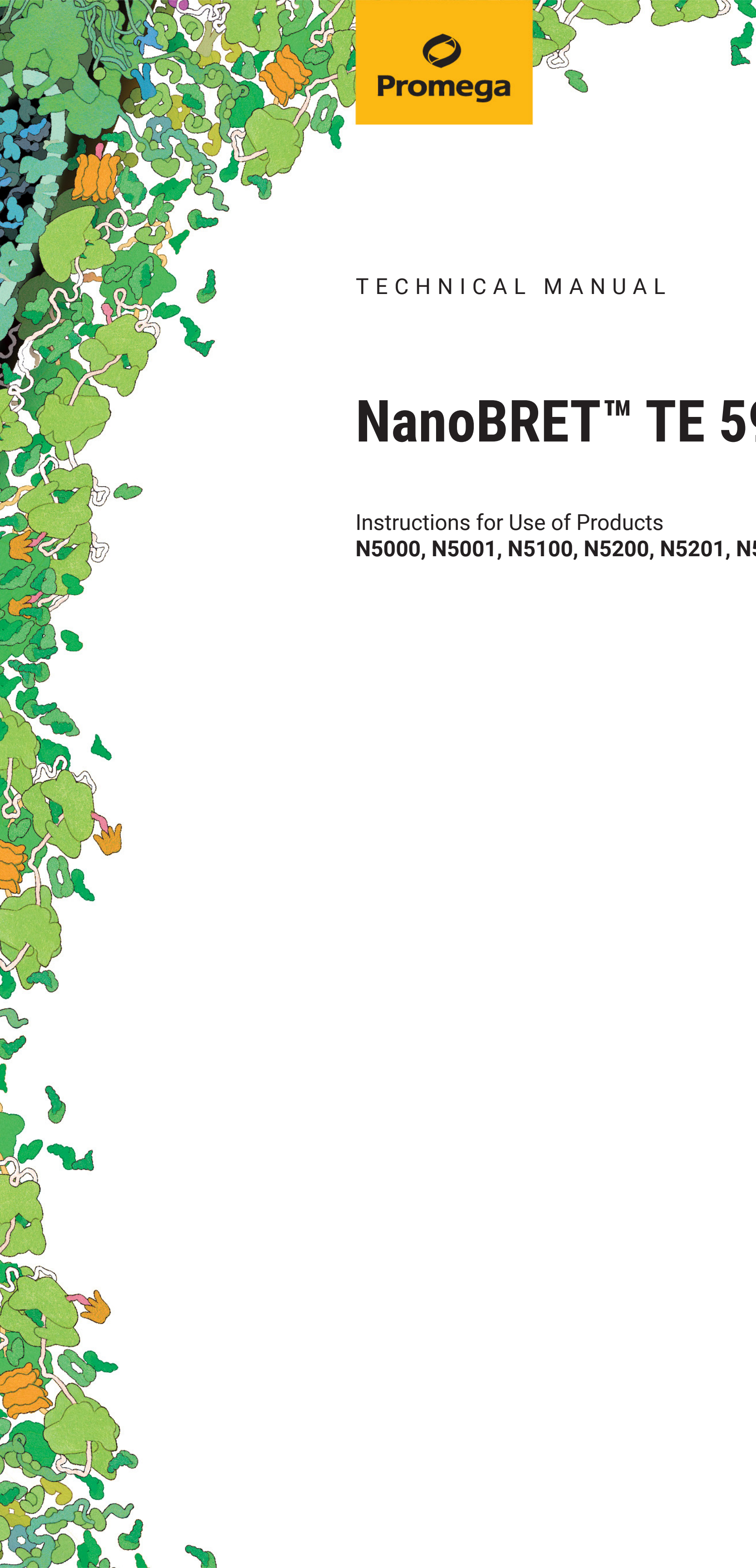

TECHNICAL MANUAL

NanoBRET™ TE 590 Dyes

Instructions for Use of Products
N5000, N5001, N5100, N5200, N5201, N5300 and N5301

7/23
TM697

NanoBRET™ TE 590 Dyes

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

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

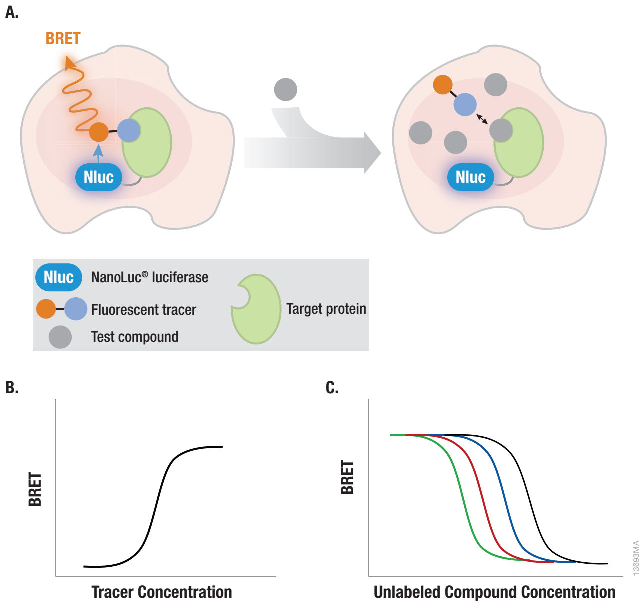

Figure 1. Illustration of the NanoBRET™ TE Assay. Panel A. Compound engagement is measured in a competitive format using a cell-permeable fluorescent NanoBRET™ tracer. Binding of the test compound results in a loss of NanoBRET™ signal between the target protein and the tracer inside intact cells. **Panel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

1. Description (continued)

The NanoBRET™ TE Assay uses four key components: 1) An expressed cellular target protein that is fused to the bright NanoLuc® luciferase; 2) a cell-permeable fluorescent tracer that specifically binds to the target protein; 3) a substrate for NanoLuc® luciferase; and 4) an extracellular inhibitor for NanoLuc® luciferase. Bioluminescence resonance energy transfer (BRET) is achieved by transferring the luminescent energy from NanoLuc® luciferase to the fluorescent tracer that is bound to the target-NanoLuc® fusion protein. Compounds that are applied to the cells and specifically engage the intracellular target-NanoLuc® fusion protein will result in a decrease in BRET. To ensure accurate assessment of intracellular target engagement, an extracellular NanoLuc® inhibitor is used to mitigate any NanoLuc® signal that may arise from cells compromised during handling, while not adversely affecting NanoLuc® luciferase expressed within healthy living cells.

In this technical manual, we describe the use of the NanoBRET™ TE 590 Dyes[a,b] to develop novel cell-permeable tracers and the considerations for optimizing conditions for intracellular target engagement assay (Figure 2). The NanoBRET™ TE 590 Dyes have consistently demonstrated robust cell permeability and optimal spectral overlap with NanoLuc® for energy transfer. These dyes contain either an amine-reactive succinimidyl ester (SE) group or an azide group that enables the use of Click chemistry (3) to couple the dye to compounds with a reactive alkyne group. In addition, three linker variants of the NanoBRET™ 590 SE dyes are available, allowing a panel of tracer candidates to be prepared from a common intermediate and providing the structural diversity needed to ensure the highest probability of success and, if required, to guide further optimization of tracer development.

To evaluate the performance of the tracer candidates in the NanoBRET™ TE assay, expression vectors encoding the target-NanoLuc® fusion proteins need to be constructed. As BRET is highly sensitive to molecular distance and orientation between the donor (NanoLuc®) and acceptor (fluorescent tracer), we recommend placing NanoLuc® at both the N and C termini of the target protein to identify the optimal donor orientation when possible. The expression vectors are then transiently transfected into cells and the BRET activity for each tracer candidate is measured. Co-incubation of excess unlabeled parent compound with the tracer can determine the nonspecific BRET signal, which is then used to calculate the assay window for each tracer. The tracer candidate with the best assay window can be further characterized to assess its intracellular affinity for the target protein and to identify the optimal concentration for rank-order and/or quantitative analysis of compound affinity at the target protein inside living cells under equilibrium conditions. For information on optimizing the NanoBRET™ TE assay for kinetic analysis of compound binding, consult reference 4 (Section 11.E).

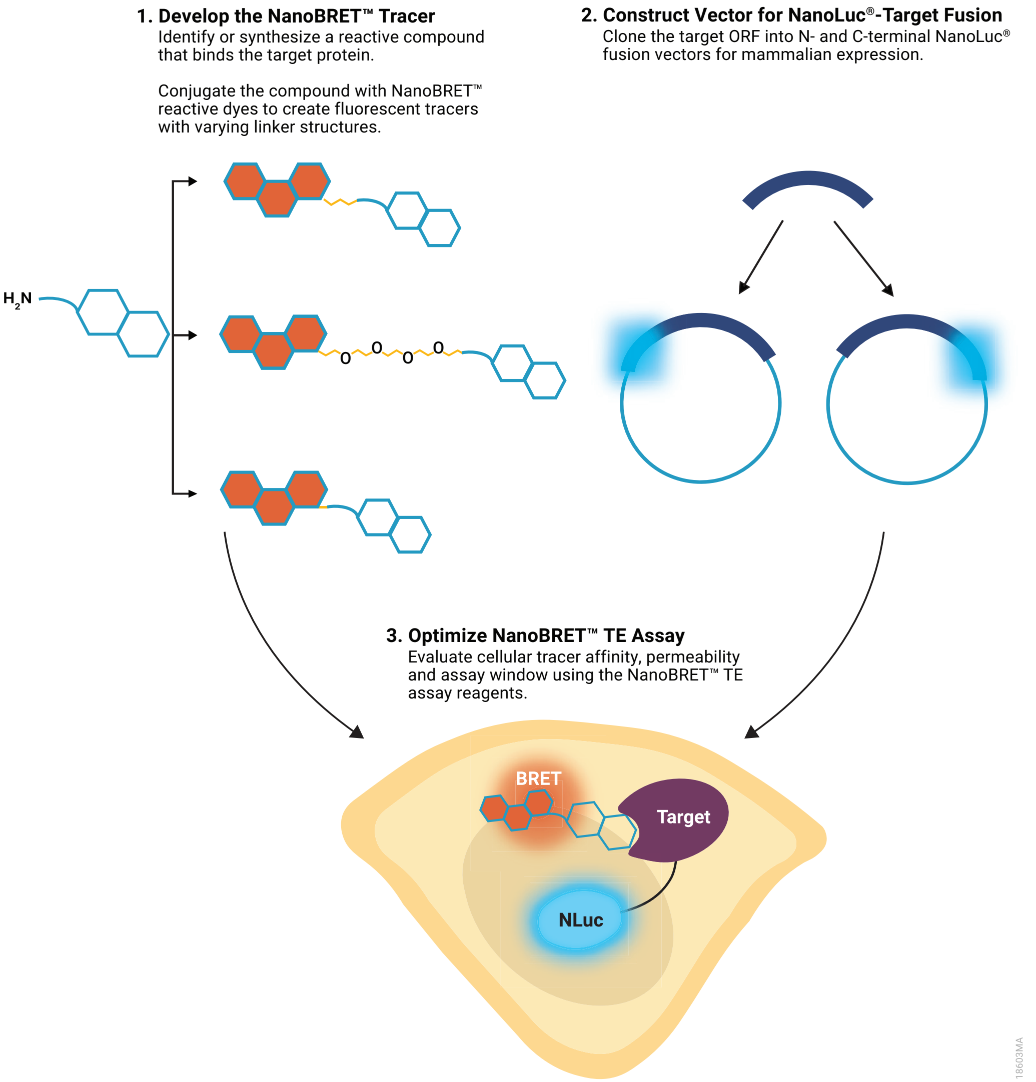

Figure 2. Workflow for developing a NanoBRET™ TE Assay. The three steps to making a NanoBRET™ TE Assay are: 1) develop multiple fluorescent tracer candidates with different linkers that can bind the target of interest; 2) construct vectors that express, in cells, a fusion of the target protein with NanoLuc® luciferase on either termini; and 3) evaluate the performance of tracer candidates and optimize the NanoBRET™ TE cellular assay using NanoBRET™ TE Assay reagents.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
NanoBRET™ 590 SE	**5mg**	**N5000**
	25mg	**N5001**
NanoBRET™ 590 C4 SE	**5mg**	**N5300**
	25mg	**N5301**
NanoBRET™ 590 PEG(O4) SE	**5mg**	**N5200**
NanoBRET™ 590 C3 Azide	**5mg**	**N5100**

PRODUCT	SIZE	CAT.#
NanoBRET™ 590 SE Bundle	**1 each**	**N5201**

Includes:

- 5mg NanoBRET™ 590 SE
- 5mg NanoBRET™ 590 C4 SE
- 5mg NanoBRET™ 590 PEG(O4) SE

Storage Conditions: Store at less than –65°C protected from light.

3. Before You Begin

3.A. Verification of NanoBRET™ Tracers

There are two main elements to the verification of NanoBRET™ tracers: 1) generating a panel of NanoBRET™ tracer candidates from a known, small molecule binder of the target protein of interest; and 2) assessing the activity of the NanoBRET™ tracer candidates against the target-NanoLuc® fusion protein in cells.

