



TECHNICAL MANUAL

# NanoBRET™ Protein:Protein Interaction System

Instructions for Use of Products  
N1661, N1662, N1663, N2583, N2584 and N2585

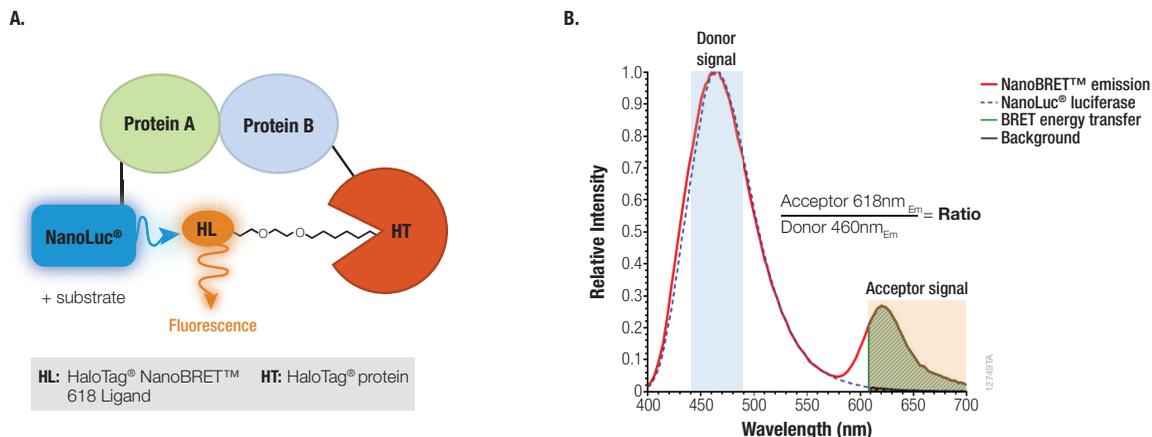
# NanoBRET™ Protein:Protein Interaction System

All technical literature is available at: [www.promega.com/protocols/](http://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](mailto:techserv@promega.com)

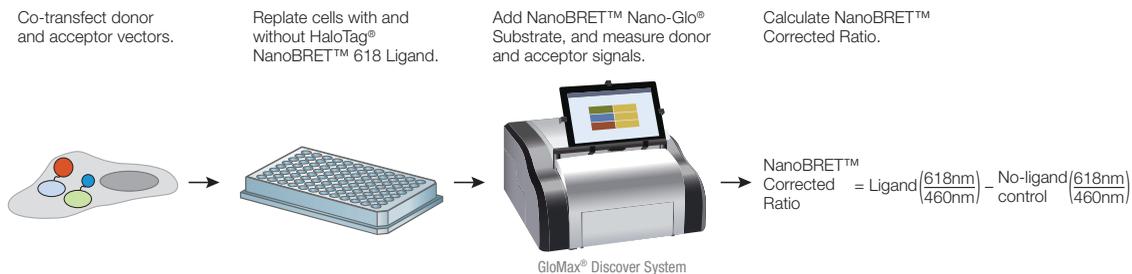
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## 1. Description

A critical key to understanding protein function is the ability to understand protein dynamic interactions within the cell. However, monitoring protein:protein interactions (PPI) in living cells and fluctuations of these interactions is very challenging, with few technologies available to do so. Here we describe the NanoBRET™ Protein:Protein Interaction (PPI) System<sup>(a-d)</sup>, a new configuration and approach to bioluminescence resonance energy transfer (BRET; 1,2). Similar to BRET, the NanoBRET™ System is a proximity-based assay that can detect protein interactions by measuring energy transfer from a bioluminescent protein donor to a fluorescent protein acceptor. As shown in Figure 1, Panel A, the NanoBRET™ assay uses a NanoLuc® fusion protein as the energy donor and a fluorescently labeled HaloTag® fusion protein as the energy acceptor (3,4). The optimized blue-shifted NanoLuc® donor paired with the red-shifted HaloTag® acceptor minimizes spectral overlap within the assay, resulting in an improved signal:background ratio when calculating the NanoBRET™ ratio (Figure 1, Panel B). This protocol provides instructions for both endpoint and kinetic NanoBRET™ detection in live cells.

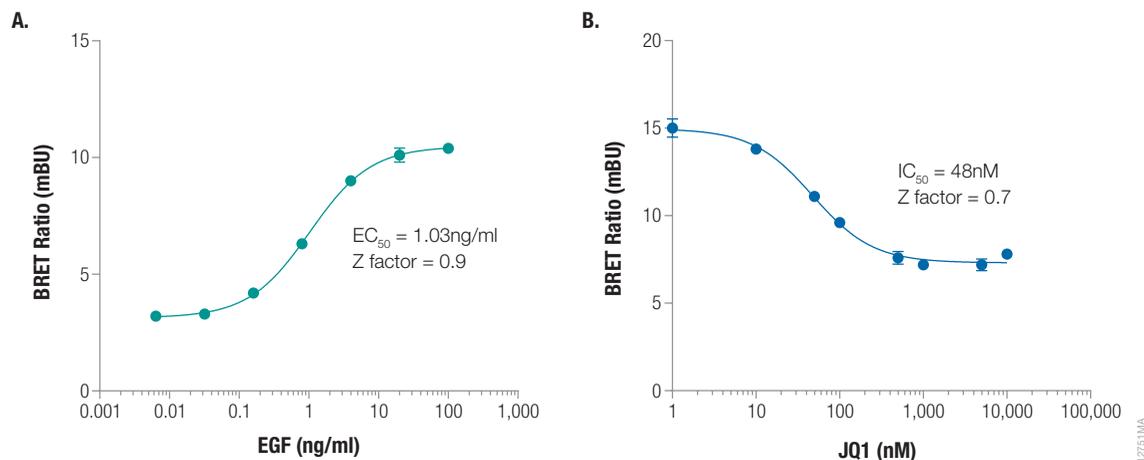


**Figure 1. The NanoBRET™ assay. Panel A.** Depiction of energy transfer from a NanoLuc®-Protein A fusion (energy donor) to a fluorescently labeled HaloTag®-Protein B fusion (energy acceptor) upon interaction of Protein A and Protein B. **Panel B.** Spectral separation of the NanoLuc® emission (460nm) and the fluorescent HaloTag® NanoBRET™ ligand emission (618nm), and calculation of the NanoBRET™ ratio.



**Figure 2. The NanoBRET<sup>™</sup> PPI Assay protocol consists of four steps.** First, both NanoLuc<sup>®</sup> and HaloTag<sup>®</sup> 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<sup>™</sup> HaloTag<sup>®</sup> 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<sup>™</sup> NanoLuc<sup>®</sup> 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<sup>™</sup> ratio is calculated, which is a subtraction of NanoBRET<sup>™</sup> ratios of the control samples from the experimental ligand-containing samples.

