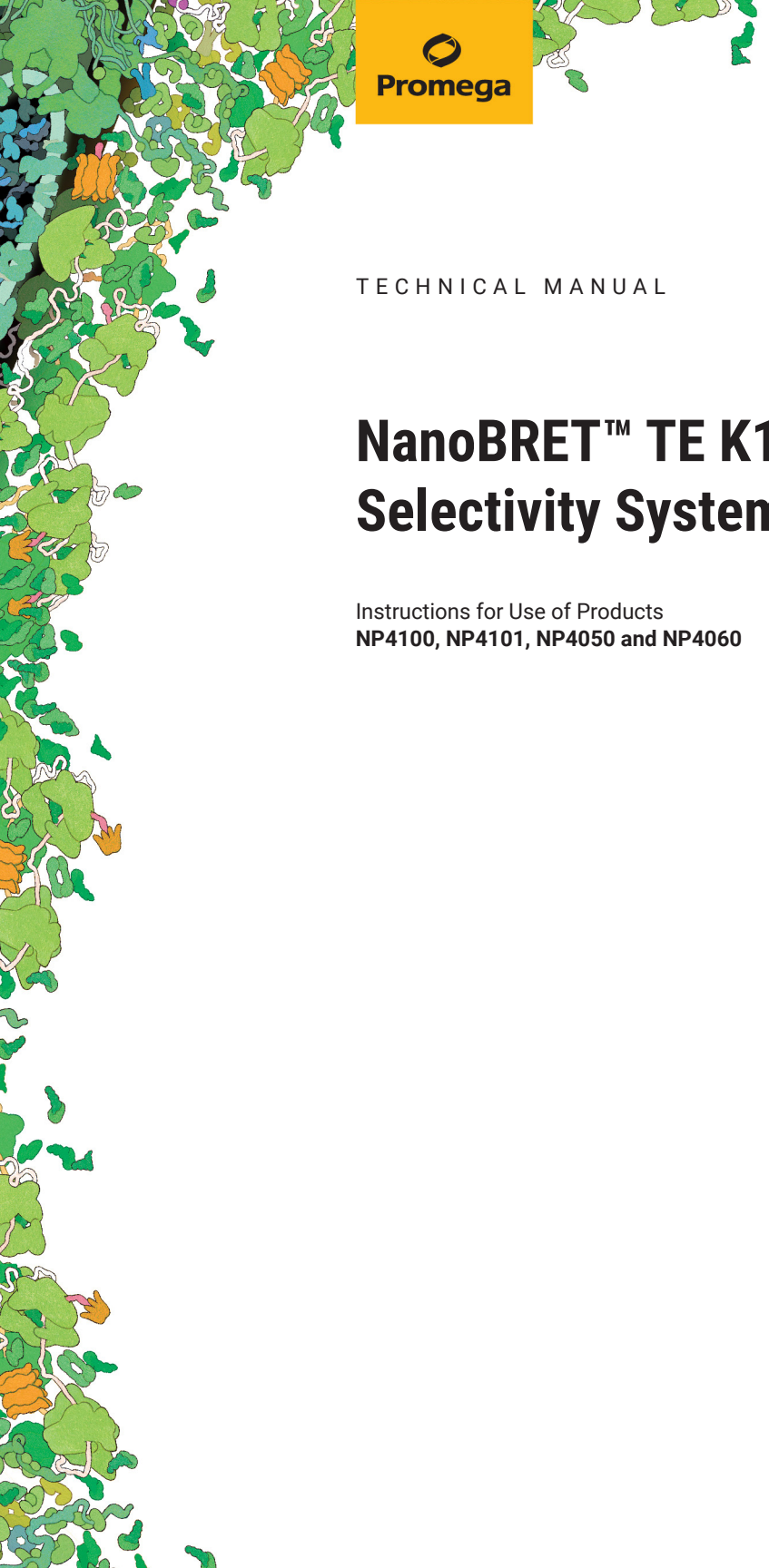

TECHNICAL MANUAL

NanoBRET™ TE K192 Kinase Selectivity System

Instructions for Use of Products
NP4100, NP4101, NP4050 and NP4060

Revised 1/24
TM683

NanoBRET™ TE K192 Kinase Selectivity System

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

1. Description

NanoBRET™ Target Engagement K192 Kinase Selectivity Systems[a–k] enable researchers to profile kinase inhibitor selectivity in live cells across a diverse panel of 192 human kinases. The foundation of this selectivity system is the NanoBRET™ Target Engagement (TE) technology, a bioluminescence resonance energy transfer (BRET) method that uses the small, extremely bright NanoLuc® luciferase (Figure 1). NanoBRET™ TE technology quantitatively measures compound binding affinity and occupancy at select target proteins within live intact cells (1). It has been successfully applied to many target classes, such as kinases (2–4). To enable a simplified live-cell kinase profiling method, TE is quantified at each kinase-NanoLuc® fusion using a single broad-spectrum NanoBRET™ tracer (K-10).

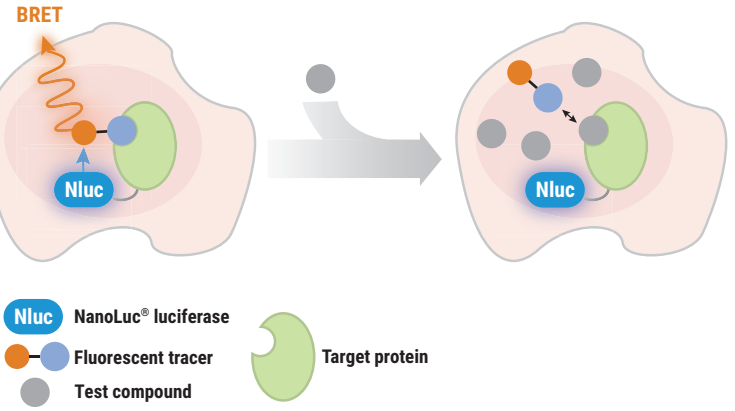

Figure 1. NanoBRET™ TE Assay Technology. The target of interest is expressed in mammalian cells as a target-NanoLuc® fusion construct. Compound engagement is measured in a competitive format using a cell-permeable fluorescent tracer. BRET is achieved by luminescent energy transfer from NanoLuc® luciferase to the cell-permeable fluorescent tracer that is bound to the target-NanoLuc® fusion protein. A test compound that is cell-permeable and competes with the tracer for target binding will result in a loss of NanoBRET™ signal. Additional details about the fundamental biophysical principles underlying the NanoBRET™ TE technology can be found in the *NanoBRET™ Target Engagement Intracellular Kinase Assay, Adherent Format Technical Manual*, #TM598.

The NanoBRET™ TE K192 Kinase Selectivity System provides a novel microplate-based workflow that determines live-cell compound occupancy across 192 kinases in a single experiment, using common laboratory equipment. The system includes the NanoBRET™ TE K192 Kinase Vector Panel[a] and the NanoBRET™ TE Intracellular Kinase Assay K-10[b–d] (Figure 2).

- The NanoBRET™ TE K192 Kinase Vector Panel contains 192 unique kinase-NanoLuc® luciferase fusion vectors that are arrayed in 96-well plates and are transfection ready. On day 1 of the experiment, the user transfects the K192 vector panel into HEK293 cells. Each kinase expressed by a vector within this panel is full-length.
- The NanoBRET™ TE Intracellular Kinase Assay, K-10, contains the necessary reagents to run the 192 kinase NanoBRET™ TE assays in a single experiment on day 2. NanoBRET™ Tracer K-10 has been optimized for maximum kinome coverage, enabling a single-reagent design for broad spectrum profiling. Furthermore, each kinase within the NanoBRET™ Target Engagement K192 Kinase Vector Panel uses the NanoBRET™ Tracer K-10 at one of four concentrations. The tracer concentrations were optimized to provide quantitative occupancy data for each kinase, further simplifying the assay. A test compound that competes with tracer for binding to the target kinase will result in a loss of BRET signal. Controls are used to calibrate the BRET competition, which allows the calculation of fractional occupancy for the test compound.

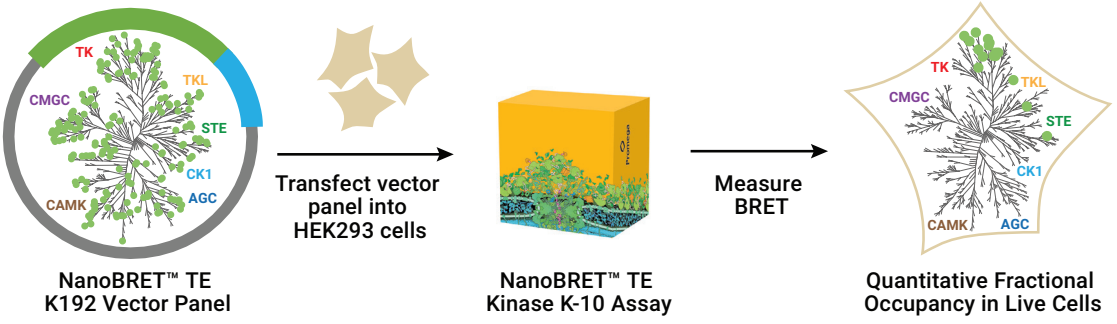

Figure 2. Overview of the NanoBRET™ TE K192 Kinase Selectivity System. Compound selectivity against a panel of 192 full-length kinases representing the kinome can be determined in live cells using this system.

