A GUIDE TO BETTER IHC

Advancing Cancer Diagnostics
Improving Lives

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BIOSYSTEMS

INTRODUCTION

This guide is to assist you with troubleshooting your immunohistochemistry (IHC) staining issues. Please keep in mind that some issues may be due to pre-staining conditions. Please refer to our **101 Steps to Better Histology** guide for troubleshooting these pre-staining conditions.

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TIPS BEFORE STARTING YOUR TROUBLESHOOTING INVESTIGATION

ENSURE YOUR STAINING PROTOCOL IS OPTIMIZED AND VALIDATED

Before troubleshooting your IHC staining, the staining protocol should be optimized and validated.

See 10 Steps to Optimizing Your IHC on page 66.

USE APPROPRIATE TISSUE CONTROLS

It is important to use well-established tissue controls for each antibody. The positive control should appropriately and consistently stain the protein of interest. Conversely, the negative control should not stain the protein of interest.

ISOLATE ONE VARIABLE AT A TIME

The most efficient and effective way to troubleshoot IHC staining issues is to isolate one variable at a time. Although it may seem more efficient to change many variables at once, if/when the issue is remedied, the issue may return because the root cause was not identified and addressed.

REFER TO THE ANTIBODY SPECIFICATION SHEET

Manufacturers provide many recommendations in their specification sheets including staining protocols and reagent storage conditions in addition to known tissue elements that should stain as well as known cross-reactivities.

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TROUBLESHOOTING TIPS

NO ANTIBODY STAINING IS PRESENT

Counterstain is present, but the slide is essentially negative including positive controls.

NOT ALL REAGENTS WERE AVAILABLE OR APPLIED

- *Postive tissue control is negative*

Verify that all necessary reagent steps were applied, especially the primary antibody and all appropriate detection reagents.

INCOMPATIBLE REAGENTS

- *Postive tissue control is negative*

Some antibodies are sensitive to carrier proteins, stabilizers, and buffers used. In particular, some antibodies may require phosphate-based (PBS) or tris-based (TBS) buffer specifically as the diluent used. Refer to the antibody specification sheet and follow the manufacturer's guidelines regarding diluents and buffers used.

See also: "INCOMPATIBLE REAGENTS/MOUNTING MEDIA" on page 21

EXPIRED REAGENTS

- *Postive tissue control is negative*

Avoid using expired reagents to ensure appropriate reactivity. Replace any outdated reagents with fresh reagents. Keep in mind that when using concentrated reagents, the shelf life of the diluted reagent is considerably less than the concentrated reagent.

AGE & STORAGE OF TISSUE SECTIONS/CONTROLS

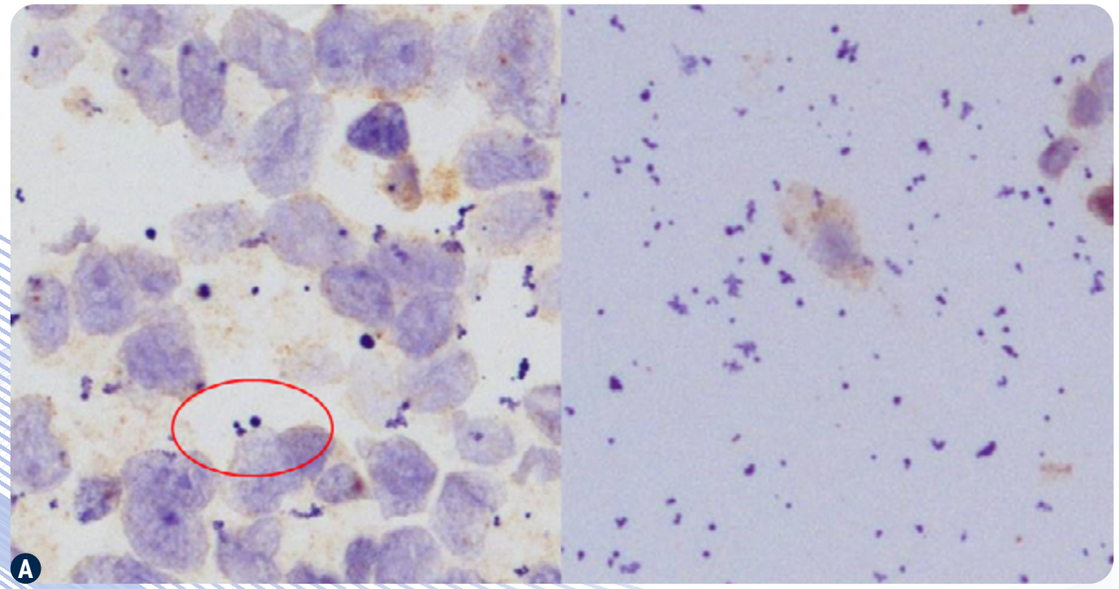
- *Positive tissue control is negative*

Ensure that the tissue sections and controls for the corresponding antibody are stored at the appropriate temperature and are not expired. In-house tissue control expiration should be determined by the laboratory based on antibody optimization, validation, and performance.

CONTAMINATION OF REAGENTS

- *Positive tissue control is negative or staining is weaker than expected.*

Check reagents for turbidity and/or contamination, especially with buffers and diluents. Replace with freshly prepared reagents.



Bacterial contamination.

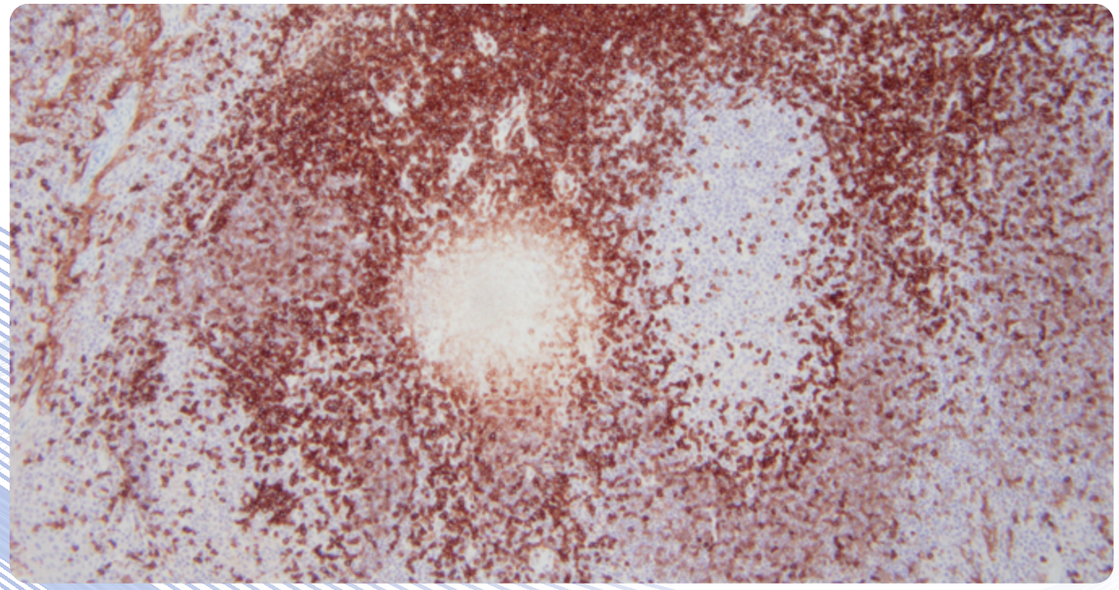
NO STAINING AND CIRCULAR ARTIFACT

Circular pockets with no staining (antibody or counterstain) is present.

