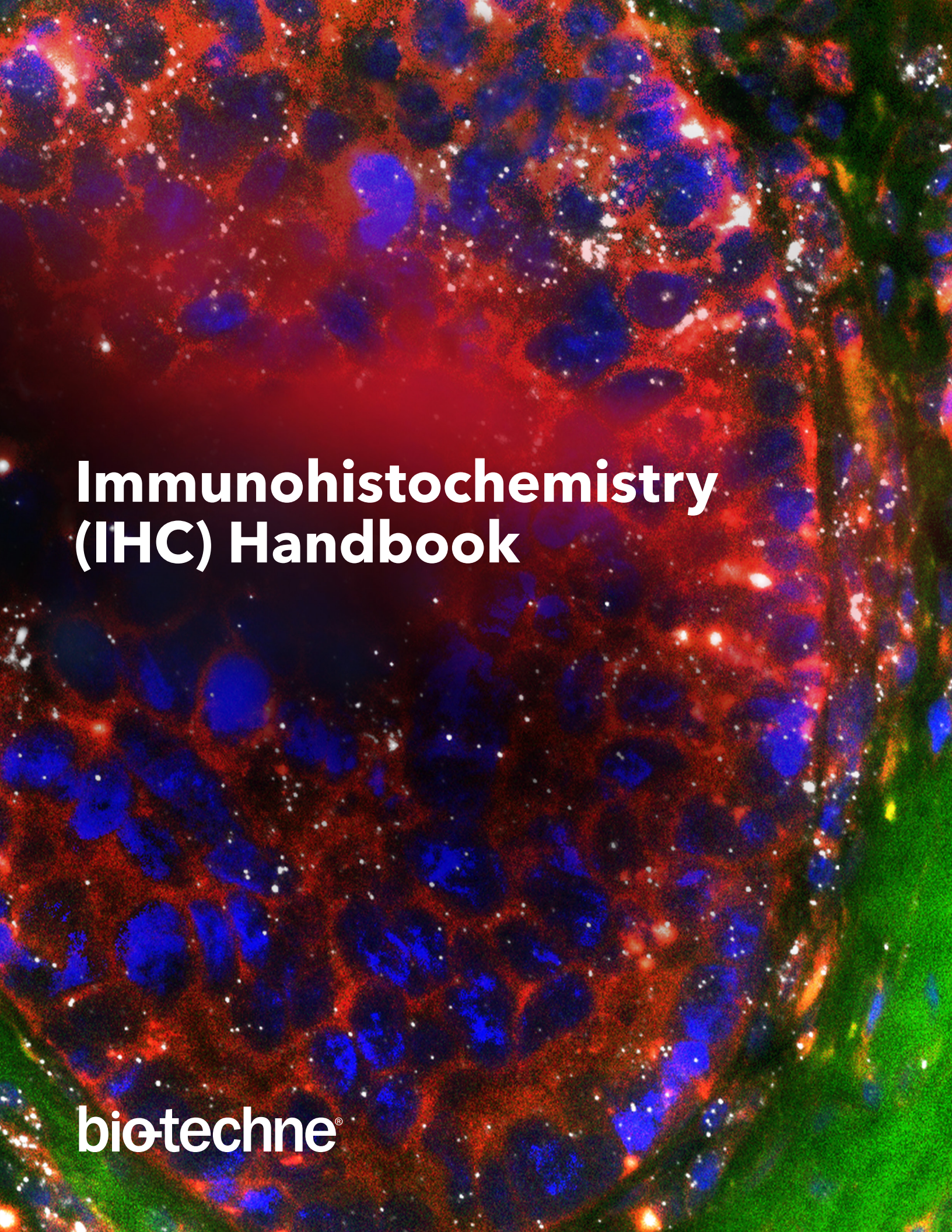


Immunohistochemistry (IHC) Handbook

bio-techne®

Data image on handbook cover provided by Ashley Oliver (Senior Research Associate, Antibody Development)

Detection of TGF beta mRNA (white), Arginase 1 protein (red), and CD204/ SR-AI/MSR protein (green) in human prostate cancer tissue using Integrated Co-Detection Workflow.

Immunohistochemistry Handbook - Fall 2022

This handbook is intended to serve as a starting point for understanding the principles behind Immunohistochemistry (IHC). Use this guide as a reference for understanding, performing, and troubleshooting IHC protocols, while developing and optimizing IHC assays.

Introduction to Immunohistochemistry

Immunohistochemistry (IHC) and immunocytochemistry (ICC) are techniques that use antibodies to detect antigens and provide semi-quantitative data about target protein expression, distribution, and localization. Both IHC and ICC are dependent on specific epitope-antibody interactions, but IHC refers to the use of tissue sections whereas ICC is performed on cultured cells or a cell suspension. The principles of IHC date to the 1930s and were first reported in the literature in 1942 when Dr. Albert Coons used a fluorescein-labeled anti-pneumococcal antibody to identify bacteria.

In both of these methods, positive marker staining is visualized via a molecular label, which can be chromogenic (enzymatic) or fluorescent. Briefly, samples are fixed to preserve cellular integrity and then subjected to incubation with blocking reagents to prevent non-specific binding of the antibodies. Samples are subsequently incubated with primary and often secondary antibodies, and the signal is visualized with a microscope

The most challenging aspect of IHC is determining and optimizing the experimental conditions required to generate a strong and specific signal for each antigen of interest. While the technique is relatively straightforward in principle, there are many variables to consider in a given experiment.

For example, visualization of an abundant protein in formalin-fixed tissue will likely require antigen retrieval and may be compatible with direct detection using a fluorochrome-conjugated primary antibody. In contrast, detection of a phosphorylation-dependent epitope in a section of frozen tissue may require signal amplification and additional blocking steps.

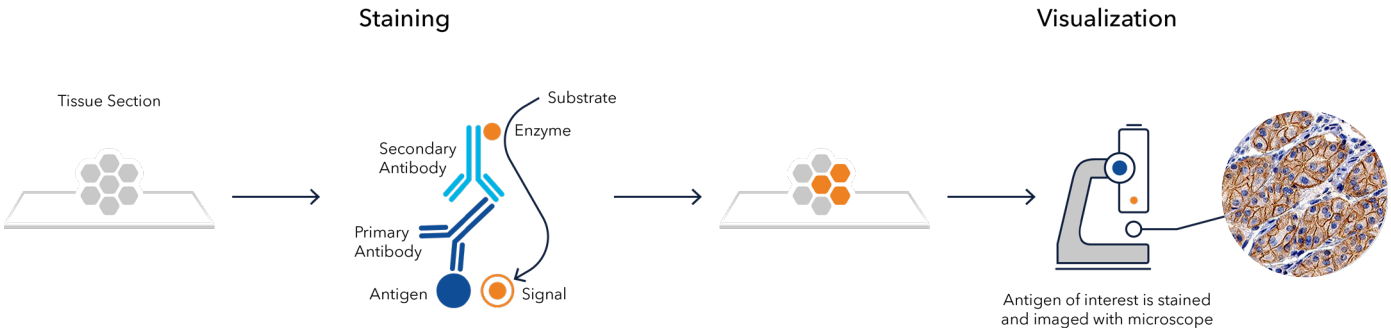


Figure 1. Traditional Chromogenic IHC Workflow. Enzymatic conversion of a chromogen substrate is required to visualize an antigen in traditional IHC assays. As an alternative, fluorescence detection can be used and is more suitable for multicolor experiments given the range of available fluorochromes and emergence of high contrast imaging.

Variables Influencing Experimental Design and Optimization

Variable	Factors
Antigen/Target	Species, expression levels, sample types, and subcellular location
Tissue	Species, type, section size, and thickness
Epitope	Dependence on conformation or post-translation modification
Sample Preparation	Paraffin-embedded or frozen
Fixation Method	Perfusion or immersion (with or without freezing)
Fixative	Aldehydes (crosslinking), alcohols (precipitating), or acetone
Blocking Reagent	Normal serum, BSA, casein, various detergents, and salt concentrations
Antigen Retrieval	None, Heat-induced Epitope Retrieval (HIER), and Protease-induced Epitope Retrieval (PIER)
Permeabilization	Triton-X 100, Tween-20, or saponin
Detection Method	Direct or indirect (with or without signal amplification)
Appropriate Controls	No primary antibody, isotype control, absorption control, tissue type control
Primary Antibody	Monoclonal or Polyclonal
Secondary Antibody	Species reactivity and label
Multiplex	Simultaneous or sequential antibody addition, and antibody host species
Labeling Method	Fluorescence or chromogenic
Label	Fluorochromes (numerous options) Chromogenic reagents: enzymes (HRP, AP) and substrates (DAB, AEC, NBT/BCIP)
Counterstain	Fluorescence: DAPI, Hoechst 33342 Chromogenic: Hematoxylin, Fast Green FCF
Mounting Reagent	Fluorescence: anti-fade mounting medium Chromogenic: aqueous mounting medium
Visualization and Analysis	Fluorescence microscope or standard light microscope

The above table is not intended to be exhaustive but rather summarizes common factors influencing IHC experimental design at Bio-Techne.

Multiplexing

The emergence of multiplex IHC has enabled a more comprehensive analysis of protein expression, complex cell-cell interactions, and spatial distribution of cell populations. While flow cytometry analysis also allows for detection of multiple markers within a cell population, it lacks contextual information on the interaction between markers or their spatial relationship. Multiplexing IHC is particularly advantageous when studying the tumor microenvironment (TME) for its prognostic and diagnostic benefits. The ability to spatially resolve multiple biomarkers simultaneously improves the understanding of the complex microenvironment consisting of various cell types, cytokines and growth factors, inflammatory factors, and extracellular matrix components. Additionally, regarding the TME, multiplexing can improve clinicians' ability to stratify cancer patients and better predict patient outcomes.

Multiplexed antibody-based imaging methods are categorized based on the type of antibody tag, such as fluorophores, metal ions, and DNA oligonucleotide barcodes. This technique can be performed using either sequential or simultaneous staining methods. Multiplex cycling platforms such as the Akoya Biosciences® PhenoCycler®, formerly CODEX®, which relies on DNA barcodes, and Canopy Biosciences® ChipCytometry, which uses fluorescently conjugated antibodies, employ consecutive cycles of reporter addition/removal or staining/bleaching, respectively. Imaging Mass Cytometry™ (IMC; Fluidigm) and multiplex ion beam imaging (MIBI) are two common technologies that utilize metal-conjugated antibodies and allow simultaneous imaging of over 40 biomarkers in a tissue section at single-cell resolution.

While IHC multiplexing allows for detection of multiple target proteins at the same time, it can also be combined with in situ hybridization (ISH) for detection of both RNA and protein within the same tissue. ISH-IHC analysis provides sensitive detection, characterization, and localization of nucleic acids and proteins while conserving the cell and tissue structural integrity.

