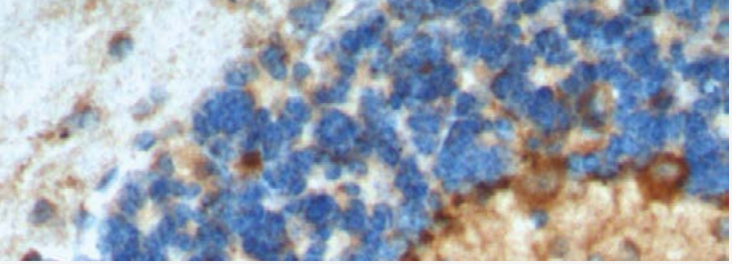

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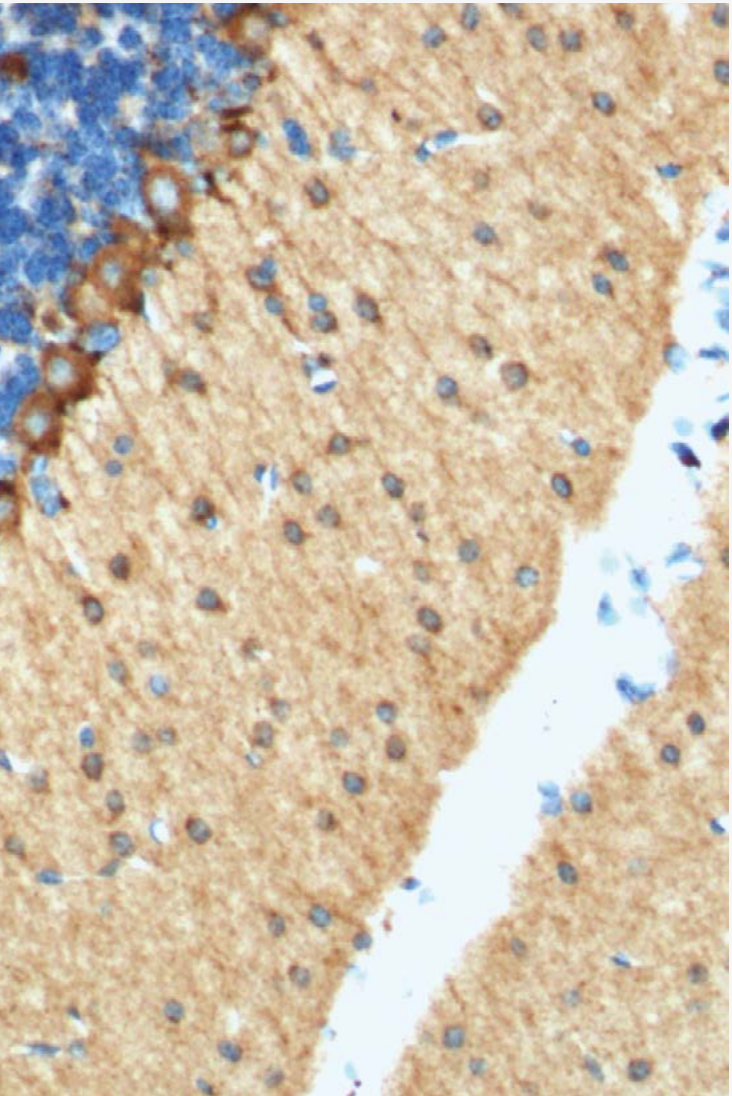
How To Optimize Your

