Novel Cloning Vectors for Stably Expressing NanoBiT® Fusion Proteins

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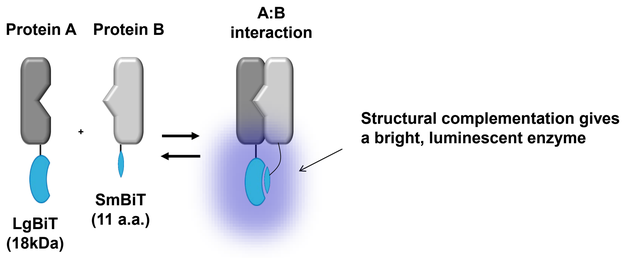
1. Introduction

Protein:protein interactions (PPIs) are essential to cellular signal transduction pathways. NanoLuc® Binary Technology (NanoBiT) is a two-subunit system based on NanoLuc® luciferase that can be applied to detecting PPIs. Large BiT (LgBiT; 18 kDa) and Small BiT (SmBiT; 11 amino acid peptide) subunits are expressed as fusions to proteins of interest, where PPI facilitates subunit complementation to give a bright, luminescent enzyme. Unlike related approaches where an enzyme or protein is simply split, LgBiT was independently optimized for structural stability, and SmBiT was selected from a peptide library specifically for the PPI application. The result is a subunit pair that weakly associates (K_D = 190µM) yet shows only threefold lower activity at saturation vs. NanoLuc *in vitro*. In contrast to many split systems, the LgBiT:SmBiT interaction is reversible, able to detect rapidly dissociating proteins. PPI dynamics can be followed in real-time in living cells using the Nano-Glo® Live Cell Reagent, a nonlytic detection reagent containing the cell-permeable furimazine substrate.

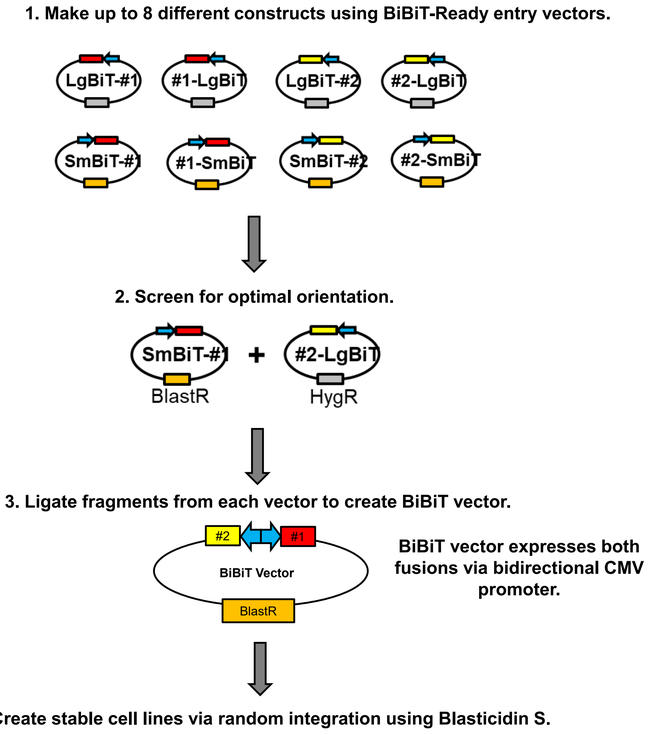
Although transient expression of fusion partners can be sufficient for many experiments, stable cell lines are often needed for maximal reproducibility. Here we describe the use of BiBiT-Ready vectors and the BiBiT approach for stable expression of LgBiT and SmBiT fusion proteins. This approach means both fusion proteins are transcribed at similar levels from the same locus following random integration of plasmid DNA into the genome, increasing the odds of finding a clone with appropriate levels of expression of both fusion proteins. We demonstrate this approach to make stable cell lines for several key membrane protein interactions: HER2:HER3, VEGFR1:VEGFR1 and CX3CR1/ARRB2. We also describe how to create a stable cell pool for CRAF:BRAF.

