

Immunohistochemistry Protocol (Paraffin)

(From Cell Signaling Technology)

***IMPORTANT:** See product data sheet for the appropriate antibody diluent and antigen unmasking procedure.

A. Solutions and Reagents

1. Xylene
2. Ethanol, anhydrous denatured, histological grade (100% and 95%)
3. Deionized water (dH₂O)
4. Hematoxylin (optional)
5. Wash Buffer:
1X TBS/0.1% Tween-20 (1X TBST): To prepare 1 L add 100 ml 10X TBS to 900 ml dH₂O. Add 1 ml Tween-20 and mix.
10X Tris Buffered Saline (TBS): To prepare 1 L add 24.2 g Trizma[®] base (C₄H₁₁NO₃) and 80 g sodium chloride (NaCl) to 1 L dH₂O. Adjust pH to 7.6 with concentrated HCl.
6. *Antibody Diluent:
7. SignalStain[®] Antibody Diluent #8112
8. TBST/5% normal goat serum (#5425): To 5 ml 1X TBST add 250 µl normal goat serum.
9. PBST/5% normal goat serum (#5425): To 5 ml 1X PBST add 250 µl normal goat serum.
1X PBS/0.1% Tween-20 (1X PBST): To prepare 1 L add 100 ml 10X PBS to 900 ml dH₂O. Add 1 ml Tween-20 and mix.
10X Phosphate Buffered Saline (PBS): To prepare 1 L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na₂HPO₄) and 2.4 g potassium phosphate, monobasic (KH₂PO₄) to 1 L dH₂O. Adjust pH to 7.4.
10. *Antigen Unmasking:
11. Citrate: 10 mM Sodium Citrate Buffer: To prepare 1 L add 2.94 g sodium citrate trisodium salt dihydrate (C₆H₅Na₃O₇•2H₂O) to 1 L dH₂O. Adjust pH to 6.0.
12. EDTA: 1 mM EDTA: To prepare 1 L add 0.372 g EDTA (C₁₀H₁₄N₂O₈Na₂•2H₂O) to 1 L dH₂O. Adjust pH to 8.0.
13. TE: 10 mM Tris/1 mM EDTA, pH 9.0: To prepare 1L add 1.21 g Trizma[®] base (C₄H₁₁NO₃) and 0.372 g EDTA (C₁₀H₁₄N₂O₈Na₂•2H₂O) to 950 ml dH₂O. Adjust pH to 9.0, then adjust final volume to 1000 ml with dH₂O.
14. Pepsin: 1 mg/ml in Tris-HCl pH 2.0.
15. 3% Hydrogen Peroxide: To prepare, add 10 ml 30% H₂O₂ to 90 ml dH₂O.
16. Blocking Solution: TBST/5% normal goat serum (#5425): to 5 ml 1X TBST add 250 µl normal goat serum.
17. Biotinylated secondary antibody.
18. ABC Reagent: (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) Prepare according to manufacturer's instructions 30 minutes before use.

19. DAB Reagent or suitable substrate: Prepare according to manufacturer's recommendations.

B. Deparaffinization/Rehydration

NOTE: Do not allow slides to dry at any time during this procedure.

1. Deparaffinize/hydrate sections:
 - a. Incubate sections in three washes of xylene for 5 minutes each.
 - b. Incubate sections in two washes of 100% ethanol for 10 minutes each.
 - c. Incubate sections in two washes of 95% ethanol for 10 minutes each.
2. Wash sections twice in dH₂O for 5 minutes each.

C. *Antigen Unmasking

NOTE: Consult product data sheet for specific recommendation for the unmasking solution.

1. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0 then maintain at a sub-boiling temperature for 10 minutes. Cool slides on bench top for 30 minutes.
2. **For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 minutes at a sub-boiling temperature. No cooling is necessary.
3. **For TE:** Bring slides to a boil in 10 mM TE/1 mM EDTA, pH 9.0 then maintain at a sub-boiling temperature for 18 minutes. Cool on the bench for 30 minutes.
4. **For Pepsin:** Digest for 10 minutes at 37°C.

D. Staining

1. Wash sections in dH₂O three times for 5 minutes each.
2. Incubate sections in 3% hydrogen peroxide for 10 minutes.
3. Wash sections in dH₂O twice for 5 minutes each.

NOTE: Consult product data sheet for recommended antibody diluent.

4. Wash sections in wash buffer for 5 minutes.
5. Block each section with 100-400 µl blocking solution for 1 hour at room temperature.
6. Remove blocking solution and add 100-400 µl primary antibody diluted in recommended antibody diluent to each section. Incubate overnight at 4°C.
7. Remove antibody solution and wash sections in wash buffer three times for 5 minutes each.
8. Add 100-400 µl biotinylated secondary antibody, diluted in TBST per manufacturer's recommendation, to each section. Incubate 30 minutes at room temperature.
9. If using ABC avidin/biotin method, prepare ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.
10. Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each.

11. Add 100-400 μ l ABC reagent to each section and incubate for 30 minutes at room temperature.
12. Remove ABC reagent and wash sections three times in wash buffer for 5 minutes each.
13. Add 100-400 μ l DAB or suitable substrate to each section and monitor staining closely.
14. As soon as the sections develop, immerse slides in dH₂O.
15. If desired, counterstain sections in hematoxylin per manufacturer's instructions.
16. Wash sections in dH₂O two times for 5 minutes each.
17. Dehydrate sections:
 - a. Incubate sections in 95% ethanol two times for 10 seconds each.
 - b. Repeat in 100% ethanol, incubating sections two times for 10 seconds each.
 - c. Repeat in xylene, incubating sections two times for 10 seconds each.
18. Mount coverslips.