The NanoBRET<sup>™</sup> PPI Assay can also be used to monitor changes in protein interactions, including induction or inhibition as shown in Figure 3. Thus, NanoBRET<sup>™</sup> assays can screen for changes to a particular PPI, resulting in a powerful live-cell assay for use in small-molecule screening (5). The NanoBRET<sup>™</sup> ratio is independent of cell number and, similar to other ratiometric assays, shows low variability and high reproducibility, as demonstrated by the Z factor calculations shown in Figure 3. Related to drug screening, we have successfully developed NanoBRET<sup>™</sup> assays for numerous epigenetic targets, transcription factors, kinases, receptors and important signaling proteins. For more information and availability, please visit: [www.promega.com/NanoBRET](http://www.promega.com/NanoBRET)



**Figure 3. Example BRET ratios showing protein interaction induction and inhibition. Panel A.** Activation of the EGFR/GRB2 interaction by EGF induction. **Panel B.** Inhibition of the BRD4/Histone H3.3 interaction by JQ1.



## 2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
<b>NanoBRET™ Nano-Glo® Detection System</b>	<b>200 assays</b>	<b>N1661</b>

This system is sufficient for approximately 200 assays performed in 96-well plates in live-cell endpoint format. This system also can be used in 384-well plates for a total of approximately 500 assays. Includes:

- 1 × 50µl NanoBRET™ Nano-Glo® Substrate
- 1 × 20µl HaloTag® NanoBRET™ 618 Ligand

PRODUCT	SIZE	CAT.#
<b>NanoBRET™ Nano-Glo® Detection System</b>	<b>1,000 assays</b>	<b>N1662</b>

This system is sufficient for approximately 1,000 assays performed in 96-well plates in live-cell endpoint format. This system also can be used in 384-well plates for a total of approximately 2,500 assays. Includes:

- 5 × 50µl NanoBRET™ Nano-Glo® Substrate
- 5 × 20µl HaloTag® NanoBRET™ 618 Ligand

PRODUCT	SIZE	CAT.#
<b>NanoBRET™ Nano-Glo® Detection System</b>	<b>10,000 assays</b>	<b>N1663</b>

This system is sufficient for approximately 10,000 assays performed in 96-well plates in live-cell endpoint format. This system also can be used in 384-well plates for a total of approximately 25,000 assays. Includes:

- 2 × 1.25ml NanoBRET™ Nano-Glo® Substrate
- 1 × 1ml HaloTag® NanoBRET™ 618 Ligand

PRODUCT	SIZE	CAT.#
<b>NanoBRET™ Nano-Glo® Kinetic Detection System</b>	<b>200 assays</b>	<b>N2583</b>

This system is sufficient for approximately 200 assays performed in 96-well plates in live-cell kinetic format. This system also can be used in 384-well plates for a total of approximately 500 assays. Includes:

- 2 × 100µl Nano-Glo® Vivazine™ Substrate
- 1 × 20µl HaloTag® NanoBRET™ 618 Ligand

PRODUCT	SIZE	CAT.#
<b>NanoBRET™ Nano-Glo® Kinetic Detection System</b>	<b>1,000 assays</b>	<b>N2584</b>

This system is sufficient for approximately 1,000 assays performed in 96-well plates in live-cell kinetic format. This system also can be used in 384-well plates for a total of approximately 2,500 assays. Includes:

- 1 × 1ml Nano-Glo® Vivazine™ Substrate
- 5 × 20µl HaloTag® NanoBRET™ 618 Ligand

PRODUCT	SIZE	CAT. #
<b>NanoBRET™ Nano-Glo® Kinetic Detection System</b>	<b>10,000 assays</b>	<b>N2585</b>

This system is sufficient for approximately 10,000 assays performed in 96-well plates in live-cell kinetic format. This system also can be used in 384-well plates for a total of approximately 25,000 assays. Includes:

- 10 × 1ml Nano-Glo® Vivazine™ Substrate
- 1 × 1ml HaloTag® NanoBRET™ 618 Ligand

**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.

**Notes:**

- Enough substrate and ligand is provided for the number of assays indicated for the NanoBRET™ Nano-Glo® Detection Systems. Because we recommend always including a set of samples without HaloTag® NanoBRET™ 618 Ligand as a negative control, you may have extra ligand.
- See Section 11, Related Products, for additional kit components, control vectors and other reagents available for purchase separately.

### 3. General Considerations

In this section, we describe critical factors for your NanoBRET™ PPI Assay, including instrumentation requirements and a strategy overview for developing and optimizing an assay.

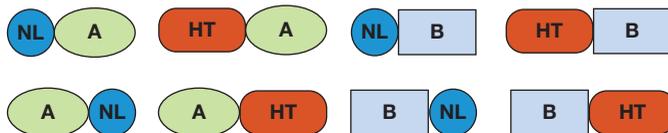
#### 3.A. Instrumentation

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 (Emission 450nm/BP 80nm) and a long pass (LP) filter starting at around 600–610nm to measure the acceptor signal (Emission 610nm/LP). **Note:** Filters outside of these ranges will miss critical measurements and compromise data quality.

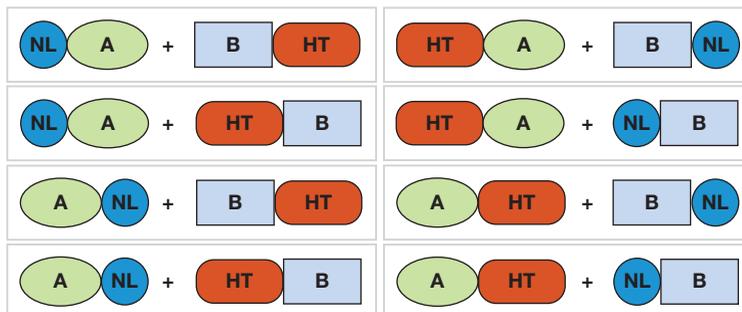
### 3.B. Strategy and Workflow for Setting Up and Optimizing a NanoBRET™ Assay

Figure 4 lists the steps necessary for generating and optimizing a NanoBRET™ assay in cases where there are no known constraints about where a particular protein can be tagged. In cases where more than one donor/acceptor combination is acceptable, these validating experiments help select the more robust configuration. In cases where the levels of one of the interacting protein partners is changing during the time of measurement, i.e., interactions, which may elicit protein degradation, we recommend the protein which is being modulated to be fixed as the donor fusion.

- 1. Generate clones** by appending NanoLuc® (NL) donor and HaloTag® (HT) acceptor tags to protein A and protein B (up to 8 possible clones).



