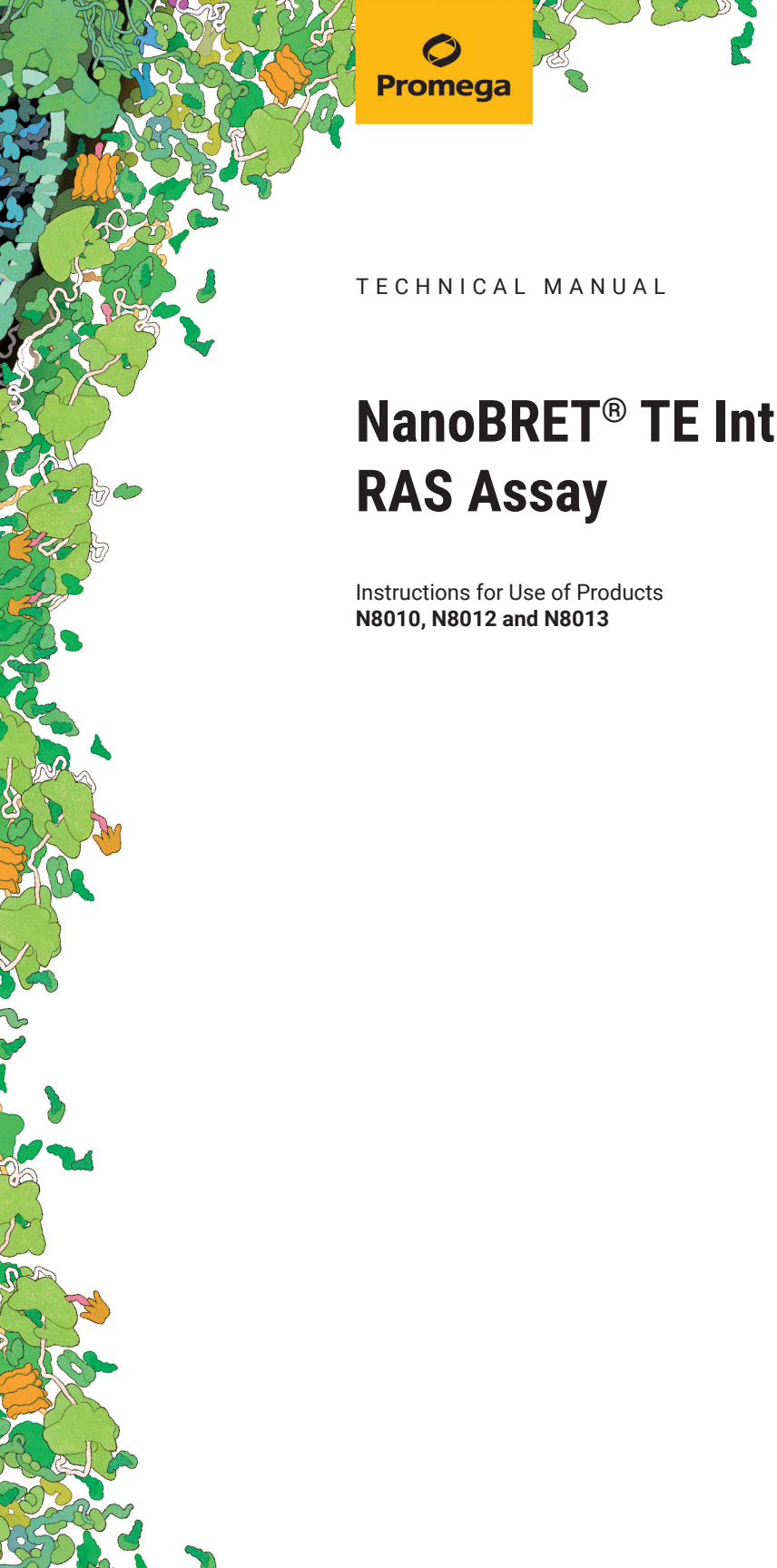

TECHNICAL MANUAL

NanoBRET® TE Intracellular RAS Assay

Instructions for Use of Products
N8010, N8012 and N8013

Revised 4/24
TM717

Promega

NanoBRET® TE Intracellular RAS 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

NanoBRET® Target Engagement (TE) Assays measure compound binding to select target proteins within intact cells and have been applied successfully to study multiple target classes (1). Here, we describe the NanoBRET® TE Intracellular RAS Assay[a–g], which measures compound binding to two major RAS family GTPases: KRAS and HRAS.

RAS proteins are small GTPases that act as biomolecular switches to regulate a number of intracellular activities including cell proliferation and survival. RAS proteins are frequently mutated in human cancers, making them important targets in drug discovery. The NanoBRET® TE Intracellular RAS Assay measures compound binding to a selected RAS target protein in the presence of cellular factors that affect target engagement potency. Specifically, the assay can quantify apparent intracellular affinity of a test compound for full-length multimeric RAS proteins inside living cells and can be used to study many RAS variants, such as KRAS, HRAS and related mutants.

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 close 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 RAS Assay uses the 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 RAS proteins in multimeric complexes can be evaluated. Specifically, a fusion protein of LgBiT and full-length RAS is co-expressed with a fusion protein of SmBiT(q) and full-length RAS in cells. When the RAS proteins interact and form multimers, 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 RAS. The BRET reporter complex is formed when the tracer binds to RAS protomers within the complex. Target engagement of unlabeled test compounds is measured as a loss of BRET signal when the tracer and test compound compete for RAS binding.

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

The NanoBRET® TE Intracellular RAS Assay can be used to study many RAS variants, including wildtype KRAS and HRAS as well as many clinically relevant KRAS and HRAS mutants. See Table 1 for a list of fusion vectors that can be used with this assay or see Section 8.F, Related Products, for a complete list of vectors and detection reagents. The assay can detect binding of both switch I/II pocket and switch II pocket inhibitors. Fusion vectors are available for the KRAS(Y96D) mutant to evaluate test compound susceptibility to a common resistance mutation for switch II pocket inhibitors.

Moreover, use of the NanoBRET® Tracer RAS at a single concentration for all RAS variants provides a simple workflow to profile test compound selectivity against specific RAS variants of interest.

