

TECHNICAL MANUAL

NanoBRET™ Target Engagement Intracellular HDAC Assay

Instructions for Use of Products
N2080, N2081, N2090 and N2170



NanoBRET™ Target Engagement Intracellular HDAC 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

The NanoBRET™ Target Engagement (TE) Assay^(a-g) measures compound binding at select target proteins within intact cells. This target engagement assay is based on the NanoBRET™ System, an energy transfer technique designed to measure molecular proximity in living cells (1). The NanoBRET™ Target Engagement Assay analyzes the apparent affinity of test compounds by competitive displacement of a NanoBRET™ tracer reversibly bound to a NanoLuc® fusion protein in cells (2). In the first step of the NanoBRET™ TE Assay, a fixed concentration of tracer is added to cells expressing the desired NanoLuc® fusion protein. Introduction of competing compounds results in a dose-dependent decrease in NanoBRET™ energy transfer for estimating intracellular affinity at the target protein. The NanoBRET™ TE Intracellular HDAC Assay analyzes compound binding to a histone deacetylase (HDAC)-NanoLuc® luciferase fusion protein.

The NanoBRET™ TE Assay uses four key components: An expressed cellular target protein that is fused to the bright NanoLuc® luciferase; a cell-permeable fluorescent tracer that specifically binds to the target protein; a substrate for NanoLuc® luciferase; and an extracellular inhibitor for NanoLuc® luciferase. Bioluminescence resonance energy transfer (BRET) is achieved by transferring the luminescent energy from NanoLuc® luciferase to the fluorescent tracer that is bound to the target protein-NanoLuc® fusion (Figure 1, Panels A and B). Compounds that are applied to the cells and specifically engage the intracellular target protein-NanoLuc® fusion will result in a decrease in BRET (Figure 1, Panels A and C). To ensure accurate assessment of intracellular target engagement, an extracellular NanoLuc® inhibitor is used to mitigate any NanoLuc® signal that may arise from cells compromised during handling, while not adversely affecting NanoLuc® luciferase expressed within healthy living cells. An overview of the NanoBRET™ Target Engagement Assay workflow is shown in Figure 2.

The NanoBRET™ TE Assays have been optimized to use a blue-shifted NanoLuc® donor and a red-shifted fluorescent tracer acceptor that have minimal spectral overlap within the assay (Figure 3). This results in optimized signal:background ratio and hence an optimized NanoBRET™ ratio.

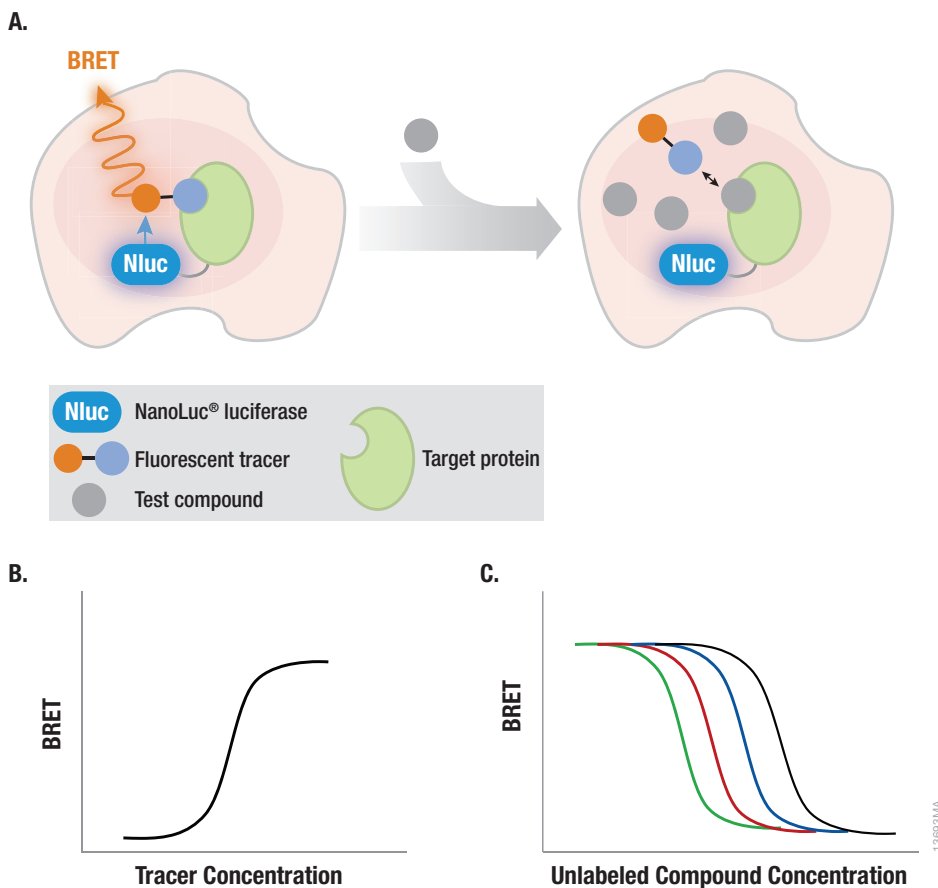


Figure 1. Illustration of the NanoBRET™ TE Assay. Panel A. Compound engagement is measured in a competitive format using a cell-permeable NanoBRET™ tracer. Binding of the test compound results in a loss of NanoBRET™ signal between the target protein and the tracer inside intact cells. **Panel B.** The optimal NanoBRET™ Tracer affinity is determined for each target protein. For analysis of target engagement by a test compound, cells are treated with a fixed concentration of NanoBRET™ Tracer that is near the EC_{50} value of the NanoBRET™ Tracer dose response curve. **Panel C.** To determine test compound affinity, cells are titrated with varying concentrations of the test compound in the presence of a fixed concentration (EC_{50} – EC_{80}) of tracer.

