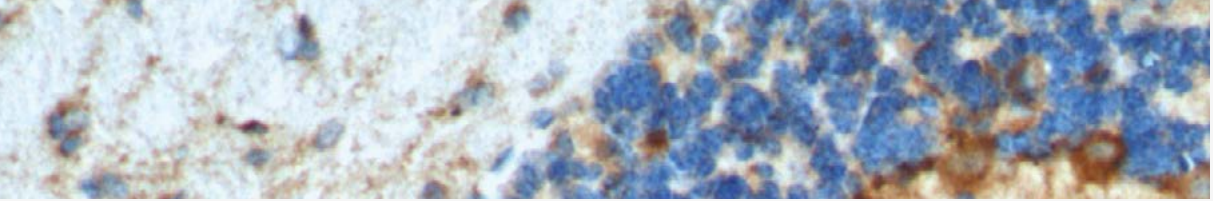

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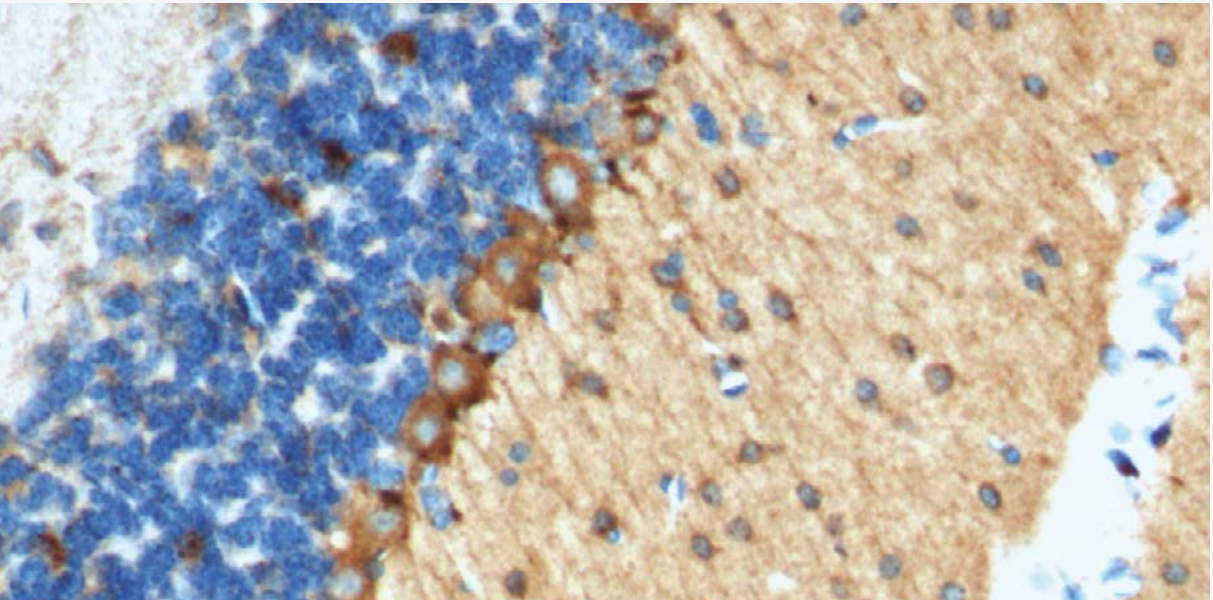
How To Optimize Your

IMMUNOHISTOCHEMISTRY EXPERIMENT

Technical Tips and Troubleshooting



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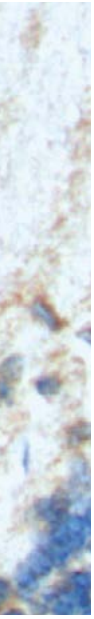