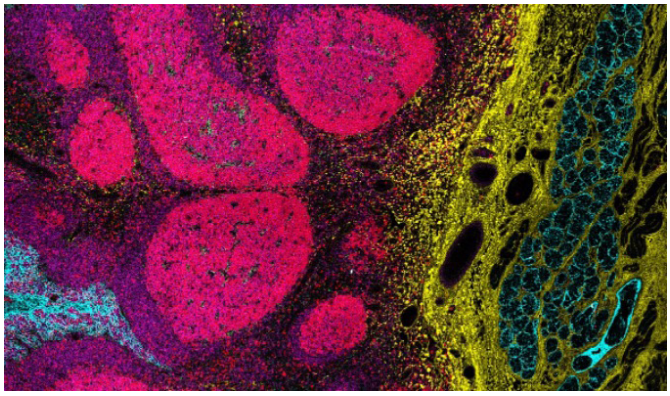
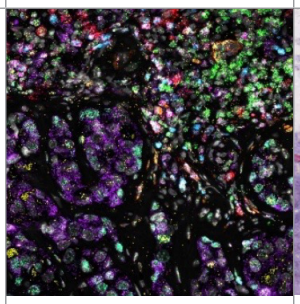
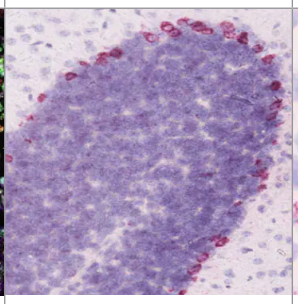
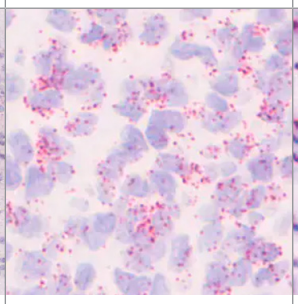
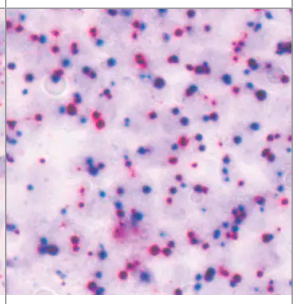


Figure 2. IMC multiplexed antibody image in human tonsil. Detection of markers using metal-conjugated antibodies in human tonsil: CD19-142 Nd (magenta), CD38-141 Pr (lime), Collagen Type I-169 Tm (yellow), E-Cadherin-158 Gd (cyan; R&D Systems, Catalog # AF748), Ki670168 Er (red). Image courtesy of verified customer review.

ISH-IHC (RNA-Protein Co-Detection)

ISH is a technique that employs probes to detect specific DNA or RNA sequences in heterogeneous cell populations such as fixed tissue sections or cells. RNA ISH reveals spatial information about gene expression within the sample. This technique is advantageous when there is no ideal antibody for a protein or protein expression is too low and an antibody is not sensitive enough. Additionally, ISH is particularly useful for identifying the cellular origin of secreted proteins, like cytokines and growth factors. ACD's RNAscope™ technology is a highly sensitive and specific ISH assay for single molecule RNA detection with single-cell resolution.

The proprietary double Z probe design amplifies target-specific signals while suppressing background noise from non-specific hybridization. RNAscope ISH technology can be employed across a number of research areas such as cancer, immuno-oncology, neuroscience, immunology, and more. Common targets for ISH include cytokines and chemokines, tumor markers, neuronal genes, splice variants, immune cell activation markers, and viral RNA.

	RNAscope	miRNAscope	BaseScope	DNAscope
Molecule Type	mRNA	Small non-coding RNAs including miRNAs, ASOs, and siRNAs	Exon junctions, splice variants, short/highly homologous RNA sequences, and point mutations	Gene copy number variations (amplifications/deletions) and gene rearrangements/ fusions
Target Size	>300 nt	17-50 nt	50-300 nt	>3000 nt (Vector/Viral) and >20,000 nt (Chromosomal)
Image Example				
	RNAscope™ HiPlex v2 12-plex target-specific marker probes used to detect immune cells, tumor cells, chemokines and cytokines in ovarian cancer tissue.	miRNAscope™ assay used to specifically detect miR-138-5p in Purkinje cells in cerebellum of the mouse brain.	Splice Variant Example showing detection of EGFRvIII+ in glioblastoma with BaseScope v2 assay.	DNAscope chromogenic duplex (red/blue) staining in ALK Break-apart positive cell line using the target probes HS-ALK-BA-5′/ HS-AK-BA-3′. Break apart events are detected through appearance of blue dots.

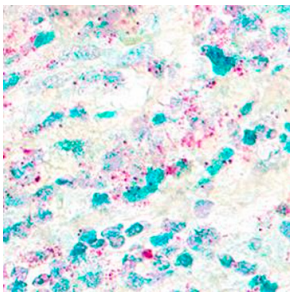


Figure 3. *In situ* detection of CD4+FoxP3+ Regulatory T cells (Tregs) in human breast cancer using RNAscope RNA-protein co-detection assay. CD4 mRNA (red) and FOXP3 protein (green) were detected in FFPE tissue sections of human breast cancer. ACD's Integrated Co-Detection Workflow was performed using ACD RNAscope Probe Hs-CD4 (ACD Catalog # 605608) and Rabbit Anti-FoxP3 Polyclonal Antibody (Novus Biologicals Catalog # NB600-245) at 1:200 dilution. Tissue was stained on Leica Bond RX using RNAscope™ 2.5 LS Reagent Kit-RED (ACD Catalog # 322150), BOND Polymer Refine Detection (DAB) and Hematoxylin, BOND Polymer Refine Red Detection and Hematoxylin and RNAscope™ 2.5 LS Green Accessory Pack (ACD Catalog # 322550). Tissue was counterstained with 50% hematoxylin (blue).

When used alongside IHC, ISH provides a more comprehensive overview of the complex molecular mechanisms involved in biological pathways. ISH and IHC techniques share a similar workflow including sample fixation and signal amplification. Thus, methods have been developed to collect RNA and protein expression data on the same tissue sample, referred to as ISH-IHC or RNA-protein co-detection, which can be performed either sequentially or following an integrated co-detection protocol.

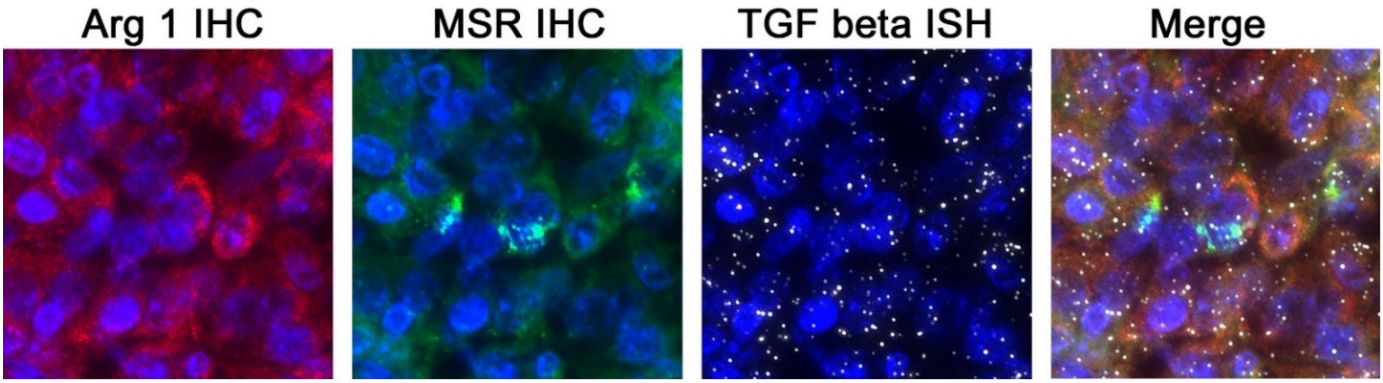


Figure 4. Detection of Arginase 1 protein (red), CD204/SR-AI/MSR protein (green) and TGFβ1 mRNA (white) in human prostate cancer tissue using Integrated Co-Detection Workflow.
ACD's Integrated Co-Detection Workflow (ICW) was performed on formalin-fixed paraffin-embedded tissue sections of human prostate cancer using Rabbit Anti-Human Arginase 1 Polyclonal Antibody (Novus Biologicals, Catalog # NBP1-32731) at a 1:50 dilution, Goat Anti-Human CD204/SR-AI/MSR Polyclonal Antibody (R&D Systems, Catalog # AF2708) at 3 µg/mL, and ACD RNAscope Probe Hs-TGFβ1 (Advanced Cell Diagnostics, Catalog # 400888). Tissue was stained using Donkey Anti-Rabbit IgG NorthernLights™ NL557-conjugated Antibody (R&D Systems, Catalog # NL004), Donkey Anti-Goat IgG NorthernLights™ NL493-conjugated Antibody (R&D Systems, Catalog # NL003), RNAscope™ LS Multiplex Fluorescent Reagent Kit (Advanced Cell Diagnostics, Catalog # 322800), and TSA Cyanine 5 (Akoya Biosciences, Catalog # NEL745001KT). Tissue was counterstained with DAPI (blue).

Applications and Benefits of ISH-IHC

- **Validate Antibody Specificity** – RNA-protein co-detection can be used to validate the specificity of the antibody by simultaneously comparing the localization of the RNA and protein signal for the same target, which qualifies as orthogonal antibody validation.
- **Visualize Source of Secreted Proteins** – Target the transcript of secreted proteins, such as cytokines or chemokines, and use protein markers to identify the cellular source.
- **Ascertain Marker Expression, Activation, and Spatial Mapping** – Detection in a morphologically relevant context is used to assess cell-cell interactions (i.e. hormone-receptor interaction, endocytosis or excretion). Immune cells that infiltrate the tissue microenvironment (TME) can be characterized using activation markers detected by ISH and immune cell markers such as CD3, CD4, CD8, CD45, and CD68 by IHC. Similarly, this approach can be applied to brain mapping to detect gene expression in neuronal subtypes using ISH alongside standard neuronal markers by IHC.
- **Increased Multiplexing Capability** - Several markers are visualized simultaneously in tissue and RNAscope probes for ISH can be employed when an adequate antibody is not available, or protein expression is too low to detect by traditional IHC.
- **Identify Transcript Variants** – Detect cell-type specific expression of splice variants and gene mutations.
- **Visualize small RNAs** – Study expression of microRNAs (miRNAs) implicated in cancer or track small interfering RNA (siRNA)/antisense oligonucleotides (ASO) therapeutics in pre-clinical models by combining cell-marker specific antibodies.

Learn More About RNAscope™ Technology From Bio-Techne

IHC WORKFLOW

Sample Preparation, Fixation, and Sectioning

Sample Preparation, Fixation, and Sectioning

Tissue Processing: Formalin-Fixed, Paraffin-Embedded (FFPE) vs. Frozen

IHC can be broadly classified into two forms based on the type of tissue processing involved: IHC- formalin-fixed, paraffin-embedded (FFPE), and IHC-frozen (Fr). Often the preservation method is closely associated with the type of fixation. Formalin-fixed tissues are commonly paraffin-embedded following fixation, while frozen tissue sections can be fixed with formaldehyde or alcohol prior to or following cryosectioning.

While paraffin embedding is thought to better preserve morphological details and offers the best option for long-term preservation of tissue samples, cryopreservation is considered to better preserve enzyme and antigen expression. The optimal method for each experiment should be determined by considering the nature of the antigen, its subcellular location, and desired method of detection, among other factors.

	Formalin-Fixed, Paraffin Embedded (FFPE)	Frozen
Fixation	Prior to embedding into paraffin	Performed either before or after cryosectioning
Common Fixative	Formaldehyde	Formaldehyde or Alcohols
Sectioning	Microtome, 4-10 µm sections	Cryostat/Cryotome, 5-20 µm sections
Storage	Ideally, fresh sections should be cut after 4 weeks due to loss of antigenic epitopes. For long-term storage (several years), coating of slide in paraffin is recommended.	Short-term: 1 year at -80°C
Advantages	• Ease of handling • Preserves structural morphology • Blocks can be stored long-term	• Preserves enzyme and antigen function • Useful for study of post-translationally modified protein, DNA, or RNA
Limitations	• Variable fixation times • Fixation can mask epitopes	• Less optimal for studying structural morphology • Ice crystals may impact tissue structure • Endogenous enzymatic activity may impact the IHC detection method

Paraffin-Embedding Tissue

Because paraffin is immiscible with water, tissue must be dehydrated before adding molten paraffin wax. Dehydration is achieved by immersion in increasing concentrations of alcohol. This approach allows for a gradual change in hydrophobicity and minimizes cell damage. Following dehydration, the tissue is incubated with xylene to clear any remaining ethanol. Paraffin is typically heated to 60 °C for embedding and is then allowed to harden overnight.

