
CELL MARQUE
Best IHC Staining Practices

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Immunohistochemistry is multifaceted. It is important to consider the impact of all aspects of the process, including fixation, microtomy, pretreatment, blocking, primary antibody, detection, and chromogen. Although troubleshooting can seem overwhelming, there is no need to worry! Cell Marque's technical team is here to help! The following pages are full of tips that will assist you with successful IHC staining.

For additional questions or support please contact Cell Marque's Technical Support Team at:

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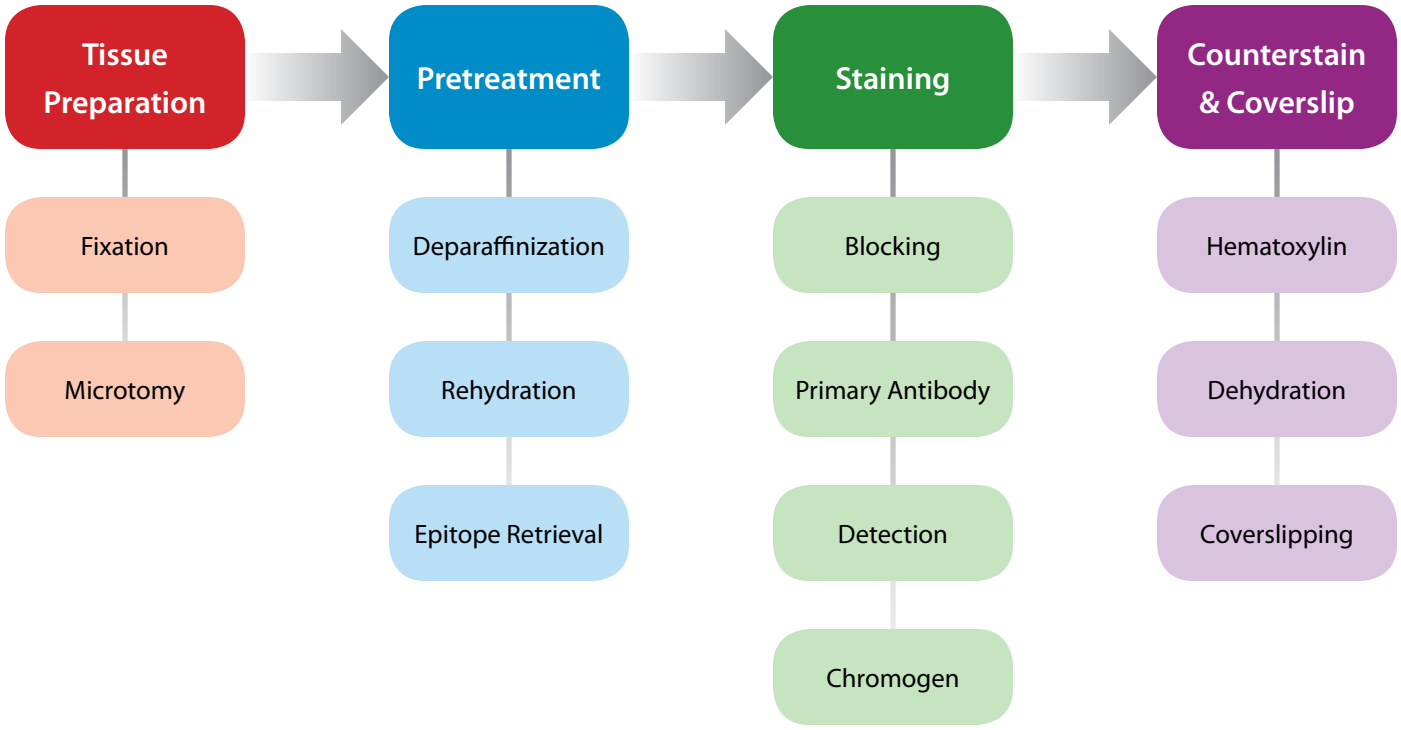
Staining Process Overview



Tissue Preparation

Fixation

Time: 12–48 hours; tissue must be fixed immediately upon removal from the body

Tech Tip: In most cases, antigenicity loss caused by overfixation can be restored by using a more intense pretreatment protocol.