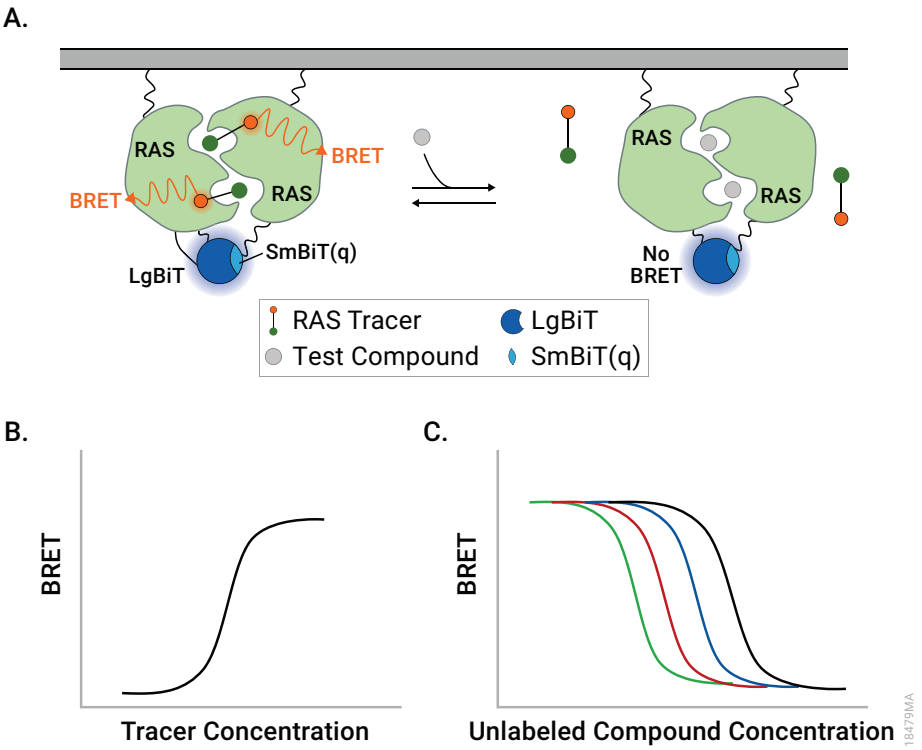

Figure 1. Overview of the NanoBRET® TE Intracellular RAS Assay. Panel A. LgBiT and SmBiT(q) fusion proteins with full-length RAS are co-expressed in live cells. Multimerization of these fusion proteins results in complementation of the SmBiT(q) and LgBiT subunits to produce functional NanoBiT® luciferase, which serves as the luminescence donor for BRET in the presence of the luciferase substrate. The cell-permeable NanoBRET® Tracer RAS binds reversibly to RAS multimers and serves as the luminescence acceptor. Together, the LgBiT and SmBiT(q) fusion proteins and fluorescent tracer create the BRET reporter complex for target engagement of the RAS multimer. Adding cell-permeable RAS ligands displace the tracer and cause a dose-dependent loss of BRET signal. **Panel B.** Tracer affinity for each RAS variant was determined by generating a NanoBRET® tracer dose response curve. The resulting curves were used to select the tracer concentration for this protocol, which is 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 concentrations of test compound in the presence of this fixed tracer concentration. As the concentration of a compound that binds to RAS increases, BRET signal decreases. Representative tracer dose response curves and compound inhibition curves for each RAS assay (Table 1) can be found in application notes (3).

1. Description (continued)

This technical manual describes the nonbinding-surface (NBS) format used by the NanoBRET® TE Intracellular RAS Assay. This assay format uses freshly harvested or suspension cells in an NBS assay plate for optimal performance of the tracer. A summary of the NanoBRET® TE Intracellular RAS Assay workflow is provided in Figure 2.

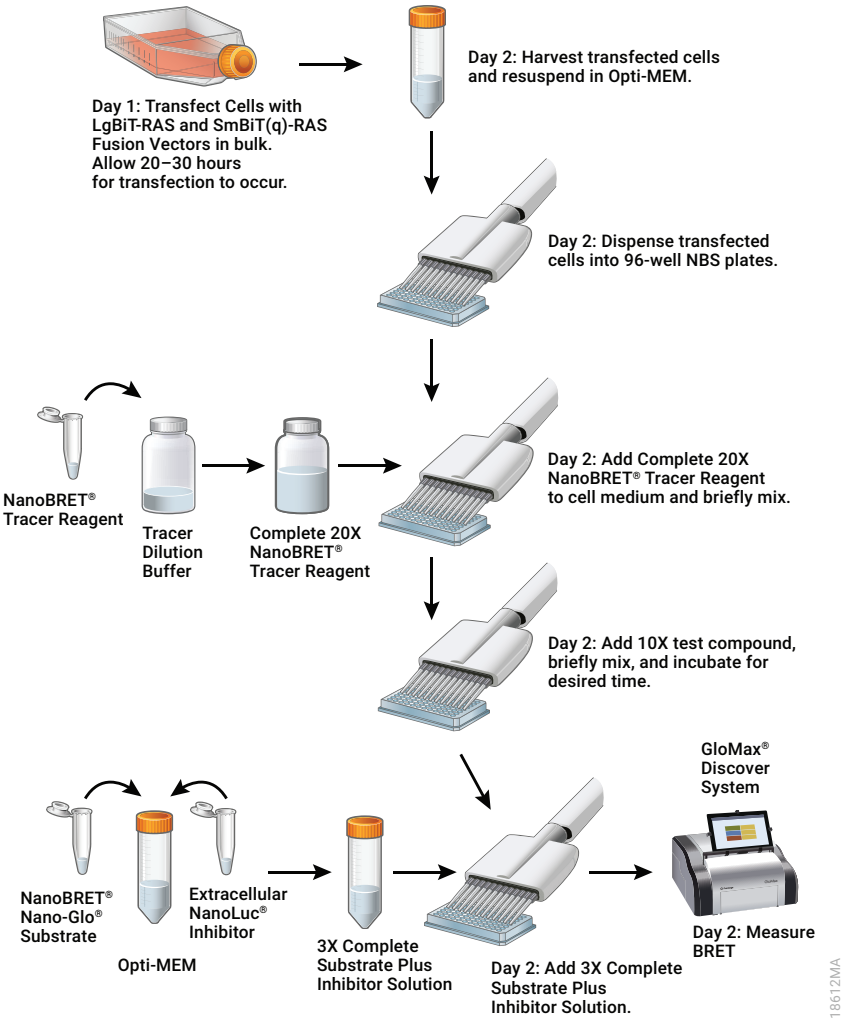

Figure 2. Overview of the NanoBRET® TE Intracellular RAS Assay. This assay must be performed in nonbinding-surface (NBS) plates using the assay format shown here.

Table 1. KRAS Fusion Vectors and Related Assays.