INADEQUATE DEPARAFFINIZATION

- *Positive tissue control may likely have the same issue*

Any wax remaining on the slide after deparaffization will prevent staining. Ensure the slide is appropriately deparaffinized and that there are no contaminants in your deparaffinization solution. Try repeating with fresh deparaffinization solution (xylene, xylene substitute, proprietary commercial deparaffinization solution, etc.) as well as fresh alcohol rinses that follow.



Residual wax has resulted in an unstained area (tonsil, CD5).

INADEQUATE DRYING OF SLIDES

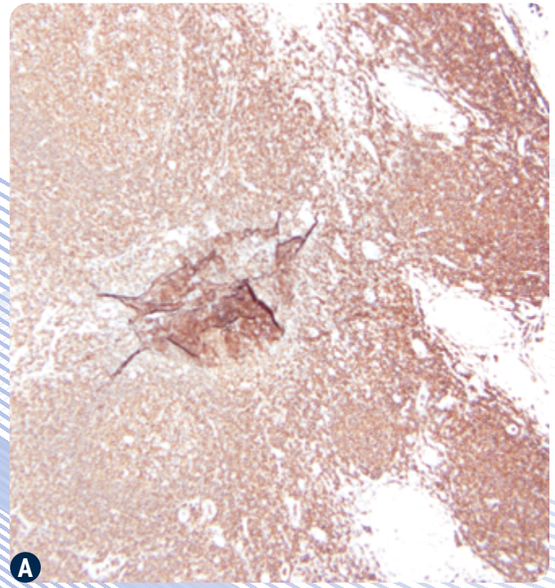
- *Positive tissue control may/may not have the issue*

Sometimes, water may be trapped under the tissue section during sectioning. If the tissue sections are not dried properly, this residual water can prevent staining. To facilitate better water drainage after sectioning, place slides in vertical racks to dry rather than lying them flat on a hot plate/slide warmer.

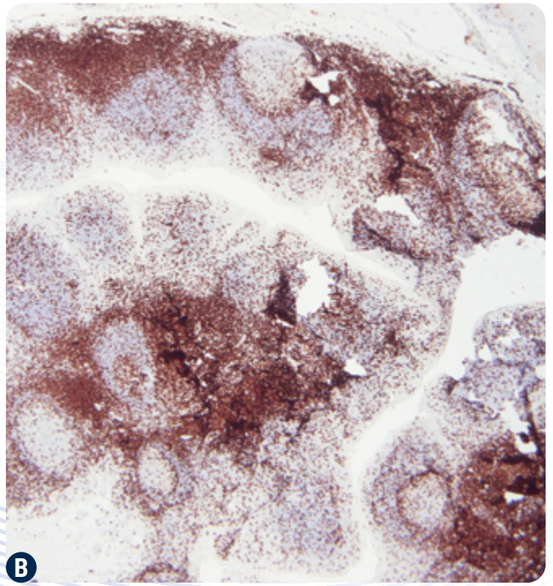
AIR BUBBLES INTRODUCED DURING SECTIONING

- *Positive tissue control likely does not have the issue and staining performs as expected*

With this circular artifact, part of the tissue appears to be raised and in a different plane. The slide may have staining on the outside of the circle and lack of staining on the inside of the circle. Sometimes, the bubble may cause tissue disruption during staining resulting in folds and darker localized staining.



A bubble under the section (from mounting) has resulted in subsequent detachment of the section during staining (tonsil, CD45).



A poor quality section, that has not been properly flattened and dried before staining, has lifted making the slide unsatisfactory (tonsil, CD3).

LOSS OF STAINING AFTER STAINING IS COMPLETED

Staining appears to have leached from the tissue section sometimes appearing as streaks of staining outside of the tissue.

IMPROPER SLIDE STORAGE CONDITIONS

- *Positive control may likely have the same issue*

Some stains may fade over time especially when slides are improperly stored such as prolonged exposure to direct sunlight, higher than ambient temperatures, etc.

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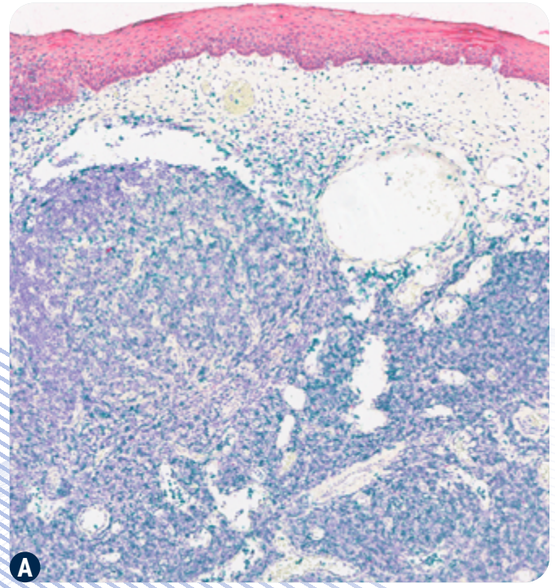
INCOMPATIBLE REAGENTS/MOUNTING MEDIA

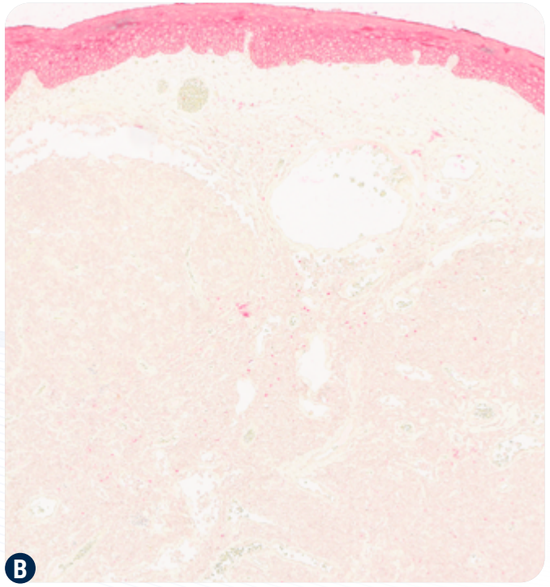
- *Positive tissue control may likely have the same issue*

Ensure that the reagents used after staining and mountant used for coverslipping are compatible with the chromogen used. Using incompatible reagents and mountants can often result in the chromogen or counterstain leaching out from tissue.

Note: *Excessive rinses of alcohol after alkaline phosphatase red chromogen staining can result in weakening or removal of red staining.*



FFPE tonsil tissue stained with Cytokeratin AE1/AE3 (red chromogen) and CD3 (green chromogen) using compatible toluene-based mounting medium. Both chromogenic stains show specific staining of cytoplasmic and membranous cellular compartments.



FFPE tonsil tissue stained with Cytokeratin AE1/AE3 (red) and CD3 (green) using incompatible aqueous mounting medium. The red AE1/AE3 staining remains present in the tissue section but the green CD3 staining and hematoxylin has completely leached from the tissue.

STAINING TOO WEAK

Staining appears to be weaker than expected.

