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Immunohistochemistry
IHC Handbook

Principle, Troubleshooting, Sample Preparation and Assay Protocols

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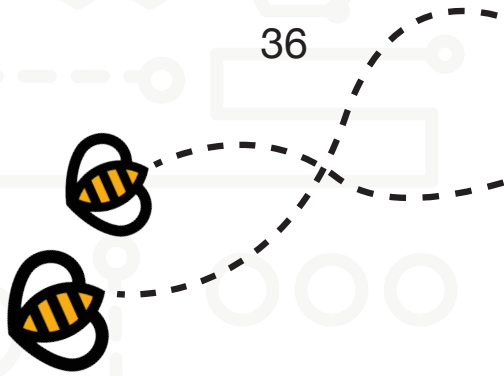


1 Introduction

Immunohistochemistry (IHC) combines biochemical, histological and immunological techniques into a powerful assay for the visualization of protein localization and distribution within cells in tissue. IHC uses specific antibodies raised against antigens in cells of a biological sample for detection.
The antibody-antigen binding can be visualized with different methods: enzymes, such as Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP), are commonly used to catalyze a color reaction. There are also numerous IHC methods that can be used to localize antigens dependent on the specimen types and the assay sensitivity. Although ICH does not provide quantitative data as with such assays as Western Blot (WB) or ELISA, IHC can provide a visual context to explain results obtained in quantitative assays.

A well-known example of demonstrating the power of this method was demonstrated in a diagnostic cancer screen. The assay was developed on the basis of research, revealing that a “tumor suppressor” gene associated with cell cycle regulation is inactivated in many cancers and in turn promotes cell proliferation. IHC and other experimental techniques have demonstrated that, depending on the type of cancer involved, this inactivation can occur via degradation, impaired synthesis or improper localization.
Further, in basic research IHC is used to demonstrate altered localization, processing, trafficking and targeting of a protein of interest within cells and tissues. For example, some proteins translocate between different cellular compartments in response to certain stimuli. Bcl-2, for instance, translocates from the cytoplasm to the nucleus in response to ischemia following a stroke. The nuclear translocation of the protein has been convincingly demonstrated by IHC (in combination with other experiments) before and after cerebral hypoxia when ischemia is induced.

2 IHC experiment design

There are a number of important considerations that must be taken into account and variables that must be optimized in order to consistently obtain robust and reproducible results from IHC experiments. The selection of suitable antibodies, the method of tissue fixation, epitope rescue, blocking procedure, antibody concentrations and suitable controls must all be considered in the design of the experiment.

In the following guide, we address these points and suggest sample protocols to help with the design of IHC experiments.

2.1 Choose the right antibody

Variable		Options	Considerations
Antibody Type	Monoclonal	Advantages • Mouse or rabbit hybridoma • Good consistency	Disadvantages • Lower affinity • False negatives common
	Polyclonal	Advantages • Several different species	Disadvantages • More non-specific reactivity • Affinity may vary between lots
Antibody Target	Entire Molecule	• Depending on location of target antigen e.g., If C-terminus is embedded in a membrane, it is not a good choice for a target.	
	C-Terminus		
	N-Terminus		

Table 1. Factors involved in selecting suitable IHC antibodies.

2.2 Monoclonal vs. Polyclonal antibodies

Monoclonal antibodies are generated from a single B-cell clone from one animal, which produces immunoglobulins specific against a single epitope. These clones are fused to a myeloma cell (B-cell cancer) to create an immortal cell called a hybridoma, which produces large amounts of homogenous antibodies. Monoclonal antibodies are easy to standardize as they show reliable consistency between batches, however the targeting of a single epitope means less cross-reactivity with other proteins which can cause false negative results.

Polyclonal antibodies are created using multiple B-cell clones of one animal, which results in a heterogeneous mix of antibodies that target several epitopes of the same antigen. As a result, polyclonal antibodies are more forgiving of changes in target proteins that can easily result from the fixation process. The fact that they recognize multiple epitopes also means that they can amplify the signal in samples in which the protein of interest is expressed at low levels. This feature also makes polyclonal antibodies the antibodies of choice when dealing with denatured proteins.

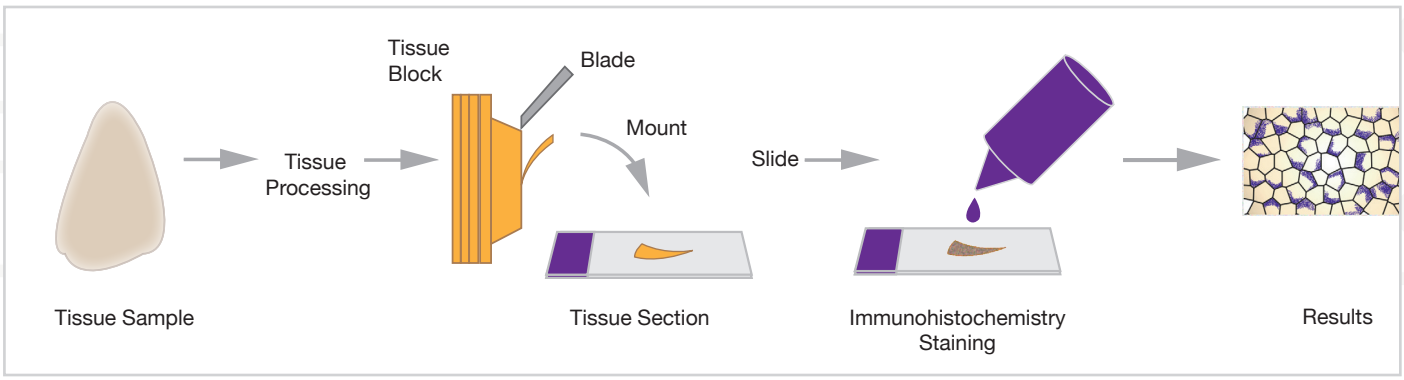
Further, while monoclonal antibodies are generated from mouse and rabbit hybridomas, polyclonal antibodies are raised in several different species including rats, goats, sheep, guinea pigs, chickens and more.
It is important to bear in mind that the primary antibody should be raised in a host specie as different from the source of your samples as possible. Otherwise, IgG secondary antibodies will bind to all the endogenous IgG in the tissue, causing high background signal.

2.3 Region of Targeted Protein

Antibodies are generated by using an array of immunogenic substances to induce an immune response in the host animal. These immunogenic substances may include full-length proteins, protein fragments, peptides and whole organisms such as bacteria or cells. If the target epitope is a protein fragment or a specific isoform or region of a full-length protein, the antibody used must be raised against an epitope that is identical to or contained within the fragment or region. For example, if the target is a membrane protein, the appropriate antibody should target the protein's extracellular domain.
Also some protein families contain a conserved motif consisting of a sequence of amino acids, where small variations within this motif can be exploited to differentiate between subtypes of proteins within the family.

3 Principle

In order to be able to use certain antibodies, samples must be processed or fixed in a very specific manner. Many antibodies only recognize proteins that have been reduced and denatured, while others only detect protein epitopes when the proteins are in their native, folded conformation. Some antibodies can only be used with unfixed, frozen tissue as they cannot bind to their target epitopes in formalin-fixed, paraffin-embedded tissues. However, an antigen retrieval step can be added to reverse the cross-links that result from fixation.
If your protein of interest is located intracellularly, the cells must be permeabilized with solvents or detergents prior to enable the antibodies access to the interior of the cell. Largely, detergents are the most popular method for permeabilizing cells, however, because they disrupt the membrane, detergents are not appropriate for cytoskeletal antigens or low molecular-weight targets such as viral antigens and certain enzymes.



[Figure 1. IHC Workflow from Sample to Results.]

In this handbook, the following topics will be covered:

1. Fixation
2. Tissue Sectioning
3. Paraffin Embedding
4. Inactivation and Blocking
5. Antigen Retrieval
6. Detection
7. Chromogens, Counterstains and Mounting Media

3.1 Fixation

Fixation preserves morphology, prevents tissue degradation, ensures antigenicity and helps to protect cells from damage during the tissue preparation process. The reproducibility and quality of IHC results are highly dependent on fixation times, temperature, pH and buffer composition—all of which must be carefully optimized and standardized for different starting materials.

