Immunohistochemistry:

An Overview
&
Steps to Better IHC Staining

By Mariammah Simmanchalam

MLS I (Histotechnologist)

PART I: AN OVERVIEW

IMMUNOHISTOCHEMISTRY (IHC)

to detect the presence of a specific protein marker

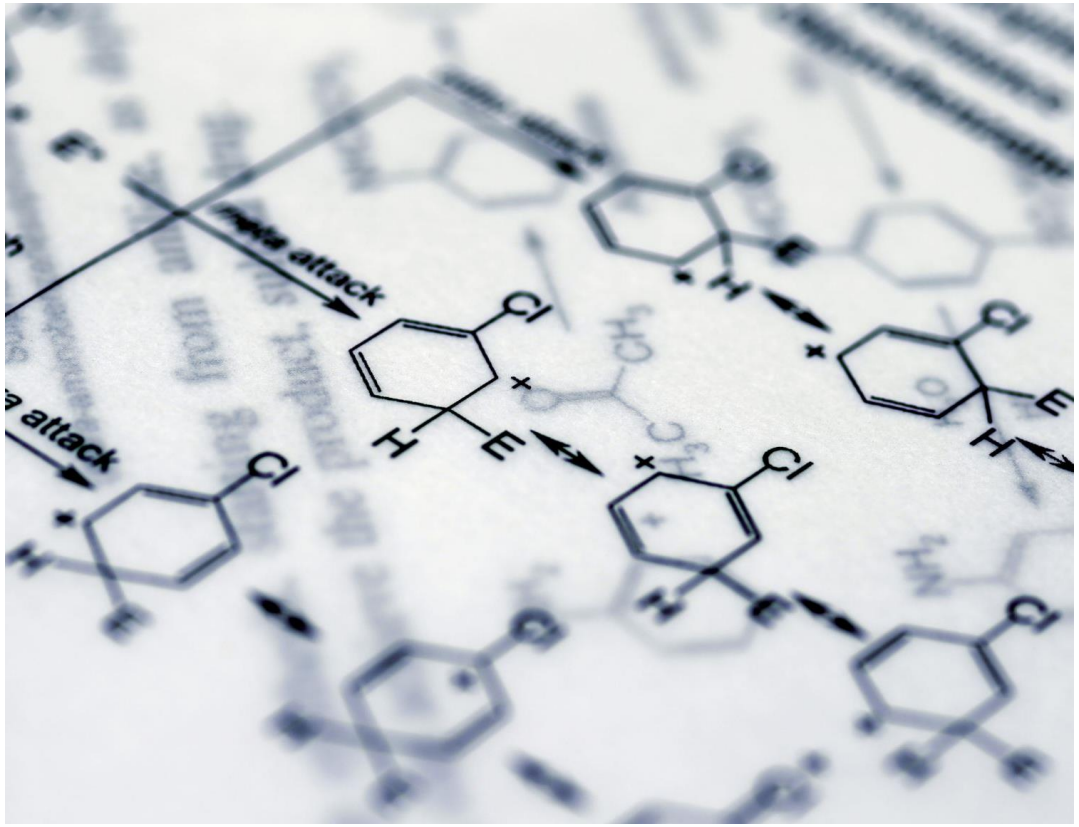
can assist with accurate tumor classification and diagnosis.

evolved to complement the Hematoxylin & Eosin (H&E) and Special Stain techniques that typically show tissue morphology (structure).

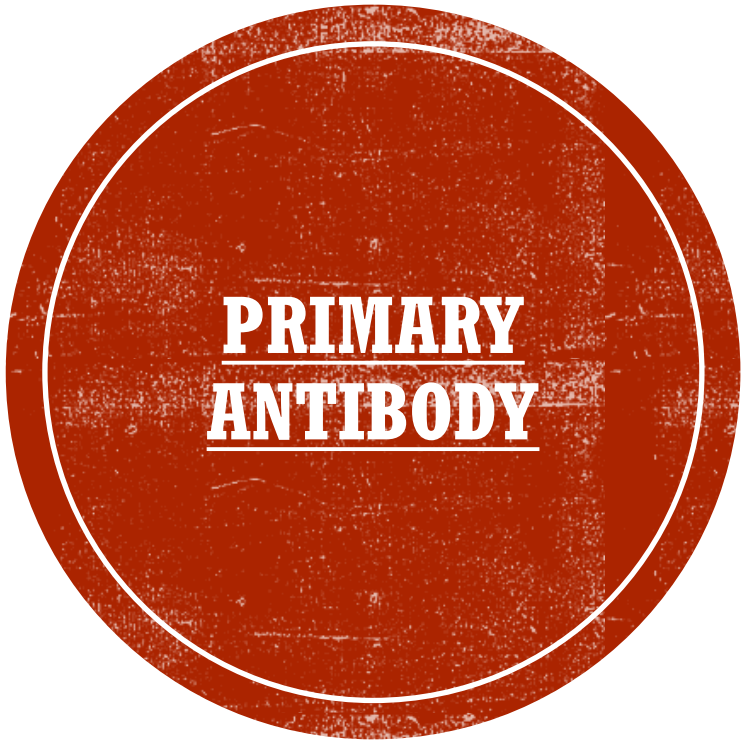
Determines the type of cell from which tumors originated

used as a diagnostic tool to assist in the diagnosis of solid tumors and cytological specimens

TARGET ANTIGEN



- proteins that are within or on the surface of a cell.
- look for the presence or absence of particular antigens to assist with diagnosis.