To generate the tracer candidates, the selected small molecule will be modified with an amine or an alkyne functional group (if one is not present already) and then coupled to the NanoBRET™ 590 dyes. Experience with organic synthesis is required to effectively synthesize and purify the tracer candidates for evaluation in the NanoBRET™ TE assay. A general protocol for tracer synthesis is described in Section 4 of this technical manual.

Once the tracer candidates are prepared, they are evaluated for BRET activity in cells expressing the target-NanoLuc® fusion protein. As proper expression of the NanoLuc® fusion protein is essential to the performance of the NanoBRET™ TE Assay, you should be familiar with molecular cloning and mammalian cell culture. Guidelines for evaluating NanoBRET™ TE tracers and optimizing NanoBRET™ TE assays are described in Sections 5–8 of this technical manual.

Note: Additional reagents are required to express the target-NanoLuc® fusion protein in cells and generate BRET signal for evaluation of tracer performance; see Section 5.A for details.

3.B. Selecting the Base Compound for Tracer Synthesis

Since the NanoBRET™ TE Assay is intended for measuring compound binding to target protein in living cells, the NanoBRET™ TE tracers must permeate live cells and function in a complex intracellular environment. Therefore, several parameters should be taken into consideration when selecting the base compound for tracer synthesis.

Reversibility: For broadest utility, reversible binding compounds are ideal tracer candidates. As a method intended to quantify target occupancy and affinity, the ideal tracer will achieve equilibrium within the assay incubation time (often 1–2 hours). Therefore, scaffolds with irreversible binding or slow intrinsic engagement rates may be suboptimal. We have found that most reversible tracers equilibrate within a 2-hour incubation window.

Target Affinity: Generally, compounds with submicromolar biochemical affinity are candidates for consideration. However, higher affinity compounds (K_d = 1–100nM) often yield the best performing intracellular tracers. Within the milieu of cells, strong affinity may be required to overcome competition from endogenous metabolites or enzymatic substrates that may compete directly with the tracer.

Ability to Functionalize at a Tolerable Position: The base scaffold must have a solvent-exposed reactive group for conjugation. This site can be determined through cocrystal structure, Structure-Activity Relationship (SAR) analysis or homology models.

Cell Permeability: Preferably, there should be previous data suggesting that the base compound is cell permeable and complies with Lipinski rule of five attributes (5).

NanoBRET is highly sensitive to molecular proximity. Accordingly, only the target-NanoLuc® fusion protein will generate a BRET signal in cells. NanoBRET™ TE tracers can, therefore, be derived from base compounds with high selectivity for the target protein or from broad-spectrum inhibitors if broader target coverage is desired.

3.C. Constructing NanoBRET™ Expression Vectors

Unless there is a known constraint, a NanoLuc® reporter can be placed at either terminus of the target protein. The optimal orientation of the NanoLuc® fusion protein will need to be empirically determined. Installation of a 4–10 residue linker between the target protein and NanoLuc® Luciferase is recommended. Standardized poly Gly/Ser linkers (e.g., Gly-Ser-Ser-Gly) are commonly used to ensure some level of flexibility. A strong, constitutively-active promoter is typically used to drive expression.

Two sets of vectors are also available for creating NanoLuc® fusions to the target protein. These vectors are compatible with the Flexi® Vector System or conventional cloning using multiple cloning sites (MCS).

Flexi® Vector System-Compatible Vectors

The pFN31 and pFC32 NanoLuc® Protein Fusion Flexi® Vectors (pFN31A, Cat.# N1311; pFN31K, Cat.# N1321; pFC32A, Cat.# N1331; pFC32K, Cat.# N1341) can be used to create NanoLuc® fusion proteins via Flexi® Cloning. For detailed information on the Flexi® Vector System, please refer to the *Flexi® Vector Systems Technical Manual* #TM254, available at: **www.promega.com/protocols**

In brief, Flexi® vectors contain a lethal gene that must be replaced with the desired gene of interest for cells to survive and colonies to form. The optimized protocols and reagents greatly reduce the overall cloning burden. The standard protocol for cloning PCR products can be used to introduce genes of interest into the NanoLuc® vectors. An alternative approach is to transfer the open reading frame (ORF) of interest from an existing Flexi®-compatible vector to the NanoLuc® vectors. Use our Find My Gene™ resource (**www.promega.com/findmygene/search.aspx**) to search a list of nearly 10,000 constructs to determine if one exists with your gene of interest. Follow the protocols in Sections 5.A and 5.B of *Flexi® Vector Systems Technical Manual* #TM254 to transfer an ORF to NanoLuc® vectors.

3.C. Constructing NanoBRET™ Expression Vectors (continued)

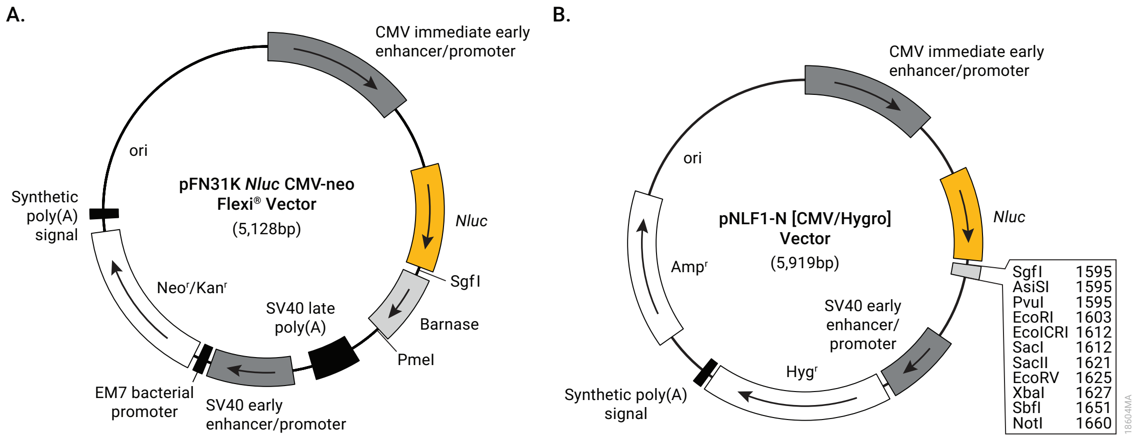

Figure 3. Vector maps of example NanoLuc® Fusion cloning vectors. Example of a Flexi® cloning vector (**Panel A**) and a MCS cloning vector (**Panel B**) for creating N-terminal NanoLuc® fusion proteins. Similar vectors are available for creating C-terminal NanoLuc® fusion proteins.

MCS Vectors

The pNLF1 NanoLuc® Protein Fusion Vectors (pNLF1-N, Cat.# N1351; pNLF1-C, Cat.# N1361) can be used to create NanoLuc® fusion proteins via conventional molecular cloning. Follow standard cloning procedures to introduce genes of interest into the MCS vectors as described for the individual vector protocols.

Note: For target proteins localized to specific organelles or to the membrane, ensure that including the NanoLuc® tag does not disrupt localization of the target protein and that the NanoLuc® tag is not cleaved during protein trafficking.

3.D. Instrument Requirements

To perform NanoBRET™ TE Assays, a luminometer capable of sequentially measuring dual-wavelength windows at the appropriate wavelengths is required. See Section 11.B for details on instrument setup.

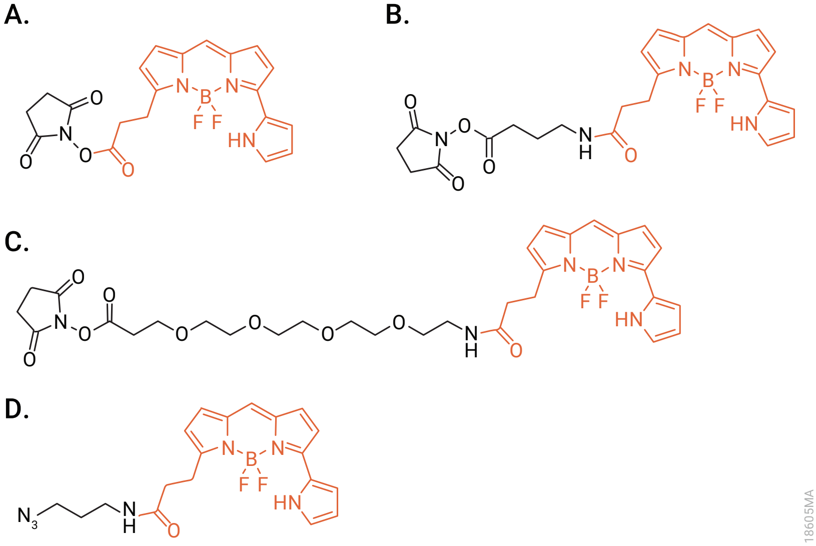

Figure 4. Structure of the NanoBRET™ 590 dyes. Panel A. NanoBRET™ 590 SE (MW: 426.2g/mol). **Panel B.** NanoBRET™ 590 C4 SE (MW: 511.3g/mol). **Panel C.** NanoBRET™ 590 PEG(O4) SE (MW: 673.5g/mol). **Panel D.** NanoBRET™ 590 C3 Azide (MW: 411.2g/mol).

4. Tracer Synthesis

NanoBRET™ reactive dyes can be used to create NanoBRET™ tracer candidates using traditional synthetic chemistry techniques. The three variants of the amine reactive dyes are available as succinimidyl esters (SE; Figure 4, Panels A–C) and are the preferred reagents for conjugates, as they produce stable amide bonds under mild coupling conditions. These reagents have been demonstrated to react quickly and quantitatively with unhindered aliphatic primary and secondary amines, including piperidine and piperazine. In addition, as the linker included in the tracer molecule could influence the permeability, aqueous solubility and BRET efficiency of the tracer, we recommend exploring a variety of linker architectures when developing NanoBRET™ tracers. The variants of the amine reactive dyes contain distinct linkers, allowing for the creation of three unique tracer structures from a common starting material and thereby facilitating the evaluation of tracers with different linkers.

The NanoBRET™ 590 Azide dye (Figure 4, Panel D) enables tracer synthesis using copper-free and copper-based azide-alkyne Click chemistry, which is characterized by efficiency, high specificity and bio-orthogonality. Click chemistry also provides an amine orthogonal coupling chemistry.

Note: The NanoBRET™ 590 dyes and their derivatives are sensitive to extreme pH and strong acids and bases are not tolerated. All protecting groups must be removed prior to reaction with the NanoBRET™ 590 dyes. Avoid temperatures >35°C during tracer synthesis and purification, to prevent decomposition.

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4.A. Materials to Be Supplied By the User

- functionalized small molecule precursor with a primary amine group or reactive alkyne group.
- amber vial or appropriate glass reaction vessel
- anhydrous dimethylformamide (DMF)
- *N,N*-diisopropylethylamine (DIPEA)
- analytical HPLC/analytical LC-MS
- preparative reverse-phase HPLC
- quenching solution: 1:1:0.01 (v/v/v) HPLC-grade water–acetonitrile–trifluoroacetic acid (TFA)
- trifluoroacetic acid (TFA) HPLC buffer: 0.1% TFA in water
- acetonitrile HPLC solvent
- anhydrous dimethylsulfoxide (DMSO, <1% water)
- $CuSO_4$
- sodium ascorbate
- Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
- deionized or higher-grade water

4.B. Synthesis of Tracer Candidate via Amine Coupling

This protocol describes tracer candidate synthesis using the NanoBRET™ 590 SE dye. Adjustment to stoichiometry will be required for other NanoBRET™ amine reactive dyes based on the molecular weight of the respective reagents. However, molar equivalent ratios should remain consistent. NanoBRET™ 590 PEG(O4) SE dye should be dissolved in 1.0ml of anhydrous DMF solvent immediately before beginning the coupling reaction.

1. Dissolve amine compound (9.8µmol, 1eq.) in 1ml of anhydrous DMF with stirring in a small amber glass vial.
2. Add 5.1µl (29.3µmol, 3eq.) of DIPEA and allow the mixture to stir for 5 minutes.
3. Add NanoBRET™ 590 SE dye (5mg, 11.7µmol, 1.2eq.) to the mixture, replace the vial cap and allow the reaction to stir in the dark for a minimum of 30 minutes.
4. The reaction can be monitored by analytical reverse-phase HPLC using the method of your choice. The reaction is considered complete by the disappearance of the starting amine compound. Additional DIPEA can be added to drive the reaction to completion.
5. Dilute the reaction mixture with a premixed quenching solution (see Section 4.A). Purify by reverse-phase preparative HPLC column chromatography using the method of your choice.
6. Analyze the dye-containing fractions by LC-MS chromatography to identify product. Pool all product fractions with purity >95% at 580nm in a tared round bottom flask.
7. Concentrate the fraction pool under reduced pressure to give the final purified product.
8. Treat the product with fresh acetonitrile and concentrate to dryness to remove spurious TFA.
9. Dry the resulting product for 2 hours to overnight under high vacuum to remove residual solvents.

   Note: Avoid lyophilization.

10. Confirm tracer identity and purity using the LC-MS method of your choice.

    Note: Residual parental compound should be quantitatively removed to avoid competition with tracer binding of target protein. Unreacted NanoBRET™ 590 dye should be removed to ensure that accurate tracer concentration measurement is achieved.