1. Description (continued)

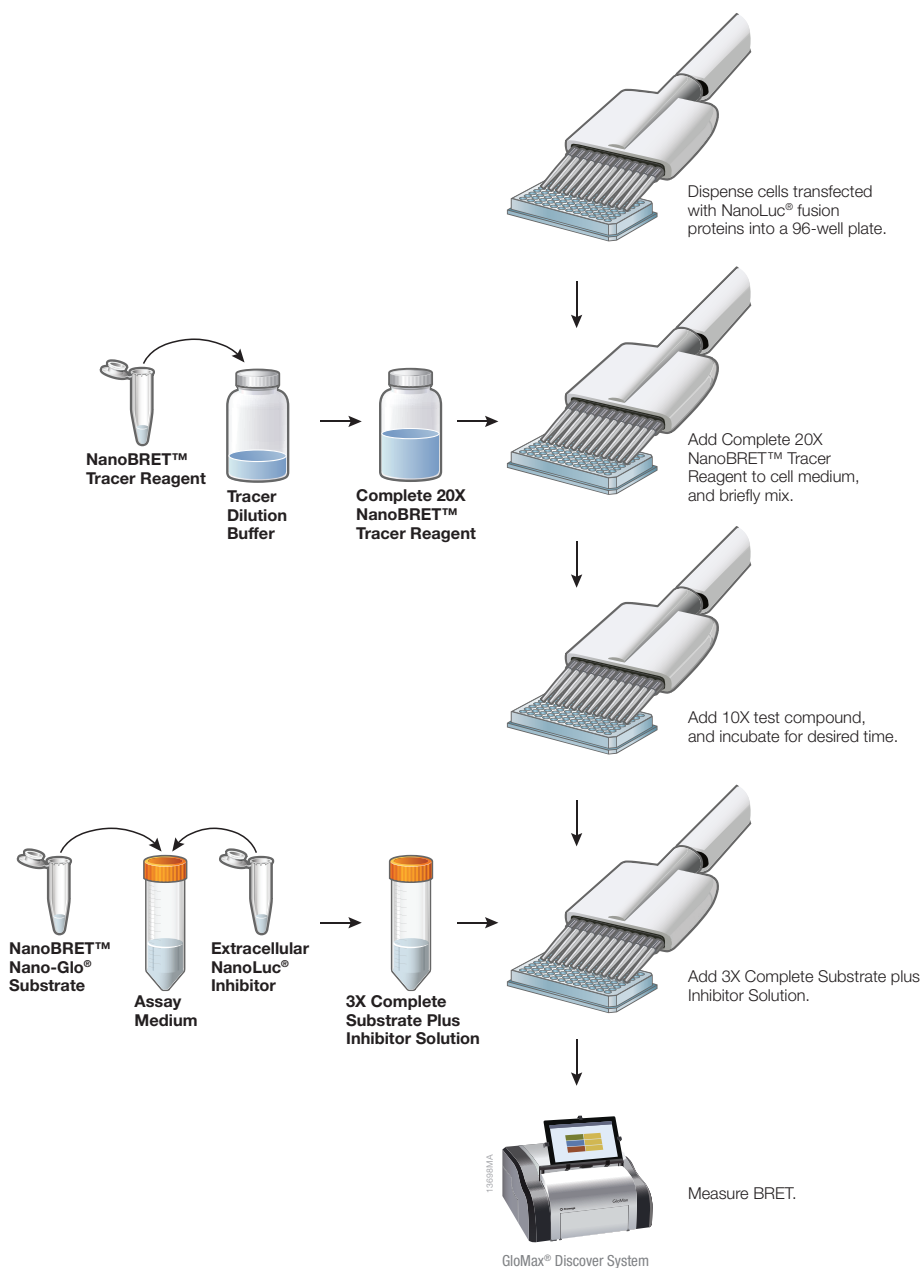


Figure 2. Overview of the NanoBRET™ Target Engagement Assay.

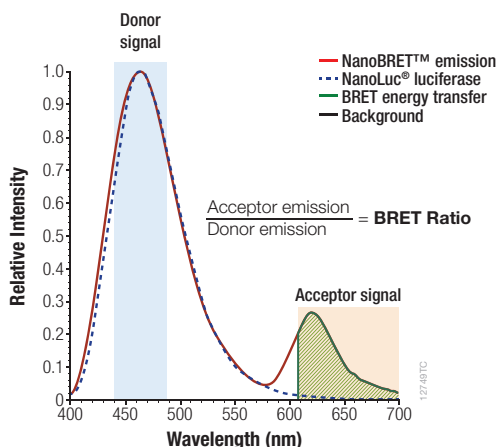


Figure 3. Spectral separation of the NanoLuc® emission (460nm) and fluorescent tracer emission (618nm), and calculation of the NanoBRET™ ratio.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
NanoBRET™ TE Intracellular HDAC Assay	100 assays	N2080

This assay system is sufficient for 100 assays performed in 96-well plates. This system can also be used in 384-well plates for a total of 250 assays. It includes:

- 20µg NanoLuc®-HDAC6 FL Fusion Vector
- 20µg Transfection Carrier DNA
- 110µl NanoBRET™ Intracellular TE HDAC Tracer, 0.1mM
- 5ml Tracer Dilution Buffer
- 50µl NanoBRET™ Nano-Glo® Substrate
- 17µl Extracellular NanoLuc® Inhibitor (30mM in DMSO)

PRODUCT	SIZE	CAT.#
NanoBRET™ TE Intracellular HDAC Assay	1,000 assays	N2081

This assay system is sufficient for 1,000 assays performed in 96-well plates. This system can also be used in 384-well plates for a total of 2,500 assays. It includes:

- 20µg NanoLuc®-HDAC6 FL Fusion Vector
- 100µg Transfection Carrier DNA
- 1.1ml NanoBRET™ Intracellular TE HDAC Tracer, 0.1mM
- 5ml Tracer Dilution Buffer
- 330µl NanoBRET™ Nano-Glo® Substrate
- 110µl Extracellular NanoLuc® Inhibitor (30mM in DMSO)



2. Product Components and Storage Conditions (continued)

Storage Conditions: Store the entire NanoBRET™ TE Intracellular HDAC Assay at less than –65°C. Alternatively, store the NanoBRET™ Intracellular TE HDAC Tracer, 0.1mM, at less than –65°C and all other components at less than –10°C. Avoid multiple freeze-thaw cycles of the vector components. Store NanoBRET™ Intracellular TE HDAC Tracer, 0.1mM, NanoBRET™ Nano-Glo® Substrate and Extracellular NanoLuc® Inhibitor protected from light.

Available Separately

PRODUCT	SIZE	CAT.#
NanoBRET™ TE Intracellular HDAC Complete Kit	1,000 assays	N2170

Includes:

- 1 NanoBRET™ TE Intracellular HDAC Assay
- 1 NanoBRET™ TE HDAC DNA Bundle

PRODUCT	SIZE	CAT.#
NanoBRET™ TE Intracellular HDAC Detection Reagents	10,000 assays	N2090

Includes:

- 10 × 1.1ml NanoBRET™ Intracellular TE HDAC Tracer
- 50ml Tracer Dilution Buffer
- 3.3ml NanoBRET™ Nano-Glo® Substrate
- 1.1ml Extracellular NanoLuc® Inhibitor

PRODUCT	SIZE	CAT.#
Intracellular TE Nano-Glo® Substrate/Inhibitor	1,000 assays	N2160

Includes:

- 330µl NanoBRET™ Nano-Glo® Substrate
- 110µl Extracellular NanoLuc® Inhibitor

PRODUCT	SIZE	CAT.#
Intracellular TE Nano-Glo® Substrate/Inhibitor	10,000 assays	N2161

Includes:

- 3.3ml NanoBRET™ Nano-Glo® Substrate
- 1.1ml Extracellular NanoLuc® Inhibitor

PRODUCT	SIZE	CAT.#
NanoBRET™ TE HDAC DNA Bundle	1 each	N2120

Includes:

- 20µg HDAC1-NanoLuc® FL Fusion Vector
- 20µg HDAC2-NanoLuc® FL Fusion Vector
- 20µg HDAC3-NanoLuc® FL Fusion Vector
- 20µg NanoLuc®-HDAC6 FL Fusion Vector
- 20µg NanoLuc®-HDAC6 (CD2) Fusion Vector
- 20µg HDAC10-NanoLuc® FL Fusion Vector

PRODUCT	SIZE	CAT.#
Tracer Dilution Buffer	50ml	N2191
Transfection Carrier DNA	100µg	E4881

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. 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. For vector sequence and feature information, visit: www.promega.com/resources/vector-sequences/

3.B. Instrument Requirements and Setup

To perform NanoBRET™ TE Assays, a luminometer capable of sequentially measuring dual-wavelength windows is required. This is accomplished 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.

