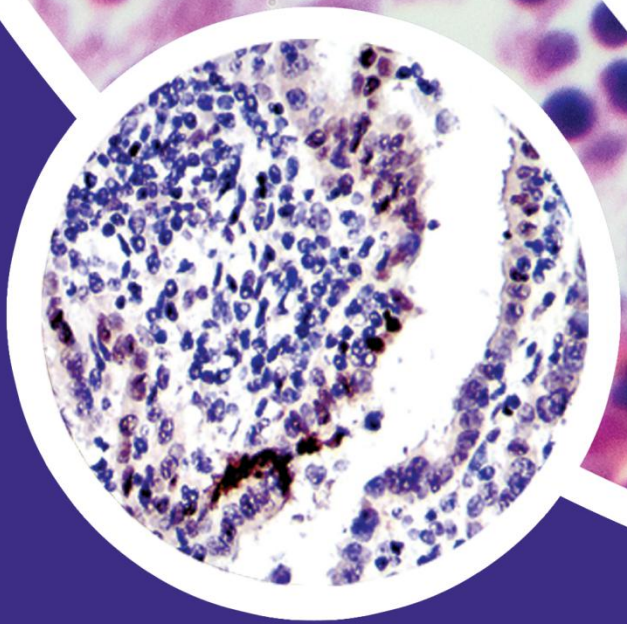
Immunohistochemistry(IHC) User Guide



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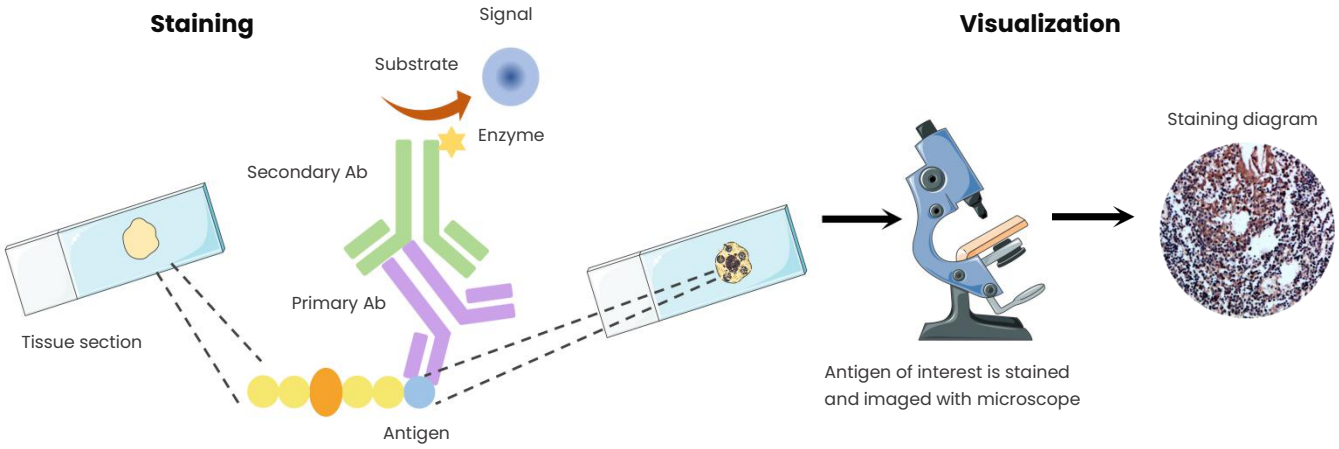
Introduction

Immunohistochemistry (IHC) is an assay that shows specific antigens in cells or tissues by the use of markers that are either fluorescent dyes or enzymes. In an IHC experiment, a primary antibody binds specifically to a protein of interest present in a tissue or cell. The antibody binding is then visualized by a detection system, which provides information about if and where the protein is present in the tissue. IHC is a common method in diagnostics to determine morphological abnormalities and the presence of biomarkers indicative of certain diseases such as cancer.