Selection of Fixing Solution

In the following commonly used fixing solutions are listed, which need to be tested whether a specific type of solution is appropriate for your detected antigen, as there is no standard fixing solution for different kinds of antigen immobilization.

(i) Acetone and Alcohol

Acetone and alcohol are both primary fixing solutions, which play a role in precipitating sugars and fats, as well as maintaining the immunologic competence. Note that alcohol is ineffective in maintaining low molecular weight protein, polypeptide and cytoplasmic proteins. However, alcohol can be mixed with glacial acetic acid, ethyl ether, chloroform and formaldehyde to overcome this downside. Acetone on the other hand is often used for frozen tissue and cytological smears as it has a strong penetrability and dehydration property.

(ii) Aldehyde

Aldehydes are di-functional cross-linking agents, which are widely used due to their strong penetrability, low contractibility and low background. They provide the cross-linking between tissues and maintain the antigen. While formalin (saturated 37% formaldehyde solution) is the most widely used, 4% paraformaldehyde is better than formaldehyde as it's the pure form of formaldehyde and methanol-free. Further, Bouin's solution (containing picric acid) is the most widely used in histology and pathology, whereas Zamboni's solution is applied to light and electron microscopic immunocytochemistry and is more suitable than formaldehyde in maintaining ultra structural organization.

(iii) Non-Aldehyde

Carbodiimide, dimethylacetamide, dimethyl-suberimidate and para-benzoquinone are widely used for the fixation of peptide hormones in tissues and are better mixed with glutaric dialdehyde or paraformaldehyde.

Fixation Methods

(i) Fixation by Perfusion

As fixation can cause cross-linking, which masks epitopes, the best way to preserve tissue morphology and target protein antigenicity is to replace the animal's entire systemic blood volume by fixative with vascular perfusion. This is followed by perfusion with sucrose solution.

(ii) Fixation by Immersion

For the majority of applications, it is not to feasible to perform fixation through perfusion, however, an alternative method is to immerse tissues in fixative solution. Biopsy

and surgical specimens, as well as other non-irrigation tissues commonly employ this fixation method. The fixation duration is generally determined by the antigen stability and the type of fixing solution used.

(iii) Snap Freezing

For cases in which standard fixation techniques cannot be used or fixation will be carried out later, tissue can be snap frozen and kept at -70°Cuntil use. For example, phosphorylation-dependent epitopes have been shown to translocate from the membrane to the cytoplasm following fixation with formaldehyde.

Exercise caution when fixating tissues

- Do not over-fix the tissues
- Store tissues appropriately after fixation
- Use adequate amounts of fixation solution and wash sufficiently after fixation
- Reduce the tissue sample size to less than 2cm×1.5cm×0.3cm (Thickness < 0.3 cm), if possible

3.2 Tissue Sectioning

(A) Slide Pre-Treatment

The following pre-treatment is designed to prevent dislocation of the sample from object mount due to elevated temperature, high pressure, radiation or other factors.

(i) Microscopic Slide

In order to remove any oil residues attached to the surface of a glass slide, it should be immersed in cleaning solution for 12 to 24 hours. After washing the slide thoroughly with distilled water, dip it into 95% alcohol for 2 hours and dry by wiping with lens paper or in an infrared oven. Avoid scratching the slide during the whole procedure.

(ii) Cell Samples

(a) Adherent Cells

Cell Climbing: adherent cells are grown on multi-aperture culture plates with coverslips*, culture vessels or chamber slides. The coverslip pre-treatment procedure should be carried out as for microcopy slides except that dipping and cleaning can be completed in 2 hours due to the reduced thickness.

Direct Cell Culture: culture adherent cells directly on culture vessels or multi-aperture culture plates

(b) Non-Adherent Cells

Cell Smear: use chemical bond to adhere the cells on a

Eccentric Cell Smears: adhere the non-adherent cells to culture vessels by cell micro-centrifugation

(iii) Tissue Section Mounting

For this application, coat the microscopic slide with 3-Amino Propyl Tri-ethoxy Si-lane (APES) in acetone and adhere the tissue by adding a drop of mounting medium (Glycerol, Gelatin). Further, incubate the coverslip at 45°, allowing the drop to spread along the edge of the slip and slowly cover the tissue entirely with the coverslip If necessary, incubate the slide at 80°C for 1 hour.

(B) Tissue Section Types

Tissue samples are typically taken from specimens of various sources: biopsy, sur-gery, animal model and autopsy. Autopsy samples are taken after an animal has died and postmortem autolysis has set in. As antigens may denature, disappear and dif-fuse, autopsy specimen especially should be fixated as soon as possible to avoid degradation of the antigen.

(i) Frozen

The most important feature for this type of tissue section is to keep the antigen's immune-competence intact, especially for cell surface antigens. Both, fresh and fixed tissues can be processed frozen. However, the tissues must be dried (or primary fixed) and stored at low temperature.

(ii) Paraffin-embedded

Paraffin-embedded tissue is usually sliced at a thickness of 2-7 µm using a mi-crotome. With appropriate staining, the section reveals clear tissue structure and exact antigen location for high-resolution microscopy. This type of section can be stored at 4°C for long-term use.

Take note when collecting, fixating and sectioning the samples:

- Use a sharp or scissors to avoid extrusion damage
- The microtome cutter should be flat, small and thin (Normal size is 1.0 cm × 1.0 cm × 0.2 cm)
- Eliminate fat tissue and calcifications
- Include normal tissue as control if necessary
- Prepare paraffin-embedded tissue or frozen tissue immediately after sectioning or store the tissues in liquid nitrogen or at -70°C

3.3 Paraffin Embedding

The process of paraffin embedding involves fixation, dehydration, transparentizing, immersion and embedding.

(A)Fixation

Please refer to the fixation section described above (3.1 Fixation).

(B) Dehydration

The dehydration process generally removes water and hardens the tissue. The dehydrating agents describes below are water-soluble and can be prepared in different volumetric ratios.

(i) Ethanol

Ethanol is the most commonly used dehydrating agent, achieving strong water separation and tissue hardening. However, since ethanol has strong penetration and contractility, its concentration should be progressively increased to avoid excessive shrinking of the tissue.

(ii) Acetone

As a usual substitute for alcohol, acetone acts as both a fixation and dehydration agent. Note that the dehydration time with acetone has to be adjusted for each tissue type, as it tends to over harden tissues.

(C) Transparentizing

After dehydration, the tissue of interest requires a transparentizing step because the dehydrating agent used in the previous step is immiscible with the paraffin in the following steps. The addition of transparent reagent helps the paraffin absorb into the tissue. Common transparent reagents are:

(i) Xylene

As the most widely used transparent reagent, xylene is miscible with both ethanol and acetone, and it acts as a fusing agent for paraffin wax. Since xylene has a strong and fast contractility to tissue, the tissue should not be immersed for an extended period of time or it will become brittle and too hard.

(ii) Benzene and Toluene

These reagents are similar to xylene. However, they have weak and slow contractility to tissue, and therefore the tissue can be immersed in these reagents for a longer time. Note that benzene and toluene show high toxicity and must be handled with care.

(iii) Chloroform

Compared to xylene, benzene and toluene, chloroform is a much gentler reagent. However, it has a small refractive index, and the tissue should thus be immersed in chloroform for a longer time than the other transparent agents in order to achieve complete penetration.

(iv) Cedar Oil

Due to minimal clarification created by cedar oil, it is an appropriate transparent agent for fine and soft tissues. Moreover, particularly hard, dense and fibrous tissues are easier to section after immersing in cedar oil. However, this oil is not useful for other common tissue sections due to its high concentration and weak penetrability.

(D) Immersion

After transparentizing, the tissue can be immersed in molten paraffin wax allowing the absorption of the wax-substituting transparent agent. Based upon the melting point of wax, immersion should be performed at 54-64°C.