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.



3.B. Instrument Requirements and Setup (continued)

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 the steps 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

4. NanoBRET[™] Target Engagement Protocol

Materials to be Supplied by the User

(Media compositions are supplied in Section 8.B.)

- HEK293 or similar cultured mammalian cells
- Dulbecco's Modified Eagle Media (DMEM; Thermo Fisher Cat.# 11995-065)
- fetal bovine serum (HyClone Cat.# SH30070.03)
- Opti-MEM[®] I Reduced Serum Medium, no phenol red (Life Technologies Cat.# 11058-021)
- white, nonbinding surface (NBS) 96-well plates (Corning Cat.# 3600) or 384-well plates (Corning Cat.# 3574)
- tissue culture equipment and reagents
- polypropylene plasticware (**Note:** Do not use polystyrene for this assay.)
- 0.05% Trypsin/EDTA (Thermo Fisher Cat.# 25300)
- FuGENE[®] HD Transfection Reagent (Cat.# E2311)
- DMSO (Sigma Cat.# 2650)
- detection instrument capable of measuring NanoBRET[™] wavelengths (e.g., GloMax[®] Discover System [Cat.# GM3000]; see Section 3)

The volumes specified for the NanoBRET™ Target Engagement Protocol are for 96-well plates. Table 1 lists the assay volumes used for both 96- and 384-well plates. Modify the reagent volumes in Sections 4.A–D as listed in Table 1 if 384-well plates are used.

Table 1. Volumes of NanoBRET™ TE Assay Reagents Used for Multiwell Plates.

Reagent to Add	Volume Per Well	
	384-Well Plate	96-Well Plate
Cell seeding	34µl	85µl
Complete 20X NanoBRET™ Tracer Reagent	2µl	5µl
10X Test compound	4µl	10µl
3X NanoBRET™ Nano-Glo® Substrate plus Extracellular NanoLuc® Inhibitor	20µl	50µl
Total assay volume	60µl	150µl



The composition of Assay Medium and Cell Culture Medium can be found in Section 8.B.

4.A. Transient Transfection of HEK293 Cells with NanoLuc®-HDAC Fusion Vector DNA

- Cultivate HEK293 cells (or desired cell type) appropriately prior to assay.
Note: If other cell types are used, optimize the transfection conditions.
- Remove medium from cell flask by aspiration, trypsinize and allow cells to dissociate from the flask.
- Neutralize trypsin using Cell Culture Medium and centrifuge at $200 \times g$ for 5 minutes to pellet cells.
- Aspirate medium and resuspend cells in Cell Culture Medium.
- Adjust density to 2×10^5 cells/ml using Cell Culture Medium.
- If HEK293 cells are used, prepare lipid:DNA complexes as follows:
 - Prepare a 10µg/ml solution of DNA in Assay Medium that consists of the following ratios: 9.0µg/ml of Transfection Carrier DNA, 1.0µg/ml of NanoLuc® fusion DNA and 1ml of Assay Medium. To accurately dilute the NanoLuc® fusion DNA, serially dilute the fusion vector with Transfection Carrier DNA to maintain the same amount of DNA (e.g, 10µg).
 - Mix thoroughly.
 - Add 30µl of FuGENE® HD Transfection Reagent into each milliliter of DNA mixture to form lipid:DNA complex. Ensure that the FuGENE® HD Transfection Reagent does not touch the plastic side of the tube; pipet directly into the liquid in the tube.
 - Mix by inversion 5–10 times.
 - Incubate at room temperature for 20 minutes to allow complexes to form.

4.A. Transient Transfection of HEK293 Cells with NanoLuc®-HDAC Fusion Vector DNA (continued)

- Mix 1 part of lipid:DNA complex (e.g., 1ml) with 20 parts of HEK293 cells (e.g., 20ml) in suspension at 2×10^5 cells/ml. Mix gently by inversion 5 times in a sterile, conical tube.
Note: Larger or smaller bulk transfections should be scaled accordingly, using this 20:1 cells to lipid:DNA complex ratio.
- Dispense cells + lipid:DNA complex into a sterile tissue culture flask and incubate 20–30 hours. We recommend a cell density of approximately 55,000– 80,000 cells/cm² during the transfection. For example, use approximately 4–6 million cells for a T75 flask.

4.B. Preparing Cells with NanoBRET™ Tracer Reagent

- Remove medium from flask with transfected HEK293 cells via aspiration, trypsinize and allow cells to dissociate from the flask.
- Neutralize trypsin using medium containing serum (e.g., Cell Culture Medium) and centrifuge at $200 \times g$ for 5 minutes to pellet cells.
- Aspirate medium and resuspend cells using prewarmed Assay Medium.
- Adjust the density to 2×10^5 cells/ml in Assay Medium.
- Dispense 85µl per well of cell suspension into white, 96-well plates. Periodically mix cells to avoid cell settling in the tube.
Optional: Dispense 90µl of cell suspension per well in triplicate as no-tracer control samples for background correction.
- Prepare Complete 20X NanoBRET™ Tracer Reagent.
 - First, prepare a 100X solution of NanoBRET™ Intracellular TE HDAC Tracer in 100% DMSO. Higher tracer concentrations may increase assay window but reduce sensitivity. Therefore, you may need to optimize the tracer concentration. See Figures 4 and 5 for example data. For target engagement assays for HDACs, we recommend a 100X tracer concentration of 100µM for a final concentration of 1.0µM tracer as a starting point.
 - Mix 1 part of 100X tracer with 4 parts Tracer Dilution Buffer to generate Complete 20X NanoBRET™ Tracer Reagent.
Note: Because the Tracer Dilution Buffer is viscous, slowly dispense the dilution buffer solution.
- Dispense 5µl of Complete 20X NanoBRET™ Tracer Reagent per well to cells. Mix the 96-well plate on an orbital shaker for 15 seconds at 700rpm. **Note:** Plate mixing may need to be optimized on different orbital shakers.
Optional: Prepare a separate set of samples without tracer for optional background correction steps.