EXPIRED REAGENTS

- *Postiive tissue control is negative or staining is weaker than expected*

See: “EXPIRED REAGENTS” on page 11

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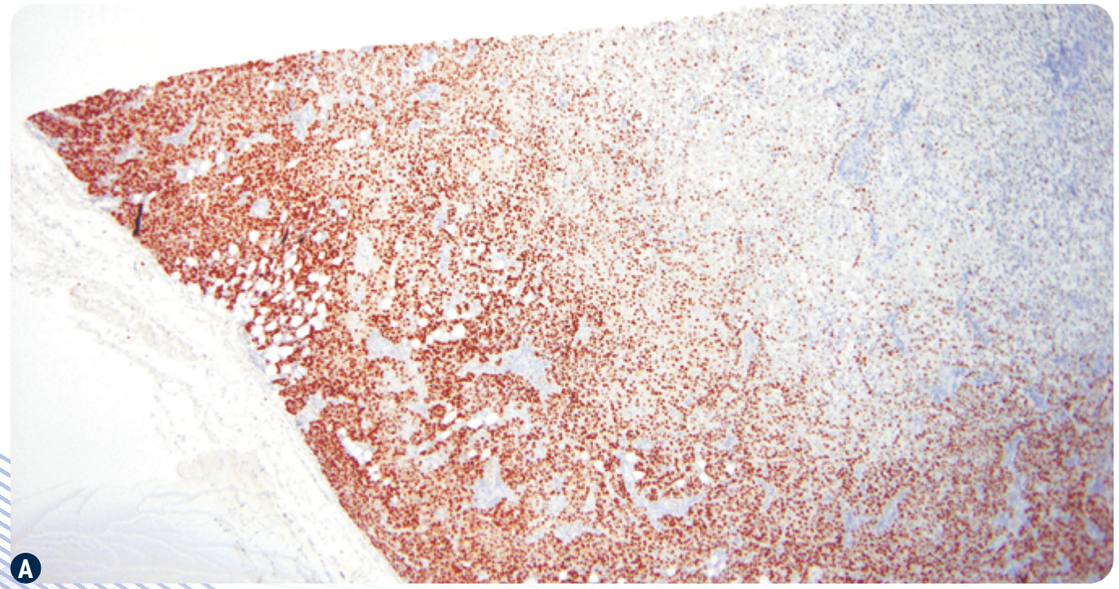
INADEQUATE TISSUE FIXATION

- *Positive tissue control performs as expected*

Poor fixation often results in stronger staining in the outer part of tissue but gets weaker towards the center of the tissue. Both the antibody and counterstain display weaker staining in the center of the tissue and this weaker staining is often seen in the H&E stain as well.



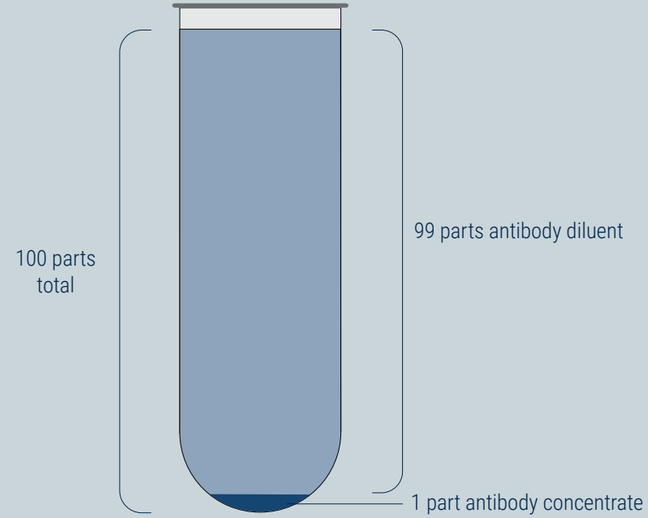
Uneven fixation (zonal fixation) has resulted in uneven staining in this section (breast tumor, ER).

INCORRECT ANTIBODY CONCENTRATION/DILUTION

- *Positive tissue control staining is weaker than expected*

Verify that the correct staining protocol was followed. If the antibody is diluted from a concentrate, check for a potential dilution error, ensure the appropriate diluent was used, and the diluted antibody passed quality control testing prior to use.

Example of 1:100 Antibody Dilution



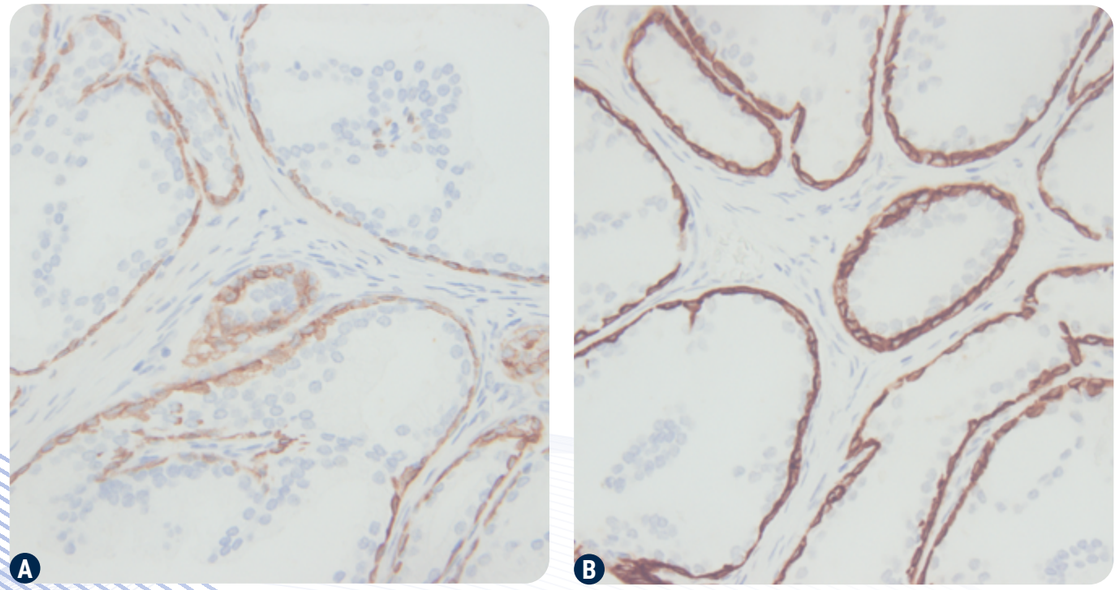
A

1:100 Antibody Dilution: 1 part antibody concentrate and 99 parts antibody diluent. Example: 10 µL antibody concentrate + 990 µL antibody diluent.

INADEQUATE OR INCORRECT ANTIGEN/EPITOPE RETRIEVAL

- *Positive tissue control staining does not perform as expected and may appear weaker than usual.*

Verify that the correct pretreatment protocol was followed including the pretreatment solution used as well as incubation time and temperature. Incorrect pretreatment may result in unexpected staining in certain tissue elements.



Prostate sections stained for Cytokeratin 34βE12. Section A shows weak staining while section B is stronger and sharper. The only difference between the two was the retrieval method used.

