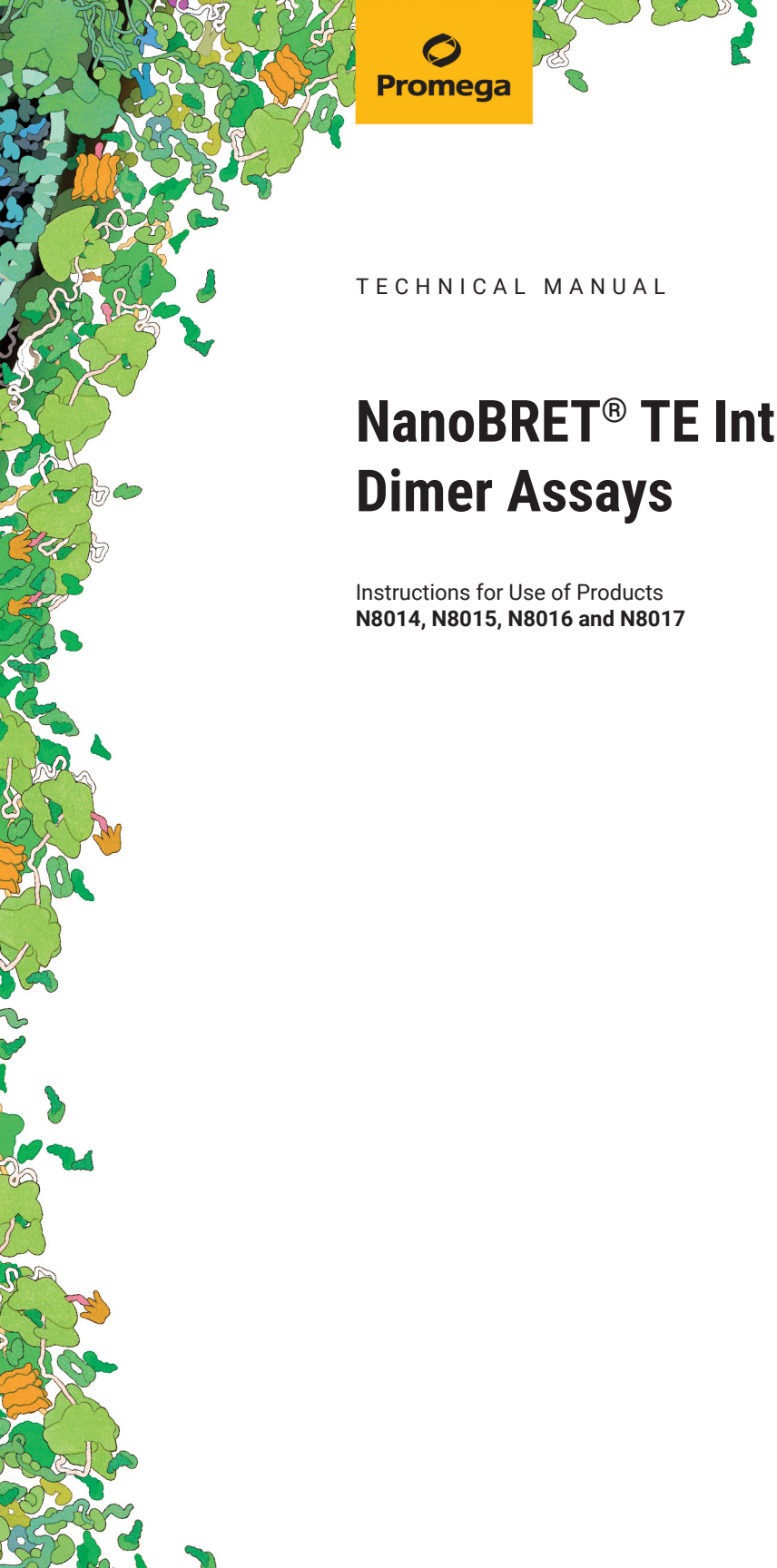

TECHNICAL MANUAL

NanoBRET® TE Intracellular RAF Dimer Assays

Instructions for Use of Products
N8014, N8015, N8016 and N8017

Revised 4/24
TM716

Promega

NanoBRET® TE Intracellular RAF Dimer Assays

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) Assays measure compound binding at select target proteins within intact cells and have been applied successfully to study multiple target classes (1). Here we describe a NanoBRET® TE method to measure compounds binding to intracellular dimeric forms of RAF kinases (ARAF, BRAF and CRAF) that are formed in the presence of the constitutively active mutant KRAS(G12C) (Figure 1). RAF kinases play an important role in signaling through the MAPK pathway, one of the most heavily mutated pathways in cancers. RAF dimers are important drug targets for various cancers, especially those driven by mutant RAS proteins. Specifically, the NanoBRET® TE Intracellular RAF Dimer Assays[a–g] measure compound engagement to the three individual RAF protomers (ARAF, BRAF or CRAF) within RAF heterodimers as well as target engagement at both BRAF and CRAF homodimers.

NanoBRET® TE assays quantify target engagement using bioluminescence resonance energy transfer (BRET), which is the transfer of energy between a luminescent donor and fluorescent acceptor that only occurs when the donor and acceptor are in proximity. Whereas the original NanoBRET® target engagement assays used intact NanoLuc® luciferase fused to a target protein as the energy donor, the NanoBRET® TE Intracellular RAF Dimer Assays use NanoLuc® Binary Technology (NanoBiT®), a structural complementation system comprised of a large luciferase subunit, LgBiT, and a small complementary peptide, SmBiT(q). This complementation approach is unique in that target engagement of RAF proteins in multimeric complexes can be evaluated. Specifically, a fusion protein of LgBiT and full-length RAF is co-expressed with a fusion protein of SmBiT(q) and full-length RAF in cells. These are co-expressed with the constitutively active mutant KRAS(G12C). When the RAF proteins interact and form multimers in the presence of KRAS(G12C), the LgBiT and SmBiT(q) subunits come together to form an active luciferase enzyme and, in the presence of a luciferase substrate, generate a bright luminescent signal that serves as the BRET energy donor (Figure 1). The energy acceptor is a cell-permeable fluorescent NanoBRET® tracer that binds specifically to one or both RAF protomers within the complex. The BRET reporter complex is formed when the tracer binds to RAF protomers within the complex and brings the fluorophore in proximity to the luciferase enzyme. Target engagement and affinity of unlabeled test compounds are measured as a loss of BRET signal when the tracer and test compound compete for binding to the RAF protomer(s).

The NanoBiT® system used in the NanoBRET® TE Intracellular RAF Dimer Assays is unique in the use of the SmBiT(q) peptide, which makes the active luciferase enzyme sensitive to the Extracellular NanoLuc® Inhibitor. This inhibitor quenches signal that can arise from compromised cells and cellular debris to ensure that the NanoBRET® signal originates from live cells.

Pairing the appropriate RAF fusion vectors with the optimized NanoBRET® TE Intracellular RAF Dimer Assay (A or BC) , enables you to evaluate target engagement of two different RAF homodimers (BRAF and CRAF) or three different RAF protomers (ARAF, BRAF and CRAF) within a RAF heterodimeric complex (Figure 2). To evaluate RAF homodimers, transfect cells with LgBiT and SmBiT(q) fusion vectors that express the same RAF protein of interest (Figure 2, Panels A and B). To selectively evaluate a specific RAF protomer in a heterodimeric complex, pair the primary protomer of interest (ARAF, BRAF or CRAF) with a second mutant RAF protein [BRAF(A481F) or CRAF(A373F)] (Figure 2 Panels C–E). These alanine-to-phenylalanine mutations are pseudokinase mutations that prevent tracer binding to the second RAF protomer but maintain the ability to form the RAF heterodimeric complex (2). In addition to measuring target engagement of a test compound to a specific RAF dimer, the five assays allow evaluation of test compound selectivity across multiple RAF dimer configurations in a simple workflow. For the remainder of this technical manual, these five assays will collectively be referred to as NanoBRET® TE RAF Dimer Assays.

