IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is a method for detecting antigens or haptens in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues

USED FOR:

A) RESEARCH

B) DIAGNOSTICS

-DIAGNOSIS

-PROGNOSIS

-THERAPY

ANTIGENS

***ANTIGEN**: Substance that can induce an immune response (proteins, polysaccharides, lipids or nucleic acids……)*

***ANTIGENIC DETERMINANT OR EPITOPE**: it is the part of an antigen that is recognized by the immune system (SPECIFIC BINDING SITE)*

***ANTIGENICITY**: Antigenicity is the capacity of a chemical structure (either an antigen or hapten) to bind specifically with a specific antibody*

LOSS OF ANTIGENICITY:

-AUTOLYSIS

-FIXATION: *-protein denaturation*

-H bond loss (protein stereochemistry modifications)

-loss or re-location of epitopes

-PARAFFIN EMBEDDING: epitopes' modifications due to heating

ANTIBODIES

Antibody type and animal where it has been raised

-POLYCLONAL

-MONOCLONAL

AFFINITY: it is the strength of binding of a antibody to its antigen. It is typically measured and reported by the equilibrium dissociation constant (KD), which is used to evaluate and rank order strengths of bimolecular interactions. The binding of an antibody to its antigen is a reversible process, and the rate of the binding reaction is proportional to the concentrations of the reactants.

$$Ab + Ag \rightleftarrows AbAg$$

$$\text{Affinity constant } K = \frac{[Ab\ Ag]}{[Ab]\,[Ag]}$$

MARKERS (label – reporter)

The antibody binding to an antigen is visualized using an appropriate detection system. The method of detection can be ***direct or indirect****, and may generate a fluorescent or chromogenic signal.*

FLUORESCENT COMPOUNDS:	***EXCITATION***	***EMISSION***
Fluorescein isothiocyanate (FITC)	*490 nm*	*525 nm*
Tetramethylrhodamine isothiocyanate *(TRITC, MRITC)*	*530 nm*	*580 nm*

-non permanent slides, no dehydration.

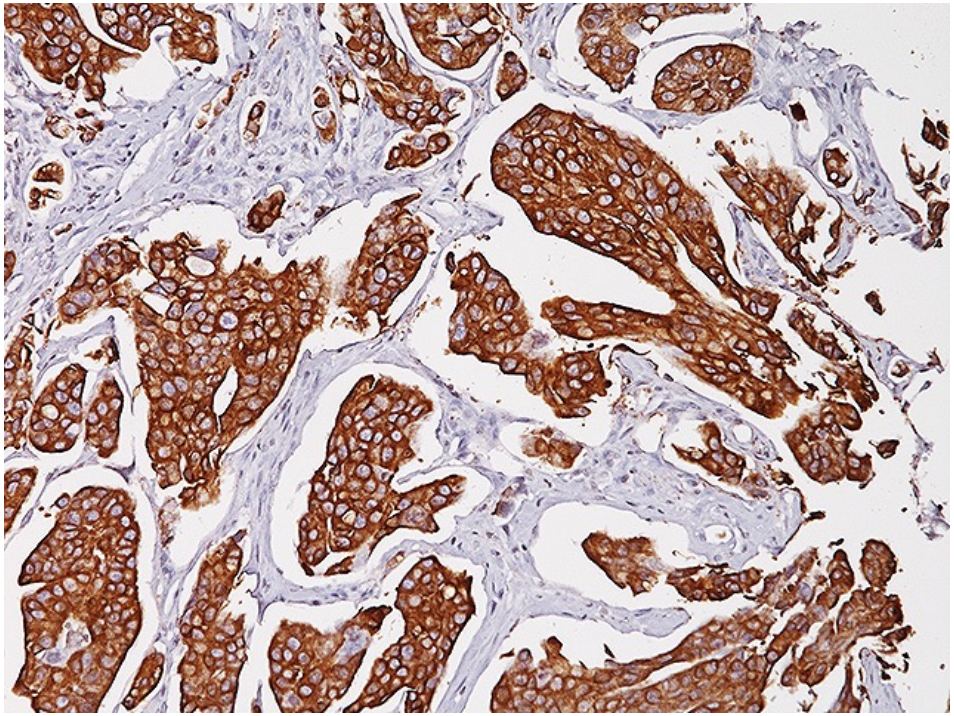
CHROMOGENIC COMPOUNDS*:*

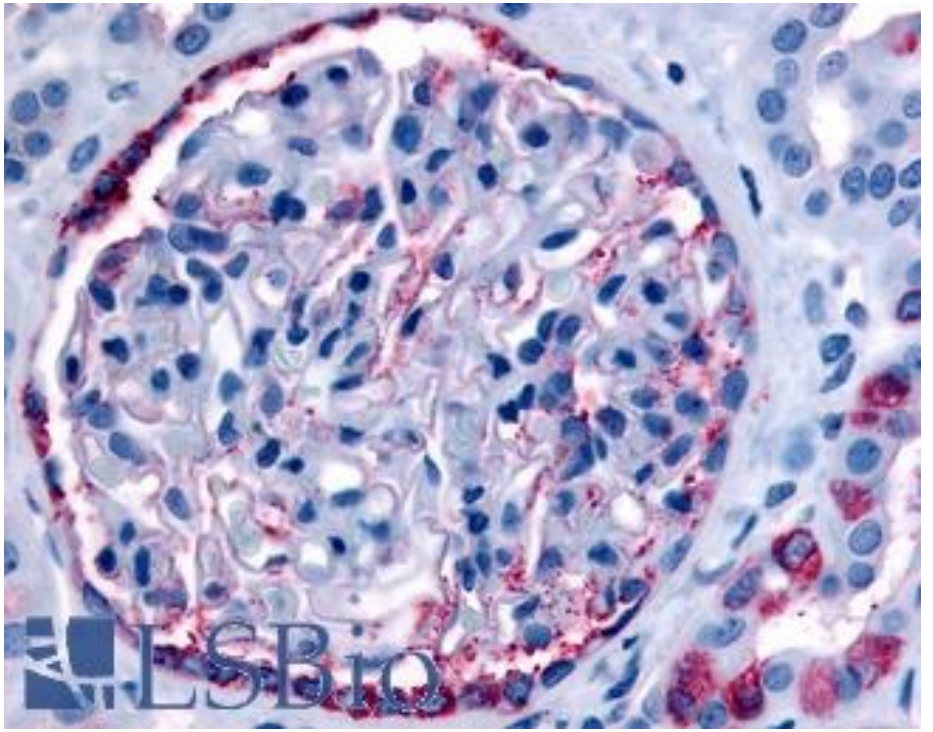
horseradish peroxidase *: It is a glycoprotein of 40 Kd including an EME group. It catalyzes the oxidation of various organic substrates by hydrogen peroxide*

