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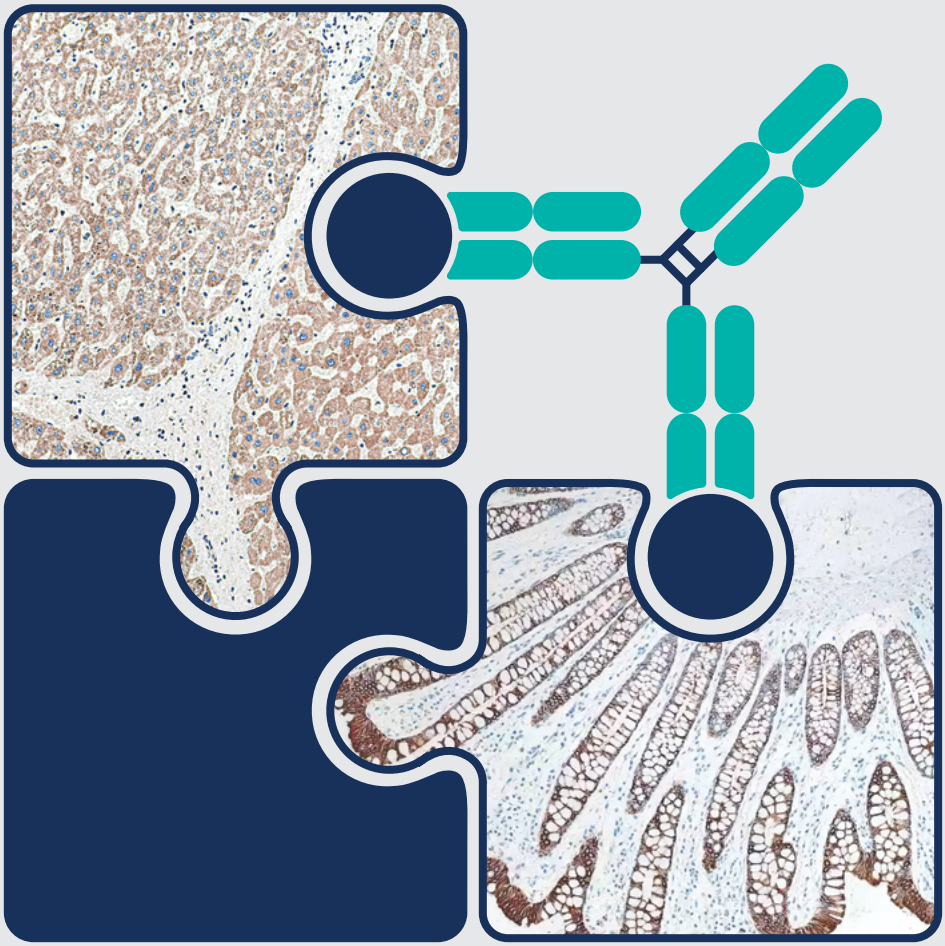
THE COMPLETE GUIDE TO

Immunohistochemistry



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ON THE COVER: Top left: IHC analysis of human liver tissue using CYC1 IHCeasy Kit (KHC0245). **Bottom right:** IHC analysis of human colon tissue using Cytokeratin 19 antibody (10712-1-AP).

Introduction to IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) enables the visualization of proteins in tissue while retaining its microstructure. It helps to demonstrate the exact position and distribution of the protein of interest in the analysed tissue section. You can then visualize direct comparisons between experimental or pathological states, e.g. between healthy and diseased tissues. During an IHC experiment, the protein of interest (antigen) is detected by the binding of a primary antibody. This antibody-antigen interaction is then visualized via chromogenic or fluorescent detection using a secondary antibody.

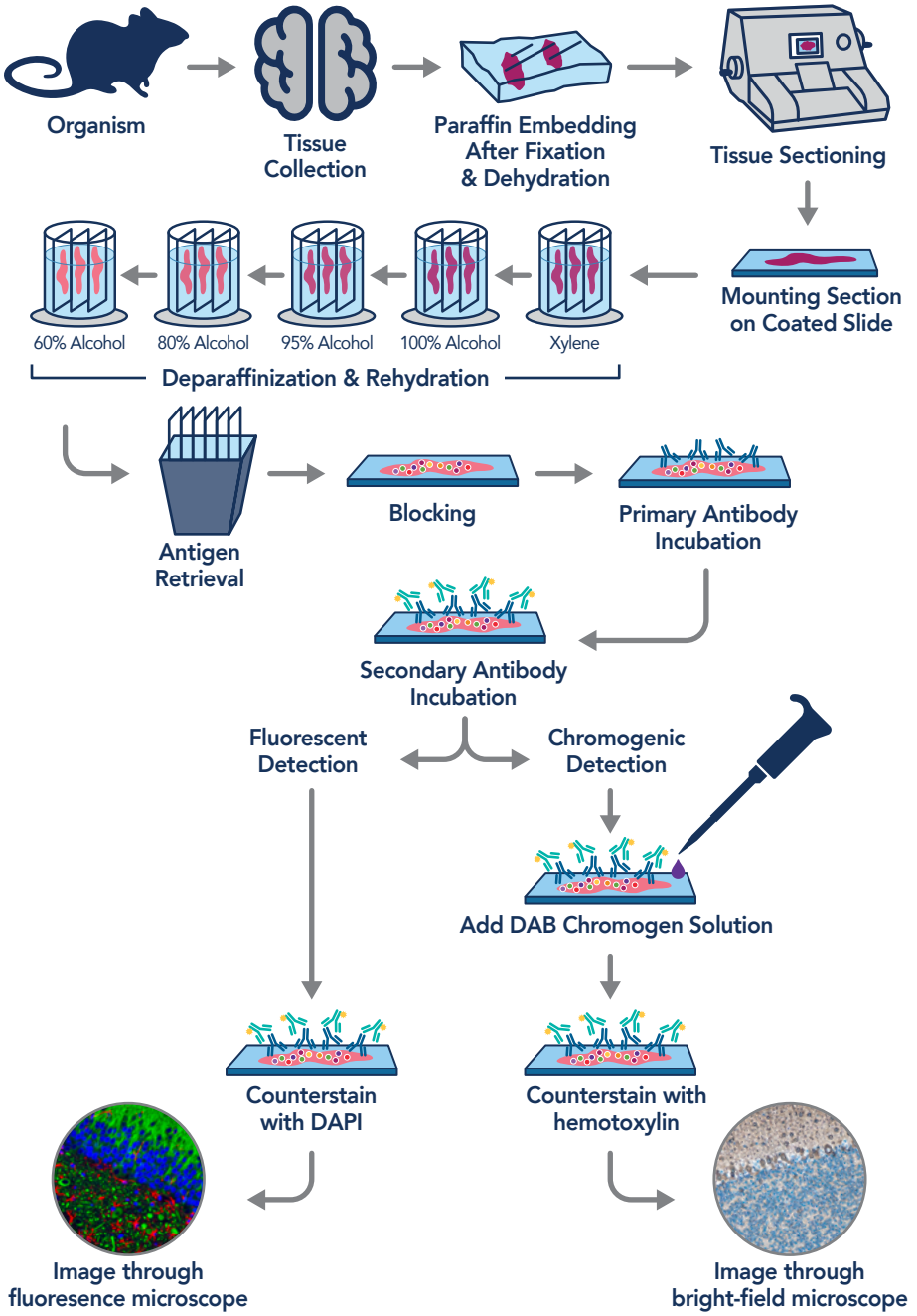
For an IHC experiment to provide the best results, several steps of the IHC protocol may require optimization to ensure specific antibody binding and optimal visualization of the target protein. An IHC protocol can be influenced by various factors, thus we must find the best working conditions to get strong and specific staining.

