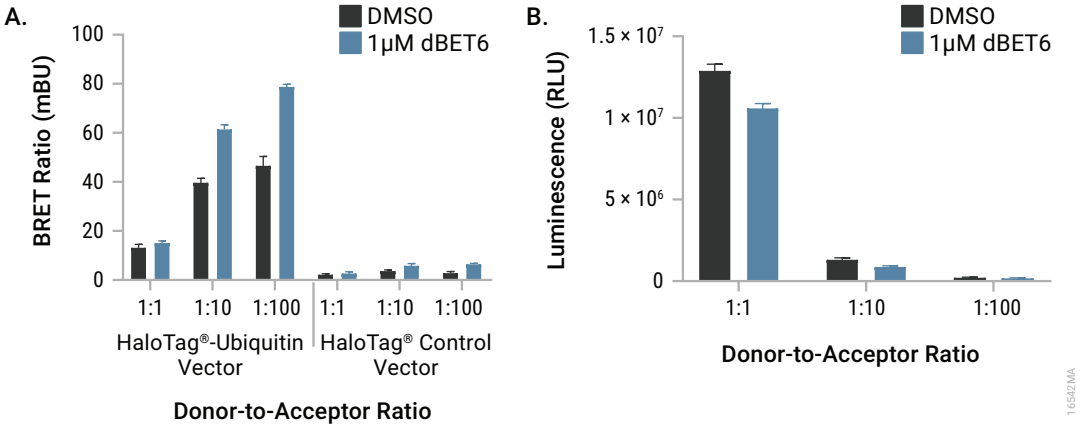

Figure 3. NanoBRET™ Ubiquitination Assay using the control HaloTag®-Ubiquitin and NanoLuc®-BRD4 fusion proteins. Panel A. Donor-to-acceptor ratios of 1:1, 1:10 and 1:100 were tested with both the specific HaloTag®-Ubiquitin Fusion Vector and HaloTag® Control Vector. Samples were treated with 1µM dBET6 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/Ubiquitin with dBET6 treatment when 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 PROTAC compound treatment. The dBET6 treatment resulted in an expected decrease in donor luminescence.

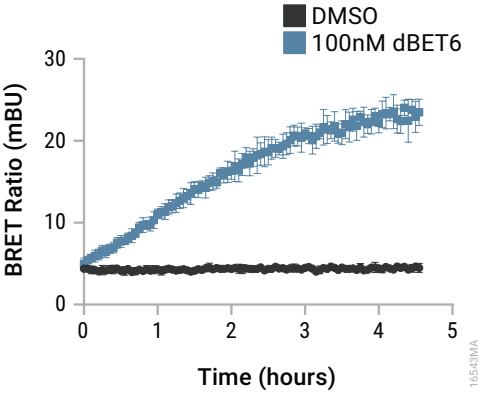

Figure 4. Measurements taken with the NanoBRET™ Ubiquitination Assay in kinetic format. The HaloTag®-Ubiquitin Fusion Vector was transiently transfected into cells stably expressing LgBiT protein and an endogenously tagged HiBiT-BRD4 fusion. Cells were treated with 100nM dBET6 or DMSO and NanoBRET™ signal was measured continuously for 4.5 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	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® Mammalian Pull-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.

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7. Troubleshooting (continued)

Symptoms	Causes and Comments
No NanoBRET™ ratio even with the NanoBRET™ Positive Control (continued)	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.
	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 increased interaction between BRD4 and ubiquitin with dBET6 treatment.

Symptoms	Causes and Comments
Ratios and raw values are different from those shown on examples (continued)	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 setup.
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 with compound concentrations to determine optimal treatment concentration. For example, using 0.1–10µM of compound.
	Target ubiquitination is time dependent. Test multiple timepoints or perform the assay in kinetic format to determine optimal time of detection.
	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.
	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 target ubiquitination 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.
	Ubiquitination may not result in target degradation. This assay detects all forms of ubiquitination but cannot distinguish between linkages, extent or poly- versus mono-ubiquitination. The target may be ubiquitinated without proteasomal degradation occurring.
	Timeframe of target RLU measurement was not sufficient to detect degradation. Compound may show degradation at timepoints longer than detecting target ubiquitination.