11. Store solid tracer materials at less than 4°C protected from light.

4.C. Synthesis of Tracer Candidate via Copper-Based Azide-Alkyne Click Chemistry

1. Charge a small amber vial with precursor alkyne (10.2µmol, 1eq.), NanoBRET™ 590 C3 Azide (5.0mg, 12.2µmol, 1.2eq.), $CuSO_4$ (1.6mg, 10.2µmol, 1eq.), sodium ascorbate (10.1mg, 50.9µmol, 5eq.) and Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (5.4mg, 10.2µmol, 1eq.).
2. Add a small stir bar and 2ml of a 50/50 mixture of DMSO/water. Cap the vial and allow the reaction to stir, protected from light, a minimum of 30 minutes.
3. The reaction can be monitored by analytical reverse-phase HPLC using the method of your choice. The reaction is considered complete when the starting alkyne compound disappears.
4. Dilute the reaction mixture with a premixed reaction quenching solution (see Section 4.A). Purify by reverse-phase preparative HPLC column chromatography using the method of your choice.
5. Analyze dye-containing fractions by LC-MS chromatography to identify product. Pool all product fractions with purity >95% at 580nm in a tared round bottom flask.
6. Concentrate the fraction pool under reduced pressure to give the final purified product.
7. Treat the product with fresh acetonitrile and concentrate to dryness to remove spurious TFA.
8. Dry the resulting product for a minimum of 2 hours under high vacuum to remove residual solvents.

   Note: Avoid lyophilization.

9. Confirm tracer identity and purity using the LC-MS method of your choice.

   Note: Residual parental compound should be quantitatively removed to avoid competition with tracer binding of target protein. Remove unreacted NanoBRET™ 590 dye to ensure that accurate tracer concentration measurement is achieved.

10. Store solid tracer materials at less than 4°C protected from light.

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4.D. Preparing NanoBRET™ Tracer Solution

The Beer-Lambert Law is used to accurately prepare tracer stock solutions:

$$A = \varepsilon \times \text{path length} \times \text{concentration}$$

1. Prepare a calculated 1mM stock solution of the tracer candidate by dissolving a known mass of the compound in an appropriate volume of DMSO.
2. Determine the stock solution concentration (C_{stock}) by measuring absorbance (A) of the dye solution at λ_{max}, 580nm:

$$C_{stock}\ (\text{mol/L}) = \frac{\text{Absorbance (A)}}{\text{Extinction Coefficient } (\varepsilon)} \times \text{dilution factor}$$

   A = average A at λ_{max} of replicates.

   Ɛ = theoretical extinction coefficient at λ_{max} (580nm): 83,000 $M^{-1}\ cm^{-1}$ for NanoBRET™ 590 dyes (6).

   Note: The path length is assumed to be 1cm. If a different path length is used for absorbance measurement, adjust the calculation according to the Beer-Lambert Law.
3. Prepare a 400µM working solution of the tracer candidate by diluting the concentrated stock solution (using C_{stock} determined in Step 2) with anhydrous DMSO.
4. The prepared stock and working DMSO solutions should be stored frozen and protected from light at less than –65°C. Avoid repeated freeze-thaw cycles.

5. Overview of NanoBRET™ TE Assay Optimization and Materials

Consider Both NanoLuc® Tagging Orientations

BRET is highly sensitive to molecular distance and orientation between the donor (luciferase) and acceptor (fluorescent tracer) partners. This feature enables a true measurement of direct molecular interactions inside intact cells. However, optimization may be warranted to identify the conditions producing optimal BRET between the donor/acceptor partners. This can generally be achieved via transient transfection of plasmids encoding target-NanoLuc® fusion proteins where both orientations of the NanoLuc® fusion can be evaluated.

Use Permeabilized Cells as a Control

While NanoBRET™ tracers are primarily used to quantify live cell target engagement, the method is also compatible with permeabilized cell analysis. Assessing NanoBRET™ signal under permeabilized cell conditions enables determination of intrinsic affinity of the tracer, which can be approximated by the EC_{50} value derived from a tracer dose response curve generated in the absence of various cellular factors that may interfere with target engagement. It is therefore generally recommended that novel NanoBRET™ tracers are evaluated in both live cells to measure the apparent affinity (or apparent K_d) of the tracer, which is reflected by the EC_{50} value derived from a tracer dose response curve in live cells and is influenced by both the intrinsic affinity of the tracer and the composite effects of the cellular factors, as well as permeabilized cells (via addition of digitonin) to confirm tracer engagement at the target protein in the absence of the cell membrane and interfering cellular factors (Figure 5).

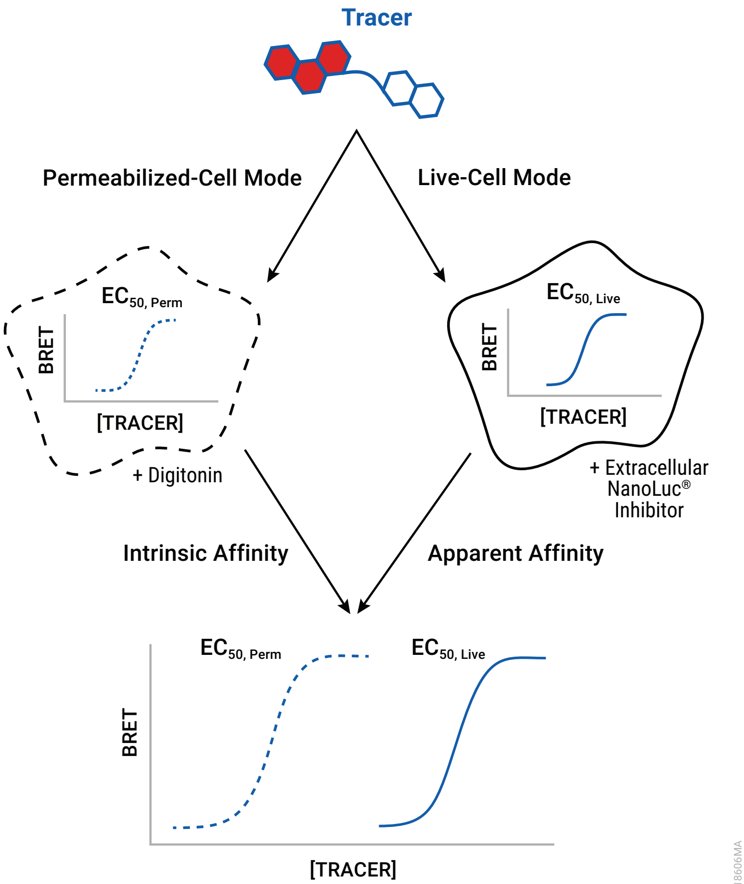

Figure 5. Determine intrinsic and apparent affinity of the tracer. Live-cell mode of the NanoBRET™ TE Assay can be used to measure the apparent affinity of the tracer inside the cell, while permeabilized-cell mode can be used to measure the tracers intrinsic affinity. The apparent affinity of the tracer may be lower (right shifted in the dose-response curve) than the intrinsic affinity due to interference from cellular factors.

5. Overview of NanoBRET™ TE Assay Optimization and Materials (continued)

Define Specific BRET Signal

Evaluating the BRET signal-to-background ratio is a critical step when evaluating novel tracers. As BRET is a sensor of molecular proximity, the BRET signal can also arise due to nonspecific colocalization of the tracer and the NanoLuc® fusion protein. This signal is often observed when tracer concentrations approach 1µM, and can confound the BRET interpretation due to direct target occupancy. Therefore, as an additional control, we recommend that a 20–100X molar excess of the nonfunctionalized parental compound of the tracers is co-incubated with the tracer to assess nonspecific BRET signal. Note that amine-functionalized precursor for the tracer candidates can negatively affect compound permeability (as demonstrated in Section 9). If a nonfunctionalized compound is not readily available, a capped amine precursor, such as an acetamide or a BOC (tert-butoxycarbonyl) group, could be used. Ideally, the NanoBRET™ signal-to-background ratio is determined in the presence and absence of the nonfunctionalized parent compound.

In general, similar reagents are used for each assay mode, with a few key exceptions, and the same transfection protocol is followed in both cases. Section 5.A lists the required reagents. Section 5.B details the transfection protocol. Section 6 describes the protocol for live-cell assay mode and Section 7 describes the protocol for permeabilized-cell assay mode.

5.A. Materials to Be Supplied By the User

- Opti-MEM™ I Reduced Serum Medium, without phenol red (Thermo Fisher Scientific Cat.# 11058-021)
- Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific Cat.# 11995-065)
- fetal bovine serum (FBS, HyClone Cat.# SH30070.03, Seradigm Cat.# 1500-050)
- 0.05% Trypsin/EDTA (Thermo Fisher Cat.# 25300)
- white, nonbinding-surface (NBS) 96-well plates (Corning® Cat.# 3600) or 384-well plates (Corning® Cat.# 3574)
- tissue-culture equipment and reagents
- polypropylene plasticware (**Note: Do not** use polystyrene plasticware for this assay.)
- FuGENE® HD Transfection Reagent (Cat.# E2311)
- NanoBRET™ Tracer Dilution Buffer (Cat.# N2191)
- Transfection Carrier DNA (Cat.# E4881)
- Intracellular TE Nano-Glo® Substrate/Inhibitor (Cat.# N2160)
- tracers: 100–400µM in DMSO (see Section 4 for tracer synthesis and preparation)
- transfection-grade plasmid encoding target-NanoLuc® fusion proteins driven by a mammalian promoter (see Section 3.C)
- DMSO (Sigma Cat.# 2650)
- detection instrument capable of measuring NanoBRET™ wavelengths (e.g., GloMax® Discover System, Cat.# GM3000; see Section 11.B)
- Digitonin (Cat.# G9441, 20mg/ml in DMSO; Sigma Cat.# D141 prepared at 50mg/ml in DMSO); **Note:** Only needed for permeabilized-cell mode.

5.B. Transient Transfection of HEK293 cells with Target-NanoLuc® Fusion Vectors in Standard Nonbinding Surface (NBS) Format.

This protocol has been optimized for HEK293 cells and can be used with live-cell and permeabilized-cell mode assays in the standard NBS format.

Note: The NBS format is the recommended option for preliminary tracer verification due to the high likelihood of NanoBRET™ tracer being compatible with the NBS plate type. However, some NanoBRET™ tracers perform equivalently with cells in the adherent (ADH) format in standard tissue culture-treated plates. Although NBS format is recommended for preliminary tracer verification, ADH format uses fewer handling steps and may be preferred if the NanoBRET™ tracer is compatible with tissue culture-treated plates. See Section 11, Appendix, for details. If other cell types are used, optimize the transfection conditions. For optimal performance, use cells that have been freshly passaged, ideally within 1–2 days.

1. Cultivate HEK293 cells (or desired cell type) appropriately prior to assay.
2. Remove medium from cell flask by aspiration, trypsinize and allow cells to dissociate from the flask.
3. Neutralize trypsin using cell culture medium (90% DMEM, 10% FBS) and centrifuge at 200 × *g* for 5 minutes to pellet the cells.
4. Aspirate medium and resuspend cells in cell culture medium.
5. Adjust density to 2×10^5 cells/ml using cell culture medium.
6. If HEK293 cells are used, prepare lipid:DNA complexes as follows:
   a. Prepare a 10µg/ml solution of DNA in Opti-MEM™ I reduced serum medium without phenol red that consists of the following ratios: 9.0µg/ml of Transfection Carrier DNA, 1.0µg/ml of target-NanoLuc® fusion vector DNA and 1ml of Opti-MEM™ I. Mix thoroughly.
   b. Add 30µl of FuGENE® HD Transfection Reagent to each milliliter of DNA mixture to form lipid:DNA complex. Ensure that the FuGENE® HD Transfection Reagent does not touch the plastic side of the tube; pipet directly into the liquid in the tube.
   c. Mix by inversion 5–10 times.
   d. Incubate at ambient temperature for 20 minutes to allow complexes to form.
7. In a sterile, conical tube, mix 1 part of lipid:DNA complex (e.g., 1ml) with 20 parts of HEK293 cells (e.g., 20ml) in suspension at 2×10^5 cells/ml. Mix gently by inversion 5 times.

   Note: Larger or smaller bulk transfections should be scaled accordingly, using this 20:1 cells to lipid:DNA complex ratio.
8. Dispense cells + lipid:DNA complex into a sterile tissue culture flask and incubate 20–30 hours at 37°C, 5% CO_2. We recommend a cell density of approximately 55,000–80,000 cells/cm^2 during transfection. For example, use approximately $4–6 \times 10^6$ cells for a T75 flask.
9. For assays in live-cell mode, continue to Section 6. For assays in permeabilized-cell mode, continue to Section 7.