IMMUNOHISTOCHEMISTRY EXPERIMENT



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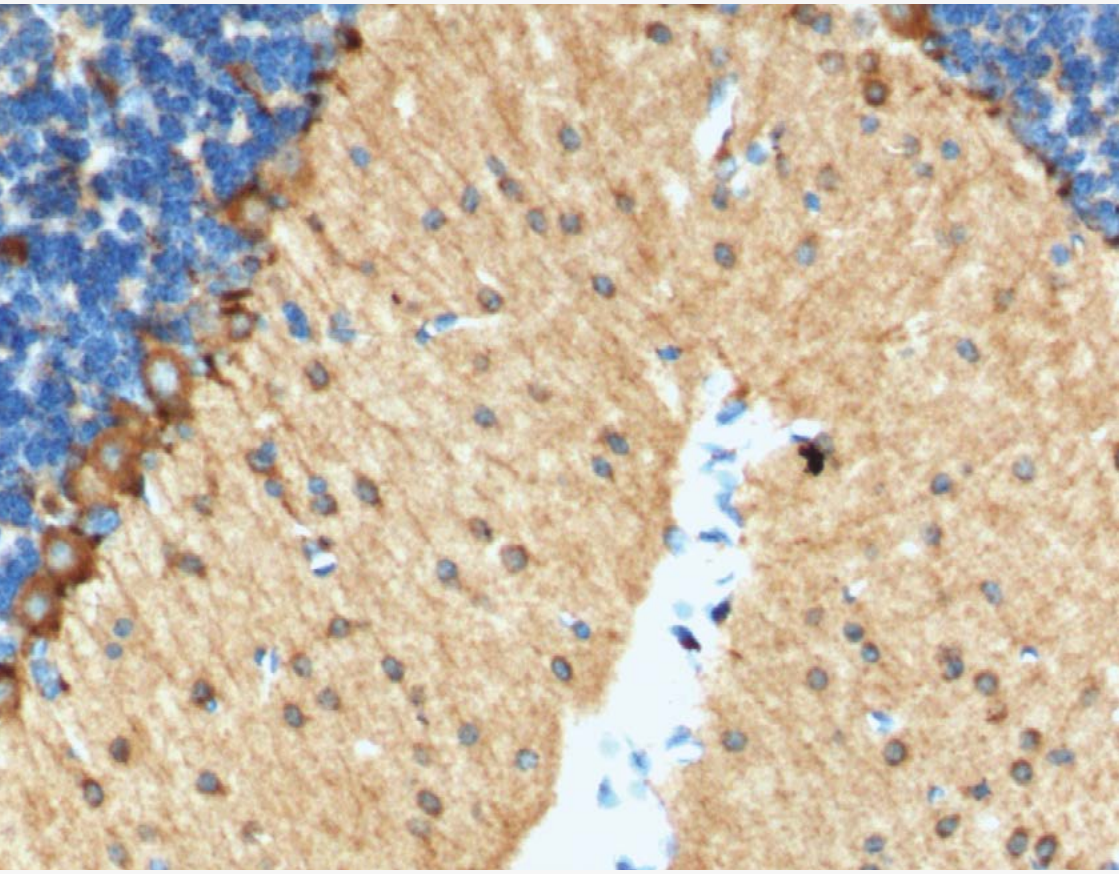


Front & Back Cover:

Immunohistochemistry of paraffin-embedded mouse cerebellum tissue slide using Adenosine A1 Receptor antibody (55026-1-AP) at a dilution of 1:200 (10x objective). Heat-mediated antigen retrieved with Tris-EDTA buffer (pH9).

CONTENTS

Introduction To IMMUNHOHISTOCHEMISTRY

Immunohistochemistry (IHC) allows you to visualize proteins in tissue while retaining its microstructure. It helps to demonstrate the exact position and distribution of the protein of interest in the analyzed tissue section. The advantage of this visualization is that it allows for comparison between, for example, healthy and diseased tissues. Briefly, in an IHC experiment, the antigen of interest is localized by the binding of an antibody. The antibody-antigen interaction is then further visualized via chromogenic or fluorescent detection.

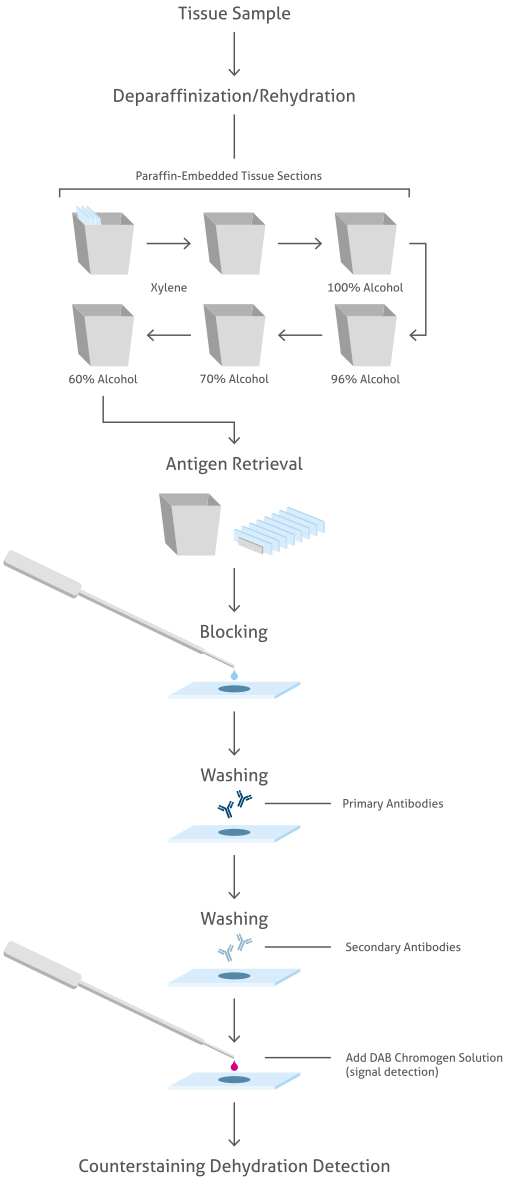
The IHC protocol contains many steps that may require optimization to ensure specific antibody binding and optimal visualization of the target protein. As IHC protocol contains many different variable factors, it is challenging to find the best working conditions to obtain strong and specific staining.