The tissue is subsequently cut with a sharp blade into ultra-thin slices using a microtome. Sections are then dried onto microscope slides and can be stored at room temperature for extended periods of time. Tissue sections must be rehydrated before commencing the IHC protocol. Paraffin is the cheapest and most commonly used substance for tissue embedding. However, tissue can also be embedded in plastic, which sets harder and allows sectioning of thinner tissue slices (1.5 µm versus 5 µm).

Fixation

All samples used in an IHC experiment must be fixed. The fixation process preserves the histologically relevant morphology of tissues for future analysis and maintains antigenicity of target molecules by preventing autolysis and target protein degradation. Fixation alters the chemical composition of tissues and often requires a compromise between preserving tissue structure and preserving the epitope. For the best results, tissue should undergo rapid and uniform fixation.

Whole animal perfusion (Figure 5A), using an animal's vascular network to disperse the fixative solution, is the preferred method for tissues from small animals including mice and rats. Alternatively, dissected tissue or organs can be directly immersed in the fixative immediately after tissue collection. (Figure 5B).

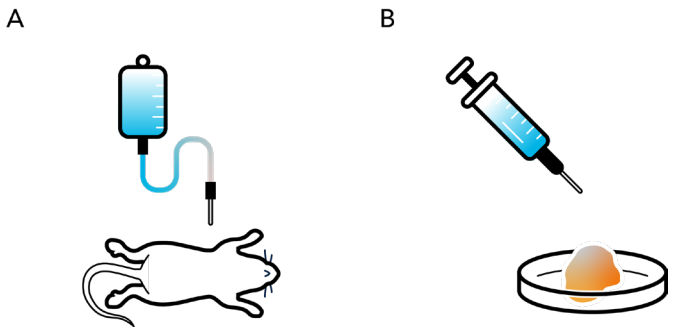


Figure 5. Fixation Methods. Perfusion (A) versus Immersion (B)

Fixation Method	Specifications	When To Use It	Advantages	Disadvantages
Perfusion Fixation	Perfusion of 4% paraformaldehyde solution through the vascular system.	For large, intact tissue of small animals.	• Rapid dispersion of fixative throughout the animal • Uniform fixation of large organs	• Technically challenging • Time consuming
Immersion Fixation	Immersion of dissected tissue (~10 mm thick) at room temperature in fixative volume 50-100x greater than the tissue volume.	For small pieces of dissected tissue.	• Quick • Technically simple	• May result in incomplete fixation of large tissue • May fail to penetrate all regions of tissue at the same rate

Type of Fixatives

Aldehydes	4% formaldehyde in phosphate-buffered saline (PBS) is the most common fixative for preserving protein targets in tissues. Formaldehyde reacts with amino groups in proteins to form methylene bridges that crosslink proteins in tissue sections. These molecular crosslinks can mask protein epitopes from antibody binding and may require the addition of an antigen retrieval step to allow antibody access to the epitope. Additionally, formaldehyde-mediated tissue fixation has been shown to induce translocation of phosphorylation-dependent epitopes from the membrane to the cytoplasm.
Alcohols	The predominant alcohols used for fixation are ≥70% methanol and ≥80% ethanol. Alcohols work by removing and replacing water molecules in tissue, which can destabilize hydrophobic bonds and alter the tertiary structure of proteins. This also causes the precipitation of soluble proteins, making alcohol-mediated fixation more appropriate for detection of membrane bound proteins. Alcohol, unlike formaldehyde, does not mask epitopes, thus antigen retrieval does not need to be done on tissue fixed with alcohol.
Acetone	Acetone is also used as a strong dehydrant and precipitant, typically applied to sections of snap-frozen tissues. Acetone fixation is generally mild and may be followed by fixation with alcohols or formaldehyde.

No single fixation condition works for all target molecules and tissues. The duration and method of fixation can impact preservation, tissue integrity, and antibody binding capacity. Ideal fixation conditions should be determined to optimally preserve tissue for each experiment. Incomplete fixation (under-fixation) of cells or tissues may allow rapid proteolytic degradation of target proteins within the tissue and can reduce specific immunoreactivity. Any issues arising from incomplete fixation, such as autolysis, cannot be reversed or fully rectified in later steps. Excessive fixation (over-fixation) may result in masking of the epitope or in strong non-specific background staining that can obscure specific labeling.

What's the difference between paraformaldehyde, formaldehyde, and formalin?

Paraformaldehyde (PFA) is the polymerized form of formaldehyde and is not itself a fixing agent. Formaldehyde can be prepared by dissolving PFA in PBS using heat and sodium hydroxide (NaOH). Formalin refers to a saturated formaldehyde solution and some commercial formalin solutions include methanol as a stabilizer to prevent formaldehyde polymerization. A 10% formalin solution is equivalent to a 3.7% formaldehyde solution.

Protocol: Formalin-Fixed, Parrafin-Embedded (FFPE)

1. Fix the tissue of interest by immersing it in 10% neutral buffered formalin (4% formaldehyde) for 4-24 hours at room temperature. Fixation time and temperature depends on tissue type/size. After fixation, wash the tissues 3x in PBS.

   Note: It is not recommended to fix tissue for >24 hrs because over-fixation can cause masking of the antigen. If necessary, tissues can be transferred to alcohol after fixation prior to starting the embedding process.

   Note: Formaldehyde-based solutions should be aliquoted and frozen, or stored at 4–8°C for up to one month.

2. Dehydrate by full immersion of tissue in the following solutions (2x for 30 minutes each):

   a) 70% Ethanol
   b) 95% Ethanol
   c) 100% Ethanol
   d) Xylene

Protocol: Formalin-Fixed, Parrafin-Embedded (FFPE) contd.

3. Embed the tissue in molten paraffin. After the paraffin solidifies keep the blocks at 4°C until sectioning.

   Note: Paraffin melts at 57°C.

4. Keep FFPE blocks chosen for sectioning tissue face down in an ice water bath, to hydrate the tissue and avoid cracking during sectioning. Certain tissues (e.g. liver or spleen) require this to be repeated after 10-20 sections.

5. Use a microtome to cut the paraffin tissue blocks into 4-10 µm thick sections and transfer them to a 37°C water bath with distilled water.

6. Pick up the floating tissue section using a clean histological slide (coated with gelatin or poly-L-lysine to improve adhesion of tissue sections) and allow mounted tissue sections to dry for about 30 min on a 37°C hot plate followed by baking them for 2-3 hours in a 40°C oven.

   Note: Slides can be safely stored at room temperature until ready for staining. Storage of cut slides for longer than 1 month is not recommended.

Protocol: Method I - Frozen Samples (Whole Animal or Dissected Tissue with Aldehyde/Formalin Fixation)

1. Fixation:

   Whole animal - Perfuse the animal with warm saline solution to flush the blood out of vasculature and immediately follow this by perfusion with freshly prepared 4% formaldehyde.

   Dissected Tissue - Immerse tissue in 4% formaldehyde (50-100x the tissue volume) for 4-24 hours at room temperature. Wash tissue with PBS.

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

2. Cryoprotection:

   Whole animal - Continue perfusion with a 10% sucrose solution.

   Dissected Tissue - Immerse dissected tissue in a 10% sucrose solution overnight at 4°C.

   Note: Tissue will sink in sucrose solution upon reaching equilibration.

3. Embed tissue in OCT cryostat sectioning medium and store at -80°C until ready for sectioning.

   Note: Tissue can be safely stored for up to 1 month.

4. When ready for sectioning, move the embedded tissue directly into the cryostat and use OCT medium to mount it to the chuck. Allow the temperature of the tissue to equilibrate with the cryostat. Cut the tissue in 5-20 µm thick sections. Mount tissue sections onto gelatin or poly-L-lysine coated slides by placing the cold sections onto warm slides.

   Note: Tissue can be safely stored for up to 1 month.

Protocol: Method II - Cryopreservation & Sectioning of Alcohol Fixed Tissues

1. After dissection, immediately snap freeze tissue with isopentane cooled by dry ice. To do this, fill the styroform box with dry ice and pour isopentane over it so that chunks of dry ice are halfway immersed in isopentane. Place a metal cryostat chuck on top of the dry ice/isopentane slurry and then mount piece of tissue on top of the chuck and cover it with OCT.

2. Wait until tissue in OCT is completely frozen (OCT turns from clear to opaque white solid material) and transfer chuck with frozen tissue into the cryostat. Allow the temperature of the tissue to equilibrate to the temperature in the cryostat.

3. Cut the tissue in 5-20 µm thick sections. Mount tissue sections onto gelatin or poly-L-lysine coated slides: place tissue sections onto cold slides and then warm the slides up to thaw tissue sections so that they can permanently adhere to the slides.

   Note: Slides can be safely stored for 6-12 months at -80°C until ready for fixing. Uncut tissue can be re-stored at -80°C.

4. Remove slides from freezer and fix with cold fixative (acetone or methanol) for 10 minutes. Proceed to staining.

How to Perform Immunostaining on Organoids

Organoids are 3D miniature versions of organs that are generated in vitro and can be derived from either tissue or stem cells. Due to their unique 3D nature, specific protocols have been designed to preserve morphology and analyze marker expression via immunostaining.

View Protocol for Harvesting, Fixing, and Immunostaining Organoids

IHC WORKFLOW

(Epitope)
Antigen
Retrieval

(Epitope) Antigen Retrieval

Formalin is a superior fixative for preserving tissue morphology, but it also adversely impacts IHC staining by masking epitopes and restricting antibody-target binding. Masking is the result of crosslinks created between amino acids both within the target antigen and between surrounding proteins. Crosslinks can impede an antibody from effectively accessing its epitope, limiting the ability to detect antigens in formalin-fixed tissue samples. This results in a weak signal or a signal that is indistinguishable from the background. Masked epitopes can be recovered with an antigen retrieval step, which works to promote epitope availability and enhance antigenicity.

What is Antigen Retrieval?

