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We use this protocol and it's working

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Whole-cell radioligand saturation binding

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ABSTRACT

This protocol details an experimental procedure used to generate results described in the manuscript Li, A., Liu, S., Huang, R., Ahn, S., & Lefkowitz, R. J. (2023). Loss of biased signaling at a G protein-coupled receptor in overexpressed systems.

Day 1

1 Grow cells on 150 mm dish to ~70% confluency

2 Wash twice with 10 mL PBS

3 Detach cells with 1 mL trypsin-EDTA + 11 mL media. Trypsinize for 5 minutes and check under microscope for complete detachment; tap dish if necessary

4 Count cells with hematocytometer and dilute collected cells to 164,000 cells/mL

5 Pipet 1 mL into each well of poly-D-lysine coated 12-well plate. Make duplicate plates for all conditions: one plate for binding and one for BCA assay

Day 2

6 Add 250 mL of 5x doxycycline for all desired concentrations to cells 14 hours before assay

Day 3 (BCA)

7 Aspirate media from BCA assay plates, wash each well with 1 mL cold PBS with MgCl2 and CaCl2, add 200 uL glycerol lysis buffer using repeater dispensing pipette. Shake in cold room

8 Fast cool centrifuge to 4 °C

9 Scrape each well with cell scraper, wiping with KimWipe and washing in cold PBS between wells. Pipet 150 uL lysate up and down 5x to wash wells and collect in Eppendorf tubes on ice

10 Centrifuge lysate for 10 minutes at 4 °C and 13,000 rpm

11 Collect 100 uL supernatant into new Eppendorf tubes on ice

12 Measure protein concentration using standard BCA assay protocol

Day 3 (Binding)

13 Prepare saturating concentration of radioligand in media on ice. For example, 12.5 uL 3H-AngII in 5 mL media, or 4 uL 3H-olmesartan in 5.5 mL media. Prepare at least 5 mL per plate. Vortex briefly to mix

14 Take 1.5 mL media with radioligand per plate and add high concentration (orders of magnitude above radioligand concentration) of nonlabelled antagonist (e.g. 3 uL of 100 mM candesartan) for nonspecific binding measurement. Vortex briefly to mix

15 Dump media from plates into sink and invert plate onto paper towel to remove remaining liquid. Add 300 uL media with radioligand per well on ice. Use top two rows of plate for total binding duplicates and bottom row for nonspecific binding. Wait ≥ 1.5 hours on ice

16 Pre-chill 50 mL per plate PBS with $MgCl_2$ and $CaCl_2$ on ice and pre-warm 0.1% SDS + 0.5 N NaOH in 37 °C water bath

17 Remove radioligand media into dedicated radioactive liquid waste vial using P1000 on ice. Remove nonspecific binding media last

18 Wash wells with 1 mL cold PBS with MgCl2 and CaCl2 on ice and collect using P1000 into radioactive liquid waste vial. Wash 4 times total

19 Remove plates from ice and add 500 uL warm 0.1% SDS + 0.5 N NaOH per well. If there is precipitate in the solution, allow it to warm for longer. After adding, wait for ≥ 15 minutes

20 Collect samples from wells using P1000 and add to labeled scintillation vials. Add 300 uL of each radioligand media mix to two scintillation vials each for total counts. Add 5 mL scintillation cocktail (Lefko-Fluor) to each vial. Cap vials

21 Wipe vials with moist KimWipe and place in racks for scintillation counter. Place racks in counter and count using 3h_cpm_3_min protocol

22 Perform second count using different protocol flag on next day

Analysis

23 Use GraphPad radioactivity calculator to calculate specific activity of radioligand. The counter efficiency of the beta counter is approximately 65%; check the latest CPA results for an exact value

24 Copy binding counts into Excel spreadsheet and label

25 Transfer protein concentrations from BCA assay into spreadsheet

26 Use GraphPad radioactivity calculator and data in spreadsheet to calculate receptor concentration in fmol/mg

27 Use GraphPad radioactivity calculator and total counts from radioligand media to calculate radioligand concentration in nM