The following guide outlines some tips and hints for each of these steps. Below, we also provide our general staining protocols.

Factors to consider in IHC experimental design	
IHC Factor	**To Consider**
Sample Type	Fixed, frozen
Antigen	Species, level of expression, subcellular location
Epitope	Conformation, post-translational modification
Primary Antibody	Monoclonal vs. Polyclonal
Blocking	Sera, BSA, commercial buffer, temperature, pH, dilution, incubation time
Secondary Antibody	Species, label type
Labelling	Chromogenic, enzymatic, fluorescent
Counterstaining	Chromogenic, fluorescent
Analysis	Microscope, software-based analysis, evaluation by eye
Controls	Secondary antibody only, antigen positive tissue, isotype control, unstained sample

The Main Steps Of IHC

Overview of the main steps of IHC using a chromogenic labeled secondary antibody on paraffin-embedded tissue slides.



GENERAL PROTOCOLS

Materials And Equipment

- Frozen or paraffin-embedded tissue
- Cryo-embedded media (e.g., OCT)
- Sucrose
- 4% Paraformaldehyde (PFA)
- Microtome
- Glass slides
- Coverslips
- Refrigerator
- Incubator
- Xylene
- Ethanol (different percentages)
- Boiling source
- Primary and secondary antibodies
- Hematoxylin
- Serum
- Citrate buffer (pH 6)
- Tris-EDTA buffer (pH 9)
- Diaminobenzidine tetrachloride (DAB)
- Microscope

Membrane Transfer			
Citrate Buffer	**For 1000 ml**	**1x TBS**	**For 1000 ml**
10 mM Tris-sodium citrate $_2H_2O$	2.9 g	20 mM Tris-base	2.4 g
1.9 mM citric acid H_2O	0.4 g	150 mM NaCl	8.7 g
Adjust pH to 6.0		Adjust pH to 7.6	
Add ddH_2O to 1000 ml		Add ddH_2O to 1000 ml	

Sample Preparation

Frozen sections of clinical samples

For fast processing of clinical samples, eliminating the fixation step by directly freezing and embedding with OCT, followed by cutting (6-8 μm thickness), is recommended as it would be time-saving and avoid the increased difficulty of sectioning caused by fixation. As for the temperature in the cryostat for unfixed tissues, please refer to the table below:

Cryostat Temperature For Unfixed Tissues	
Brain, liver and lymph node tissues	-10°C/-15°C
Thyroid, spleen, kidney and muscle tissues	-15°C/-20°C
Tissue containing fat	-25°C
Tissue containing plenty of fat	-30°C

Tip: Fresh tissue and fixation using 4% PFA in 4°C overnight are recommended.

1. Wash tissue 3 times with PBS for 5 minutes each.
2. Immerse tissue in 20–30% sucrose for 16–48 hours.
3. Place the tissue block onto a pre-labeled tissue base mold.
4. Cover the entire tissue block with cryo-embedding media (e.g., OCT).
5. Slowly place the base mold containing the tissue block into liquid nitrogen until the entire tissue block is submerged into liquid nitrogen to ensure tissue is completely frozen.
6. Store the frozen tissue block at -80°C until ready for sectioning.
7. Transfer the frozen tissue block to a cryotome cryostat (e.g., -20°C) prior to sectioning and allow the temperature of the frozen tissue block to equilibrate to the temperature of the cryotome cryostat.
8. Section the frozen tissue block into a desired thickness. Keep the section apparatus, including the blade and the blade holder, and clean by polishing with soft paper tissue.
9. Place the tissue sections onto glass slides suitable for immunohistochemistry. Sections can be stored in a sealed slide box at -80°C for later use. Before further use, equilibrate the tissue first at -20°C for 30 minutes.