Antigen retrieval is a step in the IHC process that allows the removal of crosslinks created by formalin fixation. Proteolytic-Induced Epitope Retrieval (PIER) and Heat-Induced Epitope Retrieval (HIER) are two of the most widely used antigen retrieval methods for FFPE tissue sections. PIER is a method of antigen retrieval which relies on enzymes such as proteinase K, trypsin, or pepsin to unmask antigen. HIER utilizes heat to promote epitope availability. **Because ideal retrieval conditions are influenced by the tissue type and fixation method, optimization of the retrieval protocol for each antigen is highly recommended.**

	Heat-Induced Epitope Retrieval (HIER)	Proteolytic-Induced Epitope Retrieval (PIER)
What is it?	The use of heat to retrieve antigen and restore antigenicity	The use of enzymes to retrieve antigen and restore antigenicity
How does it work?	Heat causes crosslinked protein to unfold	Enzymes degrade protein crosslinks
Tissue Prep	FFPE and Frozen tissues	FFPE tissues
Recommendation	First choice	Harsher condition, favored for heavily crosslinked samples

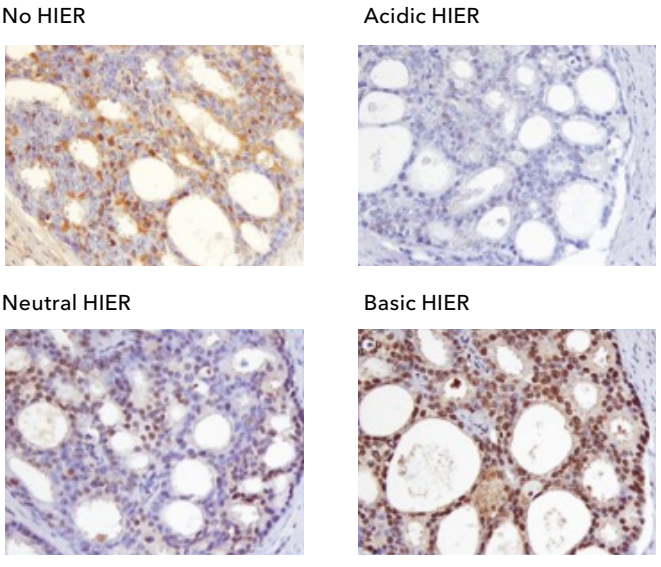


Figure 6. Antigen Retrieval Improves IHC Detection of p27. Immunohistochemical images show the detection of p27 in paraffin-embedded human prostate cancer sections following incubation of tissue for 10 minutes at 95 °C in the specified antigen retrieval solution. Compared to no HIER treatment, p27 detection was enhanced following incubation in neutral (pH 7.0) and basic (pH 9.5) HIER solution but not the acidic (pH 5.0) antigen solution. P27 was detected using Rat Anti-Human/Mouse/Rat p27/Kip1 Monoclonal Antibody (brown; R&D Systems Catalog #MAB22561) Image from R&D Systems: Antigen Retrieval Methods.

VisUCyte Antigen Retrieval Reagents: Basic (R&D Systems Catalog #VCTS021), Acidic (R&D Systems Catalog #VCTS022), Universal (R&D Systems Catalog #VCTS023)

Is Antigen Retrieval Always Necessary?

Antigen retrieval is recommended for most FFPE tissues. However, not all IHC experiments require a retrieval step. Some targets are negatively impacted by it and antigen retrieval on frozen tissue is not recommended. The fixation method and duration, in addition to the type of tissue, target antigen, and type of antibody determine the need for antigen retrieval:

- The antigen retrieval process can be too harsh and damage the tissue.
- A polyclonal antibody may enhance detection of antigen compared to a monoclonal due to its ability to bind multiple epitopes.
- A change in pH or cation concentration of antibody diluent or a simple change in the incubation conditions of primary antibody can also improve antibody affinity for its epitope without the need for a formal antigen retrieval step.

Protocol: Heat-Induced Epitope Retrieval (HIER)

Select one of the following HIER buffer options:

a) Citrate Buffer - 10mM Citric Acid, 0.05% Tween 20, pH 6.0
(also see: Pre-mixed Citrate Buffer from Novus Biologicals Catalog #NB900-62075)
b) Tris Buffered Saline (TBS) - 50mM TBS, 0.05% Tween 20, pH 9.0
c) EDTA Buffer - 1mM EDTA, 0.05% Tween 20, pH 8.0
d) VisUCyte™ Antigen Retrieval Reagents

1. Pre-heat retrieval solution in a staining dish in a vegetable steamer until temperature reaches 95-100°C.

   Note: A microwave or pressure cooker can be used as alternative heating source in place of the steamer.

2. Immerse slides in the staining dish.

3. Place the lid loosely on the staining dish and incubate it for 20-40 minutes in the steamer.

4. Remove the staining dish from the steamer and place it on the lab bench at room temperature.

5. Allow the slides to cool for 20-30 minutes before proceeding with the staining procedure.

Protocol: Proteolytic-Induced Epitope Retrieval (PIER)

Select one of 2 PIER buffer options:

a) Trypsin Working Solution, 0.05%
b) Proteinase K Working Solution, 20 µg/ml

1. Cover sections with chosen PIER buffer.

2. Incubate for 10-20 minutes at 37°C in humidified chamber.

3. Allow sections to cool at room temperature for 10 minutes.

IHC WORKFLOW

Blocking Non-Specific Binding

Blocking Non-Specific Binding

Antibody-based applications rely on the specific binding of antibody to the target epitope to generate accurate expression data. Antibodies specifically recognize the epitope of interest through intermolecular forces including hydrophobic interactions, ionic interactions, hydrogen bonding, and others. However, the same forces that govern specific interactions can also contribute to non-specific binding, i.e. binding of the primary antibody to amino acids other than those within the desired epitope of the antigen. The challenge in IHC experiments is to reduce non-specific interactions without impairing specific antibody-epitope recognition.

Causes of non-specific staining include interactions of the primary and secondary antibodies with serum proteins, ionic interactions between antibodies and tissues, and interactions with endogenous molecules capable of affecting the IHC detection system used. These issues can result in high background and an inability to visualize the antigen of interest in its appropriate cellular location. Staining problems of this type can be addressed by blocking non-specific interactions using a blocking reagent. Blocking other reactive epitopes and quenching endogenous enzymatic reactions prior to primary antibody incubation prevents non-specific binding and mitigates false positive staining (i.e., incorrect identification of positive signal).

The choice of blocking buffer is also contingent on the method of detection used. For instance, if using an alkaline-phosphatase (AP) conjugated secondary antibody, the blocking serum should be diluted in Tris buffered saline (TBS). PBS will interfere with the alkaline phosphatase reaction.

Common buffers to block non-specific interactions are serum, BSA, casein, or commercial buffers. If blocking with the serum, the host of the secondary antibody should be used to determine the species of the animal serum. For example, if the secondary antibody is goat anti-mouse, use goat serum as a blocking agent.

Autofluorescence is the endogenous level of fluorescence emitted by tissues when examined under fluorescence microscopy. Autofluorescence can impact imaging for immunofluorescence (IF) detection, especially for tissue samples with elevated levels of flavins or porphyrins.

Page 19 further describes tissues commonly associated with high non-specific binding, the affected step or detection reagent, additional steps to minimize non-specific signal, and proper background controls.

Tip: The detection method should be compatible with the sample tissue. For detecting a low abundance protein in tissues with high levels of endogenous biotin, consider using a polymer-based signal amplification method.

No Blocking + Blocking

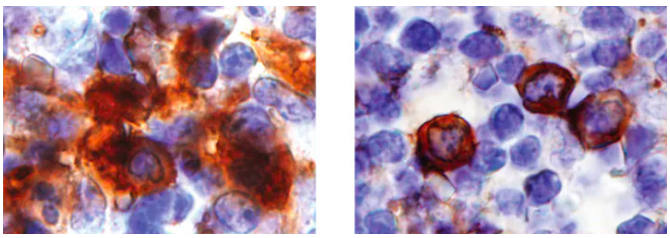
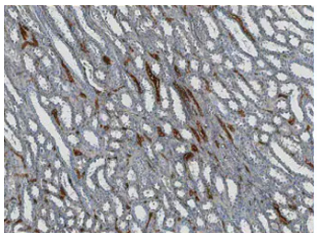


Figure 7. Blocking Non-specific Binding with Serum.
(Left) CD14 was detected in paraffin-embedded human tonsil tissue using anti-human CD14 biotinylated affinity-purified polyclonal antibody (Catalog # BAF383). Tissue was subjected to antigen retrieval using the Basic Antigen Retrieval Kit (Catalog # VCTS021). Tissue was stained using high sensitivity streptavidin conjugated to HRP (HSS-HRP) and DAB, and counterstained with hematoxylin (blue). **(Right)** Non-specific background staining is markedly reduced in a parallel experiment which included a blocking step using animal serum for 15 minutes at room temperature prior to incubation with the primary antibody.

A



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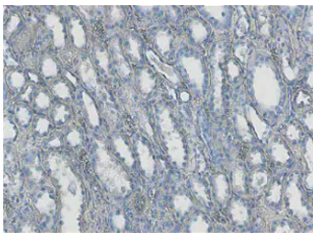
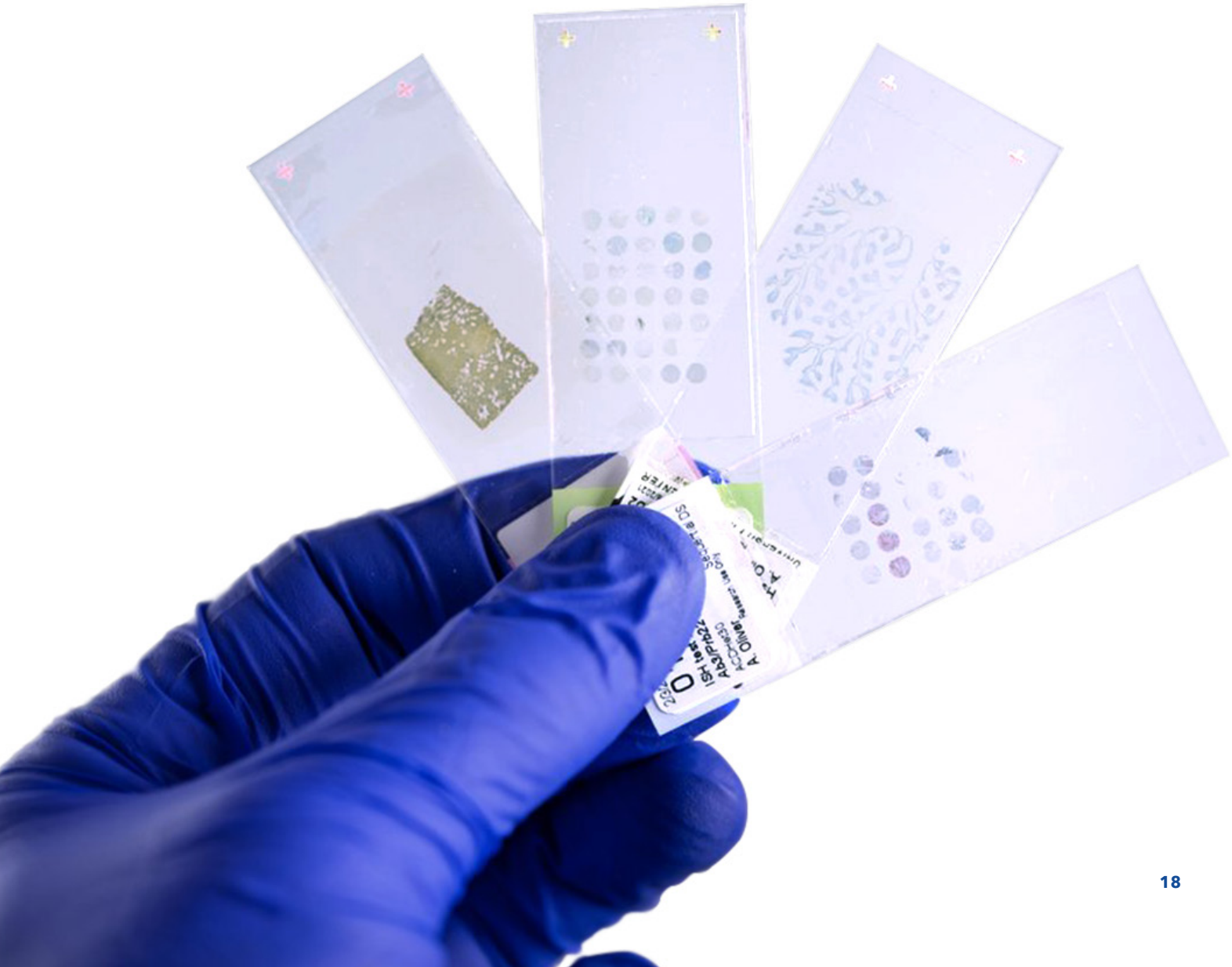


Figure 8. Quenching Endogenous Peroxidase Activity.
(A) Failing to quench endogenous peroxidase prior to staining produced a false positive signal in sections of human kidney. Tissue was stained using an Anti-Goat HRP-DAB Cell & Tissue Staining Kit (brown). **(B)** Endogenous peroxidase activity was quenched in the same tissue using 3% H_2O_2 in methanol for 15 minutes at room temperature prior to staining.