The following guide outlines the general IHC protocol, as well as some useful tips and hints on how to optimize each step.

Factors to be considered when designing an IHC experiment:

IHC Factor	Considerations
Sample type	FFPE, frozen
Antigen	Species, level of expression, subcellular location
Epitope	Conformation, post-translational modification
Blocking	Sera, BSA, commercial buffer, temperature, pH, dilution, incubation time
Primary antibody	Monoclonal vs Polyclonal
Secondary antibody	Species, label type
Signal detection	Chromogenic, fluorescent
Counterstaining	Chromogenic, fluorescent
Analysis	Microscope, software based analysis, evaluation by eye
Controls	Reagent controls, antigen controls

Overview of the IHC Workflow for FFPE Samples



General Protocols – Section 1:

MATERIALS AND EQUIPMENT

- Freshly collected tissue
- Cryo-embedding media (e.g. OCT) or paraffin
- PBS
- Sucrose
- 4% Paraformaldehyde (PFA) or acetone
- Microtome or Cryostat
- Embedding station
- Glass slides
- Coverslips
- Refrigerator
- Incubator
- Xylene
- Ethanol
- Antigen retrieval buffer
- Heating source
- Serum/BSA
- TBS
- Primary antibody
- Secondary antibody
- Diaminobenzidine tetrachloride (DAB)
- Hematoxylin
- Mounting media
- Microscope

General Protocols – Section 2:

TISSUE PREPARATION

Preparing fixed tissues for paraffin-embedded sectioning

Here we describe the procedure for preparing formalin-fixed paraffin-embedded tissue (FFPE) samples.

- For animal tissues, it is highly recommended to perform perfusion before dissection to remove blood from tissues to eliminate potential non-specific signals caused by endogenous IgG. Perfuse with ice-cold PBS to remove blood, followed by perfusion with 4% PFA until adequate fixation is achieved. Alternatively, dissect the desired tissues (<3mm thick), wash with cold PBS, place into a cassette and fix in 4% PFA or 10% formalin overnight at 4°C (no longer than 24h). The adequate volume of fixative for fixation by immersion is 50-100x of the sample size.
- Rinse with running tap water for 30 minutes.
- **Dehydration**
  - Move cassettes into 50% ethanol for 45 minutes.
  - Move cassettes into 70% ethanol for 45 minutes. (Tissues can be left in 70% ethanol for long term storage, if required.)
  - Move cassettes into 80% ethanol for 45 minutes.
  - Move cassettes into 95% ethanol for 45 minutes. Repeat this step a second time, using fresh 95% ethanol.
  - Move cassettes into 100% ethanol for 45 minutes. Repeat this step 2 more times, using fresh 100% ethanol each time.
- **Clearing**
  - Move cassettes into Ethanol : xylene (1 : 1 v/v) for 30 minutes.
  - Move cassettes into 100% Xylene for 30 minutes. Repeat this step a second time, using fresh 100% Xylene.
- **Wax infiltration**
  - Prepare beakers containing molten paraffin at 56-58°C.
  - Immerse cassettes into paraffin wax for 60 minutes. Repeat this step a second time, using fresh paraffin.

- **Embedding**
  - Move cassettes onto an embedding station.
  - Using a mold made of thick plastic or stainless steel, coat the bottom layer of the mold with molten paraffin.
  - Place the tissues on top of the base wax orienting them as desired. Partially cover with more paraffin. Place the cassette on top of the mold and completely fill the mold.
  - Place mold with tissues on a cold plate to solidify. The paraffin will solidify in 10-15 minutes.
  - Remove embedded tissues from the mold. Store at room temperature.

Sectioning tissues on a microtome

- Section paraffin blocks at the desired thickness (usually 4-5 µm) on a microtome and transfer them to a 40°C water bath containing distilled water.
- Transfer the sections onto charged slides coated with gelatin or poly-L-lysine. Place the slides in an oven to dry at 50-55°C for 4 hours (or 37°C for 6-8 hours) and store slides at room temperature until ready for use.

Preparing snap-frozen tissues for post-sectioning fixation

Here we describe how to snap-freeze tissue, section tissue on a cryostat and then postfix on a slide.

- Place the tissue block (freshly dissected, <5mm thick) onto a mold.
- Use cryo-embedding media (e.g. OCT) to fully cover the entire tissue block.
- Completely freeze the tissue on the base mold by submersion in liquid nitrogen.
- Store the frozen tissue block at -80°C until ready for sectioning. (See section on frozen sectioning below.)
- Once sectioned, fix slides by incubating them in either ice-cold acetone (-20 °C) for 20 minutes or 4% paraformaldehyde (PFA).

Preparing fixed tissues for frozen sectioning

Below is a general protocol for fixing tissues before sectioning on the cryostat.

- When possible, perfusion fixation is recommended for achieving the best tissue morphology and clear background. Perfuse with ice-cold PBS to remove blood, followed by perfusion with 4% PFA until adequate fixation is achieved. Alternatively, dissect the desired tissues and fix them in 4% PFA or 10% formalin overnight (no longer than 24 hours). The adequate volume of fixative for fixation by immersion is 50-100x of the sample size.
- Tissues should then be dehydrated for cryoprotection in fresh 20-30% sucrose, for 16-48 hours at 4°C.

- Embed the tissues in OCT medium using a mold and freeze by submersion in liquid nitrogen.
- Store the tissues at -80°C until ready for sectioning.

Sectioning tissues on a cryostat

Users must be fully trained and follow manufacturer guidelines before using the cryostat. The optimal cryostat temperatures recommended for different tissue types are displayed in the table below.

- Before sectioning, transfer the frozen tissue block to a cryostat at the recommended temperature (e.g. -20°C) and allow the frozen tissue block to equilibrate to the cryostat temperature (around 30 minutes).
- Section the frozen tissue block into a desired thickness. Use glass slides (we recommend charged slides) designed for IHC to prevent tissue slipping.
- Once cut, allow sections to dry thoroughly at room temperature. Sections can be stored in a sealed slide box at -80°C for later use. Storage might influence the antigenic potential, which varies for each protein. Therefore, minimizing storage time or using freshly prepared slides prior to staining is recommended.
- *(If necessary)* Fixation: after immediately removing from the freezer, incubate slides with either ice-cold acetone (-20 °C) or 4% PFA for 10-20 minutes. The end user must determine optimal fixation conditions for each tissue type.