7. Troubleshooting (continued)

Symptoms	Causes and Comments
Measure a decrease in NanoLuc® or HiBiT donor luminescence without detecting an increase in ubiquitination.	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.

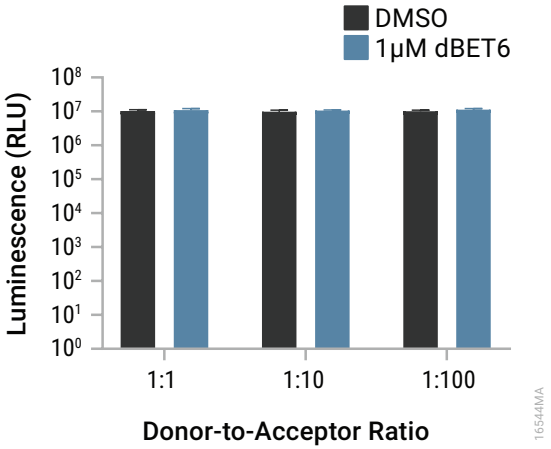

Figure 5. Multiplexing NanoBRET™ Ubiquitination Assay with the CellTiter-Glo® 2.0 Cell Viability Assay. Experimental samples for the NanoBRET™ Ubiquitination Assay from Figure 3 were multiplexed with the CellTiter-Glo® 2.0 Assay to measure cell viability. There was no effect on cell viability observed with dBET6 treatment.

8.B. Composition of Buffers and Solutions

Cell Culture Medium

90%	DMEM (GIBCO Cat.#11995-065)
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)

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8.C. 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. Machleidt, T. *et al*. (2015) NanoBRET—A novel BRET platform for the analysis of protein-protein interactions. *ACS Chem. Biol.* **10**, 1797–804.
3. 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™ Proteasomal Recruitment Starter Kit	1 each	ND2730
NanoBRET™ PPI Control Pair (p53, MDM2)	1 each	N1641

Viability Assay

Product	Size	Cat.#
CellTiter-Glo® 2.0 Cell Viability Assay	10ml	G9241

Additional kit sizes are available.

Detection Reagents

Product	Size	Cat.#
NanoBRET™ Nano-Glo® Detection System*	200 assays	N1661
NanoBRET™ Nano-Glo® Kinetic Detection System*	200 assays	N2583
NanoBRET™ Nano-Glo® Substrate*	50µl	N1571
HaloTag® NanoBRET™ 618 Ligand	20µl	G9801

*Additional kit sizes are available.

Vectors

Product	Size	Cat.#
HaloTag®-Ubiquitin Fusion Vector	20µg	N2721
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

Additional kit sizes are available.

Multimode Detection Instrument

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

Cell Lines Available upon Request

Product	Cat.#
HEK293 HaloTag-VHL Stable Cell Line	Please enquire
HEK293 HaloTag-CRBN Stable Cell Line	Please enquire

Please enquire at: **www.promega.com/c/global/forms/contact-tailored-rd-solutions-form/**

9. Summary of Changes

The following changes were made to the 6/23 revision of this document:

1. Components in Section 2 were updated and “Available Separately” products moved to Section 8.D.
2. Non-Promega catalog numbers were updated in Section 5.
3. Protocol steps in Sections 5.B and 5.C were revised.
4. The font and cover image were updated.
5. Legal statements were updated.

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[d]U.S. Pat. Nos. 8,557,970, 8,669,103, 9,777,311, 9,840,730, 9,951,373, 10,233,485, 10,633,690, 10,844,422, 11,365,436; European Pat. Nos. 2456864, 2635595, 2990478, 3181687, 3409764; Japanese Pat. Nos. 6038649, 6155424, 6227615, 6374420, 6539689; and other patents and patents pending

[e]U.S. Pat. No. 8,809,529, European Pat. No. 2635582, Japanese Pat. No. 5889910 and other patents and patents pending.

[f]U.S. Pat. Nos. 7,425,436, 7,935,803, 8,466,269, 8,742,086, 8,420,367, 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, European Pat. No. 2932267, Japanese Pat. Nos. 6751294 and 7092691 and other patents and patents pending.

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