

Screening for Inhibitors of Protein:Protein Interactions in Living Cells: Development of assays to detect modulation of proteins using NanoBRET™ technology

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Introduction

Studying the interplay between proteins in living cells allows for the development of compounds designed to inhibit protein-protein interactions (PPI) to modulate biological processes in normal and disease tissue. By using the NanoBRET system (Promega), each protein pair is tagged with either a cDNA of NanoLuc, a luminescent enzyme, or HaloTAG, a fluorescent molecular probe. Upon interaction of the protein pair and in the presence of luminescence substrate, the photon generating NanoLuc enzyme on one partner comes into close proximity with the HaloTAG fluorescence ligand on the other to generate a Bio Resonance Energy Transfer (BRET) signal within the cell, detectable in a plate reader.

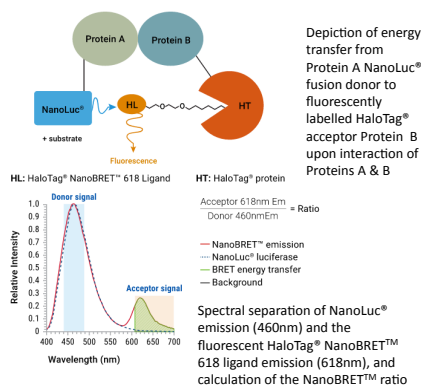
Using this system we have screened for inhibitors of the Shn-3 (Shn-3) ERK-2 PPI in HEK-293 cells. Shn-3 is a potent and essential regulator of adult bone formation. Mice lacking Shn3 profoundly increase bone mass due to augmented osteoblast activity. Shn-3 suppresses ERK phosphorylation of GSK-3 β leading to suppression of β-catenin activity. By blocking Shn-3 interaction with ERK-2 we hope to remove this brake and increase bone formation in osteoporotic patients. Selecting and screening a library of over 20,000 compounds we identified hits that on further examination were both potent and selective inhibitors of the Shn-3 ERK-2 PPI.

Methodology

HEK-293 cells were transiently transfected with two cDNA constructs; an Shn-3-NanoLuc® construct and an ERK-2 HaloTag® construct (courtesy of Promega). Briefly, 2x10⁷ cells were transferred into a T225cm² flask and allowed to settle for 4-6hrs in the incubator. The cDNA constructs were mixed with Fugene at pre-defined concentrations, added to the cells and incubated overnight. Cells were then accutased, counted and divided into two groups, (plus HaloTag® fluorescence label as the positive signal and minus HaloTag® as the negative signal). Cells were dispensed at 8,000 cells per well in 36ul media into a Greiner white 384 well plate (plus HaloTag® = columns 1 through 22, minus HaloTag® = columns 23/24) followed by 4ul of compound from a pre-prepared compound plate to generate a final assay concentration of 20uM in 0.5% DMSO (or DMSO alone in columns 23 & 24).

Cells were incubated overnight, then 10ul of 5X NanoLuc® substrate was added to each well and plates were shaken vigorously for 60 seconds. Plates were read for 1 second/well at an emission of both 460nm and 600+nm on an Envision plate reader (PerkinElmer) using appropriate emission filters. The ratio of NanoLuc® signal (@460nm) to fluorescence signal (@600nm) was determined.

Figure 1. NanoBRET™ PPI Assay Principle



Assay Development & Validation

Figure 2. Competition of MEK protein with Shn-3 for binding to ERK-2

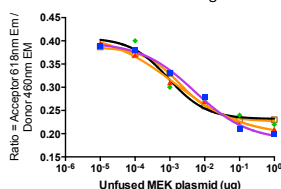
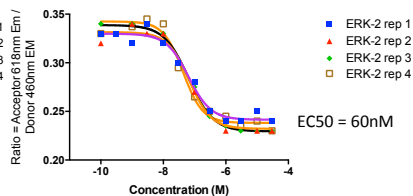


Figure 3. Competition of an ERK-2 inhibitor binding to ERK-2 and preventing the binding of Shn-3



To develop and validate the reagents in the absence of a Shn-3-ERK-2 inhibitor, we manipulated ERK-2 protein available for binding to Shn-3 by titrating it out using the over-expression of MEK within the cells (Figure 2.) or pharmacologically with an ERK-2 compound (Figure 3.). Increasing amounts of MEK cDNA were transfected together with Shn-3 and ERK-2 cDNA into cells. Increasing amounts of MEK protein interact with and titrate out ERK-2 and therefore decreased the Shn-3 interaction with ERK-2 resulting in a concentration dependent decrease in NanoBRET™. Similarly interaction of ERK-2 with a ERK-2 binding compound prevents the protein from interacting with Shn-3 in a dose dependent manner.

