Immunohistochemistry Protocol and Basic Information

Chapter · July 2019

CITATIONS
0

READS
2,367

1 author:

Subash Rimal
Institute of Agriculture and Animal Science
14 PUBLICATIONS **5** CITATIONS

SEE PROFILE

Immunohistochemistry Protocol

Prepared by:

Dr. Subash Rimal (B. V. Sc. & A. H., IAAS, TU)

Tissue Fixation

Tissue processing

(Buffer formalin (overnight))

(70%, 95%, 95%, 100%, 100%, 100% alcohol (Each 1 hour))

(Xylene1, xylene2= each 2 hour)

Paraffin infiltration (2 paraffin station each 2hour)

Block Making

Sectioning (3-5mm)

Mountaining tissue section on slide

Deparaffining

- Hot air ovan 90°c for 7-8min
- Xylene 1 for 5 minute
- Xylene 2 for 5 minute

Rehydration

- 100% Ethanol for 1 minute
- 90% Ethanol for 1 minute
- 70% Ethanol for 1 minute

Hydrated by Running Water for 2 minute

Antigen Retrieval Process

(Three whistle in Pressure Cooker)

- Process of antigen retrieval making
  - **For High P^H (P^H=9)**
    (Tris (Hydroxyl methyl) 1.2 gm + EDTA 0.37gm) in 1 litre distil water
  - **For Low P^H (P^H=6s)**
    (Trisodium citrate 0.73gm +0.5 ml of HCl(37.5%)) in 250 ml distil water

Cool down temperature for 40-45 minute

(Until normal temperature)

Directly put into buffer solution

- Preparation of buffer solution
  (Tres 1.21gm + sodium chloride (NaCl) 8gm) in 1 litre distil water

Encircle the tissue by using darko marker

Peroxidase (cover, eight minute)

Buffer wash (2 minute)

Air dry

Specific antibody marker use (20 -30 minute)

Wash by Buffer solution (2 minute)

Horse Radish Peroxidase (HRP) for 15-20 minute

Wash 2 Minute in buffer Solution

Chromogen (fresh) for eight minute

(1ml DAB + 1 drop chromogen)

Wash (2-4 minute)

Gills Hematoxylene (4-6 minute)

(Counter stain)

Wash Tap Water (5 Minute)

Dehydration (70%, 90%, 100% alcohol, Each 1 minute)

Clearing (xylene 2Tab, each 5 Minute)

DPX mountaining

Immunohistochemistry

Immunohistochemistry (IHC) is a favorite tool amongst clinicians to help diagnose a range of diseases by identifying abnormal cells, such as those in cancer. In a nutshell, IHC uses antibodies to detect proteins (antigens) that are specific to, or have altered expression in, abnormal cells within a tissue section. It can also be used as a predictor for treatment outcome. IHC isn't just a useful clinical tool, it also has great applications as a basic research tool. It can provide you with a wealth of information on the expression of specific proteins within the context of tissue structure.

Main Step in Immunohistochemistry (IHC)

1. **Tissue fixation**
   This step is pretty important as it maintains tissue structure and retains antigenicity (the availability of the antigens/proteins to be detected by antibodies). The fixation method you use depends on the tissue type you are using and your individual experimental requirements.

   - Snap frozen and acetone-fixed tissue ⟶ good antigenic expression
   - formalin-fixed and paraffin-embedded (FFPE) ⟶ clear morphology

2. **Antigen Retrieval**
   Antigen retrieval is the most important step in immunohistochemistry. Actually most formalin fixed tissues required an antigen retrieval step before immunohistochemical staining. In formalin fixed tissue, Methylene Bridge formed during fixation which covers the antibody binding sites on tissue. Antigen retrieval reagents break the protein cross-links formed by formalin fixation and there by uncover hidden antigenic sites.

   There are different methods of antigen retrievals. Heat and enzyme retrieval are both employed, with heat induced epitope retrieval (HIER) now being the most commonly used. HIER involves heating your slides in buffer at pH6 or pH9 (depending on your antibody) using a microwave or a pressure cooker.