Kinases are essential to myriad cellular process from regulation of cell physiology to signal transduction. As such, kinase activity misregulation is a common driver of various pathologies and kinases are key therapeutic targets for drug development. Historically, panels of in vitro biochemical assays have been used to evaluate compound binding selectivity across the kinome. Though biochemical methods are excellent for determining drug binding characteristics in a purified setting, they can fail to predict drug selectivity in a live-cell environment. Reasons for failure can include variable kinase activation state, the use of truncated proteins instead of full-length targets, the presence of binding partners in cells that may influence target behavior and competitive effects from high and unpredictable concentrations of intracellular ATP (3). Using a large and diverse collection of NanoBRET™ TE assays for full-length kinases demonstrated that the selectivity for FDA-approved drugs crizotinib and dasatinib is markedly improved in a live-cell environment compared to measurements from biochemical binding assays (3). This study highlights the importance of methods such as the NanoBRET™ TE K192 Kinase Selectivity System that determine drug selectivity in a cellular environment.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
NanoBRET™ TE K192 Kinase Selectivity System	**1 each**	**NP4050**

Not for Medical Diagnostic Use. Each kit contains enough DNA for 50 analyses for each of 192 kinase-NanoLuc® fusions. Includes:

- 1 each NanoBRET™ TE K192 Kinase Vector Panel
- 1 each NanoBRET™ TE Intracellular Kinase Detection Reagent, K-10 (10,000 assays)

PRODUCT	SIZE	CAT.#
NanoBRET™ TE K192 Kinase Selectivity System with Controls	**1 each**	**NP4060**

Not for Medical Diagnostic Use. Each kit contains enough DNA for 50 analyses for each of the 192 kinase-NanoLuc® fusions. Includes:

- 1 each NanoBRET™ TE K192 Kinase Vector Panel
- 1 each NanoBRET™ TE Intracellular Kinase Detection Reagent, K-10 (10,000 assays)
- 1 each NanoBRET™ TE Selectivity DNA Controls

PRODUCT	SIZE	CAT.#
NanoBRET™ TE K192 Kinase Vector Panel	**1 each**	**NP4100**

Not for Medical Diagnostic Use. Includes:

- 2 each NanoBRET™ TE K192 Vector Plate A
- 2 each NanoBRET™ TE K192 Vector Plate B
- 1 each Adhesive Foil Plate Seals

PRODUCT	SIZE	CAT.#
NanoBRET™ TE K192 Kinase Vector Panel, Small	**1 each**	**NP4101**

Not for Medical Diagnostic Use. Includes:

- 1 each NanoBRET™ TE K192 Vector Plate A
- 1 each NanoBRET™ TE K192 Vector Plate B
- 1 each Adhesive Foil Plate Seals

Storage Conditions: Store the NanoBRET™ TE K192 Kinase Vector Panel Plates and NanoBRET™ TE Selectivity DNA Controls at –30°C to –10°C. Store the NanoBRET™ TE Intracellular Kinase Detection Reagent, K-10 at less than –65°C. Alternatively, store the NanoBRET™ Tracer K-10 at less than –65°C and all other components at –30°C to –10°C. We recommend aliquoting NanoBRET™ Tracer K-10 and avoiding more than five freeze-thaw cycles. Store the NanoBRET™ Tracer K-10, NanoBRET™ Nano-Glo® Substrate and Extracellular NanoLuc® Inhibitor protected from light. Store Adhesive Foil Plate Seals at room temperature.

3. Before You Begin

3.A. NanoBRET™ Target Engagement K192 Kinase Vector Panel

There are a total of 192 kinase-NanoLuc® fusion vectors provided in this panel, which are supplied pre-arrayed in two 96-well plates. Each unique kinase-NanoLuc® fusion vector occupies a unique position in the 96-well plates. Each plate is heat sealed to maintain the integrity of each well and prevent evaporation.

The 192 kinase-NanoLuc® fusion vectors provided are in an easy-to-use, consumable format. The kinase-NanoLuc® fusion vectors are premixed with a specific carrier DNA, saving the user the work of preparing individual mixtures of the appropriate carrier DNA and kinase-NanoLuc® fusion vectors that are needed for optimal transfection and expression. This 10X formulation simply needs to be diluted prior to transfection, as described in Section 4.C.

The two 96-well plates of the NanoBRET™ TE K192 Kinase Vector Panel are labeled NanoBRET™ TE K192 Vector Plate A and NanoBRET™ TE K192 Vector Plate B. Cat.# NP4101 provides 1 copy of each plate for a total of 2 plates, and includes a sufficient amount of each kinase-NanoLuc® vector for a minimum of 25 analyses (one analysis per well of an assay plate) for each kinase. Cat.# NP4100 provides 2 copies of each plate for a total of 4 plates, and includes a sufficient amount of each kinase-NanoLuc® vector for a minimum of 50 analyses for each kinase. If you are using Cat.# NP4100 and plan to execute 25 analyses or less per kinase, you can work with one set of plates A and B at a time.

The pre-arrayed transfection-ready kinase-NanoLuc® fusion DNAs are organized in vertical columns within 96-well plates according to the concentration of the NanoBRET™ Tracer K-10 reagent that is used for the assay. The plate map and associated tracer K-10 concentrations used for each kinase in the analysis can be found in the K192 Plate Map file, available at: **www.promega.com/K192-Downloads**

3.B. NanoBRET™ TE Selectivity DNA Controls

For executing this assay, we recommend including specific control vectors that are found within the NanoBRET™ TE Selectivity DNA Controls. The set includes: 1) the NanoLuc® control vector; 2) the transfection control vector; and 3) Transfection Carrier DNA. The NanoBRET™ TE Selectivity DNA Controls are available from Promega (Cat.# NP1000), but are also included in the NanoBRET™ TE K192 Kinase Selectivity System with Controls (Cat.# NP4060). The preparation of these controls is described in Section 4.D.

Note: The NanoLuc® control vector is pNL1.1.CMV[Nluc/CMV] Vector (Cat.# N1091). The transfection control vector is NanoLuc®-HIPK2 Fusion Vector (Cat.# NV3221). The Transfection Carrier DNA is Cat.# E4881. All are available separately.

3.C. NanoBRET™ TE Intracellular Kinase Detection Reagent, K-10

In addition to the K192 vector panel and control DNAs, execution of this assay requires a few additional reagents, including:

- NanoBRET™ Tracer K-10
- NanoBRET™ Nano-Glo® Substrate
- Extracellular NanoLuc® Inhibitor
- Tracer Dilution Buffer

These reagents are provided in the NanoBRET™ TE Intracellular Kinase Detection Reagent, K-10 (Cat.# N2840), which is a component of the NanoBRET™ TE K192 Kinase Selectivity System (Cat.# NP4050) and NanoBRET™ TE K192 Kinase Selectivity System with Controls (Cat.# NP4060).

3.D. Cell Background

The NanoBRET™ TE K192 Kinase Selectivity System is a collection of 192 intracellular kinase assays that were originally optimized in a common cell background, HEK293 cells. As such, this assay panel has been validated specifically for use in either HEK293 cells (ATCC Cat.# CRL-1573) or TransfectNow™ HEK293 Cells (Cat.# NC1001, NC1002). The NanoBRET™ TE K192 Kinase Selectivity System has not been validated for use in any other cell type.

3.E. Instrument Requirements and Setup

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

- The NanoBRET™ bioluminescent donor emission occurs at 460nm. To measure this donor signal, we recommend a BP filter that covers close to 460nm with a band pass range of 8–80nm. For example, a 450nm/BP80 will capture the 410–490nm range.
  Note: A BP filter is preferred for the donor signal measurement to selectively capture the signal peak and avoid measuring any acceptor peak bleedthrough. However, a short pass (SP) filter that covers the 460nm area also can be used. This may result in an artificially large value for the donor signal and measuring the bleedthrough into the acceptor peak, which could compress the ratio calculation and reduce the assay window.
- The NanoBRET™ acceptor peak emission occurs at approximately 590–610nm. To measure the acceptor signal, we recommend a LP 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 instrument is optimized to capture the highest donor signal without reaching saturation.

Consult with your instrument manufacturer to determine if the proper filters are installed or for the information 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:

- 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. To use the EnVision® for NanoBRET™ Assays, Perkin Elmer supplies the following optics:

- EnVision Optimized Label-NanoBRET (PE Cat.# 2100-8530). It includes: dichroic mirror, 585nm (Cat.# 2100-4380); filter, 460/80nm (Cat.# 2100-5950); and filter 647/75nm (Cat.# 2100-5970).

4. General NanoBRET™ TE K192 Kinase Selectivity Assay Protocols

Details for additional materials required for the assay that need to be obtained independently are listed in Materials to Be Supplied by the User. Section 4.A discusses the treatment conditions and controls required for assay execution. Sections 4.B through 4.D provide details on preparing the working K192 panel DNAs and control DNAs, respectively. Sections 4.E–H provide details for the transfection and execution of the BRET assay, as well as guidance on data processing and quality control. Section 4.I provides an example plate layout and quick protocol that can be used to determine fractional occupancy in technical singlicate across the 192 kinases in the panel. Section 5.C lists the composition of required buffers and solutions. See Figure 3 for a schematic outlining the protocols.

Note: The protocol detailed in this technical manual has been optimized for 96-well plates only; use in 384-well plates is not currently supported.

Materials to Be Supplied by the User

- HEK293 cells (e.g., ready-to-use TransfectNow™ HEK293 Cells, Cat.# NC1001, NC1002; or prepared-by-user ATCC, Cat.# CRL-1573)
- Dulbecco's Modified Eagles Medium (DMEM; Thermo Fisher Scientific Cat.# 11995-065)
- fetal bovine serum (HyClone Cat.# SH30070.03, Seradigm Cat.# 1500-050)
- Opti-MEM™ I reduced serum medium, no phenol red (Life Technologies Cat.# 11058-021)
- white, tissue-culture treated 96-well plates (Corning® Cat.# 3917 is preferred; Greiner Cat.# 655083, alternate)
- polypropylene plasticware for tracer handling (**Note:** Polystyrene plasticware is **not** recommended for this assay).
- 0.05% Trypsin/EDTA (Thermo Fisher Cat.# 25300)
- FuGENE® HD Transfection Reagent (Cat.# E2311; **Note:** The transfection protocols used in this assay have been optimized using FuGENE® HD and may not be compatible with other transfection reagents.)
- DMSO (Sigma Cat.# D2650)
- NanoBRET™ TE Selectivity DNA Controls (Cat.# NP1000) **Note:** If the NanoBRET™ TE K192 Kinase Selectivity System with Controls (Cat.# NP4060) is being used, the NanoBRET™ TE Selectivity DNA Controls are included with that product.
- TE Buffer, 1X, Molecular Biology Grade (Cat.# V6231)
- detection instrument capable of measuring NanoBRET™ wavelengths (i.e., GloMax® Discover System, Cat.# GM3000; see Section 3.E).
- **optional:** CC1 pan-Kinase Inhibitor (control ligand; Cat.# N2661)