Tissue slide handling

Tissue sections should be placed on a glass slide. Allow adhesion of tissue to slide by air drying and additional baking in an incubator. Cut and unbaked slides can be stored at 4°C for later use. However, storage might have an effect on the antigentic potential and this varies for each protein. Therefore, it is recommended to minimize storage time or to use freshly prepared slides.

Immunohistochemistry On Paraffin Sections

Please note: All steps in the following protocol are carried out at room temperature unless stated otherwise.

Deparaffinizing and rehydration

1. Immerse slides in xylene for 10 minutes. Repeat this step again in fresh xylene for a further 10 minutes.
   (If required, repeat a third time in fresh xylene for another 10 minutes.)
2. Rehydrate sections by sequentially incubating with 100%, 95%, 80%, and 60% ethanol for 5 minutes each.
3. Rinse sections with distilled water three times for 3 minutes each.

Antigen retrieval (optional)

4. Transfer slides to a microwave-proof container and cover with citrate buffer.
5. Heat in the microwave on medium power for 10 minutes.
6. Allow slides to cool in citrate buffer for approximately 35 minutes each.

Primary antibody incubation

7. Rinse slides three times with 1x TBS for 3 minutes each.
8. Incubate slides with 3% H_2O_2 solution (diluted in distilled water) for 10 minutes to quench endogenous peroxidase activity.
9. Rinse slides three times with 1x TBS for 3 minutes each.
10. Prepare 5% normal blocking serum in 1x TBS. The serum should be derived from the same species in which the secondary antibody was raised. Block the sections for 1 hour.
    (Alternatively, use 5% BSA in 1x TBS for blocking if the corresponding serum is not available.)
11. Incubate sections with the primary antibody diluted in 1x TBS for 1.5 hours, or overnight at 4°C; the optimal antibody dilution ratio should be pre-determined by experimentation. Set up negative controls by omitting the primary antibody incubation step for one slide per each experimental condition.
12. Following primary antibody incubation, rinse slides three times with 1x TBS for 3 minutes each.

Signal Detection

Please note: Proteintech®* routinely uses EnVision Kit (Dako) for this step.

13. Apply sufficient peroxidase labeled polymer and incubate for 30 minutes.
14. Rinse slides three times with 1x TBS for 3 minutes each.
15. Prepare an appropriate volume of substrate solution prior to use by mixing one drop of Liquid DAB plus chromogen immediately with 1 ml of substrate buffer. Apply the substrate carefully and incubate for 5–10 minutes until a brown colour develops.
16. Rinse sections gently with sufficient distilled water.

Hematoxylin counterstaining (optional)

17. To stain nuclei, immerse slides in a bath of hematoxylin for 3 minutes.
18. Rinse slides gently with distilled water.
19. Transfer slides into a 1% HCl, 99% ethanol solution for 10 seconds; transfer to distilled water immediately.

Dehydration and mounting

20. Immerse slides sequentially into 60%, 80%, 95%, and 100% ethanol baths for 5 minutes each.
21. Immerse slides in xylene for 5 minutes. Repeat this step again in fresh xylene for another 5 minutes.
22. Mount the section with sufficient mounting media and cover with a coverslip Air-dry in a well-ventilated area (e.g., fume hood).

Tip: Metal ions (e.g. CuSO4, methenamine silver, cobalt chloride, ammonium nickel sulfate, nickel chloride) can enhance IHC signal.

Tip: When DAB staining is nuclear, shorten the counterstaining incubation time and wash the slide with ammonia or TBS buffer (pH 8.0).

Immunohistochemistry On Frozen Tissue

Unlike paraffin samples, frozen samples are not treated with fixative, so the antigens are not cross-linked with other proteins and therefore do not require an antigen retrieval step to unmask them for recognition by antibodies.