6. Live-Cell Mode Protocol

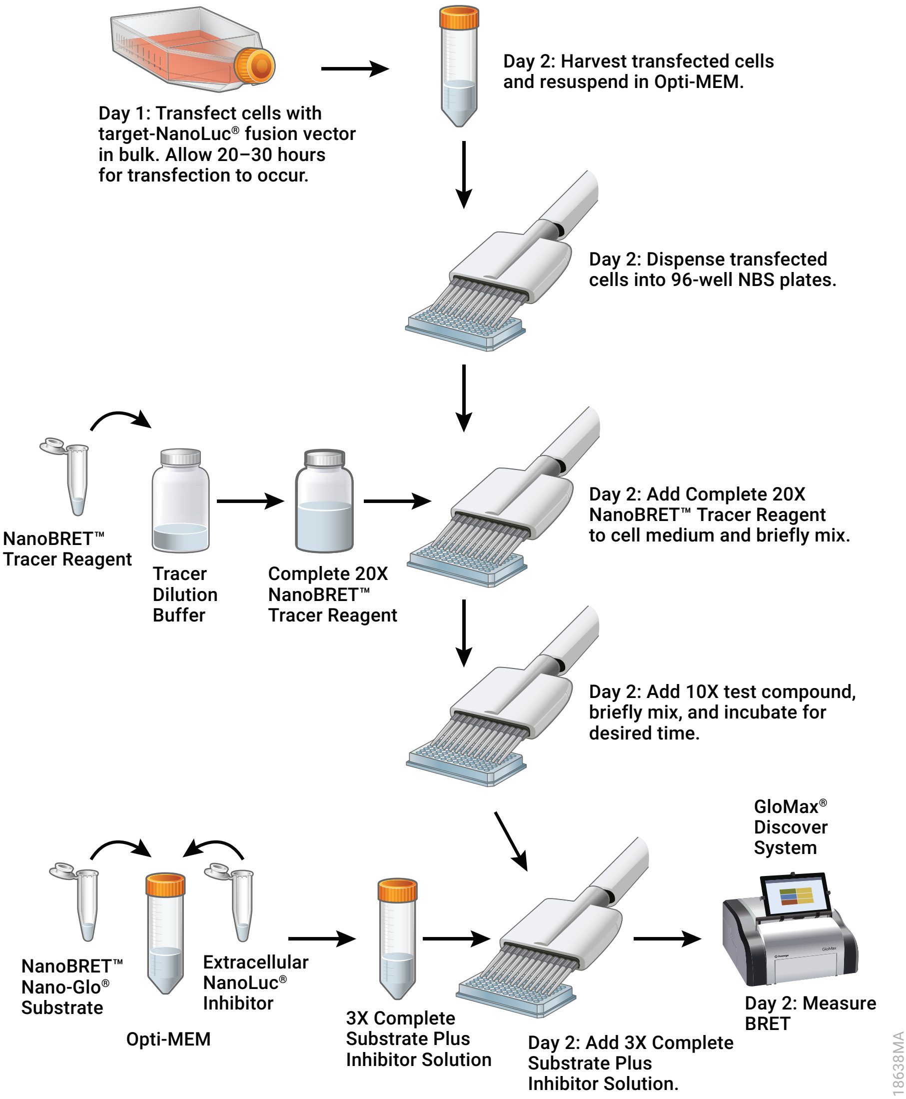

Figure 6. Overview of the live-cell assay mode for the NanoBRET™ TE Assay in NBS plates.

The volumes specified for the live-cell mode protocol are for 96-well plates. Table 1 lists the assay volumes used for both 96- and 384-well plates for live-cell mode. Modify the reagent volumes in Sections 6.A–C as listed in Table 1 if 384-well plates are used.

Table 1. Volumes of NanoBRET™ TE Assay Reagents Used for Multiwell Plates in Live-Cell Mode.

	Volume Per Well	
Tracer and Test Compound	**96-Well Plate**	**384-Well Plate**
Opti-MEM™ I reduced serum medium, without phenol red, with transfected cells	85µl	34µl
Complete 20X NanoBRET™ Tracer Reagent	5µl	2µl
10X Test Compound	10µl	4µl
assay volume	100µl	40µl
NanoBRET™ Assay Reagents		
3X complete substrate plus inhibitor solution (Section 6.C)	50µl	20µl
final assay volume	**150µl**	**60µl**

6.A. Preparing Transfected Cells and Adding Tracer

1. Remove medium from flask with transfected HEK293 cells via aspiration, trypsinize and allow cells to dissociate from the flask.
2. Neutralize trypsin using medium containing serum (90% DMEM with 10% FBS) and centrifuge at 200 × *g* for 5 minutes to pellet the cells.
3. Aspirate medium and resuspend cells using prewarmed Opti-MEM™ I reduced serum medium, without phenol red.
4. Adjust the density to 2×10^5 cells/ml in Opti-MEM™ I reduced serum medium, without phenol red.
5. Dispense 85µl of cell suspension per well into white, 96-well NBS plates. Periodically mix cells to avoid settling in the tube.
6. Perform a serial dilution of 100X tracer in DMSO. We recommend a maximum 100X tracer concentration of 200µM in 100% DMSO so that the final tracer concentration in culture medium does not exceed 2µM, which is typically the solubility limit of cell-permeable NanoBRET™ tracers.

   Notes:

   a. Always keep tracers in 100% DMSO until diluted with Tracer Dilution Buffer for the NanoBRET™ assay.

   b. When evaluating multiple tracer candidates at once, initial tests can be performed at one or two concentrations of each tracer candidate (e.g., test each tracer candidate at a concentration of 0.1µM and 1µM), instead of a full dose-response curve, to reduce workload. Once a promising tracer candidate is identified, perform a full dose-response curve to characterize tracer affinity.

   c. To characterize unlabeled test compound affinity (once an optimal tracer and tracer concentration have been identified), prepare a tracer solution in 100% DMSO at 100X the desired final tracer concentration.

6.A. Preparing Transfected Cells and Adding Tracer (continued)

7. Prepare Complete 20X NanoBRET™ Tracer Reagent by adding 4 parts of Tracer Dilution Buffer to 1 part of 100X tracer. Mix gently several times to ensure that the DMSO solution is mixed with the Tracer Dilution Buffer.

   Notes:

   a. Tracer Dilution Buffer is critical in maintaining tracer solubility in culture medium; dilute and dispense tracer as described. Do not directly dilute tracer in culture medium.

   b. Because the Tracer Dilution Buffer is viscous, slowly dispense both the Tracer Dilution Buffer and the Complete 20X Tracer Reagent.

8. Dispense 5µl of Complete 20X NanoBRET™ Tracer Reagent per well to cells in suspension.

9. Mix on orbital shaker for 15 seconds at 900rpm. Mixing may vary between orbital shakers and should be optimized for each individual unit accordingly.

10. **Optional:** Prepare a separate set of samples without tracer for optional background correction steps.

6.B. Adding Test Compounds

1. Prepare a suitable unlabeled test compound at 1,000X final concentration in 100% DMSO.

   Note: The amine-functionalized precursor for the tracer candidates should not be used as the unlabeled test compound.

2. Dilute 1,000X test compound to 10X final concentration in Opti-MEM™ I reduced serum medium, without phenol red.

3. The characterization of NanoBRET™ tracers can be performed at a single concentration of unlabeled compound for tracer dose-response curves. We recommend that a 20–100X molar excess of parental compound be co-incubated with the tracer to determine the nonspecific BRET signal.

   Note: For characterization of unlabeled test compound, create a serial dilution of test compound for IC_{50} analysis.

4. Add 10µl per well of 10X parental or test compound to the 96-well plates containing cells with 1X tracer. Mix on an orbital shaker for 15 seconds at 900rpm.

5. Incubate the plate for 2 hours at 37°C, 5% CO_2.

   Note: Test compound incubation times may vary, depending on compound binding characteristics.

6.C. NanoBRET™ Assay Protocol

1. Remove plate from incubator and equilibrate to room temperature for 15 minutes.
2. Prepare 3X complete substrate plus inhibitor solution in Opti-MEM™ I reduced serum medium, without phenol red, just before measuring BRET as described in Table 2. Mix gently by inversion 5–10 times in a conical tube.

   Note: Use 3X complete substrate plus inhibitor solution within 1.5 hours. Discard any unused solution.

Table 2. Preparing 3X Complete Substrate Plus Inhibitor Solution.

Component	96-Well Plate	384-Well Plate
NanoBRET™ Nano-Glo® Substrate	30µl	48µl
Extracellular NanoLuc® Inhibitor	10µl	16µl
Opti-MEM™ I reduced serum medium, without phenol red	4,960µl	7,936µl
final volume	**5,000µl**	**8,000µl**

3. Add 50µl of 3X complete substrate plus inhibitor solution to each well of the 96-well plate. Incubate for 2–3 minutes at room temperature.
4. Measure donor emission wavelength (e.g., 450nm) and acceptor emission wavelength (e.g., 610nm) using the GloMax® Discover System or other NanoBRET™ Assay-compatible luminometer.

   Note: We recommend measuring BRET within 10 minutes after adding complete substrate plus inhibitor solution.

6.D. Determining BRET Ratio

1. To generate raw BRET ratio values, divide the acceptor emission value (e.g., 610nm) by the donor emission value (e.g., 450nm) for each sample.

   Optional: To correct for background, subtract the BRET ratio in the absence of tracer (average of no-tracer control samples) from the BRET ratio of each sample.
2. Convert raw BRET units to milliBRET units (mBU) by multiplying each raw BRET value by 1,000 using this formula:

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

NanoBRET™ ratio with optional background correction:

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

7. Permeabilized-Cell Mode Protocol

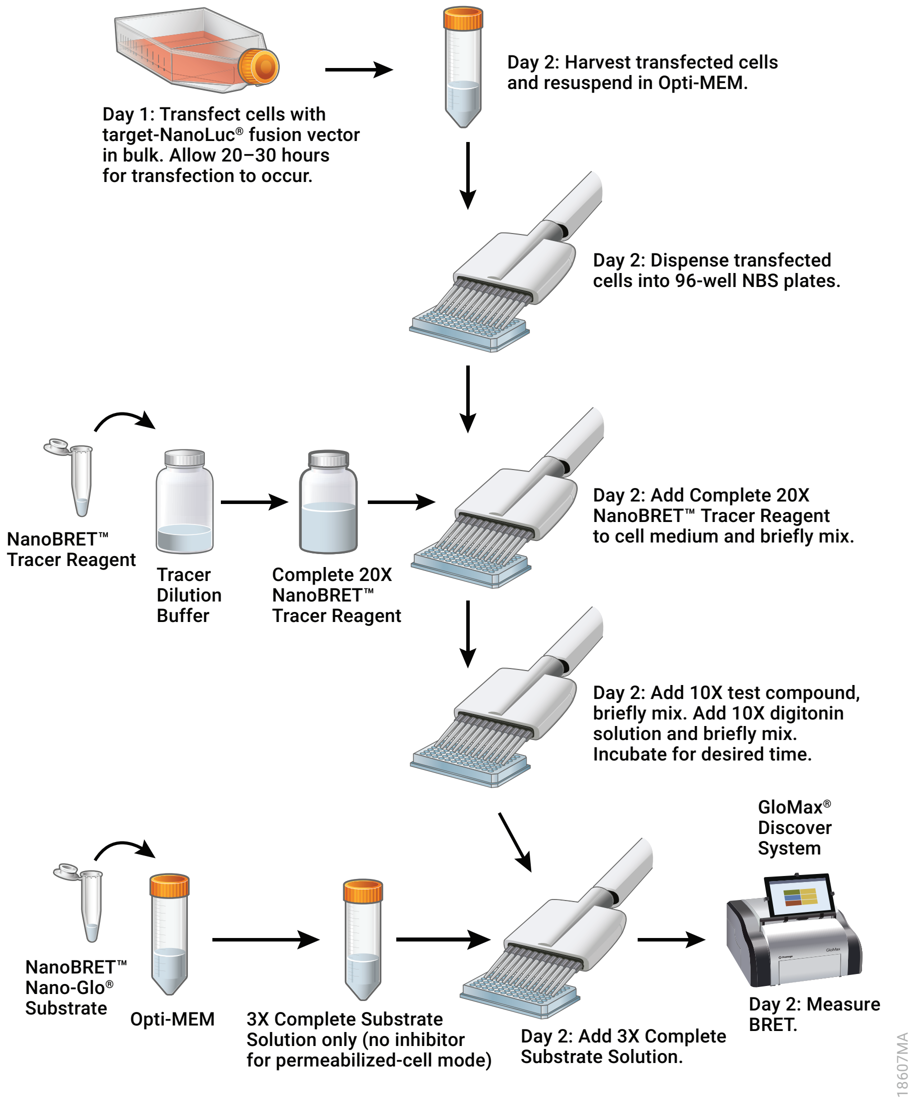

Figure 7. Overview of the permeabilized-cell mode for the NanoBRET™ TE Assay in NBS plates.

The volumes specified for the permeabilized-cell mode protocol are for 96-well plates. Table 3 lists the assay volumes used for both 96- and 384-well plates for permeabilized-cell mode. Modify the reagent volumes in Sections 7.A–D as listed in Table 3 if 384-well plates are used.

Table 3. NanoBRET™ TE Assay Reagents Used for Multiwell Plates in Permeabilized-Cell Mode.

	Volume Per Well	
Tracer and Test Compound	**96-Well Plate**	**384-Well Plate**
Opti-MEM™ I reduced serum medium, without phenol red, with transfected cells	75µl	30µl
10X Digitonin	10µl	4µl
Complete 20X NanoBRET™ Tracer Reagent	5µl	2µl
10X Test Compound	10µl	4µl
assay volume	100µl	40µl
NanoBRET™ Assay Reagents		
3X Complete Substrate Solution (Section 7.D)	50µl	20µl
final assay volume	**150µl**	**60µl**

7.A. Preparing 10X Digitonin Solution

1. Prepare 1,000X concentrated digitonin solution at 50mg/ml in DMSO.

   Note: If using Digitonin stock solution (Cat.# G9441) it is 20mg/ml and thus 400X.

