Monitoring and screening intracellular protein:protein interactions with an improved BRET assay and compatible instrument

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Principles of Bioluminescence Resonance Energy Transfer

Using BRET to study protein:protein interactions (PPI) in live cells

Current BRET configurations have limitations

- Poor spectral separation
- Poor signal-to-noise
- Low light output requires high donor expression
Improving BRET with brighter NanoLuc® Donor and red shifted HaloTag® Acceptor

NanoBRET™ Assay

NanoLuc Donor
- 19kD, Monomeric
- 100-150X Brighter than current luciferases
- Fusion partner

HaloTag Acceptor
- 34kD, Monomeric
- Binds optimized fluorescent ligand
- Fusion partner

- Proximity
- Geometry
- Spectral overlap

Optimal spectral overlap
- Increased signal
- Lower background
Comparison of NanoBRET™ with conventional BRET
PPI model: FKBP / Frb in presence of rapamycin

- Improved signal/background as compared to other BRET systems
- Greater light output with NanoLuc enables BRET at low levels of expression
NanoBRET™ assay optimization strategy

1. Append donor and acceptor tags to proteins
   - Protein A: NL, HT
   - Protein B: NL, HT

   Each N or C terminus for a total of up to 8 possible constructs

2. Find combination(s) give best ratios

3. Determine optimal donor to acceptor DNA ratio to minimize free/unbound donor to maximize dynamic range (NanoLuc donor plasmid serial dilution)

4. Validate assay by modulators (ex. inhibitor or activator) or determine assay specificity by saturation assays
NanoBRET™ Protocol Overview

**Day 1**
Co-transfect donor and acceptor vectors.

**Day 2**
- Replate cells with and without HaloTag® NanoBRET™ 618 Ligand.
- Add NanoBRET™ Nano-Glo® Substrate, and measure donor and acceptor signals.

**Day 2-3**
- Calculate NanoBRET™ Corrected Ratio.

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\text{NanoBRET™ Corrected Ratio} = \frac{\text{Ligand} \ (618\text{nm})}{\text{460nm}} - \frac{\text{No-ligand control} \ (618\text{nm})}{\text{460nm}}
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NanoBRET™ Instrumentation requirements

- Instrument capable of sequentially measuring dual filtered luminescence
- Ideal filter set-up
  - Band pass filter around 460 nm to measure the donor signal
  - Long pass filter starting at around 600 nm to measure the acceptor signal
- Promega GloMax® Discover instrument is pre-equipped with NanoBRET™ Compatible Filters
  - 450/8BP nm to measure the donor signal
  - 600/LP nm to measure the acceptor signal
  - Compatible with 96 or 384-well formats
  - Pre-loaded protocol automatically calculates BRET ratios and creates a heat map
NanoBRET™ performance on GloMax® Discover

PPI model: p53 / MDM2 in presence of Nutlin-3 inhibitor

- Excellent assay robustness in both 96 and 384-well plate formats
Multiplexing with CellTiter-Glo® 2.0
Monitor cell health or compound toxicity

- Following NanoBRET measurements, add CellTiter-Glo® 2.0 reagent to the same plate and read on the GloMax® Discover instrument.
- Nutlin-3 shows no toxicity as evidenced by no effect on cell viability.
- Decrease in BRET signal is truly due to compound inhibition – assay is ratio-metric and ratio is only derived from live cells.
Utilizing NanoBRET™ assays in epigenetic research
Study of bromodomain interactions with histones
Current high interest for drug targeting

Bromodomains
- Epigenetic readers of histone acetylation
- Regulate key developmental genes
- Implicated in numerous diseases
- Promising initial compounds for BET family bromodomain proteins (BRD2, BRD3, and BRD4) which reduce tumor growth

Challenges of current methodology
- Most current assays utilize purified domain and histone fragments
- Complexity of chromatin difficult to recapitulate in vitro assays
- Further complexity of non-BET family members with multiple points of contact with chromatin

NanoBRET bromodomain-histone interactions in live cells
- Ability to use full-length proteins or domains
- Chromatin context with all possible modifications

Monitoring interactions with chromatin in living cells
Histones-HaloTag fusions follow proper physiology

- Observed chromatin incorporation of HaloTag fusions: H2A, H2B, H3.3, and H4
- Other Histone variants available (H3.1, H3.2, H2A.X, H2A.Z, MacroH2A)

Scale bars = 10µm
Monitoring BRD4 binding to histone H3.3 or H4
NanoBRET™ measurement with optimized configuration

- Detection of specific interaction of BRD4 with Histones H3.3 and H4

- *Can we validate these assays by seeing the effect of a known inhibitor?*
Demonstrating specificity for BRD4/H4 assay

Treatment with BET inhibitor

- Expected response to IBET151 for the interaction of BRD4 with Histone 4

- IBET151 does not inhibit the interaction between Histone 4 and non-BET family CBP
**Demonstrating specificity for assays without inhibitors**

**Donor Saturation Assay**

① As the acceptor-to-donor (A/D) ratio increases, a specific BRET assay will show ratios that increase in a hyperbolic manner and reach a plateau representing complete saturation of all donors with acceptor molecules.

