TECHNICAL MANUAL

NanoBRET™ BRPF1/Histone H4 Interaction Assay

Instructions for Use of Product
N1910

Revised 12/15
TM450

NanoBRET™ BRPF1/Histone H4 Interaction 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

The NanoBRET™ BRPF1/Histone H4 Interaction Assay[a–h] monitors the interaction between human BRPF1 (Bromodomain and PHD finger containing protein 1) and histone H4 in living cells. Bromodomain-containing proteins are critical components of nuclear protein complexes involved in the recruitment of chromatin modifying enzymes and transcriptional regulation of acetylated chromatin. The protein:protein interaction (PPI) of the bromodomain of BRPF1 with acetylated histones is of great interest for drug targeting because modulation of this interaction has been implicated in disease (1,2).

This technical manual describes optimized NanoBRET™ protocols specific for the detection of the interaction of either full-length BRPF1 protein (BRPF1 FL) or only the bromodomain of BRPF1 (BRPF1 BD) with the histone H4 variant. In this assay, we have included both the full-length BRPF1 and highly studied BD alone for users that may want to understand the contribution of the bromodomain alone in addition to the full-length protein.

NanoBRET™ Protein:Protein Interaction (PPI) Assay is a proximity-based assay dependent upon energy transfer from a luminescent donor to a fluorescent acceptor. (For more information, consult the *NanoBRET™ Protein:Protein Interaction Assay Technical Manual* #TM439). For the NanoBRET™ BRPF1/Histone H4 Interaction Assay, the donor fusion protein is either NanoLuc®-BRPF1 FL or NanoLuc®-BRPF1 BD protein, and the acceptor fusion protein is Histone H4-HaloTag® coding region.

The basis of the NanoBRET™ protocol is shown in Figure 1.

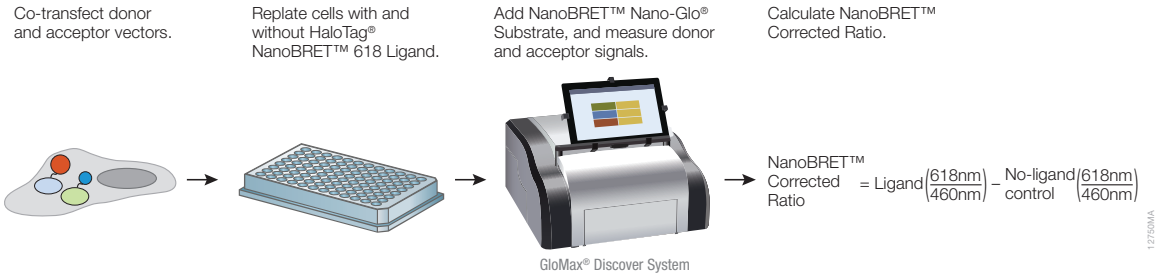

Figure 1. The NanoBRET™ PPI Assay protocol consists of four steps. First, both NanoLuc® and HaloTag® fusion vectors are transfected into the appropriate cell lines. Second, the cells are replated into either 96- or 384-well plates, and experimental samples are established, including the fluorescent NanoBRET™ HaloTag® 618 Ligand (for experimental samples) and control samples (no fluorescent ligand). If compounds are to be tested, add them at this step. Third, the NanoBRET™ NanoLuc® Substrate is added, and donor and acceptor signals are measured on an instrument capable of measuring dual-filtered luminescence equipped with appropriate filters. Fourth, the corrected NanoBRET™ ratio is calculated, which is a subtraction of NanoBRET™ ratios of the control samples from the experimental ligand-containing samples.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
NanoBRET™ BRPF1/Histone H4 Interaction Assay	**1 each**	**N1910**

This system is sufficient for 400 assays performed in 96-well plates. This system also can be used in 384-well plates for a total of 1,000 assays. Includes:

- 2 × 50µl NanoBRET™ Nano-Glo® Substrate
- 2 × 20µl HaloTag® NanoBRET™ 618 Ligand
- 20µg p53-HaloTag® Fusion Vector
- 20µg NanoLuc®-MDM2 Fusion Vector
- 20µg NanoLuc®-BRPF1 FL Fusion Vector
- 20µg NanoLuc®-BRPF1 BD Fusion Vector
- 20µg Histone H4-HaloTag® Fusion Vector

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

Note: Enough ligand and substrate are provided for each individual well when performing the NanoBRET™ BRPF1/Histone H4 Interaction Assay. Because we recommend always including a set of samples without HaloTag® NanoBRET™ 618 Ligand as a negative control, you may have extra ligand. The individual detection reagents are also available to purchase separately.

