

ABC IHC Protocol

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:

1. Biotinylated Secondary Antibody
2. Avidin-Biotin Complex Kit (e.g. Vectastain Kits from Vector Labs).
3. DAB reagents
4. Xylene
5. Ethanol
5. 1x PBS
7. Peroxidase Blocking Solution (3% H₂O₂ in PBS)
30% H₂O₂.....1 part (e.g. 10 ml)
1XPBS.....9 parts (e.g. 90 ml)
8. Coverslips
9. Methyl Green (1% in water) or Hematoxylin (Gill-1)
10. Slide racks and holders
11. Permount
12. ddH₂O
13. A standard laboratory rocker
14. Pipettors and tips

1. Place slides in a glass slide holder and place in the following solutions serially (at room temperature):

- a. Xylene: 5 minutes swishing every 2 minutes (in fume hood).
- b. Xylene: 5 minutes, swishing every 2 minutes (in fume hood).
- c. 100% Ethanol: 2 minutes, swishing every 30 seconds.
- d. 95% Ethanol: 2 minutes, rocking
- e. 80% Ethanol, 2 minutes rocking.
- f. 70% Ethanol, 2 minutes rocking.
- g. 50% Ethanol, 2 minutes rocking.
- h. ddH₂O: 3 minutes, rocking. Repeat one time.

2. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes, rocking at room temperature. Repeat once.

3. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) another 2 times, but for three minutes each this time.

4. Transfer slide holder to plastic, autoclavable container and submerge in 1X Epitope Unmasking Solution (Catalog # AA6).

5. Cover in foil and autoclave for 20-30 minutes.

6. Retrieve sample from autoclave and place on counter to let cool at room temperature for 20 minutes (Continuing submersion in Epitope Unmasking Solution (Catalog # AA6).

NOTE: For fatty tissue, such as brain or breast, use these directions for antigen retrieval:

Place slides in slide rack. Lower slide rack into 600 mL gently boiling 1X Epitope Unmasking Solution (Catalog # AA6). Boil gently for exactly 8 minutes, and then remove from the heat source (a large beaker filled with 1x Epitope Unmasking Solution (Catalog # AA6), with boiling chips on the bottom and sitting on a heat block works well). Slowly add 500 mLs of ddH₂O to bring buffer temperature down. Quickly put slide rack in glass slide holder and wash twice with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes each time.

7. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes, rocking at room temperature. Repeat once.

8. Note: Use of the Antibody Amplifier™ is optional at this point. However, ensure that the slides are completely covered by the peroxidase blocking solution.

Wipe off back and sides of slide with a kim-wipe, lay flat on bench top and cover tissue with 1-3 drops of peroxidase block (3% H₂O₂ in 1X PBS). Incubate at room temperature for 30 minutes, checking every 5-10 minutes to ensure the samples do NOT dry out (If they are starting to dry out, add more peroxidase block).

9. Put slides back into slide carrier and wash for 3 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Repeat two times.

10. Dilute primary antibody in Amplifying Antibody Dilution Buffer (Catalog # AA3). Use a minimum of 3 mLs per slide to completely submerge in the Antibody Amplifier™. The suggested dilution will vary for each antibody, and you will have to titrate out. We typically initially titrate from 1 in 1000 to 1 in 1 million. Place lid onto the Antibody Amplifier™ and place on a standard laboratory rocker. Rock overnight at 4°C. After use, store primary antibodies at -20°C. The antibodies can generally be re-used up to 4X without compromising sensitivity.

11. Put slides back into glass slide carrier and wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Repeat once.

12. Incubate slides in biotinylated secondary antibody. Secondary antibody is usually provided as part of avidin-biotin complex staining kits (e.g. Vectastain kit by Vector Labs is recommended). Secondary antibody dilution is usually 1:2000 in Amplifying Antibody Dilution Buffer (Catalog # AA3), but optimal dilution is at the discrepancy of the end-user (the use of the Antibody Amplifier™ rocking on a standard laboratory rocker often allows the user to significantly titrate out antibodies from that which is recommended by the supplier). Incubate slides

rocking at room temperature for one hour in Antibody Amplifier™. Remember that each slide needs 3 mLs for full submersion.

13. As soon as the slides are incubating, mix up an Avidin-Biotin Complex (ABC) solution (e.g. found in Vectastain kits from Vector Labs). The ABC solution is diluted 1:1000 (e.g. 5 µl A + 5 µl B in 10 mLs 1X PBS) but optimal dilution is at the discrepancy of the end-user (the use of the Antibody Amplifier™ rocking on a standard laboratory rocker often allows the user to significantly titrate out ABC solutions from that which is recommended by the supplier). Remember if using the Antibody Amplifier™, to make 3 mLs per slide. Let the mixed solution sit at room temperature for 30-60 minutes before using.

14. Put slides in glass slide carrier and wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Repeat once.

15. Incubate slides in pre-made ABC solution, rocking at room temperature for 1 hour in the Antibody Amplifier™.

16. Make up DAB solution according to the manufacturer's instructions. 100-200 µL per slide is needed.

17. Wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Repeat once.

18. Wash for 3 minutes in ddH₂O.

19. Wipe slides around tissue with kim-wipes, keeping tissue moist, and add DAB solution. Check the staining of one slide first by testing a positive control: Incubate with 100-200 µL of DAB solution, starting at 30 seconds. Monitor using the microscope at 40X. Keep note of DAB times. Wash slides as soon as staining is optimal with ddH₂O.

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.

20. Wash slides with ddH₂O for 3 minutes.

21. Counter-stain using Methyl Green: Filter 3-10 mLs of methyl green using a syringe filter into a 15 mL tube. Add 200 µL of methyl green onto tissue and incubate at room temperature for 2 minutes (or whatever time has been established). Rinse in containers of ddH₂O until slides are clean and let air dry, with slides standing vertically on their ends.

22. After tissues are completely dry, add one drop of Permount, put on coverslip, and smooth out bubbles by pushing down on the coverslip using the big/blunt end of the pipettor tip. Label slides with: date, antibody, dilution and timing. After

4-5 hours, you can clean off extra Permout with Xylene (The Xylene is optional- only if too much Permout was added, and it is covering tissue.)