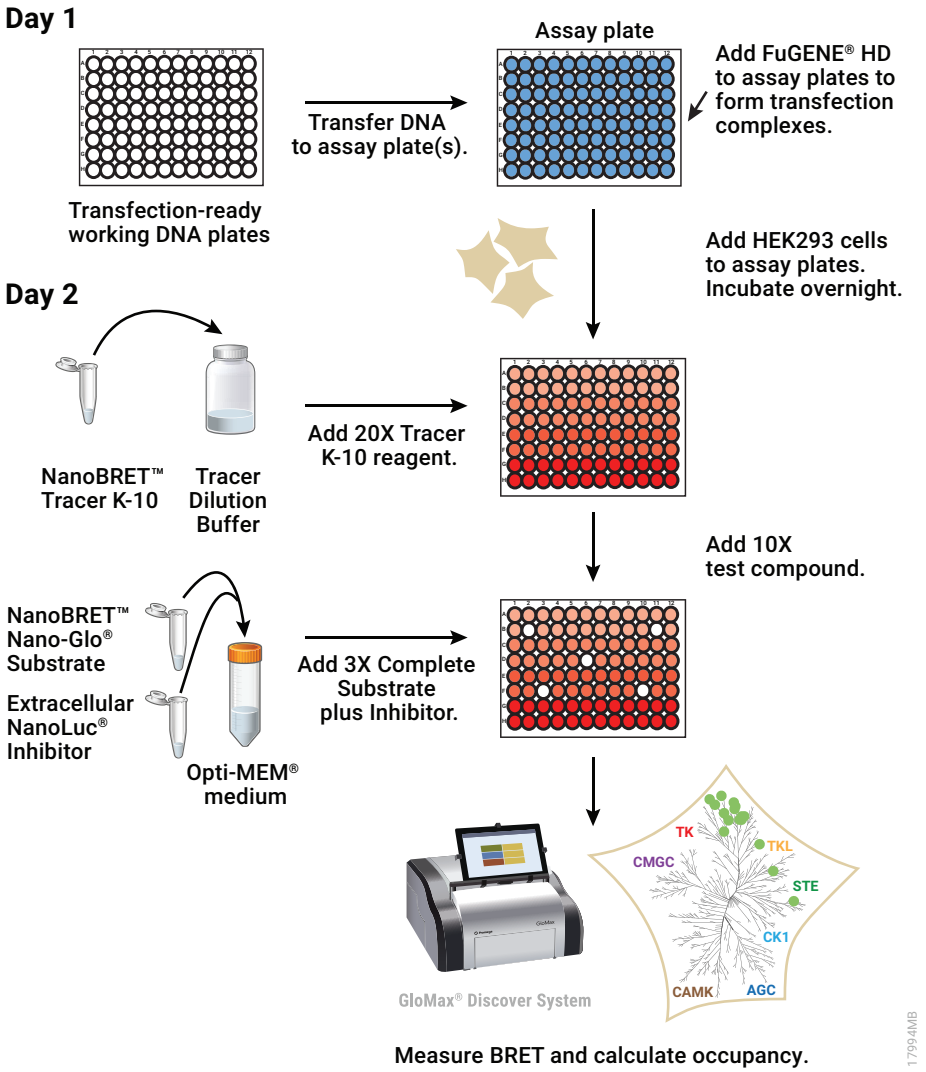

Figure 3. Schematic of the NanoBRET™ TE K192 Kinase Selectivity System protocol. On day 1, the transfection-ready kinase vectors in the NanoBRET™ TE K192 Vector Panel are transfected into HEK293 cells in assay plates. On day 2, the NanoBRET™ TE Intracellular Kinase K-10 Assay is then used to conduct the BRET analysis as follows: A single tracer (NanoBRET™ Tracer K-10) is used to form a BRET complex with each kinase in the panel, using tracer concentrations determined to provide quantitative data for each kinase. Competition of the BRET signal using a single dose of test compound allows you to determine fractional occupancy of the compound at each kinase within the panel.

4.A. Required Conditions

To calculate the fractional occupancy for a test drug at a specific dose using the NanoBRET™ Target Engagement K192 Kinase Selectivity System the following controls or conditions are required:

$BRET_{Min}$ Control: This control must be included in each experiment to define zero BRET or "100% Fractional Occupancy". The most efficient option for this control is to express the NanoLuc® control vector in cells and measure the BRET value. Adding the K-10 Tracer is not required. Specifically, the vector we recommend using as the NanoLuc® control is contained in the NanoBRET™ TE Selectivity DNA Controls (Cat.# NP1000) and is labeled pNL1.1.CMV[Nluc/CMV] Vector.

The $BRET_{Min}$ Control that expresses the NanoLuc® control vector can be placed in multiple wells on each assay plate, or it minimally needs to be placed in several wells on one of the assay plates. We recommend a minimum of two wells for the NanoLuc® control vector in each experiment. See Section 4.I for an example plate layout with NanoLuc® controls. Preparation of the NanoLuc® control vector for transfection is detailed in Section 4.D.

$BRET_{Max}$ Control: This control is needed for each kinase in the K192 panel. Specifically the $BRET_{Max}$ Control is used to define the maximum BRET or "0% Fractional Occupancy" for each kinase, and uses the K-10 Tracer + DMSO vehicle (or test compound solvent). Use of this control is detailed in Section 4.H to perform data quality control and to calculate test compound fractional occupancy.

Sample: This condition is tracer + test compound for each kinase.

4.B. Recommended Conditions

The following controls or conditions are recommended:

Donor Background Control: This control is assay medium only and is used as a reagent background control. It is used in combination with the detection reagents to measure the instrument background luminescence in the donor channel to calculate donor signal-to-background (S/B) ratio for each kinase. We recommend including this control in technical duplicate in each experiment in isolated wells to minimize well-to-well bleedthrough from the NanoLuc® signal in nearby wells. This control is used on both the transfection control plates and the NanoBRET assay plates.

Transfection Control: This control is used to determine if transfection was successful prior to executing the experiment on the full kinase panel, which can save time and expense in the event that a mistake was made during the transfection. The vector we recommend using as the transfection control is contained in the NanoBRET™ TE Selectivity DNA Controls (Cat.# NP1000) and is labeled NanoLuc®-HIPK2 Fusion Vector.

We recommend that a separate plate be prepared using the transfection control vector, treating it and the rest of the kinase DNA library identically. The Donor Background Control is also included in this transfection control plate. Transfect at least two wells for the transfection controls and include at least two wells of the Donor Background Control. Isolate these controls to minimize well-to-well signal bleedthrough from the NanoLuc® signal in the transfection control wells that may compromise the signal-to-background calculation. It is not necessary to add K-10 Tracer to the transfection control plate.

Note: Preparing the transfection control vector for use is detailed in Section 4.D. An example plate map for execution of the transfection control experiment is provided in Figure 4 in Section 4.E.

4.B. Recommended Conditions (continued)

Table 1. Summary of Control Conditions.

Control Name	Contents	Purpose
$BRET_{Min}$ Control	Cells transfected with NanoLuc® control vector. Adding tracer and test compound is not required.	For calculating minimum BRET or 100% fractional occupancy and assay window.
$BRET_{Max}$ Control	Cells transfected with each individual kinase + K-10 Tracer + vehicle.	For calculating maximum BRET or 0% fractional occupancy and assay window.
Donor Background Control	Assay medium only.	To measure instrument background luminescence and calculate donor signal-to-background ratio.
Transfection Control	Cells transfected with NanoLuc® HIPK2 Fusion Vector.	To ensure transfection was successful. Measured in a separate plate.

4.C. Preparing Transfection-Ready K192 Working DNA Plates

Dilute the DNA provided in NanoBRET™ TE K192 Vector Plate A and NanoBRET™ TE K192 Vector Plate B to a concentration that is used for transfection. The DNA in the plates are mixtures containing the kinase-NanoLuc® fusion vector mixed with either Transfection Carrier DNA (Cat.# E4882) or an appropriate regulatory protein expression vector (i.e., cyclin expression vectors for CDKs). These DNA mixtures are supplied as 10X stocks. Once the diluted, transfection-ready working DNA plates are prepared, they can be stored and re-used multiple times for subsequent experiments. We recommend the following procedure to generate transfection-ready working DNA stocks.

1. Thaw NanoBRET™ TE K192 Vector Plate A and NanoBRET™ TE K192 Vector Plate B and centrifuge at 200 × *g* for 2–3 minutes to remove condensation from the top seal.
2. Carefully remove the seal to prevent splashing and cross contamination.
3. Add 250µl of sterile, nuclease-free TE buffer to all wells of plates A and B. The plates are now transfection-ready working DNA plates.

   Notes:

   a. Change tips in between TE buffer dispenses to avoid cross contamination.

   b. If using these working DNA plates immediately to transfect cells, proceed to Section 4.D. If you will not be transfecting cells at this time, tightly seal plates A & B using supplied K192 Adhesive Foil Plate Seals or with a heat seal as described in Section 5.B. Place the clear 96-well assay plate lid on top of the sealed plate and store at –20°C. (The lid helps keep the sealing film tightly attached, reducing evaporation.)

   c. Four K192 Adhesive Foil Plate Seals are supplied in the NanoBRET™ TE K192 Kinase Vector Panel. If additional sealing films are needed, they can be purchased from Corning® (Cat.# PCR-AS-600, Axygen Aluminum Sealing Film).

4. The working DNA plates should be resealed with a new plate seal after each use and stored at −20°C. Use a new seal each time to reduce the chance of DNA cross contamination. The clear 96-well plate lid can be re-used.

   Note: For the working DNA solutions we recommend limiting the number of freeze-thaws to five or fewer for best performance. For a complete discussion of plate management and resealing conditions, see Section 5.B.

4.D. Preparing Control Vectors for Transfection

All control vectors such as the NanoLuc® control vector and the transfection control vector are supplied in purified form at 1mg/ml. These DNAs must be mixed with Transfection Carrier DNA and then diluted to a working concentration before use (Section 4.B).

Prepare the NanoLuc® Control Vector

1. Combine 1 part of pNL1.1.CMV[Nluc/CMV] Vector (Cat.# N1091; 1mg/ml) with 9 parts of Transfection Carrier DNA (1mg/ml) to prepare a 50X DNA mixture with a concentration of 1mg/ml.
2. Dilute the 50X DNA mixture 1:50 in nuclease-free TE buffer to prepare the transfection-ready working DNA solution with a concentration of 20µg/ml.