RAS Variant	Fusion Vector	Cat.#
KRAS WT	LgBiT-KRAS WT Fusion Vector	NV4561
	SmBiT(q)-KRAS WT Fusion Vector	NV4571
KRAS(G12C)	LgBiT-KRAS(G12C) Fusion Vector	NV4581
	SmBiT(q)-KRAS(G12C) Fusion Vector	NV4591
KRAS(G12D)	LgBiT-KRAS(G12D) Fusion Vector	NV4601
	SmBiT(q)-KRAS(G12D) Fusion Vector	NV4611
KRAS(G12V)	LgBiT-KRAS(G12V) Fusion Vector	NV4621
	SmBiT(q)-KRAS(G12V) Fusion Vector	NV4631
KRAS(G12A)	LgBiT-KRAS(G12A) Fusion Vector	NV4641
	SmBiT(q)-KRAS(G12A) Fusion Vector	NV4651
KRAS(G12S)	LgBiT-KRAS(G12S) Fusion Vector	NV4661
	SmBiT(q)-KRAS(G12S) Fusion Vector	NV4671
KRAS(G12R)	LgBiT-KRAS(G12R) Fusion Vector	NV4681
	SmBiT(q)-KRAS(G12R) Fusion Vector	NV4691
KRAS(G13D)	LgBiT-KRAS(G13D) Fusion Vector	NV4701
	SmBiT(q)-KRAS(G13D) Fusion Vector	NV4711
KRAS(Q61H)	LgBiT-KRAS(Q61H) Fusion Vector	NV4721
	SmBiT(q)-KRAS(Q61H) Fusion Vector	NV4731
KRAS(Q61L)	LgBiT-KRAS(Q61L) Fusion Vector	NV4741
	SmBiT(q)-KRAS(Q61L) Fusion Vector	NV4751
KRAS(Q61R)	LgBiT-KRAS(Q61R) Fusion Vector	NV4761
	SmBiT(q)-KRAS(Q61R) Fusion Vector	NV4771
KRAS(Y96D)	LgBiT-KRAS(Y96D) Fusion Vector	NV4781
	SmBiT(q)-KRAS(Y96D) Fusion Vector	NV4791
HRAS WT	LgBiT-HRAS WT Fusion Vector	NV4801
	SmBiT(q)-HRAS WT Fusion Vector	NV4811
HRAS(G12C)	LgBiT-HRAS(G12C) Fusion Vector	NV4821
	SmBiT(q)-HRAS(G12C) Fusion Vector	NV4831
HRAS(G12V)	LgBiT-HRAS(G12V) Fusion Vector	NV4841
	SmBiT(q)-HRAS(G12V) Fusion Vector	NV4851

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
NanoBRET® TE Intracellular RAS Assay	**100 assays**	**N8010**

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

- 5µg LgBiT-KRAS(G12C) Fusion Vector
- 5µg SmBiT(q)-KRAS(G12C) Fusion Vector
- 50µl NanoBRET® Tracer RAS
- 5ml Tracer Dilution Buffer
- 50µl NanoBRET® Nano-Glo® Substrate
- 17µl Extracellular NanoLuc® Inhibitor
- 20µg Transfection Carrier DNA

PRODUCT	SIZE	CAT.#
NanoBRET® TE Intracellular RAS Assay	**1,000 assays**	**N8012**

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

- 5µg LgBiT-KRAS(G12C) Fusion Vector
- 5µg SmBiT(q)-KRAS(G12C) Fusion Vector
- 500µl NanoBRET® Tracer RAS
- 5ml Tracer Dilution Buffer
- 330µl NanoBRET® Nano-Glo® Substrate
- 110µl Extracellular NanoLuc® Inhibitor
- 100µg Transfection Carrier DNA

PRODUCT	SIZE	CAT.#
NanoBRET® TE Intracellular RAS Detection Reagents	**10,000 assays**	**N8013**

Provides sufficient reagents for 10,000 assays (96-well). Includes:

- 10 × 500µl NanoBRET® Tracer RAS
- 50ml Tracer Dilution Buffer
- 3.3ml NanoBRET® Nano-Glo® Substrate
- 1.1ml Extracellular NanoLuc® Inhibitor

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

Note: The NanoBRET® TE Intracellular RAS Assay requires transfection of cells with two fusion vectors: One vector that encodes the LgBiT fusion protein and one vector that encodes the SmBiT(q) fusion protein. You may need to purchase both of the LgBiT and SmBiT(q) fusion vectors for your RAS variant of interest. See Table 1 or Section 8.F. Related Products, for a list of RAS fusion vectors.

3. Before You Begin

3.A. About the RAS Fusion Vectors

Table 1 lists a number of RAS variants (fusion vectors) available for use with this technical manual. For each RAS variant, a LgBiT-RAS and SmBiT(q)-RAS fusion vector is used with Transfection Carrier DNA to prepare the transfection complexes.

In the 100 and 1,000 kit sizes (Cat.# N8010, N8012) control vectors are supplied for the KRAS(G12C) assay. Specifically, these vectors are LgBiT-KRAS(G12C) Fusion Vector, SmBiT(q)-KRAS(G12C) Fusion Vector and Transfection Carrier DNA. Figures 3 and 4 contain data using these controls. Data for additional RAS variants used with the NanoBRET® TE Intracellular RAS Assay can be found in application notes (3). These application notes are also available on the LgBiT- and SmBiT(q)-RAS Fusion Vector product web page.

3.B. Propagating and Preparing LgBiT-RAS and SmBiT(q)-RAS Fusion Vectors

The amount of RAS fusion vector provided in each vial 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 vector transformation into *E. coli* for archival storage and cell-culture grade vector preparation.

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

For questions about vector propagation, contact Promega Technical Services: **techserv@promega.com**

3.C. Instrument Requirements and Setup

To perform the NanoBRET® TE Intracellular RAS 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, the 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 range of 410–490nm.

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 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 RAS occurs in the range of 590–610nm. 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.

3.C. Instrument Requirements and Setup (continued)

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: 6.5mm
- Measurement time: 1 second

4. NanoBRET® TE Intracellular RAS Assay Protocol

The results of the NanoBRET® TE Intracellular RAS Assay 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, 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 RAS fusion vectors. This control represents the background donor signal, which can be used to assess transfection success as described in Section 7.

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 RAS ligand, such as 20µM BI-2852. The average BRET ratio for this control represents $BRET_{Min}$ when calculating normalized BRET. Also serves as a positive control for RAS 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

- HEK293 or other cultured mammalian cells (see Note a. below)
- Dulbecco's Modified Eagle 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 needed to dilute DNA)
- LgBiT-RAS Fusion Vector (see Note b. below)
- SmBiT(q)-RAS Fusion Vector (see Note b. below)
- BI-2852 (positive control RAS ligand, MedChemExpress Cat.# HY-126247)
- 96-well white flat-bottom polystyrene NBS microplate (Corning® Cat.# 3600) (see Note c. below)
- 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
- plate mixer capable of mixing viscous reagents (see Section 8.B for details)

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 RAS (2).

b. Performing the NanoBRET® TE Intracellular RAS Assay requires LgBiT and SmBiT(q) fusion vectors for the RAS variant of interest. If you are using a system that does not contain a fusion vector for your RAS variant, you will need to purchase the appropriate LgBiT and SmBiT(q) RAS fusion vectors separately; see Section 8.F, Related Products, for a list of available LgBiT and SmBiT(q) fusion vectors.

c. Be sure to use the Corning® 96-well white flat-bottom polystyrene NBS microplate (Corning® Cat.# 3600). The assay does not work as well in conventional polystyrene or cell culture-treated plates or with plates labeled "nonbinding" from other manufacturers.