Underfixation or delayed fixation can result in:

1. Weak or No staining ➔ proteins are not preserved and thus subject to denaturation. If the target protein is denatured or distorted it will become unrecognizable by the primary antibody and there will be no antibody-epitope binding.
2. Background staining ➔ autolysis, or cellular self-digestion, can occur when tissue is removed from the body and not preserved properly or quickly enough. Primary antibody and chromogen can bind non-specifically to the resulting necrotic cells and cellular components.

Overfixation can result in:

Weak staining ➔ fixative cross-linking between proteins is not reversed; epitopes are not sufficiently retrieved using a standard pretreatment protocol.

Temperature: Room temperature

Tech Tip: Heat acts as a catalyst to speed up a chemical reaction. In the case of fixation, this involves the formation of cross-links between proteins.

Background staining ➔ while the addition of heat may shorten the standard fixation time, it will also increase the rate of autolysis.

Type: 10% Neutral Buffered Formalin (NBF)

Tech Tip: 10% NBF is the optimal fixative for preserving proteins; proteins are your antibody's target.

Weak or No staining ➔ use of other fixatives like alcohols, Mercurials, Bouin's, Schaffer's, etc. may result in a lack of adequate protein preservation. These fixatives may also damage or alter target epitopes. If the target antigen/epitope is damaged or denatured, the antibody cannot bind.

Target to Fix	Preferred Fixative
Proteins	10% NBF
Enzymes	Frozen sections
Lipids	Frozen sections, Glutaraldehyde
Nucleic Acids	Alcohol
Biogenic Amines	Bouin's, 10% NBF
Glycogen	Alcohol

Volume: 15–20x greater than the volume of the tissue specimen

Tech Tip: A tissue specimen must be fully immersed in fixative to allow for adequate and even tissue preservation.

Weak or Variable staining ➔ insufficient volume will result in limited fixative penetration; the edges of the tissue will be well preserved, but the center of the tissue will not. Staining is likely to occur optimally around the well-preserved edges, but not toward the under-preserved center.

Tissue Preparation

Microtomy

Embedding: Paraffin ensures cellular morphology is adequately supported; protocols vary

Tech Tip: Proper fixation is critical for the successful creation of model, morphologically sound tissue sections.

Under-filled cassette:

- Unstable microtome clamping causes uneven sections (thick/thin) that can result in interpretation issues for the pathologist.

Over-filled cassette:

- Misalignment of the block face causes uneven sections (thick/thin) that can result in interpretation issues for the pathologist.

Orientation of tissue in cassette:

- Depending on tissue type, inappropriate orientation can cause sectioning difficulties and interpretation issues.

Baking: 2–4 hours at 60°C

Tech Tip: Fatty tissues like breast do not adhere to microscope slides as well or as strongly as other tissues; baking overnight at 60°C is recommended to prevent tissue loss during pretreatment.

No staining ➔ If the slides are not baked adequately, water trapped underneath the tissue and/or poor tissue adhesion to the glass slide can result in damaged or lost tissue during heated pretreatment.

Sectioning:

1. **Ideal tissue thickness: 3–4µm**
2. **Blade: sharp, clean, properly angled (~5°)**
3. **Water bath: ideal temperature: 35–40°C**
4. **Positively charged slides**

Tech Tip: To ease sectioning, it is best to place the tissue block on a cold, slightly wet surface. It is important not to freeze the block however, this can create cracks and may increase separation from the paraffin wax.

Poor quality sections can cause a variety of staining and interpretation issues:

- Folds, compression, and knife lines ➔ clearance angle too great and/or dull, nicked blade
- Chatter and cracks ➔ insufficient clearance angle and/or inappropriate cutting rate
- Disruption, folds, and over-expansion of tissue ➔ water bath too hot

Weak or No staining ➔ slide adhesive in water bath interferes with IHC reagents.