- 2. Test combinations** to find the best energy transfer (up to 8 possible combinations).



- 3. Optimize transfection** of the best combinations for optimal donor to acceptor DNA ratio to minimize unbound donor and maximize dynamic range.

- 4. Validate** using an available inhibitor or activator to test specific response with optimized transfection condition. Alternatively, test specificity using saturation assays.

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**Figure 4. Generating and optimizing a NanoBRET™ assay.** Both proteins of interest are tagged with either NanoLuc® donor or HaloTag® acceptor at either the amino (N) or carboxy (C) terminus of the protein, resulting in eight potential clones (Step 1) and eight potential donor/acceptor combinations (Step 2). However, there may be instances where a tag position is known to affect protein function or a tag is preferred on a certain terminus, reducing the number of clones being prepared and tested. After finding the best donor/acceptor combination(s), transfection conditions are optimized for relative levels of donor and acceptor (Step 3). Finally, the assay can be validated by the use of a known modulator or by saturation assays (Step 4).

## 4. Before You Begin

### 4.A. Constructing NanoBRET™ Expression Vectors

Two sets of vectors exist for creating NanoBRET™ fusions to the proteins of interest (POI). These vectors are compatible with the Flexi® Vector System, which facilitates rapid cloning of all constructs of interest, or conventional vectors using multiple cloning sites (MCS).

#### Flexi® Vector System Compatible Vectors

For detailed information on the Flexi® Vector System, please refer to the *Flexi® Vector Systems Technical Manual #TM254* available at: [www.promega.com/protocols](http://www.promega.com/protocols)

In brief, Flexi® vectors contain a lethal gene that must be replaced with the desired gene of interest for cells to survive and colonies to form. The optimized protocols and reagents greatly reduce the overall cloning burden, especially when generating up to eight different constructs for a given PPI pair.

The standard protocol for cloning PCR products can be used to introduce genes of interest into the various HaloTag® vectors, NanoLuc® vectors or both. To reduce the cloning burden, each POI can be initially cloned from a PCR product into the amino (N) terminal HaloTag® entry vector pFN21A HaloTag® CMV Flexi® Vector (Cat.# G2821), and after sequencing confirmation, transferred to the remaining vectors without the need to sequence the insert for each construct. Please refer to Section 4 of the *Flexi® Vector Systems Technical Manual #TM254* for the transfer protocol.

**Note:** For vectors that append the tags on the carboxy (C) terminus, use a modified version of the Section 4.B protocol where the Carboxy Flexi® Enzyme Blend (SgfI & EcoICRI) is used in the vector digest for Step 2 instead of the Flexi® Enzyme Blend (SgfI & PmeI) as described in Technical Manual #TM254.

A simpler cloning approach is to transfer the open reading frame (ORF) of interest from an existing Flexi® compatible vector to the HaloTag® and NanoLuc® fusion vectors. Use the Find My Gene™ resource ([www.promega.com/findmygene/search.aspx](http://www.promega.com/findmygene/search.aspx)) to search a list of nearly 10,000 constructs to determine if one exists with your gene of interest. These ORFs were cloned into pFN21A HaloTag® CMV Flexi® Vector, which appends the HaloTag® protein to the N terminus of the fusion protein. Follow the protocols in Sections 5.A and 5.B of Technical Manual #TM254 to transfer an ORF to the remaining HaloTag® (C-terminal) and NanoLuc® (N- and C-terminal) fusion vectors.

#### MCS Vectors

Follow standard cloning procedures to introduce genes of interest into the various MCS vectors as described for the individual vector protocols.

#### 4.B. Instrument Setup

To perform NanoBRET™ PPI Assays, a luminometer capable of sequentially measuring dual wavelength windows is required. This is accomplished by using filters; we recommend using a band pass (BP) filter for the donor signal and a long pass filter (LP) for the acceptor signal to maximize sensitivity.

- 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 bleedthrough. 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 bleedthrough into the acceptor peak, which could compress the ratio calculation, reducing 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 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:

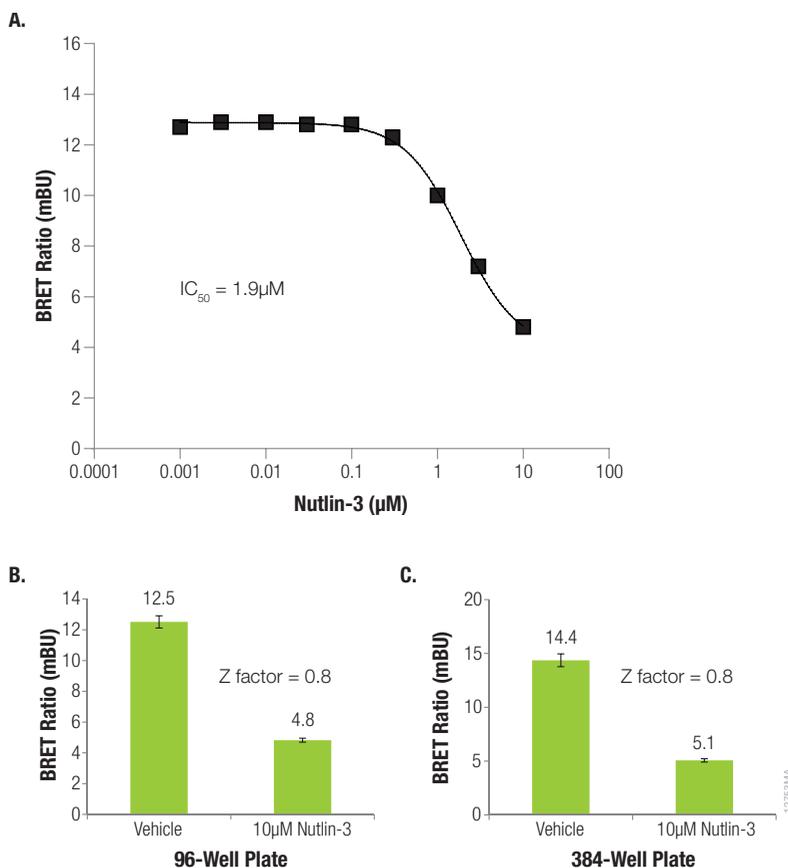
- The GloMax® Discover System (Cat.# GM3000) with preloaded filters for donor 450nm/8nm BP and acceptor 600nm LP. Select the preloaded BRET:NanoBRET™ 618 protocol from the Protocol menu.
- BMG Labtech CLARIOstar® with preloaded filters for donor 450nm/80nm BP and acceptor 610nm LP.
- Thermo Varioskan®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

#### 4.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 either available separately or included in the NanoBRET™ PPI Flexi® Starter System (Cat.# N1821) and the NanoBRET™ PPI MCS Starter System (Cat.# N1811). 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 (6; Tocris Cat.# 3984). Figure 5 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.