(E) Embedding

During the process of embedding, the paraffin-infiltrated samples from the previous step are individually enclosed in mesh cassettes, with a specific orientation in the middle of the block for subsequent sectioning. Following, the cassette is filled with molten paraffin and left to cool down. After cooling is completed, the tissue will be ready for sectioning and suitable for storage.

The treatment conditions (using ethanol and xylene as an example) are shown in the table below.

Step	Reagent	Time
1	75% Ethanol	30 min to 2 h
2	85% Ethanol	30 min to 2 h
3	95% Ethanol	2 h
4	95% Ethanol	2 h
5	95% Ethanol	2 h
6	100% Ethanol	30 mins to 1 h
7	100% Ethanol	30 mins to 1 h
8	100% Ethanol	30 mins to 1 h
9	Xylene	15 min
10	Xylene	15 min
11	Xylene	15 min
12	Paraffin Wax	30 min
13	Paraffin Wax	1 h to 2 h
14	Paraffin Wax	1 h to 2 h

Table 2. Paraffin Embedding Treatment Conditions

3.4 Inactivation and Blocking

(A) Inactivation

The activity of horseradish peroxidase (HRP) or alkaline-phosphatase (AP) used for signal visualization requires blocking or inhibition of endogenous enzymes to avoid non-specific binding and hence artifactual signal.

(i) Endogenous HRP Inactivation

- Incubate the paraffin embedded section in 3% H2O2 for 10 min
- Incubate the frozen section or cell section in solution composed of methanol and 3% H2O2 (v/v:4:1) for 30 min

(ii) Endogenous AP Inactivation

- Incubate the sample section in 0.1mM Levamisole
  (Note: Levamisole cannot inhibit the AP activation of endogenous enzymes in testine tissue)
- Incubate the frozen section or cell section in solution composed of methanol and 3% H2O2 (v/v:4:1) for 30 min

(ii) Endogenous AP Inactivation

- Incubate the sample section in 0.1mM Levamisole

(Note: Levamisole cannot inhibit the AP activation of endogenous enzymes in testine tissue)

(B) Blocking

Residual sites on the tissue section may bind to secondary antibody and produce follow-up false positive results. Therefore, serum from the same species as the secondary antibody is commonly used for blocking those sites. The blocking should be carried out at room temperature for 10-30 min (avoid excessive blocking).

3.5 Antigen Retrieval

Formaldehyde fixation usually generates methylene bridges which cross-link proteins and therefore mask the epitope of interest. It is essential to recover the antibody epitopes in order to allow the antibodies to bind, either by heat (Heat Induced Epitope Retrieval: HIER) or enzymatic digestion (Proteolytic Induced Epitope Retrieval: PIER). To find the optimal antigen recovery method, we suggest to test both HIER and PIER methods, compare their results and optimize the method as needed.

(A) HIER

The HIER method can be implemented by microwave, high pressure or water bath. It breaks the methylene bridges and exposes the epitopes to allow the antibodies to bind by continuous heating. Comparing to PIER, HIER has a gentler experimental condition in which users have more control over the experimental parameters. However, the pH and buffers for HIER must be optimized.

The following antigen retrieval reagents are required:

- M citrate buffer solution (pH 6.0)
- 0.01 MPBS buffer (pH7.0)
- 0.05 M EDTA (pH 8.0)
- 0.05 M Tris-EDTA (pH 9.0)
- 0.05 M Tris-HCl (pH1~12)

(i) Microwave Method

- Place the sample section in a microwaveable vessel with antigen retrieval reagent
- Place the vessel inside a microwave oven and heat up the sample for 5-20 min

(ii) High Pressure Method

- Place the sample section in an appropriate vessel with antigen retrieval
- Place the vessel inside a pressure cooker and heat the sample until it boils
- Once boiling starts, turn off the cooker after the sample is allowed to reach full pressure for 1-4 min

(iii) Water Bath Method

- Place the sample section in an appropriate vessel with antigen retrieval reagent
- Place the vessel and thermometer inside a water bath chamber and heat the sample to 92°C in the chamber
- Remove the sample from the chamber after it is heated at 92°C for 20-40 min

Notes

- The temperature and time should be properly controlled for the antigen retrieval methods described above.
- To avoid original protein structure restoring, do not cool the sample section by taking it out of the buffer solution.
- The higher the temperature, the shorter the heating time (and vice versa).

(B) PIER

Epitopes can be exposed by incubation with proteases, which enzymatically digest the methylene bridges. The choice for digestion enzymes depends on the antigenic components. Pepsin and bromelin are used for retrieving antigens in intercellular

(B) PIER

Epitopes can be exposed by incubation with proteases, which enzymatically digest the methylene bridges. The choice for digestion enzymes depends on the antigenic components. Pepsin and bromelin are used for retrieving antigens in intercellular substance. Other enzymes can be used for intracellular antigen exposure. PIER is suitable for retrieving more difficult epitopes, while the pH for incubation is usually known. However, PIER is a harsher method and can damage tissue morphology.

Enzyme	Working Concentration	Digestion Concentration
Trypsin	0.05% to 0.1%	37°C (10 to 40 min)*
Proteinase K	20 µg/mL	37°C (20 min)
Pepsin	0.4%	37°C (30 to 180 min)

Table 3. Digestion Enzymes for PIER.

* The reaction time can be increased for certain worn-out tissues. Fresh trypsin solution should be prepared with pH adjusted to 7.6 and used at 37°C.

3.6 Detection

IHC detection methods vary and are based on the nature of analyze reporting and binding chemistry, among other factors. Three methods are described here: immunofluorescence (IF), enzymatic and affinity.

(A) Immunofluorescence Method

	λex (nm)	λem (nm)	Color
AO	405	530 - 640	Yellowish (Green - Orange)
DAPI	358	461	Blue
EB	488	610	Red
PI	488	620	Red
Hoechst 33258	352	461	Blue
Hoechst 33342	352	461	Blue

Table 4: Common Fluorochromes for Nuclear Staining. Fluorochrome

This technique is used for the rapid identification of an antigen by exposing it to known antibodies labeled with the fluorescent dye (i.e., fluorochrome), which produces light when excited by a laser (e.g. argon-ion laser). Specific antibody binding can be determined by the production of characteristic visible light and detected by a fluorescence microscope. Tables 1 and 2 show some of the common fluorochromes and their corresponding excitation (λex) and emission wavelengths (λem) for nuclear staining and IF, respectively.

	λex (nm)	**λem (nm)**	**Color**
Alexa 488	488	497 to 643	Green
Alexa 546	530/545	610/675	Red
Alexa 647	650	668	Red
APC	650	660	Red
B-PE	546, 565	575	Orange, Red
Cy3	554	570	Red
FITC	495	525	Green
RB200	570	596	Orange
R-PE	480, 546, 565	578	Orange, Red
Texas Red	596	620	Red
TRITC	552	570	Red

Table 5: Common Fluorochromes for IF Labeling.

(B) Fluorochrome

(i) Principle

The indirect staining process involves three steps

- Primary antibody binds specifically to a target antigen
- Secondary antibody labeled with fluorophore binds to primary antibody
- Fluorophore is detected via microscopy

(ii) Tips: Operations of Fluorescence Microscope

- Operate the microscope according to the manual
- Turn on the mercury lamp for 5-15 min to stabilize the light source before use
- Intensity of high pressure mercury lamp will drop if the lamp is used for more than 90 min (typically, the lamp is continuously used for1-2 hours)
- Photo-bleaching occurs if the sample is illuminated by high pressure mercury lamp for more than 3 min (Note: the sample is generally observed within one hour after fluorescence staining)
- Observe the samples intensively to save time as the light source is limited
- Re-start the light source after turning it off for 30 min or longer

(iii) Counterstaining and Stained Sample Storage

(a) Nuclear Counterstaining

After the fluorescence staining, a counterstain should be carried out to visualize the overall structure of cells and tissues.

