Determining the EC50 of Tracer-6908 with ACVR1/ALK2-NanoLuc

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Background:

In nanoBRET assay, a fluorescently labelled tracer binds to the ATP-binding pocket of ACVR1/ALK2. This binding brings the tracer into the proximity (<10nm) of Nano-Luciferase which is fused to the C-terminus of ACVR1/ALK2. Bioluminescence resonance energy transfer (BRET) occurs where the energy released by Nano-Luciferase is transferred to the fluorophore via intermolecular forces. Nano-Luciferase and tracer emit light at 460nm and 610nm respectively. The amount of tracer bound to ACVR1/ALK2 will be proportional to BRET signal (610nm light). The direct engagement of compound molecules to ACVR1/ALK2 can be determined from the reduction in BRET signal.

Matt Robers and Jim Vasta from Promega had provided us with Tracer-6908 that was developed collaboratively with SGC. Before the tracer can be used in nanoBRET assay for compound IC50, it is necessary to determine the optimal tracer concentration. The tracer concentration needs to be sufficient to produce adequate BRET signal while at the same time low enough to not obscure the competition by test compounds. Catherine Rogers from Kilian Huber's lab offered valuable advice and kindly helped to setup the Pherastar FSX for BRET measurement.

Protocol:

Recipes:
Cell Culture Medium
90% DMEM (Life Technologies 11995)
10% FBS (HyClone SH30070.03)

Assay Medium
Opti-MEM WITHOUT PHENOL RED 100% (Life Technologies 11058)

NanoBRET Tracer Dilution Buffer
12.5mM HEPES, pH7.5 (Sigma H0887)
31.25% PEG-400 (Sigma 91893)

FuGENE® HD (Promega E2311)
Transfection Carrier DNA (Promega E4881)
Extracellular NanoLuc Inhibitor (30mM in DMSO) (CAS part # CS181047 or CS181048)
NanoBRET Nano-Glo® Substrate (CAS part # CS181045 or CS181046)

Day 1
HEK293 transfection
1) Medium was removed from cell flask via aspiration, cells were washed once with PBS and trypsinized for 5 minutes at 37°C to dissociate cells from the flask.
2) Trypsin was neutralised using growth medium and cells were pelleted via centrifugation at 500 x g for 3 minutes.
3) Medium was aspirated and cells were resuspended into a single cell suspension using cell culture medium (without antibiotic).
4) The cell density was adjusted to 2 x 10^5 cells/mL in cell culture medium.
5) Lipid:DNA complexes were prepared as following:
    a. 100uL solution of DNA in Opti-MEM without serum for every 400,000 cells.
        i. Plasmids combination:
        100ng ACVR1-c-nanoLuc + 900ng transfection carrier DNA
        100ng pEGFP-C1 + 900ng transfection carrier DNA

ii. 100uL of Opti-MEM without phenol red

b. Thoroughly mixed.

c. 3uL of FuGENE® HD was added into each 100uL of DNA mixture to form lipid:DNA complex. Caution was taken when adding FuGENE® HD so that it was pipetted directly into the liquid in the tube and did not touch the plastic side of the tube.

d. Mixture was inverted 5-10 times for thorough mixing.

e. Mixture was incubated at room temperature for 20 minutes to allow complexes to form.

6) 1 part (100uL) of lipid:DNA complex was added to 20 parts (2mL) of HEK293 cells in suspension at 2 x 10^5 cells/mL (400,000 cells total). Tube was mixed gently by 5 inversions.

7) Cells + lipid:DNA complex was dispensed into 6-well plate (200,000 cells per well). Transfection rate was checked the next day based on EGFP signal.

Day 2

Tracer preparation

1) Serial dilution of 100X Tracer-6908 was prepared in DMSO.

2) 100X Tracer-6908 was diluted in NanoBRET Tracer Dilution Buffer to obtain 20X Tracer-6908.

Cell preparation

1) Medium was removed from cell flask via aspiration, cells were washed once with PBS and trypsinized for 5 minutes at 37°C to dissociate cells from the culture plate.

2) Trypsin was neutralised using assay medium and cells were pelleted via centrifugation at 300 x g for 5 minutes.

3) Medium was aspirated and cells were resuspended into a single cell suspension using complete assay medium.

