IHC Protocol

Introduction

Immunohistochemistry (IHC) detects and visualizes antigens in tissue sections using antibodies. It preserves the spatial context of proteins within tissues and enables researchers to study cellular expression patterns, protein localization, and tissue architecture. This makes IHC a valuable tool for cancer research, neuroscience, and pathology.

Poor fixation, high background noise, or weak staining can compromise data integrity and reproducibility. This protocol provides a step-by-step guide to help you achieve consistent, optimized results using validated reagents, precise tissue sectioning, and gentle, staining-safe chromogen development.

Read on for a clear IHC step-by-step guide, troubleshooting tips, and best practices to help you consistently achieve accurate, high-quality results.

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Download the IHC Protocol

Get a printable, step-by-step guide for easy reference and offline use.

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Further Reading

Deep dive into the principles of IHC, its applications, and techniques to master the art of staining.

Immunohistochemistry: principles and practice, 3rd edition, Sheppard, Bancroft, Cell + Cell, Mammoth, Tonsington, Immunohistochemistry

Cardiovascular Pharmacy Effects of NF-κB in Lung and Big Data, Hamilton, Immunohistochemistry, Cell, Tissue, and Big Data, Hamilton, Mammoth, Rockefeller, Schlesinger, et al, 2019

Materials & Reagents

Here's everything you need to carry out our IHC protocol. In addition to Boston BioProducts' high-quality buffers and reagents, you'll need the following materials and equipment:

- Glass slides
- Coverslips
- Coverslip crystal
- Microtome
- Antibodies (primary/secondary)
- Pipettes
- Pipette and pipette tips
- Microscope

Reagent	Product Name	Link
Fixative Solution	Paraformaldehyde (4-7%, Fixative Solution)	View Product
Detergent	IGEPAL CA-630 Triton X-100 Tween 20 (10%)	View Product View Product View Product
Distilled Water	Distilled Water	View Product
Retrieval Buffer	Citrate Buffer Tris-EDTA Buffer, 10	View Product View Product
Antigen Retrieval	Proteinase K Solution (20mg/ml)	View Product
Blocking Buffer	Bovine Serum Albumin, BSA	View Product
Dripping Buffer	Dripping Buffer (PI, Strong)	View Product
Wash Buffer	Phosphate Buffered Saline, PBS (pH 7.4, Tablet, Filtered) Tris Buffered Saline, TBS (10X, pH 7.6)	View Product View Product

Boston BioProducts' Protocol

This IHC protocol walks you through tissue preparation, antigen retrieval, and visualization—even for FFPE sections and difficult samples—and includes pro-tips, troubleshooting, and recommendations at every step.

Tissue Preparation

Proper tissue preparation is crucial for successful IHC, as it preserves antigen integrity, maintains tissue morphology, and prevents degradation. Below are the steps for both frozen and FFPE tissues, plus pro-tips for handling fragile or poorly embedded tissue samples.

For fresh or frozen tissue

1. Although IHC can be performed with fresh samples, most IHC protocols are optimized for frozen or paraffin-embedded tissues.

Transfer your frozen or fresh sample block to the cryostat chamber. If using frozen tissue, allow the temperature to equilibrate first and set the tissue section to the desired thickness (typically 5-10 µm).

Keep your cryostat temperature between -15 and -21°C to prevent crystal clotting or shifting.

3. Air dry (20-1) slides before proceeding. Tissue.

4. Immerse samples in 3-7% paraformaldehyde (PF) (10% tissue volumes) for 4-24 hrs at 4°C.

5. Wash the sections in PBS to remove the fixative.

Fixation temperature and time may require optimization depending on the tissue type and size.

Into IHC protocol for paraffin sections

To ensure optimal results with embedded tissue samples you must take additional steps to process the tissue. This into IHC protocol for paraffin sections sections sets the sections.

1. **Tissue preparation**

Deparaffinize in xylene (2x10 min) Wash solution each tonsil, then rehydrate through ethanol (100% → 50%) and distilled water (2 min each).

2. **Antigen Retrieval**

Antigen retrieval is usually not required for fresh or frozen samples. However, for paraffin embedded tissue, it is required to unmask antigens. The most common methods are heat-induced epitope retrieval (HIER) and enzyme-induced epitope retrieval (EIER).

EIER is useful for antigens that may be damaged by high temperatures.

HIER: Heat citrate or Citrate Buffer or Tris EDTA Buffer (microwave, 10 min, medium power). Cool in buffer.

EIER: Incubate in Proteinase K Solution (10-20 min). Then wash in PBS.

Monitor the buffer level during heating to prevent boiling over or evaporation, which may dry the slides out.