ANTIGEN RETRIEVAL

The fixation process during paraffinization cross-links proteins. This may result in masking the epitopes, resulting in weak or false negative staining. However, this challenge can be overcome by heat-induced epitope retrieval (HIER) or proteolytic-induced epitope retrieval (PIER). Which of these to use depends on the tissue type and primary antibody.

Heat-induced Epitope Retrieval (HIER)

Antigen retrieval is carried out when the slide is heated up for a specific time in a specific buffer. Different sources such as a microwave, pressure cooker, water bath, steamer, etc are normally used for this step.

The most commonly used buffer is the citrate buffer (see recipe in general protocols). However, the choice of buffer depends on the antibody in use. Also, follow the instructions on the specific datasheet and recommendations for retrieval buffers. As a rule of thumb, an EDTA buffer is favorable when working with antibodies against phospho-tyrosines.

Proteolytic-induced Epitope Retrieval (PIER)

In the proteolytic-induced epitope retrieval (PIER) technique, epitopes are unmasked by peptidases.

Trypsin is a popular enzyme that breaks the protein cross-links, unmasks the hidden antigen, and thus increases the staining intensity and specificity of the primary antibody.

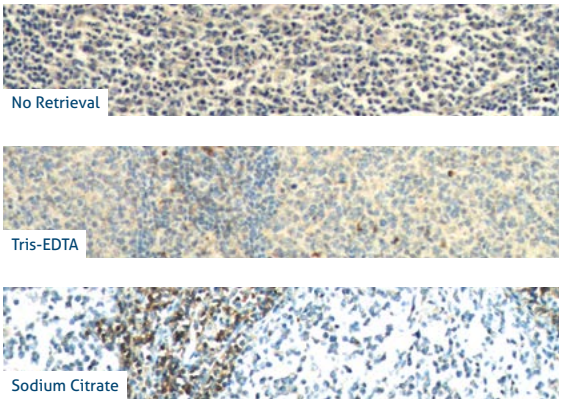
1. Prepare the trypsin and pre-heat to 37°C. Pipette the enzyme solution onto the section.
2. Place the slides in a humidified container and then into the 37°C incubator.
3. After 15 minutes, remove the slides from the incubator and transfer to a rack in a container with tap water. Rinse by running water for 3 minutes.
4. Continue with the immunohistochemical staining.

   (Other enzymes used are proteinase k, pepsin, protease, or pronase.)

	Heat-induced	Proteolytic-induced
Advantage	Smooth epitope recovery	Preferred for difficult-to-recover epitopes
Disadvantage	No impact on cell morphology	Impacts and damages, also a harsh method
Difficulties	Unequal retrieval due to unequal heating	Concentration calibration
pH*	Typically pH 6 (citrate), pH 9 (Tris-EDTA)	Typically pH 7.4
Incubation time*	Around 20 mins	Around 10 mins
Temperature*	Around 100°C	Around 37°C

*Optimal conditions always have to be determined by each laboratory and in accordance with the specific product information.

Comparison of heat-induced epitope retrieval (HIER) and proteolytic-induced epitope retrieval (PIER)



Antigen retrieval optimization of CD3 gamma antibody (60347-1-AP) on paraffin-embedded tonsillitis tissue slides.

BLOCKING

Blocking is essential for preventing non-specific binding of antibodies or other reagents to the tissue. Even if the antibody has high specificity towards the target, intermolecular forces can promote non-specific binding to other molecules. Consequently, non-specific binding prevents visualization of the antigen-antibody binding of interest. To mitigate non-specific binding, a blocking step should be carried out before incubation with the primary antibody. Different commercial buffer systems are available.

Protein Blocking

In general, serum (same species as secondary antibody) or bovine serum albumin (BSA) are used for blocking. Sera and BSA can help to prevent unspecific binding to the many hydrophobic side chains of proteins present in tissue. If you are staining with multiple antibodies, you need to use blocking serum against all used secondaries. If BSA is used, the addition of 0.1–0.5% Trition-X or Tween can help to prevent unspecific binding.

Blocking Non-Specific Ionic Bindings

Non-specific ionic bindings are due to, for example, Van der Waals interactions, dipole-dipole interactions or net charges of specific amino acid groups. In this case, altering the ionic strength of the antibody dilution buffer can help to reduce unspecific ionic bindings.