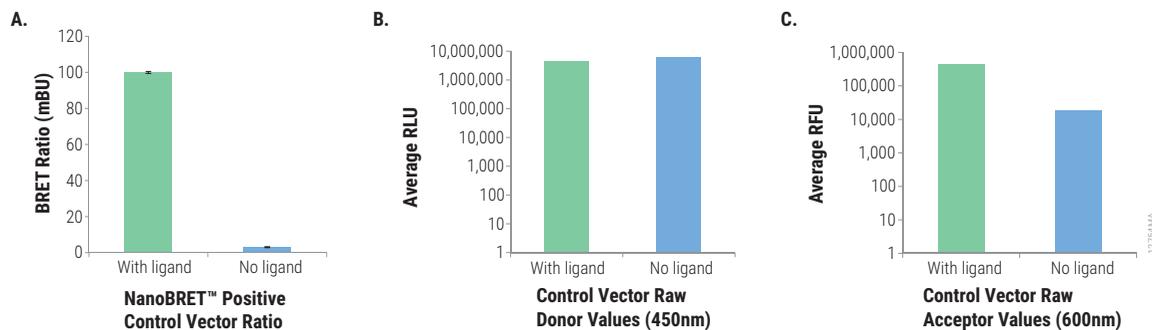


**Figure 5. Representative data for the p53/MDM2 NanoBRET™ PPI Control Pair.** Panel A is a dose-response curve (DRC) against the specific inhibitor Nutlin-3. Panels B and C show single-dose measurements and calculation of Z factors in the 96-well format (Panel B) and 384-well format (Panel C). Data generated using the GloMax® Discover System equipped with 450nm/8nm BP and 600nm LP filters.

#### 4.D. NanoBRET™ Positive Control

Another possible way of testing proper instrument setup is using the NanoBRET™ Positive Control (Cat.# N1581; available separately). This vector is an artificial system that tethers 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 shown in Figure 6.

**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 the sets of PPI partners and the NanoBRET™ Positive Control vector because the light from control plasmid might cause cross talk in adjoining wells.



**Figure 6. 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.

## 5. NanoBRET™ 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 8).
- DPBS (Gibco Cat.# 14190-144)
- 0.05% Trypsin/EDTA (GIBCO™ Cat.# 25300-054)
- FuGENE® HD Transfection Reagent (Cat.# E2311)
- HaloTag® constructs, tissue culture grade DNA
- NanoLuc® constructs, tissue culture grade DNA
- 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)
- **optional:** p53/MDM2 inhibitor Nutlin-3 (Tocris Cat.# 3984)

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

**Note:** Follow the appropriate transfection scheme as DNA amounts required are different for the various controls or the various stages of assay optimization. The five different transfection schemes are:

- Checking instrument performance by NanoBRET™ PPI Control Pair
- Checking instrument performance by NanoBRET™ Positive Control
- Assay optimization: Testing all combinations of initial protein pairs
- Assay optimization: Diluting NanoLuc® Donor DNA
- NanoBRET™ assay with optimized tag placement and DNA concentration conditions

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

## **5.A. Transient Transfection of HEK293 Cells with HaloTag® and NanoLuc® Fusion (continued)**

### **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  $4 \times 10^5$  cells/ml in cell culture medium.
4. Add 2ml of cells (800,000 cells) to one well of a six-well plate.
5. Allow the cells to attach and recover for 4–6 hours at 37°C, 5% CO<sub>2</sub>.
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 6µl of FuGENE® HD Transfection Reagent, and incubate at room temperature for 10 minutes.
9. Add transfection mixture to wells with attached cells, and express proteins for approximately 20 hours at 37°C, 5% CO<sub>2</sub>.
10. If performing an endpoint measurement without compounds or performing kinetic measurement, proceed to Section 5.B. If performing an endpoint measurement with compounds, go to Section 5.C.

### **Transfection Conditions for NanoBRET™ Positive Control**

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  $4 \times 10^5$  cells/ml in cell culture medium.
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<sub>2</sub>.
6. Prepare a transfection mixture consisting of 2µg of Transfection Carrier DNA + 0.002µg of 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. Add transfection mixture to wells with attached cells, and express proteins for approximately 20 hours at 37°C, 5% CO<sub>2</sub>.
10. If performing an endpoint measurement without compounds or performing kinetic measurement, proceed to Section 5.B. If performing an endpoint measurement with compounds, go to Section 5.C.

### Transfection Conditions for Assay Optimization: Testing All Combinations of Initial Protein Pairs

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  $4 \times 10^5$  cells/ml in cell culture medium.
4. Add 2ml of cells (800,000 cells) to one well of a six-well plate.
5. Allow the cells to attach and recover for 4–6 hours at 37°C, 5% CO<sub>2</sub>.
6. Prepare transfection mixture or mixtures consisting of 2µg of HaloTag® plasmid + 0.2µg of NanoLuc® plasmid diluted in water for each combination being tested.
7. Add 100µl of Opti-MEM™ I Reduced Serum Medium, no phenol red, to the transfection mixture, and mix well.
8. Add 6µl of FuGENE® HD Transfection Reagent, and incubate at room temperature for 10 minutes.
9. Add transfection mixture to wells with attached cells, and express proteins for approximately 20 hours at 37°C, 5% CO<sub>2</sub>.
10. If performing an endpoint measurement without compounds, proceed to Section 5.B. If performing an endpoint measurement with compounds, go to Section 5.C.

**Note:** For NanoBRET™ assay optimization, we recommend using endpoint detection.

### Transfection Conditions for Assay Optimization: NanoLuc® Donor DNA Dilution

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  $4 \times 10^5$  cells/ml in cell culture medium.
4. Add 2ml of cells (800,000 cells) to one well of a six-well plate.
5. Allow the cells to attach and recover for 4–6 hours at 37°C, 5% CO<sub>2</sub>.
6. Prepare transfection mixtures as depicted below for each selected combination from the initial screening (usually 2–4 combinations) with different amounts of HaloTag® and NanoLuc® DNA representing tenfold dilutions of the NanoLuc® DNA to reduce the amount of free donor as expressed proteins. To assemble reactions, serially dilute the NanoLuc® DNA plasmids tenfold in water. For each transfection mixture, the total amount of DNA is roughly 2µg.