No staining ➔ positively charged glass slides help your negatively charged tissue adhere. Not using these slides can result in tissue loss during heated pretreatment.

Pretreatment

Deparaffinization & Rehydration

Time:

1. **Deparaffinization: up to 45 minutes; protocols vary**
2. **Rehydration: up to 20 minutes; protocols vary**

Tech Tip: Deparaffinization and rehydration protocols can vary depending on the type/strength of reagents used as well as the intensity of the epitope retrieval procedure.

Insufficient deparaffinization can result in:

1. Weak or No staining ➔ inadequate paraffin removal. If paraffin is not removed, epitopes will not be fully exposed leaving them unrecognizable by the primary antibody.
2. Background staining ➔ paraffin traps excess chromogen. Paraffin that is not removed will also be evident under the microscope and can be distracting to pathologists.

Type:

1. **Deparaffinization: Xylene or substitute**
2. **Rehydration: Graded alcohol (100%, 95%, 50%)**

Tech Tip: Less toxic xylene substitutes (e.g. Clearene™) are not as potent and will require slightly longer protocols.

Weak or No staining ➔ inappropriate or ineffective reagent(s) are used; target epitopes are not retrieved and primary antibody cannot bind.

Weak or No staining ➔ reagents become saturated; reagents like xylene and alcohol need to be changed based on usage. If not changed regularly, potency will be lost and deparaffinization/rehydration can become ineffective; paraffin is not removed and the target epitope is still blocked.

Pretreatment

Epitope Retrieval

Time: 10–60 minutes; protocols vary

1. **HIER (heat induced epitope retrieval) ➔ 30–60 minutes**
2. **EIER (enzyme induced epitope retrieval) ➔ 10–20 minutes**

Tech Tip: Protocols vary depending on the type of retrieval (HIER or EIER) and the instrument being used. Pressure cookers allow for the shortest HIER protocols since most will achieve high temperatures of ~120°C.

Insufficient epitope unmasking can result in:

Weak or No staining ➔ epitopes are not fully exposed leaving them unrecognizable by the primary antibody; epitope-antibody binding will not occur.

Excessive epitope unmasking can result in:

Background staining ➔ tissue will become "over-exposed" with primary antibody and chromogen binding non-specifically to various cellular elements within the over-exposed tissue.

Type:

1. **HIER (heat induced epitope retrieval)**
2. **EIER (enzyme induced epitope retrieval)**

Tech Tip: It is important to follow a manufacturer's epitope unmasking recommendation. False positives/negatives can occur if not followed correctly.

Use of an incompatible epitope unmasking method can result in:

Weak or No staining ➔ some epitopes are better unmasked with either EIER or HIER. If the preferred method is not followed, inadequate epitope retrieval can occur resulting in the primary antibody being unable to bind.

Reagents/Equipment:

1. **HIER: Citrate buffer, pH 5–6 and EDTA buffer, pH 8–9**
2. **EIER (Protease): Proteinase K, Pronase, Pepsin, Trypsin**
3. **Equipment: pressure cooker, steamer, waterbath, microwave**

Tech Tip: The majority of IHC antibodies on the market will perform optimally using HIER with an EDTA (pH 8–9) buffer.

Use of an incompatible epitope retrieval reagent can result in:

Weak or No staining ➔ inadequate epitope retrieval will occur resulting in the antibody being unable to bind to its target.

Staining

Blocking

Time: 5–15 minutes

Tech Tip: Most blocking is performed prior to primary antibody incubation. In some cases however, a blocking agent may damage or destroy a target epitope. In these instances, it is recommended that blocking is performed after primary antibody incubation.

Insufficient blocking can result in:

Background staining ➔ unblocked endogenous tissue elements interact non-specifically with primary antibody, detection, and/or chromogen.

Applying a blocking agent at the wrong time can result in:

No staining ➔ blocking agent damages targeted epitope leaving the epitope unrecognizable by the primary antibody; epitope-antibody binding will not occur.

