Cellular Kinase Assays that Deliver Quantitative Compound Affinity, Occupancy, and Selectivity in Live Cells using NanoBRET

James Vasta, Cesear Corona, Jennifer Wilkinson, Morgan Thomas, Chad Zimprich, Mei Cong, Frank Fan, Kristin Huwiler, and Matthew Robers

Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711

1. Introduction

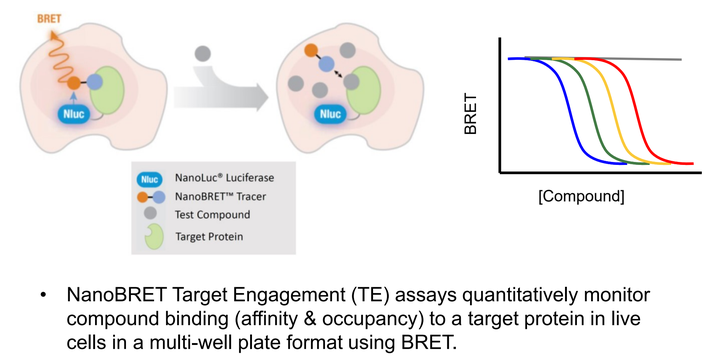
NanoBRET™ Target Engagement is a real-time biophysical technique that enables quantitative assessment of compound-target protein binding in live cells, without disruption of cellular membrane integrity. Specifically, cellular compound affinity, occupancy, and residence time can be measured in a multi-well plate format. The method utilizes energy transfer from a NanoLuc® luciferase-tagged target protein and a cell-permeable fluorescent tracer that reversibly engages the target protein of interest. The quantitative capability is achieved for select target kinases by simply adjusting the concentration of the NanoBRET tracer to an appropriate level based on its predetermined target affinity.

This technique has now been broadly applied to the kinome. Cellular assays for >300 full-length protein kinases are available, including integral membrane receptors and over 30 specific CDK-cyclin pairings. Assay conditions that enable quantitative binding measurements for each kinase have been determined. Quantitative cellular affinity for various types of kinase inhibitors will be demonstrated, including type I, II, and allosteric compounds. We will show how this cellular method can also be applied to quantitative measurement of compound selectivity against numerous kinases.

Time-dependent target-compound occupancy (or residence time) can also be obtained with using the NanoBRET method. An assessment of kinetic and equilibrium selectivity of various kinase inhibitors revealed different residence times for compounds with similar equilibrium affinity. By monitoring both cellular affinity and residence time for a compound, unique inhibitor development opportunities can be revealed.

2. NanoBRET Target Engagement

Affinity / Potency Determinations in Live Cells

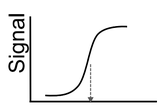


- NanoBRET Target Engagement (TE) assays quantitatively monitor compound binding (affinity & occupancy) to a target protein in live cells in a multi-well plate format using BRET.
- BRET is achieved by luminescent energy transfer from NanoLuc® luciferase to the cell-permeable fluorescent Tracer that is bound to the target-NanoLuc fusion protein.
- NanoBRET TE assays are specific for the target fused to NanoLuc, as BRET assays are governed by tight distance constraints between energy donor (NanoLuc) and energy acceptor (Tracer).

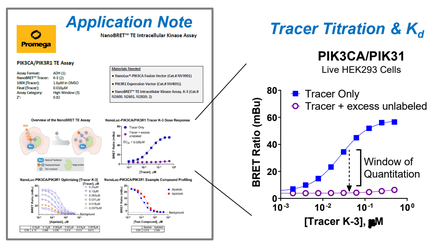
3. NanoBRET TE Kinase Assays Quantitative for Affinity & Occupancy

A. ***Conditions for Quantitation***

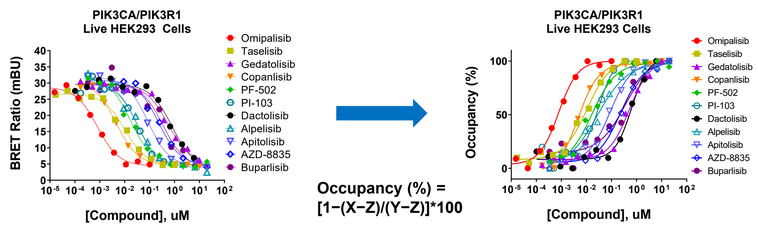
- [Tracer] ≤ K_d-apparent
- [Target] << compound K_d-apparent



B. ***Kinase Specific Application Notes Provide Assay Conditions***



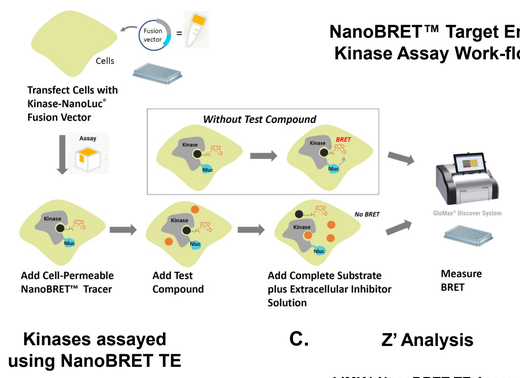
C. ***Converting BRET Ratio to Fractional Occupancy***