❖ Process of antigen retrieval making
   - **For High P^H (P^H=9)**
     (Tris (Hydroxyl methyl) 1.2 gm + EDTA 0.37gm) in 1 litre distil water
   - **For Low P^H (P^H=6s)**
     (Trisodium citrate 0.73gm +0.5 ml of 1N HCl) in 250 ml distil water
   - Only EDTA anitigen retrieval and proteolytic enzyme antigen retrieval are also used in some lab.

❖ **How to choose pH of Antigen retrieval???**

Actually it is depend upon you antibody used. Example: if you choose vimentin antibody then you should choose high pH retrieval. High pH provide clear morphological structure of cell.

3. **Blocking**

Blocking endogenous materials before staining is crucial to minimize false-positive staining. Blocking is essential for preventing non-specific binding of antibodies or other reagents to the tissue. Even if the antibody has high specificity towards the target, intermolecular forces can promote non-specific binding to other molecules. Consequently, non-specific binding prevents visualization of the antigen-antibody binding of interest. To mitigate nonspecific binding, a blocking step should be carried out before incubation with the primary antibody

❖ **Types of IHC blocking**

- **Blocking Non-Specific Ionic Bindings**

  Non-specific ionic bindings are due to, for example, Van der Waals interactions, dipole-dipole interactions or net charges of specific amino acid groups. In this case, altering the ionic strength of the antibody dilution buffer can help to reduce unspecific ionic bindings.

- **Endogenous Enzyme Blocking**

  When using a horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-conjugated antibody for detection, the endogenous levels of the enzyme have to be blocked. This normally applies to tissues such as kidney, liver, intestine, lymphoid tissue, etc. Peroxidase can be blocked with buffers containing H_2O_2 and AP can be blocked with buffers containing acetic acid or Levamisole.

4. **Antibody labeling and visualization**

IHC staining can be set up in one of two ways – as an indirect assay, or using direct labeling.

- **Indirect detection**

  With indirect detection you use a secondary antibody with a covalently attached label. This secondary antibody binds to the primary antibody during the staining process. There are two main steps to detect your antigen in this manner:

  1. Firstly you need to incubate your primary antibody (usually for one hour, but sometimes an overnight incubation may be necessary) on your tissue sample. This allows the antibody to bind to the antigen (assuming of course that the antigen is present). Once it is bound you need to wash away any excess

unbound primary antibody before incubating with a labeled secondary antibody.

2. After another period of incubation (again one hour), excess secondary antibody is washed away and the amount of label associated with the primary antibody (i.e. indirectly via the secondary reagent) is quantified.

- **Direct detection**

  This method is quicker and simpler than indirect detection, as the label is attached via a covalent bond directly to the primary antibody. This means you only need one incubation step and one round of washes. This method has several advantages over indirect detection including decreased assay variability, reduced cost (by eliminating the need for secondary antibodies) and it saves time (as the second round of incubation and wash steps are removed).

  Once you've labeled your samples, it's time to visualize! If you've gone for fluorescent labels you need to view your samples directly under a fluorescence microscope – remember to use the appropriate excitation wavelengths and filter sets based on the fluorophore you've used. However, if you've opted for an enzyme label such as horseradish peroxidase a further incubation is required with the appropriate substrate. In these cases the enzyme acts on the substrate to produce an insoluble colored component that is deposited at the site of antibody binding.

List of control Tissue for specific antibody

Antibody	Tissue Type
8-OHdG	Skin
AACT	Tonsil, Colon, Skin
AAT	Tonsil, Colon, Liver
ACTH	Pituitary
AE-1	Colon, Skin, Pancreas
AE-3	Prostate
AE-1/AE-3	Colon, Skin, Prostate, Pancreas