2. Dilute the concentrated digitonin solution to 10X final concentration in Opti-MEM™ I reduced serum medium, without phenol red. The concentration of this 10X solution is 500µg/ml.

   Note: Permeabilization is achieved rapidly at a final digitonin concentration of 50µg/ml.

7.B. Preparing Transfected Cells and Adding Tracer

1. Remove medium from flask with transfected HEK293 cells via aspiration, trypsinize and allow cells to dissociate from the flask.
2. Neutralize trypsin using medium containing serum (90% DMEM with 10% fetal bovine serum) and centrifuge at 200 × *g* for 5 minutes to pellet the cells.
3. Aspirate medium and resuspend cells using prewarmed Opti-MEM™ I reduced serum medium, without phenol red.
4. Adjust the density to 2×10^5 cells/ml in Opti-MEM™ I reduced serum medium, without phenol red.
5. Dispense 75µl of cell suspension per well into white, 96-well NBS plates. Periodically mix cells to avoid settling in the tube.

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7.B. Preparing Transfected Cells and Adding Tracer (continued)

6. Perform a serial dilution of 100X tracer in DMSO. We recommend a maximum 100X tracer concentration of 200µM in 100% DMSO so that the final tracer concentration in culture medium does not exceed 2µM, which is typically the solubility limit of cell-permeable NanoBRET™ tracers.

   Notes:

   a. Always keep tracers in 100% DMSO until diluted with Tracer Dilution Buffer for the NanoBRET™ assay.

   b. When evaluating multiple tracer candidates at once, initial tests can be performed at one or two concentrations of each tracer candidate (e.g., test each tracer candidate at a concentration of 0.1µM and 1µM), instead of a full dose-response curve, to reduce workload. Once a promising tracer candidate is identified, perform a full dose-response curve to characterize tracer affinity.

   c. To characterize unlabeled test compound affinity (once an optimal tracer and tracer concentration have been identified), prepare a tracer solution in 100% DMSO at 100X the desired final tracer concentration.

7. Prepare Complete 20X NanoBRET™ Tracer Reagent by adding 4 parts of Tracer Dilution Buffer to 1 part of 100X tracer. Mix gently several times to ensure that the DMSO solution is mixed with the Tracer Dilution Buffer.

   Notes:

   a. Tracer Dilution Buffer is critical in maintaining tracer solubility in culture medium; dilute and dispense tracer as described. Do not directly dilute tracer in culture medium.

   b. Because the Tracer Dilution Buffer is viscous, slowly dispense both the Tracer Dilution Buffer and the Complete 20X Tracer Reagent.

8. Dispense 5µl of Complete 20X NanoBRET™ Tracer Reagent per well to cells in suspension.

9. Mix on orbital shaker for 15 seconds at 900rpm. Mixing may vary between orbital shakers and should be optimized for each individual shaker accordingly.

10. **Optional:** Prepare a separate set of samples without tracer for optional background correction steps.

7.C. Adding Test Compounds

1. Prepare a suitable unlabeled test compound at 1,000X final concentration in 100% DMSO.

   Note: The amine-functionalized tracer candidate precursor should not be used as the unlabeled test compound.

2. Dilute 1,000X test compound to 10X final concentration in Opti-MEM™ I reduced serum medium, without phenol red.

3. The characterization of NanoBRET™ tracers can be performed at a single concentration of unlabeled compound for tracer dose-response curves. It is generally recommended that a 20–100X molar excess of parental compound is co-incubated with the tracer to determine the nonspecific BRET signal.

   Note: For characterization of unlabeled test compound, create a serial dilution of test compound for IC_{50} analysis.

4. Add 10µl per well of 10X parental or test compound to the 96-well plates containing cells with 1X tracer. Mix on an orbital shaker for 15 seconds at 900rpm.

5. Add 10µl of 10X digitonin solution per well. Mix the 96-well plate on an orbital shaker for 15 seconds at 900rpm.

6. Incubate the plate at room temperature, protected from light, for 20–60 minutes.

   Note: Incubations at higher temperatures and/or longer incubation times typically are not optimal for NanoBRET™ TE assays performed in permeabilized-cell mode.

7.D. NanoBRET™ Assay Protocol

1. Prepare 3X Complete Substrate Solution only (for permeabilized cell mode) in Opti-MEM™ I reduced serum medium, without phenol red, just before measuring BRET (Table 4). This solution consists of 1:166 dilution of NanoBRET™ Nano-Glo® Substrate only. Mix gently by inverting 5–10 times in a conical tube.

Table 4. Preparing 3X Complete Substrate Solution (Permeabilized-Cell Mode).

Component	96-Well Plate	384-Well Plate
NanoBRET™ Nano-Glo® Substrate	30µl	48µl
Opti-MEM™ reduced serum medium, without phenol red	4,970µl	7,952µl
final volume	**5,000µl**	**8,000µl**

Note: Because the cells are permeabilized, the Extracellular NanoLuc® Inhibitor is omitted from this solution.

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

   Note: Use 3X Complete Substrate Solution within 1.5 hours. Discard any unused solution.

3. Measure donor emission wavelength (e.g., 450nm) and acceptor emission wavelength (e.g., 610nm) using the GloMax® Discover System or other NanoBRET™ Assay-compatible luminometer.

   Note: We recommend measuring BRET within 10 minutes after adding 3X Complete Substrate Solution.

7.E. Determining BRET Ratio

1. To generate raw BRET ratio values, divide the acceptor emission value (e.g., 610nm) by the donor emission value (e.g., 450nm) for each sample.

   Optional: To correct for background, subtract the BRET ratio in the absence of tracer (average of no-tracer control samples) from the BRET ratio of each sample.

2. Convert raw BRET units to milliBRET units (mBU) by multiplying each raw BRET value by 1,000 using this formula:

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

NanoBRET™ ratio with optional background correction:

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

8. Guidelines on Assessing Tracer Performance and Assay Optimization

8.A. Assay Window

We define assay window as the raw fold change in the BRET ratio observed at a specific concentration of tracer compared to the BRET ratio in the presence of a saturating dose of unlabeled compound, and we observed a correlation between assay window and assay quality (Z´). In our experience, the performance of the NanoBRET™ TE assays can be grouped into three different assay categories based upon the likelihood that a good quality assay was achieved (Z´ > 0.5; Figure 8). High window assays in which the assay window is above threefold have an excellent probability of achieving a good Z´ score. Medium window assays in which the assay window is above twofold, but below threefold, have a good probability of achieving a good Z´ score. Low window assays in which the assay window is below twofold have a moderate probability of achieving a good Z´ score. Descriptions of the expected assay capabilities for each of the above assay categories are provided in Table 5. We do not recommend moving forward with a NanoBRET™ TE Assay with an assay window below 1.6.

Table 5. NanoBRET™ TE Assay Capabilities.

Assay Category	Assay Window (AW)[1]	Assay Capabilities
High Window	AW ≥ 3.0	• Multiple-dose compound profiling to determine IC_{50} at a fixed concentration of tracer in 96- or 384-well format. • Low- to high-throughput single-dose profiling at fixed tracer concentration. • Excellent candidate for further miniaturization with optimization.
Medium Window	3.0 > AW ≥ 2.0	• Multiple-dose compound profiling to determine IC_{50} at fixed concentration of tracer in 96-well format. • Possible candidate for medium-throughput single-dose profiling at a fixed tracer concentration with optimization. • Possible candidate for scale-down to 384-well format with optimization by the end user.
Low Window	2.0 > AW ≥ 1.6	• Multiple-dose compound profiling to determine IC_{50} at fixed concentration of tracer in 96-well format.

[1]Assay window is the raw fold change in the BRET ratio observed at the recommended concentration of tracer compared to the BRET ratio in the presence of a saturating dose of unlabeled compound.

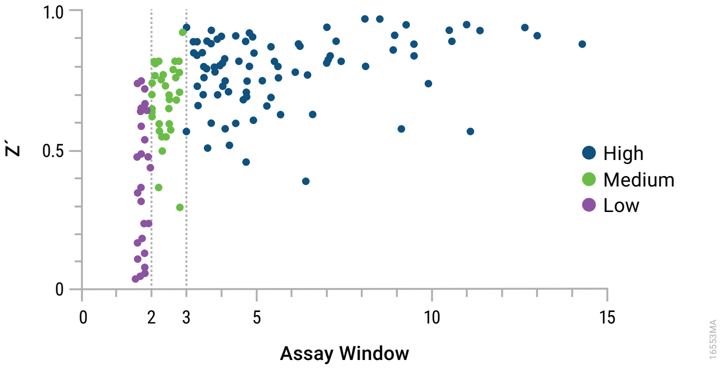


Figure 8. Correlating assay quality with assay window in the NBS format. Assay quality (Z´) was determined for over 166 target-NanoBRET™ tracer combinations tested in the NBS format by comparing BRET values (n = 4) in the presence and absence of a saturating dose of test compound. The Z´ scores were plotted versus the corresponding assay window, and the resultant data points were grouped into three different assay categories based on the likelihood that a good quality assay was achieved (Z´ > 0.5). High assay windows, above threefold, have an excellent probability of achieving a good Z´ score. Medium assay windows, above twofold and below threefold, have a good probability of achieving a good Z´ score. Low assay windows, below twofold, have a moderate probability of achieving a good Z´ score.

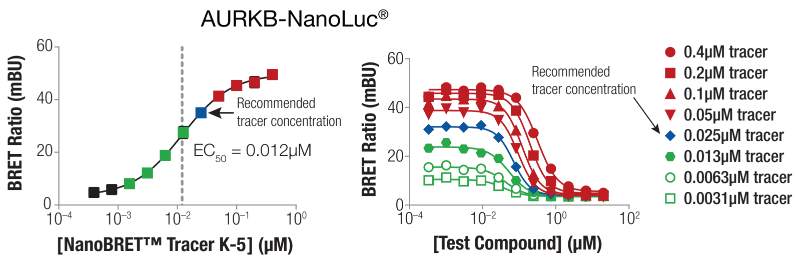
8.B. Quantitative Characterization of NanoBRET™ TE Assay

The NanoBRET™ TE assays allow for measuring the apparent affinity (apparent K_i) of a test compound for a target of interest. However, like other competitive binding assays, the IC_{50} measured for a test compound can be affected by the amount of the NanoBRET™ TE tracer that is used in the assay. Because the tracer and test compound both compete for binding to the target-NanoLuc® fusion, increasing concentrations of NanoBRET™ TE tracer can shift the apparent IC_{50} value of the test compound to higher concentrations. This relationship between the IC_{50} and the apparent K_i of a compound, termed the Cheng-Prusoff relationship, becomes most significant when the tracer concentration is in excess of its apparent affinity for the target-NanoLuc® fusion. We recommend evaluating the Cheng-Prusoff relationship (4) and quantitative capabilities for new NanoBRET™ TE tracers and assays.

The Cheng-Prusoff relationship for each NanoBRET™ TE assay can be assessed by measuring the IC_{50} values of a known inhibitor at multiple tracer concentrations. This measurement can be readily achieved using a matrix experiment where cells are cotreated with serially-diluted NanoBRET™ TE tracer and serially-diluted known inhibitor. As the concentration of the tracer is reduced below the apparent K_d of the tracer, the measured IC_{50} for the competing compound will appear insensitive to the changes in tracer concentration and generally be within twofold of the compound apparent K_i. Provided that the assay window is still adequate (ideally assay window > 2), this tracer concentration is recommended for the quantitative analysis of apparent K_i of test compounds. For a target that can be saturated when titrated with the NanoBRET™ tracer, the recommended tracer concentration is typically at or below the EC_{50} of the tracer for the target-NanoLuc® fusion protein (Figure 9, Panel A). Due to the functional performance limits of the NanoBRET™ TE tracers (typically 2µM), some targets cannot be saturated by the tracer under live-cell assay conditions. In these cases, the apparent IC_{50} value of the test compound may appear insensitive to tracer concentration and will often be a good approximation for the apparent K_i (Figure 9, Panel B).

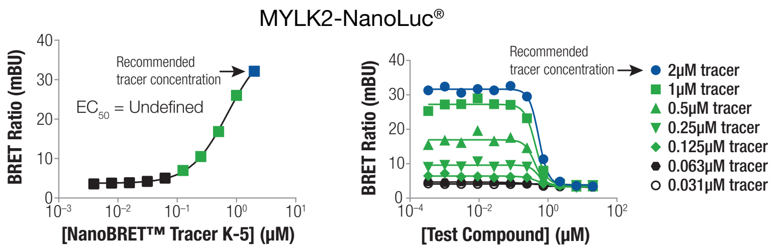
For users interested in more advanced approaches that directly measure the intracellular K_i, a linearized Cheng-Prusoff analysis can be performed for targets to which the tracer binding is saturable. For details on the linearized Cheng-Prusoff analysis, consult references 4 and 7 (Section 11.E). For users only interested in rank-order analysis or for NanoBRET™ TE assays where the assay window is inadequate at lower tracer concentrations, a subsaturating tracer concentration that is typically between the EC_{50} and EC_{80} of the tracer can be used to measure the relative potency of test compounds.