Endogenous Activity and Reactive Epitopes

Endogenous Activity

Peroxidase

- **Tissues with High Activity -** Kidney, liver, or vascular areas with red blood cells, lysosomal membranes especially in active phagocytic cells
- **Affected Step -** Chromogenic detection with HRP
- **Block -** Treat tissue with 3-10% hydrogen peroxide in methanol prior to incubation with HRP conjugated secondary antibody.
- **Background Control -** Before the antibody incubation step, incubate the sample with DAB substrate. The presence of endogenous peroxidase will be associated with the deposition of brown color.

Phosphatase

- **Tissues with High Activity -** Intestine, kidney, lymphoid
- **Affected Step -** Chromogenic detection with AP
- **Block -** Treat tissue section with 1mM Levamisole prior to incubation with AP conjugated secondary antibody. For intestinal sections, block with 1% acetic acid.
- **Background Control -** Before the antibody incubation step, incubate the sample with nitro blue tetrazolium/5-bromo-4-chloro- 3-indolyl phosphate (NBT/BCIP) substrate. The presence of endogenous phosphatases will be associated with the deposition of blue color.

Reactive Epitopes

Biotin

- **Reactive Tissues/Conditions -** Liver, kidney, spleen, heart, brain, and lung or frozen tissues
- **Affected Step -** Chromogenic detection using biotin conjugated reagents
- **Block -** Treat tissue with avidin prior to incubation with biotin-conjugated reagents. Then treat sample with biotin to block additional biotin binding sites on the avidin molecule.
- **Background Control -** Before the antibody incubation step, incubate the sample with avidin-biotin complex or streptavidin (SA)-HRP, then DAB. The presence of endogenous biotin will be associated with the deposition of brown color.

Autofluorescence

- **Tissues/Conditions with Endogenous Fluorescence -** Pancreas, brain, red blood cells, other pigmented cell types, lipofuscin, extracellular matrix components, or formalin-fixed
- **Affected Step -** IF detection, most severe in the shorter visible fluorescence wavelengths
- **Block -** For aldehyde autofluorescence, block with sodium borohydride in PBS. Choose fluorochromes emitting at unaffected wavelengths.
- **Background Control -** Prior to staining, examine tissue sections under fluorescent microscope using appropriate filter sets.

IHC WORKFLOW

Antibodies
& Detection
Methods

Antibodies & Detection Methods

One of the most important decisions that can affect the outcome of an IHC experiment is selecting an appropriate primary antibody. It is critical to choose a high-quality primary antibody that specifically binds the target antigen. A target protein's function, tissue, and subcellular localization, along with any post-translational modifications (PTMs) should be taken into consideration when choosing the appropriate antibody. For example, some proteins, e.g. AKT1, require a PTM such as phosphorylation to become activated, and to distinguish between inactive and activated AKT an antibody that specifically recognizes this activated form should be used, e.g. p Ser473.

In addition to antibody specificity, the clonality of the antibody should also be considered. Antibodies can be either monoclonal or polyclonal.

Antibody Clonality: Polyclonal vs. Monoclonal

	Polyclonal Antibody	Monoclonal Antibody	Recombinant Antibody
Antibody Production	Antibodies generated from multiple B cell clones	Antibodies generated from a single B cell clone	A type of monoclonal antibody generated in vitro using recombinant DNA technology
Epitopes Recognized	Multiple epitopes from the same antigen	A single epitope	A single epitope
Advantages	• Antigen-antibody binding is less affected by changes to antigen conformation from sample preparation and fixation • Binding to multiple epitopes can enhance signal	• Less lot-to-lot variability • Reduced non-specific binding, thus lower background staining	• Highest reproducibility and lot-to-lot consistency • Ability to be engineered, such as a species scaffold swap • Less production time compared to monoclonal antibodies
Disadvantages	• Higher background • Higher lot-to-lot variability	• Less tolerant to changes in antigen conformation from fixation or processing	• More costly development
Typical Working Conditions	Concentration range: 1.7-15 µg/mL	Concentration range: 5-25 µg/mL	Concentration range: 5-25 µg/mL

If using an antibody already validated for IHC, a basic staining protocol may be available. Keep in mind that experimental conditions such as tissue type, species, and sample preparation may differ and should all be factored in to establish the optimal staining protocol. The best antibody concentration, diluent, and incubation time should be determined to maximize the specific signal while minimizing the contribution to non-specific staining. A good starting point is to vary the antibody concentration and keep the incubation time constant (overnight at 4 °C).

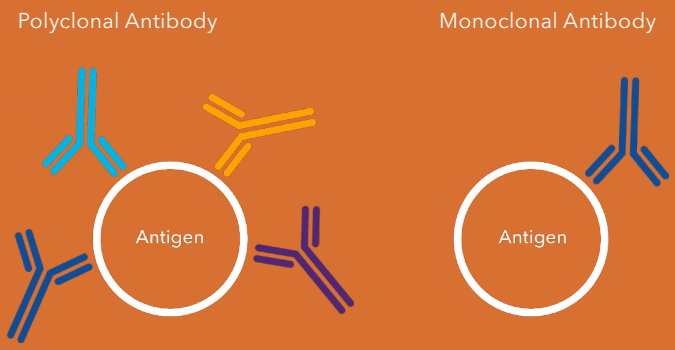


Figure 9. Polyclonal vs Monoclonal Antibody Binding.
Polyclonal Antibodies **(left)** are capable of recognizing multiple epitopes from the same antigen whereas Monoclonal Antibodies **(right)** only recognize a single epitope.

Why Choose a Polyclonal Antibody for my IHC Experiment?

Antibody binding for IHC/ICC experiments is often dependent on the target protein maintaining its native conformational state. A change in protein from its native state can impact antibody-epitope binding and affect IHC staining. Access to a desired epitope can be compromised by interactions with other proteins, post-translational modifications, temperature, pH, fixation, and salt concentration. Because polyclonal antibodies are capable of binding multiple epitopes of the same antigen, the staining result is less likely to be affected by changes in the target protein conformation state than monoclonal antibodies. In general, polyclonal antibodies are also more stable than monoclonal antibodies over a range of pH and salt concentration. Hence, polyclonal antibodies are often used for IHC/ICC experiments.

Detection Methods

Following incubation with the primary antibody, antigen expression is visualized using an appropriate detection system. The method of detection can be direct or indirect and may generate a fluorescent or chromogenic signal.

Direct vs. Indirect Direction

Direct detection involves the use of primary antibodies that are directly conjugated to a label. Frequently, IHC uses the indirect method of detection in which a secondary antibody, directed against the constant region of the primary antibody, carries the label. The indirect method is more sensitive than using a directly labeled primary antibody because multiple labeled secondary antibodies can bind to a single primary antibody.

Fluorescence vs. Chromogenic Signal

Both direct and indirect detection methods can be visualized by either immunofluorescence (IF) or by a chromogenic reaction. In IF detection, the fluorochrome conjugated antibody is excited by and emits light at specific wavelengths. In chromogenic detection, the antibody is conjugated to an enzyme (such as HRP) which converts DAB or 3-amino-9-ethylcarbazole (AEC) to a colored precipitate at the antigen site.

Signal Amplification

For detection of low abundance antigens, additional steps to amplify the antigen signal may be required. Two particular signal amplification methods for chromogenic detection make use of the high affinity complex between biotin and SA (or avidin). Biotin conjugated secondary antibodies link tissue-bound primary antibodies with either an avidin-biotin-peroxidase complex in the avidin-biotin complex (ABC) method or a SA-peroxidase complex for the labeled SA-biotin (LSAB) method. Since ABC and LSAB complexes exhibit a high enzyme-to-antibody ratio, these are more sensitive methods compared to the traditional indirect method.

Unlike ABC and LSAB, the polymer-based detection method is biotin-free. This third method of signal amplification uses a polymer backbone containing multiple enzyme molecules (e.g. HRP) and is directly conjugated to the secondary antibody. In addition to chromogenic signal amplification, tyramide signal amplification (TSA) is a highly sensitive method that can be used during either chromogenic or fluorescent IHC to enhance the detection of low-abundance targets. TSA utilizes HRP's catalytic activity for in situ labeling of the target protein or nucleic acid sequences. The basis for boosting signal amplification comes from the specific and high-density deposition of TSA dyes adjacent to the HRP-labeled antibody probe.

These signal amplification methods, along with direct and indirect detection, are described further in the table on the next page.

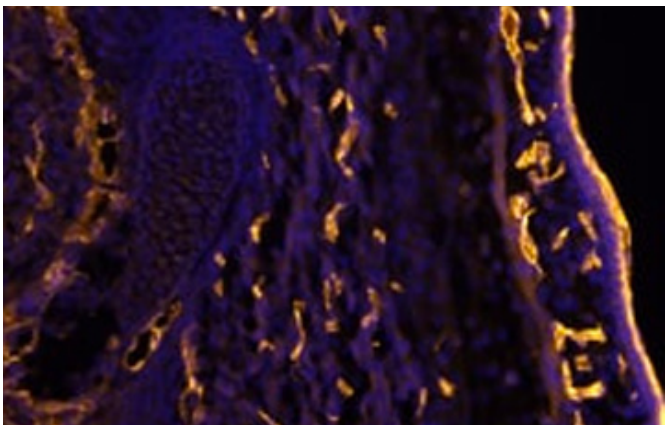


Figure 10. Example of Fluorescent IHC Detection.
CD31/PECAM 1 was detected in immersion fixed frozen sections of mouse embryo using Goat Anti-Mouse/Rat CD31/PECAM 1 Antigen Affinity-purified Polyclonal Antibody (R&D Systems Catalog # AF3628). Tissue was stained using the NorthernLights™ 557-conjugated Anti-Goat IgG Secondary Antibody (yellow; R&D Systems Catalog # NL001) and counterstained with DAPI (blue).

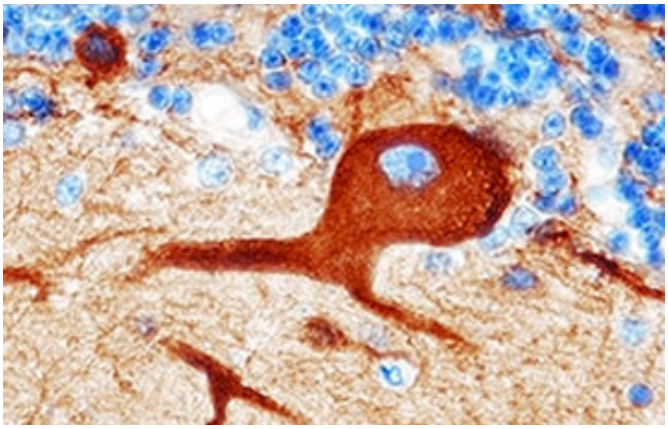
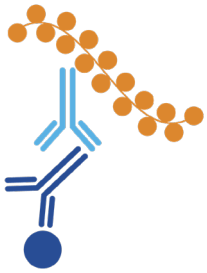
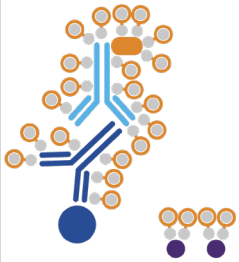


Figure 11. Example of Chromogenic IHC Detection.
beta -III Tubulin was detected in immersion fixed paraffin-embedded sections of human brain using Mouse Anti-Neuron-specific beta -III Tubulin Monoclonal Antibody (R&D Systems Catalog # MAB1195) followed by incubation with the Anti-Mouse IgG VisUCyte™ HRP Polymer Antibody (R&D Systems Catalog # VC001). Tissue was stained using DAB (brown) and counterstained with hematoxylin (blue).