Actin, Muscle	Skeletal Muscle, Tongue, Leg Muscle
Actin, Alpha-Smooth Muscle	Tongue, Small Intestine, Colon, Spleen
AFP	Fetal liver, Hepatoma, Fetus
Albumin	Liver
Androgen Receptor	Prostate
Aurora A/STK-15	MCF-7 Cell, Cancer Tissue
Bax	Mammary Carcinoma, Breast Carcinoma
Bcl2	Tonsil
Bcl6	Tonsil, Follicular lymphomas
Ber H2	Lymphoma, Pancreas
Ber EP4	Small Bowel, Colon, Small intestine
Beta Amyloid	Alzheimer's Brain
Beta Catenin	Bile Ducts/Ductules
hCG	Placenta
Bim	Skin/Skin Tumor
Bombesin	Stomach, Duodenum, Lung
BrdU	BrdU Treated Tissue
Calbindin D	Cerebellum
Calcitonin	Thyroid (parafollicular C cells)
Calretinin	Cerebellum, Testis, Thymus
Casein	Lactating Mammary Gland

Caspase3	Skin/Tumor, Embryo
Catalase	Liver, Brain (basal ganglia, thalamus, cerebellum)
Catenin	Intestine
Cathepsin D	Breast Cancer, Skin, Prostate, Liver
CC10	Lung
CD3	Tonsil, Spleen
CD4	Spleen, Thymus
CD8	Spleen, Thymus, Lung
CD11b	Spleen, Cerebellum (Microglia)
CD10	Small intestine, Kidney, Tonsil, Thymus,
CD13	Brain (Striatum)
CD14	Placenta
CD15	Tonsil
CD19	Spleen
CD20	Lymph node, tonsil, spleen
CD23	Tonsil
CD25	Tonsil, Spleen, Thymus
CD30	Tonsil, Thymus, Spleen
CD31	Skin, Kidney, Tonsil, Prostate, Liver, Heart,appendix
CD34	Kidney, Spleen
CD38	Spleen

CD40	Spleen
CD41	Spleen
CD43	Tonsil
CD44	Spleen, Thymus
CD45	Spleen, Thymus
CD54	Liver, Spleen, Lung
CD68	Skin, Kidney, Pancreas
CD79a	Tonsil, Thymus, Spleen
CD80	Spleen
CD105	Kidney
CD117 (c-Kit)	Skin, Brain, Lung, Spleen
Cdc2	Lung Tumor, Breast Carcinoma
Cdk2	Colon
Cdk4	Skin
CDw49f (Integrin-alpha6)	Skin (Basement Membrane)
CEA	Colon adenocarcinoma, Breast carcinoma
CERB2	Breast Cancer, Gastric Cancer, Adeno Cancer
Chromogranin-A	Stomach, Colon, Pancreas, Adrenal
CKR6	Lymph Node
CKR7	Lymph Node
CNPase	Brain/Glia Tumor

Collagen IV	Kidney, Skin, Placenta, Lymph Node
Collagen Type I	Placenta, Kidney
Cox2	Colon, Kidney
CX3CR1	Lymph Node
CXCR4	Skin
CYP1B1	Liver
Cytokeratin	Skin, Colon, Stomach, Intestine
Cytokeratin7	Lung, ovary, breast, skin, bladder, kidney (urothelium)
Cytokeratin8&18	Skin, Pancreas/Tumor
Cytokeratin14	Skin
Cytokeratin20	Pancreas
Defensin1-alpha (HDEFA1)	Skin
Defensin1-beta (NBD-1)	Skin
Defensin2-beta (HBD-2)	Skin
Desmin	Spleen, Tongue, Colon, Ureter, Prostate
DBA44	Spleen
Dopamine Hydroxylase	Brain (Striatum/Substantia Nigra)
E-Cadherin	Mammary Epithelium
EGFR	Placenta
EMA	Kidney, Ureter, Breast Glandular Epithelium
Estrogen	Normal Breast/Breast Cancer

ER (Estrogen Receptor)	Breast, Uterus, Prostate
F4/80	Spleen, Thymus, Lung, Liver
Factor VIII	Tonsil, Skin, Colon, Prostate, Bone Marrow
Fas	Spleen, Thymus, Colon
Fas-L	Spleen, Thymus, Colon
Flag (M2)	Heart Muscle
Fhit	Muscle
Flk-1	Colon Carcinoma, Blood Vessel Endothelium
Filaggrin	Skin
Flt-1	Kidney, Heart
FSH	Pituitary
GAPDH (V-18)	Lens, Liver, Lung, Prostate Cancer
Gastrin	Stomach (Pyloric Mucosa)
GFAP	Brain (Striatum, Cerebellum)
Glucagon	Pancreas
Glycophorine A	Bone Marrow
GRB2	Colon carcinoma
GSH2 (E-20)	Lens
HDJ2	Tonsil
Heme-Oxygenase-1 (HO-1)	Lung
Helicobacter Pylori	Helicobacter Liver