4.C. Adding Test Compounds

1. Prepare serially diluted test compound at 1,000X final concentration in 100% DMSO. Then dilute 1,000X test compound to 10X final concentration in Assay Medium.
2. Add 10µl of 10X serially diluted test compound per well of 96-well plates containing cells with 1X NanoBRET™ Tracer Reagent. Thoroughly mix plate on an orbital shaker for 15 seconds at 700rpm. **Note:** Plate mixing may need to be optimized on different orbital shakers.
3. Incubate the plate at 37°C, 5% CO₂ for 2 hours. Equilibrate plate to room temperature for ~15 minutes, then proceed to NanoBRET™ Assay Protocol, Section 4.D.
Note: Incubation times with test compound may vary, depending on the binding characteristics of the compound.

4.D. NanoBRET™ Assay Protocol

1. Prepare 3X Complete Substrate plus Inhibitor Solution in Assay Medium (Opti-MEM® I Reduced Serum Media, no phenol red or serum) just before measuring BRET. This solution consists of a 1:166 dilution of NanoBRET™ Nano-Glo® Substrate plus a 1:500 dilution of Extracellular NanoLuc® Inhibitor in Assay Medium. For a 96-well plate, mix 30µl of NanoBRET™ Nano-Glo® Substrate, 10µl of Extracellular NanoLuc® Inhibitor and 4,960µl of Assay Medium to produce 5ml of 3X Complete Substrate plus Inhibitor Solution. Mix gently by inversion 5–10 times in a conical tube. (The final concentration of Extracellular NanoLuc® Inhibitor in the 3X solution is 60µM, for a working concentration of 20µM.)
Note: Use 3X Complete Substrate plus Inhibitor Solution within 2 hours. Discard any remaining solution.
2. Add 50µl of 3X Complete Substrate plus Inhibitor Solution to each well of the 96-well plate. Incubate for 2–3 minutes at room temperature.
3. Measure donor emission wavelength (e.g., 450nm) and acceptor emission wavelength (e.g., 610nm or 630nm) using the GloMax® Discover System or other NanoBRET™ Assay-compatible luminometer (see Section 3.B).
Note: We recommend measuring BRET within 10 minutes after adding NanoBRET™ Nano-Glo™ Substrate plus Extracellular NanoLuc® Inhibitor Solution. However, you can measure BRET for up to 2 hours, but there will be some loss of luminescence signal.

4.E. Determining BRET Ratio

1. To generate raw BRET ratio values, divide the acceptor emission value (e.g., 610nm) by the donor emission value (e.g., 450nm) for each sample.
Optional: To correct for background, subtract the BRET ratio in the absence of tracer (average of no-tracer control samples) from the BRET ratio of each sample.
2. Convert raw BRET units to milliBRET units (mBU) by multiplying each raw BRET value by 1,000.

NanoBRET™ ratio equation, including optional background correction:

$$\text{BRET Ratio} = [(\text{Acceptor}_{\text{sample}} \div \text{Donor}_{\text{sample}}) - (\text{Acceptor}_{\text{no-tracer control}} \div \text{Donor}_{\text{no-tracer control}})] \times 1,000$$

4.F. BRET Data Generated using the NanoLuc®-HDAC6 Fusion Vector

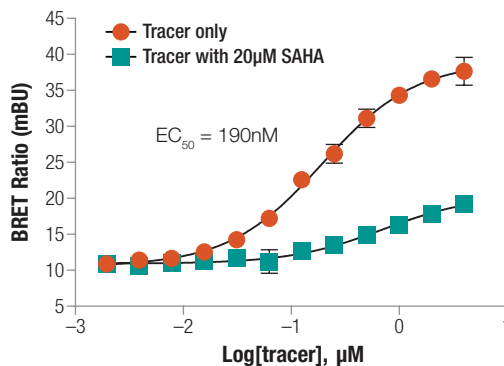
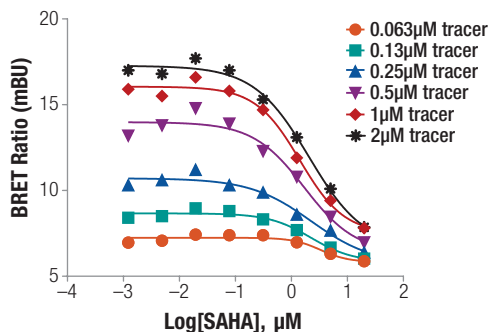


Figure 4. Apparent intracellular NanoBRET™ tracer affinity in HEK293 cells transiently expressing NanoLuc®-HDAC6 full-length fusion protein. HEK293 cells expressing NanoLuc®-HDAC6 FL fusion protein were resuspended in Assay Medium, seeded into 96-well plates and mixed with increasing concentrations of tracer. Cells were treated with a molar excess of unmodified HDAC inhibitor SAHA (20 μM) as a competitive inhibitor for 2 hours before adding 3X Complete Substrate plus Inhibitor Solution. BRET measurements were made on a GloMax® Discover System equipped with NanoBRET™ 618 filters (donor 450nm/8nm BP and acceptor 600nm LP). Raw BRET ratios were then converted to milliBRET units (mBU) and plotted vs. NanoBRET™ HDAC Tracer concentration to determine apparent intracellular affinity of the tracer.



Tracer Concentration (μM)	2	1	0.5	0.25	0.13	0.063
IC ₅₀	2.07	1.42	1.71	2.27	2.23	3.32

Figure 5. NanoBRET™ tracer competition in transiently transfected HEK293 cells expressing NanoLuc®-HDAC6 full-length fusion protein. HEK293 cells expressing NanoLuc®-HDAC6 FL fusion protein were resuspended in Assay Medium, seeded into 96-well plates and mixed with various concentrations of tracer. Cells were treated with varying concentrations of unmodified SAHA as a competitive inhibitor for 2 hours before adding 3X Complete Substrate plus Inhibitor Solution. BRET was measured using a GloMax® Discover System equipped with NanoBRET™ 618 filters (donor 450nm/8nm BP and acceptor 600nm LP). Raw BRET ratios were then converted to milliBRET units (mBU) and plotted vs. NanoBRET™ HDAC Tracer concentration to determine apparent intracellular affinity of the tracer. Our recommended concentration of 1 μM NanoBRET™ HDAC Tracer is shown in bold. Note that lower tracer concentrations result in more accurate estimation of intracellular compound affinity but a lower assay window. The use of a lower concentration of tracer may be more accurate when estimating intracellular compound affinity.