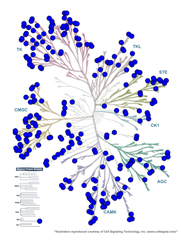
- **Quantitative compound affinity is achieved when (A):**
  - [Tracer] ≤ K_d-apparent, which is provided for each kinase
  - [Target] << compound K_d-apparent, achieved by low kinase expression.
- **NanoBRET TE Kinase-specific application notes allows quick start-up by providing assay conditions including Kd (B)**
  - Compound IC_{50} approach $K_i^{apparent}$ by using tracer concentration ≤ K_d
  - Therefore, NanoBRET TE measures more than rank order potency
- **NanoBRET TE is quantitative for compound occupancy, by (C):**
  - Using controls for background (Z) & max (Y) BRET
  - Converting BRET Ratio of Sample (X) to Occupancy
  - Fractional occupancy allows quantifying compound selectivity across many kinases

4. Homogenous, Scalable Method to Assay >340 Kinases in Live Cells

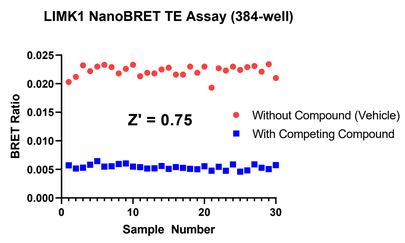
A.



B. **Kinases assayed using NanoBRET TE**



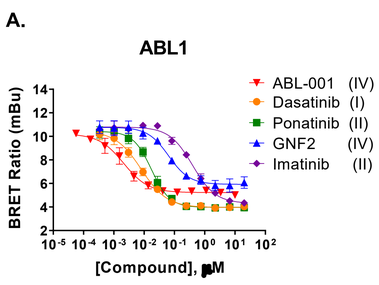
C. **Z' Analysis**



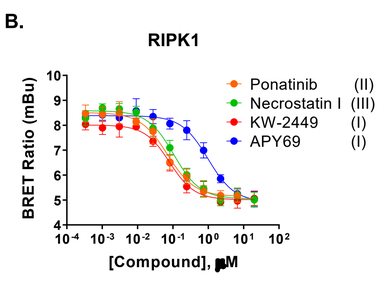
- Addition only cellular assay method performed in multi-well plates is scalable from 96-well to 384-well (A).
- >340 live cell kinase assays spanning the kinome, including full-length wild-type and mutant kinases. Each kinase has assay conditions and data available (B).
- The ratiometric BRET data and addition only format for these assays leads to excellent data quality (C).

5. Evaluate Diverse Chemical Matter and Effect of Clinical Kinase Mutations

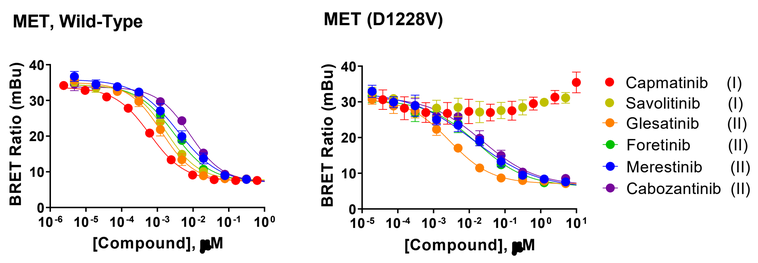
A.



B.



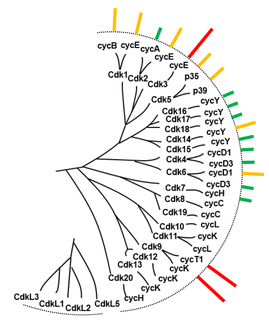
C.



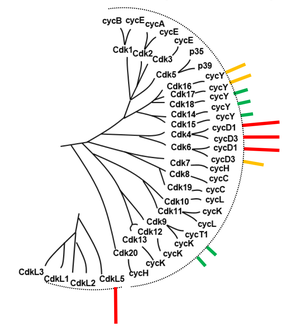
- NanoBRET TE Kinase Assays can be used to characterize type I, II, and allosteric (III, & IV) kinase inhibitors as shown for ABL1 and RIPK1 kinases (A & B).
- The ability wild-type MET and the clinical MET mutant D1228V to bind various type I & II kinase inhibitors were evaluated using NanoBRET TE. The type I inhibitors Capmatinib and Savolitinib bound potently to WT MET, as did the type II inhibitors (C). The MET (D1228V) mutant failed to bind these type I inhibitors, but retained ability to bind the type II inhibitors Glesatinib, Foretinib, Merestinib, & Cabozantinib.