CONTAMINATION OF REAGENTS

- *Positive tissue control is negative or staining is weaker than expected*

See: “CONTAMINATION OF REAGENTS” on page 13

AGE & STORAGE OF TISSUE SECTIONS/CONTROLS

- *Positive tissue control is negative or staining is weaker than expected*

See: “AGE & STORAGE OF TISSUE SECTIONS/CONTROLS” on page 13

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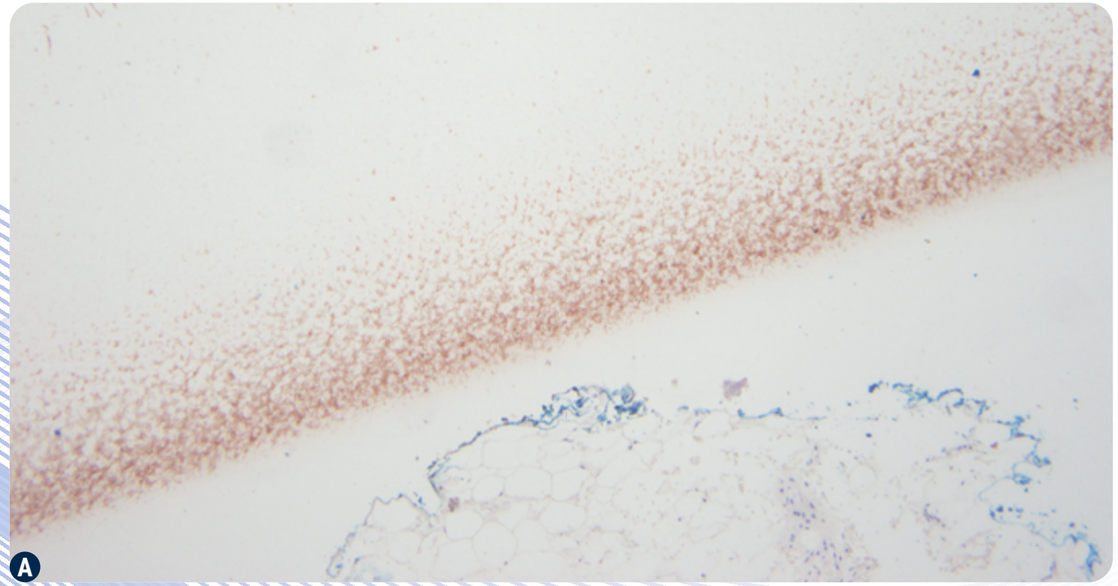
BACKGROUND STAINING ON GLASS SLIDES

Background staining all over the slide including where tissue is not present, can be uniform or patchy.

ADHESIVE ADDED TO FLOATATION BATH

- *Positive tissue control may/may not have the issue*

Adhesives (e.g. gelatin, casein) added to the sectioning water bath can negatively affect IHC. Often, this background staining results in the chromogen binding to the adhesive on the slide. It can sometimes appear as pattens such as dots, swirls, etc.



A line of thick protein-based section adhesive has stained adjacent to the section (breast, PR).

INCOMPATIBLE SLIDES

- *Positive tissue control may likely have the same issue*

Although most positively charged slides are compatible with IHC staining, some brands of charged slides may cause background staining. This background may appear as a pattern all over the slide. Repeat staining on tissue sections using a different manufacturer/brand of slide.

STAINING TOO STRONG/APPEARS OVERSTAINED

Staining appears considerably stronger than expected.

AIR DRYING ARTIFACT

- *Positive tissue control may/may not have the issue*

Ensure that the slides remain hydrated both during and after the staining process is completed. Slides should remain hydrated prior to the dehydration step that precedes clearing (if applicable) and coverslipping.

INCORRECT ANTIBODY CONCENTRATION/DILUTION

- *Positive tissue control staining is darker than expected*

See: “Incorrect Antibody Concentration/Dilution” on page 27

EXCESSIVE OR INCORRECT ANTIGEN/EPITOPE RETRIEVAL

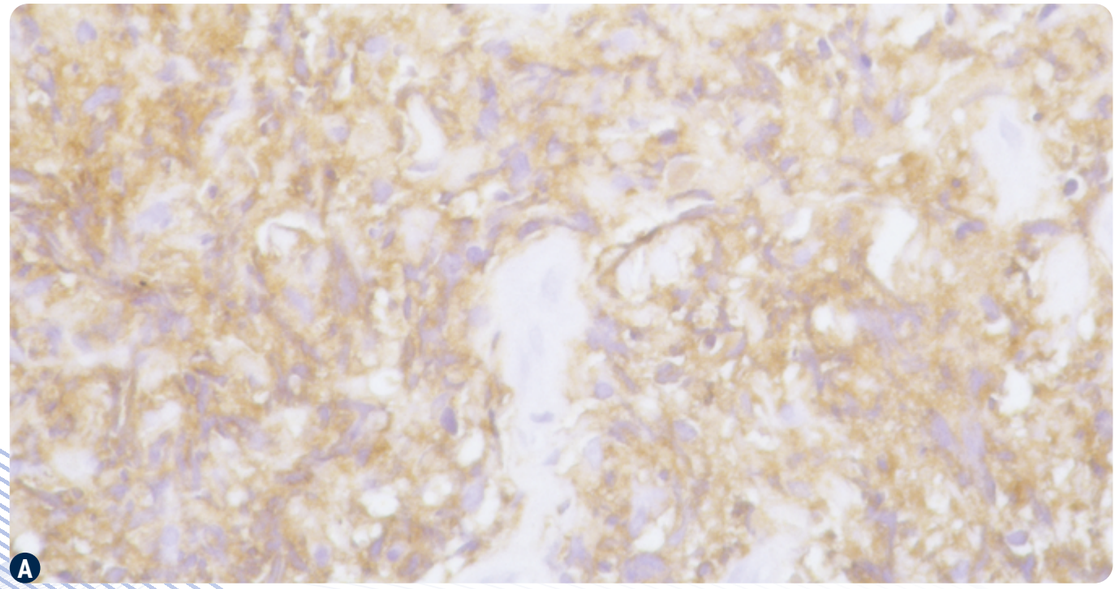
- *Positive tissue control staining does not perform as expected and may have poor morphology*

Verify that the correct pretreatment protocol was followed including the pretreatment solution used as well as incubation time and temperature. Excessive retrieval often results in a detriment to tissue morphology. Incorrect pretreatment may result in unexpected staining in certain tissue elements.

CRUSHED CELL ARTIFACT

- *Positive tissue control performs as expected*

Some tissue extraction processes can cause cellular distortion. These disrupted cells can result in extracellular staining. This can appear as background staining or staining outside of the expected tissue elements.



A high grade B cell lymphoma stained for the lymphocyte marker L26. The specimen was crushed with forceps prior to formalin fixation and paraffin embedding and shows diffuse staining of both cytoplasm and background.

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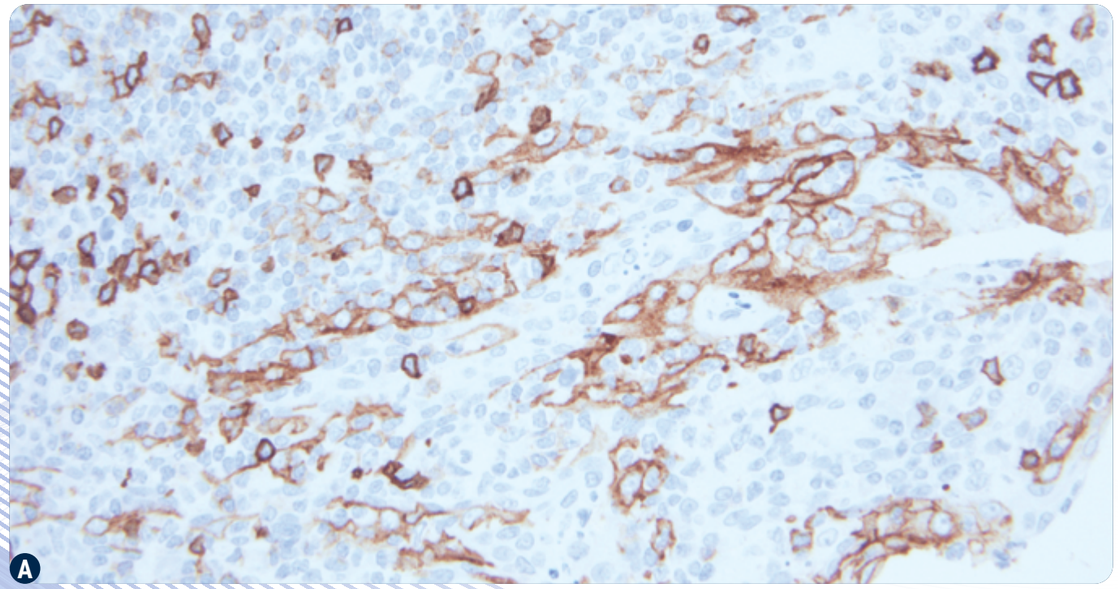
BACKGROUND STAINING OR NON-SPECIFIC STAINING

Incorrect or unexpected tissue elements stained

ANTIBODY CROSS-REACTIVITY

- *Positive tissue control may/may not have the issue*

Refer to the manufacturer's specification sheet for any known cross-reactivity for that antibody/clone and verify the appropriate tissue elements that should be stained.