5. Representative Target Engagement Data

Representative NanoBRET™ Target Engagement results are shown for several HDAC proteins. The NanoBRET™ HDAC Tracer is compatible with other members of the Class I and Class IIb HDAC proteins. NanoLuc® fusion vectors containing these classes of HDACs are available as NanoBRET™ TE HDAC Bundle (Cat.# N2120) or NanoBRET™ TE Intracellular HDAC Complete Kit (Cat.# N2170). The affinity of the tracer varies slightly among these HDACs and may be adjusted accordingly for optimal performance.

Note that lower tracer concentrations result in more accurate estimation of intracellular compound affinity but a lower assay window. Using a lower tracer concentration may be more accurate when estimating intracellular compound affinity (Figures 5 and 6).

5. Representative Target Engagement Data (continued)

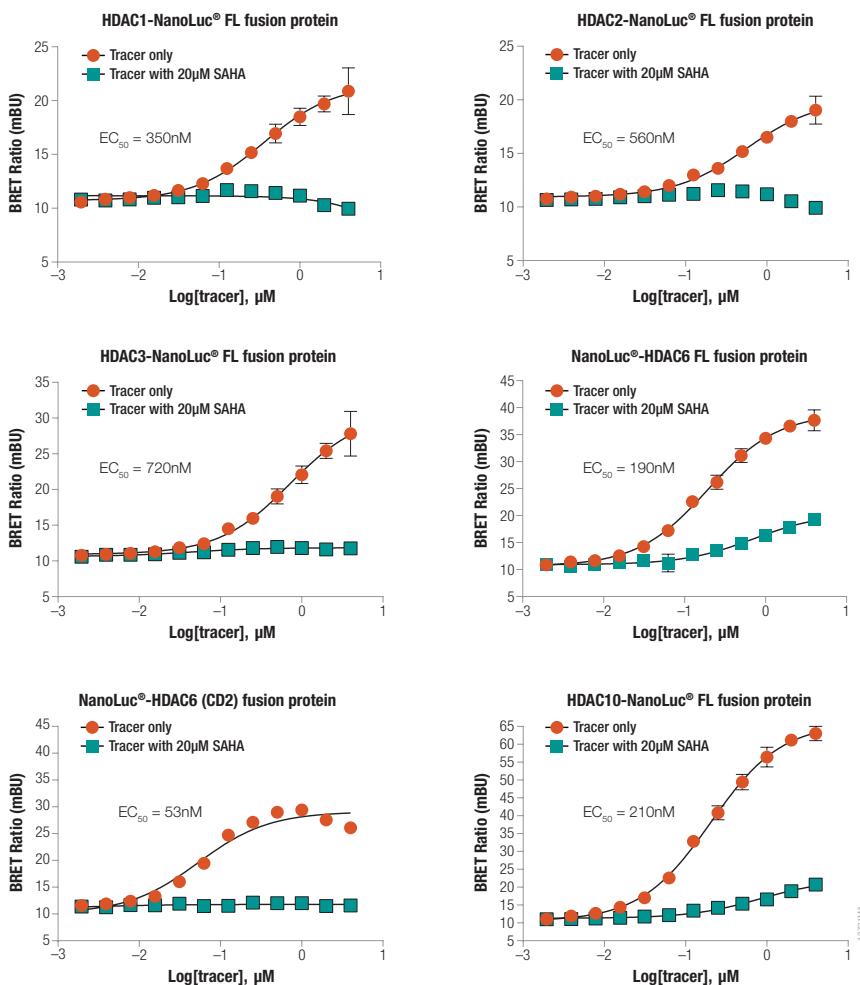


Figure 6. NanoBRET™ tracer affinity in HEK293 cells transiently expressing NanoLuc®-HDAC fusion proteins. HEK293 cells expressing individual NanoLuc® fusions of Class I or Class IIb HDAC isozymes were resuspended in Assay Medium and seeded into 96-well plates. Cells were treated with various concentrations of NanoBRET™ Tracer in the presence or absence of unlabeled SAHA as a competitive inhibitor and incubated for 2 hours before adding 3X Complete Substrate plus Inhibitor Solution. BRET was measured using a GloMax® Discover System equipped with NanoBRET™ 618 filters (donor 450nm/8nm BP and acceptor 600nm LP). Raw BRET ratios were then converted to milliBRET units (mBU) and plotted vs. NanoBRET™ HDAC Tracer concentration to determine apparent intracellular affinity of the tracer.

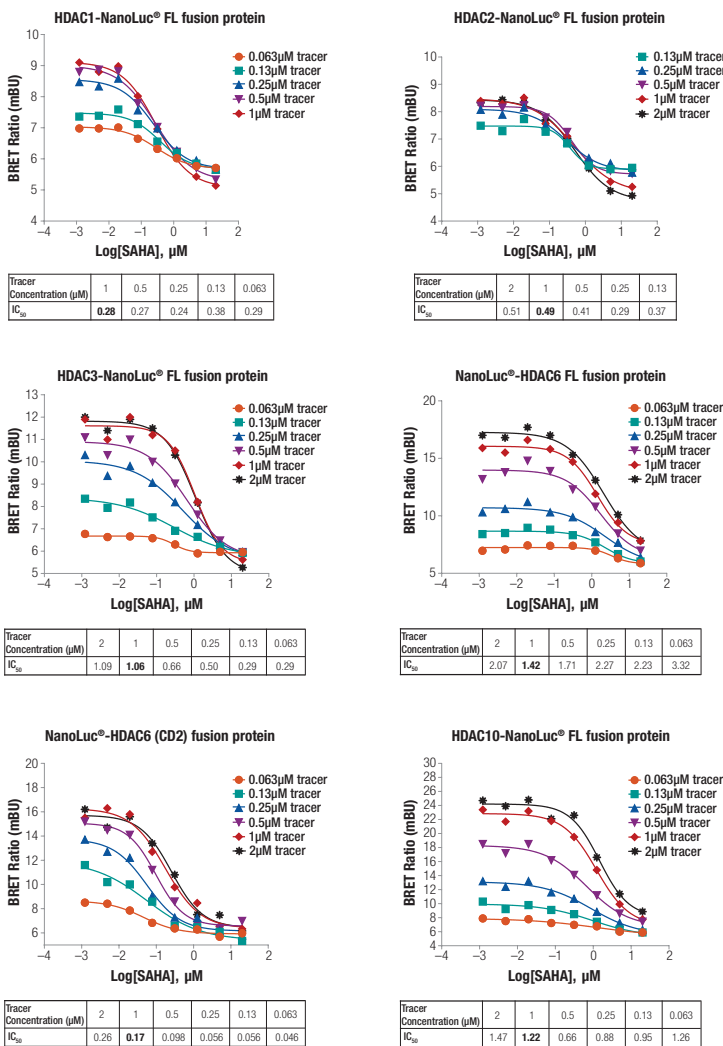


Figure 7. NanoBRET™ tracer competition in HEK293 cells transiently expressing NanoLuc®-HDAC fusion proteins. HEK293 cells expressing individual NanoLuc® fusions with Class I or Class Iib HDAC isozymes were resuspended in Assay Medium, seeded into 96-well plates and mixed with various concentrations of tracer. Cells were treated with increasing concentrations of unlabeled SAHA as a competitive inhibitor and incubated for 2 hours before adding 3X Complete Substrate plus Inhibitor Solution. BRET was measured using a GloMax® Discover System equipped with NanoBRET™ 618 filters (donor 450nm/8nm BP and acceptor 600nm LP). Raw BRET ratios were then converted to milliBRET units (mBU) and plotted over varying concentrations of unmodified SAHA. The IC₅₀ value generated using the recommended concentration of 1μM NanoBRET™ HDAC Tracer is shown in bold.