Endogenous Enzyme Blocking

When using a horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-conjugated antibody for detection, the endogenous levels of the enzyme have to be blocked. This normally applies for tissues such as kidney, liver, intestine, lymphoide tissue, etc. Peroxidase can be blocked with buffers containing H_2O_2 and AP can be blocked with buffers containing acetic acid or Levamisole.

Endogenous Biotin Blocking

Endogenous biotin is found to be especially high in tissue of the liver, kidney, or brain. Blocking is necessary if working with an avidin-biotin detection system. A direct incubation with the ABC complex or streptavidin-HRP and then DAB is also possible.

FEATURED PRODUCT

IHC Detection System, Peroxidase/DAB+, Rabbit/Mouse (KIHC-1, KIHC-5)

Two-step immunohistochemical staining technique:

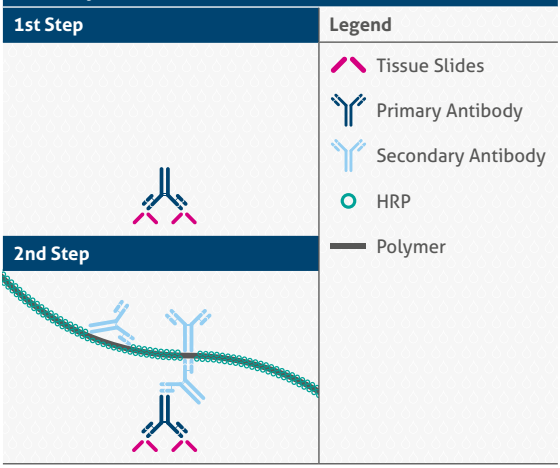
This system is based on an HRP labeled polymer that is conjugated to secondary antibodies. All reagents are ready to use, thus making it time- and cost-effective.

The secondary antibodies are mixed with anti-mouse IgG and anti-rabbit IgG. This universal reagent can be used to detect any tissue slides bound with primary antibody of mouse or rabbit origin.

Main Features

- Low background
- Strong staining
- Easy protocol

IHC Kit System



Kit Content
HRP-conjugated secondary antibody
DAB Substrate Buffer
DAB

IMMUNOSTAINING

Selecting And Optimizing Antibodies For IHC

When deciding on an antibody, the following points should be considered to increase the likelihood of high specificity and low cross-reaction and a successful staining; further optimization will then help to achieve a reliable signal: Consulting literature and antibody comparison resources, checking in-house validation data of the antibody seller, searching for independent validation data, and consulting the original manufacturer for technical service and protocol support.

Primary Antibodies For IHC

Selecting a primary antibody for IHC

The initial choice of the primary antibody can affect the whole outcome of the experiment. Thus, it is important to search for an antibody that is well validated to increase the chance of a successful experiment. Most importantly, it is helpful to purchase the antibody from the original manufacturer and not from a vendor, as only the original manufacturer has access to all validation data and can assist with superior technical support.

Main questions to ask when choosing a primary antibody supplier	
1	How many times has the antibody been cited?
2	How has the antibody been validated?
3	Has the application of interest already been tested?
4	Who is the original manufacturer?
5	Is all the data and information about the antibody publicly available?
6	What is the vendor's refund policy, delivery time, and stock availability?
7	What is the price?

Polyclonal Vs. Monoclonal Antibody

A final question to consider is whether a monoclonal or polyclonal antibody would be most suitable. For IHC, polyclonal antibodies tend to give the best results.

Benefits of polyclonal antibodies in IHC

- Heterogenous population.
- Recognise multiple epitopes.
- Stable to changes (pH, tissue, buffer, protein confirmation), stable detection.

Drawbacks of polyclonal antibodies in IHC

- Not specific to one epitope.

Benefits of monoclonal antibodies in IHC

- Homogenous population.
- Specific to a single epitope.
- Detects a single protein with high affinity, even though it shares sequences/similarites with other proteins.

Drawbacks of monoclonal antibodies in IHC

- Sensitive to even small changes (pH, tissue, buffer, protein confirmation), no stable detection.

Optimizing A Primary Antibody For IHC

The optimal conditions for the primary antibody depend on each individual experiment and therefore have to be optimized to gain a staining of high quality.

General steps on optimizing conditions for a primary antibody in IHC

- Keep incubation time and temperature, titrate different antibody dilutions.
- Specific staining, but background signal: Vary incubation time and temperature.
- High-affinity antibody with a high concentration: Incubation with a high concentration for a short time.
- High-affinity antibody with a low concentration: Increase incubation time, lower incubation temperature.
- Polyclonal antibodies, in general, can be used at a higher working dilution than monoclonal antibodies.