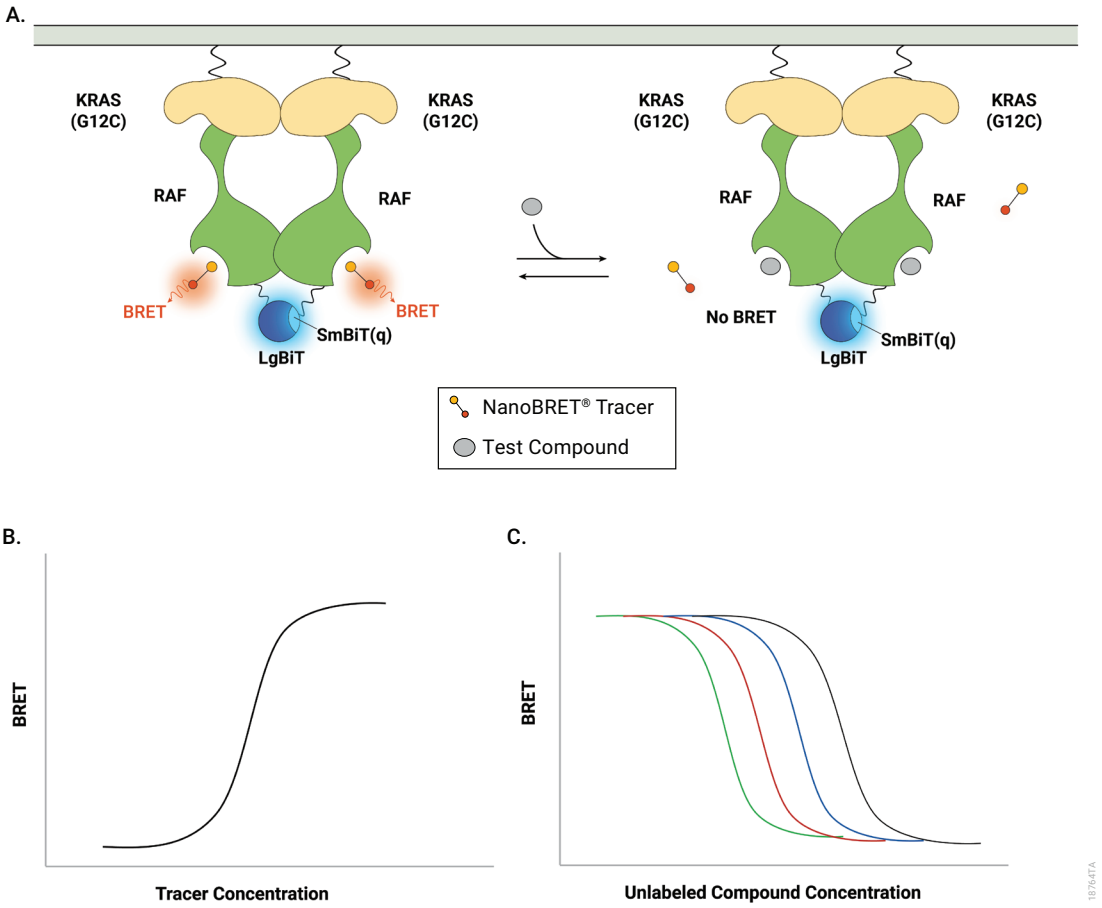


Figure 1. Overview of the NanoBRET® TE Intracellular RAF Dimer Assays. Panel A. Schematic overview of the assays. In cells transfected with RAF-LgBiT and RAF-SmBiT(q) fusion vectors, formation of RAF dimers is driven by co-expression of the KRAS(G12C) protein, bringing the SmBiT(q) and LgBiT protein subunits into proximity and creating a conditional BRET donor signal. A NanoBRET® tracer that binds to the nucleotide pocket of RAF is used to create a BRET reporter complex at the RAF dimer of interest. **Panel B.** Tracer affinity for each RAF was determined by generating a NanoBRET® tracer dose response curve; a schematic of such a dose response curve is shown. The resulting curves were used to select the tracer concentrations for this protocol, which are subsaturating and near the EC_{50} value of the tracer dose response curves. **Panel C.** To analyze target engagement and determine test compound affinity, cells are treated with varying test compound concentrations in the presence of this fixed tracer concentration. As the compound concentration that binds to the RAF increases, the BRET signal decreases.

1. Description (continued)

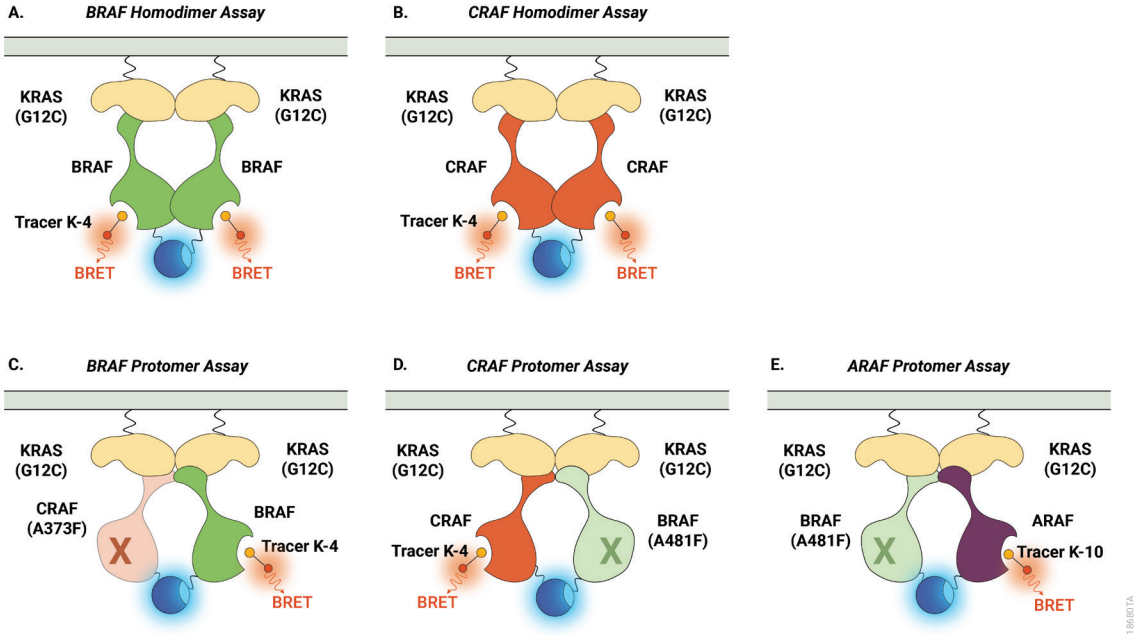


Figure 2. Assayable targets using the NanoBRET® TE Intracellular RAF Dimer Assays. Panels A and B. Schematic overviews of the two RAF homodimer configurations (BRAF and CRAF). **Panels C–E**. To evaluate a specific RAF protomer within a heterodimeric complex, the primary RAF protomer of interest is paired with a second mutant RAF protein [BRAF(A481F) or CRAF(A373F)]. These alanine-to-phenylalanine mutations are pseudokinase mutations that prevent tracer binding to the RAF protomer, but maintain the ability to form the RAF heterodimeric complex (2). This approach enables three RAF protomer configurations within heterodimers (ARAF, BRAF and CRAF).

This technical manual describes the adherent format used by the NanoBRET® TE Intracellular RAF Dimer Assays. The adherent format is compatible with cell culture-treated plates and allows target engagement measurement in adhered cells, which may be more comparable to other cell-based phenotypic assays that use adhered cells. In this adherent format, cells are seeded into assay plates on day 1 and tracer and test compound are added on day 2. A summary of the NanoBRET® TE Intracellular RAF Dimer Assay workflow is provided in Figure 3.

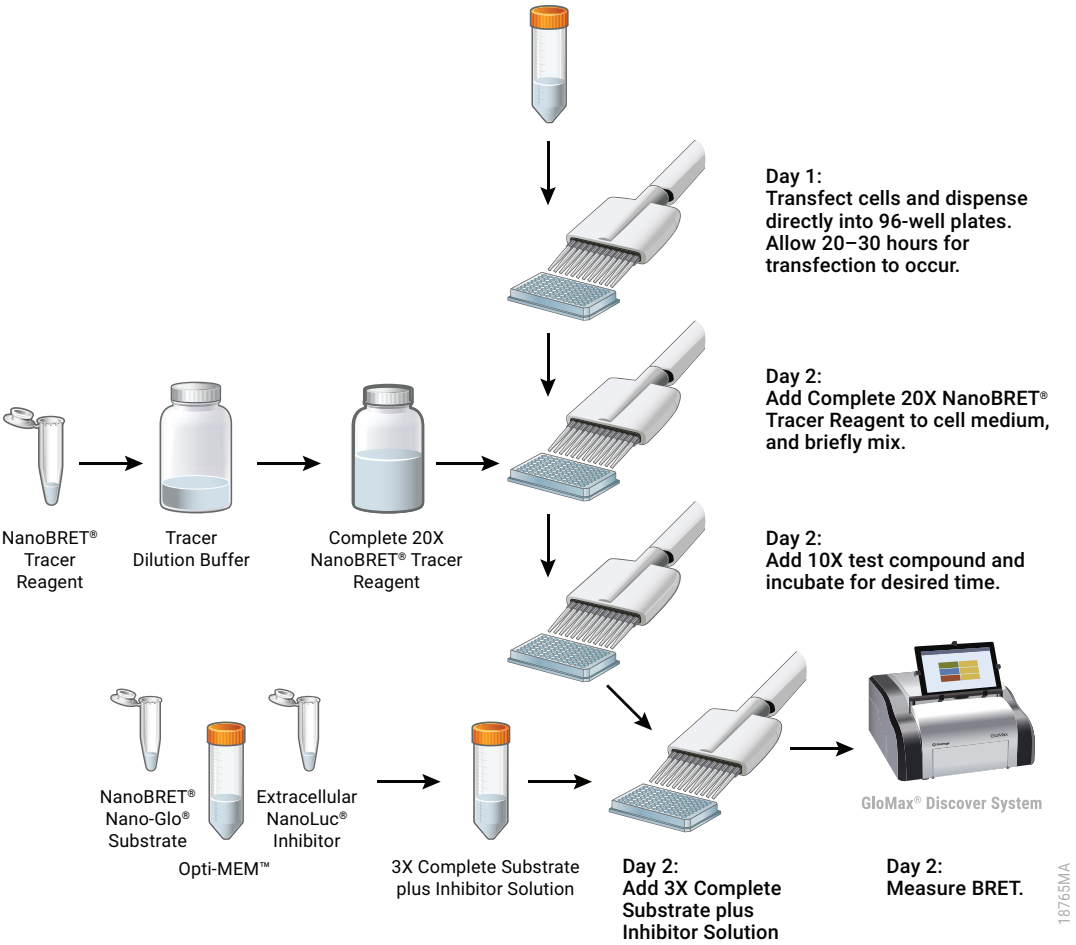


Figure 3. Overview of the NanoBRET® TE Intracellular RAF Dimer Assay workflow.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
NanoBRET® TE Intracellular RAF Dimer Assay, BC	**100 assays**	**N8014**

Provides sufficient reagents for 100 assays (96-well plate). Includes:

- 20µg BRAF-SmBiT(q) Fusion Vector
- 20µg CRAF(A373F)-LgBiT Fusion Vector
- 20µg KRAS(G12C) Expression Vector
- 30µl NanoBRET® Tracer K-4
- 5ml Tracer Dilution Buffer
- 50µl NanoBRET® Nano-Glo® Substrate
- 17µl Extracellular NanoLuc® Inhibitor

PRODUCT	SIZE	CAT.#
NanoBRET® TE Intracellular RAF Dimer Assay, BC	**1,000 assays**	**N8015**

Provides sufficient reagents for 1,000 assays (96-well plate). Includes:

- 20µg BRAF-SmBiT(q) Fusion Vector
- 20µg CRAF(A373F)-LgBiT Fusion DNA
- 60µg KRAS(G12C) Expression Vector
- 300µl NanoBRET® Tracer K-4
- 5ml Tracer Dilution Buffer
- 330µl NanoBRET® Nano-Glo® Substrate
- 110µl Extracellular NanoLuc® Inhibitor

PRODUCT	SIZE	CAT.#
NanoBRET® TE Intracellular RAF Dimer Assay, A	**100 assays**	**N8016**

Provides sufficient reagents for 100 assays (96-well). Includes:

- 20µg SmBiT(q)-ARAF Fusion Vector
- 20µg BRAF(A481F)-LgBiT Fusion Vector
- 20µg KRAS(G12C) Expression Vector
- 30µl NanoBRET® Tracer K-10
- 5ml Tracer Dilution Buffer
- 50µl NanoBRET® Nano-Glo® Substrate
- 17µl Extracellular NanoLuc® Inhibitor

PRODUCT	SIZE	CAT.#
NanoBRET® TE Intracellular RAF Dimer Assay, A	**1,000 assays**	**N8017**

Provides sufficient reagents for 1,000 assays (96-well). Includes:

- 20µg SmBiT(q)-ARAF Fusion Vector
- 20µg BRAF(A481F)-LgBiT Fusion Vector
- 60µg KRAS(G12C) Expression Vector
- 300µl NanoBRET® Tracer K-10
- 5ml Tracer Dilution Buffer
- 330µl NanoBRET® Nano-Glo® Substrate
- 110µl Extracellular NanoLuc® Inhibitor

Storage Conditions: Store all NanoBRET® TE Intracellular RAF Dimer Assay components at less than –65°C. Alternatively, store the NanoBRET® Tracer at less than –65°C and all other components at –30°C to –10°C. Avoid multiple freeze-thaw cycles of the vectors. We recommend dispensing the NanoBRET® Tracer into aliquots after first use and avoiding more than five freeze-thaw cycles. Store the NanoBRET® Tracer, NanoBRET® Nano Glo® Substrate and Extracellular NanoLuc® Inhibitor protected from light.

