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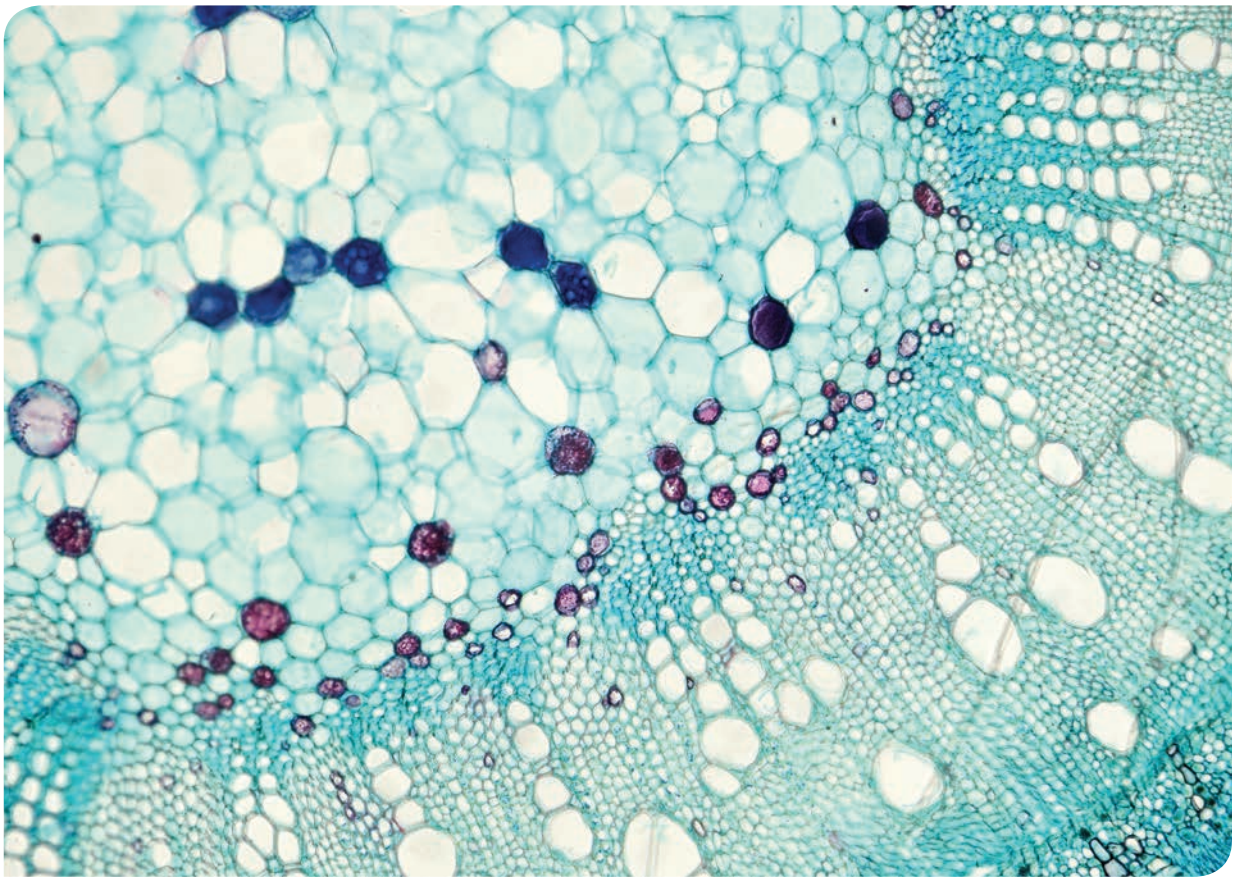

Immunohistochemistry

Tips and Tricks for your IHC-paraffin experiments

In an immunohistochemistry (IHC) experiment a primary antibody binds specifically to a protein of interest present in a tissue. 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.

Since the first IHC experiments in the 1940s much progress has been made in terms of developing different and increasingly sensitive detection systems. In the beginning secondary antibodies conjugated to enzymatic labels were almost exclusively used for visualization, while these days kits, directly conjugated primary antibodies and fluorescent labels are increasing in popularity. This trend is due to researchers wanting to detect several antigens simultaneously in one tissue specimen. This process is known as multiplexing and has many advantages, including cost and time savings and increased sample characterization.