Available Separately

PRODUCT	SIZE	CAT.#
NanoBRET™ Nano-Glo® Detection System	**200 assays**	**N1661**
	1,000 assays	**N1662**
	10,000 assays	**N1663**
NanoBRET™ Nano-Glo® Substrate	**50µl**	**N1571**
	5 × 50µl	**N1572**
	2 × 1.25ml	**N1573**
HaloTag® NanoBRET™ 618 Ligand	**20µl**	**G9801**

Transfection Reagent

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

3. Before You Begin

3.A. Preparing NanoBRET™ Expression Vectors

The amount of each plasmid DNA provided with the system is sufficient for a few initial experiments, but we strongly recommend that you archive and propagate each plasmid as transfection-ready DNA. Follow standard protocols for plasmid transformation into *E. coli* for archival storage, vector propagation and tissue-culture-grade DNA preparation.

The vectors supplied with the NanoBRET™ BRPF1/Histone H4 Interaction Assay include p53-HaloTag® Fusion Vector, NanoLuc®-MDM2 Fusion Vector, NanoLuc®-BRPF1 FL Fusion Vector, NanoLuc®-BRPF1 BD Fusion Vector and Histone H4-HaloTag® Fusion Vector. For each vector, the fusion protein is constitutively expressed by a CMV promoter and includes a kanamycin expression cassette to select for the plasmid during bacterial propagation.

3.B. Instrument Requirements and Setup

To perform NanoBRET™ PPI Assays, use an instrument capable of sequentially measuring dual-filtered luminescence values equipped with appropriate filters. The ideal filter setup will include a band pass (BP) filter centered around 460nm to measure the donor signal (e.g., Emission 450nm/BP 80nm) and a long pass (LP) filter starting at around 600–610nm to measure the acceptor signal (e.g., Emission 610nm/LP). **Note:** Filters outside of these ranges will miss critical measurements and compromise data quality.

1. The NanoBRET™ bioluminescent donor emission occurs at 460nm. To measure this 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 410nm to 490nm range.

   Note: A BP filter is preferred for the donor signal measurement to selectively capture the signal peak and avoid measuring any acceptor peak bleed-through. However, a short pass (SP) filter that covers the 460nm area also can be used. This may 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, reducing the assay window.

2. 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 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 on the 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 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 preloaded filters for 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, using donor 450nm CWL, 25mm diameter, 80nm FWHM, Interference Filter and acceptor 1 inch diameter, RG-610 Long Pass Filter

Another instrument capable of measuring dual luminescence is the PerkinElmer EnVision® Multilabel Reader with the following recommended setup:

- 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™ PPI Control Pair (p53, MDM2)

To ensure your instrument has been configured properly, we recommend testing with the NanoBRET™ PPI Control Pair (p53, MDM2; Cat.# N1641) consisting of the interacting protein partners p53 and MDM2 included in the NanoBRET™ BRPF1/Histone H4 Interaction Assay or available separately. This reference control pair can also be used as a control when running other NanoBRET™ assays. The p53/MDM2 interaction can be specifically disrupted by the commercially available compound Nutlin-3 (3; Tocris Cat.# 3984). Figure 2 shows representative data with this system generated using the GloMax® Discover System (Cat.# GM3000). In all cases, the expected biological response to Nutlin-3 was observed. For this system as well as other NanoBRET™ PPI Assays, the absolute NanoBRET™ ratios will be dependent on several factors, including the proximity of the protein partners, affinity of the interaction, relative occupancy and instrument setup. Absolute values should not be compared between systems or instruments.

