IHC (Immunoperoxidase) Protocol

Notes: 1) Do no more than 25 slides at a time, due to the timed steps.
2) If an antigen unmasking procedure is required, do either immediately after step 1 or immediately after step 3.
3) This can be a one- or two-day protocol, depending on the incubation time with primary antibody.
4) After sections are cut, heat slides at 50 C for 1 hour to melt sections to slides.

1. **Deparaffinization and hydration of tissue sections:** Place slides in slide holders and treat the tissues with the clearing agent xylene (paraffin solvent) and a series of graded alcohols. Dunk slides up and down several times at the beginning of each incubation.
100% xylene – 5 min
100% xylene – 5 min
50%/50% (xylene/100% EtOH) – 3 min
100% EtOH – 3 min
95% EtOH – 3 min
70% EtOH – 3 min
50% EtOH – 3 min
Water (MilliQ) – 2 x 3 min
*Note: Change the solutions after about 5 uses or when you see the solution is not cleanly running off the slides, indicating too much paraffin in them. Use fresh MilliQ water

2. **Quench endogenous peroxidase activity:** Use a designated container so as not to disrupt later DAB development. Wash in 0.6% H_2O_2 in methanol for 15 minutes.

3. During the above incubations, make enough fresh 1% BSA in PBS for the entire experiment (# sections X 60-100 µl X 3 steps). Make the blocking buffer by taking one third of the 1% BSA solution and adding normal serum (serum from species in which the secondary antibody was made—thus if using goat anti-rabbit, use goat serum) to a final concentration of 10%, and add Triton-X 100 to a final concentration of 0.1%.

4. 2 x 3 min PBS wash.

5. During the above washes, prepare an enclosed humidity chamber and line the bottom with paper towels soaked with water. Ensure that the racks are leveled. Up to 14 slides can fit into one chamber.

6. After the last PBS wash, dab the corners of the slide on a paper towel and wipe off the back of the slide. Then, using a vacuum hose with an attached 200 µl pipette tip, remove the PBS from around the sections. Quickly place the slide in the humidity chamber on the metal rack. Avoid drying out the sections (which leads to increased background) by doing 3-4 slides at a time.

7. **Block:** Add 60-100 µl of blocking buffer to each section, making sure that there is a ring of buffer surrounding each section (don't just allow the buffer to be held onto the edges of the section by surface tension, because it causes differential staining of the tissue edges). Incubate at RT for 25 minutes.

8. **Primary antibody:** Aspirate slides to remove most of the blocking solution. Put 60-100 µl of primary antibody diluted in 1% BSA/PBS on each section. Place in the humidity chamber for 2 hours at room temperature, or alternatively overnight at 4°C.

9. 2 x 3 min PBS wash (longer is OK).

10. **Secondary Antibody:** Incubate tissue with biotinylated secondary antibody (60-100 µl/section) diluted in 1% BSA for 30 minutes at RT.

11. During this incubation, using the Vectastain Elite ABC kit (Vector Laboratories), combine the avidin and the biotinylated horseradish peroxidase (20 µl solution "A" + 20 µl solution "B" per 1 ml PBS) to make the Avidin-Biotinylated horseradish peroxidase macromolecular Complex (ABC). This must be made at least 30 minutes in advance of use, so make it now and keep at RT.

12. 2 x 3 min PBS wash.

13. **Conjugation of horseradish peroxidase to secondary antibody**: Add enough ABC solution to cover tissues (60-100 µl/section). Incubate for 30 minutes at RT.

14. During this incubation prepare the horseradish peroxidase substrate, DAB (diaminobenzidine tetrahydrochloride). Wear protective equipment, as DAB is a suspected carcinogen. Use Biogenex DAB kit (2.5ml MilliQ H_2O + 250µl substrate buffer + 2 drops liquid DAB chromagen + 1 drop of H_2O_2). Vortex, then add 3 drops of DAB Enhancer, then vortex again.

15. 2 x 5 min PBS wash (longer is OK).

16. **Chromogenic reaction:** Cover tissue sections with DAB solution and incubate for 2-10 minutes, doing only 4 slides per development. Examine a positive control slide under the microscope during the incubation. Observe slides for color change, and when optimal staining is achieved place slides into a container with H_2O to quench the DAB reaction. Don't allow the reaction to proceed too long. Use the specified DAB rack and container. Dispose of everything that has potentially touched DAB (pipette tips, gloves, tubes, etc.) in the biohazard box.

17. 1 x 5 min dd H_2O wash (longer is OK).

18. **Counterstain (if desired):** Counterstaining can skew image analysis software, so do not counterstain sections needing quantification. Dip in methylene blue for ~90 secs (light blue stain, so allows easy distinction from immuoperoxidase stain), or Harris's modified Hematoxylin with acetic acid (Fisher). Counterstains can be re-used. Wash slides in water after counterstaining.

19. Dehydrate sections, doing the following dehydration and clearing steps (use a different set of reagents than used for the first step so that you won't get paraffin on the stained tissue sections). Dip slides up and down in solutions 20 times:
    50% EtOH – 1 min
    70% EtOH – 1 min
    95% EtOH – 1 min
    100% EtOH – 1 min
    50%/50% xylene/100% EtOH – 1 min

100% xylene – 3 min
100% xylene – 5 min

20. **Mount coverslips:** Leave slides in xylene until they are coverslipped. Use forceps to pull slides out of the rack. Coverslip with Cytoseal XYL, or similar mounting media. Allow coverslips to harden overnight, with the slides flat.

Materials:

- Fisherbrand- Superfrost/Plus slides #12-550-15
- Vector- Avidin/Biotin Blocking kit #SP-2001
- Vector- elite ABC Kit (standard) #PK6100
- Vector secondary antibodies
- Biogenex- Liquid DAB Substrate Pack #HK130-5K
- Richard Allan Scientific- Cytoseal XYL #8312-4
- Triton X-100
- Methanol
- normal animal serum (species depends on what the 2° antibody is made in)
- humid chamber for slides
- staining dishes
- counterstain (methylene blue, hematoxylin, etc.)
- BSA (Sigma A-8022 is fine)
- PBS
- Antigen Retrieval supplies, if needed