First stage - application of a primary antibody that binds specifically to the target antigen.

2 types of antibody; polyclonal and monoclonal.

Polyclonal - have an affinity with, and bind to, multiple epitopes (or parts) or the target antigen, and as such are more prone to cross-react to non-target antigens.

Monoclonal - have an affinity to only one epitope and tend to produce, cleaner, more specific staining but are less sensitive or intense.

Secondary antibodies bind to the primary antibody.

Indirect IHC.

Commonly used as multiple secondaries can bind to a single primary to amplify the staining intensity.

The detection system builds on the secondary.

Modern chromogenic detection utilizes enzymes such as Horseradish Peroxidase (HRP) that are conjugated (joined) to an antibody.

Multiple enzymes attached to the antibody are known as polymers, and they again produce more intense staining as there are more molecules for the chromogen to attach to.

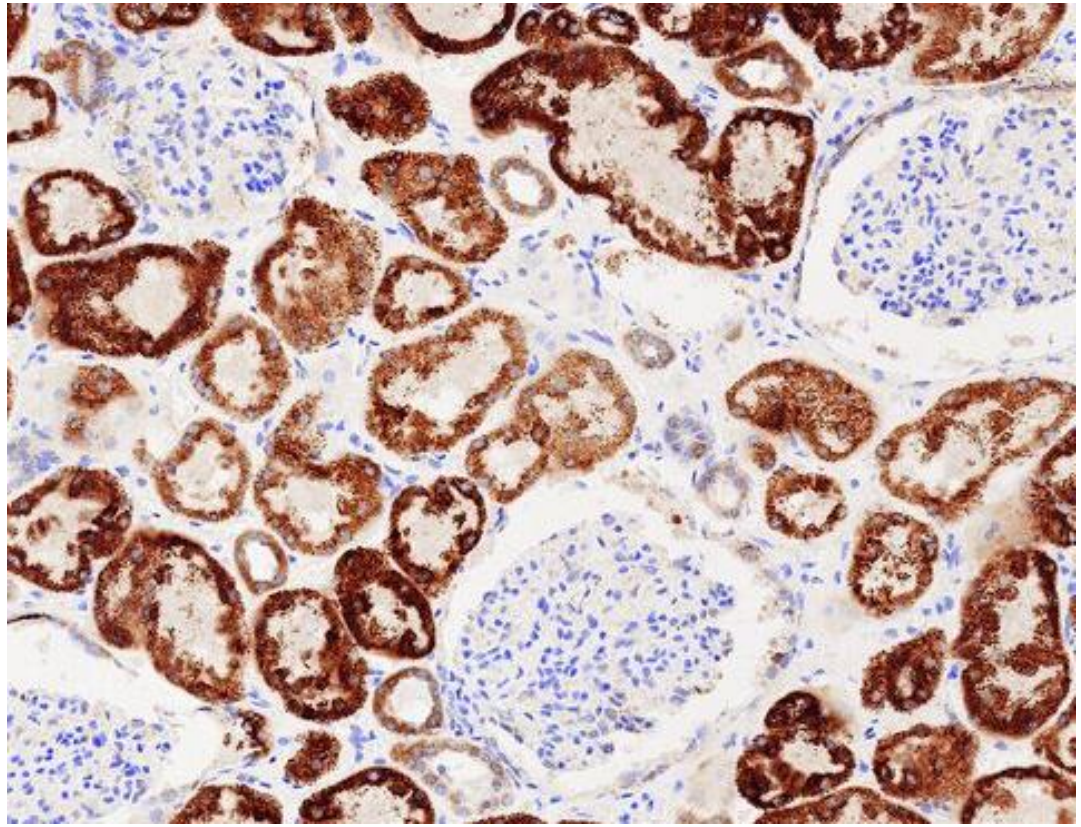
A substrate forms an insoluble colored precipitate that can be visualized under a microscope.

DAB - provides strong and permanent stains.

AP Red (or another red chromogen) is used mainly for skin sections where the brown DAB may be masked by brown melanin pigment.

DAB and AP red - used on the same tissue section to allow the pathologist to visualize two antigens on the one side.