② Non-specific interaction with a negative control protein (bystander BRET) generates much weaker ratios that plot in a linear manner.
Ability to study full-length proteins or isolated bromodomain
Inhibition of BRD4/H3.3 by IBET151

- Similar IC50s obtained for BD1 domain alone as compared to full-length protein
- **What would happen when bromodomain containing proteins also include other chromatin interacting domains with different functions?**
Differential inhibition profiles for full-length protein vs. domain
Inhibition of CBP by SGC-CBP-30

- Compound is able to displace from chromatin the isolated bromodomain of CBP
- No inhibition of the full-length CBP likely due to other chromatin interacting domains
Differential inhibition profiles for full-length protein vs. fragments
Breaking down the contributions of the BAZ2A domains

- Addition of PHD domain significantly reduces inhibition by GSK2801
Advantages of performing bromodomain assays inside of living cells by NanoBRET™

- Bromodomain proteins are very complicated
  - Multiple reader domains
  - Multiple points of contact

- Chromatin is very complicated
  - Multiple histone modifications
  - Multiple histones contacted by a single protein

- Chromatin environment cannot be recreated in any *in vitro* assay

- You get what you screen for - which may not translate to reality in living cells
  - NanoBRET™ bromodomain assays are physiological relevant
  - Able to parse out binding contributions of various domains
NanoBRET™ assays being used in compound research

Example recent publications

   Compounds shows specificity for β vs. α variants of BRPF1 interacting with Histone H3.3

   EZH2 inhibitors do not impact EZH2 interactions with other PRC2 components or chromatin occupancy

   Developing specific BRD9 non BET inhibitor and detection of cell toxicity

   Developing selective BRD7/9 inhibitor
Available NanoBRET™ Products
## NanoBRET™ Pre-Built Assays

### Pre-built catalog assay bundles

1. BRD4* + Histone H3.3 (N1830)
2. BRD9* + Histone H3.3 (N1840)
3. CBP* + Histone H3.3 (N1850)
4. BRPF1* + Histone H3.3 (N1860)
5. BRD4* + Histone H4 (N1890)
6. BRD9* + Histone H4 (N1900)
7. BRPF1* + Histone H4 (N1910)
8. cMyc + MAX (N1870)
9. G12C KRAS + BRAF (N1880)

- Include p53/MDM2 control protein pair
- Bromodomain bundles* include the full-length protein as well as the individual bromodomain (BD1 for BRD4)
- Fully tested and validated

### Pre-built custom assays (~100)

1. Other Bromodomain Assays
2. Other Epigenetic Assays
3. Transcriptional Protein Assays
4. Signaling Proteins and Kinase Assays
5. Membrane Protein Assays
6. RNA Binding Protein Assays

### Custom assay development

1. On demand assay optimization and validation
2. Option to test compounds

[www.promega.com/nanobret](http://www.promega.com/nanobret)
NanoBRET™ Build-Your-Own Assay

1. Empty NanoLuc® and HaloTag® vectors to add your own content
   - Flexi® Vector bundle (directional cloning method for rapid transfer of protein-coding regions among vectors)
   - MCS Vector bundle
     - Content as N terminal HaloTag fusion available from Find-My-Gene™ (>9,000)
2. Include p53/MDM2 control protein pair
GloMax® Discover Instrument

Learn More and Request a Demo: www.promega.com/discover
NanoBRET™ Technology enables the study of multiple types of dynamic PPI inside cells

**Binary interactions**

**Induction**

**Inhibition**

**FL proteins or domains**

**Dimerization**

**Real time kinetics**
NanoBRET™ PPI assays

- Improved BRET protein interaction method using NanoLuc® and HaloTag®
- Increase signal:background and dynamic range
- GloMax® Discover instrument fully compatible
- Screening compatible in 96- and 384-well format
- Can study full-length proteins vs. domains
- Pre-built assays for bromodomain and other high value targets
- Build-Your-Own assays vector bundles with strategy guidelines
- Assays adopted in active compound research
- Monitor multiple types of dynamic interactions within the cell
Thank you!