2. NanoBiT® Protein:Protein Interaction System

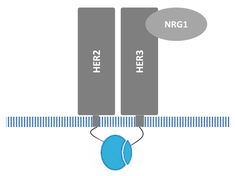


- Subunits are very small; LgBiT is structurally stable (good fusion partners)
- Very bright signal (minimizes artifacts even at low expression levels)
- Low affinity interaction of LgBiT:SmBiT, K_D = 190µM (minimize assay background)
- Live cell, nonlytic assay format (study protein dynamics in living cells)
- Reversible interaction of LgBiT:SmBiT (study association and dissociation events in real time)
- Examples of validated interactions:
  - Homo- and heterodimerization of soluble proteins (e.g., AR:AR)
  - Recruitment to activated PM receptors (e.g., EGFR:GRB2)
  - Homo- and heterodimerization of PM receptors (e.g. HER2:HER3)

3. BiBiT Approach to Stable Cell Line Development

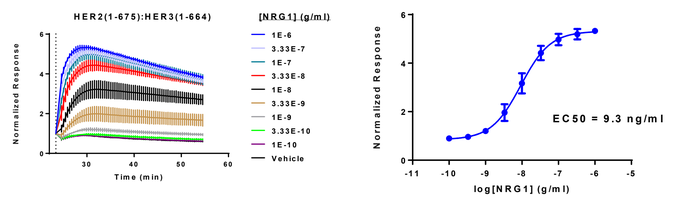


4. Creating a HER2:HER3 Stable Cell Line

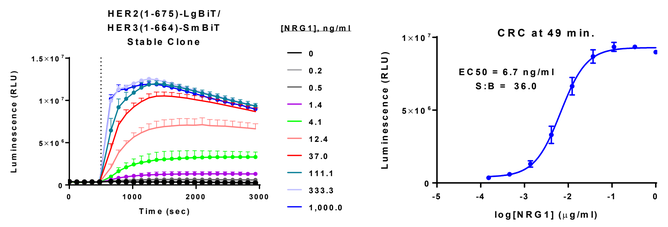


- First examined response to NRG1 treatment using transient expression
- Then stably expressed both fusion proteins in U2OS cells using BiBiT vector
  - Screened clones for maximal signal-to-background (S:B) ratio
  - Clones typically have a >S:B vs. transient expression

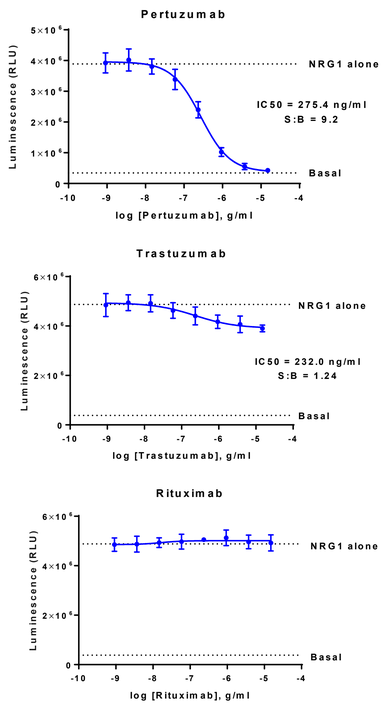
Results from Transient Expression



Results from Stable Expression Using BiBiT Vector

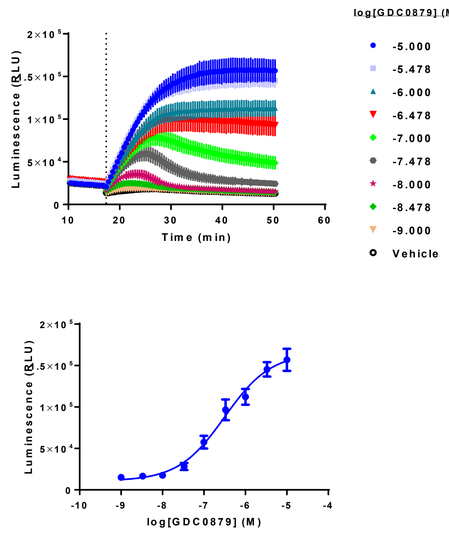


5. Inhibiting HER2:HER3 Dimerization Using mAbs



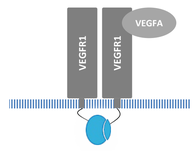
- Used the stable BiBiT clone described in Section 4
- Incubated with a dose range of mAbs for 30 minutes followed by EC80 concentration of NRG1
- Pertuzumab is known to block the dimerization of HER2:HER3
- Trastuzumab is known to bind HER2, but not known to effectively inhibit dimerization
- Rituximab as negative control. No effect on HER:HER3 dimerization