4.B. Transient Transfection of HEK293 Cells with NanoBiT® Fusion Vectors (Day 1)

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

   Notes:

   a. The transfection conditions provided in this protocol are optimized for HEK293 cells. You may need to optimize transfection conditions if using other cell types.

   b. 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 passaged within 1–2 days).

   c. For each 96-well assay plate, we recommend preparing at least 2×10^6 cells.

   d. **Optional:** Prepare and reserve a population of cells that will not be transfected in Section 4.B. These cells will serve as the nontransfected-cell controls in Sections 4.C–4.E.

2. Remove cell culture medium from each 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), and centrifuge 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.

6. To transfect HEK293 cells, prepare the 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
Opti-MEM™ I Reduced Serum Medium, without phenol red	1ml	—
Transfection Carrier DNA	8µl	8.0µg/ml
LgBiT-RAS Fusion Vector[2]	1µl	1.0µg/ml
SmBiT(q)-RAS Fusion Vector[2]	1µl	1.0µg/ml

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

[2]The amount of each control fusion vector provided with the assay 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.

(Step 6 continued, next page.)

b. For each DNA mixture prepared, add 30µl of FuGENE® HD Transfection Reagent per 1ml of DNA mixture to form the lipid:DNA complexes. 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 well.

Note: If preparing the transfection complex in a 1.5ml microcentrifuge tube, mix by inverting 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 6 (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 cells + lipid:DNA complex into a sterile cell culture flask, and incubate for at least 20 hours in a 37°C, 5% CO_2 incubator.

   Note: We recommend a cell density of approximately 55,000–80,000 cells/cm^2 during this step. For example, use approximately 4–6 × 10^6 cells for a T75 flask.

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

1. Remove the medium by aspiration from cell culture flask of transfected HEK293 cells, trypsinize cells and allow them to dissociate from the flask.

   Optional: To prepare the nontransfected-cell controls, aspirate the medium from the flask of nontransfected cells reserved in Section 4.B. Then trypsinize and allow cells to dissociate from the flask.

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

2. Neutralize trypsin by adding prewarmed cell culture medium containing serum (90% DMEM with 10% fetal bovine serum) to each flask. Transfer cell suspensions to a centrifuge tube and pellet cells by centrifuging at 200 × *g* for 5 minutes.

3. Aspirate medium and resuspend cells using prewarmed Opti-MEM™ I Reduced Serum Medium, without phenol red containing 1% fetal bovine serum.

4. Adjust the cell density to 2 × 10^5 cells/ml with prewarmed Opti-MEM™ I Reduced Serum Medium, without phenol red containing 1% fetal bovine serum.

5. Dispense 85µl of the transfected cell suspension into wells of a 96-well white flat-bottom polystyrene NBS microplate (Corning® Cat.# 3600) (see "Optional" points in Step 6).

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

6. **Optional:**
   a. Dispense 85µl of the nontransfected cell suspension into the nontransfected-cell control wells. Periodically mix cells during dispensing to prevent them from settling.
   b. To assemble the no-tracer controls, dispense 90µl of the transfected cell suspension into the no-tracer control wells of the 96-well assay plate.
   c. For the $BRET_{Min}$ controls, dispense 85µl of cell suspension into the $BRET_{Min}$ control wells of the 96-well assay plate.
   d. For the $BRET_{Max}$ controls, dispense 85µl of cell suspension into the $BRET_{Max}$ control wells of the 96-well assay plate.

   Note: Use only the Corning® 96-well white flat-bottom polystyrene NBS microplate (Corning® Cat.# 3600). The assay does not work well in conventional polystyrene or cell culture-treated plates or with "nonbinding" plates from other manufacturers.

7. Prepare the Complete 20X NanoBRET® Tracer RAS reagent using polypropylene tubes.

   Note: The tracer concentration used in this protocol (2µM) is optimized for HEK293 cells. You may need to determine the appropriate tracer concentration when using other cell types. The tracer concentration for other RAS variants can be found in application notes (3).

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

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

   b. Prepare a 100X solution of NanoBRET® Tracer RAS in 100% DMSO by combining equal volumes of 100% DMSO and NanoBRET® Tracer RAS; see example below. Mix well.

Component	Volume Per 96-Well Plate
NanoBRET® Tracer RAS	50µl
100% DMSO	50µl
Final Volume	**100µl**

   c. Prepare the Complete 20X NanoBRET® Tracer RAS reagent by slowly adding 4 parts of Tracer Dilution Buffer to 1 part of 100X NanoBRET® Tracer RAS prepared in Step 7.b; see example and Note below. Mix well.

Component	Volume Per 96-Well Plate
100X NanoBRET® Tracer RAS	100µl
Tracer Dilution Buffer	400µl
Final Volume	**500µl**

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

8. Dispense 5µl of Complete 20X NanoBRET® Tracer RAS reagent into each assay well. Do not add tracer to the no-tracer controls.

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

9. Mix the assay plate on an orbital shaker for 15 seconds at 900rpm. Visually inspect the wells to ensure that the Complete 20X NanoBRET® Tracer RAS is 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 shaker. See Section 8.B for details.

4.D. Adding Test Compounds

1. Prepare test compound at a 1,000X final concentration in 100% DMSO or appropriate test compound solvent.

   For the $BRET_{Min}$ controls or positive TE controls, prepare BI-2852 at a concentration of 20mM in 100% DMSO.

2. Dilute the 1,000X test compound to a final concentration of 10X in Opti-MEM™ I Reduced Serum Medium, without phenol red.

   For the $BRET_{Min}$ controls or positive TE controls, dilute the 20mM BI-2852 to a final concentration of 200µM in Opti-MEM™ I Reduced Serum Medium, without phenol red.

   For the $BRET_{Max}$ controls, prepare a 10X $BRET_{Max}$ control solution 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, medium or 10X $BRET_{Max}$ control to each well containing the samples and nontransfected-cell controls as described below:

   a. Add 10µl of 200µM BI-2852 to $BRET_{Min}$ controls or positive TE controls.

   b. Add 10µl of Opti-MEM™ I Reduced Serum Medium, without phenol red to no-tracer controls.

   c. Add 10µl of 10X $BRET_{Max}$ control solution to $BRET_{Max}$ controls.