Recommended Cryostat Temperatures 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

General Protocols – Section 3:

IMMUNOHISTOCHEMISTRY STAINING PROTOCOL

Below is an outline of a standard IHC protocol. Steps may need optimizing and adapting to suit individual requirements and different tissue types. All steps are carried out at room temperature unless stated otherwise.

Deparaffinizing and Rehydration (for FFPE sections only)

- Immerse slides in xylene for 10 minutes. Repeat this step in fresh xylene for 10 min.
- Rehydrate sections by sequentially incubating with 100%, 95%, 80% and 60% ethanol for 5 minutes each.
- Rinse sections with distilled water three times for 1–3 minutes each.

Antigen Retrieval (optional)

- Transfer slides to a microwave-proof container or a beaker on an electric stove and cover them with the recommended antigen retrieval buffer. If no antigen retrieval buffer is suggested, try Tris-EDTA (pH 9) first. See the "Antigen Retrieval" section for further information.
- Heat in the microwave on medium power for 10 minutes.
- Allow slides to cool in the antigen retrieval buffer for approximately 35 minutes.

Quenching and Blocking

- Rinse slides three times with 1X TBS for 3 minutes each.
- Incubate slides with 3% H_2O_2 solution (diluted in distilled water) for 10 minutes to quench endogenous peroxidase activity. For FFPE samples, this step is often not necessary. See the section on "Quenching" for more information.
- Rinse slides three times with 1X TBS for 3 minutes each.
- Prepare 1% BSA or 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 30-60 mins.

Primary antibody incubation

- Incubate sections with primary antibody diluted in 1X TBS for 1 hour, 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 experimental condition.
- Following primary antibody incubation, rinse slides three times with 1X TBS for 3 minutes each.

Chromogenic Signal Detection

- Apply sufficient horseradish peroxidase (HRP) labeled secondary antibody (we recommend a Polymer-HRP conjugate for best results) and incubate for 30 minutes at room temperature.
- Rinse slides three times with 1X TBS for 3 minutes each.
- Prepare an appropriate volume of the DAB chromogen solution based on manufacturer guidelines. Apply the substrate carefully and incubate for 5-10 minutes until a brown colour develops.
- Rinse sections gently with distilled water.
- Signal enhancement *(optional)*: Immerse slides in 0.5-1% $CuSO_4$ for 5 minutes.

Hematoxylin counterstaining (optional)

- To stain nuclei, add a few drops of Hematoxylin and incubate for 3 minutes.
- Rinse slides gently with distilled water.
- Transfer slides into a 1% HCl and 99% ethanol solution for 10 seconds and then rinse briefly in distilled water.

Fluorescent signal detection

- Add sufficient fluorophore labeled secondary and incubate at room temperature for 30 minutes in the dark. Ensure all steps following this are done in the dark or with minimal exposure to light.
- *Optional:* DAPI is an excellent nuclear counter stain. This can be added here or use mounting media with DAPI.

Dehydration and mounting

If performing fluorescence IHC, dehydration is not necessary.

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

Select the Right Kit for Your IHC Workflow

	IHC*easy* Kits Target-specific, complete IHC workflow kits containing pre-optimized primary antibody	**IHC Prep & Detect Kits** Kits for developing complete IHC workflows using any primary antibody of choice	**IHC Detect Kits** Kits for highly-sensitive chromogenic signal detection
Antigen Retrieval Buffer	✓	✓	
Washing Buffer	✓	✓	
Blocking Buffer	✓	✓	
Quenching buffer		✓	✓
Primary Antibody Dilution Buffer		✓	✓
Primary Antibody	✓		
Secondary Antibody	✓	✓	✓
Chromogen	✓	✓	✓
Signal Enhancer	✓	✓	
Counter Staining Reagent	✓	✓	
Mounting Media	✓	✓	
Control Slide *(optional)*	✓		
Step-by-Step Protocol	✓		

TISSUE PREPARATION

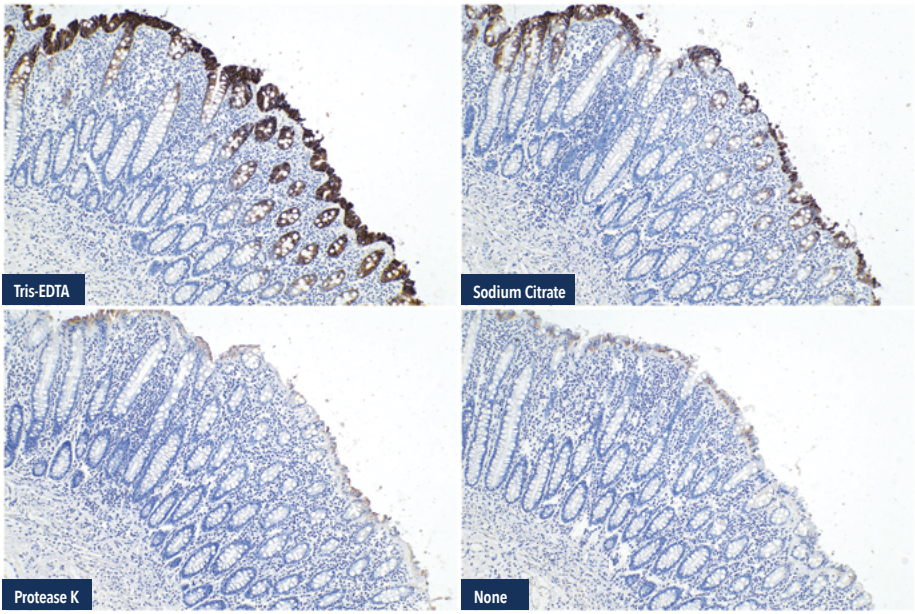
Correct processing of tissue samples prior to staining them with desired antibodies plays a critical role in determining the success of an IHC experiment. Once tissues are harvested it is important to fix or freeze them as soon as possible to prevent their degradation. Tissues can be either fixed in formalin followed by their embedding in paraffin (IHC-FFPE) or they can be frozen (IHC-Fr) followed by their fixation in formaldehyde or alcohol before or after cryosectioning. While fixation helps preserve tissue morphology and target antigenicity, embedding in a solid medium such as paraffin provides support to the tissues during sectioning.

	FFPE samples	Frozen samples
Fixation	Typically fixed with formaldehyde prior to embedding	Can be fixed with formaldehyde or alcohols either before or after sectioning
Embedding	Paraffin wax	Cryogenic embedding medium, e.g. OCT
Sectioning	Microtome	Cryostat
Advantages	• Preserves structural morphology of tissues • Easy to handle without damaging tissues • Tissue blocks can be stored for several years.	• Preserves antigenicity • Suitable for studying post-translationally modified targets • Simpler and shorter protocol
Challenges	• Can lead to masking of epitopes due to over-fixation • Lengthy and complex protocol	• Tissue structure may be impacted by ice crystals. • Thicker sections result in lower resolution and poor quality images.