Selecting And Optimizing Secondary Antibodies For IHC

In immunohistochemistry (IHC), it can be beneficial to reduce the signal-to-noise ratio when distinguishing between antibody subclass, purification, and fragments of the secondary antibody.

Subclass specificity

Polyclonal primary antibodies are mainly IgG isotypes. Primary monoclonal antibodies are occasionally of a different isotype and therefore need an isotype-specific antibody.

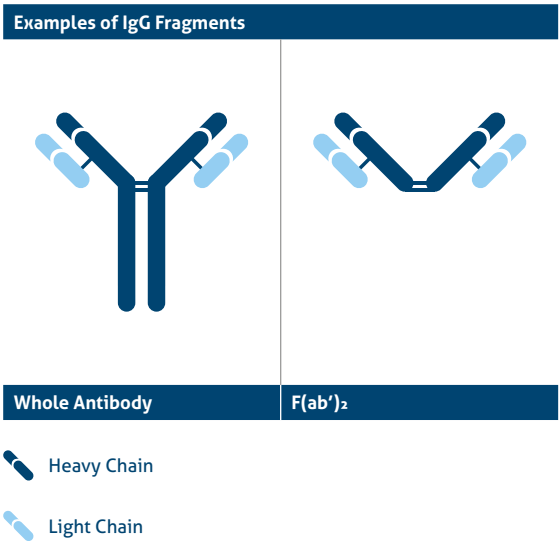
Cross-absorption

Secondary antibodies can go through an additional purification step to reduce potential cross-reactions with other species. Therefore, the secondary antibody solution is passed over different columns containing sera proteins of different species to filter out the non-specific secondary antibody.

$F(ab')_2$ fragments

High background staining can be due to the presence of Fc receptors in special tissue or cells.

Tip: If the primary antibody is a fragment, the secondary antibody should also be fragment-specific to reduce noise signal ($F(ab')_2$ fragment secondary antibodies penetrate tissue easier in IHC).



IHC Controls

It is essential to perform control stainings to ensure that the observed staining pattern is specific and authentic and not due to any, for example, cross-reaction or unspecific binding.

Positive controls

- Control tissue that is known to express the protein of interest will prove that the staining protocol and antibody are working properly.

Negative controls

- Control tissue that is known not to express the protein of interest will ensure that the observed staining pattern is due to specific signals.
- Incubate the tissue with the secondary antibody only. This control ensures that no cross-reactions or non-specific background signals are observed due to the secondary antibody and the detection reagents.
- Observing the unstained tissue under the microscope in the brightfield/fluorescence channel gives an idea about biological background signal/autofluorescence that mainly comes from mitochondria, lysosomes, and aromatic amino acid components and could be misinterpreted with positive staining.
- Incubating the tissue with a non-immune immunoglobulin of the same isotype will ensure that the observed staining is not due to unspecific binding of immunoglobulins.

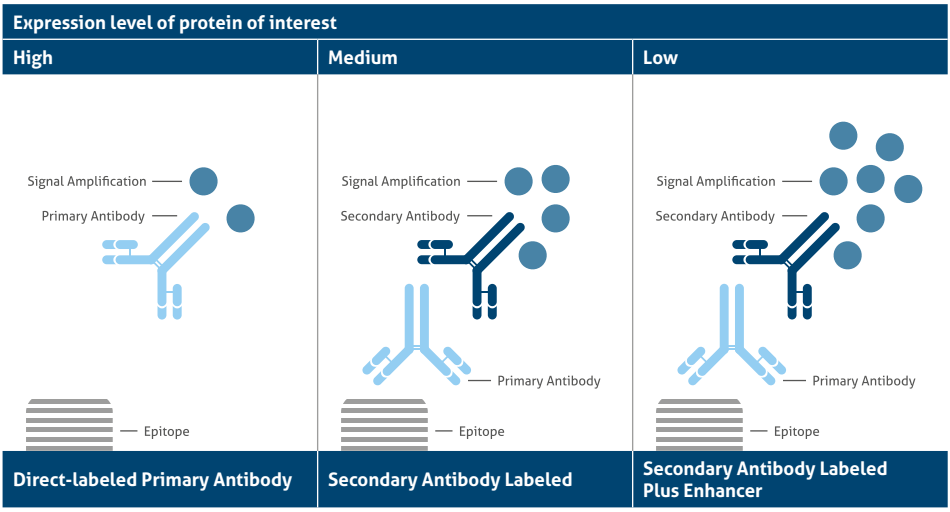
VISUALIZATION

After incubation with the primary antibody, the staining can be visualized using different detection methods. The detection technique can be direct or indirect and the signal generation can be chromogenic or fluorescent.