4. Thoroughly mix plate contents on an orbital shaker for at 900rpm for 15 second after additions in Step 3. Incubate the assays 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. Irreversible inhibitors will show time-dependent inhibition, with increasing potency over time. BRET can be measured in as little as 30 minutes.

5. Allow plate to cool to room temperature (approximately 15 minutes), then proceed to the NanoBRET® Assay protocol in Section 4.E.

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4.E. NanoBRET® Assay Protocol

1. Thaw the Extracellular NanoLuc® Inhibitor and NanoBRET® Nano-Glo® Substrate.

   Note: After first use, store the Extracellular NanoLuc® Inhibitor and NanoBRET® Nano-Glo® Substrate at –30°C to –10°C, protected from light.

2. 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 preparing. Discard unused solution.

3. Mix by gently inverting the tube 5–10 times.

4. Add 50µl of 3X Complete Substrate plus Inhibitor Solution to each well of the assay plate, including the controls. Incubate for 2–3 minutes at room temperature.

   Note: Proceed to Step 5 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 will vary depending on the assay (see Section 7).

5. Measure donor emission using a 450nm BP filter and acceptor emission using a 600nm LP filter using the GloMax® Discover System or other NanoBRET® assay-compatible detection instrument, following the instrument 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.C 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 NanoBRET® 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.

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

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 comparisons of selectivity between RAS variants 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:

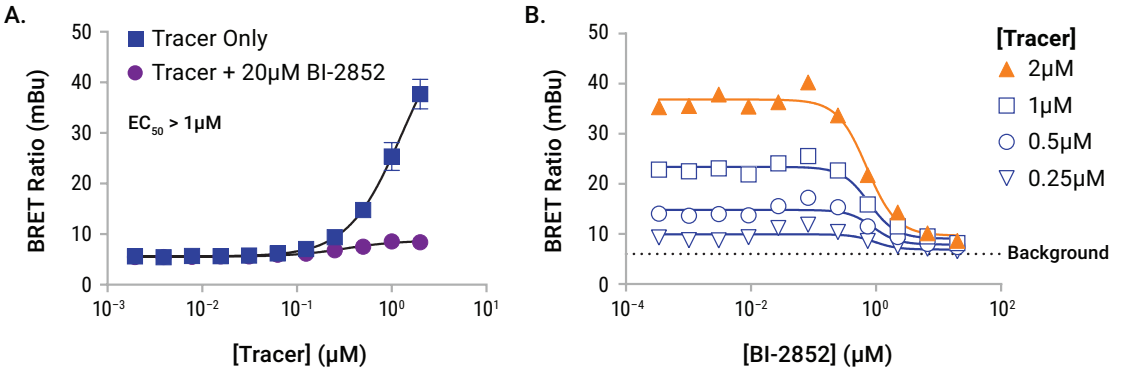
$\text{BRET}_{\text{sample}}$ is the BRET ratio for the sample;

BRET_{Min} is the average BRET ratio for controls that contain tracer and an excess concentration of a known RAS ligand, such as 20µM BI-2852. 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 BRET Data for the KRAS(G12C) Control Assay

Representative data for the KRAS(G12C) control assay are provided in Figure 3. The NanoBRET® Tracer RAS is nonsaturating up to the solubility limit of 2µM, as evident by the negligible effect of tracer concentration on apparent test compound potency (compare IC_{50} values in Panel B). Based on the data in Figure 3, we recommend a 2µM final concentration of NanoBRET® Tracer RAS.

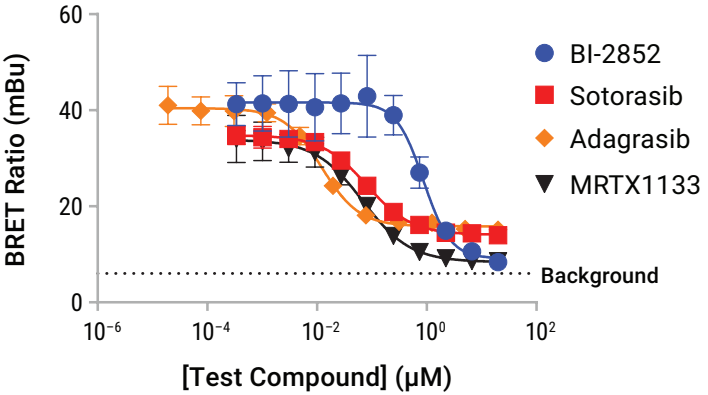


	2µM	1µM	0.5µM	0.25µM
IC_{50}	0.71	0.77	0.90	0.95

184801MA

Figure 3. Assay verification data for the NanoBRET® TE Intracellular RAS Assay. Panel A shows an example of tracer dose response curves in the presence and absence of a saturating concentration of a known RAS ligand. **Panel B** demonstrates how tracer concentration can affect apparent test compound potency. HEK293 cells expressing SmBiT(q)-KRAS(G12C) and LgBiT-KRAS(G12C) fusion proteins were resuspended in assay medium and seeded into 96-well nonbinding-surface plates. NanoBRET® Tracer RAS was added at various concentrations. Cells were incubated in the presence or absence of a saturating dose of the SI/II-pocket ligand BI-2852 (**Panel A**) or serially diluted BI-2852 (**Panel B**) for 2 hours before addition of 3X Complete Substrate Plus Inhibitor Solution and subsequent BRET measurement using the GloMax® Discover System. The recommended tracer concentration is depicted in orange in **Panel B**.

Representative compound selectivity profiling data are shown in Figure 4. Competition is observed for the SI/II pocket ligand BI-2852 and the SII-pocket ligands Sotorasib, Adagrasib and MRTX1133. Additional representative data and representative data for other RAS variants can be found in reference 3.



	BI-2852	Sotorasib	Adagrasib	MRTX1133
IC_{50}	0.87	0.080	0.012	0.068

18481MA

Figure 4. Representative compound selectivity profiling data for the KRAS(G12C) Control Assay. HEK293 cells expressing SmBiT(q)-KRAS(G12C) and LgBiT-KRAS(G12C) Fusion Vectors were resuspended in assay medium and seeded into 96-well nonbinding-surface plates. NanoBRET® Tracer RAS was added to a final concentration of 2µM. Cells were incubated with serially-diluted test compound for 2 hours before addition of 3X Complete Substrate Plus Inhibitor Solution and subsequent BRET measurement using the GloMax® Discover System.

6. Compound Selectivity Analysis Across RAS Variants

You may wish to understand not only the potency of a test compound against a specific RAS variant but also the potential engagement of the test compound across other RAS variants. The NanoBRET® TE Intracellular RAS Assay allows test compound selectivity evaluation across multiple RAS variants using the same workflow and a consistent tracer concentration of 2µM. Figure 5 shows examples of selectivity profiles for various known KRAS inhibitors across the entire suite of available RAS fusion vectors, with tabulated potency values for each ligand across the RAS variants in Table 2. Additional representative data as well as data for other RAS variants can be found in reference 3.