6. Protocol for Residence Time Analysis

This protocol is designed for kinetic analysis of compound dissociation by tracer competition in a 96-well format. To determine the optimal concentration of test compound for residence time analysis, we recommend first determining compound IC_{50} using the NanoBRET™ Target Engagement Protocol (Section 4).

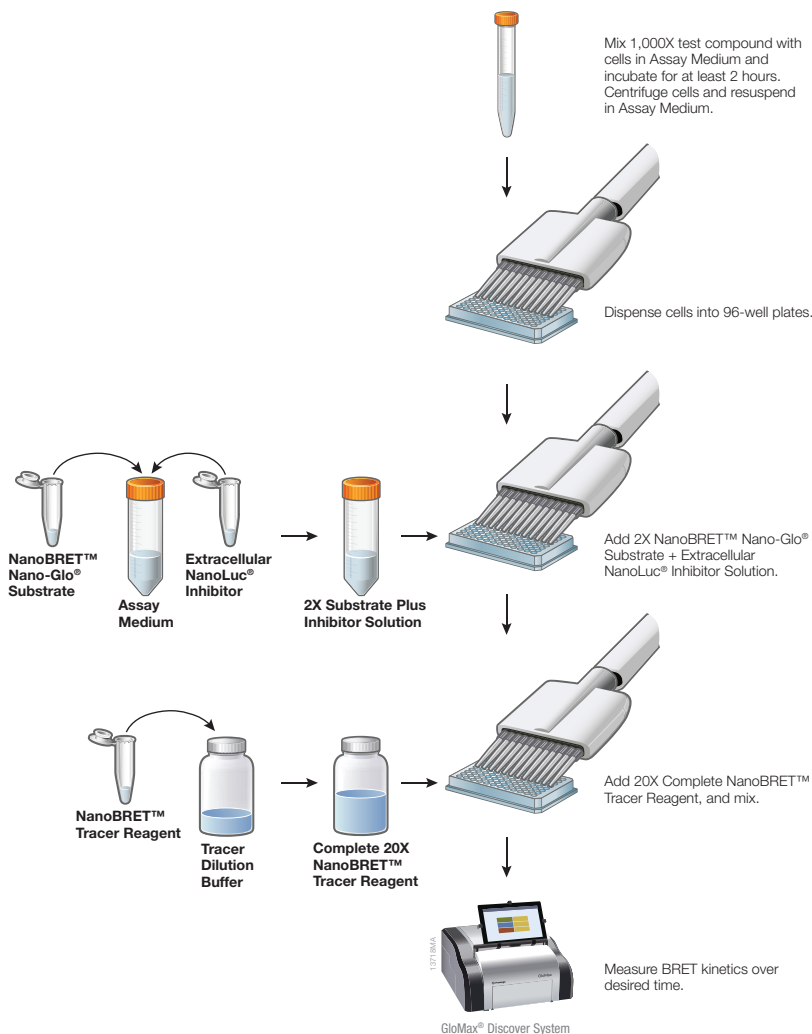
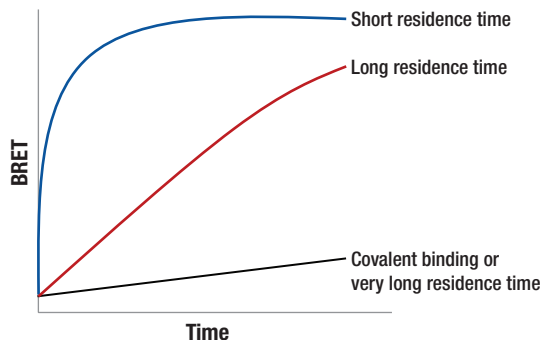


Figure 8. Illustration of NanoBRET™ Target Engagement Assay for determination of compound residence time. Cells are incubated with a near-saturating dose of test compound (e.g., IC_{80} concentration) to bind the target protein fused to the NanoLuc® fusion protein. The unbound drug is removed from the cell medium, and after dispensing the cells into multiwell plates and adding the NanoBRET™ TE Assay reagents, kinetic measurements are taken. See Figure 9 for example residence time curves.



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Figure 9. Determining compound residence time. Compound residence time is apparent from the rate of NanoBRET™ signal between the tracer and the NanoLuc® fusion protein.

Materials to be Supplied by the User

(Media compositions are supplied in Section 8.B.)

- HeLa or similar cultured mammalian cells
- Opti-MEM® I Reduced Serum Medium, no phenol red (Life Technologies Cat.# 11058-021)
- white, nonbinding surface (NBS) 96-well plates (Corning Cat.# 3600)
- tissue culture equipment and reagents
- polypropylene plasticware (**Note:** Do not use polystyrene for this assay.)
- 0.05% Trypsin/EDTA (Thermo Fisher Cat.# 25300)
- FuGENE® HD Transfection Reagent (Cat.# E2311)
- DMSO (Sigma Cat.# 2650)
- 15ml polypropylene conical tube (Corning Cat.# 430052)
- detection instrument capable of measuring NanoBRET™ wavelengths (e.g., GloMax® Discover System [Cat.# GM3000]; see Section 3)

Note: We recommend including two occupancy controls in the residence time analysis.

- **Zero target occupancy:** No-test-compound (vehicle only) control
- **Full target occupancy:** Known covalent test compound or saturating dose of test compound without washout. For example, 20µM SAHA can be used to block HDAC Tracer binding.

6.A. Adding Test Compound to Cells

1. Transfect cultured mammalian cells as instructed in Section 4.A.
2. Remove medium from flask with transfected HeLa cells via aspiration, trypsinize and allow cells to dissociate from the flask.
3. Neutralize trypsin using medium containing serum (e.g., Cell Culture Medium) and centrifuge at $200 \times g$ for 5 minutes to pellet cells.
4. Aspirate medium and resuspend cells using prewarmed Assay Medium.