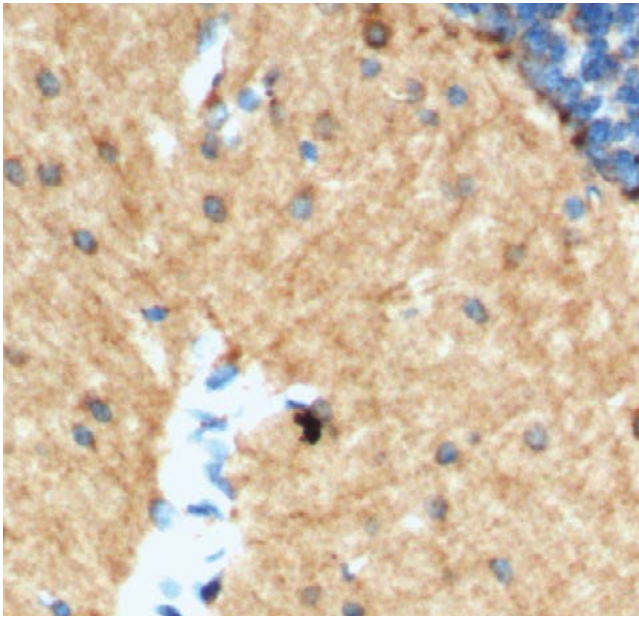

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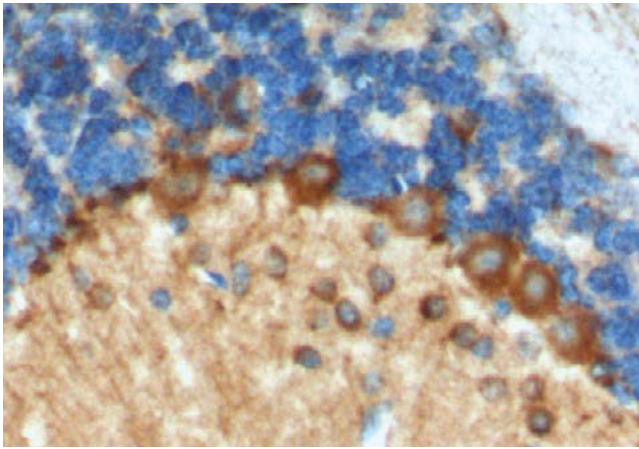
INTRODUCTION

- **Immunohistochemistry** allows the visualization of proteins in tissue while retaining its microstructure.
- **Immunohistochemistry** helps to demonstrate the exact position and distribution of the protein-of-interest.
- In an **Immunohistochemistry** experiment the antigen of interest is localised by the binding of an antibody. The antibody-antigen interaction is then further visualized by via chromogenic or fluorescent detection.



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APPLICATIONS

- Prognostic markers in cancer
- Tumours of uncertain histogenesis
- Metastasis
- Responses to treatments
- Infections
- Neurodegenerative diseases
- Muscle diseases
- Brain trauma

IHC is a crucial technique widely used in different medical research laboratories and clinical diagnostics.

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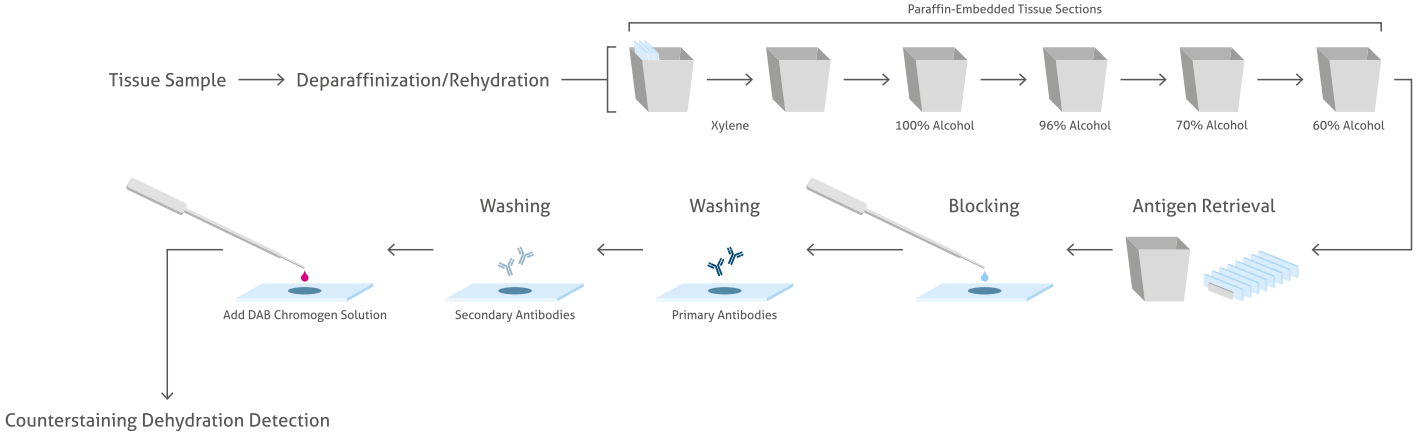
IMMUNOHISTOCHEMISTRY STEPS