6. Intracellular CDK Selectivity and Cyclin Bias for Clinical Drugs

A. ***Dinaciclib (SCH727965)***



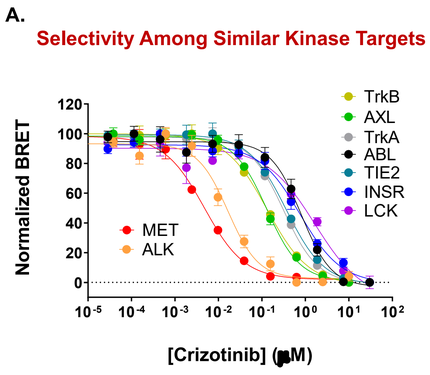
B. ***Abemaciclib (LY2835219)***



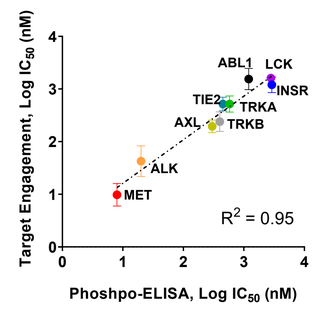
- Cellular selectivity profile of Dinaciclib identifies primary targets of CDKs 1 - 6 & 9, in addition to binding several cyclin Y CDKs (CDKs 14-18). Dinaciclib intracellular potency can depend on cyclin pairing (A).
- Cellular selectivity profile of the selective CDK inhibitor Abemaciclib reveals the primary targets CDKs 4 & 6. Also, it binds CDKs 14-18. Abemaciclib intracellular potency can depend on cyclin pairing (B).

7. Determine Cellular Compound Selectivity Against Similar Kinases

A. **Selectivity Among Similar Kinase Targets**



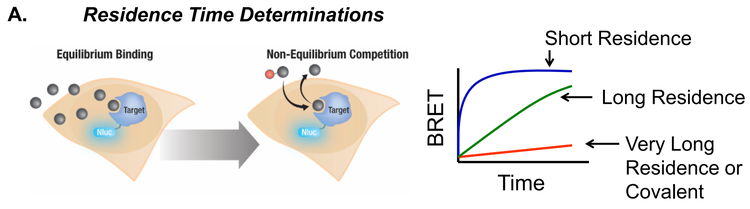
B. **Correlation to Phenotypic Data**



- **The multi-kinase inhibitor Crizotinib's cellular selectivity was determined using NanoBRET TE (A). The primary targets, MET and ALK, showed the strongest affinity.**
- **Cellular selectivity of Crizotinib could be determined against multiple kinases because NanoBRET TE**
  - Assays are quantitative for compound affinity, not just rank order potency (see panel 3)
  - Assays are available for >340 kinases
- **NanoBRET TE data correlates well with cellular functional assays such as phospho-ELISA (B)**
  - NanoBRET TE assays are more specific and scalable than some functional assays

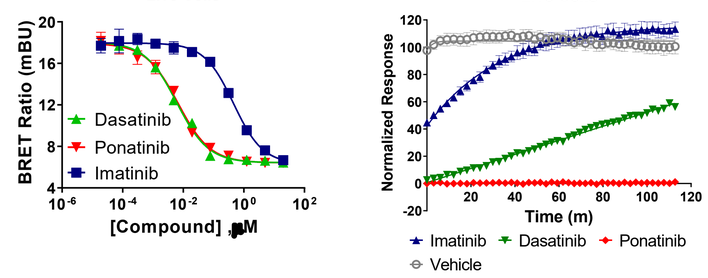
8. Intracellular Residence Time & Affinity May Not Always Correlate

A. ***Residence Time Determinations***



B. **Equilibrium Binding, ABL1 Kinase Live Cells**

C. **Residence Time, ABL1 Kinase Live Cells**



- Intracellular residence time is evaluated using NanoBRET TE via real-time kinetic analysis (A)
- The chronic myelogenous leukemia (CML) drug Imatinib (1st generation) shows weaker binding (B) & shorter residence time (C) at ABL1, compared to Dasatinib and Ponatinib (2nd & 3rd generation drugs).
- Ponatinib displays similar affinity (B) to Dasatanib, but much longer residence time (C).

9. Conclusions

NanoBRET TE cellular assays have been developed for >340 full-length kinases

- The assays are ready-to-use with application notes for each kinase
- Multi-well plate addition only assay format is simple & scalable
- BRET read-out provides excellent data quality

NanoBRET TE Kinase assays are quantitative for compound affinity and occupancy inside live cells

- Kinase inhibitor types I, II, III, and IV have been studied
- Equilibrium cellular assay measures direct binding of compound to kinase, not a downstream event

Compound selectivity inside live cells is measured using NanoBRET TE Kinase

- Quantitative compound affinity measurements against multiple kinases enable compound selectivity determination
- NanoBRET TE selectivity data is physiologically relevant, correlating to cellular functional data

Residence time in live cells is measured with NanoBRET TE

- Using both equilibrium & residence time methods, selectivity may be revealed- offering unique inhibitor development opportunities.

Corresponding author: kristin.huwiler@promega.com