Type:

1. **Endogenous elements:**
   - **Avidin-Biotin block ➔ biotin**
   - **Peroxide block ➔ peroxidase**
   - **Alkaline Phosphatase block (Levamisole) ➔ phosphatase**
2. **Background/Protein block**

Tech Tip: Liver, kidney, brain, and spleen contain the highest levels of endogenous biotin. It is important to use an avidin-biotin (A/B) block if utilizing a biotin based detection system.

No blocking or use of an incorrect blocking agent can result in:

Background staining ➔ unblocked endogenous tissue elements interact non-specifically with primary antibody, detection, and/or chromogen.

Staining

Primary Antibody

Time: 10–60 minutes
Concentration: Variable

Tech Tip: When optimizing a concentrated antibody, it is best to try a minimum of three dilutions; one dilution at the beginning of the manufacturer's recommended range, one dilution in the middle, and one dilution at the end of the range. Running several dilutions will help account for differences in detection and overall protocol as well as pathologist preference.

If the primary antibody is too diluted or the incubation time is too short, the following may occur:

Weak staining ➔ minimal epitope-antibody binding; there is not enough antibody in the solution or the primary antibodies do not have enough time to locate and bind to their targets.

If the primary antibody is too concentrated or the incubation time is too long, the following may occur:

Background staining ➔ excess antibody in the solution can bind non-specifically to various sites within the tissue specimen.

Source:

1. **Polyclonal vs. Monoclonal**
2. **Ascites vs. Supernatant**

Tech Tip: Polyclonals are a collection of antibody clones that bind to multiple epitopes on a target antigen. Monoclonals are a single antibody clone that binds to one epitope on a target antigen.

Polyclonal and ascites derived primary antibodies can be prone to:

Background staining ➔ contain additional immunoglobulins that can non-specifically bind to various elements within a tissue specimen.

Monoclonal and supernatant derived primary antibodies can be prone to:

Weak or No staining ➔ target a single epitope; destruction of that epitope during the retrieval or blocking process can minimize binding potential.

Staining Detection

Time: 15–45 minutes
Concentration: Variable

Tech Tip: Most universal detection systems contain anti-mouse/ anti-rabbit secondary antibodies.

If the detection system is too dilute or the incubation time too short, the following may occur:

Weak staining ➔ minimal detection component binding; overall weak signal.

If the detection system is too concentrated or the incubation time too long, the following may occur:

Background staining ➔ excess detection components bind non-specifically to various sites within the tissue specimen.

Handling and Storage: refrigerated at 2–8°C

Tech Tip: It is important to follow the manufacturer's detection protocol recommendation. Most 2-step detections will not work if the components are applied in the wrong order.

If the detection system is not stored according to manufacturer's recommendation, the following may occur:

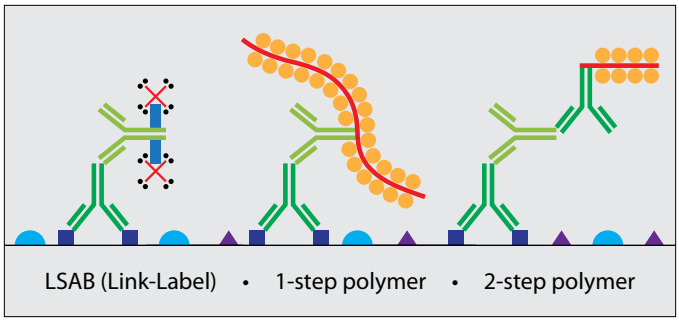
Weak or No staining ➔ contamination, degradation of detection components. If the integrity of the detection components is compromised, proper binding can be negatively impacted and overall staining diminished.

