



Immunoperoxidase Staining of Free Floating Brain Sections

Day 1.

1. Wash sections 2X with gentle rocking/agitation at 4°C, 5 min each in ice-cold TBS (0.15M NaCl, 50 mM Tris, pH 7.5). Use 6-well plate with net insert for accelerated washing. One well fits at most 10 sections. Do not overflow the well with TBS since floating sections may be caught at rims of the inserts. Be gentle lifting and lowering the inserts while transferring to prevent sections from breaking due to surface tension of the buffer.
2. Permeabilize sections in 0.5% TX-100 in TBS for 40-45 minutes at 4°C with gentle rocking/agitation.
3. Wash sections 2X, 5 min each in ice-cold TBS with gentle rocking/agitation.
4. Block sections in ice-cold vehicle (5% normal horse serum, 0.1% TX-100 in TBS) for 45 minutes at 4°C with gentle rocking/agitation.
5. Incubate sections in NeuroMab diluted in ice-cold vehicle. Dilution factors for NeuroMabs should be determined empirically, but generally NeuroMab tissue culture supernatants should be used at 1:2-1:20, purified NeuroMab IgG from 100 ng/ml to 1 µg/ml. Incubate sections overnight at 4°C with gentle rocking/agitation.

Day 2.

6. Wash sections 5X, 5 min each in ice-cold TBS with gentle rocking/agitation.
7. Incubate in ice-cold biotinylated secondary antibody (horse anti-mouse IgG, Vector Labs BA-2001, at 1:250 in vehicle) for 1 hour at 4°C with gentle rocking/agitation.
8. Just before the end of the secondary antibody incubation, make ABC reagent (Vectastain *Elite* Peroxidase System, Vector Labs PK-6100). For each 5 ml of TBS (**NO** serum), add 2 drops of ABC kit Solution A and 2 drops of Solution B. The ABC solution requires a 30 minute pre-incubation period at 4°C to work properly.
9. While the ABC solution is being activated, wash section 5X, 5 minutes each in ice-cold TBS with gentle rocking/agitation.
10. Incubate sections in ice-cold ABC solution for one hour at 4°C with gentle rocking/agitation.
11. Wash sections 3X, 5 min each in TBS with gentle rocking/agitation.
12. Wash sections 2X, 5 min each in TB (50 mM Tris, pH 7.5) with gentle rocking/agitation. While washing, prepare DAB/NAS developing solution (see below).



13. Develop sections in DAB/NAS solution. Developing duration will vary so visually monitor intensity of reaction constantly so as to avoid over developing. The optimal development time should be determined empirically for each NeuroMab.
14. To stop developing, transfer developed sections to TB and wash 2 more times with TB.
15. Mount sections on gelatin-coated microscope slides: Use 15 cm Petri dish filled with TB, submerge a slide in the buffer. Using a brush, position a section right above the desired mounting area and while holding the section with the brush, gently lift the slide out of the TB. Tilt the slide to remove excessive buffer and let it air dry for a couple of hours (overnight is better), at RT.
16. Dehydrate mounted sections successively in 70%, 95%, 100% (2X), Citrus Clearing Solvent (Thermo Scientific Richard-Allan Citrus Clearing Solvent 8301) (2X), 5 min each under a fume hood. Coverslip with Electron Microscopy Sciences DPX mountant (13510). The slides should lay flat in fume hood for 3-5 days.

DAB/NAS solution (for 100 ml)

0.04 g DAB (3-3' diaminobenzidine tetrahydrochloride)

0.3 g NAS (nickel ammonium sulfate)

Dissolve in 100 ml of TB and filter through a Whatman #2 filter.

Immediately before developing, **add 10 µl of 30% H₂O₂**

*prepare immediately before use.

*DAB is a suspected carcinogen. Wear protection gears and work under a hood when handling dry DAB powder.

*all the glassware used during preparation of DAB solution should be cleaned with bleach.