IHC uses antibodies to detect cell and tissue proteins and provide semi-quantitative data about target protein expression, distribution, and localization. Tissues are sectioned from fixed embedded or frozen blocks, and the sections are then probed with primary antibodies against the antigens of interest. Target expression can be evaluated with the corresponding labeled primary antibody (direct detection) or, more commonly, with the addition of labeled secondary antibodies. The label, either fluorescent or enzymatic, is used to visualize the antigen-antibody complex.



IHC workflow

Protocol

1. Mount and section samples

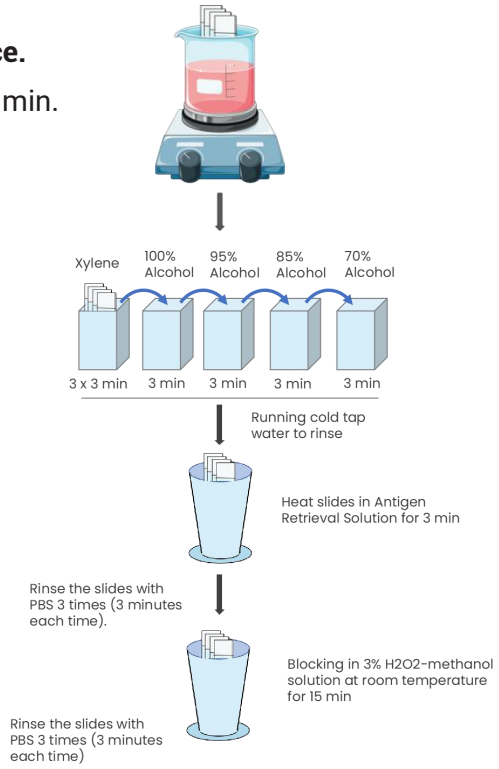
2. Heat sections on the specimen slide to improve adherence.

Put the specimen slide in the water bath and heat at 65°C 30 min.

3. Remove paraffin and rehydrate the tissue

Place the slides in a rack and perform the following washes:

- Xylene: 3 x 3 minutes.
- 100% ethanol: 3 minutes.
- 95% ethanol: 3 minutes.
- 85 % ethanol: 3 minutes.
- 70 % ethanol: 3 minutes.
- Running cold tap water to rinse.



4. Perform heat induced or protease induced epitope retrieval

Immerse slides into preheated Antigen Retrieval Solution (1x, 100°C) 10 minutes. Remove slides from the water bath, and let it cool to room temperature. Gently rinse the slides with PBS 3 times (3 minutes each time).

Tips: Antigens can be masked as a result of the fixation process, which makes antibody binding impossible. The unmasking can be reversed with a technique called epitope retrieval/antigen unmasking, which is either mediated by heat (HIER/heat-induced epitope retrieval) or proteases (PIER). The latter uses enzymes such as proteinase K, trypsin and pepsin. PIER method acts by degrading the peptides masking the epitope. However, PIER might also result in alterations to the specimen morphology or the antigen itself. Consequently, PIER is less frequently used than HIER, which acts by restoring the secondary and tertiary structure of an epitope.

5. Block endogenous peroxidases, phosphatases (for enzymatic labels) and biotin (when using biotin/avidin systems)

Put the slides in 3% H_2O_2-methanol solution (30% H_2O_2: 100% methanol=1:9) at room temperature 15 min, and gently rinse the slides with PBS 3 times (3 minutes each time).