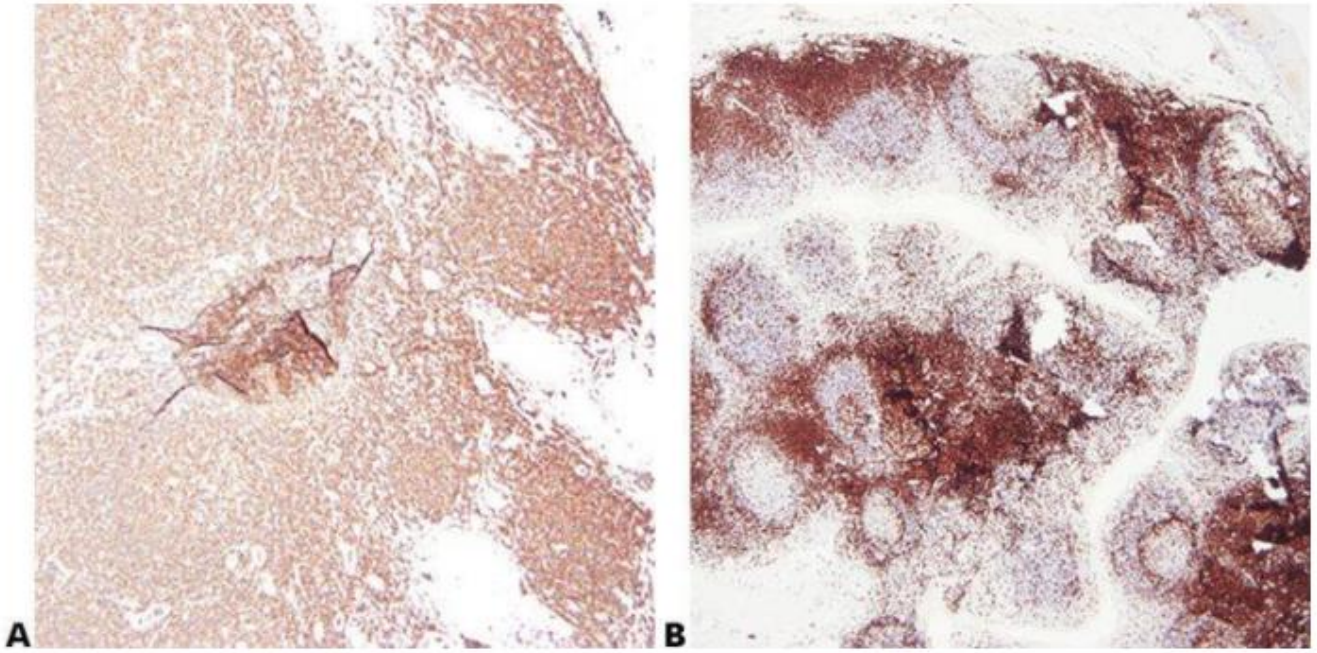
A COMPLETED IHC STAIN



- brown precipitate indicates the presence of the target antigen.
- blue background is a hematoxylin counter-stain that is often applied after the chromogen.
- The counter-stain provides a contrast to the chromogen & helps the pathologist visualize the underlying tissue structure

PART II: STEPS TO BETTER IHC STAINING

STEP 1 : USE HIGH QUALITY SECTIONS



Section A: A bubble under the section (from mounting) has resulted in the subsequent detachment of the section during staining (tonsil, CD45).
Section B: A poor quality section that has not been properly flattened and dried before staining, has lifted making the slide unsatisfactory (tonsil, CD3).

- ✔ Take particular care to use thin, flat sections that have been thoroughly dried onto the slide. Preferably use charged slides or APES coated slides for IHC.
- ✖ Uneven, poorly-adhering sections stain unevenly with variable background staining.

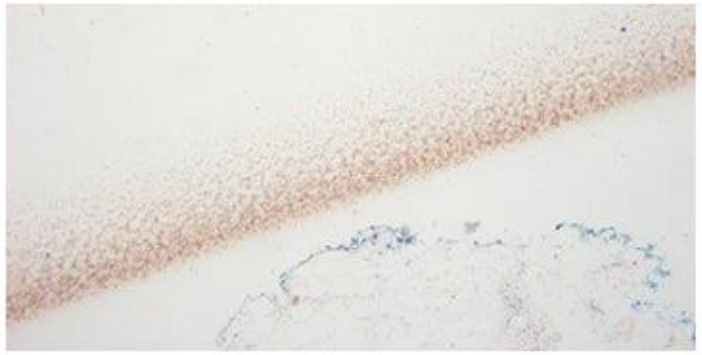
STEP 2: ENSURE OPTIMAL FIXATION

- Good quality fixation using known and consistent fixation conditions (fixative type, pH, temperature, time) produces the best results. Specimens should be checked prior to processing to determine if further fixation is required.
- Inconsistent fixation conditions, producing under-fixed or over-fixed tissues, produce variable results and make troubleshooting difficult.