Some of the counterstaining fluorochromes are:

- DAPI: classic blue counterstain which is used extensively for nucleus and chromosome staining (DAPI binds selectively to dsDNA without background staining in cytoplasm; DAPI has semi-permeability to living cells and can be used to stain fixed cells and/or tissue sections)
- Hoechst 33342: primary counterstain which is used against yellow fluorescence
- Propidium iodide: primary counterstain which is used for nucleus and chromosome staining against yellow/red fluorescence

(b) Stained Sample Storage

Following the staining, the samples should be observed and imaged immediately under a fluorescence microscope. If the imaging cannot be carried out immediately, the samples can be mounted in buffered glycerol medium and stored at 4°C for less one week. If anti-fading medium is applied to the sample, fluorescence signal may not decay significantly within one month.

(B) Enzymatic Method

The antigen of interest is detected with a specific antibody, which is labeled with an enzyme. The enzyme label later reacts with a substrate to yield a colored product. The enzymatic technique was developed with a similar principle to the IF technique, but they differ as an enzyme is used to label the antibody for the enzymatic method. The advantages of enzymatic IHC over IF IHC are:

- A fluorescence microscope is not required
- Accurate antigen location is enabled with better contrast ratio
- Stained samples can be stored for a long time
- Haematoxylin can be used as a counterstain, which enhances study of tissue morphology
- The colorimetric signal can be easily identified and observed by light microscopy
- Double or multiple stains can be implemented

(i) Labeled-Enzyme Antibody

For this method, the antibody used for antigen detection has been labeled with the enzyme before the reaction. After reacting with the targeted antigen, the labeled antigen forms an antigen-antibody complex where the enzyme catalyzes a substrate to yield an insoluble colored product. Subsequently, the product can be analyzed with microscopy or electron microscopy. The labeled-enzyme approach can be done by direct or indirect detections.

[**Figure 4**. Direct vs. Indirect IHC Detection Methods.]

(a) Direct Detection

The direct method is a one-step staining method, which involves a labeled antibody (e.g. HRP-conjugated antibody) reacting directly with the antigen of interest. The antigen-antibody-HRP complex is then able to react with a DAB substrate for staining. While the direct method is simple, rapid and highly specific, it has low sensitivity and a limited range of primary antibodies that are directly labeled. Despite the shortcomings, the direct method is commonly applied to screen monoclonal antibodies before the large-scale manufacturing process.

(b) Indirect Detection

The indirect method is a two-step process, which involves an unlabeled primary antibody that binds to the target antigen in the sample and an enzyme-labeled secondary antibody that reacts with the primary antibody. The secondary antibody must be raised against the IgG of the animal species in which the primary antibody has been raised. For instance, if the primary antibody is rabbit anti-human IgG, the enzyme labeled secondary antibody could be goat anti-rabbit IgG.

Comparing to the direct detection, the indirect detection has numerous advantages. First of all, only a relatively small number of standard conjugated secondary antibodies are needed for the indirect method. For example, a labeled secondary antibody raised against rabbit IgG, which can be purchased "off the shelf," is useful with any primary antibody raised in rabbit. With the direct method, it would be necessary to label each primary antibody for every antigen of interest. Secondly, the indirect method has greater assay sensitivity. Moreover, various kinds of controls could be designed and applied with indirect detection.

(ii) Unlabeled-Enzyme Antibody

(a) Enzyme Bridge Method

This method is based on the binding of an enzyme label to a target antigen through the antigen-antibody reactions of an immunoglobulin-enzyme bridge which consists of the following components in order:

- Specific antiserum for the tissue antigen (AnTAn)
- Antiserum against the immune globulin of the species for AnTAn
- Specific antiserum prepared against the enzyme label in the same species as AnTAn
- Enzyme label

(b) Peroxidase-Anti-Peroxidase (PAP)Method

This method involves immunization of a rabbit/goat/rat antibody with a HRP component to produce an anti-HRP rabbit/goat/rat antibody, which would then bind to another HRP part to form a stable polygon. The PAP approach excels due to its high sensitivity and low background for tissue staining.

(C) Affinity Method

The IHC sensitivity can be improved by employing a higher number of enzyme molecules bound to the tissue. In this regard, the multiple binding sites between the avidin and biotinylated antibodies have been exploited for IHC signal amplification. Avidin, an egg white protein, has four binding sites for the low-molecular-weight vitamin biotin to form a large lattice-like complex. Beside avidin, there are other methods that employ streptavidin, which is a tetrameric biotin-binding protein that is isolated from Streptomyces avidinii. The avidin and streptavidin methods work almost identically, as their structures are very similar. Avidin-Biotin Peroxidase Complex (ABC) and Labeled Streptavidin Binding (LSB) are the two most widely used affinity methods for amplifying the target antigen signal.

(i) ABC

[**Figure 5**. ABC Detection Method.]

The method involves four sequential steps:

- Incubation of primary antibody with tissue sample to allow binding to target antigen
- Incubation of biotinylated secondary antibody (which has specificity against primary antibody) with tissue sample to allow binding to primary antibody
- Pre-incubation of biotinylated enzyme (HRP or AP) with free avidin to form large ABC complexes (biotinylated enzyme and avidin are mixed together in a pre-determined ratio to prevent avidin saturation)
- Incubation of the above pre-incubated solution to tissue sample

(ii) LSB

[**Figure 6**. LSB Detection Method.]

This method uses an enzyme-labeled streptavidin to detect the bound biotinylated primary antibody on the tissue section. It can also be applied if the complex in the ABC method is too big for tissue penetration. Due to its smaller size, the enzyme-labeled streptavidin is used to enable tissue penetration. The LSB method can be

employed to replace the ABC method for the former's ability to improve sensitivity and reduce signal further. The information below describes the general staining procedure.

- Incubation of primary antibody with tissue sample to allow binding to target antigen
- Incubation of biotinylated secondary antibody (which has specificity against primary antibody) with tissue sample to allow binding to primary antibody
- Incubation of streptavidin-enzyme conjugate to tissue sample

3.7 Chromogens, Counterstains and Mounting Media

(A) Chromogens for HRP

(i) DAB

DAB (3,3'-Diaminobenzidine) is typically used as a signal enhancer in conjunction with the HRP-based immunostaining systems. The dark brown product derives from DAB is insoluble in water and alcohol, stable and suitable for long-term storage. In addition, the staining product could be observed with light microscopy or processed with OsO4 for observation with electron microscopy. Haematoxylin, methyl green and methyl blue are the suitable counterstains. Since DAB may cause skin and bladder cancers, it is necessary to use personal protective equipment to avoid contact with skin or mucosa.

(ii) AEC

After staining with AEC (3-Amino-9-Ethylcarbazole), the positive area on tissue section changes to dark red. The product derived from AEC is soluble in organic solvent and cannot be stored on a long-term basis. Similar to DAB, haematoxylin, methyl green and methyl blue are some of the suitable counterstains for AEC. Glycerin gelatin should be used as the AEC mounting medium.

(B) Chromogens for AP

(i) BCIP/NBT

Used in conjunction, BCIP (5-Bromo-4-Chloro-3-Indolyl-Phosphate)/ NBT (Nitro Blue Tetrazolium) is a widely accepted chromogenic substrate used in the AP-based immunostaining systems. After exposing to AP, the substrate changes to a blue/ violet

stain. The staining product derived from BCIP/NBT is insoluble in alcohol. Nuclear fast red and brilliant green are the suitable counterstains for BCIP/NBT.

(ii) Fast Red TR Salt

Fast Red is also used for the colorimetric detection of AP. Its product has a rose color and is soluble in alcohol. Methyl green, brilliant green and soluble haematoxylin are used for the Fast Red chromogen counterstains.

(C) Counterstains

After staining the target antigen by IHC, a secondary stain is usually applied to provide information about the overall morphology of the sample. While many of these stains show specificity for discrete antigens or cellular compartments, other stains will deliver the staining of a whole cell. Some of the most common counterstains are described as follows:

(i) Haematoxylin

Haematoxylin, a natural dye, extracted from the heartwood of the logwood tree, is used for cell nucleus staining. Differentiation refers to the process of using reagents (e.g. 1% hydrochloric acid HCl and alcohol) to remove the color caused by over-straining or non-specific staining on sample tissues. After running nuclear staining (in aluminum haematoxylin) and differentiation (in HCl and alcohol), the tissue section is transferred from an acid solution to an alkaline solution (e.g. ammonia water and disodium hydrogen phosphate solution). During this process, the section will change from red brown into blue, which is known as bluing.