3.C. NanoBRET™ PPI Control Pair (p53, MDM2; continued)

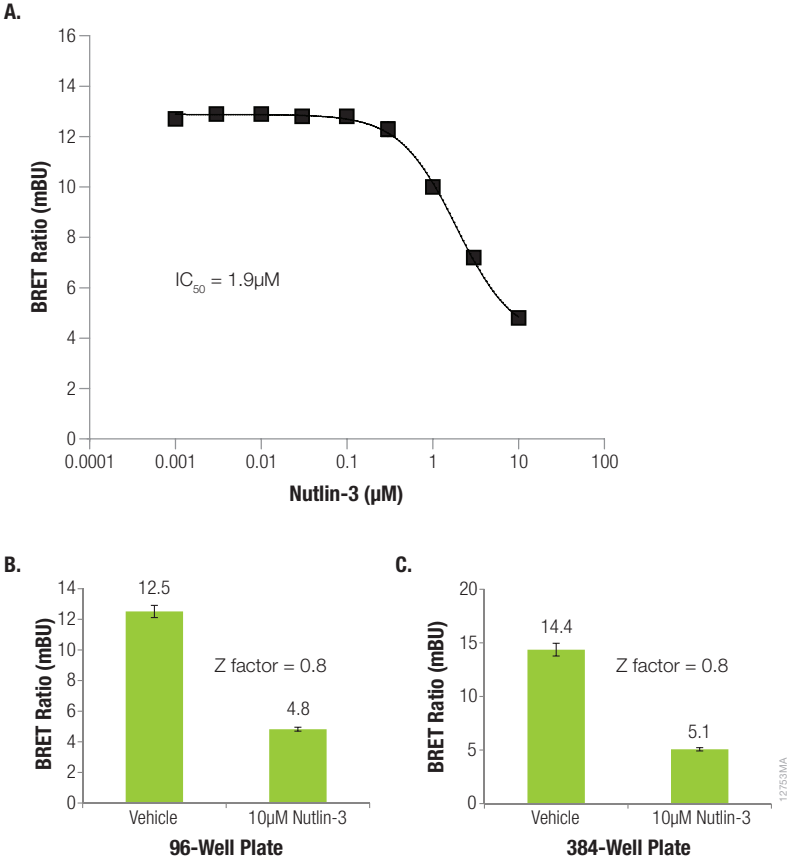

Figure 2. Representative data for the p53/MDM2 NanoBRET™ PPI Control Pair. Panel A is a dose-response curve (DRC) against the specific inhibitor Nutlin-3. Panels B and C show single-dose measurements and calculation of Z factors in the 96-well format (**Panel B**) and 384-well format (**Panel C**). Data generated using the GloMax® Discover System equipped with 450nm/8nm BP and 600nm/LP filters.

4. NanoBRET™ BRPF1/Histone H4 Interaction Assay Protocols

Materials to be Supplied by the User

- HEK293 or similar cultured mammalian cells
- white, 96-well plate (Costar Cat.# 3917) or 384-well plate (Corning Cat.# 3570)
- tissue culture equipment and reagents (see Composition of Buffers and Solutions, Section 7).
- DPBS (Invitrogen Cat.# 14190)
- 0.05% Trypsin/EDTA (Invitrogen Cat.# 25300)
- FuGENE® HD Transfection Reagent (Cat.# E2311)
- DMEM (Gibco Cat.#11995)
- fetal bovine serum (HyClone Cat.# SH30070.03)
- Opti-MEM® I Reduced Serum Medium, no phenol red (Life Technologies Cat.# 11058-021)
- DMSO (Sigma Cat.# 2650)
- Nuclease-Free Water (Cat.# P1191)
- **Optional:** p53/MDM2 inhibitor Nutlin-3 (Tocris Cat.# 3984)

4.A. Transient Transfection of HEK293 Cells with HaloTag® and NanoLuc® Fusions

The following transient transfection conditions use mammalian HEK293 cells and have been used successfully with HCT116 and HeLa cell lines. Other cell lines may require optimization. The use of stable cell lines generally is not recommended due to the need to regulate the relative expression levels of donor and acceptor proteins. However, we successfully tested a cell line stably expressing one of the partners and transiently transfected the second protein partner, but the resulting NanoBRET™ ratio had a lower dynamic range.

If using a transfection reagent other than FuGENE® HD Transfection Reagent, follow the manufacturer's recommendations but use the same relative donor-to-acceptor DNA ratios.

Two different transfection schemes are described. Follow the appropriate transfection scheme as DNA amounts required are different for the controls or the optimized BRPF1 assays.