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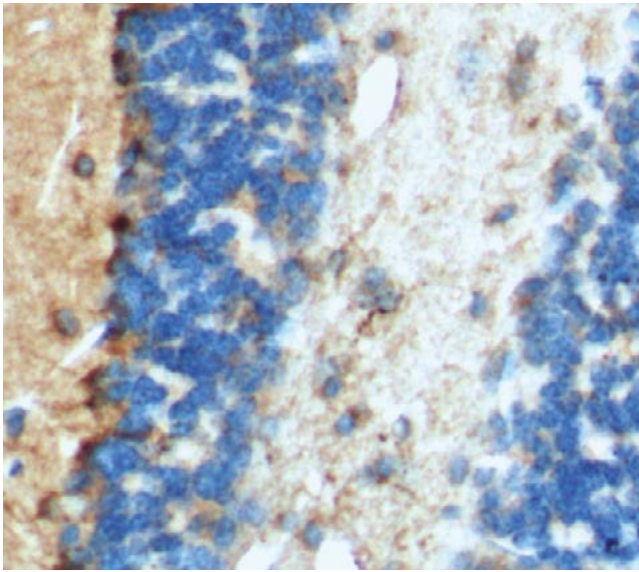
MATERIALS AND EQUIPMENT

Tissue Preparation

- Frozen/paraffin-embedded tissue
- Cryo-embedded media
- Sucrose
- 4% PFA
- Microtome
- Glass slides
- Coverslips
- Refrigerator
- Incubator
- Xylene
- Ethanol



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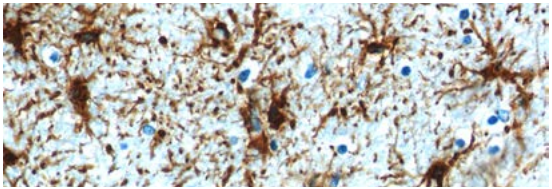
MATERIALS AND EQUIPMENT

Tissue Treatment

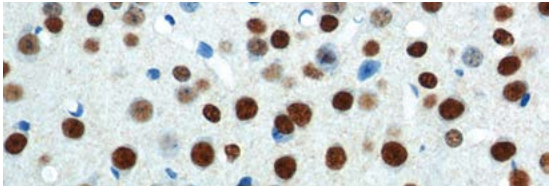
- Serum, BSA
- Citrate buffer
- Tris-EDTA buffer
- Boiling source



Immunohistochemistry of paraffin-embedded human brain tissue slide using GFAP Antibody (60190-1-IG) at dilution of 1:5000 (40x objective). Heat mediated antigen retrieved with Citric acid buffer, pH6.0.

Detection/ Visualization

- Primary and secondary antibodies
- Hematoxyline
- Citrate/Tris-EDTA buffer
- DAB solution
- Microscope



Immunohistochemistry of paraffin-embedded mouse brain tissue slide using TDP-43 Antibody (10782-2-AP) at dilution of 1:400 (40x objective).

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IHC FACTORS

IHC Factor	To Consider
Sample Type	Fixed, frozen
Antigen	Species, level of expression, subcellular location
Epitope	Conformation, post-translational modification
Primary Antibody	Mono vs. Polyclonal
Blocking	Sera, BSA, commercial buffer, temperature, pH, dilution, incubation time
Secondary Antibody	Species, label type
Labelling	Chromogenic, enzymatic, fluorescent
Counterstaining	Chromogenic, fluorescent
Analysis	Microscope, software-based analysis, evaluation by eye
Controls	Secondary antibody only, antigen positive tissue, isotype control

The IHC protocol contains many steps that may require optimization to ensure specific antibody binding and optimal visualization of the target protein.

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SAMPLE TYPE

Paraffin

- Easier (than frozen sections) to handle
- Antigen retrieval
- Damaged antigens
- Mainly used
- Short storage period

Frozen

- Good antigen recovery
- Altered morphology
- Special storage
- Challenging cutting
- Preserves the immune activity of the antigen which may have been embedded during the tissue processing

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SAMPLE HANDLING

Frozen Sections Of Clinical Samples

- Freeze and embed with cryo-embedded media.
- Temperature in the cryostat:

  -10°C/-15°C (unfixed tissues, brain, liver, lymph node)

  -15°C/-20°C (spleen, kidney, muscle tissues)

  -25°C/-30°C (fat containing tissue)

Paraffin Tissue Slide Handling

- Tissue sections are placed on glass slides.
- Adhesion by air drying, baking in an incubator.
- Cut and unbaked slides can be stored at 4°C.
- Keep the storage time short, as antigenetic potential might be affected.