Haematoxylin is sub-categorized into Mayer's Haematoxylin and Harris Haematoxylin.

(a) Mayer's Haematoxylin is reddish violet and is valued for several properties: low staining time, no perception and metal membrane as well as no post-staining differentiation.

(b) Harris Haematoxylin is purple red, widely used in H&E staining and has these advantages: fast staining, bright color, clear nuclear stains and well defined tissue morphology. Although metallic oxide may float on the Harris haematoxylin solution after a long period of time, filtering is unnecessary before use as no precipitation will appear. Differentiation and bluing should be carried out after staining with Harris Haematoxylin.

(ii) Methyl Green

Methyl green consists of metallic green microcrystalsor bright green powders. It becomes bluish green when dissolved in water. This basic dye can be easily bounded with highly polymerized DNA and changes the nucleus to green. Counterstain with methyl green takes 2 to 5 min which should be followed by washing the sample, dehydration and mounting.

(iii) Nuclear Fast Red

This counterstain will change the nucleus to red after applying to the tissue section for 2 to 5 min.

(D) Mounting Media

A mounting medium may be used to attach a coverslip or may be used to replace the coverslip. Generally, the medium selection depends on a few factors including the chemical compatibility with chromogens and counterstains, as well as the preservation period.

(i) Neutral Mounting Medium

It usually refers to an oily substance with pH 7.0 such as neutral gum (resin). Before mounting, the sample should be treated with dimethylbenzene, transparent and dehydrated for long-term storage sections.

(ii) Water-Soluble Mounting Medium

Popularly used in IF staining for short-term storage sections is 50% glycerol in water. The table below summarizes the choice of mounting medium among different enzymes, chromogens and counterstains.

Enzyme	Chromogen	Counterstain	Mounting Medium
HRP	DAB	Haematoxylin, Methyl Green, Methyl Blue	Neutral
HRP	AEC	Haematoxylin, Methyl Blue	Water Soluble
AP	BCIP/NBT	Nuclear Fast Red, Brilliant Green	Neutral
AP	Fast Red	Haematoxylin, Methyl Green, Brilliant Green	Water Soluble

Table 6. Chromogens, Counterstains and Mounting Media for HRP and AP Enzymatic Systems.

8. Controls

Every IHC experiment must include positive and negative controls. However, several other types of controls can be considered based on the type of experiment carried out.

(A) Positive Controls

A positive control validates the staining of your sample and confirms that the assay is working correctly. It will also help optimize the conditions for future experiments. To identify suitable positive controls, a good starting place is to check the antibody data-sheet and any of these may be used as your positive control. You can also check the Swiss-Prot or Omnigene database links on your antibody's datasheet. The databases often include a list of tissues that express your protein interest, which can be used for positive controls. In addition, the following resources are helpful:

- GeneCards: provides information about relative levels of expression in various tissues
- Human Protein Atlas: the protein detection database comprises different tissue types, cancers and cell lines
- Pubmed: this literature search database may give you ideas as to which tissues and cells express the protein of interest

(B) Negative Controls

Negative controls, on the other hand, reveal non-specific binding and false positive results. Obviously, negative controls should not express your protein of interest. Commonly used negative controls are knock down (KD) or knock out (KO) tissue samples.

(C) No Primary Controls

For this type of control, no primary antibody is added to the sample. These controls are indicative of non-specific binding or false positives that can result from non-specific binding of the secondary antibody. Antibody dilution buffer with no antibody added is incubated on the same sample under identical conditions.

(D) Isotype Controls

Isotype controls are antibodies of the same isotype (e.g. IgG2, IgY), clonality, conjugate and host species as the primary antibody, targeting a molecule that is not present in your sample. The target is generally a chemical or a non-mammalian protein, which is incubated with your samples with the isotype control antibody rather, than the specific primary antibody (the concentrations and experimental conditions between the primary and isotype control antibodies are the same).

(E) Endogenous Control for Transfected Cell Lines

If your experiment involves testing of recombinant protein, it is suggested that endogenous (non-transfected) positive controls are included, in order to validate your results and prove that your antibodies are working. However, the detection of recombinant proteins presents a few challenges that should be considered: first, the recombinant protein may fold differently than the native form, which could prevent the antibody from accessing its target epitope. This is especially common in cases with tagged proteins. With this in mind, tags should always be placed on either the N - or C - terminus of the recombinant protein. Secondly, it is important to make sure that the recombinant protein contains the immunogen sequence for your antibody.

4 Recommended Protocols

Tissue preparation is the key to successful IHC experiments. Since no universal tissue preparation method will be ideal for all sample and tissue types, all protocols given here are intended as a starting point from which the experimenter must optimize the protocol as needed. All conditions should be standardized in order to ensure reproducible results. Keep in mind that the sample tissue must not dry out at any point of the procedure.

4.1 IHC Paraffin Sections

[Figure 7. IHC (Paraffin Sections) Workflow]

(A) Tissue Preparation

(i) Paraformaldehyde Cooling and Dehydration

- Collect fresh tissue and place in ice-cold PBS buffer
- Wash the tissue thoroughly with PBS to remove remaining blood or other remnants (use forceps to remove connective tissues)
- Cut the tissue into slices of thickness of 3 mm or less
- Immerse the slices in 4% paraformaldehyde at room temperature for 8 min
- Replace the 4% paraformaldehyde (pre-cool at 4°C) and incubate for 6 to 7 hrs. The paraformaldehyde volume should be 20X greater than the tissue volume by weight
- Wash the tissue 3X with PBS (1 min each)
- Dehydrate the tissue by immersing the tissue sequentially as follows:

o 1X into 80% ethanol (1 hr at 4°C)
o 1X into 90% ethanol (1 hr at 4°C)
o 3X into 95% ethanol (1 hr each at 4°C)
o 3X into 100% ethanol (1 hr each at 4°C)
o 3X into dimethylbenzene (30 mins each at room temperature)

(ii) Liquid Paraffin Section

- Prepare the first portion of liquid paraffin in a suitable bath and allow the paraffin to reach 60°C
- Immerse the tissue 2X into the paraffin bath (2 hrs each)
- Prepare the second portion of liquid paraffin in a suitable bath and allow the paraffin to reach and maintain at 60°C
- Pour the second portion of paraffin into a mold
- Proceed straight to transferring the tissue from the paraffin bath to the mold with paraffin
- Incubate the tissue at room temperature until it coagulates
- Store the tissue at 4°C

(iii) Section Slicing and Incubation

- Secure the paraffin section on slicer
- Slice one to two pieces off section to adjust the slicer (section and blade must be parallel)

- Slice the remaining section carefully with ~5 µm thickness
- Carefully place the sections on the surface of a waterbath at 40°C to 50°C to unfold
- Mount the tissue section onto Poly-Lysine or APES coated glass slides
- Incubate the slides overnight at 37°C

(B) Dewaxing / Deparaffinization

Sequentially immerse paraffin sections into:

- 90% dimethylbenzene (for 7 min)
- 95% dimethylbenzene (for 7 min)
- 100% dimethylbenzene (for 7 min)
- 90% ethanol (for 7 min)
- 95% ethanol (for 7 min)
- 100% ethanol (for 7 min)
- Wash the slides with water to remove ethanol

Note: The process of dewaxing should be done in a fume hood at room temperature in summer. When the temperature is lower than 18°C, it is recommended to dewax at 50°C.