6.A. Adding Test Compound to Cells (continued)

5. Adjust the density to 2×10^5 cells/ml in Assay Medium in a 15ml sterile polypropylene conical tube. We recommend a minimum volume of 1ml of cells, ideally 5ml of cells.
6. Prepare test compound at 1,000X final concentration in appropriate solvent (e.g., 100% DMSO).
7. Add 1 μ l of 1,000X test compound to each 1ml of cells in Assay Medium and mix gently.
Note: For full-occupancy control, include a sample with a saturating dose of a positive control compound. For zero-occupancy control, add only DMSO.
8. Place the conical tube in a rack, and incubate the tube with the cap loosened at 37°C, 5% CO₂ for a minimum of 2 hours or until sample is expected to reach equilibrium.

6.B. Preparing NanoBRET™ Reagents

1. Prepare Complete 20X NanoBRET™ Tracer Reagent.
 - a. First, prepare a 100X solution of NanoBRET™ Intracellular TE HDAC Tracer in 100% DMSO. We recommend a 100X tracer concentration of 100 μ M in 100% DMSO.
 - b. Mix 1 part of 100X tracer with 4 parts Tracer Dilution Buffer to generate Complete 20X NanoBRET™ Tracer Reagent.
Note: Due to the viscosity of the Tracer Dilution Buffer, slowly dispense the dilution buffer solution.
2. Prepare a 2X solution of NanoBRET™ Nano-Glo® Substrate plus Extracellular NanoLuc® Inhibitor by diluting the NanoBRET™ Nano-Glo® Substrate 1:250 and the Extracellular NanoLuc® Inhibitor 1:750 in Assay Medium in a conical tube. For a 96-well plate, mix 20 μ l of NanoBRET™ Nano-Glo® Substrate, 6.7 μ l of Extracellular NanoLuc® Inhibitor and 4,973 μ l of Assay Medium to produce 5ml of 2X NanoBRET™ Nano-Glo® Substrate plus Extracellular NanoLuc® Inhibitor Solution. Mix gently by inversion 5–10 times.
Note: Use 2X NanoBRET™ Nano-Glo® Substrate plus Extracellular NanoLuc® Inhibitor Solution within 2 hours. Discard any remaining solution.
3. Proceed to Section 6.C.

6.C. Preparing Cells and Dispensing Reagents for the Residence Time Assay

1. After incubation with test compound is complete, centrifuge cells at $200 \times g$ for 5 minutes.
2. **Optional Wash:** To remove unbound test compound, resuspend cells in prewarmed Assay Medium, centrifuge cells and remove medium.
Note: Maintain the concentration of the positive control compound for the full-occupancy control. For the HDAC1-NanoLuc® fusion protein, include a minimum of 10 μ M SAHA.
3. Remove medium, resuspend cells in prewarmed Assay Medium, and adjust cell density to 2×10^5 cells/ml.
Note: The full-occupancy control sample is at the correct cell density.
4. Dispense 90 μ l per well of cell suspension with test compound or controls into white, 96-well plates. Periodically mix cells to avoid cell settling in the tube.
Optional: For background correction, dispense 90 μ l of cell suspension in triplicate as no-tracer control samples.

5. Add 100µl of 2X NanoBRET™ Nano-Glo® Substrate Solution to each well of the 96-well plate.
6. Dispense 10µl of Complete 20X NanoBRET™ Tracer Reagent per well to cells. Mix the 96-well plate on an orbital shaker for 15 seconds at 700rpm. **Note:** Plate mixing may need to be optimized on different orbital shakers.
Optional: Prepare a separate set of samples without tracer for optional background correction steps.
7. Proceed to Section 6.D to measure luminescence.

6.D. NanoBRET™ Detection in Kinetic Mode

1. Set a NanoBRET™ Assay-compatible instrument to perform repeat measurements for the desired time interval (see Section 3.B). The following settings would measure the kinetics over 8 hours:
 - 5-minute intervals between each measurement
 - 99 measurements
 - 0.5 second integration time
 - 25°C instrument temperature (room temperature)
2. Measure donor emission wavelength (e.g., 450nm) and acceptor emission wavelength (e.g., 610nm).
3. Process the kinetic NanoBRET™ measurements as described in Section 4.E.

6.E. An Example to Determine Compound IC₅₀ and Perform Residence Time Analysis

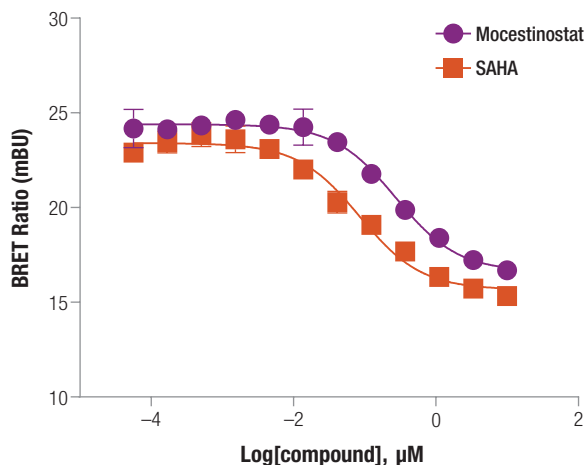


Figure 10. Determining the optimal compound treatment concentration for residence time analysis of HDAC1-NanoLuc® full-length fusion protein. HeLa cells expressing HDAC1-NanoLuc® FL fusion protein were resuspended in Assay Medium, combined with various concentrations of SAHA or mocetinostat, and incubated for 2 hours. Cells were washed, seeded in 96-well plates and Complete 20X NanoBRET™ Tracer and 2X NanoBRET™ Nano-Glo® Substrate plus Extracellular NanoLuc® Inhibitor Solution dispensed into each well. BRET was measured using a GloMax® Discover System equipped with NanoBRET™ 618 filters (donor 450nm/8nm BP and acceptor 600nm LP).

6.E. An Example to Determine Compound IC₅₀ and Perform Residence Time Analysis (continued)

Based on the results in Figure 10, 10 μ M SAHA and 10 μ M mocetinostat were used for subsequent residence time analysis in Figure 11.

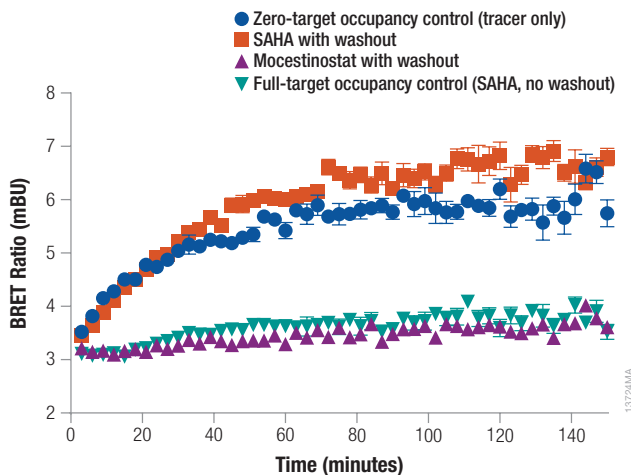


Figure 11. Residence time analysis for HeLa cells transiently expressing HDAC1-NanoLuc® full-length fusion protein. HeLa cells expressing HDAC1-NanoLuc® FL fusion protein were resuspended in Assay Medium, combined with 10 μ M SAHA or 10 μ M mocetinostat and incubated for 2 hours. After removing compound, cells were seeded in 96-well plates and Complete 20X NanoBRET™ Tracer and 2X NanoBRET™ Nano-Glo® Substrate plus Extracellular NanoLuc® Inhibitor Solution dispensed into each well. BRET was measured repeatedly using a GloMax® Discover System equipped with NanoBRET™ 618 filters (donor 450nm/8nm BP and acceptor 600nm LP).