ANTIGEN RETRIEVAL

Aldehyde-based fixatives such as paraformaldehyde or formalin act by creating cross-links between proteins in the tissue. In some cases, these cross-links mask epitopes, inhibiting antigen access by antibodies and resulting in weak or absent staining. Fortunately, this negative fixation effect can be reversed by a process called antigen retrieval. The type and method of antigen retrieval that works best depends on the tissue type and primary antibody. Our product-specific protocols state which method is best for each of our antibodies, validated using IHC.

Comparison of Different Antigen Retrieval Methods



KRT20 mAb (60183-1-Ig), human colon tissue

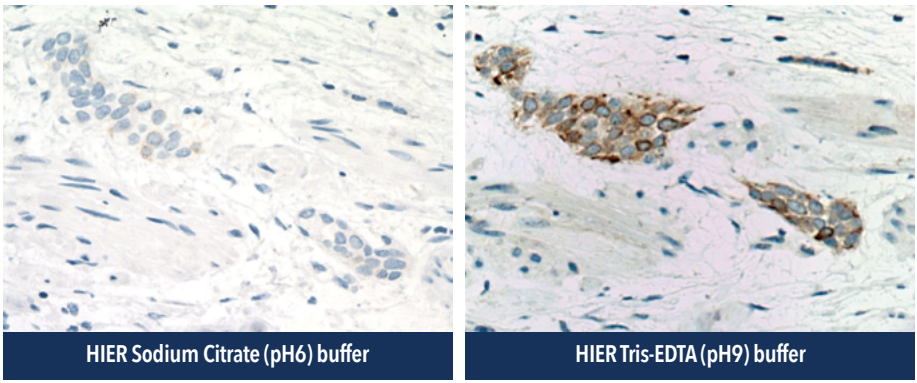
Heat-induced epitope retrieval (HIER)

HIER is carried by heating the slides in a specific buffer for a certain period of time. Different heat sources like a microwave, pressure cooker, water bath, or a hot plate are normally used for this step.

If not specified by the antibody manufacturer, first try HIER with Tris-EDTA (pH 9) buffer. If this does not produce optimal retrieval then it might be worth trying HIER with Citrate buffer (pH 6). Determine the optimal antigen retrieval conditions for your samples by seeing which buffer produces the best staining, further optimization can be achieved by varying the length of the incubation.

HIER acts by using thermal energy to break down the cross-links that bind to the surrounding proteins or peptides. Another mechanism by which HIER is thought to act is through removing calcium ions from the site of protein cross-links, a theory that is supported by the fact that some HIER buffers such as Tris-EDTA are calcium chelators.

Comparison of different HIER buffers



HIER Sodium Citrate (pH6) buffer | HIER Tris-EDTA (pH9) buffer

KRT20 mAb (60183-1-Ig), human urothelial carcinoma

General HIER protocol:

- First heat the buffer to around 95°C (near boiling).
- Apply the pre-heated buffer to the slides and incubate for 10–30 minutes.
- Remove from the heat and let the slides cool in the buffer for a further 35 minutes.

Proteolytic-induced epitope retrieval (PIER)

Proteolytic-induced epitope retrieval (PIER) technique results in a degradation of peptides to unmask the epitopes of interest. Trypsin and proteinase K are enzymes typically used in PIER, which act by breaking protein cross-links, which unmasks the hidden antigen and thus increases the staining intensity and specificity of the primary antibody.

General PIER protocol

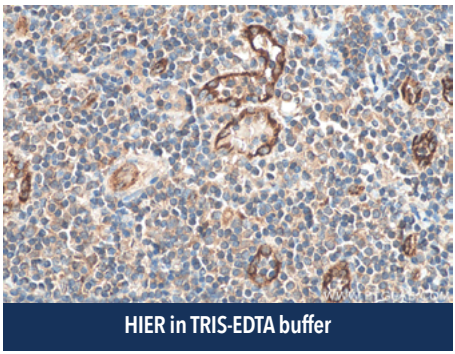
- Prepare the trypsin and pre-heat to 37°C. Pipette the enzyme solution onto the section.
- Place the slides in a humidified container and then into a 37°C incubator.
- After 15 minutes, remove the slides from the incubator and transfer to a rack in a container with tap water. Rinse in running water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

Other commonly used enzymes are proteinase k, pepsin, trypsin, and pronase.

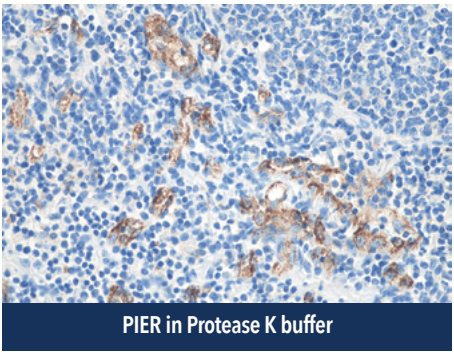
	HIER	PIER
Uses	Most common, gentle epitope retrieval	Good for difficult epitope recovery Can damage tissues
pH and Buffer	Tris-EDTA pH9 most used; Citrate buffer pH6; or other buffers at neutral pH	pH 7.4; proteinase K, trypsin, pepsin, pronase
Temperature*	95°C	37°C
Incubation Time*	~20 minutes	~10–15 minutes

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

Comparison of HIER versus PIER



HIER in TRIS-EDTA buffer



PIER in Protease K buffer

VWF pAb (27186-1-AP), human tonsilitis tissue

Buffers for heat-induced or proteolytic-induced epitope retrieval

Protease K Buffer (pH 7.4)

Tris-EDTA Buffer (pH 9.0)

Citrate Buffer (pH 6.0)

BLOCKING

Blocking is essential to prevent non-specific binding of the antibody or other reagents to the tissue. Even though the antibody might be specific, it can also show avidity for other epitopes. Antibody-antigen binding is affected by different intermolecular forces (e.g. hydrophobic binding, ionic interactions) and these forces can result in non-specific binding to endogenous molecules, serum proteins, or others. Non-specific binding then prevents visualization of the antigen-antibody binding of interest. Therefore, prior to incubation with the primary antibody, a blocking step should be carried out to block the entire epitopes on the tissue without damaging the desired epitope. Different commercial buffer systems are available.