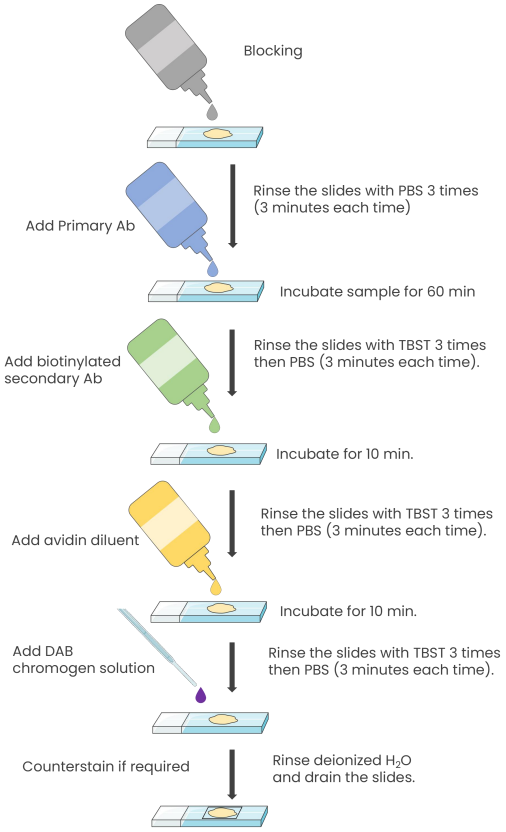
Tips: To avoid staining artifacts it is important: To block endogenous peroxidases and phosphatases prior to using alkaline phosphatase (AP)/horseradish peroxidase (HRP) antibody conjugates. To block endogenous biotin when using avidin/biotin or streptavidin/biotin detection systems. To block endogenous alkaline phosphatase activity

levamisole is most commonly used.

6. Block non-specific binding sites

Put the slides in 5% non-fat milk at 37°C 5 min, and gently rinse the slides with PBS 3 times (3 minutes each time).

Tips: Blocking should be performed prior to incubation with the primary antibody to prevent non-specific antibody binding. We use non-fat milk to block in the protocol, and serum, bovine serum albumin or gelatin can be used to block. If use serum, block with 10-20% normal serum, and use normal serum from the same species as the one in which the secondary antibody was generated in, and never use normal serum from the same species that the primary antibody that may lead to blocking of reactive sites or higher background.



7. Incubate with primary antibody

Put the slides in the wet box, and add proper volume of primary antibody diluent, then incubate at 37°C 60 min, and gently rinse the slides with TBST 3 times then PBS (3 minutes each time).

Tips: Check on the manufacturer's datasheet that the antibody has been tested in the specific immunohistochemical method intended to be used.

We recommend using a polyclonal antibody when first establishing an IHC protocol. Although antigen retrieval is possible, the efficiency of the process is variable and certain epitopes might still remain inaccessible. Therefore, a polyclonal antibody, which recognizes a multitude of epitopes due to its heterogeneous nature, provides a definite advantage over a monoclonal antibody which recognizes a single epitope.

Prior to performing the experiment check the localization of the antigen. To get an initial idea of the expected staining the relevant datasheet of the antibody supplier or other web resources can be consulted. When using an antibody for the first time always determine the optimal antibody dilution by performing staining with multiple antibody concentrations. This should be done for both the primary and secondary antibody. To ensure that the observed staining is specific include IHC staining controls in your experimental design.

8. Incubate with secondary antibody

Add proper volume of secondary antibody diluent, then incubate at 37°C 10 min, and gently rinse the slides with TBST 3 times then PBS (3 minutes each time).

Tips: We recommend the use of directly conjugated antibodies in IHC only for the detection

of very abundant target proteins (e.g., β-actin, GAPDH and α-tubulin).

For medium to low abundant proteins, we recommend using secondary antibodies for detection due to the fact that multiple secondary antibodies bind to a single primary antibody thereby leading to amplification of.

For very low abundant proteins, we suggest using biotinylated secondary antibodies in combination with conjugated avidin/ streptavidin due to a single avidin molecule being able to simultaneously bind up to four biotin molecules to amply the signal.

When using biotinylated antibodies, ensure endogenous biotin is blocked prior to primary antibody incubation. While selecting a fluorophore conjugated secondary antibody, ensure that your microscope is able to excite and detect the fluorophore appropriately.