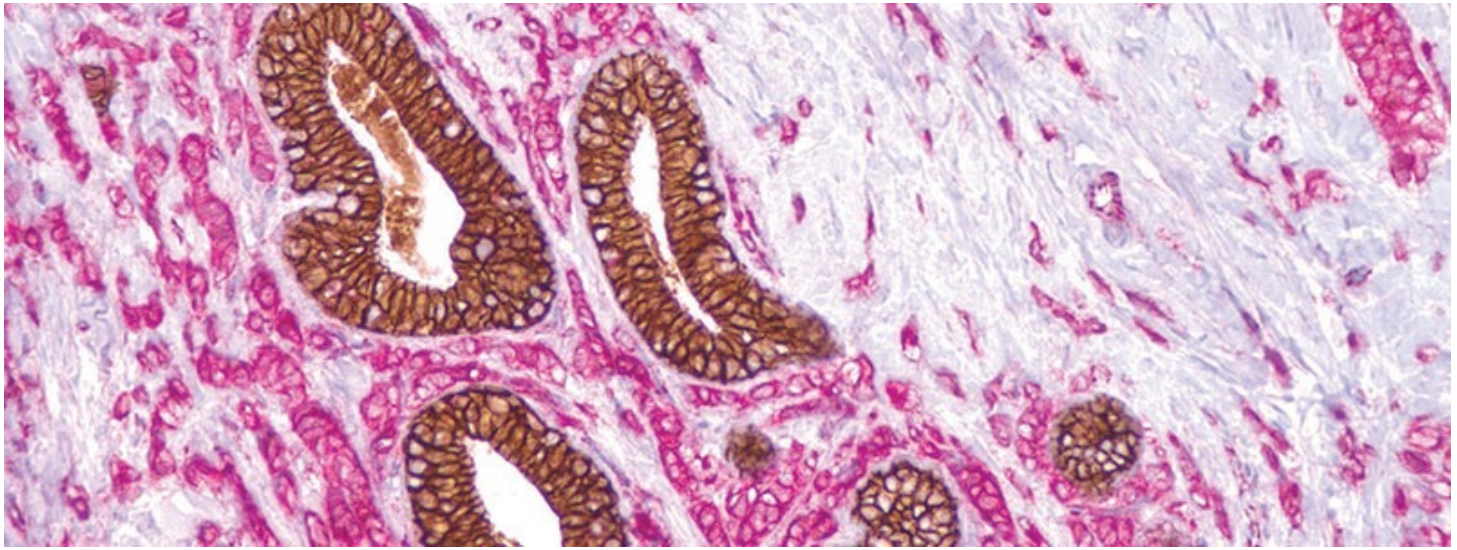
It converts 3,3' diaminobenzidine (DAB) into brown products.

Alkaline phosphatase*: it converts AEC (3-amino-9-ethylcarbazole) into a red product*

IHC

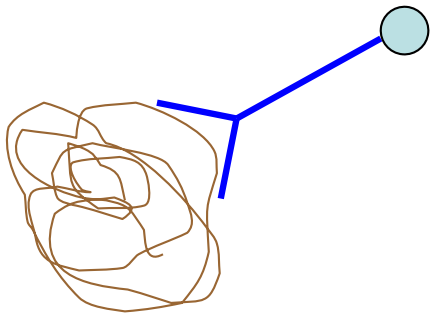






IHC DETECTION METHOD- 1

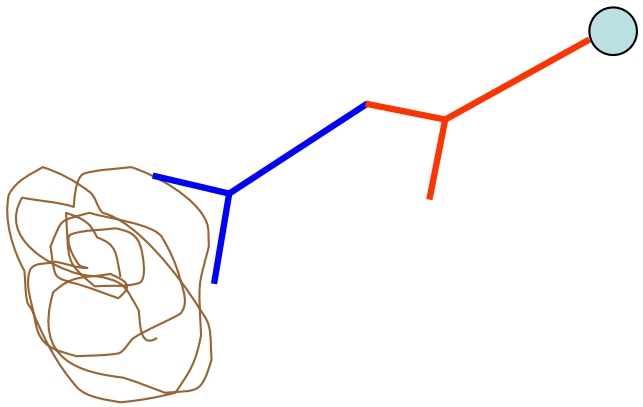
Direct detection methods *are known as a one-step process applying a primary antibody, which is directly labelled with reporter molecules, such as biotin, colloidal gold, fluorochromes, or enzymes (each primary antibody needs to be individually conjugated with fluorophores or enzymes, which increases considerably the cost of the whole process- only for highly expressed proteins-less sensitive- not used in diagnostics because of the sensitivity drawbacks)*