3. Before You Begin

The NanoBRET® TE Intracellular RAF Dimer Assays require transfection of cells with two fusion vectors, one that encodes the LgBiT fusion protein and one that encodes the SmBiT(q) fusion protein. All vectors encoding LgBiT and SmBiT(q) fusions to your RAF variant of interest are available separately and listed in Table 1.

The NanoBRET® TE Intracellular RAF Dimer Assays also require cotransfection with the KRAS(G12C) Expression Vector (Cat.# NV4941). It is supplied as a control in Cat.# N8014, N8015, N8016, N8017 and is available separately as NV4941. See Table 1.

Table 1. Vectors Required for NanoBRET® TE Intracellular RAF Dimer Assays.

Vector	Size	Cat.#
SmBiT(q)-ARAF Fusion Vector	20µg	NV4861
BRAF-SmBiT(q) Fusion Vector	20µg	NV4871
BRAF-LgBiT Fusion Vector	20µg	NV4881
CRAF-SmBiT(q) Fusion Vector	20µg	NV4891
CRAF-LgBiT Fusion Vector	20µg	NV4901
BRAF(A481F)-LgBiT Fusion Vector	20µg	NV4911
BRAF(A481F)-SmBiT(q) Fusion Vector	20µg	NV4921
CRAF(A373F)-LgBiT Fusion Vector	20µg	NV4931
KRAS(G12C) Expression Vector	20µg	NV4941

3. Before You Begin (continued)

To study target engagement of a specific RAF dimer, we recommend the combinations shown in Figure 2, Table 2 or Section 5.

Table 2. Recommended Target Assay and Fusion Vector Combinations.

Target Assay	SmBiT(q) Fusion Vector	LgBiT Fusion Vector	NanoBRET® TE RAF Dimer Assay	Notes
BRAF Homodimer	BRAF-SmBiT(q) Fusion Vector	BRAF-LgBiT Fusion Vector	BC	K-4 Tracer included in assay.
CRAF Homodimer	CRAF-SmBiT(q) Fusion Vector	CRAF-LgBiT Fusion Vector	BC	K-4 Tracer included in assay.
ARAF Protomer	SmBiT(q)-ARAF Fusion Vector	BRAF(A481F)-LgBiT Fusion Vector	A	K-10 Tracer included in Cat.# N8016, N8017.
BRAF Protomer	BRAF-SmBiT(q) Fusion Vector	CRAF(A373F)-LgBiT Fusion Vector	BC	K-4 Tracer included in Cat.# N8014, N8015.
CRAF Protomer	BRAF(A481F)-SmBiT(q) Fusion Vector	CRAF-LgBiT Fusion Vector	BC	K-4 Tracer included in assay.

3.A. Propagating and Preparing the NanoBRET® Fusion Vectors

The amount of each vector provided with the system is sufficient for a limited number of experiments. We strongly recommend that you propagate each vector in *E. coli* and purify larger amounts of low-endotoxin vector for transfection. Follow standard protocols for plasmid transformation into *E. coli* for vector propagation and cell culture-grade vector preparation.

The RAF-LgBiT Fusion Vectors, RAF-SmBiT(q) Fusion Vectors and KRAS(G12C) Expression Vector include a kanamycin expression cassette for selection during bacterial propagation. We recommend sequencing the coding regions after propagation to ensure fidelity of the protein-coding regions. For vector sequence information, visit the RAF fusion vectors product page.

For information about RAF vector propagation, visit the RAF fusion vectors product page at: **promega.com** or contact Promega Technical Services: **techserv@promega.com**

3.B. Instrument Requirements and Setup

To perform the NanoBRET® TE Intracellular RAF Dimer Assay, a luminometer capable of sequentially measuring dual-wavelength windows is required. This is accomplished using filters. We recommend using a band pass (BP) filter to measure donor signal and a long pass (LP) filter to measure acceptor signal to maximize sensitivity.

Emission of the NanoBRET® luminescent donor, NanoBiT® luciferase enzyme, occurs at 460nm. To measure donor signal, we recommend a band pass filter that covers close to 460nm with a band pass range of 8–80nm. For example, a 450nm/80nm BP filter will capture emission in the 410–490nm range.

Note: We recommend a band pass filter to measure donor signal to selectively capture the signal peak and avoid measuring acceptor peak bleedthrough. A short pass (SP) filter that covers the 460nm area can be used but may result in an artificially large value for the donor signal and reduction of the assay window.

Peak emission of the NanoBRET® Tracer K-4 and NanoBRET® Tracer K-10 occurs in the range of 610–630nm. To measure this acceptor signal, we recommend a long pass filter starting at 600–610nm.

Instruments capable of dual-wavelength measurements are equipped with a selection of filters or 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 photomultiplier tube is optimized to capture the highest donor signal without reaching instrument saturation.

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

- The GloMax® Discover System (Cat.# GM3000) with preloaded filters for donor signal (450nm/8nm BP filter) and acceptor signal (600nm LP filter). Select the preloaded BRET:NanoBRET® 618 protocol from the protocol menu.
- BMG Labtech CLARIOstar® Multimode Microplate Reader with preloaded filters for donor signal (450nm/80nm BP filter) and acceptor signal (610nm LP filter).
- Thermo Fisher Varioskan® Multimode Microplate Reader with the following filters from Edmunds Optics: 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® TE Intracellular RAF Protomer and RAF Homodimer Assay Protocol

The results of the NanoBRET® TE Intracellular RAF Protomer and RAF Homodimer Assays can be expressed several ways: as the raw BRET ratio, background-subtracted BRET ratio and/or normalized BRET. See Section 4.F for more information. When assembling the assay plate, be sure to include the necessary controls on the assay plate to perform the desired calculations and assist with troubleshooting. We recommend preparing at least three wells for each control type.

Nontransfected-Cell control: Contains cells that were not transfected and thus do not express RAF fusion proteins. This control represents the background donor signal, which can be used to assess transfection success as described in Section 8.

No-Tracer Control: Contains transfected cells but neither tracer nor test compound. The BRET signal for the no-tracer control is used to calculate the background-subtracted BRET ratio.

$BRET_{Min}$ Control: Contains transfected cells with tracer and an excess concentration of a known RAF ligand, such as 20µM TAK-632. The average BRET ratio for this control represents $BRET_{Min}$ when calculating normalized BRET. This control also serves as a positive control for RAF target engagement (positive TE control).

$BRET_{Max}$ Control: Contains transfected cells with tracer and only the solvent vehicle for the test compound(s). The average BRET ratio for this control represents $BRET_{Max}$ when calculating normalized BRET.

4.A. Materials to Be Supplied By the User

(Composition of Buffers and Solutions is provided in Section 9.D.)

- HEK293 or other cultured mammalian cells (see Note a. below)
- Dulbecco's Modified Eagle's Medium (DMEM; e.g., Thermo Fisher Scientific Cat.# 11995-065)
- fetal bovine serum (e.g., HyClone Cat.# SH30070.03 or Seradigm Cat.# 1500-050)
- Opti-MEM™ I Reduced Serum Medium, without phenol red (e.g., Life Technologies Cat.# 11058-021)
- 0.05% Trypsin/EDTA (e.g., Thermo Fisher Scientific Cat.# 25300)
- FuGENE® HD Transfection Reagent (Cat.# E2311)
- DMSO (e.g., 99.7%; Sigma Cat.# 2650)
- TE Buffer, 1X, Molecular Biology Grade (Cat.# V6231) if necessary to dilute DNA.
- 96-well tissue culture-treated white flat-bottom polystyrene microplate (e.g., Corning® Cat.# 3917 or Greiner Cat.# 655083)
- cell culture equipment and reagents
- sterile polypropylene conical tubes
- polypropylene plasticware such as troughs for diluting and dispensing reagents. **Do not** use conventional polystyrene plasticware for this assay due to adsorption of the tracer.
- detection instrument capable of measuring NanoBRET® wavelengths (e.g., GloMax® Discover System, Cat.# GM3000); see Section 3.B
- RAF-LgBiT Fusion Vector and RAF-SmBiT(q) Fusion Vector (see Note b below)
- KRAS(G12C) Expression Vector (see Note b below)
- TAK-632 (positive control RAF ligand, Selleckchem Cat.# S7291)
- plate mixer capable of mixing viscous reagents; see Section 9.B

Notes:

a. This protocol was optimized for HEK293 cells. When using other cell types, use the appropriate cell culture conditions for that cell type. You may need to optimize transfection conditions and determine the appropriate concentration of NanoBRET® Tracer K-4 or K-10.

b. Performing the NanoBRET® TE Intracellular RAF Dimer Assay requires LgBiT and SmBiT(q) Fusion Vectors for the RAF variants of interest. You will need to purchase the appropriate LgBiT and SmBiT(q) RAF Fusion Vectors separately; see Table 1, Table 2 and Section 9.F for a list of available LgBiT and SmBiT(q) Fusion Vectors.

4.B. Transient Transfection of HEK293 Cells with NanoBiT® Fusion Vectors

1. Culture HEK293 cells (or other cell type) using the appropriate cell culture conditions.

   Notes:

   a. Cell handling is very important for reproducible assay results. For optimal transfection results, use HEK293 cells that are 70–90% confluent and recently passaged (ideally within 1–2 days).

   b. For each assay plate, we recommend preparing at least 2×10^6 cells.

2. Remove cell culture medium from the cell culture flask by aspiration, trypsinize cells and allow cells to dissociate from the flask.

   Note: For best results, use trypsinization conditions that produce a single-cell suspension.

3. Neutralize trypsin by adding prewarmed cell culture medium containing serum (90% DMEM containing 10% fetal bovine serum). Centrifuge cells at 200 × *g* for 5 minutes to pellet cells.