Palatine tonsil showing the base of a tonsillar crypt stained for CD5, a lymphocyte marker that stains mainly T cells. This particular clone (4C7) cross-reacts with epithelial cells deep in the crypt.

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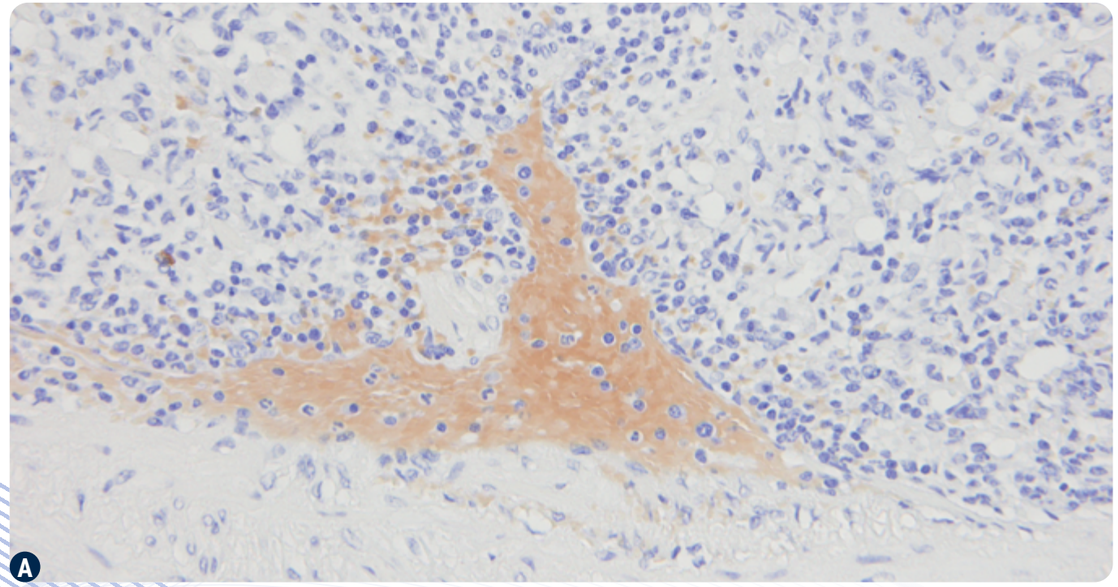
ENDOGENOUS PEROXIDASE BLOCK NEEDED

- *Positive tissue control may/may not have the issue*

Endogenous peroxidase block may need to be applied if nonspecific staining is seen especially in erythrocytes, granulocytes, monocytes, and muscle.



A

Spleen showing typical, non-specific staining of erythrocytes due to incomplete blocking of endogenous peroxidase. Here the natural peroxidase present in the red cells has reacted with the DAB chromogen.

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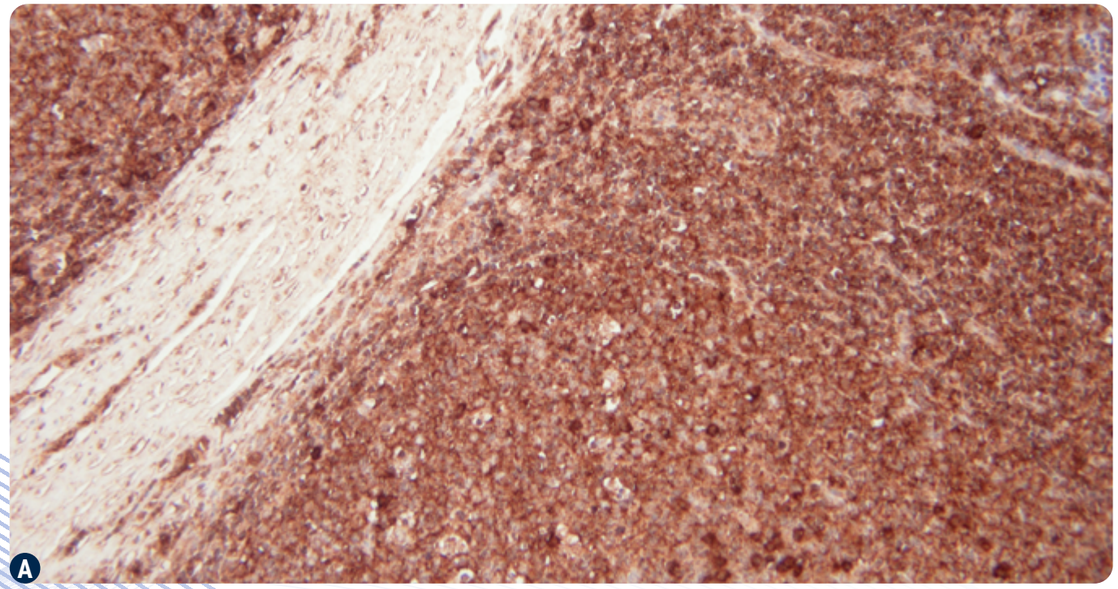
PROTEIN BLOCK NEEDED

- *Positive tissue control may/may not have the issue*

Using a protein block reagent can assist with preventing nonspecific staining. Examples of protein block include normal sera of the secondary antibody, casein, bovine serum albumin, and commercially available blocking reagents.



Normal tonsil stained for Kappa light chain showing a heavy back-ground stain due to ineffective protein block.

BIOTIN BLOCK NEEDED

- *Positive tissue control may/may not have the issue*

When using biotin-based IHC detection reagents, it may be necessary to block for endogenous biotin in tissue such liver, kidney, etc. This blocking step is not necessary when using polymer-based detection.

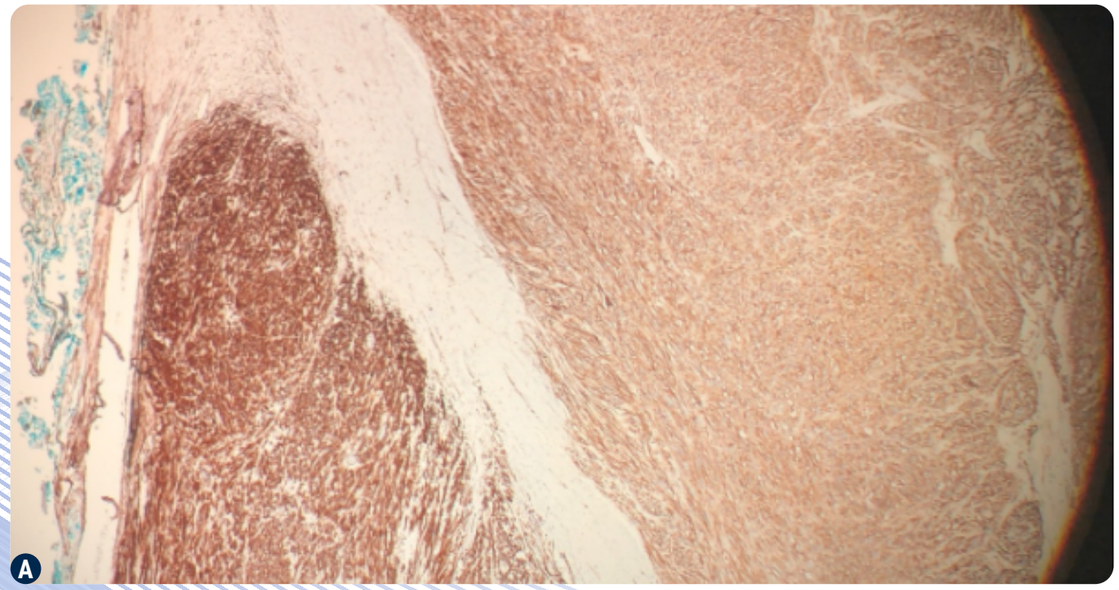
UNEVEN OR GRADIENT STAINING

Gradient staining is when the staining is stronger on one side of slide with decreased staining across slide.