A.



[Tracer] (µM)	0.4	0.2	0.1	0.05	0.025	0.013	0.0063	0.0031
Apparent IC_{50}	0.32	0.19	0.12	0.093	**0.069**	0.052	0.042	0.038
Description	Right shifted				Recommended concentration	Quantitative analysis		

B.



[Tracer] (µM)	2	1	0.5	0.25	0.125	0.063	0.031
Apparent IC_{50}	**0.54**	0.43	0.41	0.52	0.52	Poor fit	Poor fit
Description	Recommended concentration	Quantitative analysis				Low assay window	

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Figure 9. Approaches to quantifying intracellular compound affinity using NanoBRET™ TE. Examples of tracer affinity and apparent intracellular compound affinity for a target with strong tracer affinity (**Panel A**) and a target with weak tracer affinity (**Panel B**) are shown. HEK293 cells expressing individual kinase-NanoLuc® fusions were resuspended in Opti-MEM™ I reduced serum medium, without phenol red, and seeded into 96-well plates. Cells were treated with various concentrations of NanoBRET™ Tracer K-5 and unlabeled compound as a competitive inhibitor, and incubated for 2 hours before adding 3X complete substrate plus inhibitor solution. BRET was measured using a GloMax® Discover System equipped with NanoBRET™ 618 filters (donor 450nm/8nm BP and acceptor 600nm LP). Raw BRET ratios were then converted to milliBRET units (mBU) and plotted vs. NanoBRET™ Tracer K-5 concentration to determine apparent affinity of the tracer or unlabeled compound.

8.C. Additional Considerations for Assay Optimization

Orientation of NanoLuc® Luciferase in the Fusion Protein

Due to the distance and geometry components of BRET, the assay window of the NanoBRET™ TE Assay will depend in part on the orientation of NanoLuc® luciferase (the BRET donor) relative to the tracer and its binding site (the BRET acceptor). Therefore, the fusion of NanoLuc® luciferase at one terminus of the target protein may be preferred over the fusion at the other terminus. In the absence of any known constraints on tagging, we recommend testing both orientations of NanoLuc® fusion protein (e.g., fusing NanoLuc® luciferase at both N and C terminus of the target protein) to empirically determine the optimal orientation of NanoLuc® luciferase.

Expression Level of Target-NanoLuc® Fusion Protein

For first-time experiments, we recommend using a 1:10 dilution (mass:mass) of expression construct into Transfection Carrier DNA when transiently transfecting cells for the NanoBRET™ TE assay. However, for some target proteins, the BRET signal can be sensitive to protein expression level. The expression level effect on assay performance can be easily evaluated by diluting the expression plasmids into Transfection Carrier DNA. In general, we recommend using low expression levels to ensure physiological conditions and avoid ligand depletion, as long as there is still sufficient donor signal to ensure a robust BRET process (e.g., having a donor signal to background of 1,000).

Co-Expression of Regulatory Proteins

Proteins often exist within a complex and their activities can be affected by the binding to regulatory partners. In some cases, we have observed an increased assay window when the target-NanoLuc® fusion protein is co-expressed with a regulatory partner protein (8). To co-express a regulatory partner protein, the amount of Transfection Carrier DNA used during transfection can be reduced or removed to accommodate inclusion of the expression vector for the regulatory protein.

Nonbinding Surface (NBS) Assay Format vs. Adherent (ADH) Assay Format

The NanoBRET™ TE assay can be performed in two primary plate formats, ADH and NBS, where ADH uses adhered cells in a tissue-culture treated assay plate (TC plates) and NBS uses freshly harvested cells suspended in a nonbinding surface (NBS) plate. Although we recommend starting with the NBS format (described in this technical manual) due to the possibility that some NanoBRET™ tracers may behave unfavorably in TC plates, some tracers perform better in the ADH format in terms of potency. Moreover, the ADH format has multiple advantages, including workflow efficiencies that minimize cell handling and execution time, and greater comparability to other cell-based assays performed using adhered cells. Please see the Appendix, Section 11, for how to set up the NanoBRET™ TE assay in ADH format.

Note: Not all NBS plates are equivalent for running the NanoBRET™ TE assays. We have had better success with NBS plates from Corning® (e.g., Corning® Cat.# 3600). The performance of the NanoBRET™ TE assays in ADH format is typically consistent across TC plates from different vendors.

8.D. Comparing Live-Cell Mode to Permeabilized-Cell Mode

Live-cell target engagement is influenced by the composite effect of many cellular factors (e.g., plasma membrane permeation, competing intracellular metabolites and protein complexes). In contrast, permeabilized cell analysis dilutes many of these competing factors to nearly negligible levels, providing a valuable control in NanoBRET™ assay development workflows. Therefore, we recommend evaluating novel NanoBRET™ tracers in both live cells and permeabilized cells, as the permeabilized cells can serve as a control to assess tracer permeability. Tracers with good assay windows in permeabilized-cell mode but poor performance in live-cell mode could suffer from low permeability, whereas tracers with low assay window in both modes would likely have low intrinsic affinity for the target protein.

Depending on the target protein and the parental compound used for tracer design, intracellular metabolites can also compete or interfere with tracer target engagement, which can reduce the assay window in live-cell mode compared to that observed in permeabilized-cell mode. As an example, intracellular ATP concentration can affect kinase engagement by tracers that bind to the kinase ATP-binding pocket, and the loss of ATP from permeabilized cells can contribute to an improvement in apparent tracer affinity. This effect of ATP concentration on apparent tracer affinity could be assessed by depleting intracellular ATP while keeping the cells intact and/or by titrating in exogenous ATP in the permeabilized-cell mode (7).

8.E. Suggestions for Optimizing Tracer Design

If no tracer candidate was found to provide an adequate assay in live-cell mode of the NanoBRET™ TE assay, further tracer molecule optimization can improve assay performance.

Low intrinsic affinity (poor assay window in both live cells and permeabilized cells): A new parent compound with higher affinity for the target protein may lead to improved tracer affinity. Alternatively, the fluorophore conjugation site may negatively affect binding affinity and the site of conjugation could be optimized.

Low permeability (poor assay window in live cells): Linkers can affect the tracer molecule permeability. We recommend assessing multiple linker variants when developing novel NanoBRET™ tracers.

Interference by cellular metabolites (poor assay window in live cells): Increasing the intrinsic tracer affinity or permeability can help the tracer compete with endogenous metabolites for target binding.

9. **Case Study: Developing Live-Cell NanoBRET™ Tracers Targeting Aurora Kinase A (AURKA) from Amine-Reactive and Click-Reactive Precursors (9)**

1. Two candidate compounds were identified from screening DNA-Encoded Library (DEL) against aurora kinase A (AURKA) and functionalized with a terminal-free amine or alkyne for tracer synthesis (Figure 10, Panel A).

2. The functionalized compounds were conjugated to the NanoBRET™ 590 dyes as described in Section 4, generating a panel of eight tracer candidates (1C-1F and 2C-2F) (Figure 10, Panel B).

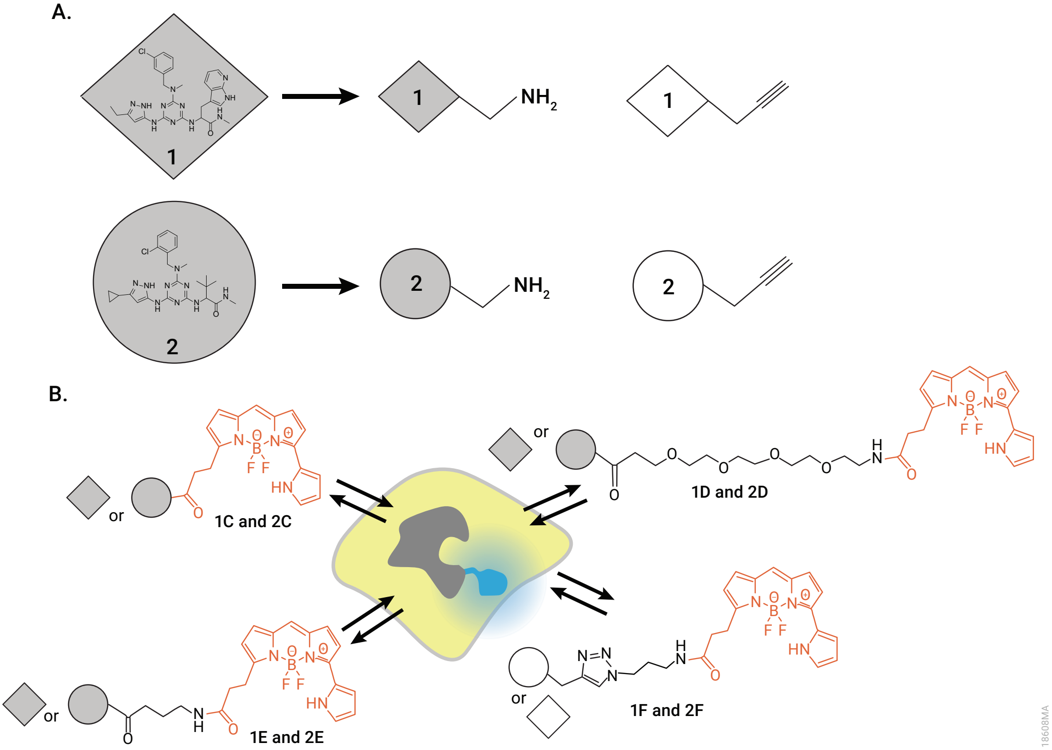


Figure 10. Small molecules and tracer candidates used in developing NanoBRET™ TE Assay for AURKA. Panel A. Two small-molecule binders of AURKA, compound 1 (diamond shape) and compound 2 (circle), were identified in a DEL screen and selected for tracer development. The amine-functionalized compounds are depicted in gray and the alkyne-functionalized compounds are depicted with empty shapes. **Panel B.** Candidate NanoBRET™ TE tracers prepared by coupling compound 1 or compound 2 to the NanoBRET™ 590 dye with a minimal linker (1C and 2C), a O4 PEG linker (1D and 2D), a 3-carbon alkyl linker (1E and 2E) or a triazole moiety formed by Click chemistry (1F and 2F).

9. Case Study: Developing Live-Cell NanoBRET™ Tracers Targeting Aurora Kinase A (AURKA) from Amine-Reactive and Click-Reactive Precursors (9; continued)

3. All eight tracer candidates were evaluated for BRET activity in HEK293 cells transiently transfected with AURKA-NanoLuc® fusion vector. The tracer candidates were assessed in both live cells and permeabilized cells (Figure 11, Panels A and B), as described in Section 6 and 7. While all eight tracer candidates generated a strong BRET signal in permeabilized cells, only tracer candidates derived from compound 2 produced specific BRET signal in live cells (Figure 11, Panel A).

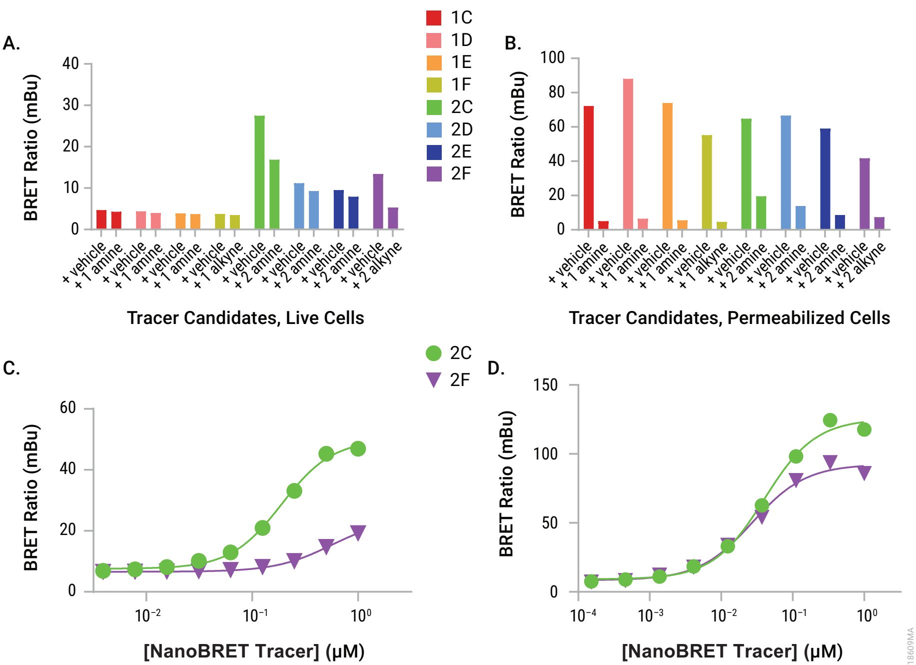


Figure 11. Evaluation of tracer candidates against AURKA-NanoLuc® Fusion protein in permeabilized and live cells. Preliminary tracer screen for AURKA in live cells (**Panel A**) and permeabilized cells (**Panel B**). Each bar represents a different tracer candidate that was tested either in the presence or absence of its respective "1" or "2" functionalized parent compound (at 10µM). Tracer affinity was measured as dose-response in live cells for 2C and 2F (**Panel C**) and in permeabilized cells (**Panel D**). Individual curves were fitted with the sigmoidal dose-response curve to determine the apparent affinity for the tracer.