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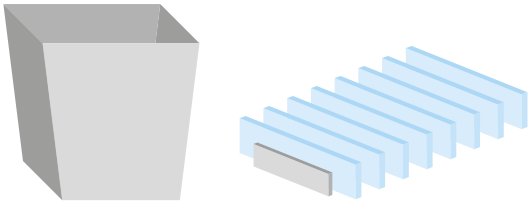
ANTIGEN RETRIEVAL

- In paraffin embedded tissue, the fixation process cross-links proteins, resulting in masked epitopes.
- Unmasking can be carried out via heat-induced epitope retrieval (HIER) or via poteolytic-induced epitope retrieval (PIER).
- It depends on the tissue type and primary antibody which retrieval technique shows better results.



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SAMPLE HANDLING

Heat-induced Epitope Retrieval (HIER)

- Heat up for a certain time in a specific buffer.
- Microwave, pressure cooker, water bath, steamer, etc.
- Citrate buffer/Tris-EDTA buffer.
- EDTA buffer for antibodies against phospho-tyrosines.

Proteolytic-induced Epitope Retrieval (PIER)

- Used enzymes are trypsin, proteinase k, pepsin, protease or pronase.
- Trypsin digestion:
  1. Prepare the trypsin and pre-heat to 37°C. Pipette the enzyme solution onto the section.
  2. Place the slides in a humidified container and then into 37°C incubator.
  3. After 15 min, remove the slides from the incubator and transfer to a rack in a container with tap water. Rinse with running water for 3 min.

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HIER VS. PIER

Comparison Of Heat-induced Epitope Retrieval (HIER) and Proteolytic Epitope Retrieval (PIER)		
	Heat-induced	**Proteolytic-induced**
Advantage	Smooth epitope recovery	Preferred for difficult-to-recover epitopes
Disadvantage	No impact on cell morphology	Impacts and damages the epitope
Difficulties	Unequal retrieval due to unequal heating	Concentration calibration
pH*	Typically pH 6 (citrate), pH 9 (Tris-EDTA)	Typically pH 7.4
Incubation time*	Around 20 mins	Around 10 mins
Temperature*	Around 100°C	Around 37°C

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

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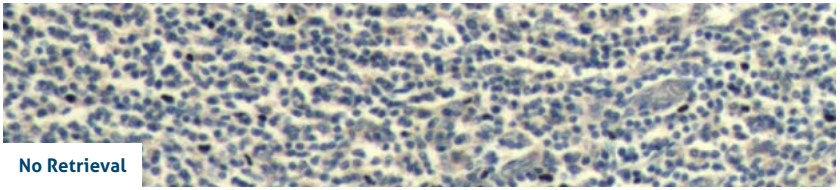
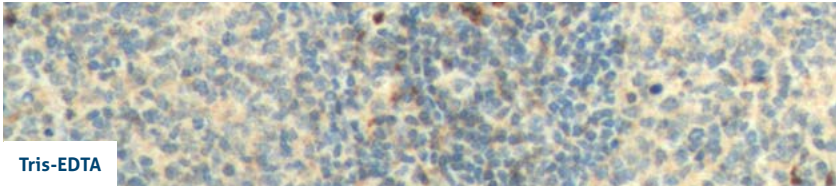
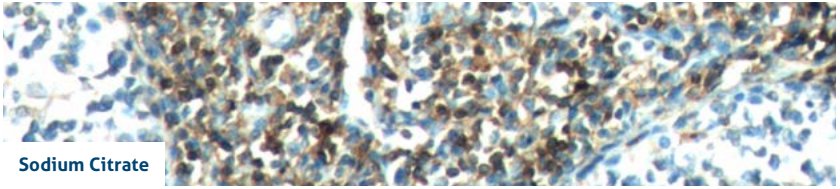
ANTIGEN RETRIEVAL



No Retrieval



Tris-EDTA



Sodium Citrate

Antigen retrieval optimization of CD3 gamma antibody (60347-1-AP) on paraffin embedded tonsillitis tissue slides.

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BLOCKING

Protein Blocking

- Serum or bovine serum albumin (BSA).
- Prevents unspecific binding to hydrophobic side chains of proteins present in tissue.
- Serum in multi-stainings, blocking serum against all used secondary antibodies is needed.
- If BSA is used, add 0.1–0.5% Trition-X or Tween.