Serum/Protein Blocking

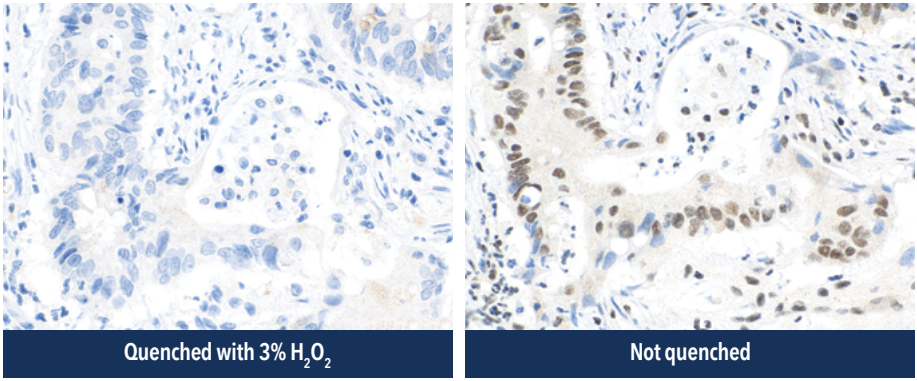
Serum (from the same species as the secondary antibody) or bovine serum albumin (BSA) is commonly used for blocking. Sera and BSA can help to prevent non-specific binding to the many hydrophobic side chains of proteins present in tissues. When multiplexing, blocking serum of all the hosts of the secondary antibodies is required (e.g. using normal goat and donkey serum). If BSA is used, the addition of 0.1-0.5% Triton-X or Tween can act as an additional blocking reagent to prevent unspecific binding.

Endogenous Enzyme Blocking (Quenching)

When using a horseradish peroxidase (HRP) or alkaline phosphatase (AP) conjugated antibody for detection, endogenous levels of the enzyme need to be blocked. This typically applies to kidney, liver, intestine and lymph tissue. HRP is blocked with buffers containing H_2O_2, and AP is blocked with buffers such as acetic acid or Levamisole.

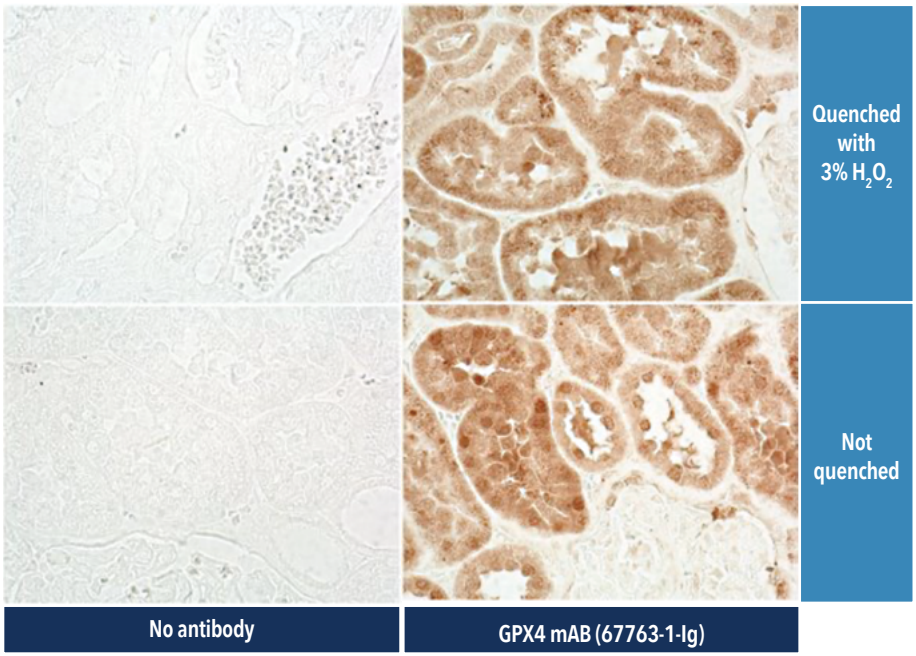
Note: With FFPE sections, quenching with H_2O_2 for HRP-DAB staining is not always necessary. When staining a tissue with any antibody for the first time, leave out the quenching step and see if there is any noticeable increase in background. H_2O_2 can be damaging to tissues if left on too long and adds further time and complexity to the protocol. Therefore, omitting this step could be beneficial. Please note that this step is imperative when using frozen sections as the endogenous enzymes are not deactivated.

Adverse Effects of Quenching



YY1 mAb (66281-1-Ig), human colon cancer tissue

No Effects of Quenching



GPX4 mAb (67763-1-Ig), human kidney tissue

Endogenous Biotin Blocking

Endogenous biotin is found to be high especially in liver, kidney and brain tissue. Biotin blocking is necessary if working with an avidin-biotin detection system.

IMMUNOSTAINING

Selecting and optimizing antibodies for IHC

High-quality antibodies are desirable for their high specificity and low cross-reactivity, generating reliable and successful staining. When choosing a primary antibody for IHC, there are certain factors that you should consider:

- Number of citations in the desired application(s)
- Consult the literature and antibody comparison resources.
- View the in-house validation data from the antibody manufacturer to ensure that the antibody is validated in the correct application in native samples.

Selecting a primary antibody for IHC

When choosing a primary antibody, the question of **clonality** always comes up regarding whether a monoclonal or polyclonal one is favorable for IHC. In general, polyclonal antibodies are more often used in IHC, but this decision always depends on the target of interest and sample type.

Advantages of *polyclonal* antibodies in IHC:

- Heterogenous population
- Recognizes multiple epitopes
- Less sensitive to changes (pH, tissue, buffer, protein confirmation), stable detection
- More likely to cross-react in rare species

Disadvantages of *polyclonal* antibodies in IHC:

- Not specific for one epitope, can lead to cross-reactivity with similar proteins
- Possibility of lot-to-lot variability

Advantages of *monoclonal* antibodies in IHC:

- Homogenous population
- Specific for a single epitope
- Detecting a single protein with high affinity, even though it shares sequence similarities to other proteins
- Good for long term projects, minimal lot-to-lot variability

Disadvantages of *monoclonal* antibodies in IHC:

- Sensitive to changes (pH, tissue, buffer, protein confirmation)
- Lower avidity can be an issue for low expressed targets
- Less chance of cross-reactivity with rare species

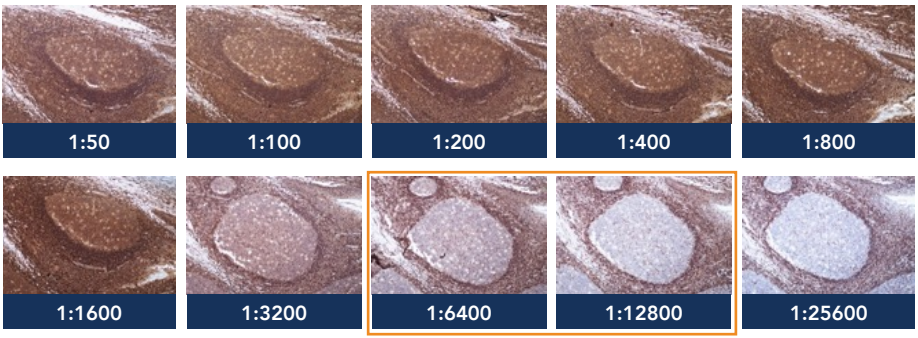
Optimizing a primary antibody for IHC

The optimal conditions for the primary antibody vary between experiments and tissue type. Therefore, these factors require optimization to generate the best results for each experiment.

