Protein-Protein interactions in Living Cells: Development of an assay to modulate Schnurri-3-ERK-2 using the Promega NanoBRET™ technology

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Introduction

Pharmacological intervention between protein-protein interactions (PPI) represents an opportunity to modulate biological processes within cells. Historically, screening for compounds that interact in PPI's has occurred in solution-based assays, however a more physiological way to study PPI is by examining the interaction in living cells. The Promega NanoBRET™ technology relies on Bioluminescence Resonance Energy Transfer (BRET) using NanoLuc® Luciferase as the BRET energy donor and HaloTag® protein labelled with the HaloTag® NanoBRET™ 618 fluorescent ligand as the energy acceptor to measure the interaction of two binding partners in cells.

Using this system we have screened for inhibitors of the Schnurri-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 beta leading to suppression of beta-catenin activity. By blocking Shn-3 interaction with ERK-2 we hope to remove this brake and increase bone formation in osteoporetic patients.

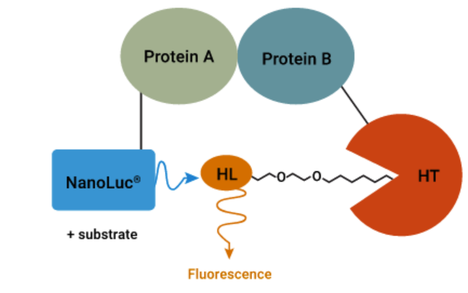
Methodology

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, 2x10^7 cells were transferred into a T225cm^2 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).

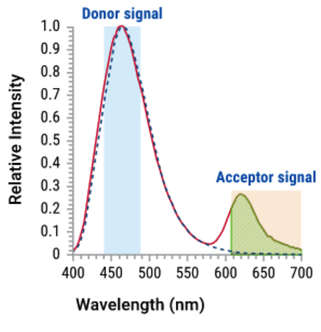
Cells were incubated overnight, then 10ul of 5X NanoLuc® substrate was added to each well and plates were shaken vigorously for 60 seconds. Plates were read for 1 second/well at an emission of both 460nm and 600+nm on an Envision plate reader (PerkinElmer) using appropriate emission filters. The ratio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

Figure.1. NanoBRET™ PPI Assay Principle



Depiction of energy transfer from Protein A NanoLuc® fusion donor to fluorescently labelled HaloTag® acceptor Protein B upon interaction of Proteins A & B

HL: HaloTag® NanoBRET™ 618 Ligand **HT:** HaloTag® protein



$\frac{\text{Acceptor 618nm Em}}{\text{Donor 460nmEm}}$ = Ratio

— NanoBRET™ emission
···· NanoLuc® luciferase
— BRET energy transfer
— Background

Spectral separation of NanoLuc® emission (460nm) and the fluorescent HaloTag® NanoBRET™ 618 ligand emission (618nm), and calculation of the NanoBRET™ ratio

Assay Development & Validation

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

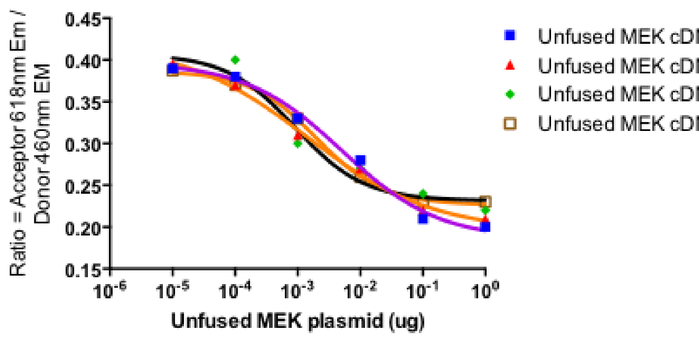
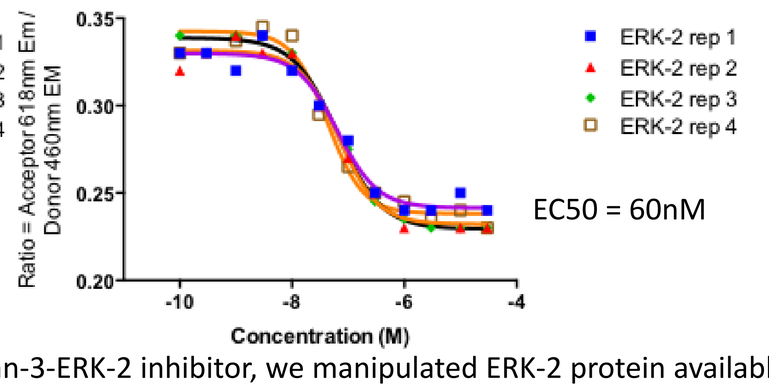


Figure 3. Competition of an ERK-2 inhibitor binding to ERK-2 and preventing the binding of Shn-3