4. Aspirate cell culture medium, and resuspend cells in prewarmed assay medium (Opti-MEM™ I Reduced Serum Medium without phenol red containing 1% fetal bovine serum).

5. Adjust cell density to 2×10^5 cells/ml using prewarmed assay medium in a sterile conical tube.

6. To transfect HEK293 cells using the FuGENE® HD Transfection Reagent, prepare lipid:DNA complex as follows:

   a. Prepare a 10µg/ml solution of DNA in Opti-MEM™ I Reduced Serum Medium without phenol red in a sterile conical tube. Mix well.

Component	Volume[1]	Final Concentration[2]
Opti-MEM™ I Reduced Serum Medium without phenol red	1ml	—
KRAS(G12C) Expression Vector[3]	9.5µl	9.5µg/ml
RAF-LgBiT Fusion Vector[3]	0.25µl	0.25µg/ml
RAF-SmBiT(q) Fusion Vector[3]	0.25µl	0.25µg/ml

[1]These volumes are appropriate to prepare approximately 1ml of transfection complex and transfect 20ml of HEK293 cells. To prepare a different volume, increase or decrease the volume of each component proportionally. If necessary to avoid pipetting volumes less than 1µl, perform serial dilution of the vectors in 1X TE buffer prior to preparing the lipid:DNA complex.

(Step 6 continued, next page.)

4.B. Transient Transfection of HEK293 Cells with NanoBiT® Fusion Vectors (continued)

[2]This ratio of vectors is optimized to produce an adequate donor signal while limiting the degree of nonspecific donor signal arising from spurious interaction of the SmBiT(q) and LgBiT fusion proteins. We do not recommend adjusting this ratio unless you are optimizing transfection conditions for a different cell line.

[3]The amount of each vector provided is limited. We strongly recommend that you propagate each vector in *E. coli* and purify sufficient amounts of low-endotoxin vector for transfection. See Section 3.A.

b. Add 30µl of FuGENE® HD Transfection Reagent per 1ml of vector mixture to form the lipid:DNA complexes. Ensure that the FuGENE® HD Transfection Reagent does not touch the side of the plastic tube; pipet directly into the liquid in the tube. Gently mix.
Note: If preparing the transfection complex in a 1.5ml microcentrifuge tube, mix by inversion 5–10 times. If preparing the transfection complex in a larger conical tube (e.g., 15ml or 50ml conical tube), mix by vortexing at the lowest speed or gently swirling the tube. Do not invert small volumes of transfection complexes in large conical tubes, as this can reduce the efficiency of complex formation and result in lower luminescent signal.

c. Incubate at room temperature for 20–30 minutes to allow the lipid:DNA complexes to form.

7. In a sterile, conical tube, mix 1 part of the lipid:DNA complex (e.g., 1ml) with 20 parts of HEK293 cells prepared in Step 5 (e.g., 20ml). Mix by gently inverting the tube 5 times.

Note: For larger or smaller bulk transfections, increase or decrease these volumes accordingly. Be sure to maintain the 20:1 ratio of cells to lipid:DNA complex.

8. Dispense 100µl of cells and lipid:DNA complex into each well of a 96-well solid white flat bottom polystyrene tc-treated microplate (Corning® Cat.# 3917 or Greiner Cat.# 655083), and incubate for 20–30 hours at 37°C, 5% CO_2.

Optional: Dispense 105µl of cells and lipid:DNA complex into three wells of the 96-well assay plate as no-tracer control samples to determine background signal to calculate normalized BRET ratios. See Section 4.F.

Note: We recommend a cell density of approximately 55,000–80,000 cells/cm^2 during this step. For example, use approximately 20,000 cells per well for a 96-well assay plate.

4.C. Preparing Cells with NanoBRET® Tracer (Day 2)

1. Prepare the Complete 20X NanoBRET® tracer reagent using polypropylene tubes as follows:

a. Thaw the NanoBRET® tracer and Tracer Dilution Buffer.

Note: At first use, we recommend dispensing the NanoBRET® tracer into aliquots; avoid more than five freeze-thaw cycles. Store the NanoBRET® tracer at less than –65°C, protected from light. Once thawed, the Tracer Dilution Buffer can be stored at room temperature.

b. Prepare a 100X solution of the recommended NanoBRET® tracer for your RAF protomer or homodimer of interest in pure DMSO according to the table below. Mix well.

Note: Each NanoBRET® tracer is provided as a 400µM stock solution in pure DMSO. Dilute the 400µM solution with an appropriate volume of pure DMSO to make the 100X solution.

	Recommended NanoBRET® Tracer	Recommended 100X Tracer Concentration
RAF Homodimer Assays		
BRAF Homodimer	K-4	50µM
CRAF Homodimer	K-4	100µM
RAF Protomer Assays		
BRAF Protomer	K-4	50µM
CRAF Protomer	K-4	100µM
ARAF Protomer	K-10	13µM

Note: These 100X tracer concentrations are provided as a starting point. Higher tracer concentrations may increase assay window but reduce sensitivity. For more information about optimizing tracer concentration, see Sections 5 and 6.

c. Prepare the Complete 20X NanoBRET® tracer reagent by slowly adding 4 parts of Tracer Dilution Buffer to 1 part of 100X NanoBRET® tracer prepared in Step 1.b. Mix well.

Note: Because the Tracer Dilution Buffer is viscous, slowly pipet the Tracer Dilution Buffer. Visually inspect the solution to ensure that the Complete 20X NanoBRET® tracer reagent is completely dispersed and tube contents are homogeneous.

Component	Volume Per 96-Well Plate
100X NanoBRET® tracer	100µl
Tracer Dilution Buffer	400µl
final volume	**500µl**

2. Dispense 5µl of Complete 20X NanoBRET® tracer reagent into each well of the 96-well assay plate prepared in Section 4.B. Do not add tracer to the no-tracer control wells.

   Note: Because the Tracer Dilution Buffer is viscous, slowly pipet the Complete 20X NanoBRET® tracer reagent.

3. Mix assay plate on an orbital shaker for 15 seconds at 900rpm. Visually inspect the wells to ensure that the Complete 20X NanoBRET® tracer reagent is completely dispersed and well contents are homogeneous. If necessary, repeat the mixing step until well contents appear homogeneous.

   Note: The plate-mixing conditions necessary to achieve homogeneity can vary, depending on the orbital shaker. See Section 9.B for more details.

4.D. Adding Test Compounds

1. Prepare test compound at a 1,000X final concentration in 100% DMSO or appropriate test compound solvent.
2. Dilute 1,000X test compound 100-fold to a final concentration of 10X in Opti-MEM™ I reduced serum medium without phenol red.

   Optional:

   a. Prepare a 200µM (10X) solution of TAK-632 in Opti-MEM™ I reduced serum medium without phenol red for the $BRET_{Min}$ or positive TE controls.

   b. Prepare a 10X $BRET_{Max}$ control by diluting the test compound solvent 100-fold into Opti-MEM™ I reduced serum medium without phenol red.
3. Add 10µl of 10X test compound to each well containing cells and NanoBRET® tracer reagent. Thoroughly mix the plate on an orbital shaker for 15 seconds at 900rpm.

   Optional:

   a. Add 10µl of 200µM TAK-632 to $BRET_{Min}$ control wells and positive TE control wells.

   b. Add 10µl of 10X $BRET_{Max}$ control to the $BRET_{Max}$ control wells.
4. Incubate the plate at 37°C, 5% CO_2 for 2 hours.

   Note: Depending on the cell permeability and binding characteristics of the test compound, you may need to optimize the incubation time. BRET can be measured in as little as 30 minutes.

4.E. NanoBRET® Assay Protocol

1. Remove plate(s) from incubator and allow to cool to room temperature for approximately 15 minutes.
2. Thaw the Extracellular NanoLuc® Inhibitor and NanoBRET® Nano-Glo® Substrate.

   Note: After first use, the Extracellular NanoLuc® Inhibitor and NanoBRET® Nano-Glo® Substrate can be stored at –30°C to –10°C, protected from light.
3. Just before measuring BRET, prepare the 3X Complete Substrate plus Inhibitor Solution in Opti MEM™ I reduced serum medium without phenol red in a conical tube as described below:

Component	Volume Per 96-Well Plate
Opti-MEM™ I reduced serum medium without phenol red	4,960µl
NanoBRET® Nano-Glo® Substrate	30µl
Extracellular NanoLuc® Inhibitor	10µl
final volume	**5,000µl**

   Note: Use the 3X Complete Substrate plus Inhibitor Solution within 1.5 hours of preparation. Discard any unused solution.
4. Mix by gently inverting the tube 5–10 times.

5. Add 50µl of 3X Complete Substrate plus Inhibitor Solution to each well of the 96-well assay plate. Incubate for 2–3 minutes at room temperature.

   Note: Proceed to Step 6 within 10 minutes of adding the 3X Complete Substrate plus Inhibitor Solution to measure BRET signal. You can measure BRET for up to 2 hours after addition, but there will be a loss of luminescent signal that is dependent upon the target.

6. Measure donor emission using a 450nm BP filter and acceptor emission using a 600 LP filter using the GloMax® Discover System or other NanoBRET® assay-compatible luminometer following the manufacturer's instructions for use. The optimal integration time can vary across instruments, but an integration time of 0.2–1 second is typically sufficient. See Section 3.B for more information.