Prolonged Proteinase K incubation can damage tissue morphology; reduce time as necessary.

Make sure you wash the slides thoroughly at this stage, as the residual detergent can interfere with antibody binding.

Permeabilization (optional)

After antigen retrieval, permeabilization is required to make staining intracellular targets as it helps antibodies penetrate cell membranes and reach intracellular antigens.

1. Incubate your tissue sections in Triton X, 0.3% or Tween 20 + PBS for 5-15 minutes at room temperature.

Triton X-100 is stronger and suited for nuclear targets, while Tween 20 is milder and is better suited for cytoplasmic staining.

2. Over permeabilization can damage tissue morphology and lead to non-specific antibody binding.

3. Wash slides in PBS or TBS before proceeding to blocking.

Make sure you wash the slides thoroughly at this stage, as the residual detergent can interfere with antibody binding.

Blocking

Effective blocking is essential to prevent non-specific binding, minimize background staining, and reduce false-positive signals.

1. Incubate your tissue sections in Block A, 10% or Tween 20 + PBS for 5-15 minutes at room temperature.

Over-blocking can reduce signal intensity. If you experience weak staining, try shortening blocking time.

2. Block slides in BSA/casein (10 min, at room temperature).

3. Wash slides in TBST.

4. Repeat.

This blocking solution should not contain serum of the host animal of the primary antibody as this could result in a high background.

Antibody Incubation

Proper antibody incubation is the step where scientists try to detect detection (with an enzyme or fluorescent conjugated primary antibody) or indirect detection (with both primary and secondary antibodies).

1. Chemogenic IHC (cIHC) uses an enzyme-conjugated secondary antibody (e.g., HRP or AP) that catalyzes a colorimetric reaction, producing a visible, permanent stain at the antigen site. This is ideal for brightfield microscopy. Fluorescent IHC uses fluorophore-conjugated antibodies that emit fluorescence when excited by a specific wavelength of light.

Direct

1. Dilute the conjugated primary antibody in TBS with 1% BSA or alternative serum) according to the manufacturer's instructions.

Before adding the antibody solution to the slides, draw around your sample with a hydrophobic barrier pen to help contain small volumes.

2. Incubate your tissue sections in the diluted primary antibody overnight at 4°C.

To prevent the slides from drying out during incubation, cover them with a humidified chamber.

3. Once complete, wash the slides for 5-15 minutes in PBS or TBS to remove any unbound antibody.

Indirect

1. Incubate your tissue sections in the diluted primary antibody overnight at 4°C.

2. After wash steps, dilute a fluorophore-conjugated secondary antibody in TBS with 1% BSA or alternative diluent according to the manufacturer's instructions.

3. Incubate at room temperature for at least 30 minutes.

4. Wash with the buffer solution PBS or TBS for 5 x 5 minutes.

Detection

Detection is the IHC reveals the presence and localization of the target antigen. Depending on the staining method, this can be visualized using chromogenic substrates (for brightfield IHC) or fluorescent signals (for fluorescence microscopy). Each method has its own advantages, requirements, signal stability, and imaging considerations.

1. Prepare your substrate solution for cIHC, or secondary antibody solution for IF-IHC.

If you are doing IF IHC, make sure your tissue sections are protected from light throughout the process to avoid photobleaching.

2. Incubate your slides for as long as necessary.

For chromogenic detection, you can monitor the staining visually and finish when you reach the desired intensity.

3. Wash slides in PBS to remove excess substrate solution or unbound antibody.

Please include a negative control to detect any non-specific secondary antibody binding.

Mounting and Visualization

Proper mounting preserves the stained tissue for imaging and, if necessary, long-term storage. Your mounting protocol may differ depending on your tissue sample and type of IHC.

1. Paraffin embedded sections require dehydration and a resin-based medium.

2. Submerge your slides in hematoxylin (10 sec-1 min).

3. Wash the slides with cool running water to blue the stain.

4. Dehydrate sequence (70% → 100%), then with xylene.

5. Mount (5 min), coverslip, and image or brightfield microscope.

Use the smallest required volume of mounting media and make sure there are no air bubbles, as these can affect image quality.

IF IHC

1. Counterstain (DAPI, 5 min), wash in PBS.

2. Mount with anti-fade medium, coverslip.

If IHC media an anti-fade mounting medium, while cIHC uses resin-based mounting for long-term storage.

3. Image using the appropriate fluorescence filter for your fluorophore.

If you are storing your slides, you can seal the edges of the coverslip with clear nail polish to prevent drying and movement.

Looking for a multiplex IHC protocol?

Multiplex IHC (mIHC) protocol workflow where you perform four differences per sequential antigen retrieval, adjacent blocking, fluorophore selection, and stripping buffer step.