STAINING AREA NOT LEVEL

- *Positive tissue control may/may not have the issue*

Staining areas that are not level can cause uneven staining or gradient staining. Note, this has more impact with manual IHC staining although some automated stainers offer built-in level indicators to verify that the staining area is level.

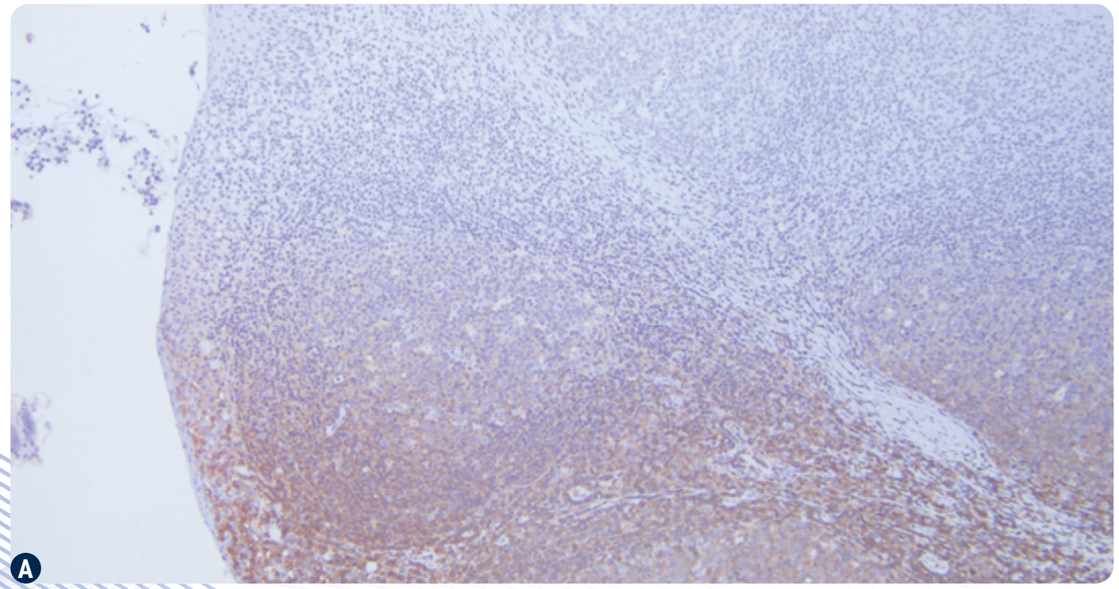


Example of uneven gradient staining.

UNEVEN REAGENT APPLICATION

- *Positive tissue control may/may not have the issue*

Ensure there is ample volume of reagents and that the entire tissue section is uniformly covered with reagents extending well past the tissue section borders. Also ensure that the tissue section is a reasonable size and does not exceed the optimal staining area on the slide.



Poor reagent flow has produced uneven staining (tonsil, CD45).

INADEQUATE TISSUE FIXATION

- *Positive tissue control performs as expected*

See “INADEQUATE TISSUE FIXATION” on page 25

AIR DRYING ARTIFACT

- *Positive tissue control may/may not have the issue.*

Air drying artifact in stained tissue often appears considerably darker than expected, particularly on the edges of tissue.

See: “AIR DRYING ARTIFACT” on page 35

ATYPICAL CHROMOGEN/COUNTERSTAIN STAINING

Staining does not appear as expected. This atypical staining may be present across the entire tissue section or limited to only certain areas of the tissue.

INCORRECT CHROMOGEN PREPARATION

- *Positive tissue control may likely have the same issue*

Verify chromogen was prepared appropriately. Try repeating the stain with freshly prepared chromogen.

INCOMPATIBLE MOUNTING MEDIA

- *Positive tissue control may likely have the same issue*

Verify the mounting media used is appropriate and compatible with the chromogen and/or counterstain. Incorrect mounting media may cause these reagents to dissociate and separate, changing their color and appearance. In some cases, these reagents may leach away from the tissue altogether resulting in the absence of expected staining.

See: “INCOMPATIBLE REAGENTS/MOUNTING MEDIA” on page 21

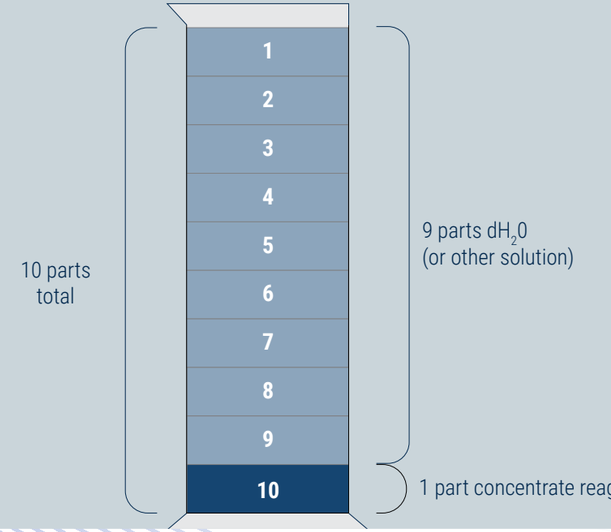
WASH BUFFER PREPARED INCORRECTLY

- *Positive tissue control may likely have the same issue*

Verify wash buffers were prepared appropriately especially when diluted from concentrate. Incorrect dilution of the wash buffer concentrate may result in the chromogen appearing as a different color than usual. With DAB, the chromogen can appear more orange, and the hematoxylin counterstain may likely be the incorrect color as well. This may affect the entire tissue section or appear as a gradient or focal staining issue.

Remember that diluting a 10x wash buffer concentrate requires 1 part wash buffer concentrate and 9 parts distilled/deionized water. It is important to add the buffer concentrate to the container first and then add the water afterwards to ensure that the concentrated reagent is appropriately mixed and diluted.

Dilution Example of 10x Concentrated Solution to 1x Working Solution



A

10x wash buffer concentrate: 1 part concentrate reagent and 9 parts dH_20. Example: 100 mL concentrate reagent + 900 mL dH_20.

TISSUE ARTIFACTS

Tissue artifacts are present in tissue independent of staining and cannot be easily removed without impacting IHC staining results. These artifacts can be verified by reviewing the section microscopically without staining and are present in the H&E stain as well.