4.F. Calculating the BRET Ratio

The assay results can be expressed by calculating the raw BRET ratio, background-subtracted BRET ratio and/or normalized BRET signal as described below:

To calculate the raw BRET ratio, divide the acceptor emission by the donor emission for each sample or control. Convert raw BRET units to milliBRET units (mBU) by multiplying each BRET ratio by 1,000.

$$\text{BRET ratio} = \frac{\text{Acceptor signal}_{\text{sample}}}{\text{Donor signal}_{\text{sample}}} \times 1{,}000$$

To calculate the background-subtracted BRET ratio, subtract the average raw BRET ratio for the no-tracer controls from the raw BRET ratio for each sample. Convert BRET units to milliBRET units (mBU) by multiplying each BRET ratio by 1,000.

$$\text{Background-subtracted BRET ratio} = \left[\frac{\text{Acceptor signal}_{\text{sample}}}{\text{Donor signal}_{\text{sample}}} - \frac{\text{Acceptor signal}_{\text{no-tracer control}}}{\text{Donor signal}_{\text{no-tracer control}}} \right] \times 1{,}000$$

To facilitate selectivity comparisons between different RAFs that may have different assay windows, consider normalizing the BRET signal of each sample to the BRET signal of two controls—one control that defines the maximum BRET value and another control that defines the minimum BRET value—as described below. The normalized BRET value represents the BRET ratio of the sample as a percentage of the maximum BRET ratio.

$$\text{Normalized BRET (\%)} = \left[\frac{(\text{BRET}_{\text{sample}} - \text{BRET}_{\text{Min}})}{(\text{BRET}_{\text{Max}} - \text{BRET}_{\text{Min}})} \right] \times 100$$

Where:

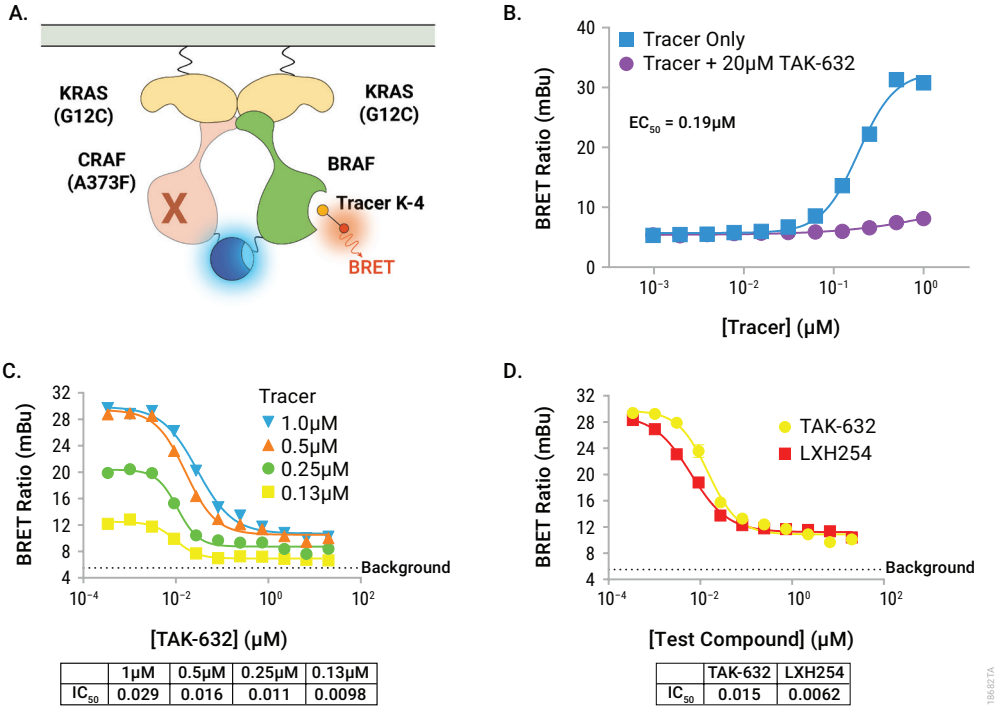
$\text{BRET}_{\text{sample}}$ is the BRET ratio for the sample.

BRET_{Min} is the average BRET ratio for controls that contain tracer and an excess concentration of a known RAF ligand, such as 20µM TAK-632. Alternatively, you can use the average BRET ratio for the no tracer controls, which contain neither tracer nor test compound.

BRET_{Max} is the average BRET ratio for controls that contain tracer and test compound vehicle but not test compound.

5. Representative Data for Individual RAF Protomers

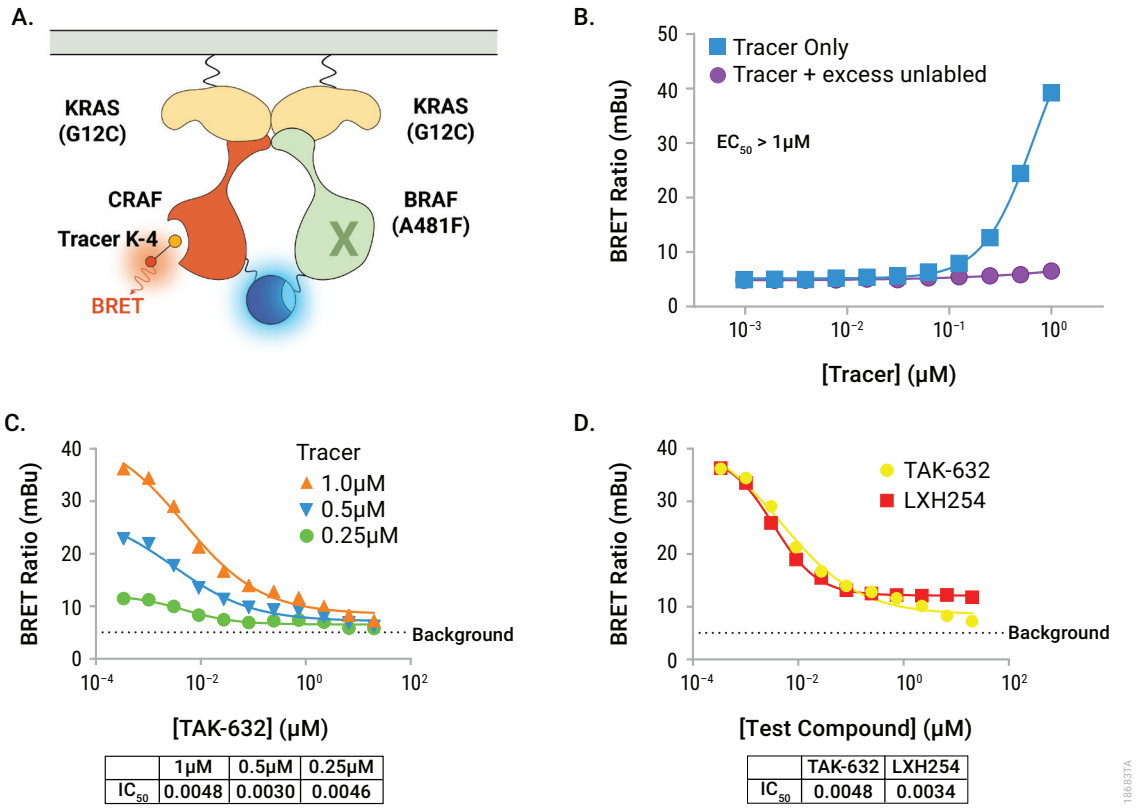
Representative data that demonstrate the use of the NanoBRET® TE Intracellular RAF Dimer Assays to evaluate target engagement of RAF protomers are provided below. In each case, a specific RAF heterodimer is induced by co-expressing untagged and constitutively active mutant KRAS(G12C). The RAF protomer to be assayed is paired with a second RAF protomer that is mutated to prevent tracer binding and facilitate measurement of target engagement specifically at BRAF (Figure 4), CRAF (Figure 5) or ARAF (Figure 6) protomer of interest. These data demonstrate tracer affinity and the influence of tracer concentration on test compound potency, as well as a recommended tracer concentration for measuring test compound affinity.



	1µM	0.5µM	0.25µM	0.13µM
IC_{50}	0.029	0.016	0.011	0.0098

	TAK-632	LXH254
IC_{50}	0.015	0.0062

Figure 4. Representative data for target engagement of the BRAF protomer in HEK293 cells using the NanoBRET® TE RAF Dimer Assay, BC. Panel A. Schematic of the NanoBRET® TE BRAF Protomer Assay. Adherent HEK293 cells were transiently transfected with the BRAF-SmBiT(q) Fusion Vector, CRAF(A373F)-LgBiT Fusion Vector and KRAS(G12C) Expression Vector at a ratio of 0.25:0.25:9.5. Cells expressing BRAF-SmBiT(q), CRAF(A373F)-LgBiT and KRAS(G12C) proteins were combined with various NanoBRET® Tracer K-4 concentrations. Cells were incubated in the presence or absence of a saturating dose of TAK-632 (**Panel B**) or serially diluted TAK-632 (**Panel C**) for 2 hours before adding 3X Complete Substrate plus Inhibitor Solution and subsequent BRET measurement using the GloMax® Discover System equipped with 450/80BP and 610/LP filters. The recommended NanoBRET® Tracer K-4 concentration for the BRAF protomer is depicted in orange in **Panel C.** An example of a compound profiling experiment at the recommended tracer concentration is provided in **Panel D.**