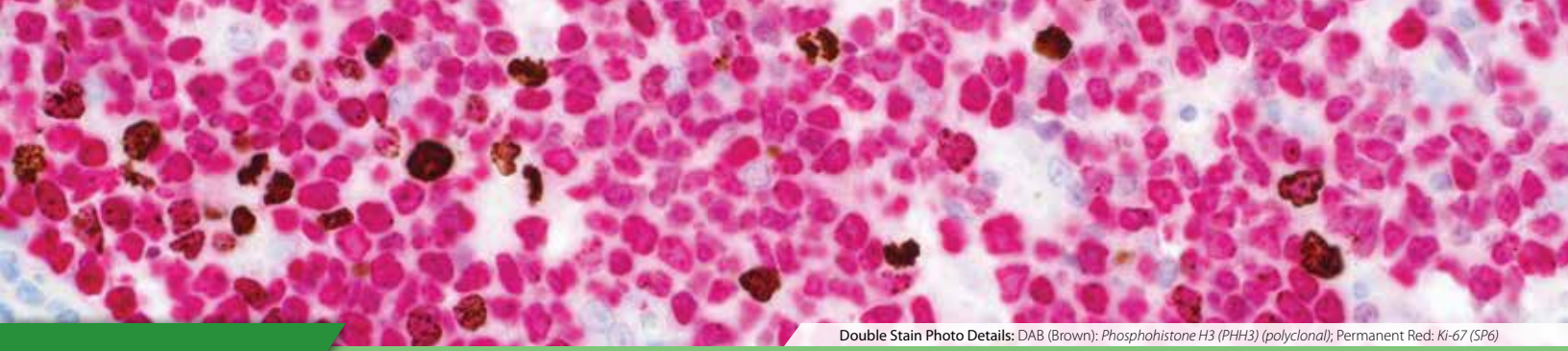
Type and Compatibility: LSAB (Link-Label) vs. 1-step polymer vs. 2-step polymer

Tech Tip: Although one-step polymer detection systems have more chromogen binding sites, the bulky polymer molecules are susceptible to steric hindrance, decreasing overall sensitivity.

Weak staining ➔ low detection sensitivity; LSAB (Link-Label) detection systems have fewer color inducing enzymes compared to the much larger polymer systems. Since there is limited opportunity for chromogen-enzyme interaction, the overall signal (staining intensity) is reduced.

Background staining ➔ high detection sensitivity; 2-step polymers utilize an additional linking or secondary antibody step. Use of secondary and tertiary antibodies increases the opportunity for non-specific binding to occur .





Double Stain Photo Details: DAB (Brown): *Phosphohistone H3 (PHH3) (polyclonal)*; Permanent Red: *Ki-67 (SP6)*

Staining Chromogen

Time: 1–10 minutes
Concentration: Variable

Tech Tip: Sticky substances/cells, like mucus/mucins, will grab onto excess chromogen resulting in non-specific staining. A short enzyme incubation during or after pretreatment can minimize this non-specific binding.

If the chromogen is too diluted or the incubation time too short, the following may occur:

Weak staining ➔ minimal enzyme-chromogen color-producing reaction.

If the chromogen is too concentrated or the incubation time too long, the following may occur:

Background staining ➔ chromogen will bind non-specifically. Chromogen is also easily trapped in folds/artifacts created during tissue processing.

Type and Compatibility:

Horseradish Peroxidase (HRP)	Alkaline Phosphatase (AP)
DAB (Brown) AEC (Red)	Permanent Red Permanent Magenta

Tech Tip: DAB and AEC will undergo an oxidation-reduction reaction when exposed to HRP; a colored precipitate results. Permanent red and Permanent magenta will lose a phosphate group when exposed to AP; a colored precipitate results.

If an enzyme is used with an incompatible chromogen (AP with DAB for example), the following may occur:

No staining ➔ color changing chemical reaction will not occur.

Handling and Storage: refrigerated at 2–8°C

Tech Tip: All chromogens are light sensitive and must be stored in opaque containers.

If the chromogen is not stored according to the manufacturer's recommendations, the following may occur:

Weak or No staining ➔ contamination or oxidation can compromise the integrity of the chromogen.