HGF	Liver
HIF-1 alpha	Prostate Tumor, Lung Tumor
Histone H2AX	Tonsil, Skin
Histone H3 (Phospho-Histone H3)	Tonsil, Skin, Spleen, Small Intestine
HPL	Placenta, Tonsil, Colon
HSA (Hepatocyte Specific Antigen)	Normal Liver or Liver Carcinoma
HSP70	Skin, Pulmonary Arteries, Smooth Muscle
IgA	Gastrointestinal mucosa (pyloric antrum recommended)
IgD	Tonsil
IgG	Tonsil, Mammary Gland, Mammary Tumor
IgM	Tonsil, Spleen
IL-1	Lung
IL-6	Lung
IL-8	Lung
Insulin	Pancreas
Insulin A	Pancreas
Integrin	Skin (Basement Membrane)
Involucin	Skin
KP-1	Colon, Small Bowel, Liver, Skin
Kappa	Tonsil, Spleen
Lambda	Tonsil

Keratin	Skin
Keratin7	Lung, ovary, endometrium, and breast carcinoma, skin
Keratin14	Skin
Keratin15	Skin
Ki67	Skin, Small Intestine, Tonsil, Thymus, Breast Carcinoma,appendix
Laminin	Skin, Kidney
LAT	Spleen
LCA	Tonsil
Ly-6A/E	Thymus
Ly-6G	Spleen
Ly-49	Spleen
Hormone	Tonsil
MAC	Placenta, Spleen
MAP2	Brain (Cerebral Cortex)
Mast Cell Tryptase	Bone Marrow, Lung, Skin, Colon
MEK 1/2 (phospho-MEK 1/2)	Breast Cancer
MHV	MHV Liver
MIP2	Skin
Mitf	Bone Marrow, Lung, Skin, Colon
MMP-2	Placenta, Skin
MMP9	

MOMA1	Lung
MRP14	Spleen
MT-1	CAPAN-1 Cells, Lung Cancer
MT-2	Tonsil
Myeloperoxidase	Tonsil
Myosin	Bone Marrow, Spleen, Tonsil
Myoglobin	Skeletal Muscle, Tonsil, Tongue
Nestin	Heart, Tongue
NeuN	Brain
Neurofilament	Brain (Cerebral Cortex)
Neutrophil Esterase	Brain, Peripheral Nerve
Nitrotyrosine	Tonsil
NOS2	Kidney, Uterus, Heart, Placenta
NOS3	Liver, Uterus
NSE (Neuron Specific Enolase)	Kidney, Brain
Occludin	Brain (Cerebral Cortex)
Osteonectin/Sparc	Lung
PSA	Cartilage, Lens
Pancreatic Polypeptide	Prostate
P53	Pancreas, Duodenum, Gastric Antrum
P63	Breast Cancer, Colon, Skin

Pancreatic Polypeptide	Breast Cancer, Colon, Skin
Panendothelial Cell Antigen	Pancreas (Pancreatic Islet Cells)
Pap 2b	Kidney, Spleen, Lung
PAR, alpha	Bladder
Pax-5	Ischemia Brain (Piriform Cortex)
Paxillin	Spleen
PCNA	Placenta
PDGF Receptor	Small Intestine, Skin, Tonsil, Lymphoma
PDGF-beta	Mammary Carcinoma
PEP-1	Mammary Tumor
PEP-13	Malignant Melanoma, Amelanotic
PLAP	Malignant Melanoma, Amelanotic
Progesterone	Placenta, Tonsil, Colon Cancer
PR (Progesterone Receptor)	Normal Breast/Breast Cancer
Prolactin	Uterus, Breast, Prostate
PS2	Pituitaryjkjuu
PSA (Prostate Specific Antigen)	Breast Cancer
Sarcomeric Actin	Prostate
Serotonin	Tongue, Tonsil
S100	Appendix, Adrenal, Bronchial Epithelium
SCF (Stem Cell Factor)	Melanoma, Skin, Colon, Brain,**APPENDIX**