IHC staining of paraffi n-embedded tissues (IHC-P)
Before starting the experiment, make sure you have selected an antibody that has been validated in IHC-P, that you have included controls in your experimental design, and that your microscope is set up to detect the antibody staining.

1. Section and mount samples
2. Heat samples
3. Remove paraffin and rehydrate the tissue
4. Perform heat induced or protease induced epitope retrieval (optional step; depending on tissue and protein of interest)
5. Perform wash
6. Block endogenous peroxidases, phosphatases (for enzymatic labels) and biotin (when using biotin/avidin systems)
7. Perform PBS wash
8. Block non-specifi c binding sites
9. Perform PBS wash
10. Incubate with primary antibody
11. Perform 3 PBS washes
12. Incubate with secondary antibody
13. Perform 3 PBS washes
14. Incubate with amplification reagent
15. Perform 3 PBS washes
16. Incubate with DAB or other development solution (for enzymatic labels only)
17. Perform ddH_2O wash
18. Counterstain
19. Dehydrate tissue sections
20. Mount coverslip

Tips for step 4 – Perform heat induced or protease induced epitope retrieval
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 antigen retrieval/antigen unmasking, which is either mediated by heat (HIER; heat-induced antigen retrieval) or proteases (PIER; proteolytic-induced antigen retrieval). The latter uses enzymes such as proteinase K, trypsin and pepsin. The 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.

In order to establish whether antigen retrieval should be performed and by which method the following guidelines should be followed:

- Perform a literature search to determine how other researchers have visualized your antigen of interest.
- Check if the antibody supplier recommends a specifi c antigen retrieval protocol.
- If no specific protocol is available, we recommend using a HIER rather than a PIER protocol.
- For HIER always start with a neutral antigen retrieval buffer, such as AbD Serotec's BUF025A and compare to a sample for which no antigen retrieval was performed.
- If the neutral staining solution did not yield a good staining, alkaline or acidic antigen retrieval buffers should be tested.

- In addition to pH, other parameters to be optimized are temperature and duration. Ideally try various pH, temperature and time combinations.
- In order to exclude artifacts caused by the HIER process always include a control sample for which staining without the HIER step was performed.

Tips for step 6 – Block endogenous peroxidases, phosphatases and biotin

To avoid staining artifacts it is important:

- To block endogenous peroxidases and phosphatases prior to using alkaline phosphatase (AP) / horseradish peroxidase (HRP) antibody conjugates. For blocking endogenous peroxidase activity AbD Serotec offers ready to use peroxide blocking reagent (BUF017B).
- To block endogenous biotin when using avidin/biotin or streptavidin/biotin detection systems. For this specifi c purpos AbD Serotec offers a ready to use avidin-biotin blocking system (BUF016).

Tips for step 8 – Block non-specific binding sites

Blocking should be performed prior to incubation with the primary antibody to prevent non-specifi c antibody binding.

- Use normal serum from the same species as the one in which the secondary antibody was generated in.
- Block with 10-20% normal serum.
- **Never** block with normal serum from the same species as the primary antibody as this would lead to the blocking of reactive sites.
- If serum is unavailable, use bovine serum albumin, non-fat milk or gelatin.

Tips for step 10 – Incubate with primary antibody

- Check on the manufacturer's datasheet that the antibody has been tested in the specific immunohistochemical method intended to be used (e.g. IHC-paraffi n).
- We recommend using a polyclonal antibody for IHC. 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 defi nite 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.

Tips for step 12 and 14 – Incubate with secondary antibody and amplifi cation reagent