Blocking Non-specific Ionic Bindings

- Non-specific ionic bindings due to e.g. Van der Waals interactions, dipole-dipole interactions or net charges of specific amino acid groups.
- Alter the ionic strength of the antibody dilution.

Endogenous Enzyme Blocking

- When using HRP or alkaline AP conjugates, the endogenous levels have to be blocked.
- HRP can be blocked with H_2O_2 and AP can be blocked with acetic acid buffers or Levamisole.

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PRIMARY ANTIBODIES FOR IHC

Main questions to ask when choosing a primary antibody supplier	
1	How many times has the antibody been cited?
2	How has the antibody been validated?
3	Has the application of interest already been tested?
4	Who is the original manufacturer?
5	Is all the data and information about the antibody publicly available?
6	What is the vendor's refund policy, delivery time, and stock availability?
7	What is the price?

The initial choice of the primary antibody can affect the whole outcome of the experiment.

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PRIMARY ANTIBODIES FOR IHC

Optimization Of Primary Antibody Conditions

- **Titrate different antibody conditions**
- **Specific staining, but background signal:**

  Vary incubation time and temperature
- **High-affinity antibody, high concentration:**

  Incubate with a high concentration, short time
- **High-affinity antibody, low concentration:**

  Increase incubation time, lower incubation temperature

Polyclonal antibodies in general can be used at a higher dilution than monoclonal antibodies.

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SECONDARY ANTIBODIES FOR IHC

Finding The Best Secondary Antibody For The Reduction Of Signal-To-Noise Ratio

- **Subclass Specificity**

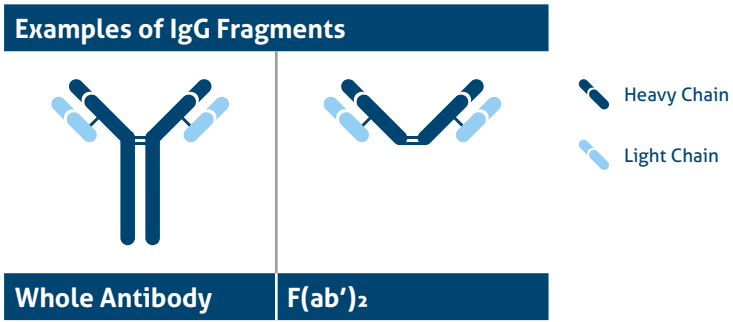
  Use of isotype specific secondary antibody.

- **Cross-absorption**

  Reduction of cross-reactions with other species.

- **F(ab')$_2$ Fragments**

  Tissue penetration is facilitated. No bkg signal due to binding to the Fc receptor (e.g., lymph nodes, spleen, macrophages, etc).



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VISUALIZATION

Direct Detection

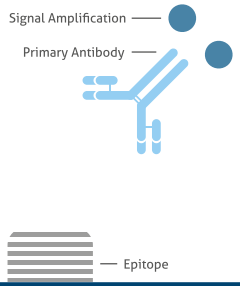
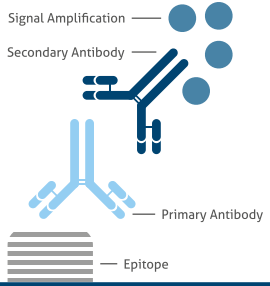
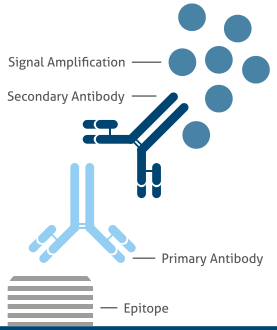
- Directly labelled primary antibody
- Low signal generation
- Highly expressed antigens

Indirect Detection

- Secondary antibody is labelled
- Signal amplification
- Medium to low expressed antigens
- High background noise

VISUALIZATION

Different Detection Systems And Signal Amplification

Expression level of protein of interest		
High	Medium	Low
		
Direct Labeled First Antibody	Secondary Antibody Labeled	Secondary Antibody Labeled Plus Enhancer

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VISUALIZATION

Chromogenic Signal Generation

- Enzyme attached to primary or secondary antibody
- Forms insoluble coloured product, when an organic substrate is added
- Horseradish Peroxidase (HRP) and Alkaline Phosphatase react with e.g., 3,3' Diaminobenzidine (DAB)

Fluorescent Signal Generation

- Fluorochrome attached to primary or secondary antibody
- Beneficial for multi-colour staining experiments
- Limited by the short lifetime of fluorescent labelled probes