- Checking instrument performance by NanoBRET™ PPI Control Pair
- BRPF1 NanoBRET™ assays

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

Transfection Conditions for NanoBRET™ PPI Control Pair

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.A. Transient Transfection of HEK293 Cells with HaloTag® and NanoLuc® Fusions (continued)

Transfection Conditions for NanoBRET™ PPI Control Pair (continued)

4. Plate 2ml of cells (800,000 cells) into a 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 of p53-HaloTag® Fusion Vector DNA + 0.2µg of NanoLuc®-MDM2 Fusion Vector DNA 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 8µl of FuGENE® HD Transfection Reagent, and incubate at room temperature for 10 minutes.
9. Add transfection mixture to wells with attached cells, and express proteins for approximately 20 hours at 37°C, 5% CO_2.
10. Proceed to Section 4.B, or if testing compounds, Section 4.C.

Transfection Conditions for NanoBRET™ BRPF1/Histone H4 Interaction Assays

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 the following DNA amounts, depending on the pair being tested:

PPI Pair	HaloTag® Plasmid	NanoLuc® Plasmid (diluted in water)
Histone H4 and BRPF1 FL	2µg of Histone H4-HaloTag®	0.02µg of NanoLuc®-BRPF1 FL
Histone H4 and BRPF1 BD	2µg of Histone H4-HaloTag®	0.02µg of NanoLuc®-BRPF1 BD

Note: These transfection conditions are optimized for the NanoBRET™ BRPF1/Histone H4 Interaction Assays. The optimal transfection conditions for other NanoBRET™ protein pairs may differ. Refer to the appropriate NanoBRET™ Interaction Assay Technical Manual for the recommended transfection parameters.

7. Add 100µl of Opti-MEM® I Reduced Serum Medium, no phenol red, to the transfection mixture, and mix well.
8. Add 8µl of FuGENE® HD Transfection Reagent, and incubate at room temperature for 10 minutes.
9. Add transfection mixture to wells with attached cells, and express proteins for approximately 20 hours at 37°C, 5% CO_2.
10. Proceed to Section 4.B, or if testing compounds, Section 4.C.

4.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.

   Note: If compounds or inhibitors are being tested, use the optional protocol in Section 4.C, which adjusts cell density and volume to accommodate compounds being tested.
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. For NanoBRET™ measurements without compound treatment, plate cells in the volumes indicated below:

   96-well format: Dispense 100µl of each pool of the cells prepared in Step 6 in at least 3–4 wells.

   384-well format: Dispense 40µl of each pool of the cells prepared in Step 6 in at least 3–4 wells.
8. Incubate plates at 37°C, 5% CO_2 a minimum of 4–6 hours to overnight (18–24 hours).
9. Proceed to Section 4.D.

4.C. Optional Protocol for Testing Compounds or Inhibitors

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 an entire 384-well plate, you need approximately 16ml of cells at this concentration.

4.C. Optional Protocol for Testing Compounds or Inhibitors (continued)

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: Dispense 36µl of each pool of the cells prepared in Step 6 in at least 3–4 wells

8. Add compounds at tenfold higher than that desired for the final concentration in assay media (e.g.,100µM for 10µM final concentration):

   96-well format: Dispense 10µl of 10X compound or vehicle to plated cells.

   384-well format: Dispense 4µl of 10X compound or vehicle to plated cells.

9. Incubate plates at 37°C, 5% CO_2 a minimum of 4–6 hours to overnight (18–24 hours). For maximum compound effect, we recommend overnight incubation.

10. Proceed to Section 4.D.

Note regarding Dose-Response Curves (DRC): If compounds or inhibitors 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 media containing 1% DMSO to keep the final concentration at 0.1% DMSO for all samples. For a vehicle or zero control, add DMSO containing media 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.

4.D. Adding NanoBRET™ Nano-Glo® Substrate and Taking NanoBRET™ Measurements

1. Prepare a 5X solution of NanoBRET™ Nano-Glo® Substrate in Opti-MEM® I Reduced Serum Medium, no phenol red. This is a 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 3.9ml of medium + 39µl 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 5X solution within 2 hours if stored at room temperature or within 4 hours if stored at 4°C.