   Note: Store the 50X concentrated DNA mixture in Step 1, and the transfection-ready working DNA solution in Step 2 at −20°C between uses. We recommend limiting the number of freeze-thaws to five or less for the transfection-ready working DNA solution for best performance.

Prepare the Transfection Control Vector

3. Combine 1 part of NanoLuc®-HIPK2 Fusion Vector (Cat.# NV3221; 1mg/ml) with 9 parts of Transfection Carrier DNA (Cat.# E4882; 1mg/ml) to prepare a 50X DNA mixture with a DNA concentration of 1mg/ml.
4. Dilute the 50X concentrated DNA mixture 1:50 in nuclease-free TE buffer to prepare the transfection-ready working solution with a DNA concentration of 20µg/ml.

   Note: Store the 50X concentrated mixture, Step 3, and the transfection-ready working DNA solution, Step 4, at −20°C between uses. We recommend limiting the number of freeze-thaw cycles to 5 or less for the transfection-ready working DNA solution, for best performance.

4.E. Transfection Workflow (Day 1)

This workflow allows preparation of transfection complexes directly in the wells of the 96-well white assay plates. To appropriately time the transfection steps, we initially recommend that one scientist prepare the transfection complexes while a second scientist simultaneously prepares the HEK293 cells. Once familiar with the workflow, a single scientist can prepare the cells while the transfection complexes are forming.

Passage HEK293 cells on the day before transfection so that cells are 75–95% confluent on the day of transfection.

Alternatively, you can use TransfectNow™ HEK293 Cells (Cat.# NC1001, NC1002) to simplify the workflow and avoid the need to routinely culture, expand and harvest HEK293 cells. To use TransfectNow™ HEK293 Cells, simply thaw and resuspend cells in assay medium as described in the *TransfectNow™ HEK293 Cells Technical Manual*, #TM690. You can skip Scientist #1 and go directly to Scientist #2, Step 4.

4.E. Transfection Workflow (continued)

Scientist #1: Prepare HEK293 Cells

1. Trypsinize the cells and inactivate the trypsin using cell culture medium.
2. Centrifuge the cells at 200 × *g* for 3–5 minutes to pellet the cells and then aspirate the supernatant.
3. Resuspend the cells in assay medium (99% Opti-MEM™ +1% FBS) at a density of 2.5×10^5 cells/ml.

Scientist #2: Prepare Transfection Complexes

4. Use the transfection-ready working DNA stocks for K192, NanoLuc® control and transfection control vectors, prepared in Sections 4.C and 4.D, as follows:

   Note: If the transfection-ready DNA stocks are frozen, we recommend thawing the DNA quickly at 37°C. Mix gently on an orbital shaker and then centrifuge the plate briefly to ensure the solution is at the bottom of the well. Each assay well requires 10µl of transfection-ready working DNA solution.

5. Estimate the required volume of transfection complex by determining the number of data points needed for each kinase, including the desired number of technical replicates and the required and recommended conditions from Sections 4.A and 4.B. For example, testing a single compound in technical singlicate at a single dose would require one well for the $BRET_{Max}$ Control and one well for the test compound Sample for a total of two wells for each kinase. This experiment would also require a minimum of two wells for the $BRET_{Min}$ Control (NanoLuc® control vector) and a recommended two wells on a separate plate for the Transfection Control. See Section 4.I for an example plate map for this type of experiment. Remember to include at least 2 wells of Donor Background Control on both one of the NanoBRET plates and the Transfection Control plate.
6. Transfer 10µl of each transfection-ready working DNA solution to the desired assay well.

   Note: Add the DNA solutions to the bottom corner of the well (rather than the center) to ensure most efficient complex formation.

   Optional: If performing a Transfection Control Experiment (Section 4.F), we recommend a minimum of technical duplicates for both the Transfection Control and the Donor Background Control. These should be set up in isolated wells of an assay plate to prevent signal bleed-through that can compromise the signal-to-background calculation. An example plate layout for the transfection control is provided in Figure 4.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
B	Empty	Transfection Control	Empty	Empty	Donor Background Control	Empty	Empty	Empty	Empty	Empty	Empty	Empty
C	Empty	Transfection Control	Empty	Empty	Donor Background Control	Empty	Empty	Empty	Empty	Empty	Empty	Empty
D	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
E	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
F	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
G	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
H	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty

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Figure 4. Example plate layout for the transfection control experiment.

7. Prepare a 20µl/ml FuGENE® HD solution by diluting FuGENE® HD to a final concentration of 20µl/ml in room temperature Opti-MEM™ medium in a sterile conical tube. Add the FuGENE® HD directly to the medium, not to the side of the tube.

   Note: For each analysis well, prepare 30µl of the diluted FuGENE® HD solution. For example, testing one compound in technical singlicate at a single dose would require two wells for each of the 192 kinases and two wells for the $BRET_{Min}$ control, for a total of 11.58ml of the FuGENE® HD solution. If the optional transfection control experiment is performed in technical duplicate, the total FuGENE® HD solution required would be 11.64ml. We recommend preparing 12ml of FuGENE® HD solution to ensure enough is available.

8. Add 30µl of 20µl/ml diluted Fugene® HD solution to each assay well containing the 10µl of transfection-ready DNA.

   Note: Add the FuGENE® HD solution on the side of the well where the DNA solutions were added previously for most efficient complex formation. Prevent tips from directly touching the DNA solution to prevent cross contamination.

9. Mix on an orbital shaker for 15 seconds at 500–600rpm.

10. Incubate for 30 minutes at room temperature to allow complexes to form.

11. Add 60µl per well of the cell suspension at 2.5×10^5 cells/ml in assay medium, as prepared in Step 3.

12. Incubate overnight at 37°C in a 5% CO_2 incubator. Allow a minimum of 16 hours for transfection to occur; 20–24 hours is ideal.

4.F. Transfection Control Experiment (Day 2)

1. Remove the transfection control plate from the incubator and allow to equilibrate to room temperature for 15 minutes.

2. Prepare 3X Complete Substrate plus Inhibitor Solution. This solution consists of a 1:166 dilution of NanoBRET™ Nano-Glo® Substrate plus a 1:500 dilution of Extracellular NanoLuc® Inhibitor in Opti-MEM™ medium without serum or phenol red. Mix gently by inversion 5–10 times in a conical tube. For example, for the transfection control experiment depicted in Figure 4, dilute 0.5µl of the Extracellular NanoLuc® Inhibitor and 1.5µl of NanoBRET™ Nano-Glo® Substrate to 250µl with Opti-MEM™ medium.

   Note: The 3X Complete Substrate plus Inhibitor Solution should be used within 1.5 hours of preparation.

3. To wells containing the Transfection Control or the Donor Background Control, add 50µl per well of the 3X Complete Substrate plus Inhibitor Solution. Incubate for 2–3 minutes at room temperature.

   Note: If the Donor Background Control was not set up in advance as in Figure 4, add 100µl per well of assay medium and 50µl per well of Complete Substrate plus Inhibitor solution to wells on the opposite side of the plate from wells containing the transfection control.

4. Measure donor emission (e.g., 450nm) for both the transfection control wells and the Donor Background Control wells using a BRET-compatible luminometer.

5. Calculate the signal-to-background (S/B) ratio in the donor channel using the following equation:

   S/B ratio = X/Y

   where X = mean donor signal for the transfection control wells and Y = mean donor signal for the background control wells.

   Note: The S/B ratio should be >1,000 to support proceeding with a larger scale assay. An ideal S/B ratio is in the range of 10,000–100,000.

4.G. NanoBRET™ Target Engagement K192 Assay Protocol (Day 2)

The NanoBRET™ Target Engagement K192 Kinase Selectivity System uses a single tracer, NanoBRET™ Tracer K-10. The concentration of the K-10 tracer used for each kinase has been optimized and then grouped into 4 bins (25nM, 100nM, 250nM and 1µM). The tracer concentration bin used for each individual kinase is available from the K192 Plate Map file at: **www.promega.com/K192-Downloads**

Dilute NanoBRET™ Tracer K-10 Reagents and Add to Cells

1. Based on the plate layout, calculate the number of data points needed for each tracer concentration bin, including implementation of controls described in Section 4.D For example, testing 1 compound in technical singlicate requires 2 wells of analysis for each kinase. An example calculation for this experiment is provided in Table 2.
2. Prepare the Complete 20X NanoBRET™ Tracer K-10 Reagents by preparing the 100X solutions of NanoBRET™ Tracer K-10 in DMSO. Dilute 1 part of each 100X solution with 4 parts of Tracer Dilution Buffer.

   Note: To prepare the Complete 20X NanoBRET™ Tracer Reagents, we recommend first adding the concentrated tracer stock and DMSO to a conical tube, then adding the Tracer Dilution Buffer to the tube. Mix and transfer the complete 20X tracer reagents into polypropylene troughs (not polystyrene) for dispensing. Example calculations for preparing the 20X Complete NanoBRET™ Tracer K-10 Reagents are provided in Table 2.

Table 2. Example Calculations for the Required Volume of Complete 20X NanoBRET™ Tracer Reagents. Each concentration bin is for an experiment in which a single compound is tested in technical singlicate.

Tracer Bin	Kinase Count	Data Points per Kinase	Total Data Points	Dilution from 400µM K-10 Stock	400µM K-10 Stock (µl)	DMSO (µl)	Tracer Dilution Buffer (µl)	Total Volume (µl)
25nM	16	2	32	1:160	0.2	31.8	128	160
100nM	40	2	80	1:40	2	78	320	400
250nM	24	2	48	1:16	3	45	192	240
1µM	112	2	224	1:4	56	168	896	1,120

Note: The calculations in Table 2 are provided as an example. We recommend preparing at least an extra 500µl of each Complete 20X NanoBRET Tracer Reagent to account for loss during dispensing, or adjusting preparation if the volumes of tracer stock required are too small to pipet accurately (e.g., the 25nM bin).

3. Add 5µl of Complete 20X NanoBRET™ Tracer Reagent per well to the transfected cells (directly into the liquid) according to the plate layout.

   Note: Adding tracer to the $BRET_{Min}$ Control (NanoLuc® Control) wells is not required.

4. Mix on an orbital shaker for 15 seconds at 900rpm.

   Note: Mixing speeds can vary between orbital shakers and should be optimized for each unit by visual inspection to ensure complete dispersal of the tracer.