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 Thoughput Screen

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

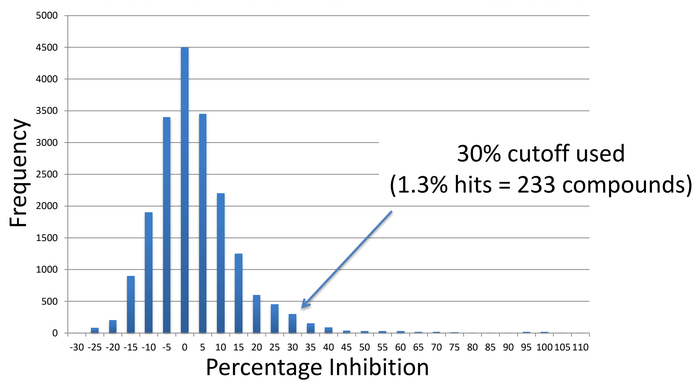


Figure 5. Z' Factors for the 56 x 384 well plates screened in the NanoBRET™ Shn-3 – ERK-2 assay

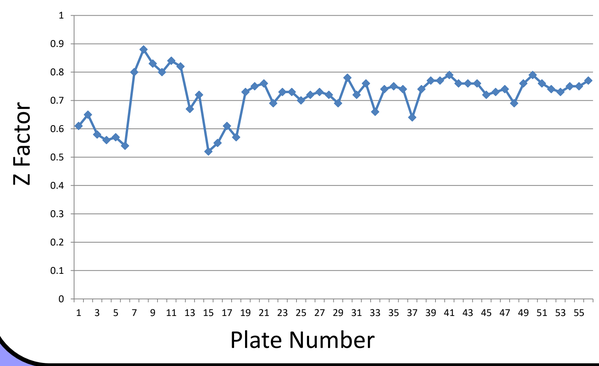
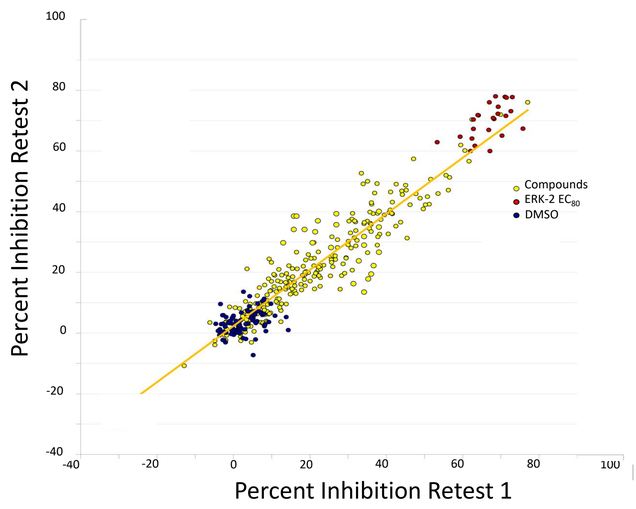


Figure 6. Retesting of compounds from primary assay



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 centred 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 EC_{50} 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

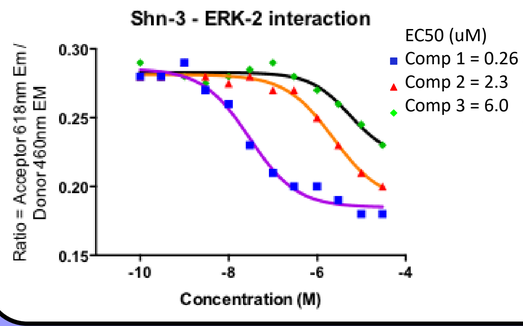


Figure 8. Dose-response profile in NanoBRET™ Shn-2 – ERK assay

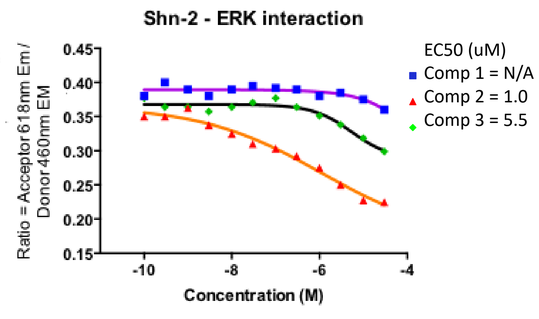
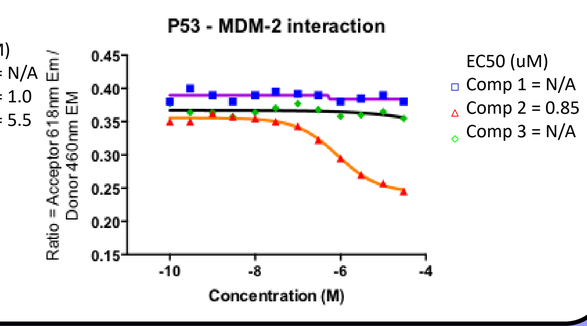


Figure 9. Dose-response profile in NanoBRET™ p53 – MDM-2 assay



Summary and Conclusion

- With the help of Promega we successfully developed a NanoBRET™ based PPI assay in order 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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