(C) Inactivation

- Immerse dewaxed paraffin section in 3% H2O2 at room temperature for 10 min
- Wash the section 3X to 5X with distilled water (total 3 to 5 min)

(D) Antigen Retrieval (Heat Induced Epitope Retrieval: HIER)

- Immerse the paraffin sections in citrate buffer
- Heat the buffer in the microwave until it is boiling
- Keep the boiled buffer in microwave for 5 to 10 min
- Repeat the heating as outlined above 1 x to 2 x
- Cool the slide until it reaches room temperature
- Wash the sections 1 x to 2 x with PBS

(E) Blocking

- Add 5% BSA blocking solution or normal goat serum to the HIER treated samples
- Incubate the samples at 37°C for 30 min
- Discard excess liquid (no washing required)

(F) Primary Antibody Incubation

- Dilute the primary antibody with antibody diluent to the concentration recommended by the antibody manufacturer
- Add the diluted antibody to the samples and incubate at 37°C overnight
- Wash the samples 2 x with PBS (20 min each)

(G) Secondary Antibody Incubation

- Dilute the biotinylated secondary antibody with antibody diluent to the concentration recommended by the antibody manufacturer
- Add the diluted antibody to the samples and incubate at 37°C for 30 min
- Wash the samples 2 x with PBS (20 min each)

(H) Staining

- Add Strept-Avidin Biotin Complex (SABC), HRP- or AP-conjugated reagents to the samples
- Incubate the samples at 37°C for 30 min
- Wash the samples 3 x with PBS (20 min each)
- Add a suitable amount of DAB reagent to the samples and incubate in the dark at room temperature for 10 to 30 min
- Monitor the tissue staining intensity under a bright-field microscope
- Wash the samples 3 x to 5 x with distilled water
- Counterstain (if required)
- Add haematoxylin to the sample
- Dehydrate
- Immerse the paraffin sections 2 x in dimethylbenzene (7 min each)
- Check the tissue staining intensity under a bright-field microscope

If there is also a lot of background staining appearing, wash the section 4 x with 0.01-0.02% TWEEN 20 PBS and 2 x with pure PBS after the SABC reaction and before DAB staining. Then use DAB to stain the samples.

4.2 IHC Frozen Sections

[**Figure 8**. IHC (Frozen Sections) Workflow]

(A) Tissue Preparation

(i) Snap Freezing and OCT Embedding

- Collect fresh tissue and place in a dish filled with ice-cold PBS buffer
- Wash the tissue thoroughly with PBS to remove blood (Use forceps to remove connective tissues)
- Cut the tissue into slices of 3 mm or less
- Immediately snap freeze the tissue in iso-pentane cooled in dry ice and keep the tissue at -70°C (Do not allow frozen tissue to thaw before cutting)
- Prior to cryostat sectioning, position the tissue in a mold (which can be simply made by using tin foil) and cover the tissue completely in Optimal Cutting Temperature (OCT) embedding medium
- Use forceps to take the bottom part of mold into liquid nitrogen for 1 to 2 min (the OCT should change to white)

(ii) Cryostat Sectioning

- Pre-cool a slicer box and the detector to -22°C and -24°C, respectively (ensure the blade is smooth)
- Place the tissue from the mold on the detector
- Immediately proceed to slicing the cryostat sections at 5-10 µm and mount them on gelatin-coated histological slides.
- Use coverslips instead of slides to take up the sliced tissue
- Ensure that the cryostat temperature is between -15°C and -23°C; the sections will curl up if the specimen is too cold or stick to the knife if the specimen is too warm.

- Air-dry the sections at room temperature for 30 min to prevent them from detaching from the slides during antibody incubations
- The slides can be stored, even unfixed, for several months at -70°C
- Frozen tissue samples saved for later analysis should be stored intact
- Immediately add 50 µL of ice-cold fixation buffer to each tissue section upon removal from the freezer
- Fix frozen section by immersing it into 4% paraformaldehyde at 2-8°C for 8 min (or ideally at -20°C for 20 min)
- Wash the section 3 x with PBS and allow it to dry at room temperature for 30 min

(B) Inactivation

- Mix H2O2 with distilled water (v/v: 1:50)
- Immerse the frozen section or cell climbing slice in diluted H2O2 at room temperature for 10 min
- Wash the section 3X distilled water (1 min each)

(C) Antigen Retrieval (Proteolytic Induced Epitope Retrieval: PIER)

- Dry the frozen sections with filter paper
- Add compound digestion solution (e.g. Trypsin solution or other enzymatic antigen retrieval solution) to the sections or slices
- Incubate the sections at room temperature for 3 to 5 min
- Wash the sections with 3 x PBS (5 min each)

(D) Blocking

- Add 5% BSA blocking solution or normal goat serum to the PIER treated samples
- Incubate the samples at 37°C for 30 min
- Discard excess liquid (no washing required)

(E) Primary Antibody Incubation

- Dilute the primary antibody with antibody diluent to the concentration r ecommended by the antibody manufacturer
- Add the diluted antibody to the samples and incubate at 37°C overnight
- Wash the samples 2 x with PBS (20 min each)

(F) Secondary Antibody Incubation

- Dilute the biotinylated secondary antibody with antibody diluent o the concentration recommended by the antibody manufacturer
- Add the diluted antibody to the samples and incubate at 37°C for 30 min
- Wash the samples 2 x with PBS (20 min each)

(G) Staining

- Add Strept-Avidin Biotin Complex (SABC), HRP- or AP-conjugated reagents to the samples
- Incubate the samples at 37°C for 30 min
- Wash the samples 3 x with PBS (20 min each)
- Add a suitable amount of DAB reagent to the samples and incubate in the dark at room temperature for 10 - 30 min
- Monitor the tissue staining intensity under a bright-field microscope
- Wash the samples 3 – 5 x with distilled water
- Counterstain (if necessary)
- Add haematoxylin to the sample
- Dehydrate
- Immerse the paraffin sections 2 x in dimethylbenzene (7 min each)
- Check the tissue staining intensity under a bright-field microscope
- If the staining background is too high, wash the section 4 x with 0.01-0.02% TWEEN 20 PBS and 2 x with pure PBS after the SABC reaction and before DAB staining. Then use DAB to stain the samples.

4.3 ICC/IF Cell Climbing Slices

[**Figure 9**. ICC/IF Workflow]

(A) Cell Climbing Slice Preparation

- Place a coverslip in a culture bottle or perforated plate
- Take out coverslip after cell growth has reached 60%
- Wash the coverslip 3 x with PBS to remove the culture medium
- Immerse the coverslip (cells face up) into cold acetone or 4% paraformaldehyde or neutral formalin for 10 to 20 min (Close the lid to prevent evaporation)
- Wash the coverslip 3 x with PBS
- Put the coverslip on filter paper (cells face up)
- Remove the liquid on the coverslip and allow it to dry for 8-10 hrs
- To thaw the slice, wash with neutral PBS at room temperature for 10-15 min (the cell climbing slice can be stored in gelatin at -20°C for one week.)

Note: This fixation procedure using paraformaldehyde and formalin fixatives may cause autofluorescence in the green spectrum. In this case, you may try fluorophores in the (i) red range or (ii) infrared range if there is an infrared detection system available.