			Signal Amplification Methods		
	Direct	Indirect	Biotin-Conjugated	Polymer-Based	TSA
Format	 Reporter enzyme or fluorochrome Labeled Primary Antibody Antigen	 Reporter enzyme or fluorochrome Labeled Secondary Antibody Primary Antibody Antigen	(Ex. ABC & LSAB)  ABC Reporter enzyme Biotin Avidin/SA Biotinylated Secondary Antibody Primary Antibody Antigen	 Reporter enzyme Polymer backbone Labeled Secondary Antibody Primary Antibody Antigen	 HRP Reporter enzyme Tyrosine Residues Labled Secondary Antibody Primary Antibody Antigen Active Tyramide
Advantages	• Quick • Less steps than indirect detection • Good for multiplexing	• Greater sensitivity • More flexibility compared to direct detection because it's possible to use different conjugated secondaries	• Greater sensitivity compared to traditional indirect detection • LSAB smaller complex size over ABC facilitates better tissue penetration	• Greater sensitivity compared to ABC and LSAB method • Fewer steps than ABC and LSAB method	• Suitable for fluorescent IHC • Multiplexing capabilities
Limitations	• Lower sensitivity • Selection of directly conjugated antibodies can be limited	• Potential non-specific binding of secondary antibodies	• Presence of endogenous biotin in tissues can increase background	• Larger size with polymer backbone may limit tissue penetration	• Can be more costly • Possible increase in background staining

IHC Staining: IF and Chromogenic

Protocol: Deparaffinization & Rehydration for Paraffin-Embedded Tissues (FFPE Tissue Sections)

1. Paraffin wax must be removed from FFPE tissue sections, and the tissue must be rehydrated before antibody staining. Xylene is the traditional organic solvent for removing paraffin.

   Deparaffinize and rehydrate by immersing the slides with:
   a) Xylene, three washes – 5 minutes each
   b) 100% Ethanol, two washes – 10 minutes each
   c) 95% Ethanol, two washes – 10 minutes each
   d) 70% Ethanol, two washes – 10 minutes each
   e) 50% Ethanol, two washes – 10 minutes each
   f) Deionized Water, two washes – 5 minutes each

   Note: Before moving to alcohol grades step, make sure to completely deparaffinize the sections. If the sections still have traces of wax, immerse in Xylene for an extra 5 minutes.

   DO NOT let the tissue dry from this point on.

Protocol: IHC Staining

Note: For IHC-Fr sections, warm slides to roomtemperature and wash slides 2x with PBS.

1. Apply an antigen retrieval protocol as needed for formaldehyde-fixed tissue sections (see page 15 for protocol).

2. Draw a circle on the slide around the tissue with a hydrophobic barrier pen (eg, PAP pen).

3. Block for endogenous activity or reactive epitopes as needed (see page 17).

   Note: For chromogenic detection, if using HRP reagents, block endogenous peroxidase activity by quenching the tissue sections with 3.0% hydrogen peroxide in methanol for at least 15 minutes. Afterwards, wash the sections by immersing them in distilled water for 5 minutes.

Optional Permeabilization & Intracellular Staining

Permeabilization of membranes is required for antibodies to access the inside of a cell for staining of intracellular antigens. Some fixatives, such as acetone and methanol, also act as permeabilization agents and IHC protocols using these fixatives may not require this additional step. Two potential permeabilization steps are listed below:

Option 1: Incubate slides in 0.1% Triton or NP-40 in PBS for 10 minutes.
Option 2: Incubate slides in 0.2 to 0.5% Tween 20, Saponin, Digitonin, or Leucoperm in PBS for 10 to 30 minutes.

The appropriate use of a permeabilization agent depends on the type of fixative, tissue size, and the cellular location of the antigen.

4. Block any non-specific binding by incubating the tissue sections with 5% animal serum in PBS + 0.3% Triton X-100 (PBS-T) for 30 minutes at room temperature.

Protocol: IHC Staining contd.

5. Add the primary antibody diluted in 1% animal serum in PBS-T and incubate at room temperature for 1-2 hours. Continue the incubation overnight at 4°C in a humidified chamber.

   Reminder: The host species of the primary antibody should differ from the source of the tissue sample.

   Note: Use the recommended dilution of the antibody specified on the datasheet. If not specified, use 3-fold serial dilutions: 2 µg/mL, 6 µg/mL and 18 µg/mL.

6. Wash sections twice with 1% animal serum in PBS-T for 10 minutes each.

7. Proceed with either the IF or chromogenic staining protocol.

Protocol: IF Staining

1. Add a fluorochrome-conjugated secondary antibody and incubate at room temperature for 1 -2 hours. Use the recommended dilution of the antibody as specified on the data sheet.

   Note: The secondary antibody should be directed against the host species of the primary antibody. For help on selecting secondary antibodies, see our Secondary Antibody Handbook.

2. Wash sections twice with 1% animal serum PBS-T for 10 minutes each.

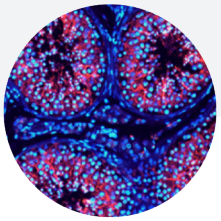
3. For nuclear staining, add DAPI solution (~2.9 µM) or other DNA binding dye (e.g. Hoechst 33342) and incubate 2-5 minutes at room temperature. Rinse 1x with PBS. DAPI has an absorption maximum at 358 nm and an emission maximum of 461 nm.

4. Apply a drop of mounting media containing a fluorescence anti-fade agent. Carefully place a coverslip on it and remove the excess mounting media, if necessary. Circle the edges of the coverslip with clear fingernail polish to prevent the cells from drying. Allow nail polish to air dry.

   Note: Some mounting media solutions have DAPI already added and will harden after exposure to air, eliminating the need to seal the edges of the coverslip.

5. Examine the cells under a fluorescence microscope using the appropriate excitation and emission filters and image as required. Slides can be stored between -20°C and 4°C in a dark slide box or slide book.

   Reminder: When examining slides using a fluorescence microscope avoid long exposures at the fluorochrome's excitation wavelength to prevent photo-bleaching.



Protocol: Chromogenic Staining (ABC amplification method)

1. Add a biotin conjugated secondary antibody and incubate at room temperature for 1 hour. Use the recommended dilution of the antibody as specified on the data sheet.

   Note: The secondary antibody should be directed against the host species of the primary antibody. For help on selecting secondary antibodies, see our Secondary Antibody Handbook.

2. Wash sections twice with 1% animal serum PBS-T for 10 minutes each.

3. Add ABC-HRP reagent and incubate at room temperature for 1 hour. Follow manufacturer's guidelines for reagent preparation.

4. Prepare a working solution of DAB and apply to tissue sections. Monitor the reaction as the chromogenic reaction turns the epitope sites brown (time of color development may vary from few seconds to 10 minutes). Proceed to the next step when the intensity of the signal is appropriate for imaging.

   Reminder: DAB is a carcinogen. Always wear gloves and work in a fume hood when working with DAB. Deactivate and clean work area after use according to manufacturer's instructions.

5. Wash sections twice in PBS for 10 minutes each.

6. Wash the sections twice in distilled water for 2 minutes each.

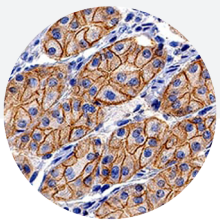
7. To counterstain nuclei, use Hematoxylin according to the manufacturer's instructions.

   Note: If using an aqueous chromogen instead of DAB (i.e. AEC, Fast Red, etc.), skip the following dehydration step and mount in aqueous media instead of organic mounting media.

8. Dehydrate tissue sections by moving slides through the following solutions twice for 2 minutes each:

   a) 95% Ethanol
   b) 100% Ethanol
   c) Xylene

9. Add mounting media to slides and top with coverslips. The DAB reaction is permanent and stable and can be analyzed under a bright-field microscope at any time.



IHC Controls

Due to the number of variables in the protocol that can introduce artifacts or affect IHC staining, inclusion of appropriate controls in each experiment is important for identifying the source of potential staining issues and for accurate interpretation of results. A good experimental design produces results that demonstrate that the antigen is localized to the correct specialized tissues, cell types, or subcellular location.

A distinct benefit of IHC is visually being able to detect or "see" your protein of interest and, more specifically, the cells or subcellular compartments expressing that target. In addition to using controls, resources and tools such as UniProt, The Human Protein Atlas, and antibodies cited in publications can all be used for analysis to help gauge where to expect target expression and localization.

Tissue Type Controls

Controls for IHC/ICC experiments include using tissue samples that are known to express (or not express) the epitope of interest. This strategy can provide a useful reference and may also be utilized during initial optimization studies. Tissue samples from different species can be included to support the species specificity of an antibody.

Tissue Control	What it Tells You	How it Helps	Commonly Used
Positive	Confirms the presence target antigen	Ensures the IHC protocol itself is working properly, even if experimental samples are negative	• Tissue or experimental condition with proven positive signal • Samples from transgenic animals that overexpress the antigen
Negative	Does not express the target antigen	Determines the degree of background staining caused by non-specific interactions. Background staining should be negligible and not resemble specific staining.	• Tissue or experimental condition with proven negative signal • Samples from knockdown or knockout tissues
Tissue Integrity	Immunoreactivity quality control	Indicates significant damage to tissue antigens, tissue is over-fixed or other failure of the staining procedure	Vimentin- stains mesenchymal and endothelial cells and is an internal quality control for antigenicity

View Tissue Control Slides From Novus

Tissue Artifact/Endogenous Tissue Background Control

Background staining may be more pronounced in particular tissues and thus it is important to use an endogenous tissue background, or tissue artifact, control. Since high background can mask positive signal from low abundance antigens or lead to artifacts being mistaken for specific staining, IHC results may be misinterpreted. Before applying primary antibodies, tissues should be examined under a bright-field or fluorescence microscope (for chromogenic or fluorescent labels, respectively) to ensure the signal is not due to the inherent properties of the tissue itself. For example, lipofuscin is an endogenous autofluorescent pigment that accumulates in lysosomes of postmitotic cells and may be confused with positive staining for both bright-field and fluorescence microscopy. Biological autofluorescence, especially in human tissues, is common with aging.

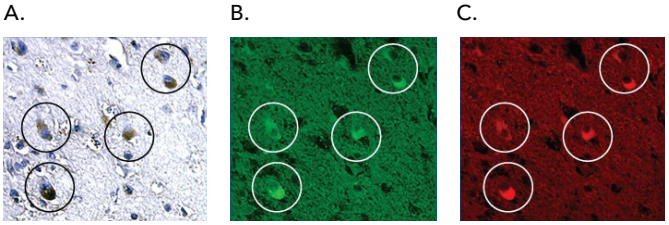


Figure 12. Lipofuscin Background in the nervous system.
Lipofuscin has autofluorescent properties that overlap with the excitation and emission spectra of commonly used fluorochromes. Circled in the micrographs above are Lipofuscin-containing neurons that may appear labeled using either bright-field microscopy (A) or fluorescence microscopy in the green (B) and red (C) spectrums.