Direct Versus Indirect Detection

In direct detection, the primary antibody contains the label. On the other hand, in indirect detection, the secondary is labeled. The choice of detection method depends on the expression level of the antigen. As primary labeled antibodies show a comparable low signal generation, they are suitable for highly expressed antigens. Direct detection is also advantageous for multi-labeling experiments. Medium expressed antigens instead show the best signal when analyzed via a secondary labeled antibody that helps to amplify the signal intensity. Indirect labeling, however, always shows a higher background noise. For very low-expressed proteins the indirect detection plus an enhancer (e.g., avidin, streptavidin) helps to further amplify the signal. This type of detection often needs some additional optimization steps to get the best signal-to-noise ratio.

Different Detection Systems and Signal Amplification

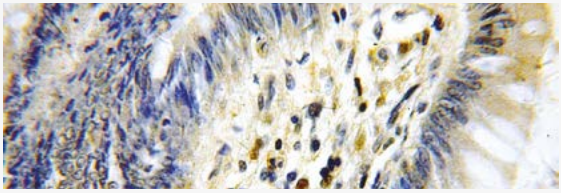


Chromogenic Versus Fluorescent Signal Generation

Signal is generated by fluorophores or enzymes. The detection method and available microscope determine the choice of label.

If working with fluorescent labels, the fluorophore can be conjugated either to the primary or secondary antibody. Especially for multi-color staining experiments, fluorescent labels are beneficial for detecting different cellular compartments at the same time. A disadvantage of fluorophores is their short lifetime. Thus, enzyme labels are often the preferred choice for visualization of tissue stainings.

If working with enzymatic labels, the enzyme is attached to the primary or secondary antibody and then forms an insoluble colored product when an organic substrate is added. The most commonly used enzyimatic labels are horseradish peroxidase and alkaline phosphatase.



Immunohistochemical staining of paraffin-embedded human colon tissue using KLF4 antibody (11880-1-AP) at a dilution of 1:50 (40x objective).

Tips, Tricks, and TROUBLESHOOTING GUIDE

No/Weak Staining

Potential cause	Suggested test or solution
The primary/secondary antibody lost its activity.	Use a new lot of antibody. Improper storage of antibody. Follow manufacturer's instruction. Normally, prepare single-use aliquots and store at -20°C. Extensive thaw-freezing cycles have damaged the antibody.
Conditions of antibody are not optimized.	Titrate the antibody concentration to optimize best working conditions. Incubate the primary antibody at room temperature or at 4°C overnight.
Protein of interest is not expressed in used tissue.	Run a positive control.
Protein of interest is low expressed in used cells.	Use signal amplification when visualizing.
Damaged epitope.	Change to another antigen retrieval buffer/technique for paraffin-embedded samples.
Antibody is not suitable for this application.	Check validation data of manufacturer.

Background Staining/ Non-specific Staining

Potential cause	Suggested solution
Too high primary/secondary antibody concentration.	Titration of antibodies to determine optimal working concentration.
Non-specific binding of primary/ secondary antibodies.	Prolong blocking step and increase concentration of blocking solution. Run positive and negative controls.
Non-specific binding of secondary antibodies.	Run control with secondary antibody only. Change to a cross-adsorbed secondary antibody or a fragment antibody.
The sample is poorly washed.	Repeat or prolong washing step.
The antibody incubation temperature/time is not suitable.	Optimize conditions.
Damaged epitope.	Change to another antigen retrieval buffer/technique for paraffin-embedded samples.

Inappropriate Cell Morphology

Potential cause	Suggested solution
Harsh antigen retrieval conditions	Optimize buffers, temperature, pH, incubation time, concentration.
Unclear tissue structure	Optimize thickness of tissue slides. Cut new sections.
Tissue is not adhesive to glass slide.	Optimize fixation. Decrease heating time or temperature during HIER.
Physically damaged cell shape.	Under-fixation. Change fixative or fixation time.

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