

## IMMUNOHISTOCHEMISTRY, PARAFFIN

**Introduction** Immunohistochemistry is used to identify the location and distribution of target antigens in cells or tissues by staining with a specific antibody. The antibody is conjugated to either a fluorescent or enzymatic label, and the location of the label seen through a microscope approximates the position of the target antigen. Most tissues cannot be viewed under a microscope because they are too thick allow light to be transmitted. The tissue can be sliced into very thin sections provided they are first processed to prevent cell damage. When the tissue is fixed with paraffin, thinner sections can be sliced compared to frozen sections. This results in better microscopic resolution and better images of what subcellular structures remain. Paraffin tissue sections are ideal for rapidly identifying cellular localization of RNA or protein. First tissue, is excised then sectioned and immediately fixed by formalin. After staining with the appropriate antibodies, the sections can be identified.

### Materials / reagents

- **Blocking buffer :**  
10% serum from host species of secondary antibody diluted in PBS, or 2% Bovine Serum Albumine (BSA) diluted in PBS.
- **Antibody buffer:**  
PBS with 2% BSA.
- **Diaminobenzidine (DAB) solution:**  
0.5 mg/ml diaminobenzidine in PBS.
- **Mounting solution:**  
Glycerol gelatin.

### Procedure

#### Fixation and paraffin embedding of tissue

##### Fixation protocol

1. Tissues to be fixed and processed should be cut to a size no larger than 3 mm thick. Let tissues fix in 10% formalin at room temperature for at least 8 hours but not to exceed 24 hours.
2. Rinse with running tap water for 1 hour.

##### Alternative fixation of tissue

Many antigenic epitopes are masked or even destroyed by 10% formalin fixation. In some cases fixation in a milder fixative such as zinc fixative for IHC is helpful to preserve the antigenic epitopes.

1. Place freshly dissected and trimmed tissues into zinc fixative and allow tissues to fix for 24-48 hours at room temperature.
2. Rinse with running tap water for 30-45 minutes.

##### Paraffin embedding protocol

If tissues are to be embedded in paraffin or plastic, all traces of water must be removed because water and paraffin are immiscible. This process is called dehydration and accomplished by passing the tissue through a series of increasing alcohol concentrations.

1. The blocks are transferred sequentially to 30%, 50%, 70%, 80%, 90%, 95% and 100% alcohols for about two hours each.
2. The blocks are then placed in a second 100% ethanol solution to ensure that all water is removed.

For paraffin embedding the tissues need to be cleared. Clearing refers to the use of an intermediate fluid that is miscible with ethanol and paraffin. The most often used clearing agent is toluene, others are benzene, chloroform or xylol.

3. Move the blocks into a 50:50 mixture of absolute ethanol and toluene for two hours.
4. The blocks are then placed into pure toluene.
5. Then move the blocks in a 50:50 mixture of toluene and paraffin.
6. Place them in an oven at 56°C – 58°C (the melting temperature of paraffin).
7. Transfer the blocks to pure paraffin and place in the oven for one hour.
8. Transfer to a second pot of melted paraffin and place in oven for an additional two-three hours.

#### Preparation of paraffin sections for immunohistochemistry

##### Sectioning Protocol

1. Section paraffin blocks at the desired thickness (usually 4-5 µm) on a microtome and float on a 40°C water bath containing distilled water.
2. Keep record of the orientation and sequence of the sections.
3. Transfer the sections onto a poly-1-lysine coated slide. Allow the slides to dry overnight and store slides at room temperature until ready for use.

##### Deparaffinization and re-hydration of tissue slide

1. Before deparaffinization, place the slides in a 55°C oven for ten minutes to melt the paraffin.
2. Deparaffinize slides in 2 changes of toluene for 5 minutes each.
3. Hydrate the slides by transferring slides twice through 100% alcohol; the 2 changes for 3 minutes each and transfer once through 95% alcohol for 3 minutes.
4. Block endogenous peroxidase activity by incubating sections in 0.3% H<sub>2</sub>O<sub>2</sub> solution in methanol for 10 minutes.

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5. Rinse for 2 changes in PBS for 5 minutes each time.
6. If the antibody staining requires antigen retrieval to unmask the antigenic epitope see section *Pretreatment of paraffin sections*. If antigen retrieval is not required proceed to section *Immunohistochemical staining of paraffin embedded tissues*.