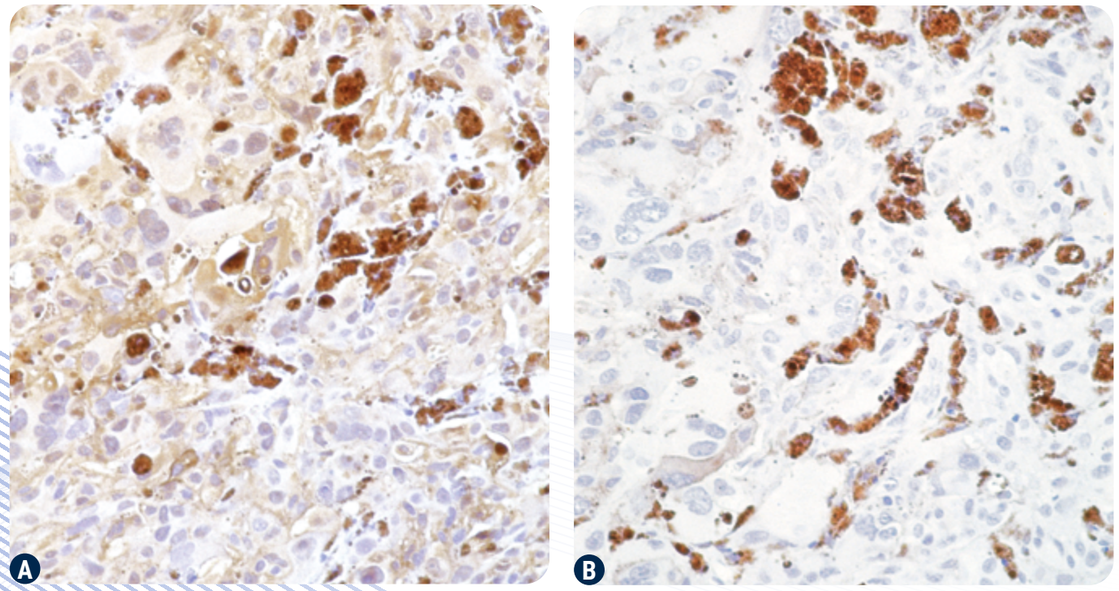
PIGMENTS IN TISSUE

MELANIN

- *Positive tissue control performs as expected.*

BROWN DEPOSITS IN TISSUE EVEN WHEN NO REAGENT APPLIED

Melanin pigment in tissue can be difficult to discern from DAB chromogen staining. Consider using a chromogen with better contrast such as fast red.



Sections from the same case of melanoma. A: Stained for S100 with DAB as the chromogen. Here it is difficult to differentiate between the brown melanin pigment and the reaction product. B: Negative control slide showing the natural color of melanin only.

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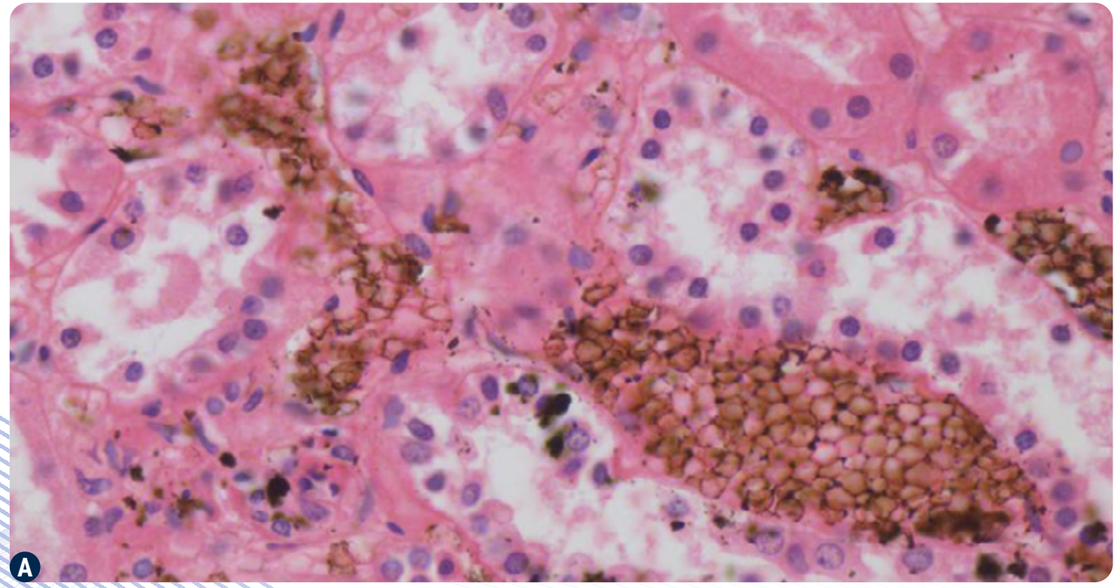
FORMALIN PIGMENT

- *Positive tissue control performs as expected*

BROWN/BLACK DEPOSITS IN TISSUE EVEN WHEN NO REAGENT APPLIED

Formalin pigment results when the pH of formalin is too low. Use neutral buffered formalin and ensure to change the reagent at appropriate intervals.



A formalin-fixed paraffin section of kidney showing the typical deposition of acid formaldehyde hematin (formalin pigment) associated with red blood cells. The pigment is brown to black in color and is birefringent under polarized light. In this case the specimen remained in fixative for an extended period before processing.

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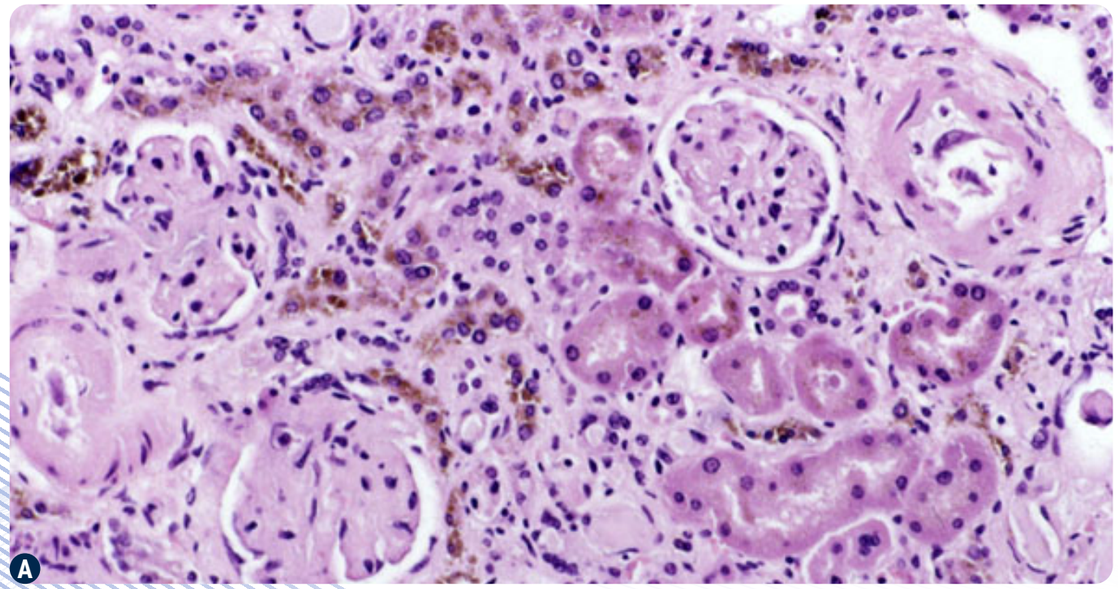
HEMOSIDERIN

- *Positive tissue control performs as expected*

GOLDEN BROWN GLOBULAR DEPOSITS IN TISSUE EVEN WHEN NO REAGENT APPLIED

Hemosiderin is iron-rich deposits in tissue that can be identified by performing a prussian blue stain if needed. If problematic when reviewing IHC staining with DAB, consider using a chromogen with better contrast such as fast red.