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

STEP 3 - AVOID SECTION ADHESION PROBLEMS

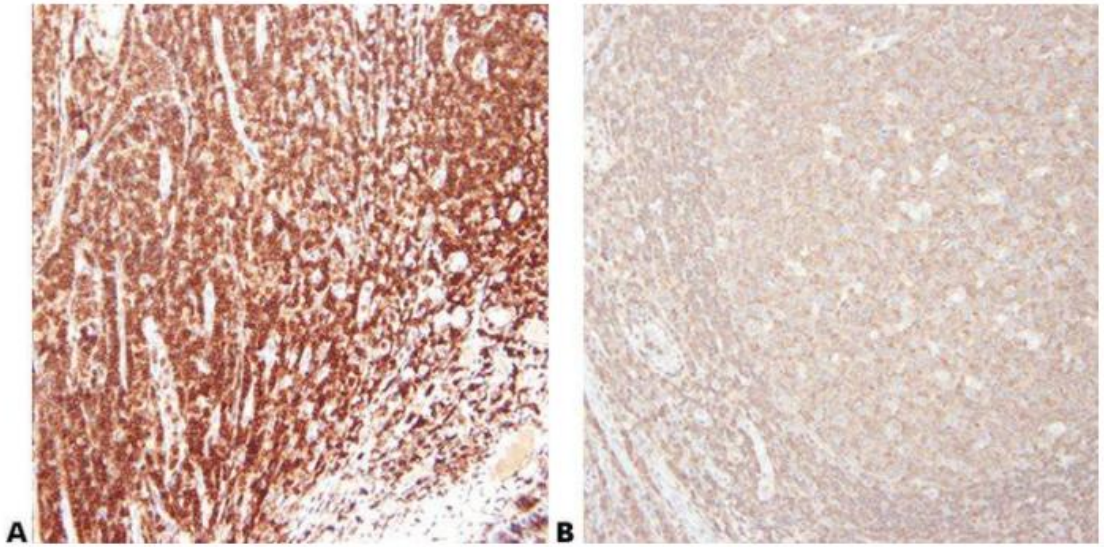
- ✔ Avoid the use of protein-based section adhesives in the flotation bath (glue, starch, or gelatin), particularly on charged slides.
- ✖ Protein-based adhesives can block the surface of the charged slide. This causes inconsistent adhesion and leads to uneven staining due to the pooling of IHC reagents beneath lifting sections.



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

STEP 4 - AVOID CONCENTRATION GRADIENTS

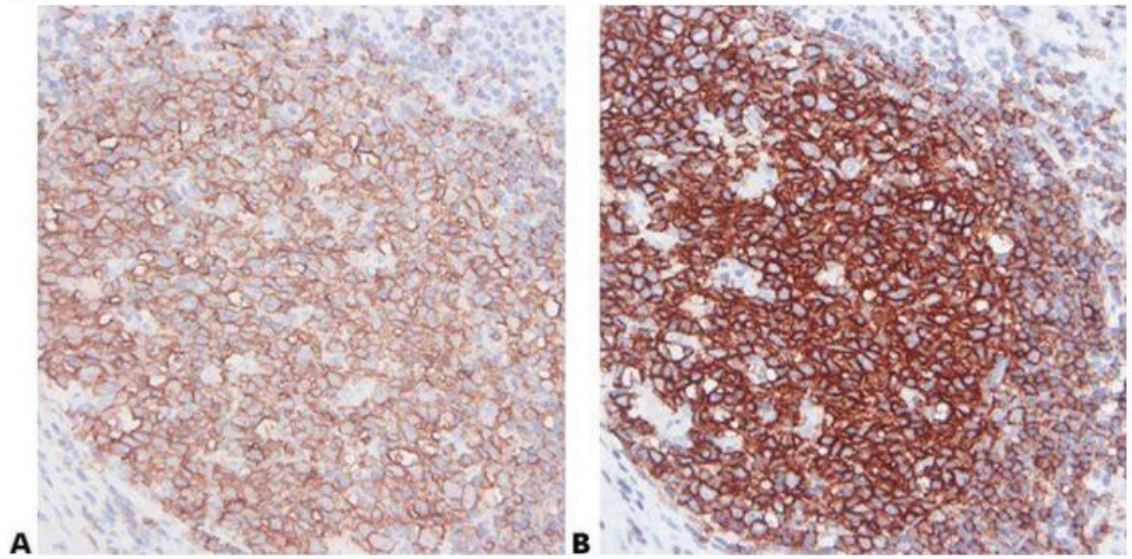
- Concentration gradients are avoided by careful application of reagents.
- "We sometimes see strong staining at one end of the slide progressing to weak staining at the other."



Sections A and B are micrographs taken from opposite ends of the same slide. One end of the slide shows strong staining (A) whereas at the other end (B) the staining was very weak. This is an extreme example of a concentration gradient created during staining (tonsil, CD45).

STEP 5 - CHOOSE ANTIBODY CAREFULLY

- Choose your primary antibody carefully with regard to its sensitivity and specificity. Be aware that antibodies sold by different suppliers often come from the same source and are repackaged/branded for sale. It is important to use the clone name when assessing an antibody.
- "We buy our antibodies based on price alone."