4. A dose-response experiment was performed for tracer candidates 2C and 2F, the two tracers generating the most robust energy transfer, to measure the apparent tracer affinity for AURKA in live cells (Figure 11, Panel C) and intrinsic affinity in permeabilized cells (Figure 11, Panel D). Tracer candidate 2C was more potent and was selected for quantitative characterization.
5. A matrix experiment where cells were cotreated with serially diluted 2C and serially diluted parent compound 2 amine was set up to assess the Cheng-Prusoff relationship for tracer 2C in permeabilized and live cells (Figure 12, Panels A and B). A tracer concentration suitable for quantitative analysis of test compound affinity was identified for 2C in both permeabilized and live cells.
6. Tracer 2C was used to evaluate both intrinsic affinity and apparent affinity for the functionalized derivatives of compound 1 and 2 (Figure 12, Panels C and D). BRET values were normalized using the equation below.

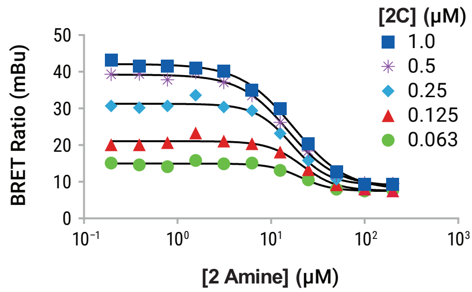
$$\text{Normalized BRET (\%)} = \frac{A - C}{B - C} \times 100$$

Where A = mBu in the presence of test compound and tracer, B = mBu in the presence of vehicle and tracer, and C = mBu in the presence of a saturating dose of inhibitor.

All of the functionalized precursors demonstrated similar intrinsic target affinity in permeabilized cells (Figure 12, Panel D); however, primary amine derivatives (1 amine and 2 amine) demonstrated weak apparent affinity in live cells, consistent with poor permeability (Figure 12, Panel C). In contrast, the alkyne-functionalized precursors demonstrated stronger apparent affinity in live cells, consistent with better permeability.

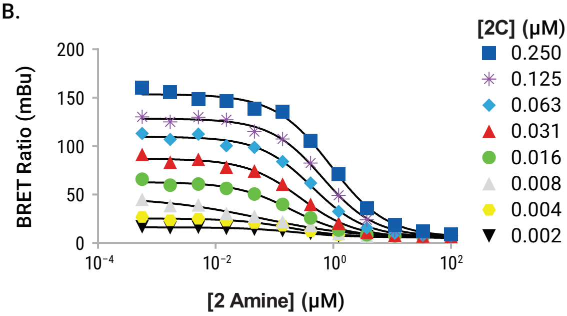
9. Case Study: Developing Live-Cell NanoBRET™ Tracers Targeting Aurora Kinase A (AURKA) from Amine-Reactive and Click-Reactive Precursors (9; continued)

A.



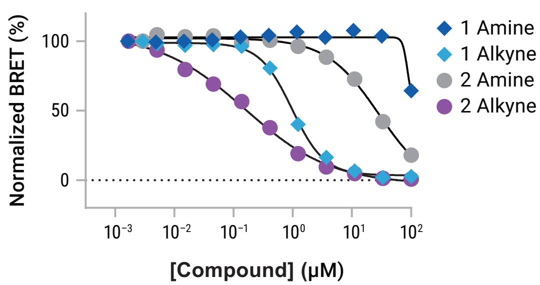
[2C] (µM)	1.0	0.5	0.25	0.125	0.063
IC_{50} (µM)	17	15	17	22	21

B.



[2C] (µM)	0.250	0.125	0.063	0.031	0.016	0.008	0.004	0.002
IC_{50} (µM)	0.91	0.66	0.44	0.28	0.20	0.057	0.19	0.24

C.



D.

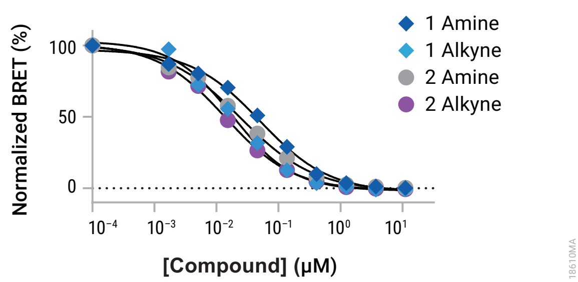


Figure 12. Determination of Target Engagement Characteristics for Functionalized Parent Compounds Using the Optimized NanoBRET™ TE Assay. Dose-response curves for parent compound 2 amine at each individual tracer concentrations for 2C in live cells (**Panel A**) and permeabilized cells (**Panel B**). Individual curves were fitted with the sigmoidal dose-response curve to determine the IC_{50}. The apparent affinity of the four functionalized parent compounds were measured in live cells (**Panel C**) versus intrinsic affinity in permeabilized cells (**Panel D**). Although each functionalized precursor demonstrated similar intrinsic affinity, the apparent affinity of the amine-functionalized precursors in live cells was compromised.

10. 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 polystyrene materials during transfer of tracer working reagents to minimize tracer adsorption
	Instrument was set up improperly. Use the correct filters for donor wavelength (450nm) and acceptor wavelength (590nm) on your instrument to accurately measure NanoBRET™ signals.
	Poor expression levels of the NanoLuc® fusion protein. Not all fusion proteins express identically in HEK293 cells, though the majority express adequately using the transfection conditions described in Section 5. If poor expression is suspected, determine the luminescence (RLU) from the donor (450nm) and acceptor (610nm) channels for cells expressing the NanoLuc® fusion (the signal) and compare that to the donor and acceptor RLU in the absence of cells expressing NanoLuc® (the background). If the signal-to-background ratio for the donor is less than 1,000, consider optimizing transfection conditions. Transfection optimization could involve increasing the ratio of the NanoLuc® fusion vector to the transfection carrier DNA while keeping the total DNA in the transfection mixture fixed. Moreover, cell density at time of harvest can affect transfectability. Newly passaged HEK293 cells (ideally within 1–2 days) should be harvested at 80–95% confluency for optimal transfectability. HEK293 cells approaching confluency do not transfect as efficiently.
	Extracellular NanoLuc® Inhibitor was added to permeabilized-cell mode assay plates. Extracellular NanoLuc® Inhibitor should not be used in the permeabilized-cell mode, as this will quench the signal.
NanoBRET™ signal observed in permeabilized-cell mode, but not in live-cell mode	The tracer may not be cell permeable or may be outcompeted by cellular metabolites that bind to target protein. Redesign tracer (Section 8.E).
Incorrect plate type used	Some NBS plates won't work with this assay. We recommend using Corning® nonbinding surface assay plates (Corning® Cat.# 3600 or Cat.# 3574).
NanoBRET™ signal observed in live-cell mode, but not in permeabilized-cell mode	Extracellular NanoLuc® Inhibitor was added to permeabilized-cell mode assay plates. Extracellular NanoLuc® Inhibitor should not be used in the permeabilized-cell mode, as this will quench the signal.

10. Troubleshooting (continued)

Symptoms	Causes and Comments
NanoBRET™ signal observed in live-cell mode but not in permeabilized mode (continued)	Target protein binding by the tracer is affected by the protein complex formed with the target protein in live cells. Cell permeabilization caused dissociation of the protein complex, affecting target engagement by the tracer. Either co-express a regulatory protein (see Section 8.C.) and/or decrease the incubation time after adding digitonin (Section 7.C, Step 6).
	Target protein is unstable in permeabilized cell mode. Decrease the incubation time after adding digitonin (Section 7.C, Step 6) or add protease inhibitors (if target protein is known to be susceptible to proteolytic cleavage after cell lysis).
	NanoBRET™ TE assay can be unstable in permeabilized-cell mode. Ensure that the assay is performed at room temperature and shorten the incubation time post-permeabilization.
NanoBRET™ signal in the presence of tracer cannot be outcompeted by the control compound	Tracer has a low off rate. Add control compound first and incubate for 30 minutes before adding the tracer and proceeding with the assay.
Higher than expected NanoBRET™ signal is observed in the presence of test compound	Brightly-colored compounds can interfere with NanoLuc® donor signal detection, causing an artificial increase in BRET values.
Observed IC_{50} value of test compound is right-shifted compared to expected value	When performing the live-cell mode assay, target engagement may result in right-shifted pharmacology relative to that observed in a biochemical assay due to myriad cellular factors. These include permeability and the presence of intracellular complexes, among other variables. Moreover, target engagement parameters for full-length targets in a cellular context may differ from that of truncated domains commonly used in biochemical assays.
	The concentration of the NanoBRET™ tracer may affect the observed IC_{50} value. Carefully select tracer concentration (see Section 8.B). Determine a more accurate compound IC_{50} by optimizing the tracer concentration.
Donor or acceptor luminescence increases or decreases when tracer is added	This phenomenon is common but generally does not affect the assay. BRET that occurs between the NanoLuc® fusion protein and fluorescent tracer may result in a dose-dependent increase in acceptor luminescence with a corresponding decrease in donor luminescence. The effect of BRET on donor and acceptor luminescence may vary depending on the target and tracer used. Ratiometric BRET analysis mitigates the influence of fluctuations in raw luminescence from NanoLuc® luciferase. (See Figure 13.)

Symptoms	Causes and Comments
Noisy data	Poor expression levels of the NanoLuc® fusion protein. Not all fusion proteins express identically in HEK293 cells, though the majority express adequately using the transfection conditions described in Section 5. If poor expression is suspected, determine the luminescence (RLU) from the donor (450nm) and acceptor (610nm) channels for cells expressing the NanoLuc® fusion (the signal) and compare that to the donor and acceptor RLU in the absence of cells expressing NanoLuc® (the background). If the signal-to-background ratio is less than 1,000, consider optimizing transfection conditions, including increasing the ratio of the NanoLuc® fusion vector to the transfection carrier DNA while keeping the total DNA in the transfection mixture fixed. Moreover, cell density at time of harvest can affect transfectability. Freshly passaged HEK293 cells (ideally within 1–2 days) should be harvested at 80–95% confluency for optimal transfectability. HEK293 cells approaching confluency do not transfect as efficiently.
	Instability of the NanoBRET™ TE assay in the permeabilized-cell mode. Maintain the same incubation time for all plates post-permeabilization.

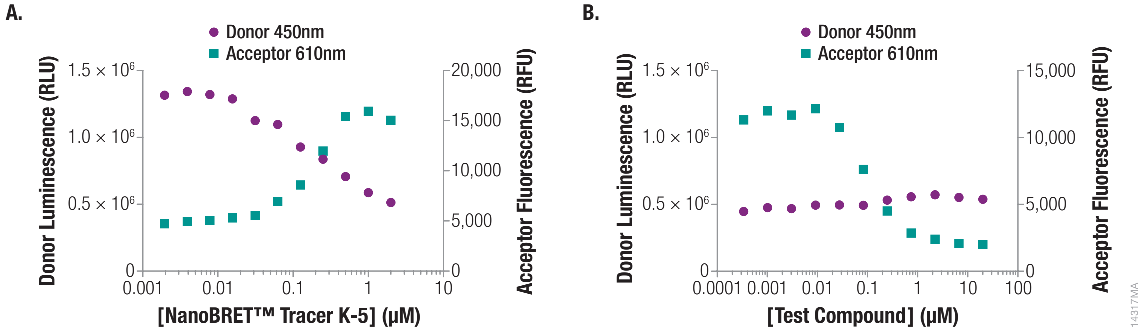


Figure 13. Potential effects of raw luminescence on donor and acceptor emission in the NanoBRET™ TE Assay in a tracer dose-response experiment or compound dose-response experiment. Panel A. The tracer dose-response experiment for the BTK-NanoLuc® fusion protein. **Panel B.** The compound dose-response experiment for the BTK-NanoLuc® fusion protein was performed using a fixed 1μM of tracer and a dilution series of the unlabeled compound.

11. Appendix

11.A. Adherent (ADH) Assay Format

The NanoBRET™ TE Assay can be performed in two primary plate formats, ADH and NBS, where ADH uses adhered cells in TC plates and NBS uses freshly harvested cells suspended in NBS plates (Figure 14). Each format offers potential advantages and disadvantages depending on the nature of the work being performed. The ADH format has the advantage of adhered cells and tissue-culture treated plates, which may be more comparable to other cell-based assays performed using adhered cells. Moreover, the ADH format creates workflow efficiencies that minimize cell handling steps and shorten the total execution time. Lastly, some tracers actually perform better in the ADH format in terms of tracer potency. In contrast, some test compounds or NanoBRET™ tracer behave unfavorably in TC plates and require assays to be performed in NBS plates for optimal performance.

The protocol in this technical manual is written for the NBS format. An abbreviated protocol for performing the NanoBRET™ TE assay in ADH format is shown in Figure 14.