Prepare 10X Test Compound or 10X $BRET_{Max}$ Control in Medium

5. Dilute the 1,000X test compound to 10X in Opti-MEM™ medium. For example, for testing in technical singlicate, prepare at least 1,920µl of each 10X test compound, with extra to account for loss during transfer.

6. For the Top Control, dilute 1 part DMSO (or test compound solvent) with 99 parts Opti-MEM™ medium. For example, for testing 1 compound in technical singlicate, prepare at least 1,920µl of this solution, with extra to account for loss during transfer.

7. Add 10µl per well of the 10X test compound or 10µl of the compound solvent solution ($BRET_{Max}$ Control) to the cells per the desired plate layout.

   Note: Adding test compound or test compound solvent solution to the $BRET_{Min}$ Control wells is not required.

8. Mix on an orbital shaker for 15 seconds at 900rpm.

9. Incubate the plate at 37°C in a 5% CO_2 incubator for 2 hours.

BRET Measurement

10. After the 2-hour incubation, allow plate to cool to room temperature for approximately 15 minutes.

11. Immediately prior to BRET measurements, prepare 3X Complete Substrate plus inhibitor solution by making a 1:166 dilution of NanoBRET™ Nano-Glo® Substrate, and a 1:500 dilution of Extracellular NanoLuc® Inhibitor in Opti-MEM™ medium without serum or phenol red. Mix gently by inversion 5–10 times in a conical tube. For example, for testing one compound in technical singlicate, there are a total of 386 wells if only required conditions (Section 4.A) are used, and 388 wells if including the optional Donor Background Control in technical duplicate (Section 4.B). For convenience, prepare a minimum of 20ml of 3X Complete Substrate plus Inhibitor Solution by diluting 40µl of Extracellular NanoLuc® Inhibitor and 120µl of NanoBRET™ Nano-Glo® Substrate to 20ml with Opti-MEM™ medium.

    Note: The 3X Complete Substrate plus Inhibitor Solution should be used within 1.5 hours of preparation.

12. Add 50µl per well of 3X Complete Substrate plus Inhibitor Solution and incubate 2–3 minutes at room temperature.

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

4.H. Perform Data Quality Analysis and Calculate Fractional Occupancy

Donor Signal and Donor Signal-to-Background (Donor S/B)

Inspect the donor emission signal (e.g., 450nm) across the entire set of 192 kinases and determine if cumulative expression was adequate.

1. Calculate the mean donor signal across the entire set of 192 kinases. Calculate the mean donor signal for Donor Background Control wells.
2. Calculate the mean donor S/B across the entire set of 192 kinases by dividing the mean donor signal across all kinases by the mean donor signal for the Donor Background Control wells.

   Note: Ideal Donor S/B across the entire set of 192 kinases will be in the range of 10,000–100,000. If the donor S/B across the entire set of kinases is <1,000 consider repeating the experiment.

Inspect the donor emission signal (e.g., 450nm) for each individual kinase and determine if expression was adequate in each well, as described in Steps 3 and 4.

3. Calculate the mean donor signal for each kinase, or the average donor signal across all wells containing an individual kinase.
4. Calculate the donor S/B for each individual kinase by dividing the mean donor signal for each kinase by the mean donor signal for the Donor Background Control wells.

   Note: An ideal donor S/B for most kinases will be 10,000–100,000. Consider excluding kinases with donor S/B less than 1,000. There are some kinases that routinely express at lower levels and will more commonly show donor S/B values less than 1,000. These include kinases such as MET, MAP3K19, HIPK3, and to a lesser extent, CDKL3, CDKL5, HIPK2, MAST4, NEK2, PLK2, PLK4, TLK2, and TXK. For these kinases, a donor S/B value less than 100 is a more appropriate criteria for exclusion.

BRET Signals and Assay Window

6. Calculate the raw BRET values for all wells by dividing the acceptor emission signal (e.g., 610nm) by the donor emission signal (e.g., 450nm).
7. Calculate the average of the BRET values for the $BRET_{Max}$ Controls (Tracer + Vehicle), the $BRET_{Min}$ Controls (NanoLuc® Control) and the Samples (Tracer + Compound). For the mean of the $BRET_{Min}$ Controls, calculate either on a plate-by-plate basis or across the entire experiment depending on the chosen plate format.

8. Calculate the assay window for each individual kinase assay by dividing the Mean BRET value for the $BRET_{Max}$ Control for each kinase by the mean BRET value for the $BRET_{Min}$ Control.

   Note: When executed by Promega scientists using the GloMax® Discover System, assay windows for most of the 192 kinases are routinely above 2. See Section 5.A for example assay window data for each kinase. Occasionally, the assay window for some individual kinases may be in the range of 1.7–2 for normal executions. In our hands, experiments in which there are >10 kinases with assay windows <2 are atypical and suggest that data quality for the entire experiment might be suspect. These ranges can be used as a starting point for quality control of individual experimental executions, but may need revision depending on the normal values obtained using your equipment and experimental setup, which may be different, depending upon the HEK293 cells or plate reader used.

Fractional Occupancy

Calculate fractional occupancy for the test drug for each kinase using the following formula:

$$\text{Occupancy (\%)} = \left[1 - \frac{(\text{Sample} - BRET_{Min})}{(BRET_{Max} - BRET_{Min})} \right] \times 100$$

Where:

Sample = Mean BRET value across all Sample (tracer + compound) wells for an individual kinase.

$BRET_{Max}$ = Mean BRET value across all $BRET_{Max}$ (tracer + vehicle) control wells for an individual kinase.

$BRET_{Min}$ = Mean BRET value of NanoLuc® control wells (calculated either on a plate-by-plate basis or across the entire experiment).

4.I. Example Plate Layout and Protocol for Fractional Occupancy Measurement Across 192 Kinases

Selectivity profiling experiments described generically in Section 4 can be configured in many ways. This section details a specific plate format that can be used to assess fractional occupancy of a single test compound in technical singlicate across all 192 kinases provided in the kit. It uses the transfection protocol described in Section 4.E and the entire experiment can be completed using a total of 6 × 96-well plates (5 plates for the BRET assays and 1 plate for the Transfection Control).

Plate Layout for Transfection and Controls

Each column of kinases from the set of working DNA plates is transfected in duplicate according to the plate map in Figure 5. Columns 1 and 12 of the plates are left as assay medium only to minimize potential edge effects. $BRET_{Max}$ Control and Sample treatments are included in adjacent columns. The $BRET_{Min}$ Control and Donor Background Controls are included in isolated wells of the fifth plate. Lastly, a Transfection Control plate is included in the experiment.

4.I. Example Plate Layout and Protocol for Fractional Occupancy Measurement Across 192 Kinases (continued)

Tracer Concentrations

Kinases that use similar tracer concentrations are grouped together on the plates for pipetting efficiency. NanoBRET™ Tracer K-10 concentrations across the plates in the analysis are depicted in Figure 6.

Day 1: Transfection

Prepare working DNA plates as described in Section 4.C for the 192 kinase panel. Prepare control vectors as described in Section 4.D. Transfect HEK293 cells according to the plate map shown in Figure 5 using the transfection protocol described in Section 4.E. Remember to include all controls described in Sections 4.A–B, including the Transfection Control plate.

1. For each analysis well, transfer 10µl of the transfection-ready working DNA solution to the bottom corner of the well.
2. Prepare 20µl/ml of FuGENE® HD solution in Opti-MEM™ medium. Add 30µl of the FuGENE® HD solution per well, transferring to the same side of the well as the DNA solution. Avoid touching the DNA solution to minimize cross contamination.
3. Mix and incubate the plates for 30 minutes to allow the transfection complexes to form.
4. Meanwhile, prepare an HEK293 cell suspension in assay medium (99% Opti-MEM + 1% FBS) at 2.5×10^5 cells/ml. Alternatively, if using a TransfectNow™ HEK293 Cells, thaw and resuspend as directed in Technical Manual #TM690.
5. After the 30-minute incubation, transfer 60µl of the cell suspension to each well.
6. Incubate overnight at 37°C in a 5% CO_2 incubator.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Assay Medium	LRRK2	LRRK2	AURKA	AURKA	AXL	AXL	CLK1	CLK1	BRSK1	BRSK1	Assay Medium
B	Assay Medium	MAPK6	MAPK6	AURKC	AURKC	FGFR3	FGFR3	SBK3	SBK3	MAP3K10	MAP3K10	Assay Medium
C	Assay Medium	IRAK3	IRAK3	AURKB	AURKB	FLT3	FLT3	NEK9	NEK9	MAP3K9	MAP3K9	Assay Medium
D	Assay Medium	TEK	TEK	NUAK1	NUAK1	IGF1R	IGF1R	NEK3	NEK3	MYLK3	MYLK3	Assay Medium
E	Assay Medium	TNK1	TNK1	LATS2	LATS2	INSR	INSR	NIM1K	NIM1K	PHKG1	PHKG1	Assay Medium
F	Assay Medium	GAK	GAK	RPS6KA3	RPS6KA3	LIMK2	LIMK2	STK36	STK36	STK33	STK33	Assay Medium
G	Assay Medium	MAPK4	MAPK4	SNF1LK2	SNF1LK2	TEC	TEC	ULK2	ULK2	STK4	STK4	Assay Medium
H	Assay Medium	AAK1	AAK1	MYLK2	MYLK2	TIE1	TIE1	ULK3	ULK3	TLK1	TLK1	Assay Medium

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	Assay Medium	FGFR1	FGFR1	BRAF(V600E)	BRAF(V600E)	BMP2K	BMP2K	MARK4	MARK4	CLK4	CLK4	Assay Medium
B	Assay Medium	FGFR2	FGFR2	IRAK4	IRAK4	NEK5	NEK5	PRKAA1	PRKAA1	MAPK8	MAPK8	Assay Medium
C	Assay Medium	MUSK	MUSK	ITK	ITK	STK16	STK16	PRKAA2	PRKAA2	MAPK9	MAPK9	Assay Medium
D	Assay Medium	NTRK1	NTRK1	JAK2 (V617F)	JAK2 (V617F)	TBK1	TBK1	RPS6KA1	RPS6KA1	IKBKE	IKBKE	Assay Medium
E	Assay Medium	RET	RET	MAP3K11	MAP3K11	ULK1	ULK1	RPS6KA2	RPS6KA2	LATS1	LATS1	Assay Medium
F	Assay Medium	NTRK2	NTRK2	PTK2	PTK2	MAP4K2	MAP4K2	RPS6KA4	RPS6KA4	PRKX	PRKX	Assay Medium
G	Assay Medium	TNK2(Iso1)	TNK2(Iso1)	PTK6	PTK6	WEE1	WEE1	RPS6KA6	RPS6KA6	CSNK2A2	CSNK2A2	Assay Medium
H	Assay Medium	LTK	LTK	PTK2B	PTK2B	MYLK4	MYLK4	SIK1	SIK1	HIPK4	HIPK4	Assay Medium