High Throughput Screen

Figure 4. Frequency Distribution of Compound Inhibition of the Shn-3 – ERK-2 Interaction

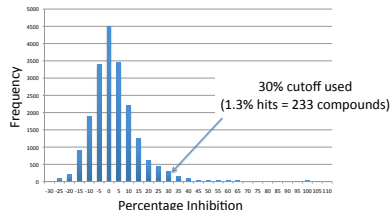


Figure 5. Z' Factors for the 56 x 384 well plates screened in the NanoBRET™ Shn-3 – ERK-2 assay

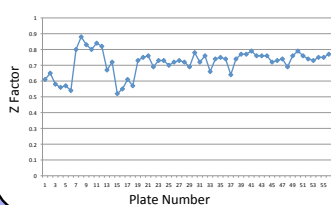
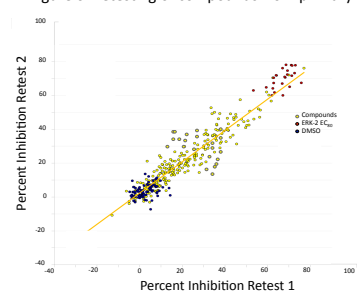


Figure 6. Retesting of compounds from primary assay



In total 56 x 384 well plates were screened in the primary assay (~20,000 compounds) at 20uM, with Z factors over 0.5 (Figure 5). The distribution of the activity of all compounds was centered around zero (Figure 4) and using a 30% cutoff 233 compounds (1.3%) were taken forward for retesting. The compounds were retested at 20uM in the primary assay and 69 with activity over 35% (Figure 6.) were taken forward for EC₅₀ testing in the Shn-3 – ERK-2 assay (Figure 7. below) in addition to testing in a selectivity Shn-2 – ERK NanoBRET™ assay and a non-selectivity p53 – MDM-2 NanoBRET™ assay.

Selectivity and Non-Specific Technology Assay

To examine the selectivity of compounds with the help of Promega we developed a counter screen by transiently expressing Shn-2 NanoLuc® and ERK HaloTag® (Figure 8.). Furthermore to exclude compounds that non-specifically interacted with the NanoLuc®-HaloTag® technology we purchased an unrelated PPI assay kit containing p53 NanoLuc® and MDM-2 HaloTag® (Figure 9.). Compounds were profiled as dose-response curves in the primary assay, in the Shn-2 - ERK and P53 – MDM-2.

Figure 7. Dose-response profile in NanoBRET™ Shn-3 – ERK-2 assay

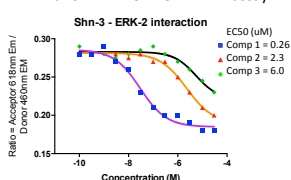


Figure 8. Dose-response profile in NanoBRET™ Shn-2 – ERK assay

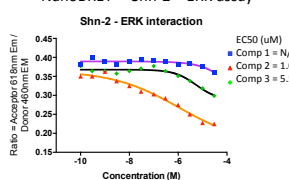
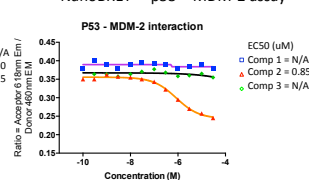


Figure 9. Dose-response profile in NanoBRET™ p53 – MDM-2 assay



Summary and Conclusion

- We successfully developed a NanoBRET™ based PPI assay to study the interaction of Shn-3 with ERK-2
- We used the assay to screen ~20,000 compounds and identified a small number of compounds that appeared to specifically interact with Shn-3 and block it's binding to ERK-2
- Hits in the primary assay were dose dependent, however some compounds interacted with Shn-2
- A small number of hits also blocked the interaction of P53 with MDM-2 suggesting these compounds either bind non-specifically to proteins or interact with the NanoBRET™ system
- **The NanoBRET™ system opens interesting new opportunities for drug discovery to examine protein – protein interactions in native environments within living cells**

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