

ABC IHC Protocol: Mouse on Mouse
For use when detecting on mouse tissues with mouse
monoclonal antibodies

Materials needed:

1. Mouse-on-Mouse (M.O.M.) kit from Vector Labs (PK-2200)
2. DAB reagents
3. Xylene
4. Ethanol
5. 1x PBS
6. Peroxidase Blocking Solution (3% H₂O₂ in PBS)
 30% H₂O₂.....1 part (e.g. 10 ml)
 1XPBS.....9 parts (e.g. 90 ml)
7. Coverslips
8. Methyl Green (1% in water) or Hematoxylin (Gill-1)
9. Slide racks and holders
10. Permount
11. ddH₂O
12. A standard laboratory rocker
13. Pipettors and tips

Procedure:

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 # SKC-903) for 5 minutes, rocking at room temperature. Repeat once.
3. Wash with 1X Amplifying IHC Wash Buffer (Catalog # SKC-903) 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 # SKC-905).
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 # SKC-905).

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

Place slides in slide rack. Lower slide rack into 600mLs gently boiling 1X Epitope Unmasking Solution (Catalog # SKC-905). Boil gently for exactly 8 minutes, and then remove from the heat source (a large beaker filled with 1x Epitope Unmasking Solution (Catalog # SKC-905), with boiling chips on the bottom and sitting on a heat block works well). Slowly add 500mLs 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 # SKC-903) for 5 minutes, rocking at room temperature. Repeat once.
8. Wipe off back and sides of slide with a kimwipe, 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 # SKC-903). Repeat two times.
 10. Incubate sections for 1 hour in working solution of M.O.M.[™] Mouse Ig Blocking Reagent
M.O.M.[™] Mouse Ig Blocking Reagent working solution: add 2 drops of stock solution to 2.5mL of 1x PBS.
 11. Wash with 1X Amplifying IHC Wash Buffer (Catalog # SKC-903) for 5 minutes, rocking at room temperature. Repeat once.
 12. Incubate tissue sections for 5 minutes in working solution of M.O.M.[™] diluent prepared as described. M.O.M.[™] Diluent working solution: add 600µL of Protein Concentrate stock solution to 7.5mL of 1x PBS.
 13. Tip excess of M.O.M.[™] diluent off sections. Dilute primary antibody in M.O.M.[™] diluent topped up with Amplifying Antibody Dilution Buffer (Catalog # SKC-902). 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.
 14. Put slides back into glass slide carrier and wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # SKC-903). Repeat once. Apply working solution of M.O.M.[™] Biotinylated Anti-Mouse IgG Reagent prepared as described. Incubate sections for 10 minutes M.O.M.[™] Biotinylated Anti-Mouse IgG Reagent working solution: add 10µL of stock solution to 2.5mL of M.O.M. diluents prepared above.
 15. 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. 5µL A + 5µL B in 10mLs 1X PBS) but optimal dilution is at the discrepancy of the end-user. 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.
 16. Put slides in glass slide carrier and wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # SKC-903). Repeat once.
 17. Incubate slides in pre-made ABC solution, rocking at room temperature for 1 hour in the *Antibody Amplifier[™]*.
 18. Make up DAB solution according to the manufacturer's instructions. 100-200µL per slide is needed.
 19. Wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # SKC-903). Repeat once.
 20. Wash for 3 minutes in ddH₂O.
 21. Wipe slides around tissue with kimwipes, keeping tissue moist, and add DAB solution. Check the staining of one slide first by testing a positive control: Incubate with 100-200µL 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.

22. Wash slides with ddH₂O for 3 minutes.
23. Counter-stain using Methyl Green: Filter 3-10mLs of methyl green using a syringe filter into a 15mL tube. Add 200µL 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.
24. After tissues are completely dry, add one drop of Permount, put on coverslip, and smooth out bubbles. Label slides with: date, antibody, dilution and timing. After 4-5 hours, you can clean off extra Permount with Xylene.