

Materials:

5HT (serotonin-HCL) from RBI S-011

TALON metal affinity resin Clontech 8901-2; do NOT use other brands.

PCR tubes 0.5 ml thermotube from Advantec, to be obtained via VWR (ADVAAB-0350); these are low binding tubes for keeping diluted protein

Reagents:

IBTX 125I-abungarotoxin 2000Ci/mmol (=50 μ Ci/250 μ l) or 200 nCi/ μ l

calculation of specific activity: 2000Ci=1 mmol. So 200 nCi/ μ l=0.1 pmol/ μ l

diluted IBTX in 100 μ l assay-mix represents 0.1 μ l IBTX: 0.01 pmol (=90 pg IBTX) = 20 nCi (=44400 cpm)

radioligand for 10 tubes (10 x 5 μ l): 1 μ l IBTX + 50 μ l PBS

BSA50 50 mg/ml BSA; store in 0.5 ml aliquots at -20 °C

BB binding buffer: 0.5 mg/ml BSA in PBS; mix 50 ml of PBS with 0.5 ml BSA50 and 0.2 ml 25% Tween-20

B alternative binding buffer: 1 mg/ml BSA in 100mM NaCl-20 mM TrisCl pH8

ligand	range (M)	MW	IC50	
nicotin	10-5- 10-9	162	10-7	0.1 M 16.2 μ l + 916 μ l H2O
acetylcholine	10-3 - 10-8	181.7	10-4/5	0.2 M: 150 mg in 4.135 ml H2O
5HT	10-1 - 10-6	212.68	10-3	1 M: 212.7 mg/ml 0.001M HCl
a-bungarotoxin	10-5 - 10-10	7907	10-8	1 mM = 7.91 mg/ml

Protocol:

1. Make serial dilutions of ligands, e.g. nicotine, of 10-5 to 10-9 M in duplicate in 90 μ l BB
2. Add 10 μ l (2 ng) of (mutant) protein (0.2 ng/ μ l), e.g. AchBP-6HIS
3. incubate for 5-10 min.
4. Add 5 μ l of radioligand mix and incubate for 1h.
5. Add 20 μ l of Talon-beads and incubate for 45 min.
6. Wash 3-4 x with BB
7. Resuspend beads in 200 μ l BB and transfer to large liquid scintillation vial which contains 0.3 ml 0.15 M imidazole/0.1% SDS, and shake a few seconds.
9. Add 8 ml liquid scintillation cocktail and count in liquid scintillation counter
10. Calculate results using Scatchard plots.

Alternative protocol:

If you have many samples, the whole assay can conveniently be done in microtitre plates (low binding, e.g. Greiner 655 180) You can use 2 columns of 8 for making a duplicate dilution series of 2x8, so 6 samples can be tested on one plate.

Mount a 96-well filter apparatus, with 2 sheets of Whatman 3MM (or GB002) and one sheet of nitrocellulose (top), all prewetted with BB. The nitroceloluse solely acts as a smooth surface for collecting the beads, not for binding.

Transfer contents of microplate to the filter apparatus using an 8-multichannel pipet.

Wash wells 3x with 100 μ l BB, also transfer washes to the filter apparatus.

Finally wash the filters 2x with 100 μ l BB. Continue with step 7 as above.

Commentary:

- slightly acid solution for 5HT is recommended
- The presence of Tween in binding buffer reduces non-specific binding
- Suspension of beads in imidazole gives better reproducibility as the protein - IBTX complex comes off the beads, providing better geometry for counting.
You may as well use smaller scintillation vials, but you have to check reproducibility then.
- If you make protein dilutions, use the low binding tubes e.g. the ones mentioned above. Normal Eppendorf tubes just bind proteins gradually (if $< 100 \text{ ng}/\mu\text{l}$). Alternatively, make protein dilutions using binding buffer. Do not freeze protein, but keep at $4 \text{ }^\circ\text{C}$.
- If you are not sure about the protein concentration, it should be as low as to never bind more than 5% of the free label (IBTX), without competitor. You can determine this experimentally by counting $5 \mu\text{l}$ of your diluted radioligand