4) The cell density was adjusted to 2 x 10^5 ells/mL in assay medium.

5) Cell suspension was dispensed into each well using multi-channel pipette at the following volume:

19ul or 38ul for 384-well microplate

95ul for 96-well microplate

Incubation with Tracer-6908

1) 20X Tracer-6908 was added to each well with cells using multi-channel pipette at the following volume:

1ul or 2ul for 384-well microplate

5ul for 96-well microplate

2) Microplates were mixed for 30 seconds at 200rpm.

3) The plate was then incubated at 37°C humidified box with 5% CO_2 for 2 hours.

NanoBRET measurement

1) The plate was removed from incubator and allowed to cool down to room temperature for 15 minutes.

2) Less than 20 minutes before NanoBRET measurement, NanoLuc substrate solution was prepared by diluting Nano-Glo substrate (1:166) and Extracellular NanoLuc Inhibitor (1:500) in assay medium.

3) 10ul of NanoLuc substrate solution was added to each well.

4) BRET signal was measured using Pherastar FSX with the following settings:

Top optic

610/460 luminescent optical module simultaneous dual emission

3600/1879 gain

0.1s setting time

1s to normalize the results

NanoBRET calculation

1) NanoBRET signal in mBRET units was calculated using the following formula:

$$\left(\frac{Sample\ 610nm}{Sample\ 410nm} - \frac{No\ tracer\ 610nm}{No\ tracer\ 410nm}\right) \times 1000$$

Results:

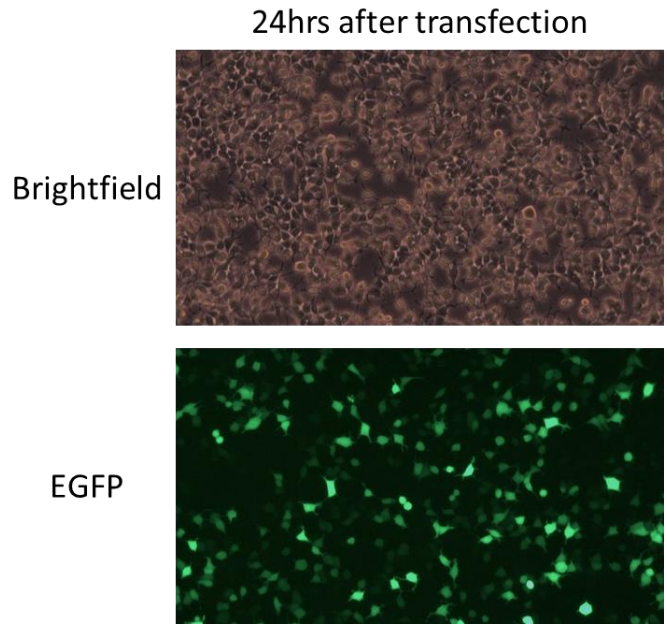

Figure 1. EGFP signal in transfected cells. HEK293 were transfected efficiently. Cells can be harvested for nanoBRET assay.

