

### **Immunoperoxidase Labeling of Free-Floating Perfusion-Fixed Brain Sections**

Day 1.

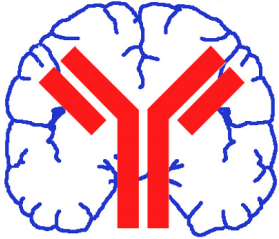
For antibody screening, prior to the primary antibody step, all incubation steps can be accelerated using 6-well plates with net inserts containing 10-15 sections in each well.

- 1) Wash: transfer sections to 6-well inserts and rinse twice with ice-cold (4°C) TBS (recipe below) with gentle rocking/agitation at room temperature (RT) for 5 min each.
- 2) Permeabilization: transfer sections into ice-cold (4°C) TBS containing 0.5% Triton X-100 and 10 µg/mL Avidin (egg white avidin, Thermo Fisher Scientific catalog # A887) with gentle rocking/agitation at 4°C for 45 min.
- 3) Wash: transfer sections into fresh ice-cold (4°C) TBS and wash twice with gentle rocking/agitation at RT for 5 min each.
- 4) Block: incubate sections in ice-cold (4°C) vehicle (recipe below) containing 18.75 µg/mL Biotin (Sigma-Aldrich catalog # B4501-1G) with gentle rocking/agitation at 4°C for 45 min.
- 5) Primary antibody incubation: transfer sections into ice-cold (4°C) vehicle containing diluted NeuroMab(s) and incubate with gentle rocking/agitation at 4°C overnight.

Note: antibody concentrations and incubation conditions should be determined empirically for each combination of target sample, NeuroMab, secondary antibody and detection system but, as a general guide, NeuroMabs should be used between 0.1 and 10 µg/mL.

Day 2.

- 6) Wash: transfer sections into ice-cold (4°C) TBS and wash a total of five times with gentle rocking/agitation at RT for 5 min each.
- 7) Secondary antibody incubation: transfer sections into ice-cold (4°C) vehicle containing biotinylated anti-mouse antibodies diluted as per manufacturer's recommendations (e.g., 0.5 µg/mL of biotinylated horse anti-mouse IgG H+L antibody, Vector Labs catalog # BA-2001) and incubate with gentle rocking/agitation at 4°C for 1 h. Alternatively, a cocktail of subclass-specific secondary antibodies matched to the NeuroMab IgG subclass (i.e., IgG1, IgG2a or IgG2b at 0.5 µg/mL each) can be used for optimal signal detection and lowest background (Manning et al., PLoS One 7:e38313, 2012, <http://www.ncbi.nlm.nih.gov/pubmed/22675541>).
- 8) ABC preparation: immediately before next round of washes, make ABC reagent (Vectastain Elite Peroxidase System, Vector Labs catalog # PK-6100) according to manufacturer's instructions (i.e., a 1:1 ratio of Solution A and Solution B mixed and incubated at 4°C for 30 min).



- 9) Wash: transfer sections into ice-cold (4°C) TBS and wash a total of five times with gentle rocking/agitation at RT for 5 min each.
- 10) ABC incubation: transfer sections into ice-cold (4°C) ABC solution and incubate with gentle rocking/agitation at 4°C for 1 h.
- 11) Wash: transfer sections into ice-cold (4°C) TBS and wash a total of three times with gentle rocking/agitation at RT for 5 min each.
- 12) Wash: transfer sections into ice-cold (4°C) TB (recipe below), wash twice with gentle rocking/agitation at RT for 5 min each. During washes, prepare DAB/NAS solution (recipe below).
- 13) DAB/NAS development: transfer sections into DAB/NAS solution and develop, taking care to visually monitor the intensity of the reaction so as to avoid over-developing. To stop reaction, transfer sections to TB and wash twice more with TB at RT.
- 14) Mounting: transfer sections into a 15 mL Petri dish containing TB, carefully mount onto gelatin-coated microscope slides and dry using a slide warming tray (Fisher Scientific) for 20-30 min.
- 15) Dehydration: transfer slides with sections through 70%, 95% and 100% ethanol (twice), followed by Richard-Allan Citrus Clearing Solvent (Thermo Fisher Scientific catalog # 8301), for 10 min each in a fume hood.
- 16) Coverslipping: pipet DPX mountant (Electron Microscopy Sciences catalog # 13510) on each slide according to manufacturer's instructions, place glass coverslip on top and lay flat to set in fume hood overnight.

### Recipes:

TB: 50 mM Tris pH 7.5

TBS: 150 mM NaCl in 50 mM Tris pH 7.5

Vehicle: 5% normal horse/goat serum (secondary antibody host) and 0.1% Triton X-100 in TBS

DAB/NAS solution (100 mL, prepare immediately before use):

- 1) Dissolve 0.04 g DAB (3-3' diaminobenzidine tetrahydrochloride) and 0.3 g NAS (nickel ammonium sulfate) in 100 mL of TB and filter through a Whatman #2 filter.
- 2) Immediately before developing, add 10  $\mu$ L of 30% H<sub>2</sub>O<sub>2</sub>.
- 3) Note that DAB is a suspected carcinogen. Wear protective gear and work under a hood when handling dry DAB powder. Also, all glassware used during preparation of DAB solution should be cleaned with bleach.

Reference: Rhodes et al., *J Neurosci* 15:5360, 1995 (<https://www.ncbi.nlm.nih.gov/pubmed/7623158>)