ABC IHC Protocol 8/1/2008

The following outlines a DAB/peroxidase protocol, using avidin-biotin as an amplifying step, on paraffin-embedded tissues, to allow optimal colorimetric detection of markers for immunohistochemistry.

Materials needed:

-***Antibody Amplifier***^{TM} (one Antibody Amplifier fits a maximum of 12 slides)
-Amplifying Antibody Dilution Buffer (1x) cat# AA3
-Primary antibody (try to use the same LOT of antibody for all slides in a project)
-Amplifying IHC Wash Buffer (20x) cat # AA4 (you will need to dilute 20 fold in ddH_2O for working dilution)
-Epitope Unmasking Solution (10x) cat# AA5 (you will need to dilute 10 fold in ddH_2O for working dilution)
-Vectastain Kit (Vector Labs; contains biotinylated 2°, reagents A and B)
-DAB reagents
-Xylene
-Ethanol
-1x PBS
-Peroxidase Blocking Solution (3% H_2O_2 in PBS):
    30% H_2O_2 -------------------------- 2 ml
    1X PBS ---------------------------- 18 ml
-Coverslips
-Methyl Green (1% in water) or Hematoxylin (Gill-1)
-Slide racks and holder:
-Permount
-ddH_2O

Approximate time involved: 4 hours day 1; 4-5 hours day 2;

Procedure:

1. Put slides in a glass slide holder and place in the following solutions serially (at RT):
   a. xylene: 5 min., swishing every 2 min. (IN FUME HOOD)
   b. xylene: 5min., swishing every 2 min. (IN FUME HOOD)
   c. 100% ETOH: 2 min., swishing every 30 sec.
   d. 95% ETOH: 2 min.,rocking.
   e. 80% ETOH: 2 min., rocking.
   f.70% ETOH: 2min., rocking.
   g. 50% ETOH: 2 min., rocking.
   h. dd H_2O: 2 X 3min., rocking.

2. Wash with 1X Amplifying IHC Wash Buffer (cat # AA4) 2 x 5 min., rocking at RT.

3. Wash 2 X 3 min. in 1X Amplifying IHC Wash Buffer (cat # AA4)

4. Transfer slide holder to plastic, autoclavable container and emerse in 1X Epitope Unmasking Solution (cat# AA5).

5. Autoclave for 20-30 min., while covered with foil.

6. Retrieve sample from autoclave and place on counter to let cool at RT 20 min. (continuing emersion in Epitope Unmasking Solution (cat #AA5).

NOTE: for fatty tissue, such as brain or breast, use these directions for antigen retrieval: place slides in slide rack. Lower slide rack into gently boiling 1X Epitope Unmasking Solution (cat# AA5) (about 600 mls of buffer). We use a large beaker on a heat block. Boil gently for exactly 8 min. Remove from heat source. Slowly add 500 mls dd H_2O to bring buffer temp down. Quickly put slide rack in glass slide holder and wash 2 X 5 min in 1X Amplifying IHC Wash Buffer (cat # AA4)

7. Wash with 1X Amplifying IHC Wash Buffer (cat # AA4) 2 x 5 min., rocking at RT.

8. Wipe off back and sides of slide with a kimwipe, lay flat on benchtop (in a humidity chamber if desired) and cover tissue with 1-3 drops of peroxidase block (3% H_2O_2 in PBS). Incubate RT 30 min., checking every 5-10 min. to make sure samples don't dry out. If starting to dry, add more peroxidase block.

9. Put slides back into slide carrier and wash 3 X 3min. in 1X Amplifying IHC Wash Buffer (cat # AA4)

10. Dilute 1° antibody in Amplifying Antibody Dilution Buffer (cat# AA3)**.** Use a minimum of 3 mls per slide for Antibody Amplifier. The dilution will, of course, vary for each antibody, but in our experience, you can use at least 5x and often >20x the dilution you typically use for the standard sedentary method. Place lid onto the ***Antibody Amplifier***^{TM}, and place on a standard laboratory rocker. Rock overnight at 4^oC.

After use, store primary antibody at -20°C.

12. Put slides back into glass slide carrier and wash 2 X 5 min. in 1X Amplifying IHC Wash Buffer (cat # AA4).

13. Incubate slides in biotinylated secondary antibody. Secondary is provided as part of the Vectastain kit by Vector Labs. Secondary antibody dilution is 1:2000 in Amplifying Antibody Dilution Buffer (cat# AA3). Incubate slides rocking at RT 1 hour in ***Antibody Amplifier***^{TM}. Remember that each slide needs 3 mls.

14. As soon as slides are incubating in secondary antibody, mix up an ABC solution (found in Vectastain kit). The ABC solution is diluted 1:1000. Remember if using *Antibody Amplifier*^{TM}, to make 3mls per slide. Let the mixed solution sit RT 30-60 min. before using.

15. Put slides in glass slide carrier and wash 2 X 5 min. in 1X Amplifying IHC Wash Buffer (cat # AA4).

16. Incubate slides in premade ABC solution, rocking at RT 1 hr in the ***Antibody Amplifier™***.

17. Make up DAB solution according to manufacturer's instructions. You need 100-200 ul per slide. Incubate RT 20-30 min.

18. Wash 2 X 5min in 1X Amplifying IHC Wash Buffer (cat # AA4).

19. Wash 1 X 3 min dd H_2O.

20. Wipe slides around tissue with kimwipe, keeping tissue moist, and add DAB solution. Check the staining of one slide first by testing a positive control: Incubate with 100-200 ul of DAB solution, starting at 30 seconds. Check using the microscope at 40X. Keep note of DAB times. Wash slides as soon as staining is optimal with dH_2O.

> **NOTE:** All slides that you are comparing must be incubated for the same time period with DAB once this time is established with the positive control.

21. Wash slides in ddH_2O 1 X 3 min.

22. Counterstain using Methyl Green: Filter 3-10mls of methyl green using a syringe filter into a 15 ml tube. Add 200 ul of methyl green onto tissue and incubate at RT 2 min (or whatever time you have established). Rinse in containers of ddH_2O until slides are clean and let air-dry, with slides standing vertically on their ends.

23. After tissues are completely dry, add one drop of Permount, put on coverslip, and smooth out bubbles using big end of pipettor tip. Label slides with: date, antibody, dilution, and timing. After 4-5 hours, clean off extra permount with xylene (xylene is optional-only if covering tissue).