2. 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.

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

   Note: You can use the same plate to determine cell viability and determine the effect of compounds on cell viability (i.e., toxicity) by multiplexing with CellTiter-Glo® 2.0 Assay (Cat.# G9241). After taking NanoBRET™ measurements, keep the plate and follow the protocol in Section 9.

4.E. 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-acceptor control samples. To factor in donor-contributed background or bleed-through, subtract the no-acceptor control mean from the Experimental mean for the corrected NanoBRET™ ratio.

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

4. **Optional:** Generate Z´ and Z factor calculations to gauge assay consistency (4). A Z´ factor estimates assay consistency by comparing the mean and standard deviation values of the experimental samples and a baseline control such as the no-ligand control.

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

In the presence of a modulator, such as an inhibitor, 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. In the following example, the treated sample represents an inhibitor. However, if measuring an enhancer, reverse the values in the formula to ensure a positive value.

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

5. Assay Validation Data

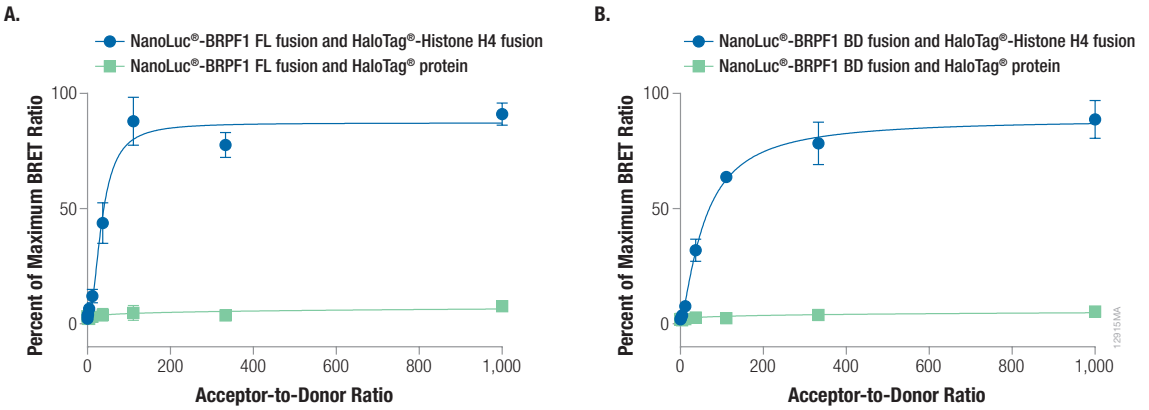

Figure 3. Donor Saturation Assay (DSA) showing specificity for detecting the interaction of BRPF1 FL or BRPF1 BD with Histone H4. HEK293 cells were transfected with a constant amount of NanoLuc® donor DNA and paired with increasing amounts of Histone H4-HaloTag® Fusion Vector acceptor DNA to represent increasing amounts of acceptor-to-donor (A:D) ratios. **Panel A** shows the interaction of full-length (FL) BRPF1 with Histone H4. **Panel B** shows the interaction of only the BD (bromodomain) of BRPF1 with Histone H4. The negative control for both reactions is HaloTag® protein alone used as a mock acceptor in place of the Histone H4-HaloTag® fusion. After transfection and protein expression, the cells were plated in a 96-well format, and NanoBRET™ measurements were captured on a GloMax® Discover System equipped with 450nm/8nm BP and 600nm LP filters. The data are plotted as percent of the maximum BRET ratio obtained for the specific protein pairs against A:D ratios.

Both NanoBRET™ BRPF1/Histone H4 Interaction Assays in Figure 3 generate hyperbolic curves, which indicate detection of a specific BRET interaction. Nonspecific interaction with the negative control protein alone (bystander BRET) generates much weaker linear ratios.