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	Assay Medium	STK10	STK10	LIMK1	LIMK1	ABL2	ABL2	CLK2	CLK2	CDK1	CDK1	Assay Medium
B	Assay Medium	FGFR4	FGFR4	EPHA1	EPHA1	BMX	BMX	DYRK1A	DYRK1A	CDK2	CDK2	Assay Medium
C	Assay Medium	MAP4K1	MAP4K1	EPHA4	EPHA4	BTK	BTK	DYRK1B	DYRK1B	CDK3	CDK3	Assay Medium
D	Assay Medium	MERTK	MERTK	EPHA6	EPHA6	FER	FER	ERN1	ERN1	CDK4	CDK4	Assay Medium
E	Assay Medium	MET	MET	EPHA7	EPHA7	FES	FES	ERN2	ERN2	CDK5	CDK5	Assay Medium
F	Assay Medium	RON	RON	EPHB1	EPHB1	JAK3	JAK3	HIPK2	HIPK2	CDK6	CDK6	Assay Medium
G	Assay Medium	TYRO3	TYRO3	EPHB4	EPHB4	SRMS	SRMS	HIPK3	HIPK3	CDK7	CDK7	Assay Medium
H	Assay Medium	LCK	LCK	FYN	FYN	TXK	TXK	ICK	ICK	CDK9	CDK9	Assay Medium

Plate 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	Assay Medium	CDK10	CDK10	CDKL1	CDKL1	NEK11	NEK11	DAPK2	DAPK2	BRSK2	BRSK2	Assay Medium
B	Assay Medium	CDK14	CDK14	CSNK2A1	CSNK2A1	NEK1	NEK1	MAP3K2	MAP3K2	MARK2	MARK2	Assay Medium
C	Assay Medium	CDK15	CDK15	CDKL3	CDKL3	NEK2	NEK2	PLK2	PLK2	MELK	MELK	Assay Medium
D	Assay Medium	CDK16	CDK16	CDKL5	CDKL5	NEK4	NEK4	PLK3	PLK3	CSNK1A1L	CSNK1A1L	Assay Medium
E	Assay Medium	CDK17	CDK17	JNK3	JNK3	PAK4	PAK4	PLK4	PLK4	CSNK1D	CSNK1D	Assay Medium
F	Assay Medium	CDK18	CDK18	MAPK11	MAPK11	MAP4K3	MAP4K3	STK35	STK35	CSNK1G2	CSNK1G2	Assay Medium
G	Assay Medium	CDKL2	CDKL2	MAPK14	MAPK14	STK11	STK11	STK17B	STK17B	SIK3 FL	SIK3 FL	Assay Medium
H	Assay Medium	CDK20	CDK20	NLK	NLK	SLK	SLK	TLK2	TLK2	SNRK	SNRK	Assay Medium

Plate 5

	1	2	3	4	5	6	7	8	9	10	11	12
A	Assay Medium	CAMK1	CAMK1	RIOK2	RIOK2	PAK6	PAK6	RIPK1	RIPK1	Assay Medium	Assay Medium	Assay Medium
B	Assay Medium	CAMK2A	CAMK2A	MAP4K5	MAP4K5	AKT2	AKT2	RIPK2	RIPK2	Assay Medium	Donor Background Control	Assay Medium
C	Assay Medium	CAMK2D	CAMK2D	MAST3	MAST3	PKMYT1	PKMYT1	TNNI3K	TNNI3K	Assay Medium	Donor Background Control	Assay Medium
D	Assay Medium	CHEK2	CHEK2	MAST4	MAST4	PRKACA	PRKACA	MLTK	MLTK	Assay Medium	Assay Medium	Assay Medium
E	Assay Medium	DCLK3	DCLK3	STK32B	STK32B	PRKACB	PRKACB	MAP3K12	MAP3K12	Assay Medium	Assay Medium	Assay Medium
F	Assay Medium	MKNK2	MKNK2	STK3	STK3	PRKCE	PRKCE	MAP3K19	MAP3K19	Assay Medium	$BRET_{Min}$ Control	Assay Medium
G	Assay Medium	PHKG2	PHKG2	STK38	STK38	SGK1	SGK1	MAP3K21	MAP3K21	Assay Medium	$BRET_{Min}$ Control	Assay Medium
H	Assay Medium	MAP3K3	MAP3K3	STK38L	STK38L	WEE2	WEE2	MAP3K4	MAP3K4	Assay Medium	Assay Medium	Assay Medium

Transfection Control Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
B	Empty	Transfection Control	Empty	Empty	Donor Background Control	Empty	Empty	Empty	Empty	Empty	Empty	Empty
C	Empty	Transfection Control	Empty	Empty	Donor Background Control	Empty	Empty	Empty	Empty	Empty	Empty	Empty
D	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
E	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
F	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
G	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
H	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty

$BRET_{Max}$ Control (Tracer + Vehicle)	$BRET_{Min}$ Control (NanoLuc® Control)	"Sample" (Tracer + Compound)	Donor Background Control	Transfection Control	Assay Medium

Figure 5. Kinase locations and drug treatment conditions. Example plate map for transfection of K192 vectors, and control conditions for measurement of fractional occupancy for 192 kinases in technical singlicate.

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Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Tracer	LRRK2	LRRK2	AURKA	AURKA	AXL	AXL	CLK1	CLK1	BRSK1	BRSK1	No Tracer
B	No Tracer	MAPK6	MAPK6	AURKC	AURKC	FGFR3	FGFR3	SBK3	SBK3	MAP3K10	MAP3K10	No Tracer
C	No Tracer	IRAK3	IRAK3	AURKB	AURKB	FLT3	FLT3	NEK9	NEK9	MAP3K9	MAP3K9	No Tracer
D	No Tracer	TEK	TEK	NUAK1	NUAK1	IGF1R	IGF1R	NEK3	NEK3	MYLK3	MYLK3	No Tracer
E	No Tracer	TNK1	TNK1	LATS2	LATS2	INSR	INSR	NIM1K	NIM1K	PHKG1	PHKG1	No Tracer
F	No Tracer	GAK	GAK	RPS6KA3	RPS6KA3	LIMK2	LIMK2	STK36	STK36	STK33	STK33	No Tracer
G	No Tracer	MAPK4	MAPK4	SNF1LK2	SNF1LK2	TEC	TEC	ULK2	ULK2	STK4	STK4	No Tracer
H	No Tracer	AAK1	AAK1	MYLK2	MYLK2	TIE1	TIE1	ULK3	ULK3	TLK1	TLK1	No Tracer

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Tracer	FGFR1	FGFR1	BRAF(V600E)	BRAF(V600E)	BMP2K	BMP2K	MARK4	MARK4	CLK4	CLK4	No Tracer
B	No Tracer	FGFR2	FGFR2	IRAK4	IRAK4	NEK5	NEK5	PRKAA1	PRKAA1	MAPK8	MAPK8	No Tracer
C	No Tracer	MUSK	MUSK	ITK	ITK	STK16	STK16	PRKAA2	PRKAA2	MAPK9	MAPK9	No Tracer
D	No Tracer	NTRK1	NTRK1	JAK2 (V617F)	JAK2 (V617F)	TBK1	TBK1	RPS6KA1	RPS6KA1	IKBKE	IKBKE	No Tracer
E	No Tracer	RET	RET	MAP3K11	MAP3K11	ULK1	ULK1	RPS6KA2	RPS6KA2	LATS1	LATS1	No Tracer
F	No Tracer	NTRK2	NTRK2	PTK2	PTK2	MAP4K2	MAP4K2	RPS6KA4	RPS6KA4	PRKX	PRKX	No Tracer
G	No Tracer	TNK2(Iso1)	TNK2(Iso1)	PTK6	PTK6	WEE1	WEE1	RPS6KA6	RPS6KA6	CSNK2A2	CSNK2A2	No Tracer
H	No Tracer	LTK	LTK	PTK2B	PTK2B	MYLK4	MYLK4	SIK1	SIK1	HIPK4	HIPK4	No Tracer

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Tracer	STK10	STK10	LIMK1	LIMK1	ABL2	ABL2	CLK2	CLK2	CDK1	CDK1	No Tracer
B	No Tracer	FGFR4	FGFR4	EPHA1	EPHA1	BMX	BMX	DYRK1A	DYRK1A	CDK2	CDK2	No Tracer
C	No Tracer	MAP4K1	MAP4K1	EPHA4	EPHA4	BTK	BTK	DYRK1B	DYRK1B	CDK3	CDK3	No Tracer
D	No Tracer	MERTK	MERTK	EPHA6	EPHA6	FER	FER	ERN1	ERN1	CDK4	CDK4	No Tracer
E	No Tracer	MET	MET	EPHA7	EPHA7	FES	FES	ERN2	ERN2	CDK5	CDK5	No Tracer
F	No Tracer	RON	RON	EPHB1	EPHB1	JAK3	JAK3	HIPK2	HIPK2	CDK6	CDK6	No Tracer
G	No Tracer	TYRO3	TYRO3	EPHB4	EPHB4	SRMS	SRMS	HIPK3	HIPK3	CDK7	CDK7	No Tracer
H	No Tracer	LCK	LCK	FYN	FYN	TXK	TXK	ICK	ICK	CDK9	CDK9	No Tracer