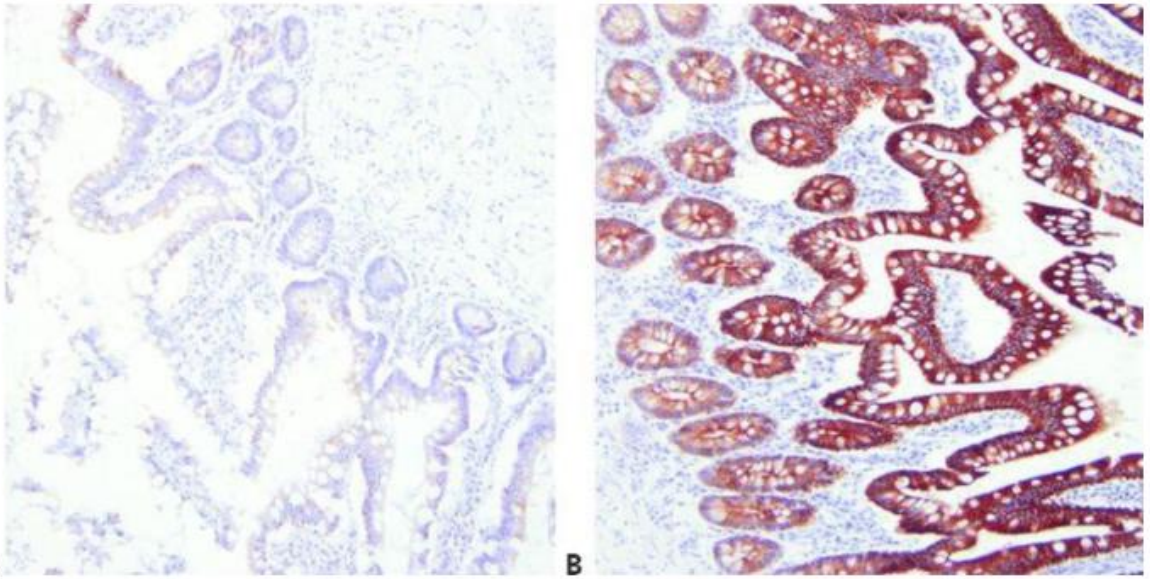


These sections of human tonsil from the same block have been stained with the B cell marker CD20 using primary monoclonal antibodies from different sources (suppliers). In each case, the recommended pretreatment and optimized dilution were used. There is an obvious difference in the quality of the results achieved.

STEP 6 - READ SPECIFICATION SHEETS

✔ Know your primary antibody. Always check the specification sheet to determine the suitability of your method for a particular antibody. Specification sheets should be updated when a new batch of antibody is purchased.

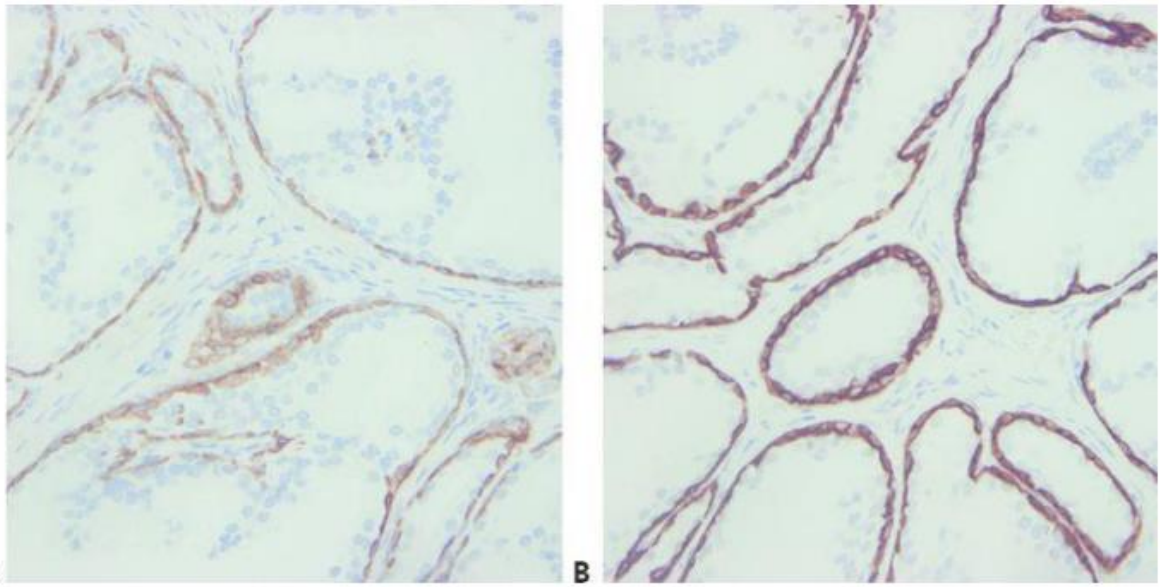
✖ "We don't have access to the antibody specification sheets in our laboratory."



These sections of intestine have been stained for Cytokeratin AE1/AE3. Different retrieval conditions were used for each section. Section A shows unacceptable weak staining, while section B shows strong precise staining.

STEP 7 - OPTIMIZE RETRIEVAL METHODS

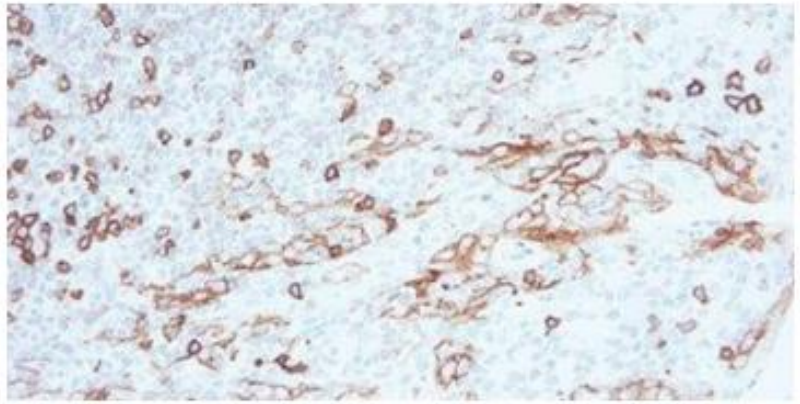
- ✔ Choose appropriate unmasking conditions for the primary antibody being used, the tissue being stained, and the fixation employed (pH, reagent, reaction conditions).
- ✖ The same retrieval technique is used for all primaries on the assumption that there is a successful universal HIER method.