Counterstain & Coverslip

Hematoxylin: 30 seconds–10 minutes; depends on concentration and pathologist preference

Tech Tip: The main difference between the 2 most common types of hematoxylin is concentration: Mayer's = 1gm/L and Gill's = 2gm/L.

If the hematoxylin incubation time is too long or too short, then the following may occur:

Dissatisfied pathologist ➔ difficulty distinguishing cellular morphology.

If the hematoxylin is exposed to light, then the following may occur:

Dissatisfied pathologist ➔ oxidation creating crystalline precipitates that are observed under the microscope; these crystalline structures can be distracting and, in some instances, make accurate diagnosis difficult.

Dehydration and Mounting:

1. **Dehydration: graded alcohol (3–10 minutes) and xylene or xylene substitute (5–15 minutes)**
2. **Mounting Media: non-aqueous (permanent chromogens) vs. aqueous (non-permanent chromogens)**

Tech Tip: Non-permanent chromogens like AEC are soluble in alcohols. It is important to air-dry these slides and use an aqueous mounting media.

No or inadequate dehydration can result in:

Cloudy appearance ➔ water is not fully removed creating a hazy appearance to the tissue; cellular morphology can also be affected.

Use of the wrong dehydration method or inappropriate mounting media can result in:

Weak staining ➔ non-permanent chromogens can fade if used with alcohols or with non-aqueous mounting mediums.

Methods & Controls

Methods

Automation/Instrumentation

Tech Tip: There are 2 types of automated platforms:

"Open" systems that allow the use of any vendor's detection, antibodies, buffers, and ancillaries.

"Closed" systems that require use of the manufacturer's detection, buffers, and ancillaries; only antibodies from other vendors can be used with these instruments.

Operator error can result in:

Weak or No Staining ➔ no reagent or incorrect reagent is applied, incorrect protocol set-up (mapping), and/or a possible reagent mix-up.

An instrument malfunction can result in:

Weak or No staining ➔ no reagent or incorrect reagent is applied.
Variable staining ➔ insufficient or uneven washing.

Manual Staining

Tech Tip: It is important to never let pretreated tissue dry-out; always keep your slides in wash buffer in-between the steps of your manual staining procedure.

Operator error can result in:

Weak or No staining ➔ no reagent or incorrect reagent application or a possible reagent mix-up.
Variable staining ➔ insufficient or uneven washing.

Controls

Tech Tip: A positive control can be any tissue type, normal or diseased, that demonstrates an antibody's immunoreactivity; you do not have to use the same tissue type as the patient tissue.

Positive control: tissue known to be immunoreactive to the primary antibody.

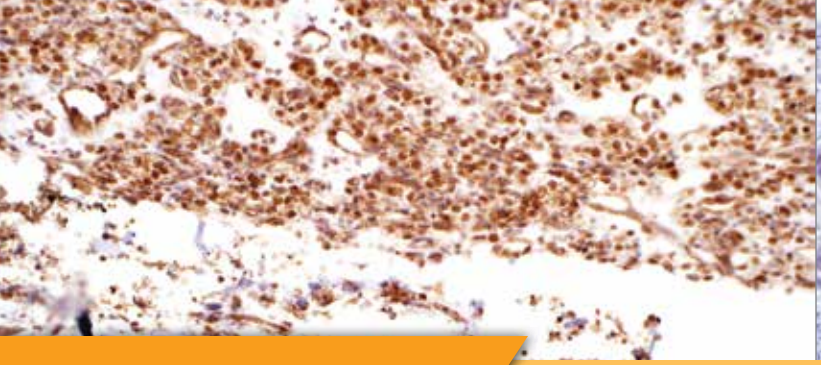
- Verifies that reagents were applied properly and staining was performed correctly
- Included every time a staining run is performed with a particular antibody
- Guards against false negative staining