Desired Reporter Ratio	Amount Used
1:1 (NanoLuc® to HaloTag®)	1µg HaloTag® plasmid + 1µg NanoLuc® plasmid
1:10 (NanoLuc® to HaloTag®)	2µg HaloTag® plasmid + 0.2µg NanoLuc® plasmid
1:100 (NanoLuc® to HaloTag®)	2µg HaloTag® plasmid + 0.02µg NanoLuc® plasmid
1:1,000 (NanoLuc® to HaloTag®)	2µg HaloTag® plasmid + 0.002µg NanoLuc® plasmid

### Transfection Conditions for Assay Optimization: NanoLuc® Donor DNA Dilution (continued)

7. Add 100µl of Opti-MEM™ I Reduced Serum Medium, no phenol red, to the transfection mixture, and mix well.
8. Add 6µl of FuGENE® HD Transfection Reagent, and incubate at room temperature for 10 minutes.
9. Add transfection mixture to wells with attached cells, and express proteins for approximately 20 hours at 37°C, 5% CO<sub>2</sub>.
10. If performing an endpoint measurement without compounds, proceed to Section 5.B. If performing an endpoint measurement with compounds, go to Section 5.C.

**Note:** For NanoBRET™ assay optimization, we recommend using endpoint detection.

### Transfection Conditions for a NanoBRET™ PPI Assay with Optimized Tag Placement and DNA Concentration

Follow the optimized and validated protocol for your assay. For assay validation suggestions, see Section 10.

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  $4 \times 10^5$  cells/ml in cell culture medium.
4. Add 2ml of cells (800,000 cells) to each well of six-well plates 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.
5. Allow the cells to attach and recover for 4–6 hours at 37°C, 5% CO<sub>2</sub>.
6. Prepare a transfection mixture consisting of the optimized pair and relative DNA concentrations. For example, prepare 2µg of HaloTag® plasmid + 0.02µg of NanoLuc® plasmid (diluted in water) if the 1:100 dilution yielded the best results.
7. Add 100µl of Opti-MEM™ I Reduced Serum Medium, no phenol red, to the transfection mixture and mix well.
8. Add 6µl of FuGENE® HD Transfection Reagent, and incubate at room temperature for 10 minutes.
9. Add transfection mixture to wells with attached cells, and express proteins for approximately 20 hours at 37°C, 5% CO<sub>2</sub>.
10. If performing an endpoint measurement without compounds or performing kinetic measurement, proceed to Section 5.B. If performing an endpoint measurement with compounds, go to Section 5.C.

### 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. Centrifuge cells at  $125 \times g$  for 5 minutes. Discard cell culture medium and resuspend cells in an equal volume of assay medium (Opti-MEM™ I Reduced Serum Medium, no phenol red + 4% FBS).

- Count to estimate cell density, and adjust density to  $2 \times 10^5$  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 5.C, which adjusts cell density and volume to accommodate compounds being tested.

- 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).

- 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 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.

- Incubate plates at 37°C, 5% CO<sub>2</sub> for a minimum of 4–6 hours to overnight (18–24 hours).
- If performing an endpoint measurement, proceed to Section 5.D. If performing a kinetic measurement, go to Section 5.E.

### 5.C. Optional Protocol for Testing Compounds or Inhibitors Using Endpoint Detection

**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 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.

- For each well in a six-well plate, remove medium from cells, and wash with 1ml of DPBS. Discard.
- Add 0.5ml of 0.05% trypsin-EDTA and incubate at room temperature until cells lift from well bottom.
- Add 2ml of cell culture medium to neutralize trypsin, mix to collect and resuspend cells and transfer cell suspension to a 15ml conical tube.
- Centrifuge cells at  $125 \times g$  for 5 minutes. Discard cell culture medium, and resuspend cells in an equal volume of assay medium (Opti-MEM™ I Reduced Serum Medium, no phenol red + 4% FBS).
- Count to estimate cell density, and adjust density to  $2.2 \times 10^5$  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.

### 5.C. Optional Protocol for Testing Compounds or Inhibitors Using Endpoint Detection (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. Add 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 medium (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<sub>2</sub> a minimum of 4–6 hours to overnight (18–24 hours). For maximum compound effect, we recommend overnight incubation.
10. Proceed to Section 5.D.

### 5.D. Adding NanoBRET™ Nano-Glo® Substrate and Taking Endpoint 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. The kit contains sufficient Substrate volume 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.
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 4.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 10.C.

## 5.E. 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<sub>2</sub> to equilibrate substrate luminescence.
4. **Optional:** Prepare a 10X concentration of test compound in Opti-MEM™ I Reduced Serum Medium, no phenol red.  
**96-well format:** Add 10µl to each well for a final concentration of 1–10µM.  
**384-well format:** Add 8µl to each well for a final concentration of 1–10µM.
6. **Optional:** 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.
5. Immediately collect kinetic measurements of donor emission (460nm) and acceptor emission (618nm) every 3–5 minutes up to 6 hours after adding test compounds using a NanoBRET™ PPI Assay-compatible luminometer (see Section 4.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 10.C.

## 5.F. 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.

$$\frac{618\text{nm}_{\text{Em}}}{460\text{nm}_{\text{Em}}} = \text{Raw NanoBRET}^{\text{TM}} \text{ Ratio} = \text{BU}$$

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

$$\frac{618\text{nm}_{\text{Em}}}{460\text{nm}_{\text{Em}}} = \text{BU} \times 1,000 = \text{mBU}$$

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 bleedthrough, subtract the no-acceptor control mean from the Experimental mean for the corrected NanoBRET™ ratio.

$$\text{Mean mBU experimental} - \text{Mean mBU no-ligand control} = \text{Mean corrected mBU}$$

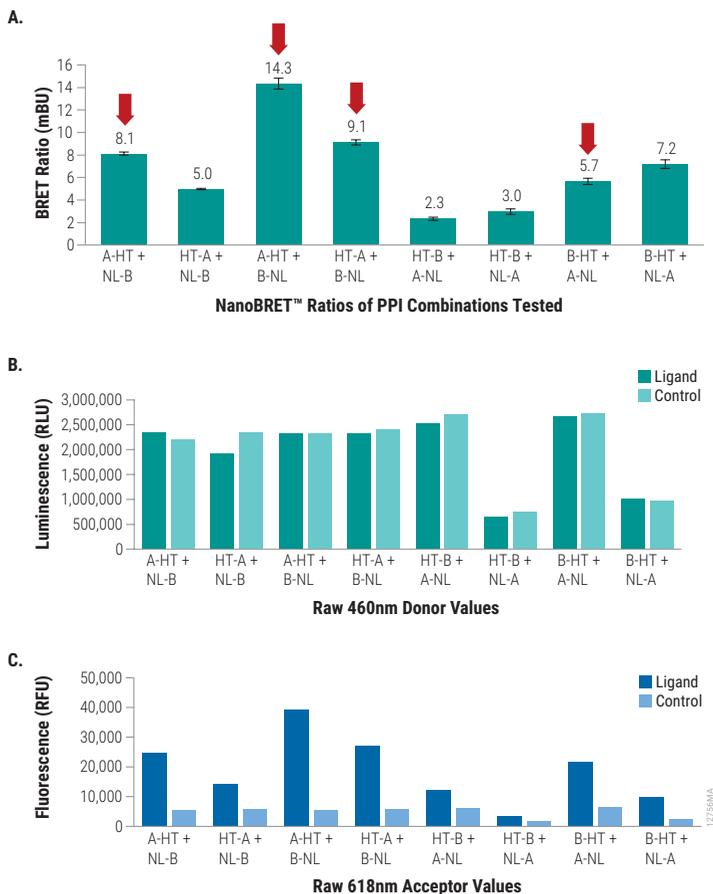
4. **Optional:** Generate Z' and Z factor calculations to gauge assay consistency (7). A Z' factor estimates assay consistency by comparing the mean and standard deviation (STDV) values of the experimental samples and a baseline control such as the no-ligand control.