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.

STEP 8 - CONSIDER ANTIBODY CROSS-REACTIVITY

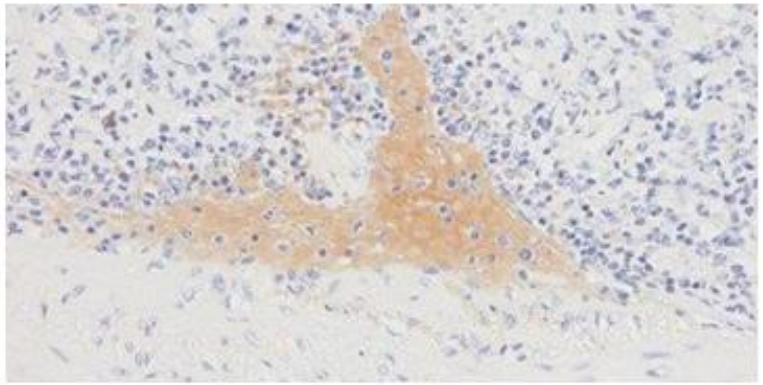
- ✔ Be aware of any potential problems with antibody cross-reactivity (read the specification sheet).
- ✖ No attempt is made to explain unexpected positive staining.



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.

STEP 9 - BLOCK ENDOGENOUS PEROXIDASE

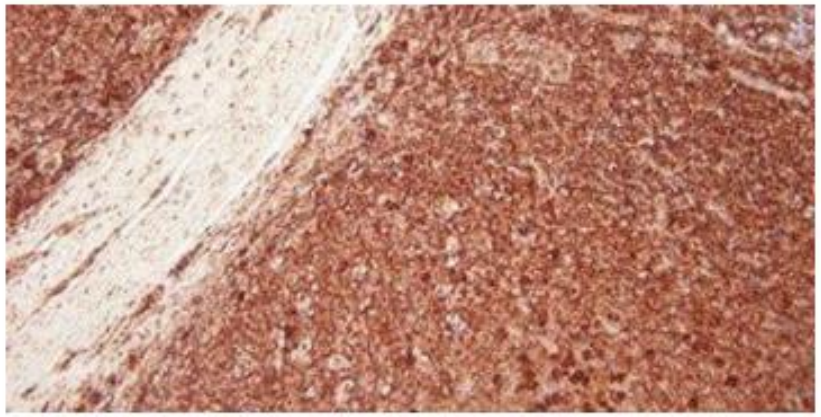
- For peroxidase-based detection systems, always use a peroxidase-blocking step.
- Non-specific staining is often seen in erythrocytes, granulocytes, monocytes, and in muscle. This is due to incompletely-blocked endogenous peroxidase.



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.

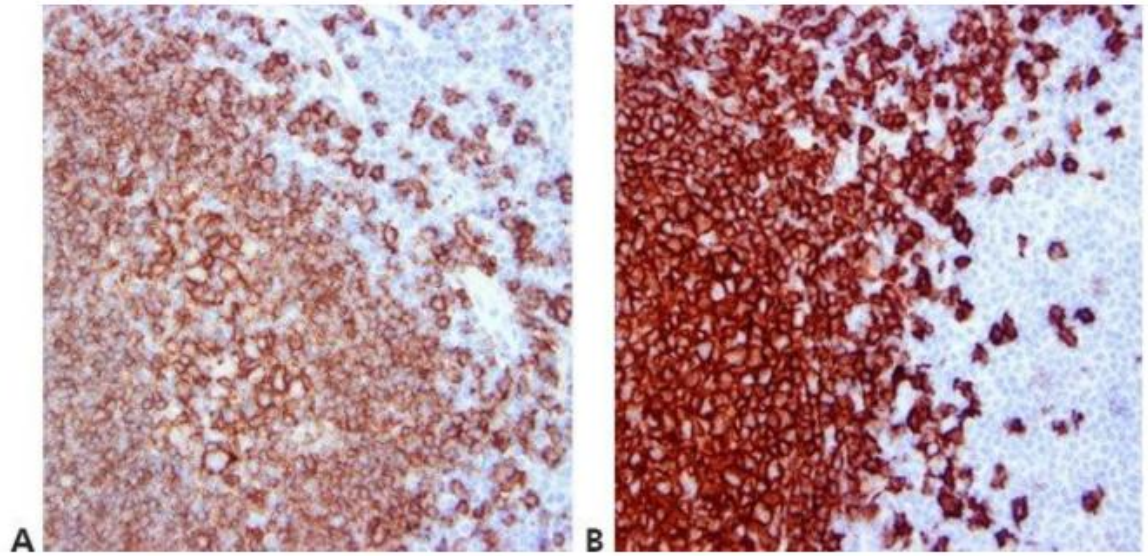
STEP 10 - AVOID BACKGROUND STAINING

- Appropriate protein block is always used.
- Generalized background staining is sometimes seen due to ineffective protein block.