IHC DETECTION METHOD- 2

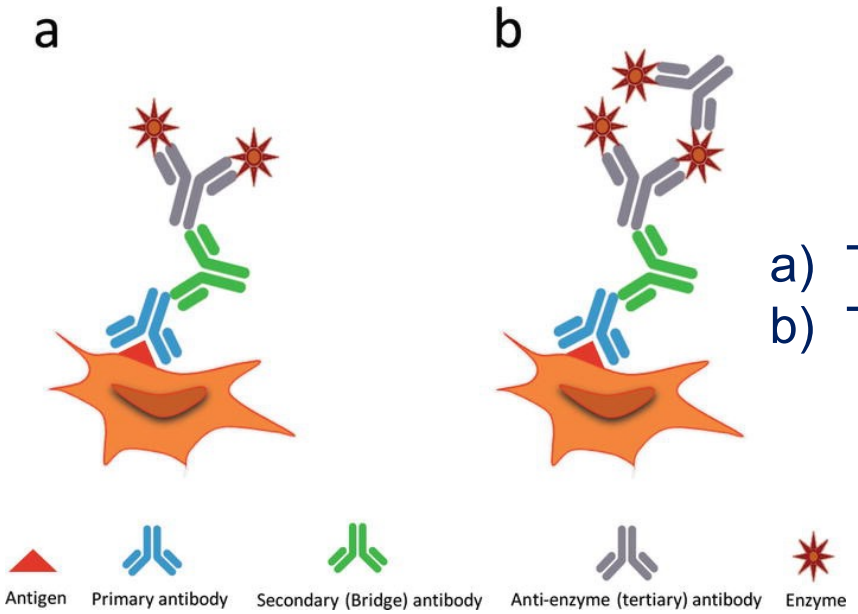
INDIRECT DETECTION METHODS: *This system employs an unlabeled primary antibody as the first layer and the secondary antibody, which is raised against the primary antibody and is labeled with different fluorophores or enzymes.*

In indirect methods, primary antibodies retain full avidity because they remain unlabeled. Indeed, higher number of labels per molecule of primary antibody is achieved in indirect compared to direct detection methods. The later stems from the fact that at least two labeled secondary antibodies can bind to each primary antibody molecule. These factors result in increased reaction intensity and the higher sensitivity in indirect staining methods.



IHC DETECTION METHOD- 3

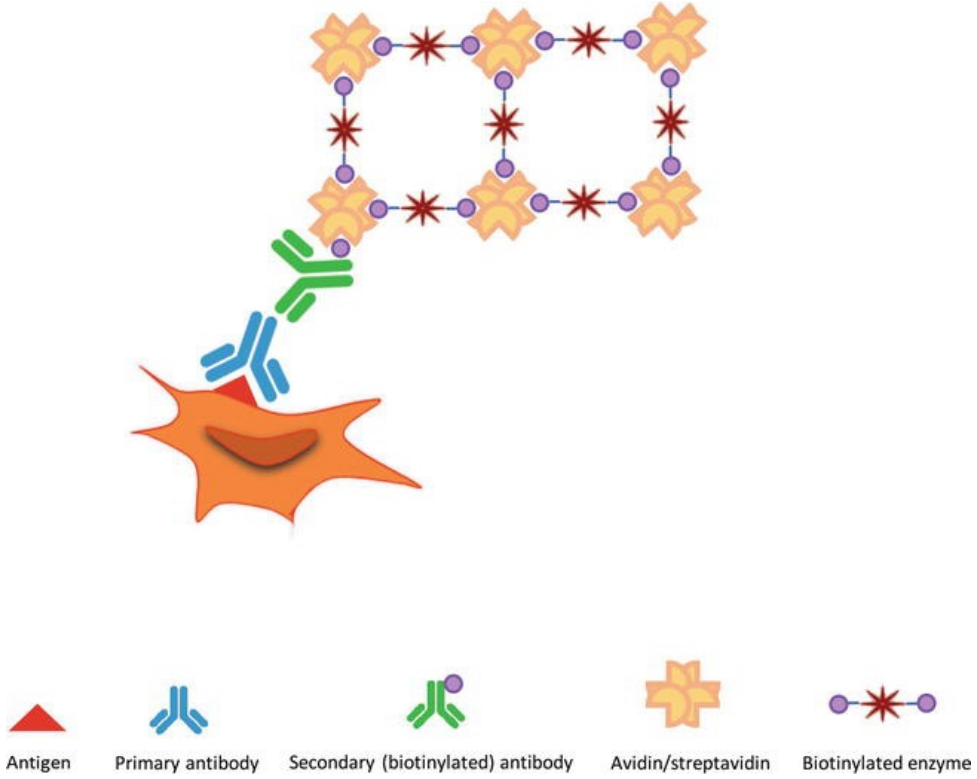
PAP – PEROXIDASE-ANTI-PEROXIDASE –BRIDGE METHOD- Taking advantage of the bivalent properties of IgG binding, a second-step antibody with binding specificity to primary antibody and tertiary antienzyme antibody complexed with the enzyme bridges two layer. The bridge antibody is usually used in excess, so that one of its two identical binding sites interacts with enzyme-coupled tertiary antibody, while the other site interacts with primary antibody. The tertiary antienzyme antibody has the same animal species of origin as the primary antibody. The bridge methods are collectively called as soluble enzyme-antienzyme methods