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

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.

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

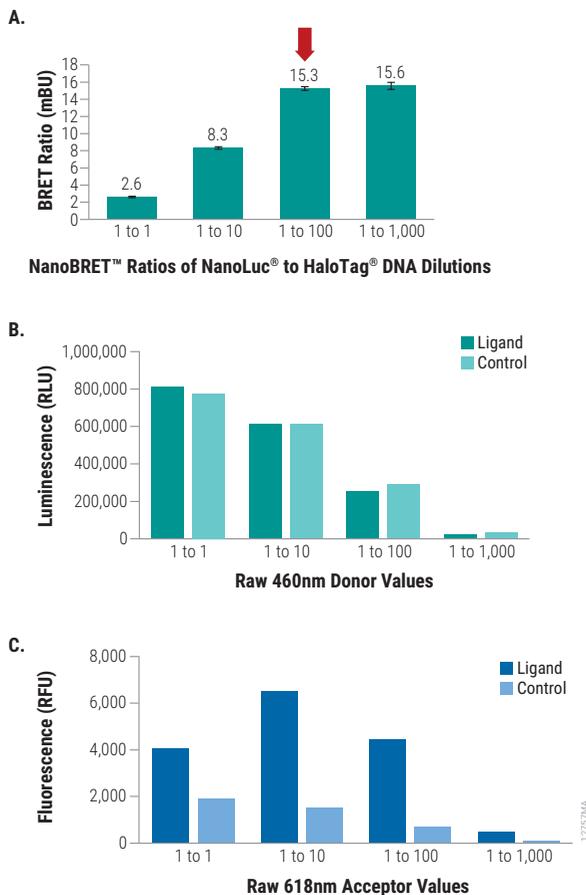
## 6. Representative Data



**Figure 7. Representative results testing all possible PPI combinations when both proteins (A or B) have been tagged with NanoLuc® (NL) donor or HaloTag® (HT) acceptor at either N- or C-terminus.** For the initial screening all samples were transfected with 1:10 NL:HT DNA ratio. The combinations marked by arrows on the NanoBRET™ ratios graph (**Panel A**) were chosen for further optimization based on calculated ratios, and raw donor (**Panel B**) and acceptor values (**Panel C**).

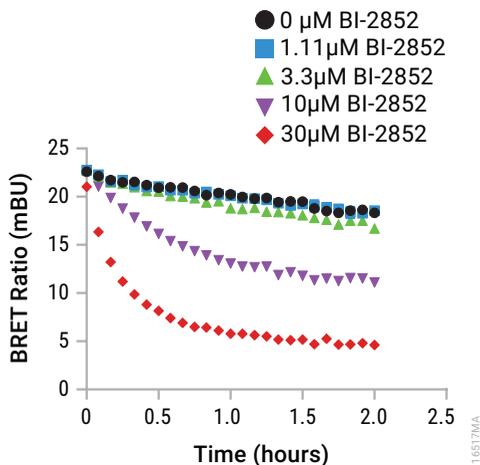
## 6. Representative Data (continued)

Although the last combination (labeled B-HT + NL-A) in Figure 7, Panel A, yields a larger ratio, it was not chosen due to the reduced donor values (Figure 7, Panel B) that will be lower at higher DNA dilutions. If possible, always choose the combinations where the raw donor values are well above the limit of detection for the instrument.



**Figure 8. One of the top combinations from the initial screen shown in Figure 7 (HT-A + B-NL) was chosen for serial dilution of NL DNA to find the optimal amount yielding the best dynamic range.** Equal amounts of NL and HT DNA (1:1) and 1:10, 1:100, and 1:1,000 dilutions of NL relative to HT DNA were used.

The 1:100 dilution marked by an arrow on the NanoBRET™ ratios graph was chosen as the optimal NL to HT DNA ratio. Note that the 1:1,000 dilution also yielded a similar ratio, but the raw donor values are extremely low running into the risk of operating too close to the instrument limit of detection, which is likely to increase variability.



**Figure 9. Live-cell kinetic measurement of the interaction between NanoLuc®-KRAS 2B G12D and BRAF RBD-HaloTag® fusion proteins.** NanoLuc®-KRAS 2B G12D and BRAF RBD-HaloTag® constructs were transfected into HEK293 cells. Cells were treated with varying concentrations of BI-2852 and the NanoBRET™ signal was measured every 5 minutes for 2 hours in a GloMax® Discover System. Live-cell kinetic data shows an expected decrease in interaction between KRAS 2B G12D and BRAF RBD when treated with increasing concentrations of BI-2852.

## 7. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: [www.promega.com](http://www.promega.com). E-mail: [techserv@promega.com](mailto:techserv@promega.com)

<b>Symptoms</b>	<b>Causes and Comments</b>
Unable to generate NanoBRET™ ratio, even with control vector or control pair.	<p>Improper instrument setup.</p> <ul style="list-style-type: none"> <li>• Make sure luminometer has the proper filters: 460nm/8–80nm BP for donor signal; 600–610nm LP for acceptor signal.</li> <li>• Make sure the gain on the PMT is set to detect donor signal without instrument saturation.</li> </ul> <p>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 proteins by labeling the proteins with a fluorescent HaloTag® ligand, separating them on SDS-PAGE and scanning the bands with a fluorescence scanner (see the <i>HaloTag® Mammalian Pull-Down and Labeling Systems Technical Manual #TM342</i>).</p>
Unable to generate NanoBRET™ ratio, even with control vector or control pair (continued).	<p>Suboptimal pairing of protein partners. Test all possible combinations to find optimal pair.</p> <p>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 negative control from the ratio of the experimental samples.</p> <p>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 10.C).</p> <p>Unexpected biology or lack of proper stimulation. Some PPI might be dependent on specific biological events or stimuli to activate specific pathways. Ensure proper pathway activators are added. If possible, check proper phenotypical responses by other means.</p>
Poor Z' and Z factor values.	<p>Too much variability in assay. Optimize all parameters of the assay for 0.5–1 Z' values. If the assay parameters have been optimized, consider automated dispensing to reduce variability.</p> <p>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.</p>

## Symptoms

Ratios and raw values are different from the examples shown.

