Appendix B3

Protocol for the Competitive Ligand Binding Assay

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Competitive Ligand Binding Assay

1. Caution: this protocol requires the use of radioactivity. Proper handling and disposal of all radioactive samples should be followed as outlined by the institution's safety office.
2. Prepare TEGD buffer by adding DTT to a final concentration of 1 mM to TEG buffer.
3. Add BSA (carrier protein) to a final concentration of 1 mg/ml.
4. Thaw receptor on ice. Using the appropriate dilution factor, add the receptor. As a rule of thumb, ~25 ml of TEGD+receptor is required per 96-well plate (240 µl per tube). Keep on ice until adding to tubes in step 7.
5. Label rack of 96 1 ml glass test tubes (Marsh Scientific).
6. Add 5 µl of radiolabeled compound at appropriate concentration to each tube using 8-channel pipettor.
7. Pipet 5 µl of unlabeled competitor into each tube. A typical assay may involve 5 concentrations of competitor plus solvent alone, with each concentration being run in quadruplicate. For example: A1-D1 are DMSO, A2-D2 have compound A at 10^{-10}M, A3-D3 have compound A at 10^{-9}M, ... and A6-D6 have compound A at 10^{-6}M. A similar scheme is set up for compound B (A7-D12), compound C (E1-H6), and compound D (E7-H12). Typically, compound A is the 'cold' version of the radiolabeled compound.
8. Transfer 240 µl of TEGD+receptor to each tube, using 8-channel pipettor.
9. Label and place an empty test tube rack on ice. Vortex each test tube from step 8 and place in new rack, ensuring that the order of tubes remains unchanged.
10. Place cover on glass tube rack and incubate at 4°C for 24 hrs.
11. At time of harvest, fill head of harvester with millipore-filtered water and place the head of the harvester (Packard Filtermate 196) into position.
12. Put the positioning bracket in place. Invert the 'wash' filter place and place it within the bracket so position A12 is now in the top left. Gently close the harvester unit by pulling down the lever.
13. Turn the pump on.
14. Push and lock the 'cold' vacuum circuit on the harvester. Placing the collection tray against the intake vacuum inlets and wash for 30 sec.
15. Dry the wash plate by opening the harvester and applying continued suction for 10 sec.
16. Place a new filter plate in the harvester. Rinse the plate once with 50 ml of cold TEG buffer using the 'cold' circuit.
17. Switch to 'hot' circuit. Place the rack of 96 test tubes underneath the head unit and raise into place until all the liquid has passed through the harvester.
18. Wash with 3 x 50 ml of cold TEG buffer, leaving harvester on 'hot' circuit for all three washes. Dry as in step 15.
19. Wash harvester as in steps 11-14.
20. Label and date the filter plate. Place in radioactive hood for 10 min.
21. Put back seal on each plate and add 50 µl of Microscint20 (Packard) to each well. Remember that the plate is now inverted (tube A1 is now bound to filter location A12).
22. Put top seal on plate and incubate at room temperature at least 30 min. Count the plate using the Receptor Binding Protocol on the TopCount Scintillation counter (Packard).

TEG Buffer

10 mM Tris
1.5 mM EDTA
10% Glycerol
pH 7.6

GST Purification

Bacterial overexpression

1. Transform *E. coli* BL 21 cells with appropriate pGEX vector. Allow colonies to grow all day.
2. Pick 2-3 colonies at the end of the day and allow them to grow overnight in 3 ml LB-Amp.
3. Perform miniprep. Add more LB-Amp (~2 ml) to starter cultures and place in shaker incubator all day (6-8 hr) at 37°C and 225 rpm.
4. Check miniprep using appropriate restriction enzymes. Select a single colony for overexpression.
5. Inoculate 50 ml of LB-Amp with 500 µl of starter culture. Incubate overnight at 37°C and 225rpm.
6. Inoculate 500 ml of LB-Amp with 5 ml of culture. (Often ~6L (i.e. 12 flasks) are inoculated.) Grow at 37°C and 225 rpm.
7. Induce culture with 0.5 mM IPTG (final conc.) when culture reaches $O.D._{600}$ of 1.0 (~3.5 hr). Grow induced culture for 3.5 hr at 37°C.
8. Pellet 500 ml culture by centrifuging 10 min at 5000 rpm using a Beckman JA-14 rotor. Remove supernatant, and a second 500 ml culture can be added to the same tube and centrifuged as above.
9. Remove supernatant and store at –80°C.

Fusion protein extract – should be done at 4°C or on ice

10. Resuspend pellet (from the equivalent of 1L of culture) in 25 ml of resuspension buffer containing protease inhibitors and 5 mM DTT added fresh. Transfer resuspended cells into 50 ml centrifuge tube.
11. Disrupt cells by sonication. Keep tube on ice at all times. Use the pulse mode at setting 3 and sonicate cells for 3x20 sec.
12. Add Tween20 to a final concentration of 0.3%. Incubate at 4°C under constant shaking for 30-60 min.
13. Centrifuge at 20,000g (15,000rpm using the SS-34 rotor) for 30 min at 4°C.
14. Filter supernatant to eliminate cellular debris that did not pellet.
15. Transfer supernatant to a 50 ml tube.

Protein purification – degas all buffers and GSH matrix before setting up the column

16. Add 10 ml of packed matrix to 20 ml glass column. Place the adaptor at an appropriate distance from the top of the matrix in order to reduce the void volume. Hook the column up to the peristaltic pump in the following order: buffer, pump, followed by the column.
17. Be sure that there are NO air bubbles in the lines or the column.
18. Equilibrate the matrix with 5x bed volume (50 ml of equilibration buffer). Keep the flow rate at 0.5 ml/min. Steps 15-17 should be done in advance to allow the column to properly equilibrate.
19. Stop the pump between buffer transfers and wait briefly before transferring the collection line to a different buffer.
20. Place the collection line into the 50 ml tube containing the crude protein. (A 250 µl aliquot of the crude sample should be saved for subsequent analysis.) Keep flow rate at 0.5ml/min.
21. Collect the flowthrough and save a sample for subsequent analysis.
22. Wash the column with 10x bed volume (80-100 ml) of wash buffer.
23. Elute fusion protein with 2x bed volume (20 ml) of elution buffer.
24. Collect eluate in a 50 ml tube.
25. Concentrate protein to 0.5 mg/ml using the Amicon 50,000 MWCO spin column.
26. Check protein concentration using the Bradford method.

Equilibration/Resuspension Buffer

50 mM HEPES
3 mM EDTA
50 mM NaCl
10% glycerol
pH 7.5

Add prior to use (final conc.)
10 ug/ml Pepstatin A (from 1 mg/ml stock in ethanol)
10 ug/ml Leupeptin (from 1 mg/ml stock in water)
100 ug/ml PMSF (from 10 mg/ml stock in isopropanol)
5 mM DTT (from 1M stock in water)

Column Wash Buffer

50 mM HEPES
3 mM EDTA
150 mM NaCl
10% glycerol
pH 7.5

Elution Buffer

50 mM HEPES
3 mM EDTA
150 mM NaCl
10% glycerol
10 mM glutathione
pH 8.0