Plate 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Tracer	CDK10	CDK10	CDKL1	CDKL1	NEK11	NEK11	DAPK2	DAPK2	BRSK2	BRSK2	No Tracer
B	No Tracer	CDK14	CDK14	CSNK2A1	CSNK2A1	NEK1	NEK1	MAP3K2	MAP3K2	MARK2	MARK2	No Tracer
C	No Tracer	CDK15	CDK15	CDKL3	CDKL3	NEK2	NEK2	PLK2	PLK2	MELK	MELK	No Tracer
D	No Tracer	CDK16	CDK16	CDKL5	CDKL5	NEK4	NEK4	PLK3	PLK3	CSNK1A1L	CSNK1A1L	No Tracer
E	No Tracer	CDK17	CDK17	JNK3	JNK3	PAK4	PAK4	PLK4	PLK4	CSNK1D	CSNK1D	No Tracer
F	No Tracer	CDK18	CDK18	MAPK11	MAPK11	MAP4K3	MAP4K3	STK35	STK35	CSNK1G2	CSNK1G2	No Tracer
G	No Tracer	CDKL2	CDKL2	MAPK14	MAPK14	STK11	STK11	STK17B	STK17B	SIK3 FL	SIK3 FL	No Tracer
H	No Tracer	CDK20	CDK20	NLK	NLK	SLK	SLK	TLK2	TLK2	SNRK	SNRK	No Tracer

Plate 5

	1	2	3	4	5	6	7	8	9	10	11	12
A	No Tracer	CAMK1	CAMK1	RIOK2	RIOK2	PAK6	PAK6	RIPK1	RIPK1	No Tracer	No Tracer	No Tracer
B	No Tracer	CAMK2A	CAMK2A	MAP4K5	MAP4K5	AKT2	AKT2	RIPK2	RIPK2	No Tracer	Donor Background Control	No Tracer
C	No Tracer	CAMK2D	CAMK2D	MAST3	MAST3	PKMYT1	PKMYT1	TNNI3K	TNNI3K	No Tracer	Donor Background Control	No Tracer
D	No Tracer	CHEK2	CHEK2	MAST4	MAST4	PRKACA	PRKACA	MLTK	MLTK	No Tracer	No Tracer	No Tracer
E	No Tracer	DCLK3	DCLK3	STK32B	STK32B	PRKACB	PRKACB	MAP3K12	MAP3K12	No Tracer	No Tracer	No Tracer
F	No Tracer	MKNK2	MKNK2	STK3	STK3	PRKCE	PRKCE	MAP3K19	MAP3K19	No Tracer	$BRET_{Min}$ Control	No Tracer
G	No Tracer	PHKG2	PHKG2	STK38	STK38	SGK1	SGK1	MAP3K21	MAP3K21	No Tracer	$BRET_{Min}$ Control	No Tracer
H	No Tracer	MAP3K3	MAP3K3	STK38L	STK38L	WEE2	WEE2	MAP3K4	MAP3K4	No Tracer	No Tracer	No Tracer

Transfection Control Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
B	Empty	Transfection Control	Empty	Empty	Donor Background Control	Empty	Empty	Empty	Empty	Empty	Empty	Empty
C	Empty	Transfection Control	Empty	Empty	Donor Background Control	Empty	Empty	Empty	Empty	Empty	Empty	Empty
D	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
E	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
F	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
G	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
H	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty

Tracer after each concentration	25nM	250nM	100nM	1,000nM	No Tracer

Figure 6. Tracer K-10 concentration positioning for the 192 kinase fractional occupancy analysis in technical singlicate. Tracer does not need to be added to the Transfection Control plates or to the $BRET_{Min}$ and Donor Background Control wells.

Day 2: Transfection Control, BRET Determination and Data Analysis

7. Test transfection success by reading the donor signal for the transfection control plate and calculating the Mean Donor S/B for the transfection control according to Section 4.H. If the Mean Donor S/B is ≥1,000, proceed with the remainder of the experiment. If the Mean Donor S/B is <1,000, consider repeating the transfection.
8. Prepare Complete 20X NanoBRET™ Tracer K-10 Reagents as described in Section 4.G and add 5µl to cells according to the plate map described in Figure 6. Mix on an orbital shaker for 15 seconds at 900rpm.

   Note: Tracer does not need to be added to $BRET_{Min}$ or Donor Background Control wells.
9. Prepare 10X test compound and 10X vehicle solutions as described in Section 4.G and add 10µl to the cells according to the plate map described in Figure 5. Mix on an orbital shaker for 15 seconds at 900rpm.

   Note: Test compound or test compound solvent solution does not need to be added to the $BRET_{Min}$ or Donor Background Control wells.
10. Incubate plates for 2 hours in a 37°C incubator with 5% CO_2. After the incubation, re-equilibrate plates to room temperature for 15 minutes.
11. Prepare 3X Complete Substrate plus Inhibitor Solution as described in Section 4.G and add 50µl per well. Incubate for 2–3 minutes at room temperature.
12. Measure donor emission signal (e.g., 450nm) and acceptor emission signal (e.g., 610nm) as described in Section 4.G, Step 13.
13. Perform data quality analysis for expression and assay window as described in Section 4.H and ensure that the experiment meets the recommended acceptance criteria.
14. Calculate fractional occupancy for the test compound at each kinase as described in Section 4.H.

5. Appendix

5.A. Example Assay Window and Selectivity Data for Control Compounds

The NanoBRET™ TE K192 Kinase Selectivity System provides unique information regarding the intracellular selectivity of test compounds across a representative set of protein kinases. However, due to the distance and geometry components of BRET, each individual kinase assay within the panel may exhibit a different assay window. Within this panel of 192 assays, the assay windows range from ~twofold to 25-fold, with a median assay window of fourfold. We have generally observed consistent assay windows for each individual kinase assay across many experimental executions, with typical assay window coefficient of variation (CV) of ~8%. For some individual kinases (particularly those with large assay windows), the assay window may vary to a greater extent between experiments, but this variation generally does not impact the measurement of fractional occupancy for test compounds. Example assay windows are provided in the K192 Assay Performance file, available at: **www.promega.com/K192-Downloads**

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Due to myriad factors present inside cells (e.g., intracellular ATP, protein complexes and variable kinase activation states), the selectivity behavior of test compound observed in live cells can be very different compared to the selectivity behavior in cell-free kinase assays. To ensure that the NanoBRET™ TE K192 Kinase Selectivity System is performing properly, we recommend including control compounds. We have extensively tested two control compounds with unique selectivity behaviors. These include the CC1 pan-Kinase Inhibitor, a promiscuous kinase ligand that binds to every kinase within the selectivity panel, and crizotinib, which demonstrates greater intracellular selectivity for a small subset of kinases. Occupancy data and specific doses for each of these control compounds is provided in the K192 Assay Performance file: **www.promega.com/K192-Downloads**

To build confidence in the fidelity of experimental execution, we recommend initially testing both of these control compounds and comparing to the data in the K192 Assay Performance file: **www.promega.com/K192-Downloads** for general agreement. Subsequently, one or both these controls can be used occasionally to calibrate new users or potentially included in each run as a routine internal control for expected assay behavior.

5.B. Managing Plate Evaporation and Resealing

The NanoBRET™ TE K192 Kinase Selectivity System is provided as concentrated set of DNA that is premixed with an appropriate carrier DNA to facilitate transfection. The NanoBRET™ TE K192 Kinase Vector Plates are heat sealed to minimize evaporative loss during storage and shipping. However, plastic storage plates such as these are breathable. Thus, even during storage they may demonstrate some degree of liquid loss over time, which can vary from well-to-well. We have found that adding TE buffer to the concentrated DNA plates according to the instructions described in Section 4.C will recover DNA transfectability.

After diluting the stock DNAs to prepare the working DNA plates, these plates can be resealed and retain transfection capacity for at least 6 months with at least five freeze-thaw cycles. If possible, we recommend resealing the working DNA plates using a heat seal. We recommend using a Porvair Sciences miniSeal II (Porvair Part # 500090), with Porvair Sciences Thermal Sealing Film (Porvair Part# 229571), and heat seal for 1.9 seconds at 190°C. For other heat sealers, optimizing the sealing conditions may be required. If resealing using a heat sealer isn't possible, we recommend at a minimum that the plates are resealed with an adhesive foil seal provided with NanoBRET™ TE K192 Vector Panel. Additional seals can be obtained from Corning®: (i.e., Axygen Aluminum Sealing Film, Corning® Cat.# PCR-AS-600). Ensure that a seal has been created around each individual well using firm compression with fingertips. Use a fresh adhesive foil seal each time to minimize DNA cross contamination. In addition, we recommend that plates sealed manually using an adhesive foil seal are capped with the included plastic plate lid for most effective long-term sealing. The plastic plate lid can be re-used. This should lessen the extent of evaporative loss over time and extend the usable lifetime of the working DNA plates.

When thawing transfection-ready working DNA plates for use in subsequent transfections, always centrifuge the working DNA plate to remove any condensation from the top seal. Then remove seal carefully to prevent splashing and DNA cross contamination.

5.C. Composition of Buffers and Solutions

cell culture medium

90% DMEM
10% fetal bovine serum

assay medium

99% Opti-MEM™ I Reduced Serum Medium, no phenol red
1% fetal bovine serum

5.D. Frequently Asked Questions

1. Can the NanoBRET™ Target Engagement K192 Kinase Selectivity System be performed in another cell type?

   Answer: Though technically the NanoBRET™ Target Engagement K192 Kinase Selectivity System could be performed in a cell type other than HEK293 cells, it would require revalidation of all individual kinase assays in the alternative cell line to ensure that the tracer concentrations used for each kinase remain quantitative. Moreover, there is no guarantee that each individual kinase will perform adequately in the alternative cell background. For these reasons, it may be impractical to consider running the assay in another cell line.

2. Do I need to worry about evaporation of liquid from the NanoBRET™ TE K192 Vector Plates?

   Answer: To minimize evaporation of the DNA stocks in the NanoBRET™ TE K192 Vector Plates, the plates have been heat sealed, capped with a plastic lid, and stored in a vacuum sealed bag. Still, minor evaporation may occur due to the inherent breathability of plastic. In our experience, as long as the DNA stocks in the original plates are initially diluted with 250µl of molecular biology grade TE buffer according to Section 4.C, the DNA stocks will perform properly for transfection.

3. Can I make transfection complexes in bulk?

   Answer: Yes, see an example application using bulk transfection complexes in our *STAR Methods* article (6).