6. Compound Selectivity Analysis Across RAS Variants (continued)

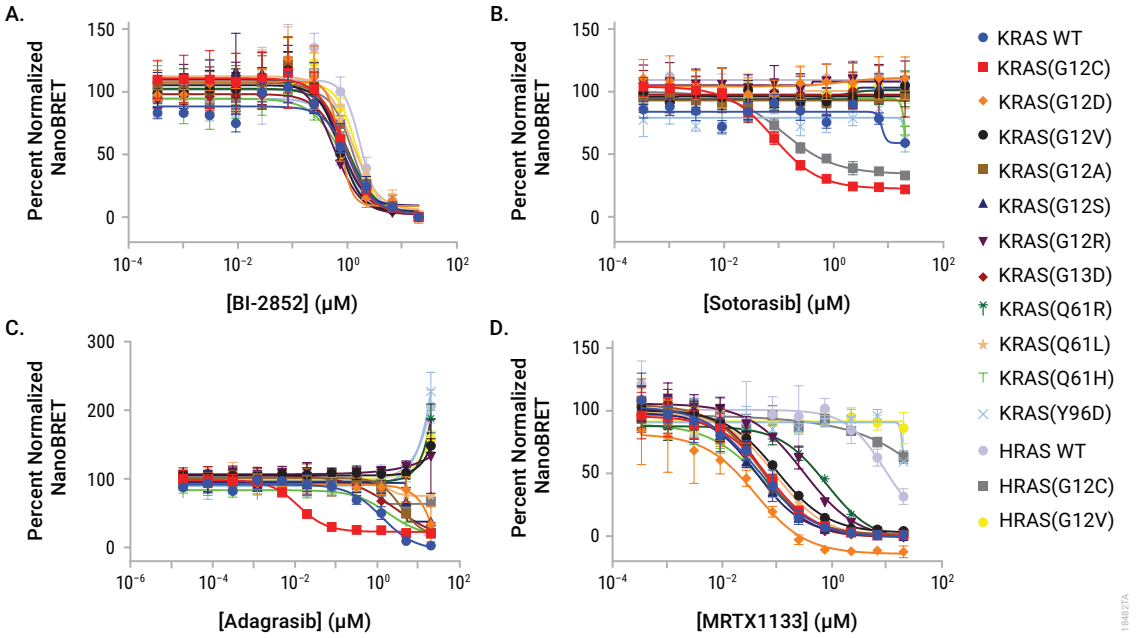


Figure 5. Selectivity profile examples for RAS ligands. HEK293 cells expressing SmBiT(q)-RAS and LgBiT-RAS fusion proteins were resuspended in assay medium and seeded into 96-well nonbinding-surface plates. NanoBRET® Tracer RAS was added to a final concentration of 2µM. Cells were incubated with serial dilutions of test compound for 2 hours before adding 3X Complete Substrate Plus Inhibitor and measuring BRET using the GloMax® Discover System. Normalized BRET was calculated as described in Section 4.F using the average BRET ratio of DMSO vehicle-treated cells as $BRET_{Max}$ and the average BRET ratio of cells treated with 20µM BI-2852 as $BRET_{Min}$. **Panel A.** The SI/II-pocket ligand BI-2852 demonstrates competition of BRET signal for all RAS variants with similar potency. Thus, BI-2852 serves as an ideal positive control for target engagement across the RAS variants. **Panel B.** The SII-pocket ligand Sotorasib demonstrates strong selectivity for G12C hotspot RAS mutants. **Panel C.** The SII-pocket ligand Adagrasib is selective for KRAS(G12C) but also demonstrates weak engagement of other KRAS variants. Note that Adagrasib demonstrates an increase in BRET at very high concentrations, which is likely associated with cell toxicity (see Section 7, Troubleshooting, for more information). **Panel D.** The SII-pocket ligand MRTX1133 potently engages KRAS(G12D) but also engages many KRAS variants.

Table 2. Tabulated Potency Values. Potency values are shown for each ligand across the RAS variants (see also Figure 5).

RAS Assay	BI-2852 IC_{50} (µM)	Sotorasib IC_{50} (µM)	Adagrasib IC_{50} (µM)	MRTX1133 IC_{50} (µM)
KRAS WT	1.1	>10	1.3	0.047
KRAS (G12C)	0.87	0.080	0.012	0.068
KRAS (G12D)	0.73	>10	>10	0.040
KRAS (G12V)	0.74	>10	>10	0.100
KRAS (G12A)	0.94	>10	3.8	0.051
KRAS (G12S)	0.73	>10	>10	0.043
KRAS (G12D)	0.55	>10	>10	0.300
KRAS (G13D)	0.86	>10	2.7	0.059
KRAS (Q61R)	1	>10	>10	0.78
KRAS (Q61L)	1.2	>10	>10	0.093
KRAS (Q61H)	0.84	>10	1.8	0.056
KRAS (Y96D)	0.8	>10	>10	>10
HRAS WT	1.7	>10	>10	>10
HRAS (G12C)	1.2	0.012	>10	>10
HRAS (G12V)	1.5	>10	>10	>10

7. Troubleshooting

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

Symptoms	Causes and Comments
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 the NanoBRET® Tracer RAS to minimize adsorption.
	Incorrect plate type was used. We strongly recommend the 96-well white flat-bottom polystyrene NBS microplate (Corning Cat.# 3600). The assay does not work as well with conventional polystyrene or cell culture-treated plates nor with plates labeled as "nonbinding" from other manufacturers.
	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 improperly. 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 LgBiT-RAS and SmBiT(q)-RAS 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 LgBiT-RAS and SmBiT(q)-RAS fusion vector to Transfection Carrier DNA 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 LgBiT-RAS and SmBiT(q)-RAS 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 LgBiT-RAS and SmBiT(q)-RAS 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. Make sure that the CMV promoter provides sufficient expression levels in your cell type.
Donor or acceptor luminescence changes when tracer is added	This phenomenon is common but generally does not affect the assay. Figure 6 shows representative raw luminescence data from donor (450nm) and acceptor (600nm) channels when NanoBRET® Tracer RAS is titrated. BRET that occurs between the LgBiT-RAS and SmBiT(q)-RAS 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 effects of fluctuations in raw luminescence from NanoBiT® 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 and the RAS target engagement assay in Sections 4.C–4.E.
	Pipetting was inaccurate when preparing the Complete 20X NanoBRET® Tracer RAS reagent in Section 4.C. The Tracer Dilution Buffer is viscous and can be difficult to accurately pipet. Be sure to pipet the Tracer Dilution Buffer and Complete 20X NanoBRET® RAS Tracer RAS Reagent slowly to ensure the proper volume is dispensed.