a) Two step bridge immunostaing method
b) Three-step bridge immunostaing method

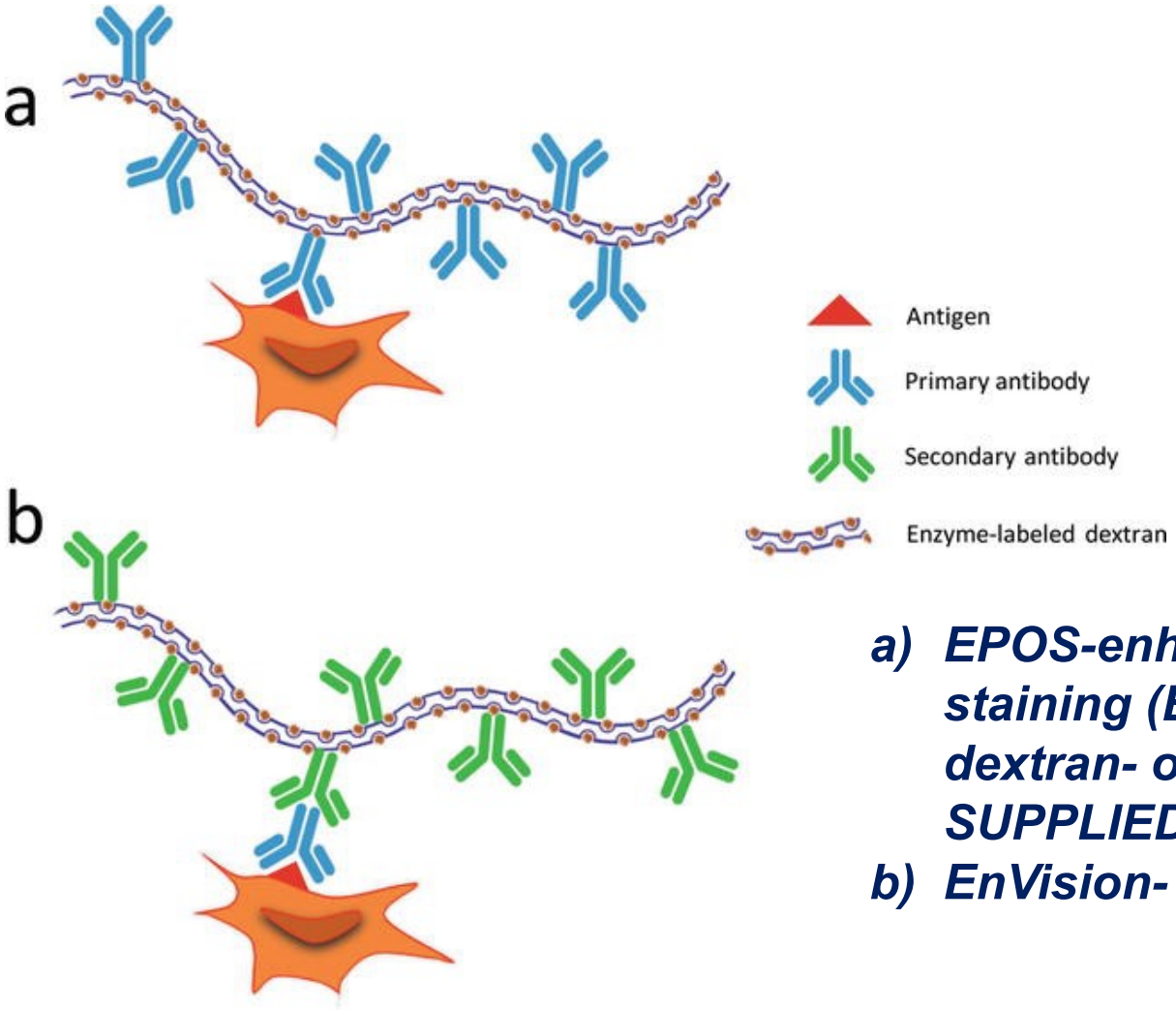
IHC DETECTION METHOD- 4

For signal amplification, the avidin-biotin complex (ABC) method was developed, in which a preformed avidin-biotin-peroxidase complex is used as the detection layer. This technique induces three different layers; an unconjugated primary antibody, a biotinylated secondary antibody, and finally a large complex of enzyme-labelled biotin and avidin, which is attached to the biotin molecules conjugated to the secondary antibodies.



IHC DETECTION METHOD- 5

Polymer method *– it is a chain polymer-conjugated. Improved sensitivity of this technology is based on using synthetic or natural polymers that increase the capacity for incorporating ligands or enzymes to be coupled to linker antibodies e technology.*



a) *EPOS-enhanced polymer one-step staining (EPOS) system . Polymer is dextran- one step IHC. NOT FOR USER SUPPLIED PRIMARY Ab*
b) *EnVision- more user friendly*

SENSITIVITY AND EFFICIENCY

SENSITIVITY: *the sensitivity of a detection system refers to the lowest concentration of antigen that are detected. It depends on:*

-Antigen nature*: related to multiple antigen determinants (epitopes)*

-Antigen accessibility*: possible use of antigen retrieval to unmask the antigen*

-Detection method*: direct, indirect PAP, ABC, EnVision*

Antibody efficiency*: It is the minimal amount of Ab needed to detect the antigen*

SPECIFICITY

SPECIFICITY: *it is the capability to immunoreact only with the specific antigen*

IMMUNOLOGIC ASPECIFICITY:

-Sequence homology in different proteins

-Partially similar antigens (ie. glycoproteins, phosphorylated groups , sulfurated groups, ecc…)

-Contamination with other antibodies (impure antigen used for immunization, autoantibodies, antibodies against infectious agents….)

NON IMMUNOLOGIC ASPECIFICITY:

- Non-specific bonds of the primary antibody

-Necrotic areas, tissue margins, connective tissue (fibrotic proteins)

- peroxidase similar (erythrocytes and neutrophils …)

-Endogenous biotin (kidney, pancreas, liver)

-Peroxidase and avidin non specific bonds (glycoproteins)

Blocking non immunologic- non specificiity

-Endogenous peroxidase or peroxidase similar (erythrocytes and neutrophils …): treatement with H_2O_2 in methanol prior to AR

-Endogenous biotin (kidney, pancreas, liver)- Prior to primary Ab decoration incubate sections ***in avidin solution for 15 minutes*** *followed by brief rinse in PBS, and then incubate sections* ***in biotin solution for 15 minutes*** *(all at room temperature). Briefly rinse in PBS and continue the protocol with primary antibody. The first step is the incubation with avidin solution and this will allow* ***avidin to bind any avidin binding sites on biotin in the tissue****, and the second step is the incubation with biotin and this step is* ***to saturate all the biotin-binding sites left open on the avidin****.*

-Endogenous alkaline phosphatase *(intestinal mucosa, liver, spleen, kidney, placenta). Block adding 1 mM levamisole to substrate. Alternatively, placenta and intestinal isoenzymes can be blocked with Acetic acid 20% @ 4°C 15 min. Prior to blocking non-specific reaction*