9. Incubate with amplification reagent

Add proper volume of avidin diluent, then incubate at 37°C 10 min, and gently rinse the slides with TBST 3 times then PBS (3 minutes each time).

When performing experiments with multiple fluorescent labels, ensure that each fluorophore can be spectrally separated. This ensures that one fluorophore does not get detected in another fluorophore's channel. For this purpose, we recommend mocking up the fluorophore excitation and emission spectra with the help of a spectrum viewer at the experimental design stage.

10. Incubate with DAB or other substrate solution (for enzymatic labels only)

Add 1-5 drops of DAB chromogen solution to cover the entire tissue section and incubate for 3-5 minutes, and rinse in deionized H_2O and drain the slides.

Tips: In immunohistochemistry the enzymatic labels horseradish peroxidase (HRP) and alkaline phosphatase (AP) are mainly used. In an immunoenzymatic staining, a colored precipitate is formed due to the reaction of an enzyme with its substrate.

Factors should be considered:

- The precipitate color varies depending on the enzyme and chromogenic substrate combination. For example selecting DAB (3,3'Diaminobenzidine) as an HRP substrate leads to a brown staining while choosing AEC (3-Amino-9-Ethylcarbazole) results in a red one.
- When selecting chromogens do not simply select on staining color alone. Other factors to consider are the chromogen's staining efficiency, staining intensity, and compatibility with organic mounting media (see mounting media section).
- When designing experiments with multiple enzymatic labels care has to be taken so that the final precipitate colors are spectrally differentiable. *e.g., AP/Fast Red (red) and HRP/DAB (brown) is a good combination while AP/Fast Red (red) and HRP/AEC (red) is not.* Also take care that the counterstain does not have the same color as the precipitate.

The common chromogens for both HRP and AP and the resulting precipitate colors

Substrate		Precipitate color
HRP	3,3'-Diaminobenzidine (DAB)	Brown
	3-Amino-9-Ethylcarbazole (AEC)	Rose-red
	4-Chloro-1-Naphthol (CN)	Blue
	P-Phenylenediamine Dihydrochloride/ pyrocatechol (Hanker-Yates reagent)	Blue-Black
AP	Fast Red TR	Bright red
	New Fuchsin	Red
	Fast Blue BB	Blue

11. Counterstain

Add 1–5 drops of hematoxylin to cover the entire tissue section and incubate for 5 minutes, and rinse in deionized H2O and drain the slides.

Tips: Like controls, counterstaining is crucial for every IHC experiment, as the counterstain provides background contrast and puts the observed staining into perspective.

Especially for multi-color experiments, care should be taken when selecting counterstains to ensure that they are spectrally differentiable from the color of the antibody staining. In the interest of time and for your convenience, we recommend using mounting media that contain counterstains.

The common chromogenic and fluorescent counterstains as below

Counterstains		Precipitate color	Counterstain
Chromogenic	Hematoxylin	Blue	Nucleus
	Fast red	Red	Nucleus
	Methylene blue	Blue	Nucleus
	Methylene green	Blue/green	Nucleus
	Toluidine blue	Blue	Nucleus
Fluorescent	DAPI	Blue	Nucleus
	DRAQ5™	Red	Nucleus
	Hoechst	Blue	Nucleus
	Propidium iodide	Blue/green	Nucleus
	Sytox® Green	Green	Nucleus
	Wheat germ agglutinin (WGA)	Variable; depending on which dye was conjugated to WGA	Plasma membrane
	Phalloidin	Variable; depending on which dye was conjugated to WGA	Filamentous Actin

12. Dehydrate tissue sections (only needed when organic mounting media are used)

Cover stained tissue with a coverslip of an appropriate size. Place slides vertically on a filter

paper or towel to drain excess mounting medium and allow them to dry.

13. Mount coverslip

Add drops of resinene, then seal with coverslip. Visualize tissue under a light microscope.