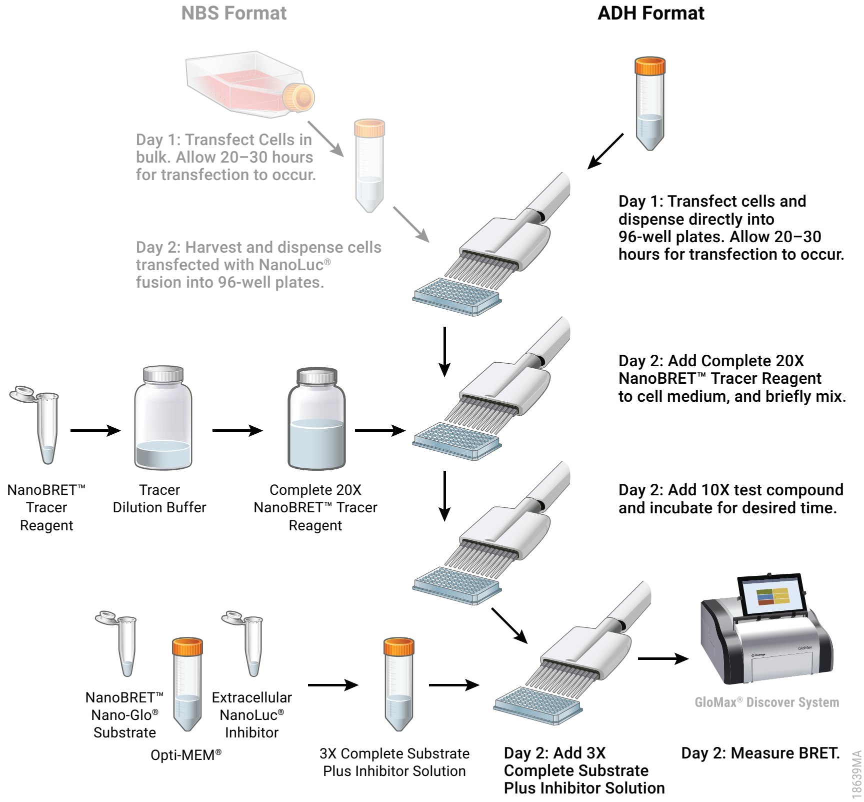


Figure 14. Overview of the NanoBRET™ TE Assay in ADH format compared to NBS format (shaded area).

Materials to Be Supplied By the User

- Opti-MEM™ I Reduced Serum Medium, without phenol red (Thermo Fisher Scientific Cat.# 11058-021)
- Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific Cat.# 11995-065)
- fetal bovine serum (HyClone Cat.# SH30070.03, Seradigm Cat.# 1500-050)
- 0.05% Trypsin/EDTA (Thermo Fisher Cat.# 25300)
- white, tissue culture treated 96-well plates (Corning Cat.# 3917)
- tissue culture equipment and reagents
- polypropylene plasticware (**Note:** Do not use polystyrene plasticware for this assay.)
- FuGENE® HD Transfection Reagent (Cat.# E2311)
- NanoBRET™ Tracer Dilution Buffer (Cat.# N2191)
- Transfection Carrier DNA (Cat.# E4881)
- Intracellular TE Nano-Glo® Substrate/Inhibitor (Cat.# N2160)
- tracers, 100–400µM in DMSO (see Section 4 for tracer synthesis and preparation)
- transfection-grade plasmid encoding target-NanoLuc® fusion proteins driven by a mammalian promoter (see Section 3.C)
- DMSO (Sigma Cat.# 2650)
- detection instrument capable of measuring NanoBRET™ wavelengths (e.g., GloMax® Discover System, Cat.# GM3000; see Section 11.B)
- Digitonin (Cat.# G9441, 20mg/ml in DMSO; Sigma Cat.# D141 prepared at 50mg/ml in DMSO; **Note:** Only needed for permeabilized-cell mode).

Composition of Buffers and Solutions

cell culture medium	assay medium
90% DMEM	99% Opti-MEM™ I Reduced Serum Medium, no phenol red
10% fetal bovine serum	1% fetal bovine serum

Transient Transfection of HEK293 Cells with Target-NanoLuc® Fusion Vectors

1. Cultivate HEK293 cells (or desired cell type) appropriately prior to assay.

   Notes:

   a. This protocol has been optimized for HEK293 cells. If other cell types are used, optimize the transfection conditions.

   b. For optimal performance, users should use cells that have been freshly passaged, ideally within 1–2 days.

2. Remove medium from cell flask by aspiration, trypsinize and allow cells to dissociate from the flask.
3. Neutralize trypsin using cell culture medium and centrifuge at 200 × *g* for 5 minutes to pellet cells.
4. Aspirate medium and resuspend cells in assay medium.

11.A. Adherent (ADH) Assay Format (continued)

5. Adjust density to 2×10^5 cells/ml using assay medium.

   Note: Exceeding this number of cells can result in poor signal-to-background levels.

6. If HEK293 cells are used, prepare lipid:DNA complexes as follows:

   a. Prepare a 10µg/ml solution of DNA in Opti-MEM™ I reduced serum medium, without phenol red, that contains: 9.0µg/ml of Transfection Carrier DNA, 1.0µg/ml of target-NanoLuc® Fusion Vector DNA and 1ml of Opti-MEM™ I medium.

   b. Mix thoroughly.

   c. Add 30µl of FuGENE® HD Transfection Reagent to each milliliter of DNA mixture to form a lipid:DNA complex. Ensure that the FuGENE® HD Transfection Reagent does not touch the plastic side of the tube; pipet directly into the liquid in the tube.

   d. Mix by inversion 5–10 times.

   e. Incubate at ambient temperature for 20 minutes to allow complexes to form.

7. In a sterile conical tube, mix 1 part of lipid:DNA complex (e.g., 1ml) with 20 parts of HEK293 cells (e.g., 20ml) in suspension at 2×10^5 cells/ml. Mix gently by inversion 5 times.

   Note: Larger or smaller bulk transfections should be scaled accordingly, using this 20:1 cells to lipid:DNA complex ratio.

8. Dispense 100µl cells + lipid:DNA complex (for live-cell mode) or 90µl cells + lipid:DNA complex (for permeabilized-cell mode) per well into a sterile tissue-culture treated 96-well assay plate and incubate for 20–30 hours. We recommend a cell density of approximately 55,000–80,000 cells/cm^2 during the transfection. For example, use approximately 20,000 cells/well for a 96-well (Corning® Cat.# 3917) assay plate.

9. Proceed with live-cell mode (see Section 6) or with permeabilized-cell mode (see Section 7) but omit the replating steps (Step 1–5, Section 6.A and Steps 1–5, Section 7.B).

11.B. Instrument Setup

Measurement of luminescence with dual-wavelength windows is typically accomplished using filters. We recommend using a band pass (BP) filter for the donor signal and a long pass filter (LP) for the acceptor signal to maximize sensitivity.

- The NanoBRET™ bioluminescent donor emission occurs at 460nm. To measure this donor signal, we recommend a band pass (BP) filter that covers close to 460nm with a band pass range of 8–80nm. For example, a 450nm/BP80 will capture the 410nm to 490nm range.
  Note: A BP filter is preferred for the donor signal measurement to selectively capture the signal peak and avoid measuring any acceptor peak bleed-through. However, a short pass (SP) filter that covers the 460nm area also can be used. This may result in an artificially large value for the donor signal and measuring the bleedthrough into the acceptor peak, which could compress the ratio calculation and reduce the assay window.
- The NanoBRET™ acceptor peak emission occurs at approximately 590–610nm. To measure the acceptor signal, we recommend a long pass filter starting at 600–610nm.

Instruments capable of dual-luminescence measurements are either equipped with a filter selection or the filters can be purchased and added separately. For instruments using mirrors, select the luminescence mirror. An integration time of 0.2–1 second is typically sufficient. Ensure that the gain on the PMT is optimized to capture the highest donor signal without reaching instrument saturation.

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

- The GloMax® Discover System (Cat.# GM3000) with preloaded filters for donor 450nm/8nm BP and acceptor 600nm LP. Select the preloaded BRET:NanoBRET™ 618 protocol from the Protocol menu.
- BMG Labtech CLARIOstar® with preloaded filters for donor 450nm/80nm BP and acceptor 610nm LP
- Thermo Varioskan® with filters obtained from Edmunds Optics, using donor 450nm CWL, 25mm diameter, 80nm FWHM, Interference Filter and acceptor 1 inch diameter, RG-610 Long Pass Filter.

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

- Mirror: Luminescence – Slot4
- Emission filter: Chroma Cat.# AT600LP- EmSlot4
- Second emission filter: Chroma Cat.# AT460/50m – EmSlot1
- Measurement height: 6.5mm
- Measurement time: 1 second

11.C. Achieving Adequate 96-Well and 384-Well Format Plate Mixing

Due to the viscosity of the Tracer Dilution Buffer, pay special attention to plate mixing to ensure that the Complete 20X NanoBRET™ Tracer Reagent is adequately dispersed in the assay. 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 formats, most commercially available orbital shakers will be capable of dispersing the tracer reagent, though optimizing the shaking force may be required (typically by visual inspection). For the 384-well assay format, we recommend using specific equipment that creates a mixing vortex in the wells of 384-well plates. We have experience with horizontal shakers such as the Vibra-Translator™ 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.

11.D. Preparing Stable Cell Lines Expressing NanoLuc® Fusion Proteins

Some users may prefer to use stable cell lines for the NanoBRET™ assay as opposed to cells that have been transiently transfected. This approach can help to shorten the assay workflow and improve day-to-day assay variability. However, the NanoLuc® expression vectors recommended for transient transfection use relatively strong constitutive promoters (e.g., CMV). To avoid overexpression in stable cell lines, we recommend the use of attenuated promoters (e.g., CMV deletion promoters, the PGK promoter or the TK promoter) for appropriate expression of the NanoLuc® fusion protein.

An alternative to stable cell lines is the use of assay-ready cells that have been pretransfected with the NanoLuc® fusion protein of interest. Assay-ready cells can be prepared in advance, in bulk, aliquoted and cryopreserved, after which individual tubes can be thawed on demand and used immediately in the NanoBRET™ TE assay. This approach can also help to shorten the assay workflow and improve day-to-day assay variability.

11.E. References

1. Machleidt, T. *et al.* (2015) NanoBRET-A novel BRET platform for the analysis of protein-protein interactions. *ACS Chem. Biol.* **10**, 1797–804.
2. Robers, M.B. *et al.* (2015) Target engagement and drug residence time can be observed in living cells with BRET. *Nat. Commun.* **6**, 10091.
3. Kolb, H.C., Finn, M.G. and Sharpless, K.B. (2001) Click Chemistry: Diverse Chemical Function from a Few Good Reactions. *Angew. Chem. Int. Ed. Engl.* **40** 2004–21.
4. Robers, M.B. *et al.* (2019) Quantitative, real-time measurements of intracellular target engagement using energy transfer. *Methods Mol. Biol.* **1888**, 45–71.
5. Lipinski, C.A. *et al.* (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug. Deliv. Rev.* **46**, 3–26.
6. Anthropological Genetics: Theory, Methods and Applications, Michael H. Crawford, ed. (2006) University of Cambridge Press, Table 10.1.
7. Vasta, J.D.*et al.* (2018) Quantitative, wide-spectrum kinase profiling in live cells for assessing the effect of cellular ATP on target engagement. *Cell Chem. Biol.* **25**, 206–14.
8. Wells, C.I. *et al.* (2020) Quantifying CDK inhibitor selectivity in live cells. *Nat. Commun.* **11**, 2743.
9. Teske, K.A. *et al.* (2023) DELs enable the development of BRET probes for target engagement studies in cells. *Cell Chem. Biol.* **https://doi.org/10.1016/j.chembiol.2023.06.019**

11.F. Related Products

NanoBRET TE 590 Dyes Companion Reagents

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

*Additional sizes are available.

Intracellular TE Nano-Glo® Substrate/Inhibitors

Product	Size	Cat.#
Intracellular TE Nano-Glo® Substrate/Inhibitor	1,000 assays	N2160
Intracellular TE Nano-Glo® Vivazine/Inhibitor	1,000 assays	N2200

Additional sizes are available.

NanoLuc® Vectors

Product	Size	Cat.#
pFN31A Nluc CMV-Hygro Flexi® Vector	20µg	N1311
pFN31K Nluc CMV-neo Flexi® Vector	20µg	N1321
pFC32A Nluc CMV-Hygro Flexi® Vector	20µg	N1331
pFC32K Nluc CMV-neo Flexi® Vector	20µg	N1341
pNLF1-N [CMV/Hygro] Vector	20µg	N1351
pNLF1-C [CMV/Hygro] Vector	20µg	N1361

Transfection Reagents

Product	Size	Cat.#
FuGENE® HD Transfection Reagent	1ml	E2311
ViaFect™ Transfection Reagent	0.75ml	E4981
FuGENE® 4K Transfection Reagent	1ml	E5911

Additional sizes are available.

11.F. Related Products (continued)

Detection Instrument

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

For Research Use Only. Not For Use in Diagnostic Procedures

NanoBRET™ Dyes Limited Use Label License

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[b]U.S. Pat. Nos. 10,067,149 and 10,024,862, European Pat. No. 2932267, Japanese Pat. Nos. 6751294 and 7092691 and other patents and patents pending.