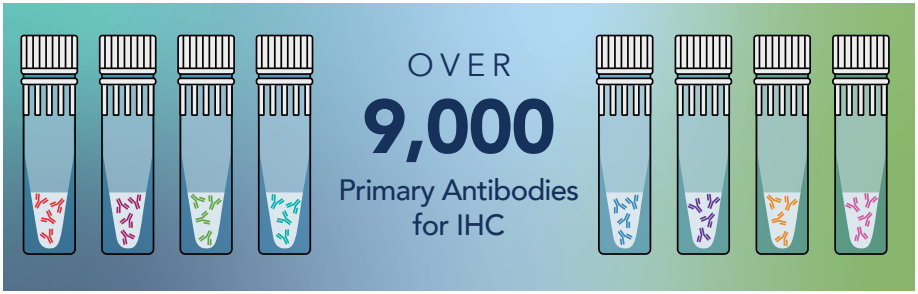
Below are some general steps on how to optimize your primary antibody in IHC:

- Try different antibody dilutions based on manufacturer guidelines. Alternatively, dilutions of 1 in 50 or 1 in 300 are good starting points for optimization. Make sure you keep the temperature and incubation time the same. You may need to go even lower to get the best staining.
- If you have specific staining, but high background signal, try varying incubation time and temperature. Here, shorter incubations at room temperature are recommended.
- If your antibody has a high-affinity antibody with a high concentration, try incubating for a shorter time.
- If your antibody has a high affinity with a low concentration, try increasing the incubation time and lowering the incubation temperature.
- Polyclonal antibodies in general can be used at a higher working dilution than monoclonal antibodies, due to monoclonals often having higher specificity for the target and being diluted at a higher concentration.

Optimization of antibody concentration



CD68 mAb (66231-2-Ig), human tonsillitis tissue



Selecting and optimizing secondary antibodies for IHC

Selecting the right secondary antibody for your needs is essential to help reduce the signal-to-noise ratio, leading to better quality staining. Below are some of the points to be aware of when choosing a secondary antibody:

- **Subclass specificity:** Polyclonal primary antibodies are mainly IgG isotypes. Primary monoclonal antibodies are occasionally of a different isotype and therefore require an isotype-specific secondary antibody. For example, mouse-derived antibodies have four IgG subclasses: IgG1, IgG2a, IgG2b, and IgG3. To select an antibody that targets all subclasses, ensure it is against the "heavy and light" (H+L) chain.
- **Cross-adsorption:** Secondary antibodies can go through an additional purification step to reduce potential cross-reactions with other species. Here, the secondary antibody solution is passed through different columns containing sera 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 certain tissue or cells (lymph nodes, spleen, macrophages etc). A whole antibody can bind to the Fc region, while an F(ab')2 fragment does not, ensuring higher specificity. F(ab')$_2$ fragments also allow for better tissue penetration due to their smaller size.

IHC controls

To ensure that the observed staining pattern is specific and not due to any cross-reactivity or non-specific binding, it is important to include control slides as part of your experimental design. Controls must be run alongside the main experiment.

Reagent controls

Secondary only control

- Process the slide as normal but omit the primary antibody.
- Ensures signal detected is specific for the target
- Test all fluorophores in isolation

Endogenous only control

- Process the slide as normal but omit the primary AND secondary antibody.
- Reveals level of autofluorescence/ artefacts

Isotype control

- Replace the primary antibody with a non-immune IgG of the same species, isotype and concentration as the primary antibody.
- Ensures observed signal is specific of FAB paratrope-epitope binding

Antigen controls

Positive control

- Control tissue that is known to express the protein of interest.
- Confirms the protocol is correct
- Validates negative results

Negative control

- Control tissue that is known not to express the protein of interest.
- Ensures that the observed staining pattern is due to specific signals
- Use **www.uniport.org** or **www.pax-bd.org** to find endogenous negative controls in tissues.
- CRISPR knockouts or siRNA knockdown cell lines are good negative controls.
- Checking mRNA levels of target proteins can also serve as a good negative control.

SIGNAL DETECTION

After incubation with a primary antibody, there are a few choices when it comes to signal detection: chromogenic or fluorescent, and direct or indirect.

Chromogenic Detection

Chromogenic IHC antibodies commonly use conjugations with enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase. When these enzymes encounter their substrate, they produce a dark precipitate, which can then be visualized using a light microscope. DAB (3,3'-diaminobenzidine) is the substrate of HRP and is the most used chromogen. DAB is insoluble, extremely stable, and heat resistant, thus making it a good choice for many researchers.

Substrates and Colors for Chromogenic Staining

Enzyme	Substrate – Color
HRP – Horseradish Peroxidase	DAB – Brown DAB and Nickel – Black AEC – Red TMB – Blue
AP – Alkaline Phosphatase	Fast Red – Red NBT and BCIP – Black to Purple

For detecting low-abundance targets, additional steps are often necessary for amplifying the primary antibody signal. While methods like avidin-biotin complex (ABC) detection or labeled streptavidin-biotin (LSAB) detection exploit the high-affinity complex between biotin and avidin for signal amplification, polymer-based amplification is biotin-free and utilizes a polymer backbone with multiple enzyme molecules.

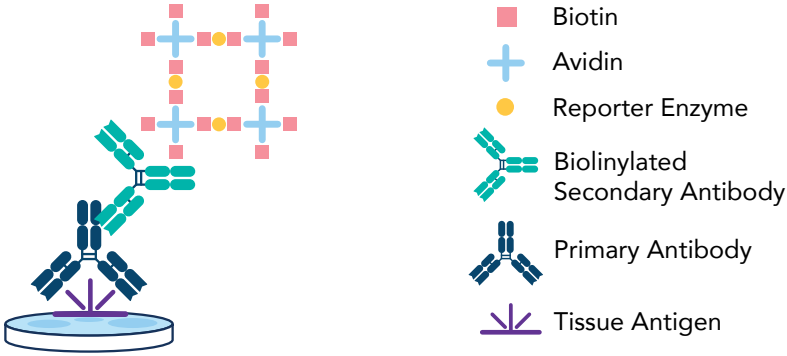
Avidin-Biotin Complex (ABC) Detection

- Utilizes biotin labeled secondary antibodies
- Large avidin–biotin–enzyme complexes bind to biotinylated secondaries.
- Addition of chromogenic enzyme substrate leads to signal amplification due to the presence of large multi-enzyme complexes.

Advantages: Highly sensitive

Limitations:

- High background resulting from the presence of endogenous biotin
- Large size of the ABC complex may hinder tissue penetration.



