

## Immunohistochemical Staining Protocol – USING DAKO KIT

**[This is the specific staining method we use at ProHisto, LLC]**

### Materials needed:

Kits (these are expensive, but in our opinion well worth the money, as they give very good staining):

If your primary antibody is a mouse monoclonal, use: DAKO Envision + System, HRP (DAB), cat#K4006 – Try to use same LOT of antibody for all slides in project

If your primary antibody is a rabbit polyclonal, use: DAKO Envision + System, HRP (DAB), cat#K4011– Try to use same LOT of antibody for all slides in project

ProHisto **Antibody Amplifier™** (one Antibody Amplifier fits a maximum of 12 slides)

Primary antibody (try to use the same LOT of antibody for all slides in a project)

Amplifying Antibody Dilution Buffer (ProHisto, Cat# AA3)

Amplifying Wash Buffer 20X (ProHisto, Cat# AA4)

Epitope Unmasking Solution (10X) (ProHisto, Cat# AA5)

Xylene

Ethanol

H<sub>2</sub>O<sub>2</sub>

Coverslips 24 X 50 mm No. 1 thickness, Chase Scientific Glass

Methyl Green (1% in water) or Hematoxylin (Gill-1, Fisher Cat#23-245653)

Slide racks and holder: Wheaton Glass 20 Slide Staining Dish with Removable Rack (Fisher Sci. Cat# 08-812)

-You would be best off getting a case of 6 to start off...

Permount (Fisher cat# SP15-100)

### **IMPORTANT:**

-Do not allow the tissue sections to dry out at any point during this protocol.

-Try to always take 1 or 2 extra slides through with your protocol. When doing the DAB stain, take your first extra slide through, stain them for 1 min with DAB, then adjust accordingly from there. If you feel you need to tweak the time (e.g.: to 2 min), do so with the second extra slide. After doing this, you are ready to finish the staining (i.e. DAB and methyl green) with your test slides.

-Also take an extra slide through and add only secondary antibody to it (another negative control). Another good negative control is to preabsorb before adding the antibody to the tissue. You can preabsorb with the peptide used to make the antibody (often available from the same company as you bought the antibody from).

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. ddH<sub>2</sub>O: 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 ddH<sub>2</sub>O 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. Take each individual slide, and with a kimwipe, wipe off the back of the slide, and around the tissue. Be sure not to touch the tissue, and that it keeps wet. Cover tissue with 1-3 drops of Peroxidase Block (comes with the DAKO kit). Incubate at room temperature 30 minutes, checking every 5-10 minutes to make sure samples don't dry out. If you observe some drying, place more Peroxidase Block onto the tissue.

9. Place slides back into the slide carrier and wash 3 x 3 min. in 1x Amplifying Wash Buffer (Cat# AA4).

10. Place slides into the **Antibody Amplifier™** (here, the **Antibody Amplifier™** is required), and cover each slide with approximately 3 mls of the Amplifying Antibody Dilution Buffer (Cat#AA3)/1° antibody. 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™**, and place on a standard laboratory rocker. Rock overnight at 4°C.

- Notes: This is the step that is the most important, and why the **Antibody Amplifier™** was developed. The use of the **Antibody Amplifier™** this way will improve sensitivity, specificity, and efficiency. Because you use much less antibody compared with the sedentary technique, you will also save money!

11. The next morning, take slides out of the **Antibody Amplifier™** (this can be done with any pointy object... we use a 1000 ul pipette tip) and place in the glass slide rack. Wash 2 x 10 min. in 1x Amplifying Wash Buffer (Cat# AA4).

12. Wipe off the back of slides and around tissues as above with a kim wipe. Add 1-3 drops of Labeled Polymer (comes with DAKO kit) straight from the bottle, enough to cover tissue. Incubate at room temperature for 30 minutes. Add polymer as needed so that the tissue does not dry out.

- Note: If you are not using the DAKO kit, you will be adding the secondary antibody here. In this case, use the **Antibody Amplifier™** here. We have had reports that you can use 100x less concentrated secondary, without compromising staining, but the specific concentration to use is left up to the discrepancy of the individual investigator.

13. As soon as slides are incubating, mix up the DAB solution. The ratio of the solution is 1 ml solution 3a (Buffered Substrate in DAKO kit): 1 drop 3b (liquid DAB and chromagen in DAKO kit). Mix well. Make enough so there is 200 ul per slide. Let the mixed solution sit at room temperature 30-60 minutes before using.

14. After incubation with Labeled Polymer, put slides in glass slide rack, and wash 2 x 5 minutes in 1x Amplifying Wash Buffer (Cat# AA4).

15. Wash slides in ddH<sub>2</sub>O 1 x 3 minutes.

16. Wipe back of slides and around tissue with kimwipe. Do each slide individually. Use the positive control to test DAB (this can also be an extra tissue slide of ones you are staining... eg. colon tissue from mouse#1... take two of

these through, so that you can test DAB time with the second tissue piece). Place 100-200 ul DAB solution onto the tissue, and carefully monitor under a microscope. You should see individual cells starting to light up (brown). This can take anywhere from 15 seconds to 5 minutes, depending on the antibody, the antibody concentration you used, etc. After you get optimal DAB staining, wash in ddH<sub>2</sub>O. You can keep each slide in a slide rack in the ddH<sub>2</sub>O until you are finished staining all slides.

- You will have to get a feel for this DAB staining. It should be such that there is maximal 'signal to noise'. If all tissue turns brown, this is usually too much.

#### 17. Wash slides in ddH<sub>2</sub>O

- Counterstain using 1% Methyl Green: Filter 3 mls of methyl green using a syringe filter into a 15 ml tube. Check with your extra slide first by incubating for 30 seconds, rinsing in ddH<sub>2</sub>O until all green is off the slide (usually have to use 2 sequential washes of ddH<sub>2</sub>O), and looking under a microscope.
  - Note that the methyl green is usually very weak at the beginning. It usually has to sit for a few months before it gets 'good'. At the beginning, it often takes greater than 5 minutes for it to stain the tissue. After sitting for a few months, it gets more concentrated, and usually works within 30 seconds.
  - An alternative counterstain that many people use is hematoxylin.

**18.** If you used methyl green to counterstain, then let slides/tissues dry, then mount with permount by adding 1-2 drops, placing a coverslip, and pushing the coverslip down to get rid of any bubbles. Let dry. You are now finished

**19.** If you use hematoxylin, you need to dehydrate the tissue. Do this by:

- Hematoxylin 2 min.
- Rinse with tap ddH<sub>2</sub>O
- Rinse with Tris pH7.6 for 30 seconds
- Rinse with ddH<sub>2</sub>O
- Dehydrate with 30% ETOH for 1 minute
- 70% ETOH for 1 minute
- 95% ETOH for 1 minute
- Isopropanol for 1 minute
- Second Isopropanol---Fresh for 1 minute
- Xylene for 1 minute
- Second Xylene for 1 minute
- Mount with Permout as above