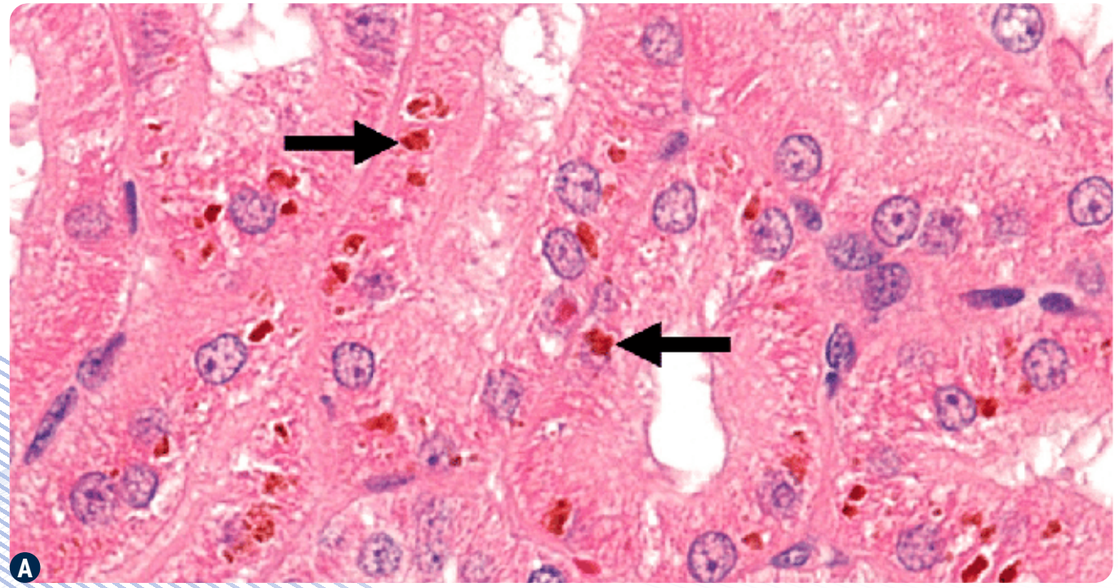
Hemosiderin image of a kidney viewed under a microscope. The brown areas represent hemosiderin. Image source: Wikipedia.

LIPOFUCSIN

- *Positive tissue control performs as expected*

FINELY GRANULAR YELLOW TO BROWN DEPOSITS IN TISSUE EVEN WHEN NO REAGENT APPLIED

Lipofucsin is lipid-containing pigment as part of the oxidative process related to aging. It can be identified by performing lipid stains such as Sudan stains or Oil Red O if needed. It gives acid-fast coloration with carbol fuchsin and stains with ferric ferricyanide (Schmorl method I), methyl green, and the periodic acid-Schiff (PAS) stain. Similar to other pigments, consider using a chromogen that provides better contrast such as fast red.

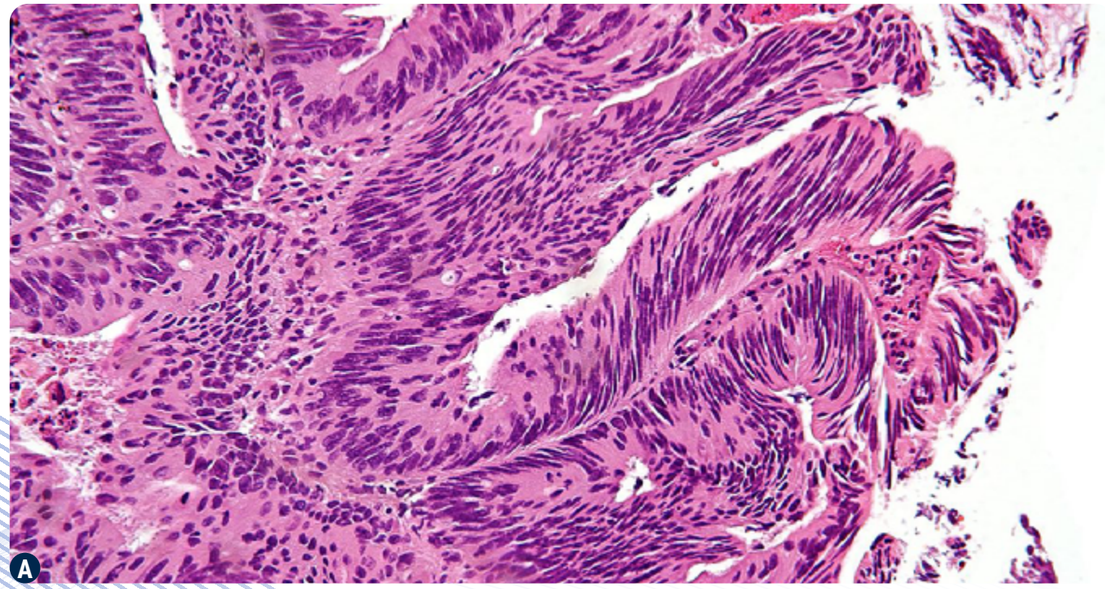


Kidney: Brown pigment deposition in the tubular epithelium. Brown pigment deposition occurs occasionally in the proximal tubular epithelium. The deposited pigments are considered to be lipofuscin. Image source: Wikipedia.

CAUTERY ARTIFACT

- *Positive tissue control performs as expected.*

Used during surgical tissue extraction, electrocautery heats the tissue by facilitating blood clotting to stop bleeding. This cautery artifact makes the cell nuclei appear darker and stretched out compared to normal cells. Some antibodies are sensitive to this heat resulting in either a loss of expected staining or background staining depending on the antibody.



Positive margin with cautery artifact. Image source: librepathology.org.

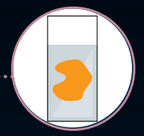
10 STEPS TO OPTIMIZING YOUR IHC

Content based on 101 Steps to Better Histology by Geoffrey Rolls

PRE-STAIN

1 ENSURE OPTIMAL FIXATION

Use known, consistent fixation conditions to produce the best results. Check specimens prior to processing.



2 EFFECTIVELY PROCESS TISSUE

Ensure processing schedule is suitable for tissue size/type.

STAINING PREPARATION

3 CHOOSE ANTIBODY CAREFULLY AND USE APPROPRIATE CONTROLS

Consider sensitivity, specificity, and suitability for your methodology.

4 USE HIGH QUALITY SECTIONS AND CONTROLS

Use thin, flat sections that have been thoroughly dried onto slides.

5 USE AN APPROPRIATE DETECTION SYSTEM

Choose an appropriate detection system that will provide precise, specific staining with adequate sensitivity.

6 REFER TO THE SPECIFICATION SHEETS

Review recommended use such as pretreatment, staining protocols, etc. as applicable.

STAINING

7 OPTIMIZE THE PROTOCOL

Select antibody & detection protocol conditions to start the optimization process.

8 OPTIMIZE RETRIEVAL METHODS

Choose appropriate unmasking conditions for the primary antibody, tissue, and fixation.

9 OPTIMIZE COUNTERSTAINING

Standardize counterstain to provide appropriate staining without obscuring positive staining.

10 STANDARDIZE THE PROTOCOL

Standardize/validate assay to achieve consistent, reproducible results.

CONSIDERATIONS

PREVENT NON-SPECIFIC STAINING

Carefully select antibodies to avoid cross-reactivity, block endogenous peroxidase, and ensure signal-to-noise ratio is optimized.

STANDARDIZE WASHING STEPS

Use standardized washing steps throughout (duration, volume, and form of agitation).

MAINTAIN OPTIMAL TISSUE MORPHOLGY

When optimizing protocols, obtain optimal sensitivity & specificity while maintaining good tissue morphology.

Advancing Cancer Diagnostics
Improving Lives

LEICA BIOSYSTEMS

Leica Biosystems is a global leader in workflow solutions and automation, integrating each step in the workflow. As the only company to own the workflow from biopsy to diagnosis, we are uniquely positioned to break down the barriers between each of these steps. Our mission of “Advancing Cancer Diagnostics, Improving Lives” is at the heart of our corporate culture. Our easy-to-use and consistently reliable offerings help improve workflow efficiency and diagnostic confidence. The company is represented in over 100 countries and is headquartered in Nussloch, Germany.

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