Antibody Controls

The specificity of the antigen-antibody interaction is critical for accurate detection of the antigen. Due to the complex nature of tissue and the multifactorial effect of sample preparation on the staining procedure, antibody controls help to confirm the presence of the antigen of interest. A few recommended antibody controls include:

No Primary Antibody Control:	The primary antibody is absent from the diluent during the primary antibody incubation step. This control will show the contribution of all other components, such as the secondary antibody and detection reagents, to background staining (Figure 13).
Isotype Control:	Should be used to confirm the specificity of the primary antibody. For this control, the primary antibody should be replaced with a non-immune immunoglobulin of the same isotype and at the same concentration as the primary antibody. Example isotypes are IgG1, IgG2a, IgG2B, and IgM. All other conditions and protocol steps should remain the same. This control will demonstrate if the staining by the primary antibody is a result of an interaction with the antigen binding site (paratope) or due to non-specific interaction with the immunoglobulin molecule.
Absorption Control:	Also used to demonstrate that an antibody binds specifically to the antigen of interest. For this control, the primary antibody is first inactivated by preincubation with the immunogen (antigen) and then used in place of the primary antibody in the IHC protocol. The antigen to antibody solution should be prepared at a molar ratio of 10:1 to fully saturate the antibody and then incubated in the IHC antibody diluent overnight at 4°C. The staining pattern produced by the primary antibody can be compared to that produced by the pre-absorbed antibody. The optimal immunogen for absorption is small, purified peptide. With antibodies raised against the whole protein, addition of the antibody plus protein may result in higher non-specific staining. This is due to the antigen itself binding to the tissue. Thus, it is important to note that an absorption control using whole protein may not always confirm the specificity of an antibody and results should be analyzed with discretion.

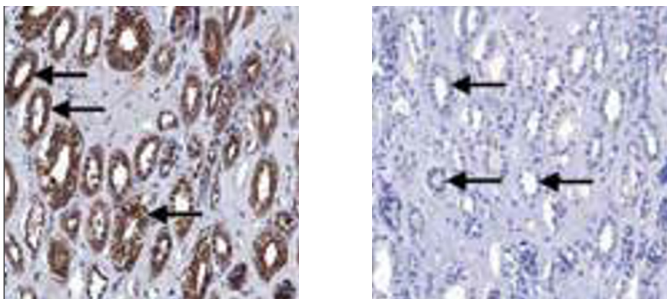


Figure 13. Primary Antibody Control in Human Kidney.
Neprilysin/CD10 was detected in a FFPE section of human kidney using Goat Anti-Human Neprilysin/CD10 Polyclonal Antibody (R&D Systems Catalog # AF1182). Tissue was stained with an anti-Goat HRP-DAB Cell & Tissue Staining Kit (brown) and counterstained with hematoxylin (blue). Neprilysin/CD10 staining (brown) is lost if the primary antibody is absent from the diluent in a parallel experiment.

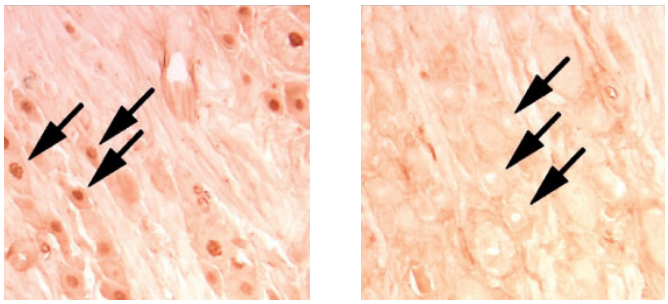
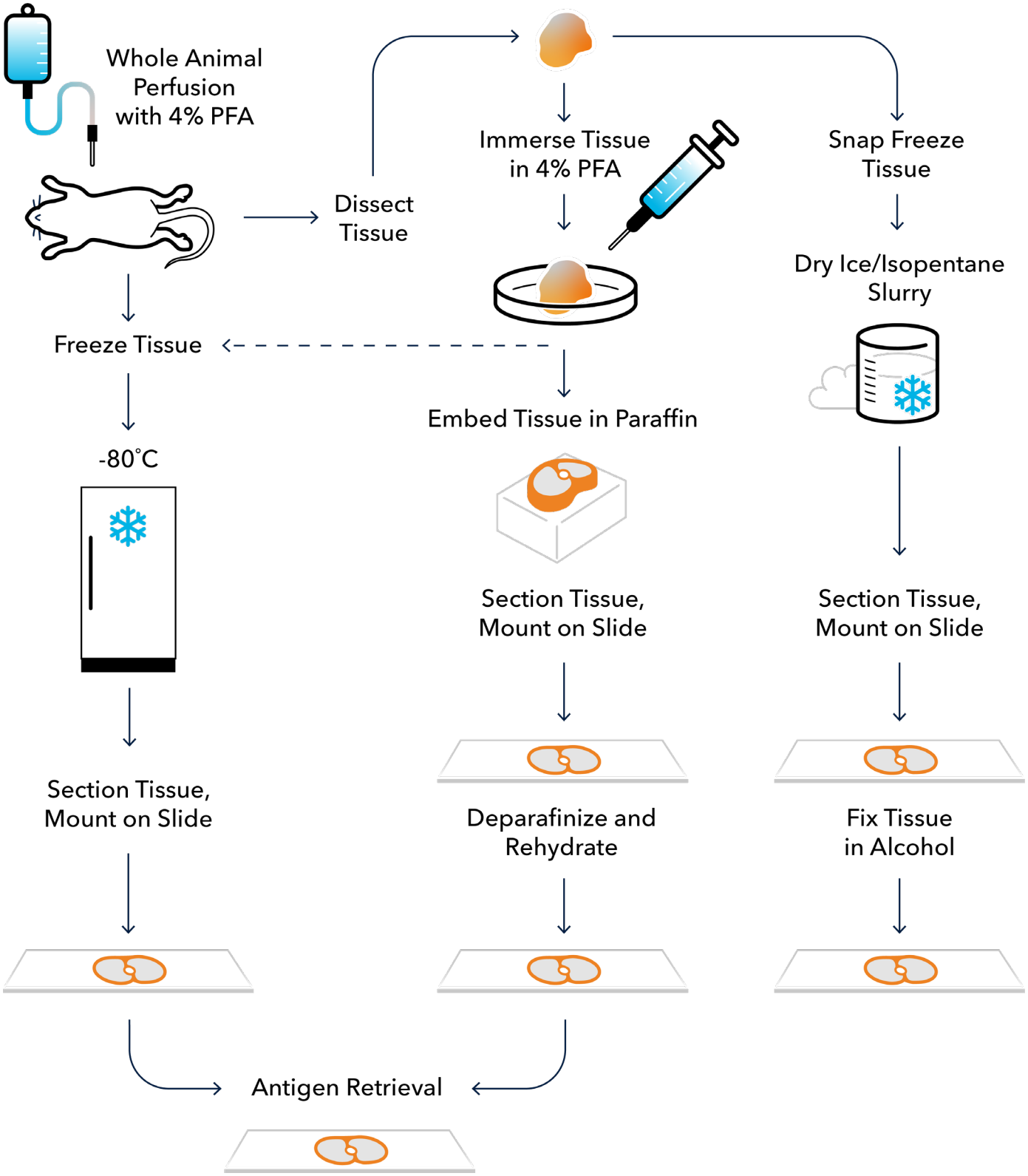


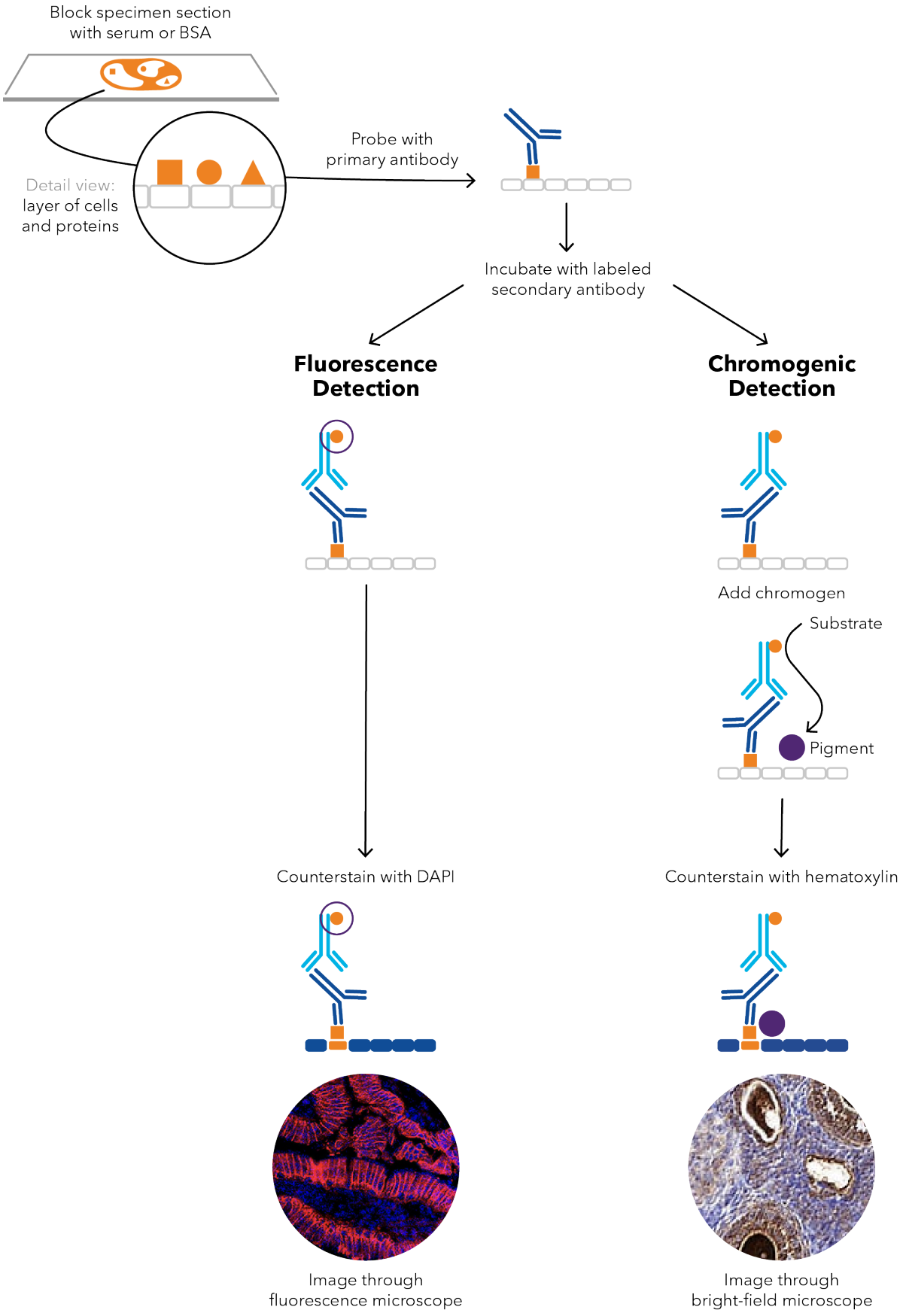
Figure 14. Absorption Control in Rat Dorsal Root Ganglion.
(A) A cryostat section of rat dorsal ganglion stained for phospho-MSK1 (S212) using Anti-Human phospho-MSK1 Affinity-Purified Polyclonal Antibody (R&D Systems Catalog # AF1036). **(B)** Nuclear staining (indicated by arrows) is abolished if the antibody is first pre-absorbed with the S212 phosphorylated immunogen.

Visual Workflow

Traditional IHC - Sample Preparation & Fixation



Traditional IHC - Sample Preparation & Fixation contd.