7. Troubleshooting (continued)

Symptoms	Causes and Comments
High variability in NanoBRET® signal (continued)	Mixing was insufficient, especially when preparing or adding the Complete 20X NanoBRET® Tracer RAS 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 RAS reagent to ensure that well contents are homogeneous. Perform additional mixing if needed.
	Donor luminescence was low due to poor expression of the LgBiT-RAS and SmBiT(q)-RAS 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 7). 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.
	The SI/II-pocket ligand BI-2852 may show a subtle increase in BRET ratio over certain concentration ranges before causing a reduction in BRET signal. The exact mechanism is not yet clear, but this could be due to cooperativity effects in binding the RAS dimers. Other related compounds with a similar binding mechanism may demonstrate a similar increase in BRET signal.
	Test compound is colored or fluorescent. Compounds of this nature can be difficult to characterize using the NanoBRET® TE Intracellular RAS Assay due to interference with the BRET signal.
Test compound shows saturable but incomplete reduction of the BRET signal compared to BRET signal in the presence of BI-2852.	Test compound is selective for only one state of the RAS protein. The NanoBRET® Tracer RAS can bind to both active and inactive states of the RAS protein. If the test compound binds only to the active or inactive state of the RAS protein, the compound may demonstrate incomplete competition with the tracer and a partial reduction of BRET signal compared to an SI/II-pocket ligand like BI-2852. This is common for certain switch-II pocket binders such as Sotorasib and Adagrasib and is clearly visible in the test compound dose response curves for the KRAS(G12C) Assay (see Figure 4 and reference 4 for details).

Figures 6 and 7 contain data to support Section 7, Troubleshooting (see details above). Figure 6 demonstrates the potential behavior of donor and acceptor signals during normal execution of the NanoBRET® Intracellular TE RAS Assay. Figure 7 shoes the potential effect of a cytotoxic compound on assay behavior.

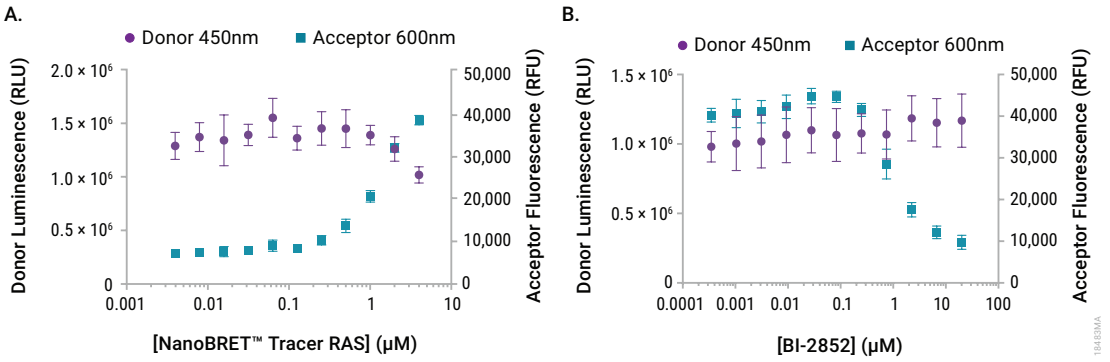


Figure 6. Potential effects of tracer and test compound concentrations on raw donor or acceptor emission in the NanoBRET® TE Intracellular RAS Assay. HEK293 NanoBRET® TE Intracellular RAS Assay. HEK293 cells expressing LgBiT-KRAS(G12C) and SmBiT(q)-KRAS(G12C) fusion proteins were resuspended in assay medium and seeded into 96-well nonbinding surface plates. **Panel A.** Cells were incubated with varying NanoBRET® Tracer RAS concentrations (0.002–2µM) for 2 hours before addition of 3X Complete Substrate Plus Inhibitor Solution and subsequent BRET measurement using the GloMax® Discover System. **Panel B.** Cells were incubated in the presence of 2µM NanoBRET® Tracer RAS and a dilution series of BI-2852 (0.0003–20µM) for 2 hours before addition of 3X Complete Substrate Plus Inhibitor Solution and subsequent BRET measurement using the GloMax® Discover System.

7. Troubleshooting (continued)

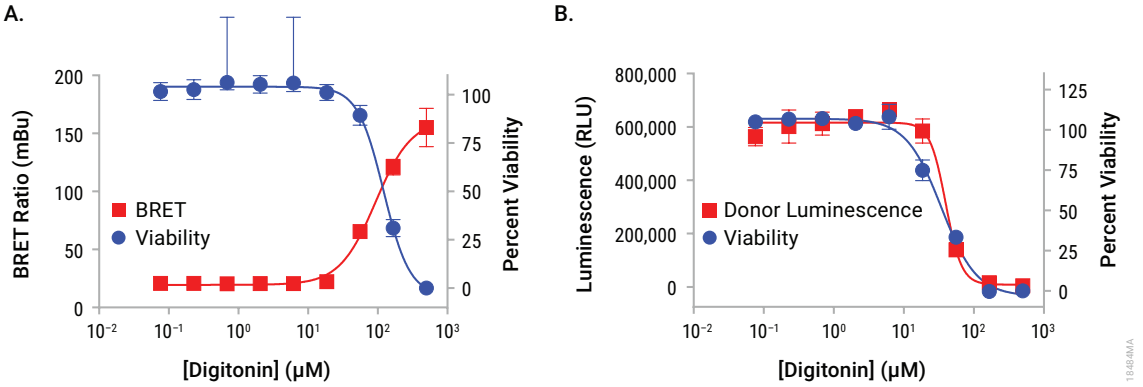


Figure 7. Effect of a cytotoxic compound on BRET signal and donor luminescence. HEK293 cells expressing LgBiT-KRAS(G12C) and SmBiT(q)-KRAS(G12C) fusion proteins were resuspended in assay medium and seeded into 96-well nonbinding surface plates. Digitonin was tested as an acutely cytotoxic compound that disrupts membrane integrity. For BRET and donor luminescence measurements, NanoBRET® Tracer RAS was added to a final concentration of 2µM, 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 on a GloMax® Discover System. **Panel A.** Digitonin causes a distinct increase in the 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 NanoBiT™ luciferase by the Extracellular NanoLuc® Inhibitor.

8. Appendix

8.A. Additional Information on NanoBRET® Technology and NanoBiT® Luciferase Fusion Proteins

Donor luminescence for the NanoBRET® TE Intracellular RAS Assay is generated by a functional luciferase enzyme formed by structural complementation of the LgBiT subunit and SmBiT(q) peptide. Interaction of these subunits produces a functional NanoBiT® luciferase, which demonstrates luminescence properties similar to those of intact NanoLuc® Luciferase. The NanoBRET® TE Intracellular RAS Assay uses a blue-shifted NanoBiT® 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 NanoBiT® 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 RAS assay, SmBiT(q) is not as suitable to measure protein:protein interactions as the conventional SmBiT peptide and should not be used as a general replacement for the SmBiT peptide.