Fixation and tissue processing	
Under-fixation with formalin	Reduced immunostaining in central areas of sections
Over-fixation with formalin	Non-specific binding of antibodies by free-aldehyde group
Alcohol fixation	Most of CD and some growth factor peptides are poorly reactive
Mercury-based fixatives (B5, Zenkers)	CD4, CD5, CD10, CD23, (CD30) loose immunoreactivity
Dehydration, non-polar solvents and paraffin embedding	Possible change on the conformation of some antigens
Decalcification by 10% formic acid or 5% nitric acid	Decreased antigenicity on many antigens, particularly CD markers
Protease (PIER) and heat-induced epitope retrieval (HIER)	
PIER at incorrect pH and/or temperature	Antigens not retrieved (masked)
PIER with strong enzymatic digestion	Potential destruction of antigens
HIER at pH 3.0–6.0	Decrease in staining of Ki67 (Mib1) and ER
HIER on biotin-rich tissues (e.g. mitochrondrion-rich cells)	Unmasking of endogenous biotin
HIER by zinc sulphate, citrate (pH 6) and TRIS (pH 9) buffer solutions	Non-specific staining of nuclear proteins
Endogenous enzymes (peroxidase)	
Absence of endogenous enzyme inhibition	Non-specific background staining
Strong endogenous enzyme inhibition	Destruction of some antigens (i.e. CD4)
Avidin–biotin system	
High ionic attraction of avidin	Non-immune binding to nucleic acids, phospholipids and glycosamin
Binding of avidin to endogenous biotin	Strong background and false positive staining of liver, lung, spleen, tissue, mammary gland, kidney, brain, gestational and post-partum endometrial cells, myelin and mast cells
Primary antibody	
Improper reaction buffer	Background staining or absence of antigen binding
Hydrophobicity, polymerisation and aggregation of immunoglobulins	High background staining
Interaction with protein polar groups in tissue sections	High background staining
Protein–antibody complement-mediated binding	High background staining
Attraction of the Fc fragment to basic groups of collagen fibres	Non-specific staining
Binding of immunoglobulins to the cellular Fc receptors[19 108]	Non-specific staining
High concentrations of antibodies	Background staining or absence of antigen binding (prozone phenon
Bacterial contamination of antibodies	Antibody agglutination
Detection system	
Incorrect pH of the reaction buffer	Absence of staining
Ionic charges of the polymers	Background staining
Spontaneous agglutinations of the antisera	Absence of staining

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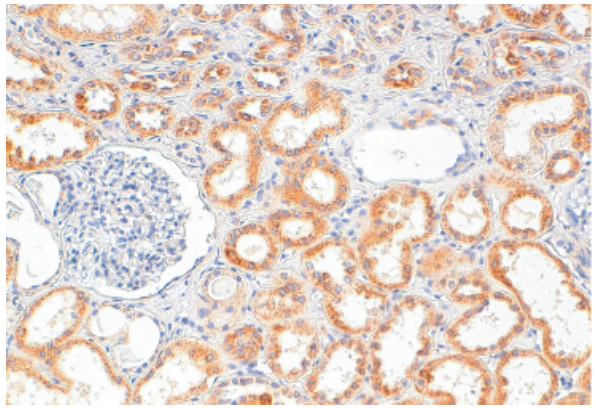
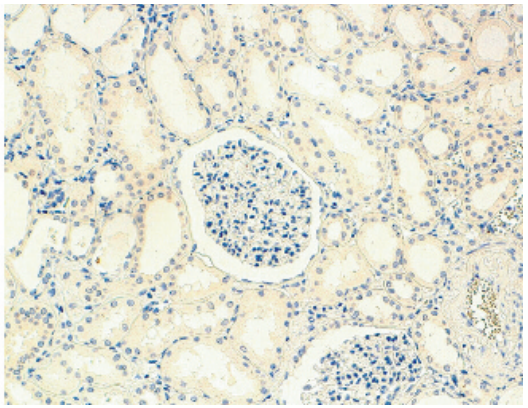
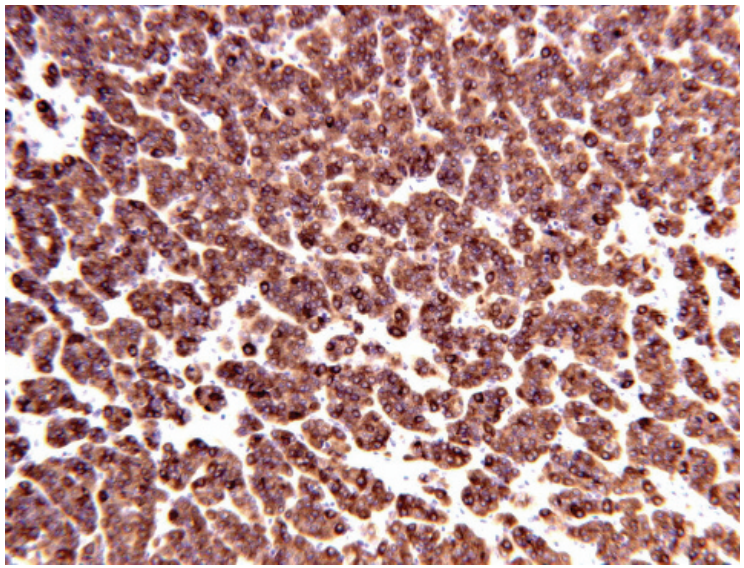
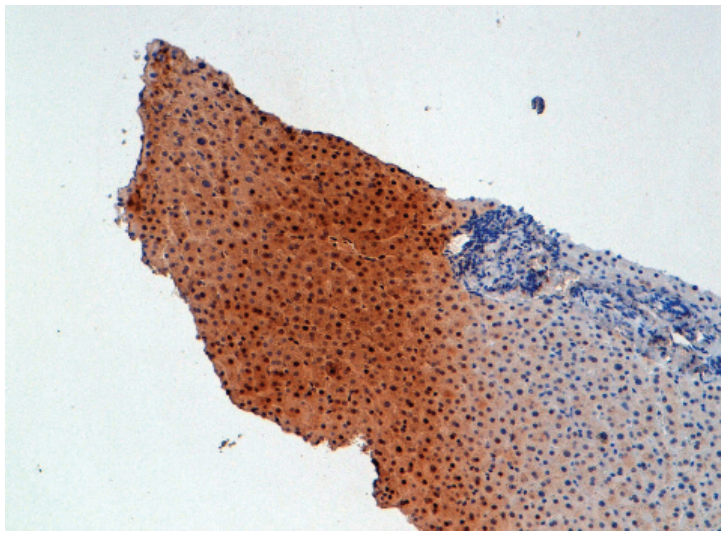
Renal tubules, rich in endogenous biotin, bind avidin, which results in a strong cytoplasmic staining (A). A blocking procedure prevents such spurious staining (B).