(B) Inactivation

- Mix H2O2 with distilled water (v/v: 1:50)
- Immerse the frozen section or cell climbing slice in the diluted H2O2 at room temperature for 10 min
- Wash the section 3 x distilled water (1 min each)

(C) Antigen Retrieval (Proteolytic Induced Epitope Retrieval: PIER)

- Dry the cell slices with filter paper
- Add compound digestion solution (e.g. Trypsin solution or other enzymatic antigen retrieval solution) to the slices (we recommend the addition of 0.1% Triton to the samples before the digestion, as this reduces surface tension and allows reagents to easily cover the entire sample)
- Incubate the slices at room temperature for 10 min
- Wash with 3 x PBS (10 min each)

(D) Blocking

- Add 5% BSA blocking solution or normal goat serum to the PIER treated samples
- Incubate the samples at 37°C for 30 min
- Decant excess liquid and dry the samples with filter paper (no washing required)

(E) Primary Antibody Incubation

- Dilute the primary antibody with antibody diluent to the concentration recommended by the antibody manufacturer
- Add the diluted antibody (Recommended concentration: 0.4 µg to 2 µg) to the samples and incubate at 4°C overnight
- Wash the samples 3 x with PBS (15 min each)

(F) Secondary Antibody Incubation

- Dilute the biotinylated secondary antibody with antibody diluent to the concentration recommended by the antibody manufacturer
- Add the diluted antibody to the samples and incubate at 37°C for 30 min
- Wash the samples 3 x with PBS (8 min each)

(G) Staining

- Add Strept-Avidin Biotin Complex – Fluorescence Iso-Thio-Cyanate (SABC-FITC) or Strept-Avidin Biotin Complex – Cyanine-3 (SABC-Cy3) reagents to the samples
- Incubate the samples in the dark at 37°C for 30 min
- Wash the samples with 2 x with PBS for 2 hrs in total
- Seal the slices with water soluble sealing reagent
- Monitor the staining intensity under a fluorescence microscope
- Counterstain by adding DAPI staining solution to the sample
- Check the staining intensity under a fluorescence microscope
- For slide storage without significant decay in fluorescence signal, add 20 µL of anti-fade solution to the sample followed by a cover glass (avoid bubbles)

5 Troubleshooting Guide

The following guide serves as a checklist for the possible causes and solutions with respect to some of the most commonly encountered problems from the IHC assays.

1. No or Weak Staining

	Possible cause	Solution to the problem
1	Slides lose signal over time during storage	- Prepare slides with fresh tissue - Store slides at 4°C - Do not bake slides before storage sections vs. frozen samples) - Perform Western blot in both its native and denatured forms to ensure that the antibody detects the native form
2	The antibody used is not suitable for IHC procedures which detect proteins in its native conformation	- Check the antibody datasheet to make sure that it has been validated for IHC applications - Check the antibody is applicable to the right IHC samples (paraffin sections vs. frozen samples) - Perform Western blot in both its native and denatured forms to ensure that the antibody detects the native form
3	Fixation procedures (using formalin and paraformaldehyde fixatives) have masked the epitope that the antibody recognizes	- Use different antigen retrieval methods to unmask the epitope (HIER or PIER) - Fix the sections in a shorter time
4	The primary and/or secondary antibody have lost its activity due to improper storage, dilution or excessive freezing and thawing	- Run positive controls to ensure that the primary and/or secondary antibody is working properly - Store the antibodies according to manufacturer instructions - Avoid contamination and light on antibodies

	Possible cause	Solution to the problem
5	Insufficient deparaffinization	- Increase the deparaffinization time - Use fresh dimethylbenzene
6	The protein is located in the nucleus and the antibody cannot penetrate the nucleus	Add a permeabilizing agent (e.g. Triton X) to the blocking buffer and antibody dilution
7	The PBS buffer is contaminated with bacteria that damage the phosphate groups on the protein of interest	- Add 0.01% azide to the PBS antibody storage buffer - Use fresh sterile PBS
8	The primary antibody and the secondary antibody are not compatible	- Use a secondary antibody raised against the species in which the primary was raised (e.g. if the primary antibody was raised in mouse, an anti-mouse secondary antibody should be used) - Check that the isotypes of the primary and secondary antibody are compatible
9	The protein is not present in the tissue of interest or is not sufficiently expressed	- Run positive controls to ensure that target protein is present in the tissue - Include an amplification step in your protocol - Use a higher antibody concentration
10	Insufficient antibody to detect the protein of interest	- Use a higher antibody concentration - Incubate for a longer time (e.g. over night at 4°C)
11	Tissues dry out	Cover the tissues in liquid at all time during the experiment

2. High Background

	Possible cause	Solution to the problem
1	The blocking serum is incorrect	Make sure to block according to the provided protocol
2	Blocking is insufficient (Do not over-block the tissue because antigenic sites may be masked)	Increase blocking incubation period Change blocking reagent: (a) For sections: 10% normal serum (1 hr) (b) For cell cultures: 1-5% BSA (30 mins)
3	The primary antibody concentration is too high	- Titrate the antibody to determine the optimal concentration - Incubate at 4°C
4	Non-specific binding by secondary antibody	- Run a secondary control without primary antibody: If you see staining with your secondary only: (a) Change your secondary antibody or (b) Use secondary antibody that has been pre-adsorbed against the immunoglobulin of the species from which your samples were obtained. - Block your sample with serum from the same species as the host in which the secondary antibody was raised
5	Endogenous peroxide or phosphatase is active	Quench the endogenous peroxidase or phosphatase activity by enzyme inhibitors: (a) Peroxidase: H2O2 and methanol (v/v: 0.3%:99.7%) (b) Phosphatase: 2 mM Levamisol
6	Too much amplification (Refer to solution #8 from no/ weak staining)	- Reduce amplification incubation time - Dilute the secondary antibody

	Possible cause	Solution to the problem
7	Too much substrate was applied (enzymatic detection)	- Further dilute the substrate - Reduce substrate incubation time - Choose substrate of higher S/N ratio e.g. Metal-enhanced DAB
8	Tissue section is not thin enough for reagent penetration	Prepare thinner sections
9	Incubation temperature is too high	Incubate samples at 4°C
10	Primary antibody was raised in the same species as source of tissue (therefore, secondary antibody recognizes and binds to everywhere on the entire tissue because it was raised against that species)	- Use primary antibody raised against a species which is different from the source of tissue - Use biotinylated primary antibody and conjugated streptavidin for the detection system
11	Secondary antibody binds endogenous IgG	Include control slide stained without the primary antibody to confirm whether the secondary antibody is the source of the background
12	Fixation reagents are still present (Due to insufficient tissue washing)	Wash the tissues extensively with PBS buffer
13	Reaction between chromogens and PBS buffer in tissue or cell samples	Before incubating with the substrate, use Tris buffer to wash the samples
14	Membrane damage by permeabilization	- Use a less stringent detergent such as Tween 20 (instead of Triton X) - Remove permeabilizing agent from your buffers

	Possible cause	Solution to the problem
15	Insufficient deparaffinization	- Increase the deparaffinization time - Use fresh dimethylbenzene
16	High levels of endogenous biotin in biotin-based detection systems for samples (e.g. liver and kidneytissues)	- Perform biotin block after normal blocking procedure (before primary antibody incubation) - Use polymer-based detection
17	Use of polyclonal primary antibody	Use monoclonal primary antibody to reduce cross-reactivity

6 FAQs

1. What is immunohistochemistry?

Immunohistochemistry (IHC) is the identification and localization of antigens or proteins in tissue preparations through the use of antibodies labeled with enzymes or fluorescent dyes. The antibody-antigen complexes are generally visualized after a reaction step with a substrate that produces a calorimetric result or with specialized imaging equipment to detect fluorescence.

2. How do you determine the starting IHC antibody concentrations?

Optimal antibody concentration will give you good staining with minimum background. It is generally a good idea to conduct a titration experiment with new antibodies using a range of concentrations that includes the manufacturer's suggested dilution. For example, if the manufacturer suggests a dilution of 1:100, you might also test 1:50, 1:200, 1:500 and 1:1000. This should help you determine the ideal dilution for your conditions.

For antibodies with no suggested dilution, it is advisable to begin with titration experiments in the following ranges based on the antibody format:

- Tissue culture supernatant: neat to 1/10
- Ascites: 1/100
- Whole anti-serum: 1/50 to 1/100
- Purified antibody: 5 µg/mL

3. What is the best way to mount slides?

Tissue sections should be mounted on positively charged or APES (Amino-Propyl-Triethoxy-Silane) coated slides. After mounting the sections, the slides should be left at room temperature overnight to allow water that may be trapped under the section to dry out. In the event that tissue sections adhere to the slide, it may be incubated at 60°C for a few hours.

4. How should IHC slides be stored?

Slides with paraffin-embedded tissue sections can be kept for up to 3 years at 2-8°C, depending on the antigen in question. Frozen tissue sections from snap-frozen tissue blocks should be carefully wrapped in aluminum foil and stored at -20°C or lower for up to six months.