8.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 RAS is adequately dispersed when preparing and adding the Complete 20X NanoBRET® Tracer RAS reagent. 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). Though this assay has not been optimized for a 384-well plate format, if you wish to use a 384-well plate, we recommend using 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 equipment manufacturer to determine if your plate mixer is suitable for 384-well applications that use viscous reagents.

8.C. Extinction Coefficient of the NanoBRET® Tracer RAS

The NanoBRET® Tracer RAS uses the NanoBRET® 590 fluorophore. The concentration of NanoBRET® Tracer RAS was determined using an extinction coefficient of 83,000$M^{-1}cm^{-1}$ at 590nm (5).

8.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

8.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. Robers, M.B. *et al.* (2019) Quantitative, real-time measurements of intracellular target engagement using energy transfer. *Methods Mol. Biol.* **1888,** 45–71.
3. Application Notes for the NanoBRET® TE Intracellular RAS Assay. Access online at: **www.promega.com/RASTEvectors**
4. Vasta, J.D. *et al.* (2022) KRAS is vulnerable to reversible switch-II pocket engagement in cells. *Nat. Chem. Biol.* **18**, 596–604.
5. Anthropological Genetics: Theory, Methods and Applications, Michael H. Crawford, *ed.* (2006) University of Cambridge Press, Table 10.1.

8.F. Related Products

NanoBRET® Fusion Vectors

Product	Size	Cat.#
LgBiT-KRAS WT Fusion Vector	20µg	NV4561
SmBiT(q)-KRAS WT Fusion Vector	20µg	NV4571
LgBiT-KRAS(G12C) Fusion Vector	20µg	NV4581
SmBiT(q)-KRAS(G12C) Fusion Vector	20µg	NV4591
LgBiT-KRAS(G12D) Fusion Vector	20µg	NV4601
SmBiT(q)-KRAS(G12D) Fusion Vector	20µg	NV4611
LgBiT-KRAS(G12V) Fusion Vector	20µg	NV4621
SmBiT(q)-KRAS(G12V) Fusion Vector	20µg	NV4631
LgBiT-KRAS(G12A) Fusion Vector	20µg	NV4641
SmBiT(q)-KRAS(G12A) Fusion Vector	20µg	NV4651
LgBiT-KRAS(G12S) Fusion Vector	20µg	NV4661
SmBiT(q)-KRAS(G12S) Fusion Vector	20µg	NV4671
LgBiT-KRAS(G12R) Fusion Vector	20µg	NV4681
SmBiT(q)-KRAS(G12R) Fusion Vector	20µg	NV4691
LgBiT-KRAS(G13D) Fusion Vector	20µg	NV4701
SmBiT(q)-KRAS(G13D) Fusion Vector	20µg	NV4711
LgBiT-KRAS(Q61H) Fusion Vector	20µg	NV4721
SmBiT(q)-KRAS(Q61H) Fusion Vector	20µg	NV4731
LgBiT-KRAS(Q61L) Fusion Vector	20µg	NV4741
SmBiT(q)-KRAS(Q61L) Fusion Vector	20µg	NV4751
LgBiT-KRAS(Q61R) Fusion Vector	20µg	NV4761
SmBiT(q)-KRAS(Q61R) Fusion Vector	20µg	NV4771
LgBiT-KRAS(Y96D) Fusion Vector	20µg	NV4781
SmBiT(q)-KRAS(Y96D) Fusion Vector	20µg	NV4791
LgBiT-HRAS WT Fusion Vector	20µg	NV4801
SmBiT(q)-HRAS WT Fusion Vector	20µg	NV4811
LgBiT-HRAS(G12C) Fusion Vector	20µg	NV4821
SmBiT(q)-HRAS(G12C) Fusion Vector	20µg	NV4831
LgBiT-HRAS(G12V) Fusion Vector	20µg	NV4841
SmBiT(q)-HRAS(G12V) Fusion Vector	20µg	NV4851

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8.F. Related Products (continued)

Intracellular TE Nano-Glo® Substrate/Inhibitors

Product	Size	Cat.#
Intracellular TE Nano-Glo® Substrate/Inhibitor	100 assays	N2162
	1,000 assays	N2160
	10,000 assays	N2161
Intracellular TE Nano-Glo® Vivazine™/Inhibitor	1,000 assays	N2200
	10,000 assays	N2201

NanoBRET® Target Engagement Assay Reagents

Product	Size	Cat.#
Tracer Dilution Buffer	50ml	N2191
Transfection Carrier DNA	5 × 20µg	E4881
	2 × 100µg	E4882

Transfection Reagents

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

Luminometers

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

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

9. Summary of Changes

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

1. In Section 4, the definition of $BRET_{Max}$ control was revised.
2. In Section 4.D, additional details were added to Step 2, and Step 3 and 3.C were revised.
3. NanoBRET was updated to a registered trademark.

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Researchers shall have no right to modify or otherwise create variations of the nucleotide sequences of the subunits of NanoBiT® Technology. No other use of this product or its derivatives is authorized without the prior express written consent of Promega.

In addition, researcher must:

(1a) use Nano-Glo®-branded luminescent assay reagents (LARs) for all determinations of luminescence activity of this product; or

(1b) contact Promega to obtain a license for use of the product with LARs not manufactured by Promega.

For uses of this product for energy transfer (such as bioluminescence resonance energy transfer), researcher must:

(2a) use NanoBRET®-branded luminescent assay reagents (LARs; e.g., NanoBRET® Nano-Glo® Substrate), Intracellular TE Nano-Glo® Substrate/Inhibitor, or Intracellular TE Nano-Glo® Vivazine™/Inhibitor for all determinations of luminescent activity; and

(2b) use NanoBRET®-branded energy acceptors (e.g., NanoBRET® tracers, NanoBRET® dyes, BRET-optimized HaloTag® ligands) for all determinations of energy transfer activity; or

(2c) contact Promega to obtain a license for use of the product for energy transfer assays using energy acceptors not manufactured by Promega. No license is needed if the energy transfer acceptor is a genetically encoded autofluorescent protein.

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[d]U.S. Pat. Nos. 9,797,889, 9,797,890, 10,107,800 and 11,493,504, European Pat. Nos. 2970412 and 3783011, Japanese Pat. No. 6654557, and other patents pending.

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

[f]U.S. Pat. Nos. 10,067,149 and 10,024,862, Japanese Pat. Nos. 6751294 and 7092691 and other patents and patents pending.

[g]Patent Pending.

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