Labeled Streptavidin-Biotin (LSAB) Detection

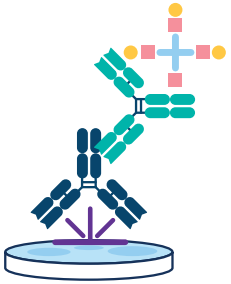
- Used instead of ABC detection whenever avidin-biotin-enzyme complexes are too large to penetrate target tissues
- Utilizes a streptavidin-enzyme conjugate

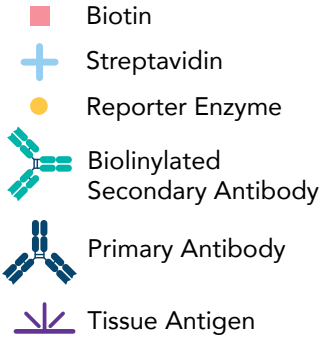
Advantages:

- 10X times more sensitive than the ABC method
- Less background and better tissue penetration

Limitations:

- High background resulting from the presence of endogenous biotin





Polymer-based Detection

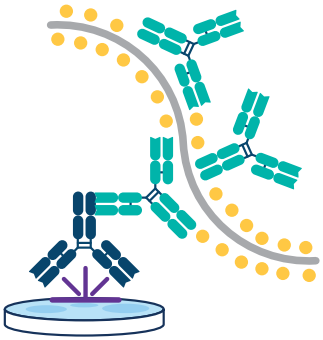
- Preferred method of detection when endogenous biotin, present in some tissues, leads to high background with the ABC or LSAB methods
- Utilizes secondary antibodies directly conjugated to a polymer backbone containing multiple enzyme molecules

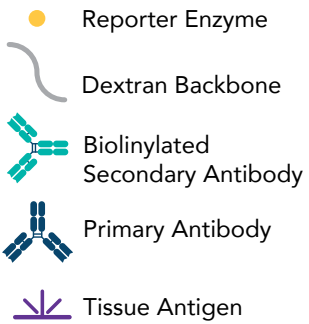
Advantages

- More sensitive compared to the ABC and LSAB methods
- Shorter protocol compared to the ABC and LSAB methods

Limitations

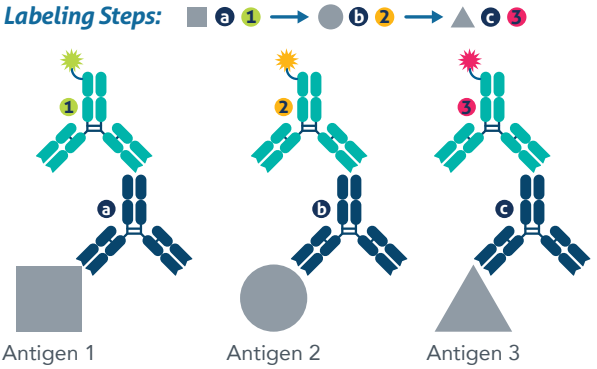
- May hinder tissue penetration due to the larger size of the polymer backbone





Fluorescence Detection

As an alternative to enzymatic detection, fluorescent signal detection during IHC can be performed using fluorophore-conjugated antibodies. The basic premise behind fluorescence detection is that the conjugated fluorophore is excited by a certain wavelength of light, which then undergoes a change in conformation, resulting in a lowering of energy state called the "Stokes shift." Light is then is emitted at a longer wavelength. Specialized fluorophores emit distinct excitation/emission spectra, meaning that you can image multiple target proteins in the same sample using different detection fluorophores, which is called multiplexing. Multiplexing can be done by using primary antibodies that are raised in different hosts, and can thus be targeted by different secondaries. Alternatively, this can be performed using directly conjugated primary antibodies (see the section below).



Primary Antibody

- a Rabbit-anti-antigen 1
- b Mouse-anti-antigen 2
- c Goat-anti-antigen 3

Secondary Antibody

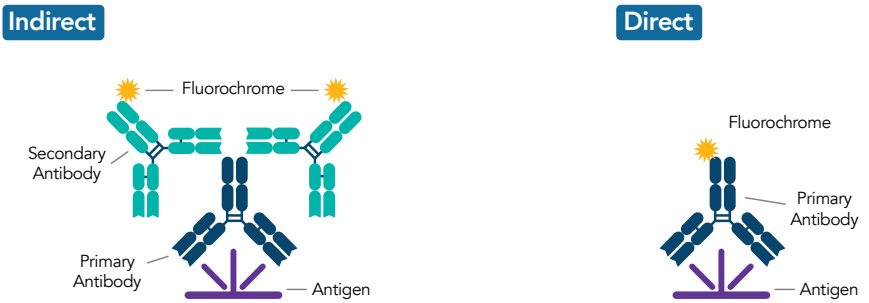
- 1 Donkey-anti-rabbit A488
- 2 Donkey-anti-mouse A546
- 3 Chicken-anti-goat A594

Chromogenic vs Fluorescent Signal Detection

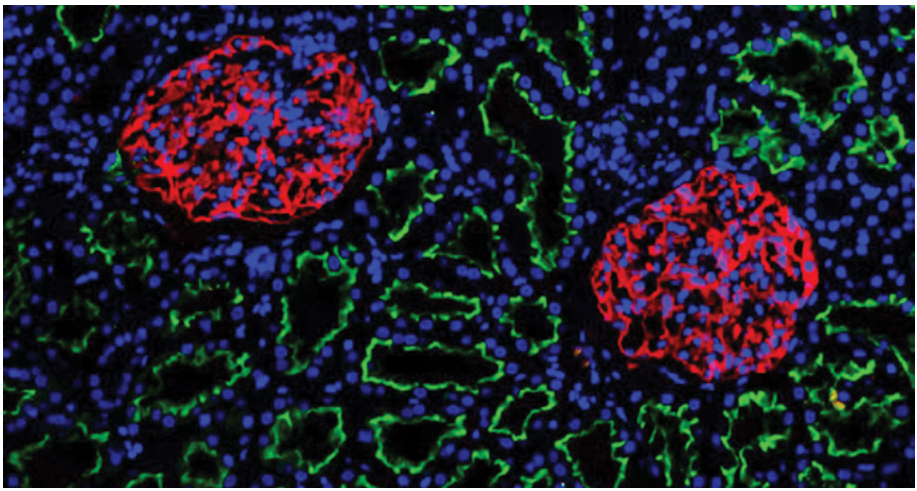
	Fluorescence	Chromogenic
Advantages	Excellent Multiplexing	Greater sensitivity
	Target Co-localization	Longer lasting signal
	Higher Dynamic Range	Easier imaging
Limitations	Staining will fade over time	Poor multiplexing/co-localization
	Lower sensitivity	Low dynamic range
	More complex imaging	Longer protocol

Indirect vs Direct Detection

Direct detection is an approach where a primary antibody is directly conjugated with an enzyme or a fluorophore, whereas indirect detection uses a labeled secondary antibody. The choice of the detection method depends on the expression level of the antigen. Directly labeled primary antibodies have slightly lower signal generation and are therefore best suited for highly expressed antigens. Direct detection is also advantageous for multiplexing experiments that analyze multiple protein targets simultaneously in the same sample, as this negates the need to find primary antibodies raised in different species. Medium-expressed antigens show the best signal when analyzed via a secondary labeled antibody, as this helps to amplify the signal intensity. For very low expressed proteins, indirect detection plus an enhancer (e.g. streptavidin or a polymer) helps to further amplify the signal.