Renal oncocytoma. Neoplastic cells are rich in mitochondria. Endogenous biotin (the coenzyme of oxidative enzymes) is retrieved following the HIER procedure, thus causing spurious staining when using avidin-based immunohistochemical methods.



The free edge of histological sections can show non-specific uptake of immunohistochemical staining reagents.

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IHC METHOD

- *CUTTING OF THE SECTIONS*
- *ADHESION OF THE SECTIONS ON THE SLIDE*
- *DEWAXING*
- *HYDRATION*
- *PROCEDURES TO INCREASE THE EXPOSURE OF THE ANTIGEN*
- *BLOCKING*
- *INCUBATION WITH THE PRIMARY ANTIBODY*
- *INCUBATION WITH THE SECONDARY ANTIBODY*
- *AVIDIN-BIOTIN - PEROXYDASE- COMPLEX*
- *CROMOGENOUS REACTION-*
- *COUNTERSTAIN*
- *-DEHYDRATION-*
- *CLEARING*
- *MOUNTING*

METHOD DESCRIPTION

*-**SECTION CUT**: tissue section on the slide are left in oven for 30 – 60 min @ 56-60°C, or at lower temperature for longer time.*

*-**DEWAXING***

-3 – 4 times in xylene for 4 min

-1 time in EtOH Abs

ENDOGENOUS PEROXIDASE BLOCK (optiona):

- 0.5% H_2O_2 in methanol for 30 min

HYDRATION:

-2 times in EtOH Abs for 4 min

-2 times in EtOH 95% for 4 min

-2 times in water for 4 min

-1 time in buffer for10 min

ANTIGEN RETRIEVAL

Most formalin-fixed tissues require an antigen retrieval step before immunohistochemical staining.

The longer is the fixation the higher is the masking of the antigenic site.

Methylene bridges formed during fixation cross-link proteins and mask antigenic sites.

Antigen retrieval methods break these methylene bridges and expose antigenic sites, allowing antibodies to bind.

The two methods for antigen retrieval are ***heat induced epitope retrieval*** *(HIER) and* ***enzymatic retrieval.***

ANTIGEN RETRIEVAL- ENZYMATIC

TRYPSINIZATION:

-Trypsin 0.1% in Tris-HCl pH 7.8 including $CaCl_2$ for 15 min (up to 2h)

DIGESTION WITH DNAse:

-DNAse I 5 mg/ml in Tris-HCl pH 7.4 with $MgSO_4$ 0.01M for 15-30 min.

After the enzymatic digestion, slides are rinsed thoroughly with buffer solution

ANTIGEN RETRIEVAL- HIER

*Heat-induced epitope retrieval is most often performed using a **pressure cooker, a microwave, or a vegetable steamer.** Some labs use a water bath set to 60°C and incubate the slides in retrieval solution overnight. This is useful when working with tissue sections that fall off the slide when heated at higher temperatures; in particular bone, cartilage, and skin.*

*FOR **HIER** THE FOLLOWING BUFFERS ARE USED:*

- ***Sodium citrate buffer** (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0)*
- ***1 mM EDTA**, pH 8.0*
- ***Tris-EDTA buffer** (10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0)*

AFTER TREATMENT RUN COLD WATER FOR AROUND 10 MINUTES AND CONTINUE WITH IHC PROTOCOL

BLOCKING ASPECIFIC REACTION AND STAINING

-TISSUE SLIDES SHOULD NOT DRY (unuformely stained): *humid chamber*

-BLOCK of immunoglobulins aspecific bonds:

-10 min BSA 1% in PBS *(CAN BE INCREASED UP TO 5%)*

-10 min with serum of the animal where secondary Ab was raised

-10 min with purified immunoglobulins of the animal where secondary Ab was raised

Incubation with BSA 1% is made before decoration with the primary Ab. Excess is removed without rinsing.

-BLOCK of glycoproteins aspecific bonds (optional): *for instace the peroxydase can stain slightly nuclei*

-Incubation with a 5% skimmed milk solution for 10 min (BLOTTO).

-WASHING TO REMOVE ASPECIFIC BONDS:

-Wash with buffer including 0.1% of detergents (Triton X-100 o Tween 20) or NaCl 0.5M.

-HOW TO BLOCK ALDEHYDE GROUPS FROM FIXATIVES

- Sodium borohydride 0.1 mg/ml for 10 min.

DECORATION WITH PRIMARY Ab

- Choose the dilution of the primary antibody, for diagnostic Ab it is already reported or prediluted Ab are used For research check the proper one 1:50, 1:100, 1:500

-Ab shall be diluted in the proper diluting solution or in 0.1% di BSA

-Incubation is usually carried out at r.t. in a humid chamber for 30-60 min, or @ 4°C o.n. with the higher Ab dilution.

Washing

-10 min in buffer (with 0,1% detergent, optional)

DECORATION WITH BIOTINILATED SECONDARY Ab

-30 min r.t. diluted as reported by the provider in dilution buffer (optional 0.1% BSA)

Washing

-10 min in buffer (PBS including 0,1% detergent, optional)

COMPLX AVIDIN-BIOTIN-PEROXYDASE

-Mix equal volumes of the solution containing avidin and of the one including biotinilated peroxydase just before use (30 min).