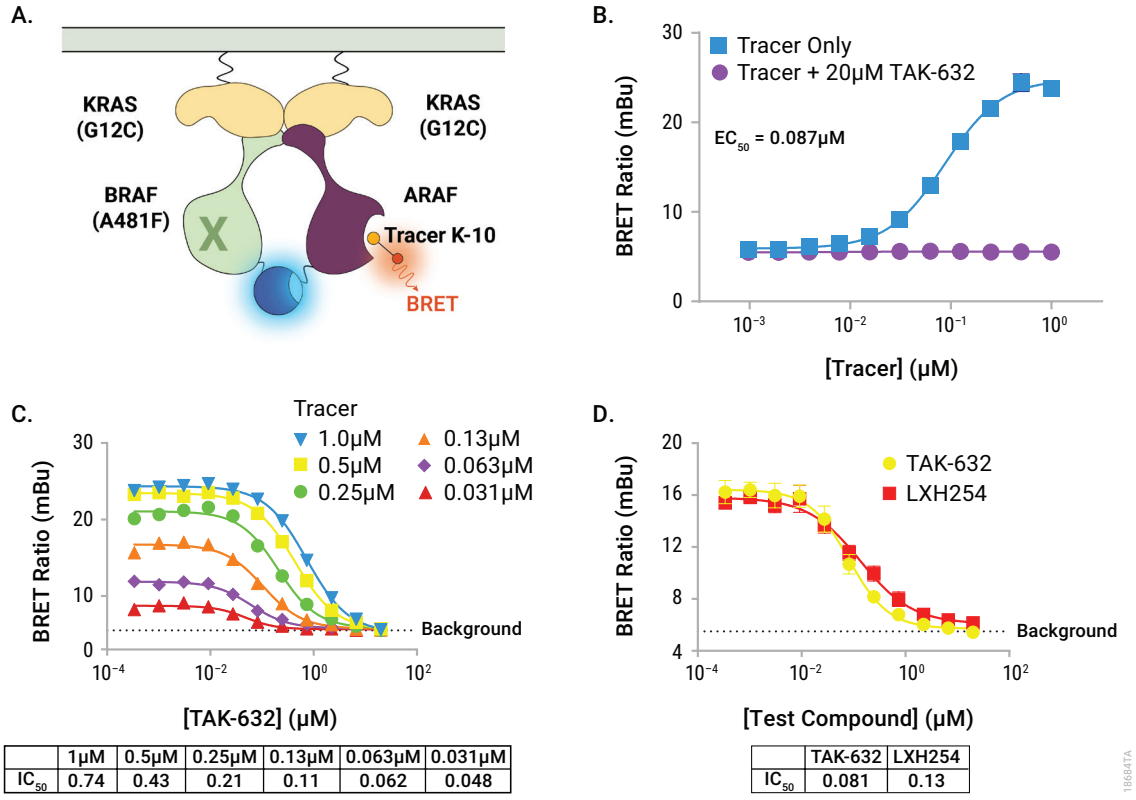


	1µM	0.5µM	0.25µM
IC_{50}	0.0048	0.0030	0.0046

	TAK-632	LXH254
IC_{50}	0.0048	0.0034

Figure 5. Representative data for target engagement of the CRAF protomer in HEK293 cells using the NanoBRET® TE RAF Dimer Assay, BC. Panel A. Schematic of the NanoBRET® TE CRAF Protomer Assay. Adherent HEK293 cells were transiently transfected with the CRAF-LgBiT Fusion Vector, BRAF(A481F)-SmBiT(q) Fusion Vector and KRAS(G12C) Expression Vector at a ratio of 0.25:0.25:9.5. Cells expressing CRAF-LgBiT, BRAF(A481F)-SmBiT(q) and KRAS(G12C) proteins were combined with various NanoBRET® Tracer K-4 concentrations. Cells were incubated in the presence or absence of a saturating dose of TAK-632 (**Panel B**) or serially diluted TAK-632 (**Panel C**) for 2 hours before addition of 3X Complete Substrate plus Inhibitor Solution and subsequent BRET measurements on a GloMax® Discover System equipped with 450/80BP and 610/LP filters. The recommended NanoBRET® Tracer K-4 concentration for the CRAF protomer is depicted in orange in **Panel C**. An example of a compound profiling experiment at the recommended tracer concentration is provided in **Panel D**.

5. Representative Data for Individual RAF Protomers (continued)



	1µM	0.5µM	0.25µM	0.13µM	0.063µM	0.031µM
IC_{50}	0.74	0.43	0.21	0.11	0.062	0.048

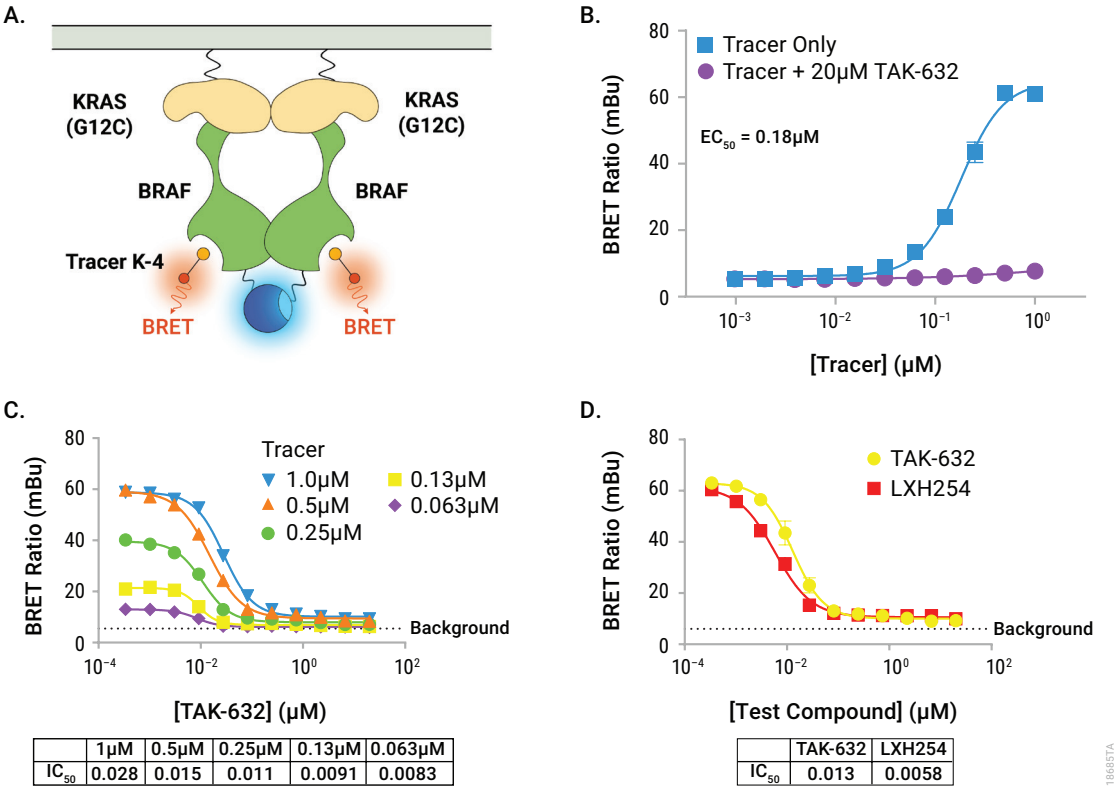
	TAK-632	LXH254
IC_{50}	0.081	0.13

186847A

Figure 6. Representative data for target engagement of the ARAF protomer in HEK293 cells using the NanoBRET® TE RAF Dimer Assay, A. Panel A. Schematic of the NanoBRET® TE Intracellular ARAF Protomer Assay. Adherent HEK293 cells were transiently transfected with the SmBiT(q)-ARAF Fusion Vector, BRAF(A481F)-LgBiT Fusion Vector and KRAS(G12C) Expression Vector at a ratio of 0.25:0.25:9.5. Cells expressing SmBiT(q)-ARAF, BRAF(A481F)-LgBiT and KRAS(G12C) proteins were combined with various NanoBRET® Tracer K-10 concentrations. Cells were incubated in the presence or absence of a saturating dose of TAK-632 (**Panel B**) or serially diluted TAK-632 (**Panel C**) for 2 hours before addition of 3X Complete Substrate plus Inhibitor Solution and subsequent BRET measurements on a GloMax® Discover System equipped with 450/80BP and 610/LP filters. The recommended NanoBRET® Tracer K-10 concentration for the ARAF protomer is depicted in orange in **Panel C**. An example of a compound profiling experiment at the recommended tracer concentration is provided in **Panel D**.

6. Representative Data for RAF Homodimers

Representative data demonstrating the use of the NanoBRET® TE Intracellular RAF Dimer Assay to evaluate target engagement of RAF homodimers are provided in Figures 7 and 8.



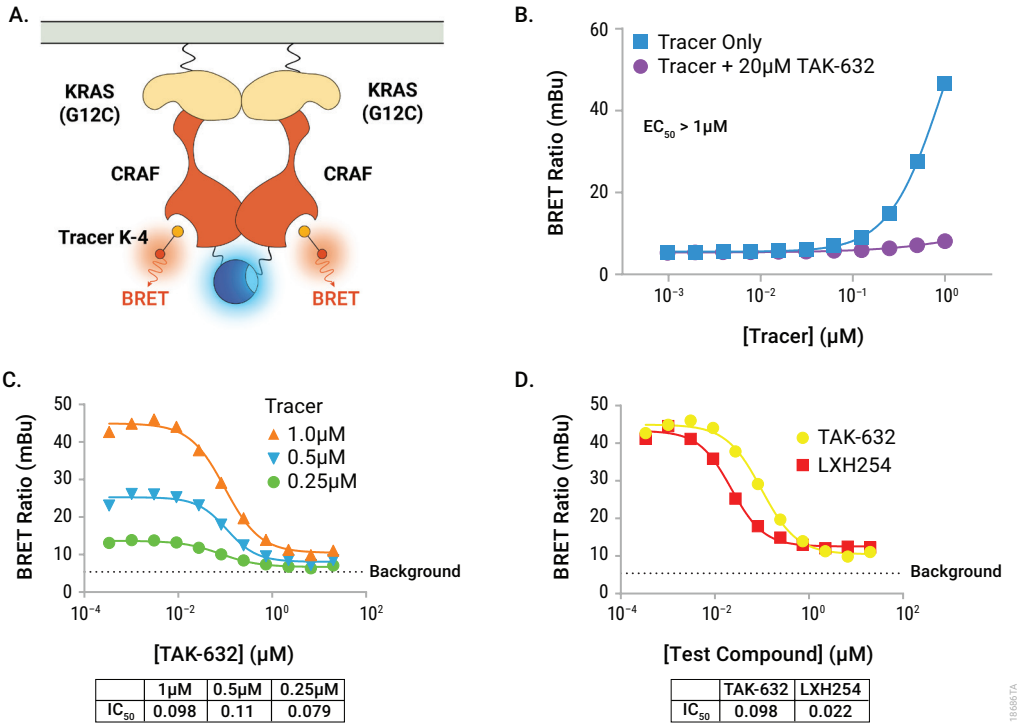
	1µM	0.5µM	0.25µM	0.13µM	0.063µM
IC_{50}	0.028	0.015	0.011	0.0091	0.0083

	TAK-632	LXH254
IC_{50}	0.013	0.0058

186857A

Figure 7. Representative data for target engagement of the BRAF homodimers in HEK293 cells using the NanoBRET® TE RAF Dimer Assay, BC. Panel A. Schematic of the NanoBRET® TE BRAF Homodimer Assay. Adherent HEK293 cells were transiently transfected with the BRAF-SmBiT(q) Fusion Vector, BRAF-LgBiT Fusion Vector and KRAS(G12C) Expression Vector at a ratio of 0.25:0.25:9.5. Cells expressing BRAF-SmBiT(q), BRAF-LgBiT and KRAS(G12C) proteins were combined with various NanoBRET® Tracer K-4 concentrations. Cells were incubated in the presence or absence of a saturating dose of TAK-632 (**Panel B**) or serially diluted TAK-632 (**Panel C**) for 2 hours before addition of 3X Complete Substrate plus Inhibitor Solution and subsequent BRET measurements on a luminometer equipped with 450/80BP and 610/LP filters. The recommended NanoBRET® Tracer K-4 concentration for the BRAF homodimer is depicted in orange in **Panel C**. An example of a compound profiling experiment at the recommended tracer concentration is shown in **Panel D**.