Troubleshooting Guide

Lack of staining

Possible case	Solution
Lack of antigen.	Check protein expression by in situ hybridization (in some rare cases translation may be blocked even though mRNA is detected).
Antibodies do not work due to improper storage.	Follow storage instructions on the datasheet. In general, aliquot antibodies into smaller volumes sufficient to make a working solution for a single experiment. Store aliquots in a manual defrost freezer (-20 to -70°C) and avoid repeated freeze-thaw cycles.
Inactive primary or secondary antibodies.	Test reporter system independently to assess reagent viability.
Inadequate tissue fixation.	Try increasing the fixation time or try a different fixative.
Tissue overfixation.	Reduce the duration of the immersion or post-fixation steps. If immersion-fixation cannot be avoided (e.g., collection of postmortem tissues or biopsies in pathology lab), antigens may be unmasked by treatment with antigen retrieval reagents.
Incompatible secondary and primary antibodies.	Use a secondary antibody that will interact with the primary antibody. For example, if the primary antibody was raised in rabbits, use an anti-rabbit secondary antibody.
Antigen was destroyed before incubation with the primary antibody.	If quenching of endogenous peroxidase was done prior to the addition of primary antibodies, block peroxidase after incubation with the primary antibody.
Epitope altered during fixation or embedding procedure.	Try restoring immunoreactivity through various antigen retrieval techniques. Embed tissue at 58°C or below.
Antigen retrieval was ineffective.	Increase the time of treatment or change the treatment solution.
Reagents omitted or used in wrong order.	Repeat staining and confirm that correct reagents are used and are added in the correct order.

High background

Possible case	Solution
High concentration of primary and or secondary antibodies.	Titer antibody to determine optimal concentration needed to promote a specific reaction of the primary and the secondary antibodies.
Hydrophobic interactions of the antibody and proteins in the tissue.	Lower the ionic strength of the antibody diluent (particularly, monoclonal antibodies respond well to reducing the salt concentration).
Non-specific binding of primary and/or secondary reagents to tissues.	Use blocking step just prior to primary antibody incubation (we use 1% BSA with 10% normal donkey serum, or non-fat dry milk is another option).
Non-specific binding of secondary antibody.	Use an antibody that has had cross-reactive IgG species removed (absorbed against sample species).

Tissue dried out.	Avoid letting the tissue dry during the staining procedure.
Reagents sticking to old or poorly prepared slides.	Start over with freshly prepared or purchased slides.
Background due to ionic interactions.	Increase the ionic strength of the diluent buffer.

Cell/Tissue morphology destroyed

Possible case	Solution
Antigen retrieval methods are too harsh.	Empirically determine the conditions that preserve tissue morphology while restoring the immunoreactivity of the antigen.
Tissue sections falling off slide (more common with frozen sections).	Increase fixation time. Empirically determine an additional or alternative fixative. Use freshly prepared, adequately charged slides.
Tissue section appears torn or folded. Air bubbles under section.	Re-cut sections using a sharp blade, or ignore damaged areas when analyzing the results.
Poor resolution of tissue morphology.	Cut thinner tissue sections. Ice crystals may have destroyed morphology of frozen sections.
Underfixation has physically damaged the tissue or cells during the staining process.	Increase fixation time and/or add a post-fixation step. Increase the fixative/tissue ratio. Cut smaller pieces of tissue for more thorough immersion-fixation.
Autolysis of tissue leading to staining of necrotic debris.	Increase the fixation time, ratio. Consider using cross-linking fixative.

Inappropriate staining

Possible case	Solution
Fixation method is inappropriate for the antigen.	Try a different fixative or increase the fixation time.
Antigen retrieval may be inappropriate for this antigen or tissue.	Try different antigen retrieval conditions.
Electrostatic charge of the antigen has been altered.	Try adjusting the pH or cation concentration of the antibody diluent.
Delay in fixation caused diffusion of the antigen.	Fix tissue promptly. Try a cross-linking fixative rather than organic (alcohol) fixative.

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