-Incubate for 60 min

Washing

-10 min with buffer

Chromogenic reaction

-Dilute DAB stock solution (5mg/ml) 30:1 in,

-Add 2 drops of 30% H_2O_2 (100µl) for 100 ml DAB solution

*-Incubate **5**-15 min*

-Wash for 5min with tap water

-Distilled water

COUNTERSTAIN

- Mayer's hematoxylin for 3 min (shorter time or diluted hematoxylin for nuclear staining)

-Tap water for5 min

DEHYDRATION

-Alcohol 95%	*1X*	*4 min*
-Alcohol 100%	*2X*	*4 min*

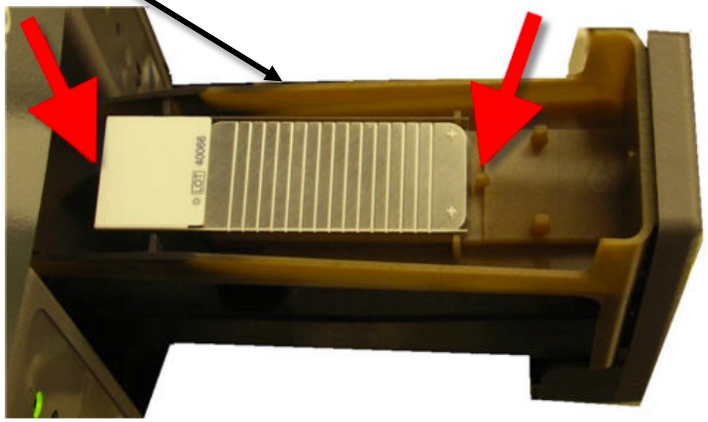
CLEARING

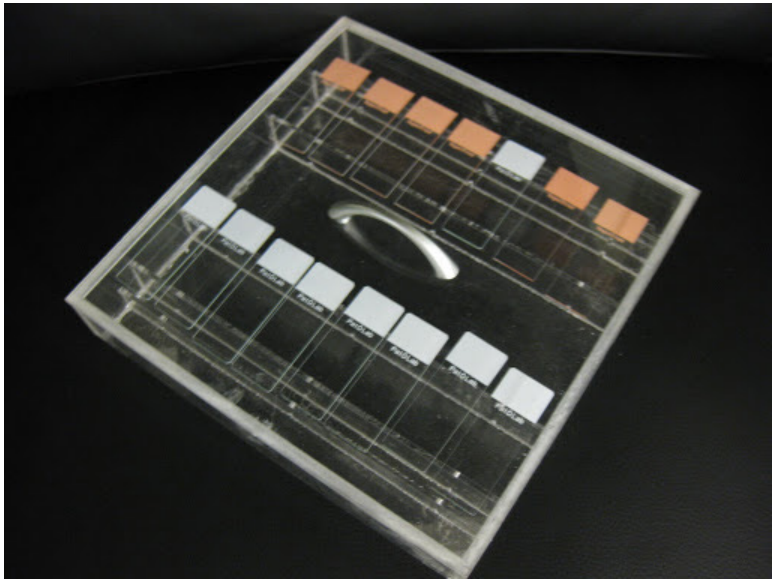
-Xylene	*2X*	*per 4 min*

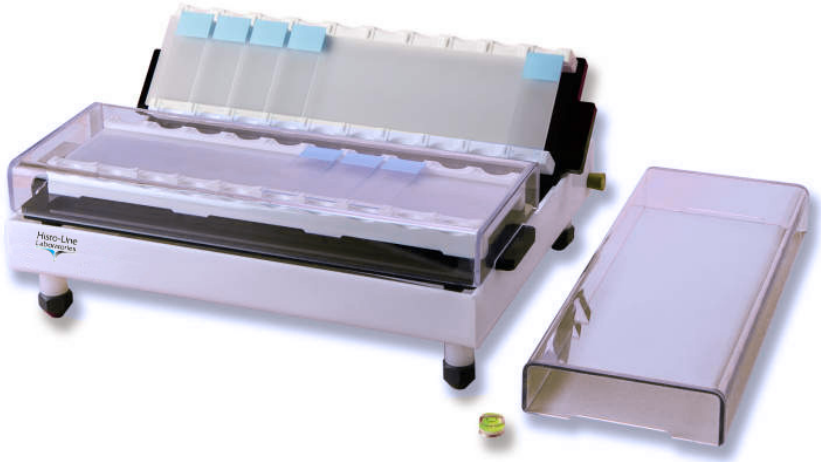
MOUNTING

Application

-IHC,
-ISH
-Double staining: IHC+ISH, IHC+IHC, ISH+ISH






Histo-Line
Laboratories

CONTROLS

DIAGNOSTIC

-Positive controls

-Internal controls

-Negative controls

RESEARCH

-Logic controls

-Negative Controls

1. Specific- tissue or cells expected to be negative by Ab (May be also a portion of the same sample)
2. Non Specific

Non specific negative control

No use of the primary Ab

Use of dilution solution as Ab.

To investigate on the background of the detection system.

It is better to replace the primary Ab with non-immune serum of the same species and isotype of the primary Ab.

Pre-adsorption test

Use:

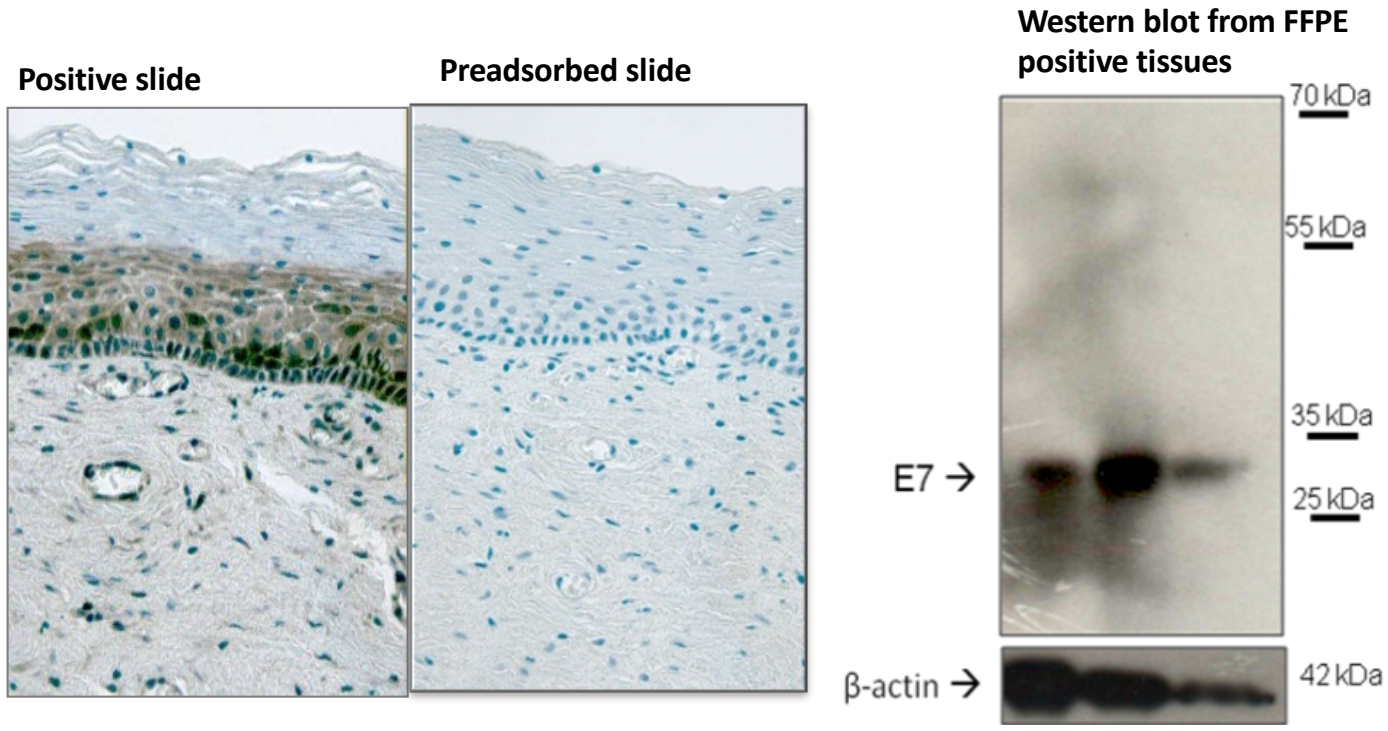
-to test the specificity of the Ab

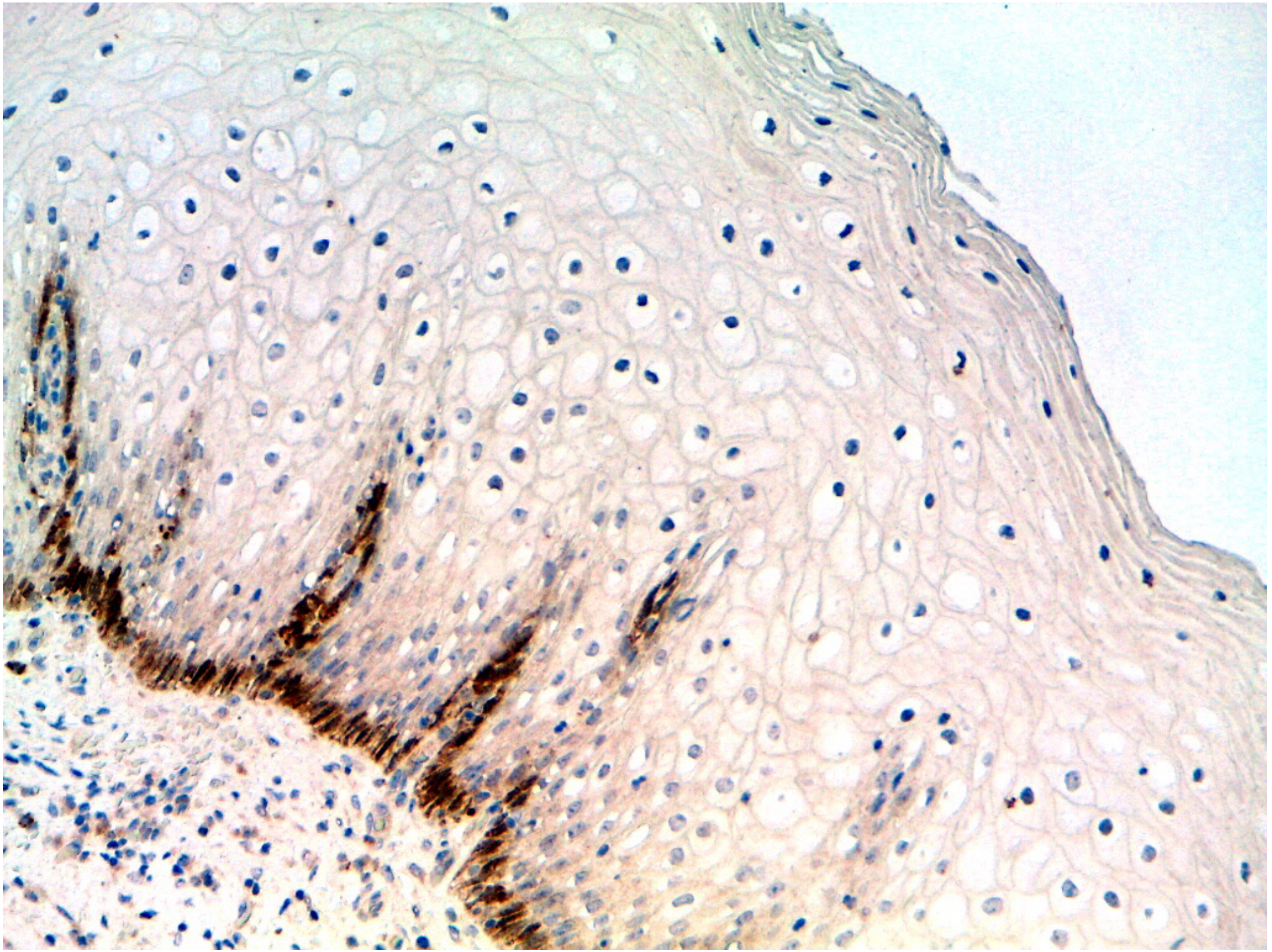
Protocol:

The Ab is blocked by the immunization peptide used to generate the Ab

The test is based on the incubation i solution of the primary Ab + immunization peptide before immunodecoration.

MAb against HPV E7 protein





Results

1. **Percentage of positive cells**
2. **Localization -** *-membrane, nuclear, cytoplasm)*
3. **Intensity of stain**

In research:

H score (histo)= [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)]

The score can vary in the range 0 - 300.

Cases can be classified as positive or negative according to specific cut-off.

IHC- Limits

1. *Many antibodies with different sensitivity and specificity*
2. *Result depends on preanalytical factors*
3. *Semi-quantitative subjective interpretation*.