- We recommend the use of directly conjugated antibodies in IHC only for the detection of very abundant target proteins (e.g. beta-actin and alpha-tubulin).
- For medium to low abundant proteins, we recommend using secondary antibodies for detection. We make this recommendation due to the fact that multiple secondary antibodies bind to a single primary antibody thereby leading to amplifi cation of the signal.
- For very low abundant proteins, we suggest using biotinylated secondary antibodies in combination with conjugated avidin/streptavidin. We make this recommendation due to the high signal amplification of this method, which is due to a single avidin molecule being able to simultaneously bind up to four biotin molecules.
- When using biotinylated antibodies, ensure endogenous biotin is blocked prior to primary antibody incubation. For this specific purpose AbD Serotec offers a ready-to-use avidin-biotin blocking system (BUF016).
- For your convenience, we also offer the Histar Detection kit series, STAR3000, which provides linking and labeling reagents for the visualization of cellular antigens in tissue specimens. The Histar Detection kits are biotin-free systems based on a novel polymer-based labeling technology.
- When selecting a fluorochrome conjugated secondary antibody, ensure that your microscope is able to excite and detect the fluorochrome appropriately. This can be done by looking up the excitation and emission data of the fluorochrome of interest and the lasers/filters included in your microscope.
- Select photostable fl uorochromes such as Alexa Fluor® and DyLight Fluor® dyes rather than FITC and PE, which are highly susceptible to fading/photobleaching.
- When performing experiments with multiple fluorescent labels, ensure that each fluorochrome can be spectrally separated and that one fluorochrome does not get detected in another fluorochrome's channel (a process known as bleed-through). For this purpose we recommend mocking up the fluorochrome excitation and emission spectra with the help of a spectrum viewer at the experimental design stage.

Tips for step 16 – Incubate with DAB or other development solution

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. This reaction converts a chemical compound called chromogen into the precipitate (see below formula; Agilent Technologies 2009).

1. Enzyme (E) + Substrate (S) = ES complex (rather transient)

2. ES → E + Product (P)

Factors to consider:

- 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 that the final precipitate colors are spectrally differentiable (for example 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 (see counterstain section).

The most popular chromogens for both HRP and AP and the resulting precipitate colors are shown below (adapted from Agilent Technologies 2009).

HRP substrate	Precipitate color
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 substrate	Precipitate color
Fast Red TR	Bright red
New Fuchsin	Red
Fast Blue BB	Blue

Tips for step 18 – Counterstain

Like controls, counterstaining is crucial for every IHC experiment, as the counterstain provides background contrast and puts the observed staining into perspective (e.g. by visualizing nuclei).

- 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 (e.g. DRAQ5™ should be used as a nuclear counterstain rather than DAPI when using antibodies conjugated to blue emitting fl uorochromes such as Alexa Fluor® 405 or DyLight® 405).
- In the interest of time and for your convenience, we recommend using mounting media that contain counterstains.

The most commonly used chromogenic and fluorescent counterstains are shown below (adapted from Paul 2013).

Chromogenic counterstains	Color	Counterstain for
Hematoxylin (4 types - Harris's, Mayer's, Carazzi's, and Gill's)	Blue	Nucleus
Fast red	Red	Nucleus
Methylene blue	Blue	Nucleus
Methylene green	Blue/green	Nucleus
Toluidine blue	Blue	Nucleus

Fluorescent counterstains	Color	Counterstain for
DAPI	Blue	Nucleus
DRAQ5™	Red	Nucleus
Hoechst 33258/33342	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

Tips for step 20 – Mount coverslip

Mounting media are essential for making permanent slides. They make the specimen adhere to the microscope slide and firmly place it between coverslip and slide. Mounting has the purpose of protecting the specimen from damage while adding contrast during microscopy. Two types of mounting media exist:

1. Aqueous (hydrophobic; examples of aqueous mounting media are glycerol and glycerol jelly)
2. Organic solvent based (hydrophilic; examples of organic solvent based media include Euparal and *Canada Balsam*)
   Mounting media can be further differentiated into media that solidify or stay liquid (Kim). In general organic solvent based media solidify, while aqueous ones remain in a liquid state.

Factors to consider:

- Do not use organic mounting media for fl uorescent labels.
- When using organic mounting media care should be taken as some precipitates, formed as the result of the chromogen substrate reaction, are alcohol soluble. One of those examples is the red precipitate formed as the result of the reaction of horseradish peroxidase (HRP) and 3-Amino-9-ethylcarbazole (AEC) (Renshaw 2007).
- For wide-fi eld microscopy mounting media that solidify should be used.
- For 3D imaging mounting media that stay liquid have to be used (North 2007). When using mounting media that remain liquid, the sides must be sealed with nail polish to prevent the medium from leaking.
- To prevent photobleaching (a process in which the chemical destruction of a fluorochrome results in the loss of fluorescence) slides should be stored in the dark.
- Further bleaching can be prevented by using mounting media containing antifade reagents. However as not every fluorochrome is compatible with every antifade reagent; we recommended to check the manufacturer's datasheet prior to use.

References:

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