

Immunohistochemistry (IHC) Staining Protocol

1. **Rehydration:** Rehydrate tissues before starting DAB (3,3' -diaminobenzidine tetrahydrochloride) staining.
 - a. Immerse slides in xylene (mixed isomers) twice, 10 min each.
 - b. Immerse slides in 100% ethanol twice, 10 min each.
 - c. Soak slides in 95% ethanol for 5 min.
 - d. Soak slides in 70% ethanol for 5 min.
 - e. Soak slides in 50% ethanol for 5 min.
 - f. Rinse slides with deionized water.
 - g. Rehydrate slides in wash buffer for 10 min, then drain excess buffer.
2. **Antigen Retrieval:** Place the slides in antigen retrieval solution (usually a citrate buffer at pH 6.0), and heat in a microwave oven or autoclave to re-expose the antigenic determinants masked during fixation.
3. **Washing:** Rinse slides with PBS 3 times (1 min each), then drain.
4. **Peroxidase Blocking:** To inhibit endogenous peroxidase activity, incubate samples with 1–3 drops of peroxidase blocker (3% H₂O₂ in water or methanol) for 15 min.
5. **Washing:** Rinse slides with PBS 3 times (1 min each), then drain.
6. **Hydrophobic Barrier (Optional):** Use a barrier pen to surround the tissue with the hydrophobic barrier.
7. **Blocking:** Apply blocking solution(5% BSA) and incubate at room temperature (RT) for 30 min.
8. **Washing:** Rinse slides with PBS 3 times (1 min each), then drain.
9. **Primary Antibody Incubation:** Remove blocking solution, apply primary antibody, and incubate at 37°C for 2 hr or at 4°C overnight.
10. **Washing:** Rinse slides with PBS 3 times (1 min each), then drain.
11. **Secondary Antibody Incubation:** Apply secondary antibody and incubate at RT for 30–60 min.
12. **Washing:** Rinse slides with PBS 3 times (5 min each), then drain.
13. **DAB Development:** Add 1–5 drops of DAB substrate to fully cover the tissue section. Incubate for 2–10 min. Monitor staining intensity under a microscope. A colored precipitate forms at antigen sites as HRP converts DAB into an insoluble product.

Caution: DAB is hazardous. Wear gloves, safety goggles, and work in a fume hood. Follow MSDS guidelines.

Note: If necessary, DAB enhancer can be used to enhance the DAB chromogen solution.

14. **Washing:** Rinse slides with PBS 3 times (5 min each), drain, then rinse with deionized water and drain.
15. **Counterstaining:** The stained tissue can either be left without nuclear counterstaining, or counterstained with hematoxylin nuclear counterstaining to better observe the tissue morphology.
16. **Microscopy:** Apply a coverslip of appropriate size, remove excess liquid, and observe staining under a microscope.