6. Creating a CRAF:BRAF Stable Pool

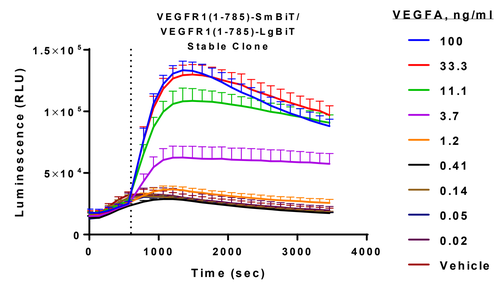


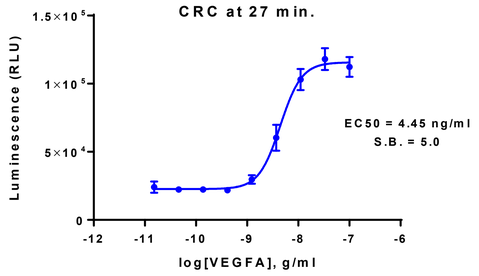
- HEK293 cells were transfected with a BiBiT vector encoding CRAF-SmBiT and BRAF-LgBiT fusion proteins.
- Transformed cells were selected using 2ug/ml Blasticidin S to create a stable pool.
- The stable pool was assayed using GDC0879 to induce dimerization of the NanoBiT® fusion proteins, which were monitored in real time.

7. VEGFR1:VEGFR1 Stable Cell Line



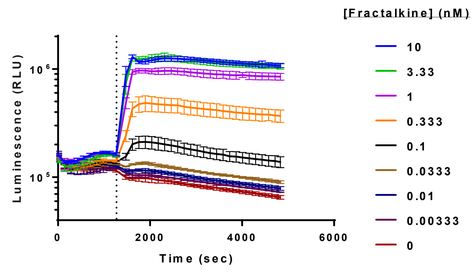
- Stable expression of both fusion proteins in U2OS cells using BiBiT vector
- Monitor VEGFR1 dimerization in real time after adding VEGFA

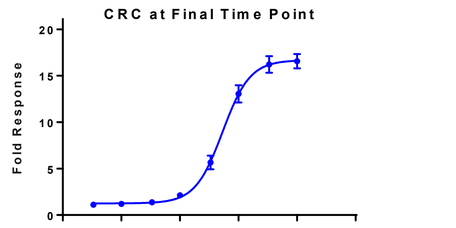




8. CX3CR1:ADRB2 Stable Cell Line

- Stable expression of CX3CR1-LgBiT & SmBiT-βarr2 in HEK293 cells using BiBiT vector
- Treatment with soluble fractalkine agonist at indicated time point (dotted line)
- Potential bioassay for antibodies targeting CX3CR1 or CX3CL1 tor the treatment of autoimmune disease





9. Conclusions

NanoBiT Protein:Protein Interaction System

- Small fusion partners: SmBiT,11 a.a.; LgBiT,18 kDa
  - LgBiT evolved for structural stability
- Extremely bright for expression at or near endogenous levels
- Low affinity interaction between LgBiT and SmBiT minimizes assay background
- Live-cell, nonlytic assay format to study protein dynamics in real time
- Reversible system to study protein association/dissociation events in real time

BiBiT-Ready Cloning System

- Clone into BiBiT-Ready vectors and determine optimal orientation
- Create single BiBiT construct encoding both fusion proteins with:
  - Bidirectional CMV promoter
  - Random integration and selection using Blasticidin S
  - Equivalent levels of transcription of both fusions from the same locus
- Vectors compatible with existing NanoBiT® cloning vectors for transfer
- Proven performance with multiple PPI pairs