6. Troubleshooting

Symptoms	Causes and Comments
Unable to generate NanoBRET™ ratio, even with control pair.	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 the gain on the PMT is set to detect donor signal without instrument saturation.
	Lack of expression of protein partners. Check expression of NanoLuc® fusions by measuring luminescence or reading light at 460nm. Check expression of fluorescent HaloTag® fusion using a fluorometer, reading at 618nm, or quantifying bands by SDS-PAGE analysis using a fluorescence scanner (see the *HaloTag® Mammalian Pull-Down and Labeling Systems Technical Manual* #TM342).
	Improper relative amounts of HaloTag® and NanoLuc® vectors. Follow the recommended relative amounts of HaloTag® and NanoLuc® vectors indicated in the protocol.
	Incorrect 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 negative control from the ratio of the experimental samples.
	Poor cell health or compound toxicity. Ensure cells are still viable at the time of measuring the assay by performing the CellTiter-Glo® 2.0 Assay (see Section 9).
Poor Z´ and Z factor values.	Too much variability in assay. Consider automated dispensing to reduce variability.
	A weak modulator is present. A weak inhibitor can produce a small delta between treated and untreated samples, resulting in a suboptimal Z factor value that is not due to assay robustness.
	NanoLuc® signal is low, close to the instrument's limit of detection. The recommended amount of donor DNA has been optimized for detection on most commonly used instruments. If the luminometer being used has reduced sensitivity, increase the amount of NanoLuc® donor DNA to 0.2–0.5µg. Keep the HaloTag® acceptor DNA at 2µg.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
Ratios and raw values are different from the examples shown.	Absolute raw values and ratios may vary among instruments and assay setups. Confirm that the expected biological response is observed, such as the effect of Nutlin-3 on the interaction of p53 and MDM2 Control Protein Pair.
	Absolute raw values and ratios will vary among PPI systems. Absolute NanoBRET™ values depend on the proximity of the protein partners, the affinity of the interaction, the relative occupancy with other interacting proteins and the instrument setup. When possible, check specificity with a known modulator of the BRPF1/Histone H4 interaction.
Unable to get proper protein expression.	Suboptimal transfection conditions. • Follow the recommended relative amounts of HaloTag® and NanoLuc® vectors. • Optimize transfection conditions if using a different cell line or transfection reagent. • Use only transfection-quality DNA.
	Poor cell health due to too many passages. Do not passage cells above 40 passages.
Unable to detect signal reduction with known compound inhibitor.	Compound is too dilute or there is insufficient time to detect inhibition in live cells. Increase the concentration of inhibitor, treat overnight or both to see maximum effect.
	Full-length BRPF1 was used rather than BRPF1 bromodomain. If compound was developed in vitro against BD, it may not disrupt the interaction of BRPF1. Test BD alone versus BRPF1 FL protein.
	Compound affects enzymatic activity between proteins without disrupting the PPI.
Discolored HaloTag® NanoBRET™ 618 Ligand	The HaloTag® NanoBRET™ 618 Ligand is typically a 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 closed form, the ligand is colorless. When added to medium, the ligand converts to the open usable form. To confirm chemical integrity, dilute 1µl of HaloTag® NanoBRET™ 618 Ligand in 1ml of Opti-MEM® I Reduced Serum Medium, no phenol red, and check fluorescence by exciting the fluorophore at 593±4nm and reading the emission at 621±4nm. If using the GloMax® Discover System, use the green channel (Ex: 525nm, Em: 580–640 nm).

7. Composition of Buffers and Solutions

Cell Culture Medium

- 90% DMEM (Gibco Cat #11995)
- 10% FBS (HyClone Cat.# SH30070.03)

Assay Medium

- 96% Opti-MEM® I Reduced Serum Medium, no phenol red (Life Technologies Cat.# 11058)
- 4% FBS (such as HyClone Cat.# SH30070.03)

8. References

1. Filippakopoulos, P. and Knapp, S. (2014) Targeting bromodomains: Epigenetic readers of lysine acetylation. *Nat. Rev. Drug Discov.* **13**, 337–56.
2. Demont, E.H. *et al.* (2014) 1,3-dimethyl benzimidazolones are potent, selective inhibitors of the BRPF1 bromodomain. *ACS Med. Chem. Lett.* **5**, 1190–5.
3. Vassilev, L.T. *et al.* (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **303**, 844–8.
4. Zhang J.H., Chung, T.D. and Oldenburg, K.R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4**, 67–73.

9. 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 4 shows example data.