## Causes and Comments

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 such as an inhibitor or by Donor Saturation Assay (DSA; Section 10.B). Despite following proper developmental procedure, there may be rare instances where lack of specificity results in low assay window, highly variable suboptimal assays.

Unable to get proper protein expression.

Suboptimal transfection conditions.

- Optimize transfection conditions and the relative ratios of donor to acceptor DNA.
- Optimize transfection conditions if using a different cell line or transfection reagent.
- Use only transfection-quality DNA.

Errors in clone generation. Use sequencing to confirm that clones have proper start site, linker and end sequence.

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 protein used rather than domain or region. If compound was developed in vitro against the domain or region alone, it may not disrupt the interaction of the full-length proteins. Test region or domain alone versus full-length protein.

When using kinetic detection, be sure to first test and optimize tag orientation and protein expression levels in endpoint detection format.

Compound affects enzymatic activity between proteins without disrupting the PPI.

## 7. Troubleshooting (continued)

### Symptoms

Discolored HaloTag® NanoBRET™ 618 Ligand

### Causes and Comments

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).

Unexpected luminescence values in NanoBRET™ Nano-Glo® Kinetic Assay

Luminescence increased at the beginning of a kinetic measurement because the Nano-Glo® Vivazine™ Substrate was not properly stabilized. Nano-Glo® Vivazine™ Substrate must be stabilized in the assay plate for 30–60 minutes prior to compound addition and assay measurement. For more information on the use of Nano-Glo® Vivazine™ Substrate, consult the *Nano-Glo® Endurazine™ and Vivazine™ Live Cell Substrates Technical Manual #TM550*.

The NanoBRET™ Nano-Glo® Kinetic Detection System is designed for use with Nano-Glo® Vivazine™ Substrate. We do not recommend using other NanoLuc® substrates for kinetic detection of NanoBRET™ protein:protein interactions.

## 8. Composition of Buffers and Solutions

### Cell Culture Medium

- 90% DMEM (GIBCO Cat.#11995)
- 10% fetal bovine serum (HyClone Cat.# SH30070.03)

### Assay Medium

- 96% Opti-MEM™ I Reduced Serum Medium, no phenol red (Life Technologies Cat.# 11058)
- 4% fetal bovine serum (HyClone Cat.# SH30070.03)

## 9. References

1. Pflieger, K.D. and Eidne, K.A. (2006) Illuminating insights into protein-protein interactions using bioluminescence resonance energy transfer (BRET). *Nat. Methods* **3**, 165–74.
2. Mercier, J.F. *et al.* (2002) Quantitative assessment of beta 1- and beta 2-adrenergic receptor homo- and heterodimerization by bioluminescence resonance energy transfer. *J. Biol. Chem.* **277**, 44925–31.
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5. 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.
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8. Bacart, J. *et al.* (2008) The BRET technology and its application to screening assays. *Biotechnol. J.* **3**, 311–24.

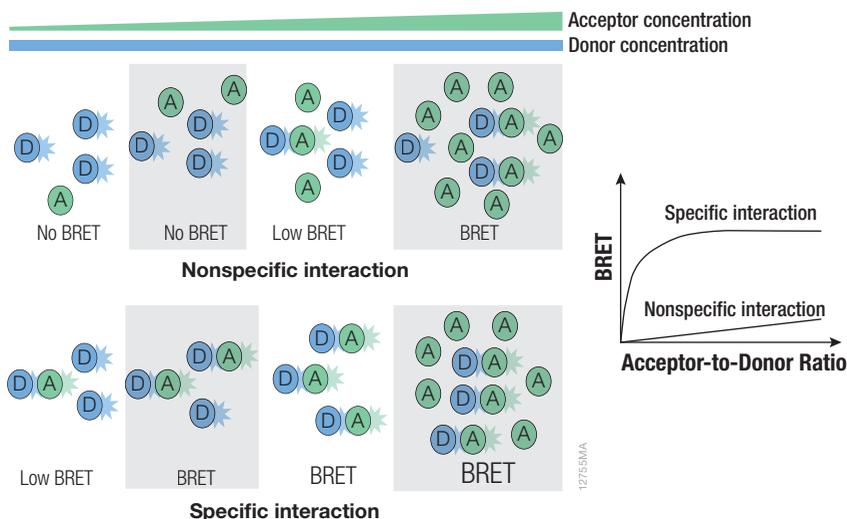
## 10. Appendix

### 10.A. Assay Validation Recommendations

If more than one pair combination work and a known modulator (inhibitor or enhancer) is available, test all potential combinations to see which one responds best to expected biology.

## 10.B. Donor Saturation Assay (DSA) Protocol

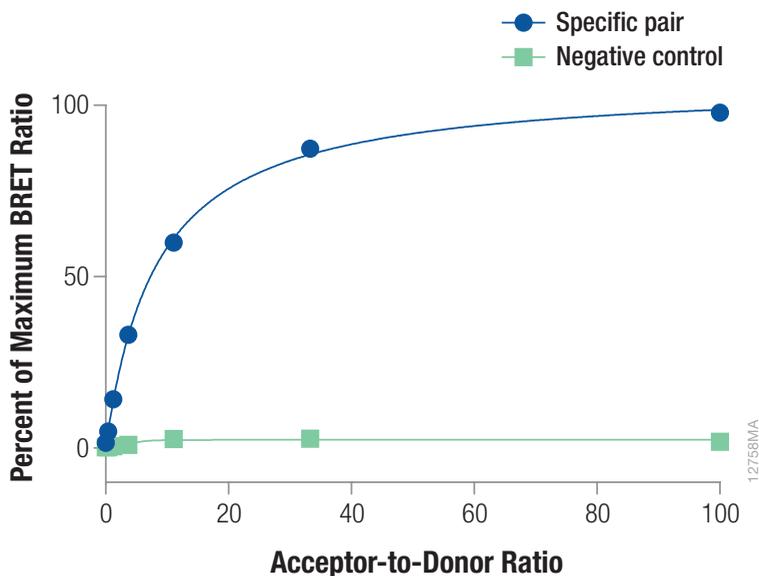
To validate a NanoBRET™ assay in the absence of known inhibitors, ascertain assay specificity by performing a Donor Saturation Assay (DSA; 8) using the donor/acceptor combination(s) identified in the initial screening. (See Figure 10 for an illustration of the principle of a DSA.)