SLUG	Brain
SMA (Smooth Muscle Actin)	Tongue
SMAD2 (phospho-SMAD2)	Tongue, Small Intestin, Colon, Ureter, Prostate, Skin
Somatostatin	Embryo
Somatostatin Receptors	Pancreas, Brain
Sos-1	Pancreas, Brain
SOX-10	Placenta
Survivin Ab-3	Embryo
SV40	Stomach/Lung Tumor
Synaptophysin	Tumor, Mammary Carcinoma
Tau	Brain, Adrenal, Pancreas,appendix
TCR, beta	Alzheimer's Brain
Testosterone	Spleen, Thymus
TGF-beta RI	Testis
TGF-beta1 LC	Prostate
TH (Tyrosine Hydroxylase)	Placenta
Thyroglobulin	Brain (Striatum, Substantia Nigra)
TNF, alpha	Thyroid
Thymine Dimer	Skin, Lung
TLT-1	Skin (UV Damage)
TSH	Spleen

TTF-1(Thyroid Transcription Factor)	Pituitary
Tubulin III, beta (Tuj1)	Lung, Thyroid, Pituitary
Ubiquitin	Brain (Cerebral Cortex)
UCHL	Alzheimer's/Parkinson's Brain
UCP-1	Tonsil
UCP-2	Pancreas
ULEX	Pancreas
VCAM-1	Prostate, Tonsil
VE-Cadherin	Vessels in Tissue
VEGF	Kidney
VEGF R1	Pancreas, Kidney, Adenocarcinoma
VEGF R2 (Flk-1)	Skin, Brain, Hey Cells, Angiosarcoma
VIP (Vaso Intestinal Polypeptide)	Kidney, Heart, Prostate Carcinoma, Tumor,
Vimentin	Gastric Antrum, Small Intestine, Brain (Amygdala)
vWF (von Willebrand Factor)	Colon, Prostate, Tonsil, Uterus, Ureter
WT-1 (Wilms' Tumor)	Tonsil, Skin
	Kidney (Glomeruli), Testis

References:

1. https://www.researchgate.net/publication/315673693_Pax8_Napsin_A_and_CD10_as_Immunohistochemical_Markers_of_Canine_Renal_Cell_Carcinoma
2. https://www.ncbi.nlm.nih.gov/pubmed/21204715
3. https://journals.sagepub.com/doi/10.1177/0300985817698211
4. https://journals.sagepub.com/doi/pdf/10.1177/0300985817698211
5. http://www.ihcworld.com/epitope_retrieval.htm

6. https://www.abcam.com/products?keywords=antigen+retrieval+buffer&gclid=CjwKCAjw1f_pBRAEEiwApp0JKA4XTJAVhRp91fNAY8mKBWLJsP1VzWINl_4ZH1hRcgQw5VyAfdOpUBoCF7MQAvD_BwE
7. https://www.rndsystems.com/resources/protocols/antigen-retrieval-methods
8. https://bitesizebio.com/20929/getting-started-with-immunohistochemistry/
9. https://www.thermofisher.com/np/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-immunohistochemistry.html
10. https://www.abcam.com/kits/antigen-retrieval-for-ihc
11. http://www.enzolifesciences.com/ENZ-ACC113/antigen-retrieval-reagent-ph-9-10x/
12. http://www.enzolifesciences.com/ADI-950-280/ihc-enzyme-antigen-retrieval-reagent/pdf/
13. https://journals.sagepub.com/doi/pdf/10.1177/43.2.7822775
14. https://bitesizebio.com/13466/immunohistochemistry-basics-blocking-non-specific-staining/
15. https://www.abcam.com/kits/blocking-for-ihc
16. https://www.ptglab.com/support/immunohistochemistry-protocol/selecting-antibodies-for-ihc/
17.

Prepared by:

Dr. Subash Rimal

B. V. Sc. & A.H.

IAAS, TU

Thank You