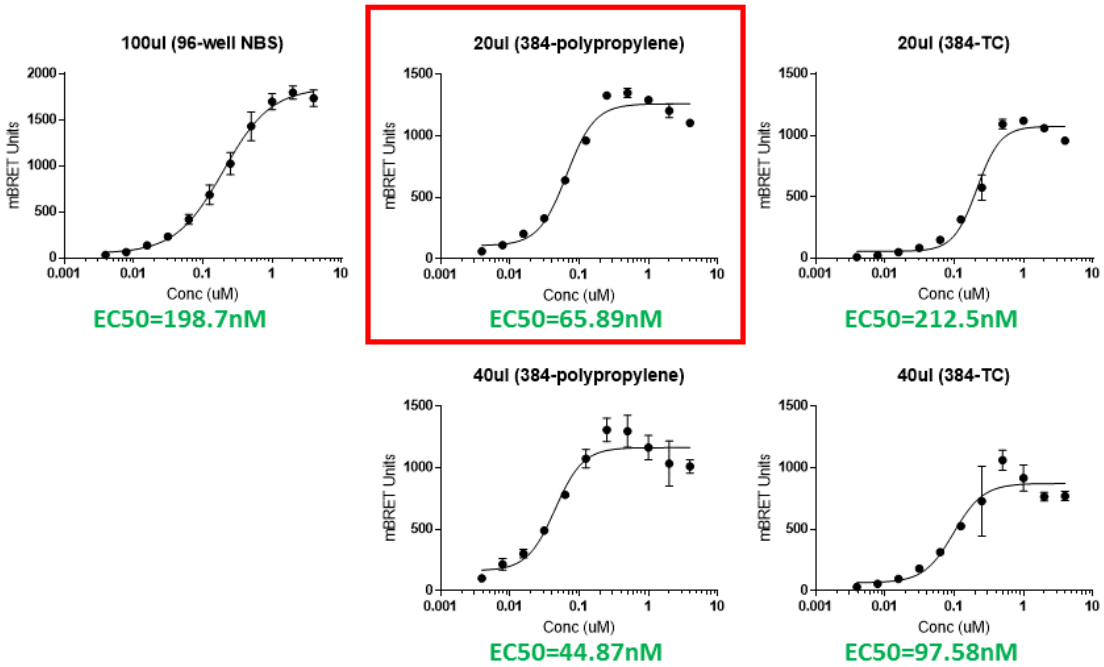

Figure 2. NanoBRET EC50 of Tracer-6908 with ACVR1/ALK2-c-nanoLuc transiently expressed in HEK293 cells at different volume and different microplates. Final chosen assay condition is bracketed in red rectangle.

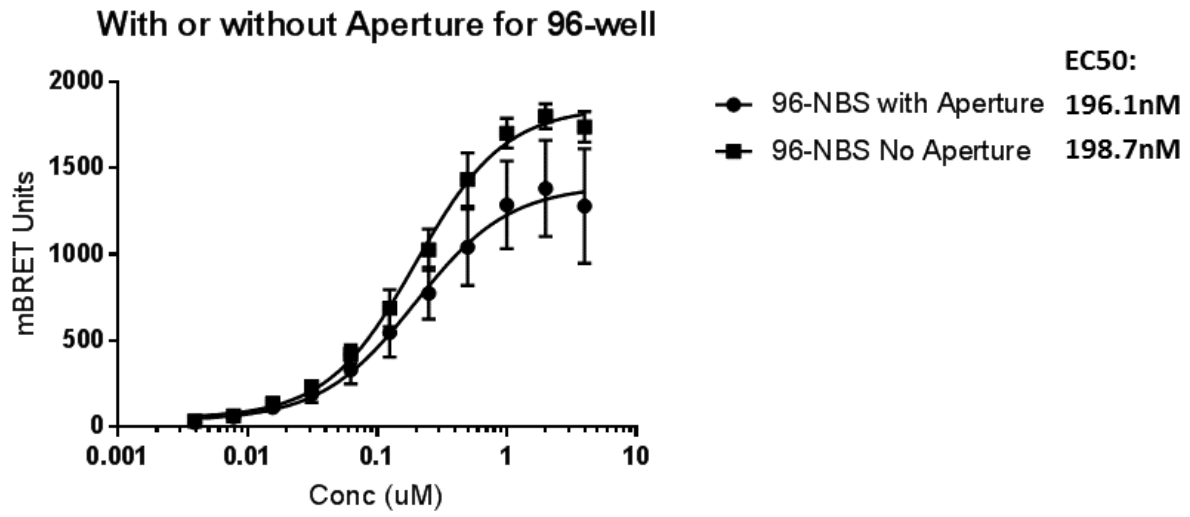

Figure 3. BRET measurements by Pherastar FSX with and without 384-well aperture. Aperture "spoons" for 384-well and 1536-well microplates can be installed onto the machine to minimise spill-over signal from adjacent wells.

Conclusions:

1) The EC50 of Tracer-6908 with ACVR1/ALK2-c-nanoLuc differs depending on the microplate and assay volume.

2) Care must be taken when acquiring BRET measurements with aperture "spoon" installed. The 96-well microplate BRET measurements acquired with 384-well aperture "spoon" contained large standard deviation. Removal of the aperture "spoon" drastically reduced the standard deviation, with minimal change to the EC50 value estimated from the curve.

3) 20ul assay volume in 384-well polypropylene microplate was chosen for ACVR1/ALK2 nanoBRET assays in the future because it will be most cost efficient. In that setting, the EC50 of Tracer-6908 is 65.89nM.