**Figure 10. Principle of a DSA.**

In a scenario where the amount of donor is held constant while the amount of acceptor is gradually increased, nonspecific BRET can result from proteins being in close proximity, and the signal will increase linearly with increasing amounts of acceptor. However, a specific BRET signal will increase in a hyperbolic manner and reach a plateau representing complete saturation of all donors with acceptor molecules.

To perform this DSA protocol, cells are transfected with a constant amount of the NanoLuc® donor DNA and variable amounts of HaloTag® acceptor DNA to represent increasing amounts of acceptor-to-donor ratios. An optional negative control sample could be transfected with unfused or unrelated DNA as a mock donor or an acceptor that is known to be in the same cellular space (e.g., nucleus, cytoplasm or membrane). If the interaction of the protein pair is real, the DSA curve will show saturation, while a negative control would yield a linear or flat curve as shown in Figure 11.



**Figure 11. DSA representation of a specific BRET pair versus a negative control.**

The following example protocol assumes a previously chosen NanoLuc® donor:HaloTag® acceptor ratio of 1:100 and a series of transfections will be performed keeping the donor constant while gradually diluting the acceptor in carrier DNA to equate a range of acceptor-to-donor ratios. Adjust appropriately for other NanoLuc® donor:HaloTag® acceptor ratios.

1. Add 1ml of cells ( $4 \times 10^5$  cells/ml) in cell culture medium to nine wells of a 12-well plate.
2. Allow the cells to attach and recover for 4–6 hours at 37°C, 5% CO<sub>2</sub>.
3. When ready to transfect, dilute NanoLuc® donor to 0.01µg/µl in water.
4. Prepare a NanoLuc® donor DNA master mix in medium using 0.5ml of Opti-MEM™ I Reduced Serum Medium, no phenol red + 10µl of diluted NanoLuc® donor.
5. Dilute HaloTag® acceptor and Transfection Carrier DNA to 1µg/µl.

**10.B. Donor Saturation Assay (DSA) Protocol (continued)**

6. Serially dilute acceptor into carrier DNA 1:3 (e.g., 4µl of previous dilution + 8µl of carrier DNA) for points 2–8 of the dilution scheme listed in Table 1. Note that for Point 1, only HaloTag® acceptor DNA is used (no carrier DNA) and for Point 9, only carrier DNA is used.

**Table 1. Dilution Scheme.**

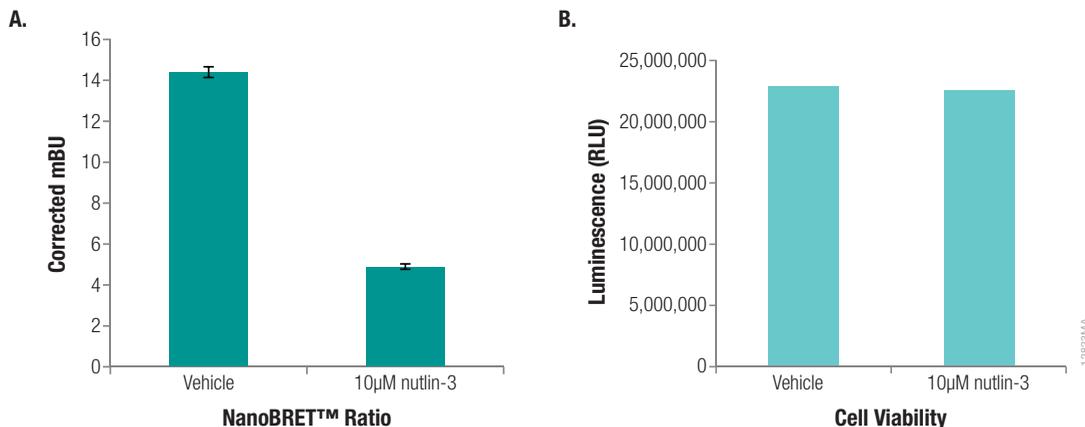
<b>Point</b>	<b>Amount of NanoLuc® Donor (ng)</b>	<b>Amount of HaloTag® Acceptor (ng)</b>	<b>Acceptor-to-Donor Ratio</b>
1	10	1,000	100
2	10	333	33.3
3	10	111	11.1
4	10	37	3.7
5	10	12.3	1.2
6	10	4.1	0.41
7	10	1.4	0.14
8	10	0.46	0.046
9	10	0	

7. To assemble transfection mixtures (nine total) combine:
- 1µl of proper HaloTag® acceptor dilution or transfection carrier DNA
  - 50µl of NanoLuc® donor DNA master mix
  - 4µl of FuGENE® HD Transfection Reagent
8. Incubate transfection mixtures for 10 minutes at room temperature, and add entire mixtures to the appropriate wells in the 12-well plate.
9. Incubate at 37°C for approximately 20 hours, and follow the protocol in Section 5.B.
10. Plot NanoBRET™ ratios against acceptor-to-donor ratio to determine the shape of the curve. A hyperbolic curve indicates a specific assay; a linear curve indicates a nonspecific NanoBRET™ assay.

### 10.C. Multiplexing with the CellTiter-Glo® 2.0 Assay

In some cases, you may want to determine the cell viability or compound toxicity or both in addition to performing 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, and thus indicates the presence of metabolically active cells. Figure 11 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 System, select 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.



**Figure 12. 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.

## 11. Related Products

### NanoLuc® Vectors

Product	Size	Cat.#
pFN31A <i>Nluc</i> CMV-Hygro Flexi® Vector	20µg	N1311
pFN31K <i>Nluc</i> CMV-neo Flexi® Vector	20µg	N1321
pFC32A <i>Nluc</i> CMV-Hygro Flexi® Vector	20µg	N1331
pFC32K <i>Nluc</i> 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

### Control Vectors

Product	Size	Cat.#
NanoBRET™ Positive Control	1 each	N1581
NanoBRET™ PPI Control Pair (p53, MDM2)	1 each	N1641

### 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

## Detection Reagents

Product	Size	Cat. #
NanoBRET™ Nano-Glo® Substrate	50µl	N1571
	5 × 50µl	N1572
	2 × 1.25ml	N1573
Nano-Glo® Vivazine™ Live Cell Substrate	100µl	N2580
	1ml	N2581
	10 x 1ml	N2582
HaloTag® NanoBRET™ 618 Ligand	20µl	G9801

## NanoBRET™ PPI Starter Systems

Product	Size	Cat. #
NanoBRET™ PPI Flexi® Starter System	1 each	N1821
NanoBRET™ PPI MCS Starter System	1 each	N1811

## 12. Summary of Changes

The following changes were made to the 10/23 revision of this document:

1. Updated y-axis labels for Figures 6, 7 and 8.
2. Updated a table column header in Section 5.A.



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