6. Representative Data for RAF Homodimers (continued)



	1µM	0.5µM	0.25µM
IC_{50}	0.098	0.11	0.079

	TAK-632	LXH254
IC_{50}	0.098	0.022

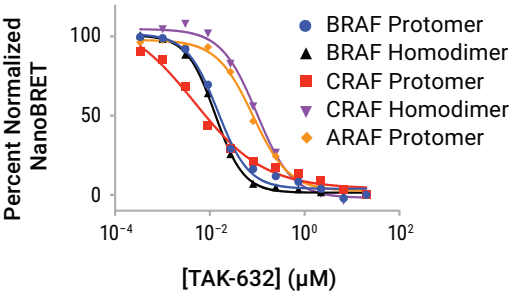
Figure 8. Representative data for target engagement of the CRAF homodimers in HEK293 cells using the NanoBRET® TE RAF Dimer Assay, BC. Panel A. Schematic of the NanoBRET® TE CRAF Homodimer Assay. Adherent HEK293 cells were transiently transfected with the CRAF-SmBiT(q) Fusion Vector, CRAF-LgBiT Fusion Vector and KRAS(G12C) Expression Vector at a ratio of 0.25:0.25:9.5. Cells expressing CRAF-SmBiT(q), CRAF-LgBiT and KRAS(G12C) proteins were combined with various NanoBRET® Tracer K-4 concentrations. Cells were incubated in the presence or absence of a saturating dose of TAK-632 (**Panel B**) or serially diluted TAK-632 (**Panel C)** for 2 hours before addition of 3X Complete Substrate plus Inhibitor Solution and subsequent BRET measurements on a GloMax® Discover System equipped with 450/80BP and 610/LP filters. The recommended NanoBRET® Tracer K-4 concentration for the CRAF homodimer is depicted in orange in **Panel C**. An example compound profiling experiment at the recommended tracer concentration is shown in **Panel D**.

7. Analysis of Compound Selectivity Across RAF Dimer Variants

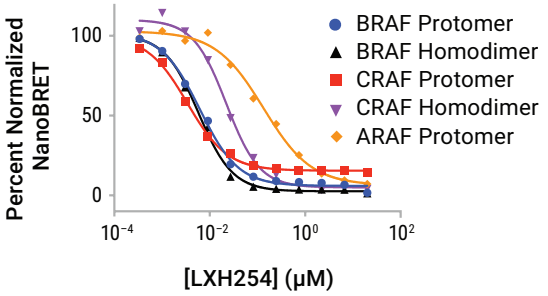
You may wish to gain an understanding of both the affinity of a test compound against a specific RAF protomer or RAF homodimer, and the potential engagement of the compound across other RAF configurations. The NanoBRET® TE Intracellular RAF Dimer Assays can be used to evaluate test compound selectivity across multiple RAF configurations using a similar workflow and up to two different NanoBRET® tracers. In Figure 9, examples of selectivity profiles for various known RAF inhibitors across a panel of NanoBRET® TE Intracellular RAF Dimer Assays are shown. Representative data for each RAF protomer or RAF homodimer can be found in Sections 5 and 6.

Both RAF dimer inhibitors TAK-632 and LXH254 are capable of inhibiting the BRET signal from all RAF homo- or heterodimers with potencies in the nanomolar range. Both compounds preferentially interact with BRAF regardless of the RAF protomer partner, as well as the CRAF protomer when partnered with BRAF. Both compounds also show comparatively weaker binding to CRAF within homodimers as well as the ARAF protomer when partnered with BRAF.

A.



B.



C.

Assay	TAK-632 IC_{50} (µM)	LXH254 IC_{50} (µM)
BRAF Protomer	0.015	0.0062
BRAF Homodimer	0.013	0.0058
CRAF Protomer	0.0048	0.0034
CRAF Homodimer	0.098	0.022
ARAF Protomer	0.081	0.13

18687MA

Figure 9. Examples of selectivity profiles for RAF ligands. Adherent HEK293 cells expressing various configurations of RAF protomers or RAF homodimers were treated with the recommended concentration of NanoBRET® Tracer K-4 or K-10 (see Figures 4–8). Cells were incubated with serially diluted TAK-632 (**Panel A**) or LXH254 (**Panel B**) for 2 hours before addition of 3X Complete Substrate plus Inhibitor and subsequent BRET measurements using a GloMax® Discover System. BRET values were normalized between the BRET of DMSO vehicle-treated samples ($BRET_{Max}$) and the BRET after competition with 20µM TAK-632 ($BRET_{Min}$) as described in Section 4.F. Potency values are provided in **Panel C**.

8. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com** E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
NanoBRET® signal without test compound is weak or close to instrument background	Tracer was adsorbed to plasticware surface. Use polypropylene materials and avoid conventional polystyrene materials when preparing or working with reagents containing NanoBRET® Tracer to minimize adsorption.
	Incorrect plate type was used. We recommend 96-well solid white flat-bottom polystyrene TC-treated microplates (Corning® Cat.# 3917 or Greiner Cat.# 655083). This assay may not work well with conventional polystyrene plates.
	The 3X Complete Substrate plus Inhibitor Solution was not used within 1.5 hours of preparation. Prepare fresh 3X Complete Substrate plus Inhibitor Solution for each experiment and use the solution within 1.5 hours; discard any unused solution.
	NanoBRET® signal was not measured promptly after adding the 3X Complete Substrate plus Inhibitor Solution. For best results, measure BRET signal within 10 minutes of adding the solution. BRET signal can be measured for up to 2 hours after addition, but there will be some loss of signal.
	Instrument was set up incorrectly. Use the correct filters for the donor wavelength (450nm BP) and acceptor wavelength (600nm LP) on your instrument to accurately measure NanoBRET® signal. See Section 3.B for more information.
	Expression levels of the RAF-LgBiT and RAF-SmBiT(q) fusion proteins were poor due to low transfection efficiency of HEK293 cells. If you suspect expression was low due to poor transfection, measure the donor signal for cells expressing the fusion proteins and compare that to the background donor signal in nontransfected cells. If the signal-to-background ratio in the donor channel is less than 1,000, prepare a new population of HEK293 cells and carefully repeat the transfection as described in Section 4.B. If the signal-to-background ratio is still low after repeating the transfection, consider increasing the ratio of RAF-LgBiT and RAF-SmBiT(q) fusion vectors to KRAS(G12C) Expression Vector while keeping the total amount of DNA in the lipid:DNA complex constant in Section 4.B.

Symptoms	Causes and Comments
NanoBRET® signal without test compound is weak or close to instrument background (continued)	Expression levels of the RAF-LgBiT and RAF-SmBiT(q) fusion proteins were poor in cell types other than HEK293 due to low transfection efficiency. If you suspect expression was low due to poor transfection, measure the donor signal for cells expressing the fusion proteins and compare that to the background donor signal in the absence of cells expressing the fusion proteins. If the signal-to-background ratio in the donor channel is less than 1,000, consider optimizing transfection reagent and conditions for your cell type. The optimal transfection reagent and conditions can vary greatly depending on the cell type.
	Expression levels of the RAF-LgBiT and RAF-SmBiT(q) fusion proteins were poor in cell types other than HEK293 due to poor expression from the CMV promoter. The CMV promoter is a strong promoter in most but not all cell lines. Check to be sure that the CMV promoter provides sufficient expression levels in your cell type.
Donor or acceptor signal changes when tracer is added	This phenomenon is common but generally does not affect the assay. Figure 10 demonstrates representative data showing raw luminescence from donor (450nm) and acceptor (600nm) channels when NanoBRET® tracer is titrated. BRET that occurs between the RAF-LgBiT and RAF-SmBiT(q) fusion proteins and fluorescent tracer may result in a dose-dependent increase in acceptor signal with a corresponding decrease in donor signal. Ratiometric BRET analysis mitigates the effect of fluctuations in raw luminescence from active luciferase.
High variability in NanoBRET® signal	Cell handling is very important for reproducible assay results. To reduce variability, be sure that HEK293 cells transfected in Section 4.B are recently passaged (within 1–2 days) and at 70–90% confluency. Use single-cell suspensions of HEK293 cells for transfection in Section 4.B.
	Pipetting was inaccurate when preparing the Complete 20X NanoBRET® tracer reagent in Section 4.C. The Tracer Dilution Buffer is viscous and can be difficult to pipet accurately. Be sure to pipet the Tracer Dilution Buffer and Complete 20X NanoBRET® tracer reagent slowly to ensure the proper volume is dispensed.

8. Troubleshooting (continued)

Symptoms	Causes and Comments
High variability in NanoBRET® signal (continued)	Mixing was insufficient, especially when preparing and adding the Complete 20X NanoBRET® tracer reagent in Section 4.C. The Tracer Dilution Buffer is viscous, making it difficult to achieve complete mixing. Visually inspect the assay wells after adding the Complete 20X NanoBRET® tracer reagent to cells to ensure that well contents are homogeneous. Perform additional mixing if necessary.
	Donor luminescence was low due to poor expression of the RAF-LgBiT and RAF-SmBiT(q) fusion proteins. This can result in noisy BRET data due to a reduced signal-to-background ratio in both the donor and acceptor channels.
Increased NanoBRET® signal after adding test compound	Test compound is cytotoxic. Cytotoxic compounds that cause cell lysis often demonstrate an artificial increase in BRET signal (Figure 11). This is caused by quenching of luminescence by the Extracellular NanoLuc® Inhibitor, which may result in a net increase in the BRET ratio. Evaluate your test compound for cytotoxic effects at the concentration that caused the increase in the BRET ratio.
	Test compound is colored or fluorescent. Compounds of this nature can be difficult to characterized using the NanoBRET® TE Intracellular RAF Protomer and RAF Homodimer Assays due to interference with the BRET signal.
High background signal	The 3X Complete Substrate plus Inhibitor Solution did not contain the Extracellular NanoLuc® Inhibitor. The Extracellular NanoLuc® Inhibitor mitigates signal that arises from NanoBiT® luciferase released from cells compromised during handling. BRET signal measured in the absence of this inhibitor includes luminescence by both intracellular and extracellular NanoBiT® luciferase, resulting in higher background.

In Figure 10, example data are provided demonstrating possible effects on the raw donor or acceptor signals during the normal execution of the assay. In Figure 11, example data demonstrating the possible effects of a cytotoxic compound on BRET are provided.