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5.E. 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
Weak expression levels (mean 450nm donor signal-to-background <1,000)	Cell status at the time of transfection. Ensure that the cells were passaged on day prior to transfection, and that the cell confluency was appropriate (70–90%).
	Inaccurate DNA stock solution preparation. Ensure the DNA has been properly diluted to the working concentration and stored properly to reduce evaporation. See Sections 4 and 5 for details.
	Inaccurate transfection complex preparation. Rely on the transfection control samples to ensure that each experiment results in appropriate transfection levels prior to executing the full 192 kinase profiling experiment. This can save reagent when aberrant transfections occur.
Noisy assay window (BRET S/B), generating coefficient of variation (CV) >20%	Weak expression levels. Ensure that the donor (450nm channel) RLU for each kinase are >1,000 above background (see Section 4.A and 4.F for details).
	Inconsistent dispensing of tracer. Ensure that liquid handlers are accurately delivering the tracer to each well.
Negative fractional occupancy (%) of test compound	Inaccurate dispensing of tracer for $BRET_{Max}$ controls (100% BRET, or 0% fractional occupancy controls). Ensure liquid handling is accurately dispensing the NanoBRET™ tracer.
	Autofluorescent or light-scattering properties of the test compound. Optical effects may increase the BRET value. This is often determined by using an irrelevant BRET control assay. If the compound has the same effect on an irrelevant BRET assay, this is likely a spurious optical effect. Potential control assays could be found in Section 5.H, Related Products, and include those BRET assays for HDACs, BET BRDs, E3 ligases CRBN and VHL, or any other BRET assay for a target that should not bind the test compound.

Symptoms	Causes and Comments
Negative fractional occupancy percent of test compound (continued)	Although rare, global/nonspecific impacts on kinase activation state may be observed. Nonspecific kinase inhibitors may indirectly affect the target of interest, thus altering the activation state of the kinase. In some cases, altering the kinase activation state may increase the apparent affinity of the NanoBRET™ tracer leading to a nonspecific increase in BRET. Run specific Nano BRET™ kinase assays in digitonin-treated cells to determine if this increase in BRET is due to such nonspecific pathway influences as described in earlier studies (3,5).
Unexpectedly low fractional occupancy (percent) of the test compound	Inaccurate dispensing of test compound. Ensure liquid handling is accurately dispensing the compound.
	Poor compound solubility. Ensure that the compound is soluble as a 10X solution.
	Discordance between a cell-free and live cell target engagement assay. If comparing NanoBRET™ to a cell-free assessment of target occupancy, consider the effect of permeability or [ATP], which can interfere with target engagement. The composite effect of these variables may shift the occupancy results in a live cell versus an acellular system. Follow up experiments in digitonin-treated cells may be needed to address the impact of [ATP] or permeability as described in earlier studies (3,5).
Unexpectedly high fractional occupancy (percent) of the test compound	Inaccurate dispensing of test compound. Ensure liquid handling is accurately dispensing the compound.
	Discordance between a cell-free and live-cell target engagement assay. If comparing NanoBRET™ to a cell-free assessment of target occupancy, consider the impact of target activation state. If the compound preferentially engages an active or inactive kinase state, this may impact intracellular engagement to an unpredictable extent.

5.F. Example Data Processing and Fractional Occupancy Calculation

In Figure 7, we provide example raw data for the CDK6 assay tested with a single dose of test compound (palbociclib), as well as the processing of that data to calculate fractional occupancy.

5.G. Extinction Coefficient of NanoBRET™ Tracers

NanoBRET™ Tracer K-10 uses the NanoBRET™ 590 fluorophore. The concentration of Tracer K-10 was determined using an extinction coefficient of 83,000M^{-1} cm^{-1} at 590nm. See Table 10.1 in reference for details (7).

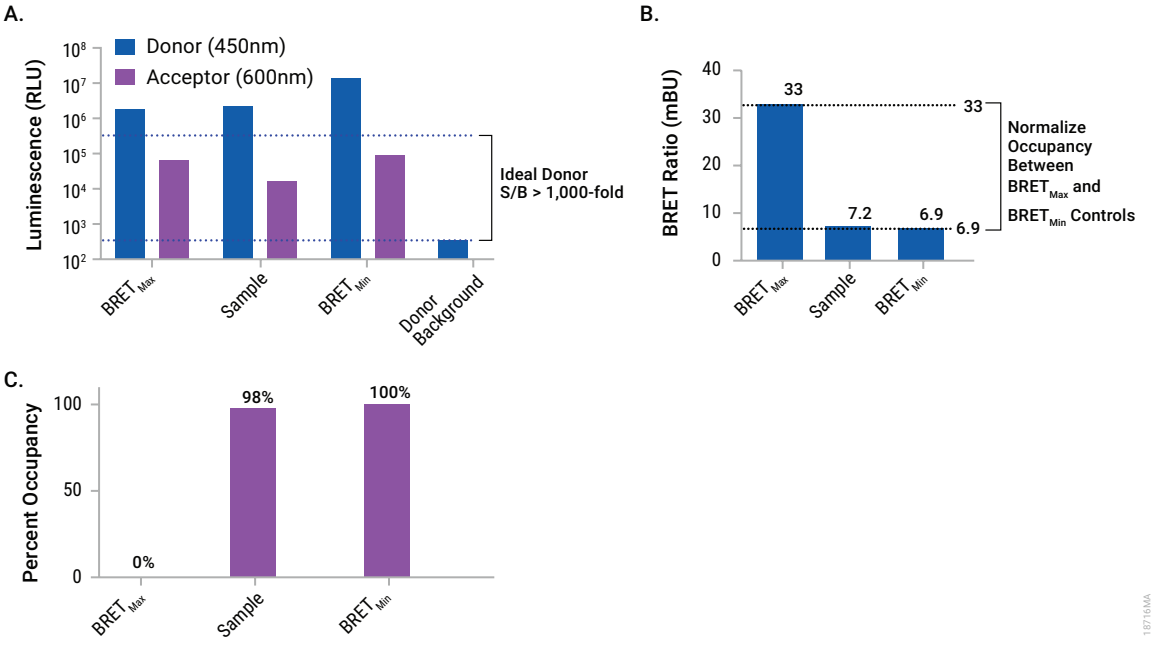

Figure 7. Example raw data and processing for the CDK6 assay. HEK293 cells expressing NanoLuc®-CDK6 and Cyclin D1 in a 96-well assay plate were combined with 1µM NanoBRET™ Tracer K-10 and treated with a sample (500nM palbociclib) or DMSO vehicle ($BRET_{Max}$ Control). The NanoLuc® control vector was included in the experiment as the $BRET_{Min}$ Control and a Donor Background Control was included to assess quality of the expression. After a 2-hour incubation, Complete NanoBRET™ Nano-Glo® Substrate plus Extracellular NanoLuc® Inhibitor was added and BRET measurements were made on a luminometer equipped with 450/80BP and 610/LP filters. **Panel A.** Example raw donor (450nm) and acceptor (600nm) are plotted for both the $BRET_{Max}$ and $BRET_{Min}$ Controls, as well as the palbociclib-treated sample. The sample and controls demonstrated donor signals that were > 1,000-fold above the Donor Background Control. **Panel B.** BRET ratios were calculated as mBRET values for the $BRET_{Min}$ and $BRET_{Max}$ Controls as well as the palbociclib-treated sample. **Panel C.** Percent occupancy was calculated as described in this protocol, using the BRET values for $BRET_{Max}$, $BRET_{Min}$ and sample from **Panel B.**

5.H. References

1. Machleidt, T. *et al.* (2015) NanoBRET-A novel BRET platform for the analysis of protein-protein interactions. *ACS Chem. Bio.* **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. 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.
4. Wells, C.I. *et al.* (2020) Quantifying CDK inhibitor selectivity in live cells. *Nat. Commun.* **11**, 2743.
5. Robers, M.B. *et al*. (2020) Quantifying target occupancy of small molecules within living cells. *Annu. Rev. Biochem.* **89**, 557–82.
6. Robers, M.B. *et al.* (2021) Single tracer-based protocol for broad-spectrum kinase profiling in live cells with NanoBRET. *STAR Protocols* **2**, 100822. **https://doi.org/10.1016/j.xpro.2021.100822**
7. In: *Anthropological Genetics: Theory, Methods and Applications*, Michael H. Crawford, *ed.* (2006) University of Cambridge Press.

5.I. Related Products

NanoBRET™ TE Intracellular CDK Selectivity Assay

Product	Size	Cat.#
NanoBRET™ TE CDK Vector Panel A	1 each	NP5000
NanoBRET™ TE CDK Vector Panel B	1 each	NP5100
NanoBRET™ CDK Selectivity System A	1 each	NP5050
NanoBRET™ CDK Selectivity System B	1 each	NP5150
NanoBRET™ TE Selectivity DNA Controls	1 each	NP1000

NanoBRET™ TE Intracellular Kinase Assays

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

Additional assay sizes are available. For a complete listing of the available kinase TE expression vectors and their NanoBRET™ tracer compatibility, visit: **www.promega.com/kinasevectors**

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5.I. Related Products (continued)

NanoBRET™ 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

Other NanoBRET™ Target Engagement Assays

Product	Size	Cat.#
NanoBRET™ Target Engagement Intracellular HDAC Assays	100 assays	N2080
NanoBRET™ TE Intracellular HDAC Complete Kit	1,000 assays	N2170
NanoBRET™ Target Engagement Intracellular BET BRD Assays	100 assays	N2131
NanoBRET™ TE Intracellular BET BRD Complete Kit	1,000 assays	N2180
NanoBRET™ TE Intracellular E3 Ligase Assay, CRBN	100 assays	N2910
NanoBRET™ TE Intracellular E3 Ligase Assay, VHL	100 assays	N2930

Additional assay sizes are available.

Transfection Reagents and Accessories

Product	Size	Cat.#
FuGENE® HD Transfection Reagent	1ml	E2311
	5 × 1ml	E2312
Transfection Carrier DNA	5 × 20µg	E4881
	2 × 100µg	E4882
TransfectNow™ HEK293 Cells	1 × 0.5ml	NC1001
	2 × 1ml	NC1002

Detection Instrument

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

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6. Summary of Changes

The following changes were made to the 1/24 revision of this document:

1. Corrected percentages in Table 1, Section 4.B for $BRET_{Min}$ and $BRET_{Max}$ controls.
2. Corrected text in Section 4.H, Step 4 to "Donor Background Control".

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

[i]U.S. Pat. Nos. 9,056,885 and 10,139,400, European Pat. No. 2782916, and other patents pending.

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

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