1. Equilibrate CellTiter-Glo® 2.0 Reagent to room temperature.
2. Following NanoBRET™ measurements, add 125µl of CellTiter-Glo® 2.0 Reagent per well of the plate, and mix on a plate shaker at 500–700rpm for 5 minutes.
3. Incubate the plate at room temperature for 30 minutes to allow cell lysis and quenching of NanoLuc® signal.
4. After the 30-minute incubation is complete, measure total luminescence on a luminometer. If using the GloMax® Discover Instrument, read the plate by selecting the CellTiter-Glo® protocol.
5. If determining compound toxicity, compare the luminescence (RLU) of vehicle-containing samples versus compound-containing samples. Note that even if some toxicity is observed, the NanoBRET™ ratio is only derived from the live cells in the NanoBRET™ assay. Unless total cell death is observed, a reduction in NanoBRET™ signal by an inhibitor is most likely due to actual disruption of the interaction and not cell death.

9. Multiplexing with the CellTiter-Glo® 2.0 Assay (continued)

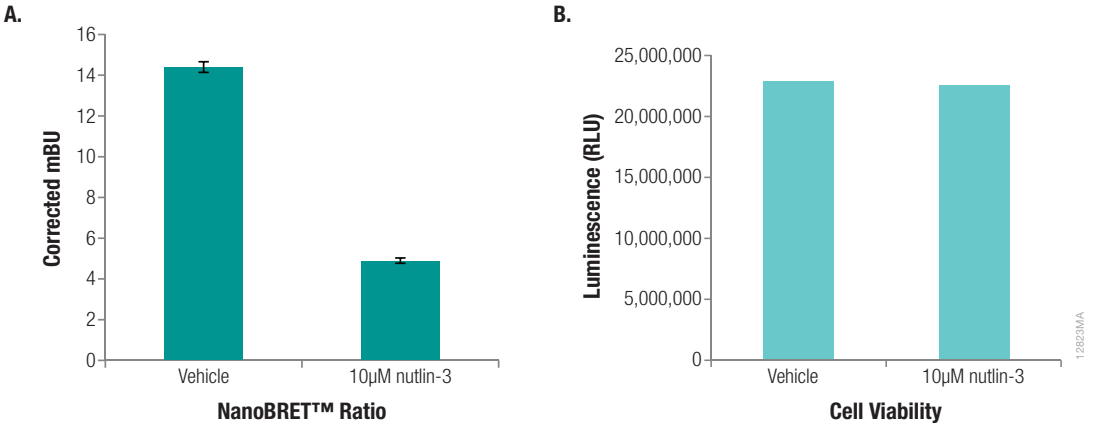

Figure 4. Example data for multiplexing the NanoBRET™ PPI Assay with the CellTiter-Glo® 2.0 Assay. **Panel A.** The NanoBRET™ PPI Control Pair (p53-HaloTag® Fusion Vector and NanoLuc®-MDM2 Fusion Vector) were assayed in the NanoBRET™ Assay with and without the modulator nutlin-3. **Panel B.** Following NanoBRET™ measurements, the same samples were assayed for cell viability using the CellTiter-Glo® 2.0 Reagent.

10. Related Products

NanoLuc® Vectors

Product	Size	Cat.#
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
pNLF1-secN [CMV/Hygro] Vector	20µg	N1371

HaloTag® Fusion Vectors

Product	Size	Cat.#
pFN21A HaloTag® CMV Flexi® Vector	20µg	G2821
pFC14K HaloTag® CMV Flexi® Vector	20µg	G9661

Multimode Detection Instrument

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

Transfection Reagent

Product	Size	Cat.#
FuGENE® HD Transfection Reagent	1ml	E2311
	5 × 1ml	E2312

Cell Viability Assay

Product	Size	Cat.#
CellTiter-Glo® 2.0 Assay	10ml	G9241
	100ml	G9242
	500ml	G9243

11. Summary of Change

The following change was made to the 12/15 revision of this document:

Updated the Chroma emission filters to use with the PerkinElmer EnVision® Multilabel Reader.

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[c]U.S. Pat. No. 8,809,529 and other patents pending.

[d]U.S. Pat. No. 7,867,726, Japanese Pat. No. 4748685 and other patents and patents pending.

[e]U.S. Pat. Nos. 8,557,970 and 8,669,103 and other patents pending.

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

[g]Patents Pending.

[h]Licensed under EP1295121 and EP1088233.

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