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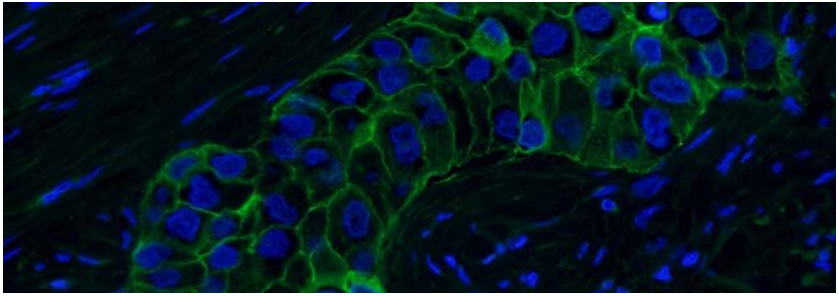
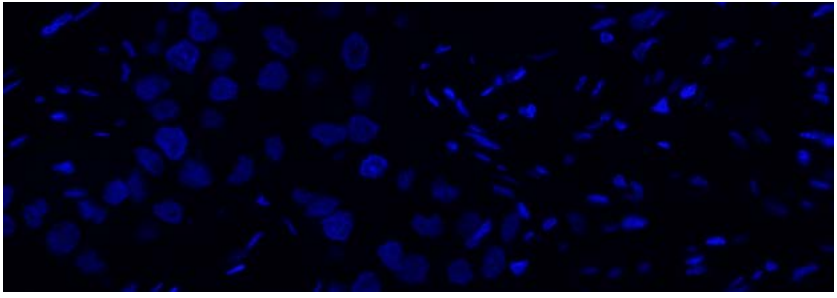
VISUALIZATION





IHC staining of human breast cancer tissue with E-Cadherin (Cat.Nr. 60335-1-Ig) and Alexa® 488 Goat anti-mouse IgG (H+L) (Cat.Nr. SA00006-1), nuclei was counterstained with DAPI, 40x objective.

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CONTROLS

Positive Control

- Control tissue that is known to express the protein of interest.
- Detection of false negative results.
- Helps to validate the protocol.

Negative Control

- Control tissue that is known not to express the protein of interest.
- Incubation just with the secondary antibody.
- Observation of unstained tissue in brightfield/ fluorescence channel gives an idea about biological background signal/autofluorescence.
- Incubation with a non-immune immunoglobulin of the same isotype.

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TROUBLESHOOTING

No/Weak Staining

Potential cause	Suggested test or solution
The primary/secondary antibody lost its activity.	Use a new lot of antibody. Improper storage of antibody. Follow manufacturer's instruction. Normally, prepare single-use aliquots and store at -20°C. Extensive thaw-freezing cycles have damaged the antibody.
Conditions of antibody are not optimized.	Titrate the antibody concentration to optimize best working conditions. Incubate the primary antibody at room temperature or at 4°C overnight.
Protein of interest is not expressed in used tissue.	Run a positive control.
Protein of interest is low expressed in used cells.	Use signal amplification when visualizing.
Damaged epitope.	Change to another antigen retrieval buffer/technique for paraffin-embedded samples.
Not suitable for this application.	Check validation data of manufacturer.

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TROUBLESHOOTING

Background Staining/ Non-specific Staining

Potential cause	Suggested solution
Too high primary/secondary antibody concentration.	Titration of antibodies to determine optimal working concentration.
Non-specific binding of primary/ secondary antibodies.	Prolong blocking step and increase concentration of blocking solution. Run positive and negative controls.
Non-specific binding of secondary antibodies.	Run control with secondary antibody only. Change to a cross-adsorbed secondary antibody or a fragment antibody.
Sample is poorly washed.	Repeat or prolong washing step.
Incubation temperature/time is not suitable.	Optimize conditions.
Damaged epitope.	Change to another antigen retrieval buffer/ technique for paraffin-embedded samples.

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TROUBLESHOOTING

Inappropriate Cell Morphology

Potential cause	Suggested solution
Harsh antigen retrieval conditions	Optimize buffers, temperature, pH, incubation time, concentration.
Unclear tissue structure	Optimize thickness of tissue slides. Cut new sections.
Tissue is not adhesive to glass slide.	Optimize fixation. Decrease heating time or temperature during HIER.
Physically damaged cell shape.	Under-fixation. Change fixative or fixation time.

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