ISH-IHC (RNA-Protein Co-Detection)

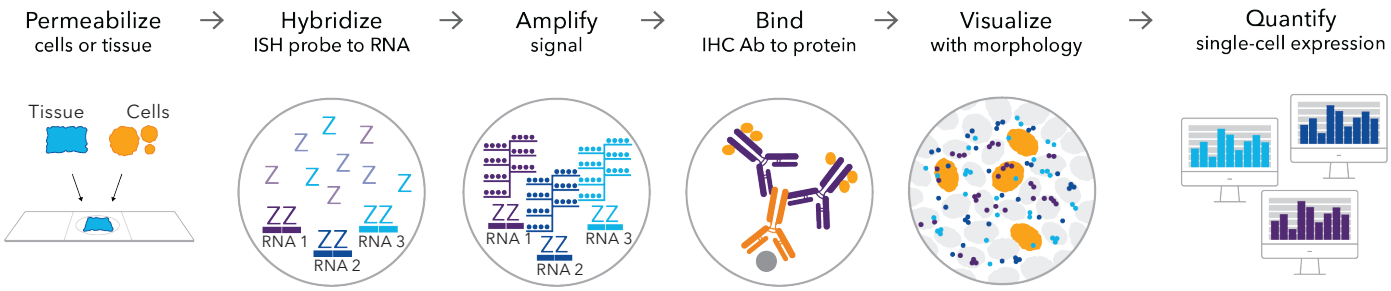


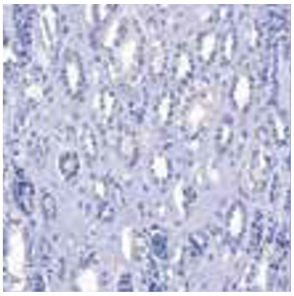
Figure 15. Advanced Cell Diagnostic's (ACD's) Integrated Co-Detection Workflow (ICW) for simultaneous detection of protein and RNA. The ICW allows for inclusion of wider range of antibodies to be combined with RNA ISH enabling researchers to acquire more data and conserve precious samples.

Troubleshooting Guide

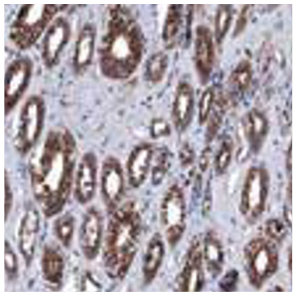
Problem: Lack of Staining / No Signal		
Possible Cause	**Explanation**	**Recommendation(s)**
Antigen not present	Protein isn't expressed in tissue or under experimental conditions.	Check protein expression by Western Blot or in situ hybridization (in some rare cases translation may be blocked even though mRNA is detected).
Tissue fixation	Over-fixation can cause epitope masking.	Reduce the time or concentration of the fixative. Apply an antigen retrieval step.
	Under-fixation can cause heavy edge staining with little to no positive signal in middle of your specimen. It can also result in autolysis and target protein degradation.	Increase the time or concentration of the fixative. Alternatively, try a different fixative.
Paraffin-Embedding	Epitope altered during embedding process and not recognized by primary antibody.	Switch to using frozen sections. Embed tissue at 58 °C or below.
Ineffective Antigen Retrieval	Epitope masking prevents binding of primary antibody.	Increase treatment time or change procedure (either new buffer or different method).
Permeabilization	Inadequate penetration of antibodies and buffers into the tissue sections.	Use 0.5-1.0% Triton (or Tween-20) detergent in the buffers.
Antigen Damaged	Antigen was destroyed before incubation with the primary antibody.	If quenching of endogenous peroxidase was done prior to the addition of primary antibodies, block peroxidase after incubation with the primary antibody.
Antibody Storage	Antibodies do not work due to improper storage.	Follow storage instructions on the datasheet. In general, aliquot antibodies into smaller volumes sufficient to make a working solution for a single experiment. Store aliquots in a manual defrost freezer (–20 to –70 °C) and avoid repeated freeze-thaw cycles.

Problem: Lack of Staining / No Signal contd.		
Possible Cause	**Explanation**	**Recommendation(s)**
Antibody Application	Epitope isn't saturated with antibody and/or hasn't reached binding equilibrium.	Increase the concentration or incubation time of the primary or secondary antibody.
Antibody Compatibility	Primary antibody isn't reactive in the tissue sample.	Confirm the species reactivity of the primary antibody.
	Primary antibody binds an epitope only exposed when protein is unfolded.	Confirm the antibody can be used for assays in which the protein is in its native conformation.
	Incompatible primary and secondary antibody.	Verify that the secondary antibody will interact with the species of the primary antibody.
Reagents	Missing reagents or added improperly/used in wrong order.	Repeat staining procedure and verify all reagents are added in correct order.
Microscope Adjustments (Fluorescence)	Fluorochrome isn't effectively excited or emission isn't captured by filters.	Verify fluorochrome is compatible with filter sets and appropriate settings are used. Also, increase the camera exposure time.

No Antigen Staining

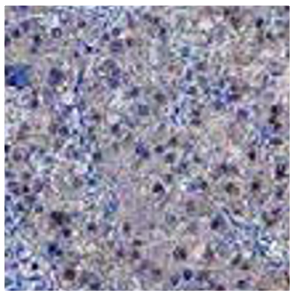


Good Antigen Staining

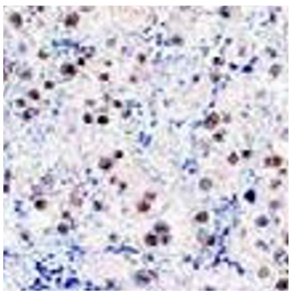


Problem: High Background		
Possible Cause	**Explanation**	**Recommendation(s)**
Tissue Fixation	Over-fixation can cause strong non-specific staining.	Reduce the time or concentration of the fixative.
Blocking Step	Inadequate blocking.	Increase the incubation time or concentration of serum in the blocking buffer.
Endogenous Activity/Reactivity	Tissue may have high autofluorescence, biotin, peroxidase activity, etc.	Check slides prior to staining and use the appropriate blocking/quenching steps. Try a more compatible detection method.
Antibody Application	Primary or secondary antibody concentration too high.	Titrate antibodies to find the optimal signal to background staining.
	Non-specific binding of primary or secondary antibody.	Increase the amount or incubation period of washes. Reduce the secondary antibody incubation period.
Antibody Compatibility	Secondary antibody is non-specifically binding the tissue sample.	Select a secondary antibody pre-adsorbed against the species of the experimental sample. For example, a secondary antibody adsorbed against mouse immunoglobulin or serum is recommended when staining mouse tissue.
Detection Method	DAB overstaining.	Reduce the time of incubation with DAB chromogen.
	Endogenous peroxidases are activating the DAB reaction.	Quench with hydrogen peroxide.
	Endogenous biotin is activating the SA-biotin complex using the ABC signal amplification method.	Block endogenous biotin.
Slide Condition	Reagents sticking to old or poorly prepared slides.	Use freshly prepared or purchased slides.
	Tissue dried out.	Avoid letting the tissue dry during the staining procedure.
Buffer Condition	Background from ionic interactions.	Increase the ionic strength of the blocking and antibody buffers.
Spectral Overlap (Fluorescence)	Multiple fluorochromes have overlapping emission spectra.	Select compatible fluorochrome conjugates.

High Background



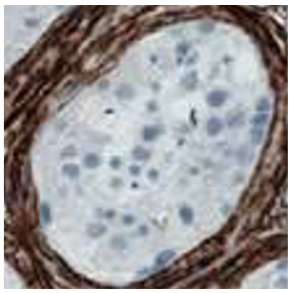
Low Background



Problem: Poor Tissue Morphology/Quality		
Possible Cause	**Explanation**	**Recommendation(s)**
Tissue Preparation	Tissue section appears torn or folded. Air bubbles under section.	Re-cut sections using a sharp blade or adjust the cutting speed. Ignore damaged areas when analyzing the results.
	Thick sections cause poor resolution of tissue morphology.	Cut thinner tissue sections.
	Frozen sections - Ice crystals may have destroyed morphology.	Repeat procedure or try paraffin embedded procedure.
Tissue Fixation	Under-fixation can lead to tissue damage.	Increase fixation time or fixative/tissue ratio. Try alternative fixing reagent.
	Under-fixation leads to tissue sections falling off slides (more common with frozen sections).	Increase the fixation time or use alternative fixative. Use freshly prepared, adequately charged slides.
	Autolysis of tissue leading to staining of necrotic debris.	Increase the fixation time, ratio. Consider using cross-linking fixative.
Antigen Retrieval	Methods are too harsh and damage tissue.	Determine optimal conditions (buffers and incubation periods). Try a different method.

Poor Tissue Morphology

Good Tissue Morphology



Problem: Uneven or Inappropriate Staining		
Possible Cause	**Explanation**	**Recommendation(s)**
Tissue Preparation	Thickness of tissue section is variable.	Re-cut sections using a sharp blade.
Tissue Fixation	Under-fixation with alcohols can produce staining artifacts.	Increase fixing time. Try a different fixative.
	Delay in fixation causes antigen diffusion.	Fix tissues immediately. Try a cross-linking fixative over an organic (alcohol) fixative.
Deparaffinization	Incomplete removal of paraffin wax.	Use fresh xylene.
Antibody Compatibility	Primary antibody may bind epitopes on other antigens.	Switch from a polyclonal to a monoclonal antibody. Try a different monoclonal antibody.
Permeabilization	Cell membrane damage and membrane proteins removed.	Use detergent at a lower concentration or apply a less stringent detergent (Tween-20).
Slide Condition	Tissue dried out.	Avoid letting the tissue dry during the staining procedure.

Basic Buffer & Reagent Recipes

Sample Preparation & Fixation

10X PBS

For 1 L:
80 g NaCl
2 g KCl
11.5 g Na_2HPO_4-$7H_2O$
2 g KH_2PO_4
Add Distilled water up to 1000 mL
Mix to dissolve
Adjust pH to 7.4 with 1N NaOH

4% Formaldehyde

For 1 L:
100 mL 10X PBS
700 mL Distilled water
Add PBS and water to a beaker on a stir plate in a ventilated hood. Heat while stirring to 60°C.
40 g paraformaldehyde powder
Add 1 N NaOH dropwise to raise pH until the solution clears
Cool and filter sterilize
Add Distilled water up to 1000 mL
Adjust pH to approximately 6.9 with small amounts of dilute HCl

10% Sucrose

For 100 mL:
10 g Sucrose
10 mL 10X PBS
Add Distilled water up to 100 mL
Mix to dissolve
Filter Sterilize

Antigen Retrieval Buffers

HIER Buffers

- **Citrate Buffer (10mM Citric Acid pH 6.0)**
  1.92 g Citric acid (anhydrous)
  Add Distilled water up to 1000 mL
  Mix to dissolve
  Adjust pH to 6.0 with 1N NaOH
- **TBS (50mM TBS pH 9.0)**
  6.1 g Tris
  8.8 g Sodium Chloride (NaCl)
  Add Distilled water up to 1000 mL
  Mix to dissolve
  Adjust pH to 9.0 with HCl. Mix well.
- **EDTA Buffer (1mM EDTA, pH 8.0)**
  0.37 g EDTA
  Add Distilled water up to 1000 mL
  Mix to dissolve
  Adjust pH to 8.0 using 1N NaOH
  Mix well. Store this solution at room temperature

PIER Buffers

- **Trypsin Working Solution, 0.05%**
  1 mL Trypsin stock solution (0.5%)
  1 mL Calcium chloride stock solution (1%)
  Add Distilled Water up to 8 mL
  Adjust pH to 7.8 with 1N NaOH
- **Proteinase K Working Solution, 20 µg/mL**
  1 mL Proteinase K Stock Solution (20X)
  19 mL TE Buffer, pH 8.0. Mix well

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Additional Resources

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