7. Troubleshooting

Symptoms

NanoBRET™ signal without test compound is weak or close to background

Causes and Comments

Tracer was adsorbed to plasticware surface. We recommend using nonbinding surface plates. Use polypropylene materials to minimize tracer adsorption and avoid using polystyrene.

Suboptimal tracer concentration. Consider optimizing the concentration of tracer for your experiment. Only dilute NanoBRET™ tracers to 20X in Tracer Dilution Buffer prior to adding the tracer to the culture medium. The tracer will precipitate at concentrations above 1 μM in Assay Medium or other aqueous environments.

Instrument was set up improperly. Use the correct filters for donor wavelength (450nm) and acceptor wavelength (610nm) on your instrument to accurately measure NanoBRET™ signals.

Low protein expression levels. To ensure that the NanoLuc® fusion protein is expressed at appropriate levels, compare the donor (450nm) and acceptor (610nm) luminescence to the background signal in the absence of cells expressing NanoLuc® luciferase but in the presence of the NanoBRET™ Nano-Glo® Substrate. Both donor and acceptor luminescence values should be significantly above the background from the substrate. Optimize transfection conditions to improve expression of the NanoLuc® fusion protein.

Observed IC₅₀ value is right-shifted compared to expected value

Excessively high tracer concentration in the cell medium. Cell-based analysis of target engagement may result in right-shifted pharmacology. The concentration of the NanoBRET™ tracer may affect the IC₅₀ value, so carefully select tracer concentration. Optimize tracer concentration to determine the accurate compound IC₅₀ value.

Donor or acceptor luminescence alters when tracer is added

Donor or acceptor luminescence increased or decreased after adding tracer. This phenomenon is common but generally does not affect the assay. See Figure 12 for representative data showing raw luminescence from donor (450nm) and acceptor (610nm) channels when a NanoBRET™ Tracer is titrated. BRET that occurs between the NanoLuc® fusion protein and fluorescent tracer may result in a dose-dependent increase in acceptor luminescence with a corresponding decrease in donor luminescence. The effect of BRET on donor and acceptor luminescence may vary, depending on the target and tracer used. Ratiometric BRET analysis mitigates influences of fluctuations in raw luminescence from NanoLuc® luciferase.

7. Troubleshooting (continued)

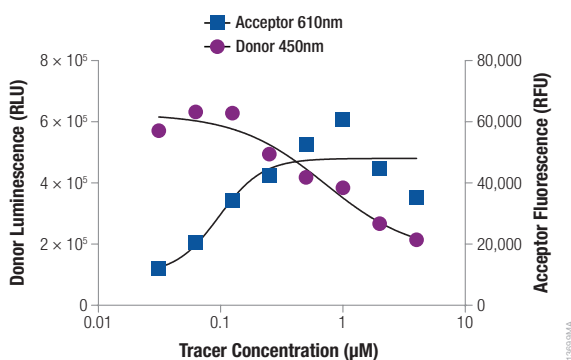


Figure 12. Potential effects of raw luminescence on donor and acceptor emission in the NanoBRET™ TE Assay.

8. Appendix

8.A. Preparing Stable Cell Lines Expressing NanoLuc® Fusion Proteins

The NanoLuc® expression vectors use relatively strong constitutive promoters. To avoid over expression in stable cell lines, we recommend use of attenuated promoters for appropriate expression of the NanoLuc® fusion protein. Please contact our Custom Assay Services for custom preparation of stable cell lines expressing NanoLuc® luciferase fusion proteins at: www.promega.com/products/pm/custom-assay-services/custom-assay-services-home/

8.B. Composition of Buffers and Solutions

Cell Culture Medium

- 90% DMEM (Thermo Fisher Cat #11995-065)
- 10% fetal bovine serum (FBS; HyClone Cat.# SH30070.03)

Assay Medium

- 100% Opti-MEM® I Reduced Serum Medium, no phenol red (Life Technologies Cat.# 11058-021)

8.C. References

- Machleidt, T. *et al.* (2015) NanoBRET-A novel BRET platform for the analysis of protein-protein interactions. *ACS Chem. Bio.* **10**, 1797–1804.
- Robers, M.B. *et al.* (2015) Target engagement and drug residence time can be observed in living cells with BRET. *Nature Comm.* **6**, 10091.

8.D. Related Products

NanoBRET™ Target Engagement Assays

Product	Size	Cat.#
NanoBRET™ TE Intracellular BET BRD Assay	100 assays	N2130
	1,000 assays	N2131

NanoBRET™ Protein:Protein Interaction Assays

Product	Size	Cat.#
NanoBRET™ BRD4/Histone H3.3 Interaction Assay	1 each	N1830
NanoBRET™ BRD4/Histone H4 Interaction Assay	1 each	N1890
NanoBRET™ BRD9/Histone H3.3 Interaction Assay	1 each	N1840
NanoBRET™ BRD9/Histone H4 Interaction Assay	1 each	N1900
NanoBRET™ BRPF1/Histone H3.3 Interaction Assay	1 each	N1860
NanoBRET™ BRPF1/Histone H4 Interaction Assay	1 each	N1910
NanoBRET™ CBP/Histone H3.3 Interaction Assay	1 each	N1850

Epigenetics

Product	Size	Cat.#
HDAC-Glo™ Class IIa Assay	10ml	G9560
HDAC-Glo™ 2 Assay	10ml	G9590
HDAC-Glo™ I/II Assay	10ml	G6420
	5 × 10ml	G6421
	100ml	G6422
HDAC-Glo™ I/II Screening System	10ml	G6430
	5 × 10ml	G6431
SIRT-Glo™ Assay	10ml	G6450
SIRT-Glo™ Screening System	5 × 10ml	G6471
MTase-Glo™ Methyltransferase Assay	400 assays	V7601
	2,000 assays	V7602

Multimode Detection Instrument

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



8.D. Related Products (continued)

Transfection Reagent

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

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^(c)U.S. Pat. Nos. 8,557,970 and 8,669,103 and other patents pending.

^(d)U.S. Pat. Nos. 8,809,529; 9,139,836; and other patents pending.

^(e)U.S. Pat. No. 9,056,885 and other patents pending.

^(f)Patents Pending.

^(g)Patent Pending.

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