**Pretreatment of paraffin sections:**

Antigen retrieval is a heat based antigen unmasking technique that can be used prior to immunohistochemical staining of archival formalin-fixed paraffin-embedded tissue sections. While some antibodies recognize the formalin-fixed antigen, the majority of monoclonal antibodies will not stain formalin-fixed tissues. In this protocol the Sodium Citrate Antigen Retrieval method is described:

1. Place slides in a glass slide holder and fill in the rest of the rack with blank slides (10 total) to ensure even heating.
2. Place rack in 600 ml of 10 mM Sodium Citrate in a glass 2L beaker. Mark a line at the top of the liquid on the beaker.
3. Microwave for 20 minutes total, replacing evaporated water every 10 minutes.
4. Cool slides for 20 minutes in the beaker.
5. Wash four times in distilled water and one time in PBS.

**Immunohistochemical staining of paraffin embedded tissues:**

*Please read entire procedure before staining sections. Perform all incubations in a humidified chamber and do not allow sections to dry out. Controls should also be run and must be matched to the species and isotype of each primary antibody to be tested.*

1. Label slides with a solvent resistant pen and demarcate the tissue if required.
2. Block non-specific binding by incubating with **blocking buffer** for 30-60 min at room temperature in a humidified chamber.
3. Remove blocking buffer.
4. Dilute the primary antibody in the **antibody buffer**. Apply the diluted antibody to the tissue sections on the slide. Incubate for 1 hour at room temperature in a humidified chamber.
5. Rinse slides for 3 changes in PBS, 2 minutes each time.
6. Dilute the secondary antibody in the antibody buffer. Apply to the tissue sections on the slide and incubate for 30 minutes at room temperature.
7. Rinse slides for 3 changes in PBS, 2 minutes each time.
8. According to the second antibody an appropriate conjugate should be used.
9. Rinse slides for 3 changes in PBS, 2 minutes each time.
10. Prepare **DAB solution**. For blue stain, mix two parts DAB solution + one part 3% NiCl<sub>2</sub>. For orange-brown stain, use the DAB solution without NiCl<sub>2</sub>.
11. Apply the appropriate DAB solution, allow slides to incubate for 5 minutes or until the desired color intensity is reached.
12. Transfer slide to a glass dish. Add ~5 µl 0.3% H<sub>2</sub>O<sub>2</sub>. It may take seconds to several minutes for staining to occur, depending on the abundance of the antigen and the quality of the antibody. Observe the extent of staining under a stereomicroscope and, when the color is appropriately developed, stop the reaction by washing with several changes of PBS.
13. Dehydrate through 4 changes of alcohol (95%, 95%, 100% and 100%).
14. Clear in 3 changes of toluene and cover slip using **mounting solution**.

**Safety**

- Samples of tissue, serum or blood origin should be handled to guidelines for prevention of transmission of blood borne diseases.
- Some enzyme substrates are considered hazardous, due to potential carcinogenicity. Handle with care and refer to Material Safety Data Sheets for proper handling precautions.
- Reagents which contain preservatives may be toxic if ingested, inhaled, or in contact with skin.
- Formalin has been implicated as a causative agent for strong allergy reactions (contact dermatitis with prolonged exposure) and may be a carcinogen. It should be used with care and always in a well ventilated environment.
- Be wary of all organic solvents. Most are implicated as carcinogenic agents. Heed all precautions for the proper use of these compounds.

**Notes**

- When making buffer(s) fresh before you start, there is no need to add sodium azide to the buffer.
- Pre-incubation of the sample with 5% BSA for 10 min. prior to the primary antibody reaction may decrease background staining. For best results with animal tissues, use 5 to 10% normal serum from the same species as the host of the secondary antibody.
- Allow the slides to drain, shake off excess fluid with a brisk motion and carefully wipe each slide around the sections.
- Dilute the primary antibody or negative control reagent to its optimal dilution. The antibody buffer

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alone may be used as a negative control. A positive control slide (a tissue known to contain the antigen under study) should also be run.

**Technical assistance**

For technical assistance and more information please contact Hycult Biotech directly at [support@hycultbiotech.com](mailto:support@hycultbiotech.com).

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