

IHC staining protocol

Paraffin, frozen and free-floating sections

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Paraffin and frozen sections

Reagents can be applied manually by pipette, or the protocol can be adapted for automated and semi-automated systems if these are available.

Carry out incubations in a humidified chamber to avoid tissue drying out, which will lead to non-specific binding and high background staining. A shallow plastic box with a sealed lid and wet tissue paper in the bottom is an adequate chamber. Keep slides off the paper and lay flat so that the reagents don't drain off.

Cut a plastic serological pipette into lengths to fit your incubation chamber then glue them in pairs to the bottom of the chamber, with each pair about 4 cm apart. This provides a level and raised surface for the slides to rest on, away from the wet tissue paper.

Dilutions of the primary and secondary antibody are listed on the datasheets or are determined by testing a range of dilutions. Adjust dilutions appropriately from the results obtained.

For enzymatic methods, horseradish peroxidase (HRP) or alkaline phosphatase (AP) are the most commonly used enzymes. A number of chromogens are used with these enzymes.

Method

Perform antigen retrieval before commencing with immunostaining if necessary.

1. If using a HRP conjugate for detection, blocking of endogenous peroxidase can be performed but we recommend waiting until after the primary antibody incubation.

H₂O₂ suppresses endogenous peroxidase activity to reduce background staining. Check for the presence of endogenous peroxidases by incubating a tissue slide after re-hydration in a solution of DAB. If areas of the section appear brown under the microscope, a blocking step should help reduce staining.

Some epitopes are modified by peroxide, leading to reduced antibody-antigen binding. Incubate sections with peroxide after the primary incubation to avoid this problem. Peroxide can be diluted in TBS or water. Methanol is useful for blood smears or other peroxidase-rich tissues; peroxide diluted in methanol tends to reduce tissue damage caused by the reaction in aqueous solutions.

For other tissue, dilute in TBS or water. Reduced binding of some antibody-antigen pairs, in particular cell surface proteins, has been observed after methanol/peroxide incubation. (If using AP or fluorescent detection, omit peroxidase quenching as it only applies to HRP conjugates).

2. Wash the slides 2x5 min in TBS plus 0.025% Triton X-100 with gentle agitation.

0.025% Triton X-100 in the TBS reduces surface tension, allowing reagents to cover the whole tissue section with ease. It is also believed to dissolve Fc receptors and reduce non-specific binding. We recommend TBS over PBS to get a cleaner background.

3. Block in 10% normal serum with 1% BSA in TBS for 2 h at room temperature.

The secondary antibody may cross react with endogenous immunoglobulins in the tissue. Minimize this by pre-treating the tissue with normal serum from the species in which the secondary was raised.

The use of normal serum before the application of the primary also eliminates Fc receptor binding of both the primary and secondary antibody. BSA is included to reduce non-specific binding caused by hydrophobic interactions.

4. Drain slides for a few seconds (do not rinse) and wipe around the sections with tissue paper.

5. Apply primary antibody diluted in TBS with 1% BSA.

Dilute the primary antibody to the manufacturer's recommendations or to a previously optimized dilution. Most antibodies will be used in IHC-P at a concentration of 0.5–10 µg/mL.

The primary antibody should be raised in a species different from the tissue being stained. Eg if you had mouse tissue and your primary antibody was raised in a mouse, an anti-mouse IgG secondary antibody would bind to all the endogenous IgG in the mouse tissue and cause high background. Use of mouse monoclonals on mouse tissue is discussed in our mouse-on-mouse protocol.

6. Incubate overnight at 4°C.

Overnight incubation allows use of antibodies of lower titer or affinity by allowing more time for the antibodies to bind. Regardless of the antibody's titer or affinity for its target, once the tissue has reached saturation point no more binding can take place. Incubate overnight to ensure this occurs.

7. Rinse 2x5 min TBS 0.025% Triton with gentle agitation.

8. If using an HRP conjugate for detection, incubate the slides in 0.3% H₂O₂ in TBS for 15 min.

Develop the colored product of the enzyme with the appropriate chromogen. The choice depends on enzyme label, the preferred colored end product and whether aqueous or organic mounting media is used.

AEC, Fast Red, INT or any other aqueous chromogen are alcohol soluble. Use a suitable aqueous mounting media and do not dehydrate and clear.

Detection step

For enzymatic detection (HRP or AP secondary conjugates)

1. Apply enzyme-conjugated secondary antibody to the slide, diluted in TBS with 1% BSA, and incubate for 1 h at room temperature.
2. Develop with chromogen for 10 min at room temperature.
3. Rinse in running tap water for 5 min.
4. Counterstain (if required).

Some commonly used counterstains are hematoxylin (blue), nuclear fast red, or methyl green. When using fluorescent detection, DAPI (blue) or propidium iodide/PI (red) can be used.

5. Dehydrate, clear and mount.

AEC, Fast Red, INT or any other aqueous chromogen are alcohol soluble. Use a suitable aqueous mounting media and do not dehydrate and clear.

Dehydrate and clear sections developed using DAB, New Fuchsin, Vega Red, NBT, TNBT or any other organic chromogen developed sections by running the rehydration steps in our deparaffinization protocol. Mount sections in a suitable organic mounting medium.

Sections mounted in organic mounting media have a better refractive index than those mounted in aqueous mounting media. This provides a sharper microscopic image if an organic mounting medium is used.