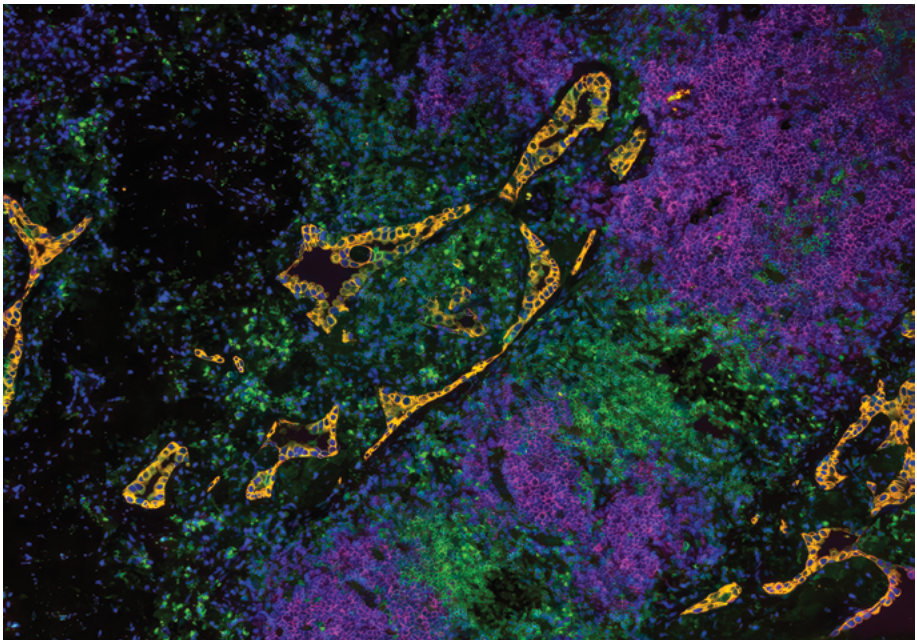


Indirect Staining

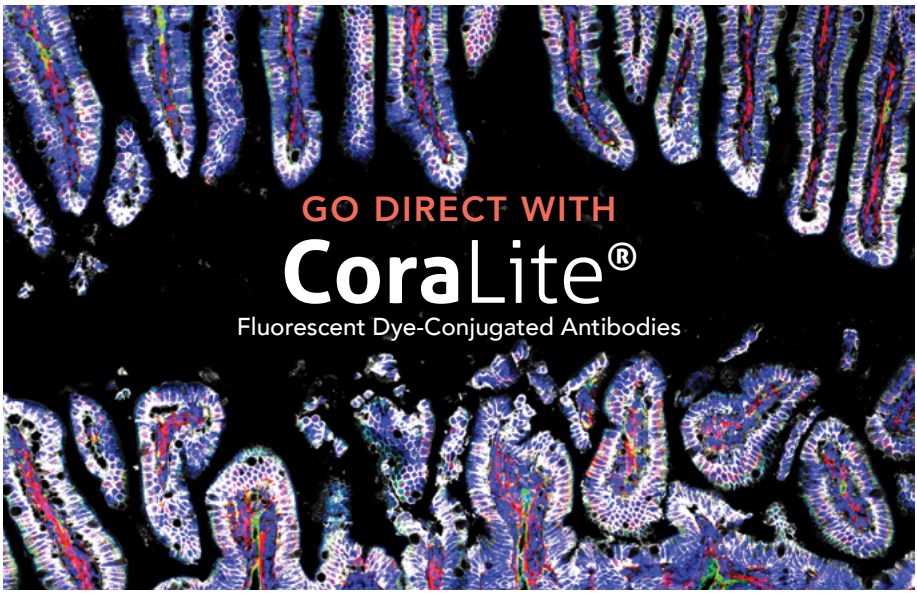


▲ IF analysis of human kidney tissue using PTPRO antibody (67000-1-Ig, Clone: 2F2B4) and CoraLite594-Conjugated AffiniPure Goat Anti-Mouse IgG(H+L), ACE2 antibody (21115-1-AP, green) and CoraLite488-Conjugated AffiniPure Goat Anti-Rabbit IgG(H+L).

Direct Staining



▲ IF analysis of human lung cancer tissue using CoraLite® Plus 488 CD3 antibody (CL488-60181, Clone: 3F3A1), CoraLite®555 pan-keratin antibody (CL555-26411, orange), and CoraLite® Plus 647 CD20 antibody (CL647-60271, Clone: 4A7G3, Magenta).



Common

TROUBLESHOOTING TIPS

No/Weak Staining

POTENTIAL CAUSE	SUGGESTED SOLUTION
Primary/secondary antibody lost activity	Use new lot of antibody. Improper storage – follow manufacturer's instructions.
Antibody conditions not fully optimized	Titrate antibody concentrations. Adjust incubation times.
Protein of interest not expressed	Run a positive control.
Protein of interest has low expression	Use signal amplification.
Damaged epitope	Try a different epitope retrieval technique.

Background staining / Non-specific staining

POTENTIAL CAUSE	SUGGESTED SOLUTION
Primary/secondary concentration too high	Titrate antibody concentrations. Decrease incubation times.
Non-specific antibody binding	Prolong blocking step. Increase concentration of blocking solution.
Poor sample washing	Repeat or prolong washing step. Increase concentration of blocking solution.
Damaged epitope	Try a different epitope retrieval technique.
Endogenous enzymes or biotin/lectin	Perform a quenching step.

Inappropriate cell morphology

POTENTIAL CAUSE	SUGGESTED SOLUTION
Harsh antigen retrieval conditions	Optimize buffers, temperature, pH, incubation time, concentration.
Folded or torn tissue sections	Optimize thickness of tissue slides. Re-cut sections using sharper blade.
Damaged epitope	Under-fixation. Change fixative or fixation time. Ice crystals can sometimes damage morphology during freezing. Try using FFPE tissues.

Uneven staining

POTENTIAL CAUSE	SUGGESTED SOLUTION
Basic slides or out of date slides	Used charged slides.
Dried out sections	Avoid drying of tissue sections during the staining procedure.
Delay in fixing tissues	Fix tissues immediately after collection to avoid antigen diffusion.
Incomplete deparaffinization	Use fresh xylene.
Distilled water loosens sections	Use PBS wash buffer instead of distilled water. Do not pipette wash buffer directly on tissues.
Issues with section adhesion	Avoid using protein-based section adhesives on charged slides to prevent inconsistent adhesion.

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