5. What is the stability of IHC slides after staining?

This depends on your fixation and mounting protocols as well as the type of stain used. For example, slides stained using chromogenic methods are stable for years, whereas those using immune-fluorescent methods are stable on the order of months after staining if held at 4°C and protected from light. The longevity of fluorescent stains can be improved by using a fluorescence-protective medium and sealing the preparation from air with nail polish.
Additionally, adding an antioxidant to the mounting medium can prevent photo-bleaching due to reactive oxygen species (ROS).

6. Should I use frozen or paraffin sections?

Sample preparation goes hand-in-hand with the fixation method, which, in turn, is influenced by the detection technique (i.e., fluorescence vs. chromogenic). Generally, tissue that has been immersion-fixed in formaldehyde must be paraffin-embedded and then cut by a microtome. However, formaldehyde fixation is not appropriate with phosphorylation-dependent epitopes, which have been shown to translocate from the membrane to the cytoplasm following this type of fixation. In these cases, tissue may need to be snap frozen and sectioned with a cryostat.

7. Should I use IHC or IF?

When deciding between immunohistochemistry (IHC) and immunofluorescence (IF), these considerations should be taken into account:

- IHC is used if a fluorescence microscope is not available.
- If co-localization studies are being conducted, IF is a better option because fluorescence can be imaged using a confocal microscope which allows superior localization of labeled proteins within the cellular/tissue setting.
- The signal amplification needed for the experiment: IHC is usually much more sensitive than IF.
- Simplicity: IHC requires an additional step in which the enzyme is reacted with the substrate while IF does not.
- Stability: IHC stains can be preserved for years, if not indefinitely, whereas IF slides are stable only for a period of 1-6 months, depending on the fluorophore used.

8. How does IHC sensitivity compare to IF?

IHC allows superior sensitivity due to two major factors:

- Excellent signal to noise ratio for high density antigens (>10,000 molecules per cell) can be achieved with a simple indirect IHC. An equivalent IF immunostaining is less sensitive since none of the fluorophore for which filters are commonly available can compare (with the possible exception of Cy3). Other fluorophores (e.g. Pe,Cy5 or APC) either fade rapidly (Pe) or require special filters that are usually not available on standard epifluorescence microscopes.
- For low-density antigens (2,000 < x<10,000molecules per cell), the only reliable and widely available signal amplification methods are with IHC.

9. Should I use Alkaline Phosphatase(AP)or Horseradish Peroxidase(HRP)? How do their sensitivities compare?

HRP and AP are enzyme probes used to detect target proteins through chromogenic, chemiluminescent or fluorescent outputs. HRP produces abundant reaction products in a short amount of time at physiological pH (7.6) and has higher specific enzyme activity as well as immunological reactivity than AP. However, HRP is associated with nonspecific staining resulting from endogenous peroxide activity in some tissues, which can be addressed with peroxidase inhibitors.

Since paraffin inhibits endogenous peroxidase activity, HRP is also the method of choice for paraffin-embedded samples. HRP can be degraded not only by microorganisms but also antibacterial agents used against them. HRP should not be used with reagents containing sodium azide.
AP, on the other hand, has an optimal pH range of 9.0 -9.6. AP reaction rates are linear so if the sensitivity is insufficient, the reaction can be allowed to proceed for a longer time period. Another advantage for using AP is that it is not inhibited by microorganisms or antibacterial agents, such as sodium azide or thimerosal.
While NBT/BCIP substrate for AP is the most sensitive, it is not widely used because the reaction is slow and does not allow adequate nuclear counterstain plus it can produce a diffuse signal. Further, it is incompatible with permanent mounting media.
Development using DAB substrate for HRP is far more common because the reaction is fast, deposition is precise, and results are better in color contrast with nuclear stains.

10. Do I need detergent for cell permeation? Ionic vs non-ionic?

Detergents are a class of molecules with unique biochemical properties that enable the disruption of hydrophobic-hydrophilic interactions between molecules in biological samples. If your target antigen has an intracellular location, you will need to use a detergent to allow the antibody access to intracellular compartments.
Ionic detergents consist of a hydrophobic chain and a charged head group (either anionic or cationic). Because ionic detergents, such as SDS (anionic),disrupt protein-protein interactions, they are not recommended for studies that involve functioning proteins or protein-protein interactions.
Nonionic detergents consist of uncharged and hydrophilic head groups. While they disrupt protein-lipid and lipid-lipid associations (cellular membranes), they are mild surfactants because they do not break protein-protein interactions. This allows proteins to be solubilized and isolated in their native (active) form, retaining the protein interactions.
Unfortunately, there is no ideal detergent for all applications as the results may also vary with the conditions and buffers used. Generally, moderate concentrations of mild (nonionic, i.e., Triton X and Tween) detergents are sufficient to permeabilize cell membranes and facilitate the extraction of soluble/native proteins. Using certain

buffer conditions, various detergents effectively penetrate between the membrane bilayers at concentrations sufficient to form mixed micelles with isolated phospholipids and membrane proteins. It is best to try out with several detergents and even mix some of the detergents to determine the optimal agent for your experiment.

11. How long should I incubate the primary antibody?

Incubation for an insufficient time period will not produce adequate signal. However, incubations that are too lengthy can result in non-specific staining. The incubation time will vary for each antibody and must be individually determined. Generally, antibodies with known high affinity (i.e., monoclonal) should be used at high dilutions with overnight incubations. Since polyclonal antibodies have varying affinities, one must iteratively determine its optimal incubation.

12. What is the stability for diluted antibody?

Carefully read the manufacturer's instructions and ensure that you store your antibodies properly and protect them from contamination. Optimal storage conditions are unique to each antibody but some general guidelines can be followed. Antibodies must be stored at an appropriate temperature and pH range and frequently in the presence of concentrated (~ 1M) substances such as glycerol or sucrose in order to retain activity and prevent aggregation. Antimicrobial agents are sometimes used to prevent contamination, though this is inappropriate for assays in which HRP is used. When antibodies are stored in aqueous form at 4°C, a typical shelf life is one month. With this in mind, it is a good idea to aliquot the antibody before diluting and keep the aliquots at -20°C (see exception below) in which case the aliquots are stable for years.

Enzyme-conjugated antibodies should generally be kept at 4°C and never be frozen. Conjugated antibodies, whether conjugated to fluorochromes, enzymes, or biotin, should be stored in dark vials or wrapped in foil because exposure to light can compromise the activity of conjugates. Fluorescent conjugates in particular are susceptible to photo-bleaching and should be protected from light during all phases of an experiment.

13. Should I freeze my antibodies?

Antibodies are vulnerable to some degrees of freezing conditions most likely because of the damage incurred during ice crystal formation, which causes antibody to lose its biological activity. With this in mind, freeze-thaw cycles should be avoided. Aliquoting the antibodies and keeping them at -20°C resolve the freeze-thaw issue.

Again, enzyme-conjugated antibodies should generally not be frozen. Instead, they should be kept at 4°C, protected from light and contamination.

14. How should samples be prepared for IHC?

Tissue and cell samples must be harvested and prepared carefully for each IHC study. In order for the incubation steps to work properly, whole tissues must be cut into ultra-thin (5-10 µm) slices or cut into smaller pieces for whole mount IHC. Sample preparation is also closely linked to the method of fixation. Careful consideration must be given to the requirements of the detection technique that will be used (fluorescence vs. chromogenic).

For example, tissue that has been immersion-fixed in formaldehyde must be paraffin-embedded and cut using a microtome. However, some tissues and epitopes cannot be fixed with formaldehyde and therefore must be snap frozen, then sectioned with a cryostat and fixed with alcohol.

15. How should I design the IHC controls?

Appropriate controls are critical to validate the IHC experiment and enable accurate interpretation of results. A well-designed IHC experiment shows that: 1) the antigen is localized to the correct specialized tissues, cell types or subcellular location; 2) the optimization of experimental conditions (i.e., fixation, blocking, antibody incubation, and antigen retrieval steps) generates a robust and specific signal. Positive and negative controls must be included to validate staining and identify or rule out experimental artifacts. In addition, variations in antibody specificity, experimental conditions, biological conditions between tissue types should be carefully considered.

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