For fluorescent detection

1. Apply fluorophore-conjugated secondary antibody to the slide diluted in TBS with 1% BSA.
2. Incubate for 1 h at room temperature.

These steps should be done in the dark to avoid photobleaching.

3. Rinse 3x for 5 min with TBS.
4. Mount using compatible mounting medium and add coverslip.

Immunostaining: free-floating sections

Materials and reagents

Peroxidase block (40 mL)

- 20 mL 0.2M phosphate buffer
- 8 mL methanol
- 80 μ L Triton-X100
- 2 mL hydrogen peroxide
- Make up to 40 mL with ddH₂O

Blocking buffer

- 0.1M Phosphate buffer
- 0.3% Triton
- 1% serum from secondary antibody host species

Method

1. Cut 30–40 μ m sections on a freezing microtome. Collect sections into petri dishes or a multi-well plate containing 1-2 mL 0.1M phosphate buffer (PBS).
2. Store sections at 4°C for no more than a week. For longer storage, add azide, wrap the petri dish or multi well plate in cling film and store at 4°C for no more than 2 months. If left too long the sections become harder to mount.
3. Deactivate endogenous peroxidases by incubating in peroxidase block for 15 min with gentle agitation.
4. Wash (3x15 min) in 0.1M PBS/0.3% Triton.
5. Incubate the sections in blocking buffer for at least 1 h with gentle agitation.
6. Wash (3x15 min) in 0.1M PBS/0.3% Triton.
7. Add the primary antibody and incubate at 4°C overnight with gentle agitation.
8. Wash (3x15 min) in 0.1M PBS/0.3% Triton.
9. Add secondary antibody either for 2 h at room temperature or overnight at 4°C with gentle agitation.
10. Wash (3x15 min) in 0.1M PBS (no Triton).
11. Wash once briefly in 0.1M acetate buffer to remove PBS.

Acetate buffer must be made up fresh.

12. Place sections in solution for DAB reaction. Monitor DAB reaction on microscope (2–10 min).

13. Stop the reaction once the background is high enough by placing sections into 0.1M acetate buffer.
14. Wash (3x15 min) in 0.1M PBS.
15. Sections can be kept in cold room for a couple of days until mounted and coverslipped.
16. Air dry sections in fume hood. Place sections (2x10 min) in histoclear.
17. Mount from the third 10 min histoclear incubation.
18. Coverslip using Ralmount. Use an appropriate mounting medium for sections labeled with fluorescent detection.

Signal amplification

To achieve a stronger signal, various strategies have been developed to add more enzyme or fluorophore to the target of interest.

Avidin-biotin complex (ABC)

This technique uses the high affinity of avidin for biotin, an enzyme co-factor in carboxylation reactions. Avidin has four binding sites for biotin and binding is essentially irreversible.

In brief, the primary antibody is bound to the protein of interest. A biotinylated secondary antibody is then bound to the primary antibody. In a separate reaction, a complex of avidin and biotinylated enzyme is formed by mixing the two in a ratio that leaves some of the binding sites on avidin unoccupied. This complex is then incubated with the tissue section after the antibody incubations. The unoccupied biotin-binding sites on the complex bind to the biotinylated secondary antibody. This attaches more enzyme to the target than is possible using an enzyme-conjugated secondary or primary antibody.

The components of the avidin-biotin complex are commercially available in kits and the complex can be used with our biotinylated antibodies. The presence of endogenous biotin in tissues can bind the avidin-biotin complex and cause background staining. Such tissues include kidney, liver, brain, prostate, colon, intestines, and testes.

Labeled streptavidin biotin (LSAB)

This method is similar to ABC as it uses the interaction of streptavidin (similar to avidin in binding affinity) and biotin. The primary antibody is followed by a biotinylated anti-Ig secondary antibody, followed by streptavidin conjugated to an enzyme or fluorophore. Streptavidin produces less non-specific background staining than avidin since it is non-glycosylated (unlike avidin) and so doesn't interact with lectins or other carbohydrate-binding proteins. For a comparison to ABS in which LSAB was shown to be 4–8 times more sensitive see Giorno R (1984), *Diagn Immunol.* 2:161–6.

HRP polymer

Both avidin-biotin methods (ABC and LSAB) are losing favor to new polymer-enzyme-antibody products that consist of a secondary antibody (eg anti-mouse and/or rabbit

IgG) attached to a polymer-enzyme complex. This is partly because the issue of endogenous biotin is avoided.

EXPOSE detection kits for IHC use small linker-based detection modules that can penetrate tissues better than the large complexes used in conventional polymer-based detection, resulting in higher sensitivity.

Tyramide signal enhancing (TSE)

One of the most effective amplification procedures is the patented and licensed method, TSE (also known as TSA or CSA). It is useful for detection of sparse antigens that other systems have difficulty detecting, and for improving results obtained with poorly-performing antibodies.

The method relies on a peroxidase-catalyzed reaction to covalently attach the tyramide portion of tyramine-protein conjugates to the area around the protein of interest, after applying a primary antibody and secondary-HRP conjugate. The covalently attached protein cannot be washed off, even if the slides are treated to remove the antibodies, since the tyramide bond is covalent. A signal is obtained by directing antibody-enzyme or fluorophore conjugate against the protein portion of the tyramine-protein conjugate.

The kits are expensive and multiple steps are time consuming.