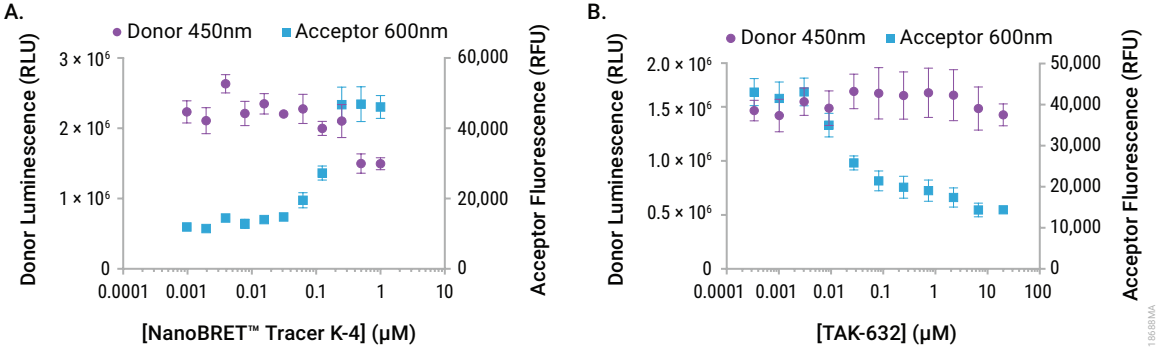


Figure 10. Potential effects of tracer and test compound concentrations on raw donor or acceptor emission in the NanoBRET® TE Intracellular RAF Dimer Assay. HEK293 cells expressing BRAF-SmBiT(q), CRAF(A373F)-LgBiT and KRAS(G12C) were resuspended in assay medium and seeded into 96-well plates. **Panel A.** Cells were incubated with varying NanoBRET® Tracer K-4 concentrations (0.0001–1µM) for 2 hours before addition of 3X Complete Substrate plus Inhibitor Solution and subsequent BRET measurements using a GloMax® Discover System. **Panel B.** Cells were incubated in the presence of 0.5µM NanoBRET® Tracer K-4 and a dilution series of TAK-632 (0.0003–20µM) for 2 hours before addition of 3X Complete Substrate plus Inhibitor Solution and subsequent BRET measurements using a GloMax® Discover System.

8. Troubleshooting (continued)

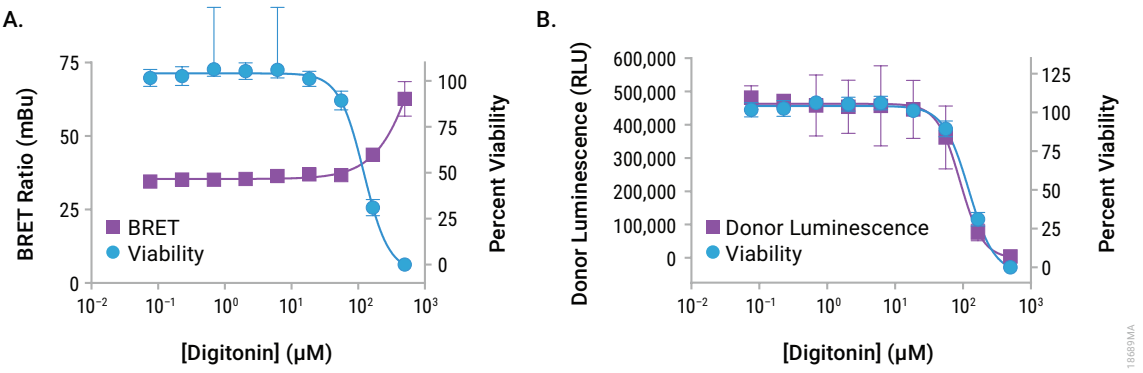


Figure 11. Effect of a cytotoxic compound on BRET signal and donor luminescence in the NanoBRET® TE Intracellular RAF Dimer Assay. Adhered HEK293 cells expressing BRAF-SmBiT(q), CRAF(A373F)-LgBiT and KRAS(G12C) were treated with digitonin, an acutely cytotoxic compound that disrupts membrane integrity. For BRET and donor luminescence measurements, NanoBRET® Tracer K-4 was added to a final concentration of 0.5µM, and digitonin was added as a threefold dilution series (0.076–500µM). Cells were incubated for 2 hours before addition of 3X Complete Substrate plus Inhibitor Solution and subsequent measurement using the GloMax® Discover System. For cell viability measurements, nontransfected HEK293 cells were treated with an identical dilution series of digitonin for 2 hours, after which viability was measured using the CellTiter-Glo® Luminescent Cell Viability Assay and a GloMax® Discover System. **Panel A.** Digitonin causes a distinct increase in BRET signal over the same concentration range that reduces cell viability. **Panel B.** Cell lysis due to digitonin treatment causes a decrease in donor luminescence due to quenching of the active luciferase by the Extracellular NanoLuc® Inhibitor.

9. Appendix

9.A. Additional Information about NanoBRET® Technology and NanoBiT® Fusion Proteins

Donor luminescence for the NanoBRET® TE Intracellular RAF Dimer Assays is generated by a functional luciferase enzyme formed by complementation of the LgBiT subunit and SmBiT(q) peptide. Interaction of these subunits produces a functional active luciferase, which demonstrates luminescence properties similar to those of intact NanoLuc® luciferase. The NanoBRET® TE Intracellular RAF Dimer Assays use a blue-shifted active luciferase enzyme as the BRET donor and a red-shifted fluorescent tracer as the BRET acceptor to minimize spectral overlap, resulting in a higher signal-to-background ratio.

When the SmBiT(q) peptide is used to complement the LgBiT subunit, the resulting active luciferase is sensitive to inhibition by the Extracellular NanoLuc® Inhibitor, which mitigates any NanoBRET® signal that may arise from extracellular debris and ensures accurate assessment of intracellular target engagement. Though the SmBiT(q) peptide provides sensitivity to the Extracellular NanoLuc® Inhibitor and enables a truly live-cell RAF assay, SmBiT(q) has not been broadly tested for measuring protein:protein interactions as the conventional SmBiT peptide and is not recommended as a general replacement for the SmBiT peptide.

9.B. Achieving Adequate Plate Mixing

Due to the viscosity of the Tracer Dilution Buffer, special attention must be given to plate mixing to ensure that the NanoBRET® Tracer is adequately dispersed when preparing the Complete 20X NanoBRET® Tracer Reagent and when adding the Complete 20X NanoBRET® Tracer Reagent to the assay plate. Creating a sufficient mixing vortex requires that the orbit of the mixer is smaller in diameter than the inside diameter of each well. For 96-well plate formats, most commercially available orbital shakers are capable of dispersing the tracer reagent, though optimization of the shaking force may be required (typically by visual inspection). If you want to attempt to perform the assay in a 384-well assay format, we recommend using specific mixing equipment that creates a mixing vortex in the wells of 384-well plates. We have experience with horizontal shakers such as the VibraTranslator™ line of products from Union Scientific. Consult your mixing apparatus manufacturer to determine the suitability of your plate mixer for 384-well applications that use viscous reagents.

9.C. Extinction Coefficient of the NanoBRET® Tracers

The NanoBRET® Tracer K-4 and NanoBRET® Tracer K-10 use the NanoBRET® 590 fluorophore. The concentrations of NanoBRET® Tracers K-4 and K-10 were determined using an extinction coefficient of 83,000$M^{-1}cm^{-1}$ at 590nm. See reference 4 for more information.

9.D. Composition of Buffers and Solutions

assay medium

99%	Opti-MEM™ I reduced serum medium without phenol red
1%	fetal bovine serum

cell culture medium

90%	DMEM
10%	fetal bovine serum

Promega

9.E. References

1. Robers, M.B. *et al.* (2015) Target engagement and drug residence time can be observed in living cells with BRET. *Nat. Commun.* **6**, 10091.
2. Hu, J. *et al.* (2011) Mutation that blocks ATP binding creates a pseudokinase stabilizing the scaffolding function of kinase suppressor of RAS, CRAF and BRAF. *Proc. Natl. Acad. Sci. USA.* **108**, 6067–72.
3. Robers, M.B. *et al.* (2019) Quantitative, real-time measurements of intracellular target engagement using energy transfer. In: *Systems Chemical Biology. Methods in Molecular Biology*, Ziegler, S., Waldmann, H. eds. Humana Press, New York, NY. **1888**, 45–71.
4. In: *Anthropological Genetics: Theory, Methods and Applications* (2006). Crawford, M.H. ed. University of Cambridge Press. Chapter 10, Table 10.1.

9.F. Related Products

NanoBRET® TE RAF Fusion and Expression Vectors

Product	Size	Cat.#
ARAF-SmBiT(q) Fusion Vector	20µg	NV4861
BRAF-SmBiT(q) Fusion Vector	20µg	NV4871
BRAF-LgBiT Fusion Vector	20µg	NV4881
CRAF-SmBiT(q) Fusion Vector	20µg	NV4891
CRAF-LgBiT Fusion Vector	20µg	NV4901
BRAF(A481F)-LgBiT Fusion Vector	20µg	NV4911
BRAF(A481F)-SmBiT(q) Fusion Vector	20µg	NV4921
CRAF(A373F)-LgBiT Fusion Vector	20µg	NV4931
KRAS(G12C) Expression Vector	20µg	NV4941

NanoBRET® Tracers

Product	Size	Cat.#
NanoBRET® Tracer K-4, 400µM	300µl	N2492
NanoBRET® Tracer K-10, 400µM	300µl	N2642

NanoBRET® Target Engagement Assay Reagent

Product	Size	Cat.#
Tracer Dilution Buffer	50ml	N2191

NanoBRET® TE Intracellular Kinase Assays

Product	Size	Cat.#
NanoBRET® TE Intracellular Kinase Assay K-3	100 assays	N2600
NanoBRET® TE Intracellular Kinase Assay K-4	100 assays	N2520
NanoBRET® TE Intracellular Kinase Assay K-5	100 assays	N2500
NanoBRET® TE Intracellular Kinase Assay K-8	100 assays	N2620
NanoBRET® TE Intracellular Kinase Assay K-9	100 assays	N2630
NanoBRET® TE Intracellular Kinase Assay K-10	100 assays	N2640
NanoBRET® TE Intracellular Kinase Assay K-11	100 assays	N2650

Additional assay sizes are available.

Intracellular TE Nano-Glo® Substrate/Inhibitors

Product	Size	Cat.#
Intracellular TE Nano-Glo® Substrate/Inhibitor	100 assays	N2162
Intracellular TE Nano-Glo® Vivazine™/Inhibitor	1,000 assays	N2200

Additional sizes are available.

Transfection Reagent

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

Additional sizes are available.

Detection Instrument

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

For Research Use Only. Not For Use in Diagnostic Procedures.

10. Summary of Changes

The following changes were made to the 4/24 revision of this document:

1. In Section 4.C, Step 1, Note b. “heterodimer” was changed to “homodimer”.
2. NanoBRET was updated to a registered trademark.

Promega

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[d]U.S. Pat. Nos. 9,797,889, 9,797,890 and 10,648,971, Japanese Pat. Nos. 6654557 and 7280842 and other patents and patents pending.

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

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

[g]Patent Pending.

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