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

STEP 11 - USE AN APPROPRIATE DETECTION SYSTEM

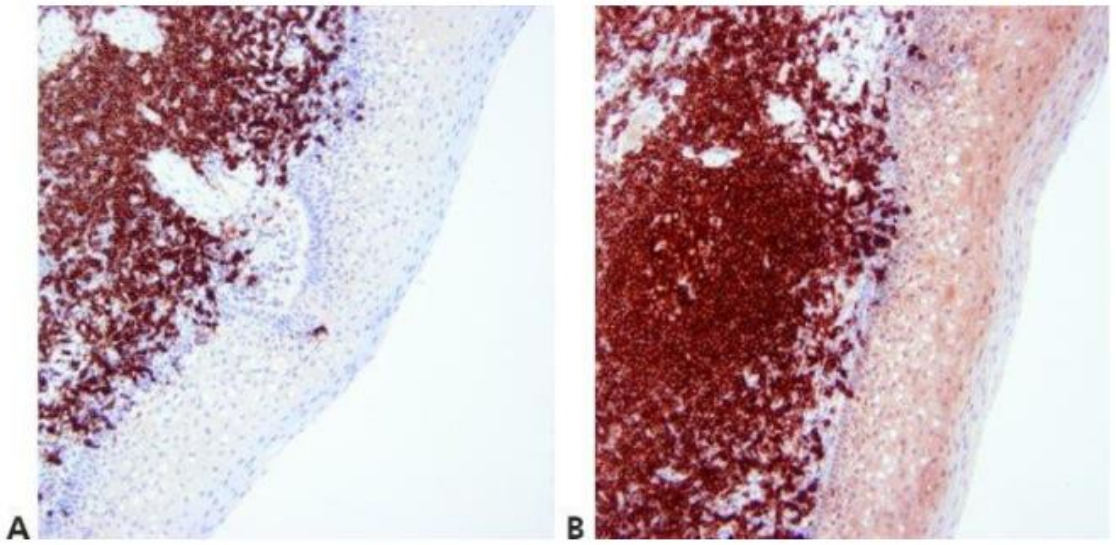


Sections A and B are from the same specimen but have been stained using different detection systems. Note the difference in the intensity and precision of the stains (tonsil, CD20).

- ✔ Choose an appropriate detection system that will provide precise, specific staining with adequate sensitivity.
- ✖ "We have been using the same detection system for a long time and see no reason to change. Sometimes our stains are weak and are not as sharp as we would expect."

STEP 12 - STANDARDIZE WASHING STEPS

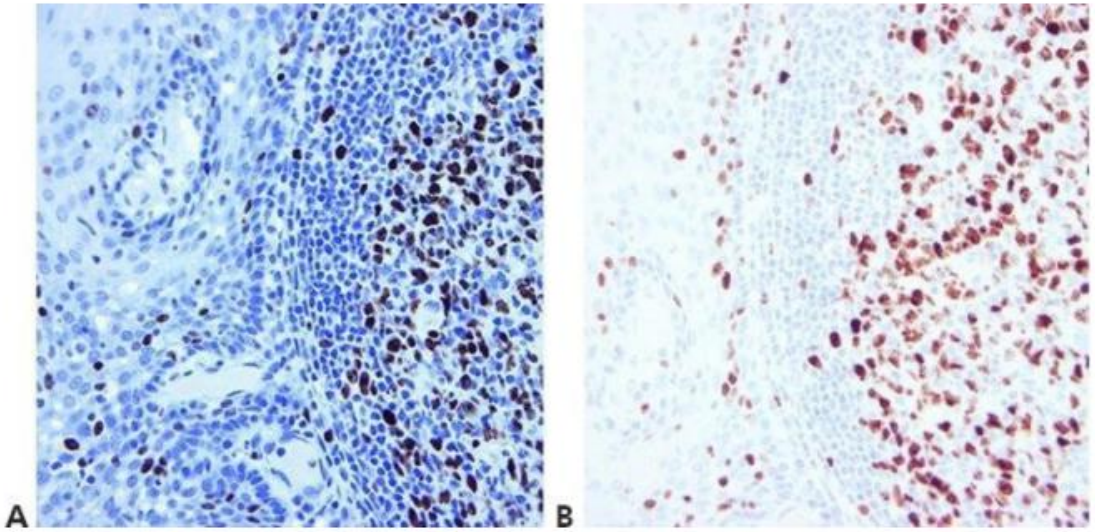
- ✔ Use standardized washing steps throughout (duration, volume, and form of agitation). This will ensure the consistency of results.
- ✖ Results are very variable within runs with the same antibody and between runs on different days. This can be due to different washing techniques used by different operators.



Sections A and B are from the same specimen and have been stained manually using the same reagents. Note the difference in the level of background staining in the stratified epithelium. This is probably due to a difference in the efficiency of the washing technique used (tonsil, CD20).