1. **Tissue preparation**

Follow the tissue section steps in the protocol above for tissue sectioned, deparaffinized, or paraffin-embedded.

2. **Antigen retrieval**

Following tissue preparation, multiplex IHC may require you to perform antigen retrieval multiple times to prevent epitope masking or signal loss because rounds of staining.

Choose a retrieval method that may be validated in the above IHC protocol(s) as these steps as necessary.

3. **Blocking**

Apply blocking steps relevant to each antibody to minimize cross-reactivity and background staining.

Depending on your detection system, you may need to block endogenous peroxidases, phosphatases, and Fc receptors.

4. **Sequential or simultaneous antibody staining**

- **Sequential staining:** Apply the first primary antibody, followed by its secondary antibody, then proceed to the next antibody.
- **Simultaneous staining:** If fluorophore-conjugated primary antibodies are used, mixes specific suspension to prevent bleed-through.

5. **Stripping for sequential staining**

If using multiple rounds of staining, stripping steps may be required between each cycle to remove primary antibody complex.

- Incubate the tissue sections in a stripping buffer.
- Wash with PBS.
- Repeat blocking and staining as necessary.

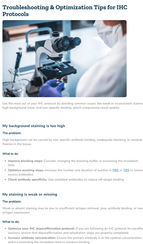
6. **Signal amplification (optional)**

For low-abundance targets, apply tyramide signal amplification (TSA) to enhance signal intensity while maintaining specificity.

7. **Mounting and Visualization**

Use an anti-fade mounting medium to prevent your sections for imaging and storage according to the relevant method.

Troubleshooting & Optimization Tips for IHC Protocols



Get the most out of your IHC protocol by avoiding common issues like weak or nonexistent staining, high background noise, and poor reproducibility, which compromise result quality.

My background staining is too high

The problem:

High background can be caused by non-specific antibody binding, inadequate blocking, or residual fixative in the tissue.

What to do:

- **Improve blocking steps:** Consider changing the blocking buffer or increasing the incubation time.
- **Optimize washing steps:** Increase the number and duration of washes in PBS or TBS to remove excess antibodies.
- **Check antibody specificity:** Use validated antibodies to reduce off-target binding.

My staining is weak or missing

The problem:

Weak or absent staining may be due to insufficient antigen retrieval, poor antibody binding, or low antigen expression.

What to do:

- **Optimize your IHC staining protocol:** If you are following an IHC protocol for paraffin sections, ensure the deparaffinization and rehydration steps are properly completed.
- **Check your antibody concentration:** Titrate your primary antibody for optimal performance, and try extending the incubation time to enhance binding.
- **Check tissue fixation:** Over-fixation with formalin can mask antigens, so if necessary, reduce fixation time or perform antigen retrieval.
- **Try signal amplification:** If you are following a multiplex IHC protocol, make sure you do the signal amplification step using TSA.

I have high non-specific staining

The problem:

Non-specific staining can arise due to antibody cross-reactivity, bleed-through in IF IHC, or insufficient blocking between rounds of staining in multiplex IHC protocols.

What to do:

- **Optimize antibody selection:** To prevent cross-reactivity, ensure that the primary antibodies you choose are from a different host species.
- **Adjust fluorophore selection:** Ensure proper spectral separation to minimize channel overlap and bleed-through in multiplex IHC.
- **Use an effective stripping buffer:** If sequential staining is performed in a multiplex IHC protocol, use a dedicated stripping buffer between rounds to remove previously bound antibodies.

Why Boston BioProducts?

Whether you're working on routine tissue sections or challenging samples, Boston BioProducts' reagents and buffers are designed to help you achieve consistent, high-quality results. Our optimized antigen retrieval buffers ensure effective epitope unmasking, while our blocking solutions minimize background staining without compromising signal intensity. Plus, our stabilizing buffers help preserve tissue morphology and antibody binding, so you can trust your results every time.

Try integrating Boston BioProducts' high-quality IHC buffers into your workflow, you can enhance signal clarity, improve specificity, and streamline your protocol for more reliable, reproducible results. Explore our staining solutions, high-quality results are only a click away.

FAQs

- **When is antigen retrieval necessary in IHC?**

This is one of the most common questions in IHC applications. Since tissue fixation conditions and the antibody used, it is recommended that, unless the source of the primary antibody specifically states that retrieval is unnecessary, you should perform it. Heat-induced epitope retrieval (HIER) is the most commonly used method for paraffin sections, as formalin fixation can create protein cross-links that can mask antibody binding sites.

- **What are the key differences between paraffin-embedded and frozen tissue IHC?**
- **What's the Difference Between IHC and ICC?**

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