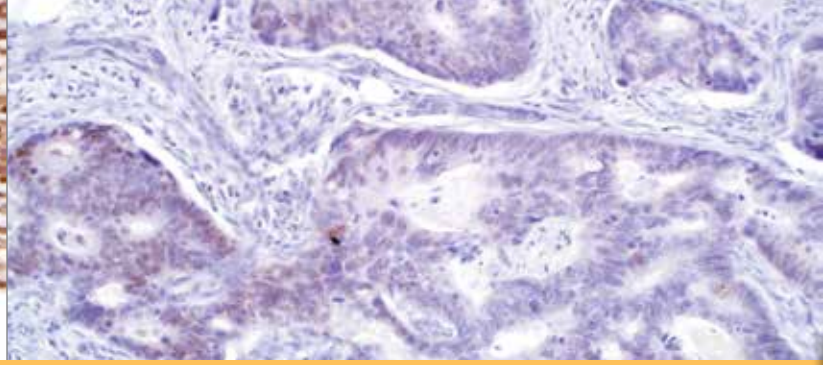
If a positive tissue control is negative ➔ reagent did not get applied, reagents were mixed-up, wrong or insufficient pretreatment, reagents were expired or stored improperly, and/or there could be a tissue fixation issue.

Negative control: patient tissue where primary antibody is NOT applied.

- Included every time a staining run is performed with a particular antibody
- Guards against false positive staining

If a negative tissue control is positive ➔ endogenous (biotin, peroxidase, phosphatase) elements may be picking up chromogen or interacting non-specifically with components within the detection system.





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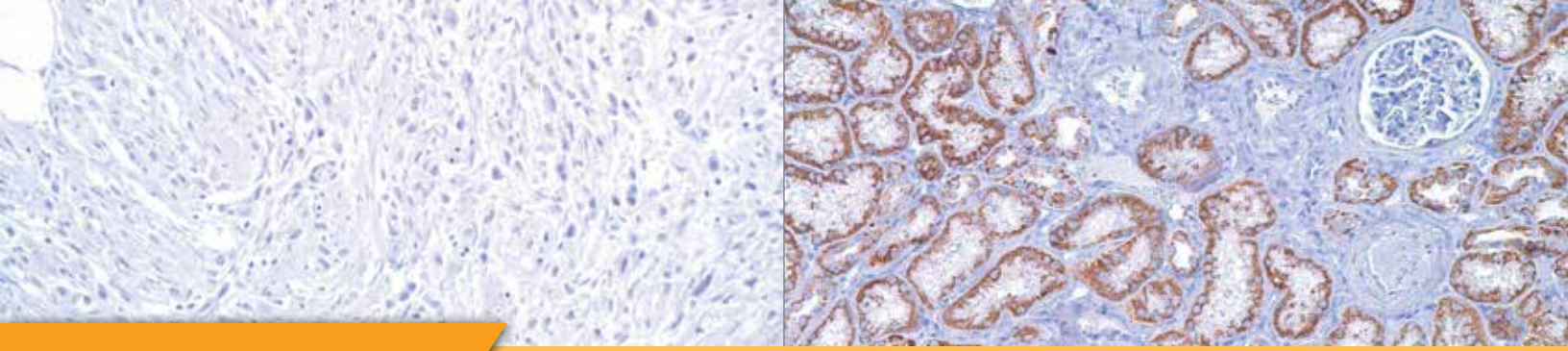
Background Staining

Weak Staining



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No Staining

False Positive Staining

Troubleshooting Tips

1. Consider the entire staining process and understand the impact of all potential variables.

Tissue Preparation:	Staining:
• specimen handling	• pretreatment
• storage	• blocking
• fixation	• primary antibody
• processing	• detection
• microtomy	• chromogen
• slide preparation	• counterstain/coverslip

2. When performing a troubleshooting experiment, isolate one variable at a time.

3. Consult the manufacturer's "Instructions-for-use" (IFU) to ensure you are following all product recommendations.

4. Contact your trusted technical support representative sooner rather than later.

5. Ask the right questions, such as those in the example below.

Troubleshooting Form

What is the problem?

_____

_____

What is the protocol you are using?

_____

_____

What type of controls are you using and how are they staining?

_____

_____

Has the problem been verified?

_____

_____