STEP 13 - OPTIMIZE COUNTERSTAINING

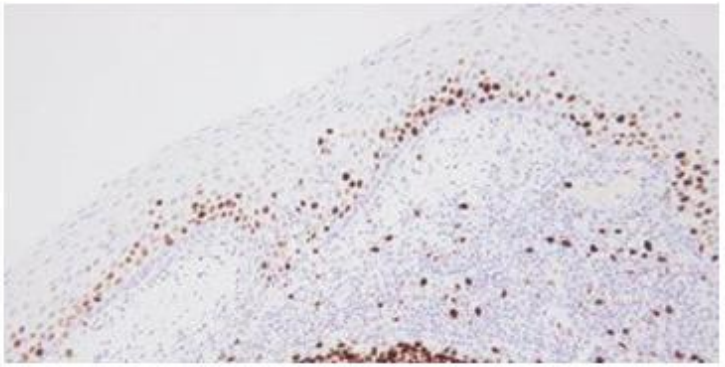
- The level of nuclear counterstain is carefully regulated and standardized so as not to obscure positive staining. The counterstain should provide the best possible contrast between chromogen and background tissue elements. An appropriate counterstain is chosen for the chromogen used.
- Nuclear counterstain is sometimes very strong. This can obscure weak specific staining.



Tonsil stained for Ki67, a nuclear marker for proliferating cells. The two sections are from the same specimen showing different levels of hematoxylin counterstaining. Slide A shows staining that is too strong and would obscure a weak positive reaction. Slide B shows a better level of staining.

STEP 14 - USE APPROPRIATE CONTROLS

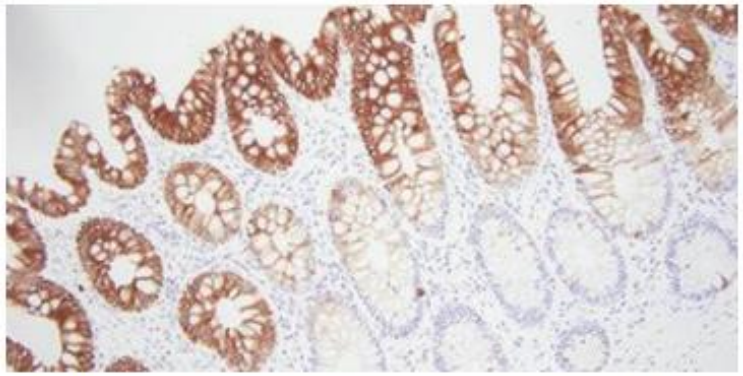
- Always use appropriate positive and negative controls that are carefully examined to validate results. Internal positive and negative controls are also important and provide an excellent means of ensuring quality assurance in IHC.
- "We only do controls when our method doesn't seem to work. If we did them for every run, people wouldn't bother to look at them."



Tonsil stained for Ki67. This was the negative control slide, and the nuclei should not be stained. The primary antibody was mistakenly applied to this slide instead of the negative control reagent.

STEP 15 - EVALUATE RESULTS CAREFULLY

- ✔ Know what to look for and where to look when evaluating your test sections and controls after staining.
- ✖ If staining is observed in test sections, it is assumed the stains are satisfactory.



Intestine stained for AE1/AE3. Unexpected weak staining of the crypt epithelium has occurred. On investigation, it was found that CK20 had been wrongly used as the primary antibody.

BONUS TIP: CHOOSING THE RIGHT ANTIBODIES

Overlooked step is to choose antibodies that work for immunohistochemistry.

Search for specific antibodies from literature, vendors, as well as your peers.

Things to keep in mind with antibodies.

What might work well in one laboratory might not be optimal for your laboratory.

Each antibody needs to be tested with your staining system.

Antibodies over time can lose their staining intensity.

Exposure to air and light can cause this.

BONUS TIP: PRE-STAINING STANDARDIZATION IS CRITICAL

Any pathologist, lab manager, or histotechnologist will readily acknowledge that preparation for IHC staining begins the moment tissue is acquired.

The literature documents optimal conditions for tissue fixation, processing, and sectioning to ensure morphology and antigenicity are maintained.

Further improvements in maintaining consistency to correctly control for these factors could be pursued.

This may involve a laboratory establishing itself at the site of collection, recognizing that accessioning and sample preparation begins here.

HOWEVER, THERE ARE A NUMBER OF GOOD PRACTICES THAT ARE KNOWN TO ESTABLISH AND MAINTAIN HIGH QUALITY AND CONSISTENT RESULTS FROM IHC STAINING.

ABSENCE OF STAINING OR WEAK STAINING

Ab too dilute-check Ab titration, increase concentration, lengthen incubation time, increase temp. of reaction, check amount of rising buffer

Inadequate fixation-avoid delay of fixation (>30min) or over fixation (48hrs)

Reagent not working - check exp. dates, storage temp,

Prolonged or excessive heating-optimize antigen retrieval time

Insufficient incubation time - lengthen incubation time to achieve desired intensity of staining, add heat, increase conc. of Ab

BACKGROUND STAINING

Incomplete removal of paraffin-use only complete deparaffinized slides

Poorly fixed or necrotic tissue

Thick preparation-Cut section at 3µm-5µm

Inappropriately conc. Ab- check titration, decrease conc., incubation time, temp. of reaction

Incomplete rinsing of slides-follow protocol for proper slide rinsing

THANK YOU FOR LISTENING