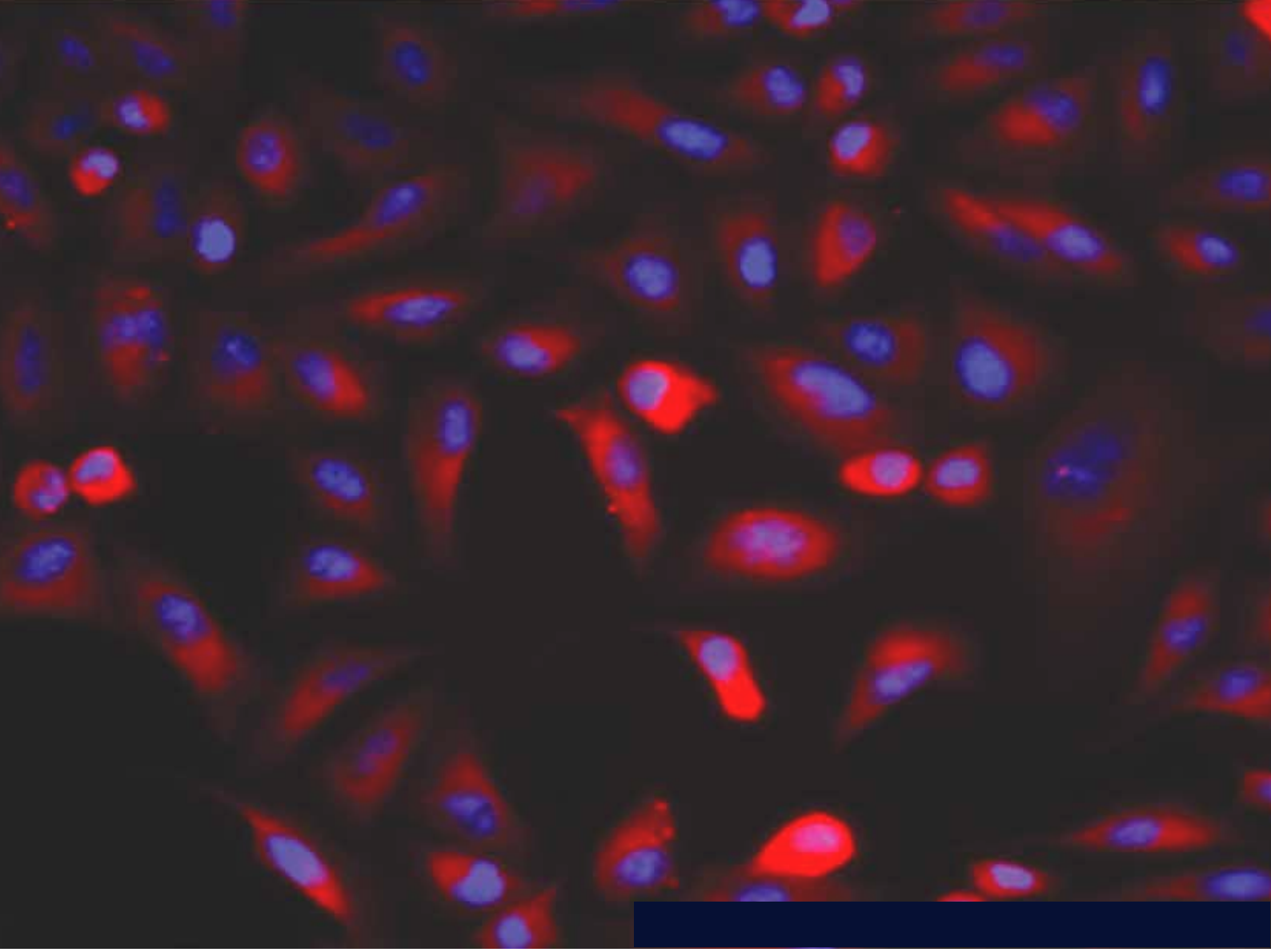


IMMUNOHISTOCHEMISTRY GUIDE

AssayGenie

www.assaygenie.com

CONTENTS

1. INTRODUCTION

Immunohistochemistry (IHC) is a technique widely used in histology and pathology laboratories. IHC enables the visual detection of proteins and other antigens in tissue, providing a method for the detection of abnormal cells or the characterization proteins within a biological sample.

Although considered less quantitative than other immunoassays such as ELISA or Western blot, IHC enables the localisation of protein expression in the context of intact tissue and therefore provides a means for assessing the progression and treatment options of diseases such as cancer.

IHC is dependent on the specificity of the antibody used. A highly specific antibody will ensure binding of the protein of interest in the tissue section. Visualisation of the antibody-antigen interaction can be achieved using either chromogenic or fluorescent detection. Chromogenic detection relies on the use of an antibody that is conjugated to an enzyme. This enzyme cleaves its substrate to produce a coloured product at the location of the protein. In contrast for fluorescent detection the antibody used is conjugated to a fluorophore, which can be visualised using a fluorescent microscope.

1.1 ANTIBODY SELECTION

Choosing the correct primary antibody, either monoclonal or polyclonal is the key to a successful and specific IHC assay with each having unique advantages and disadvantages. Monoclonal antibodies are generated from a single B-cell clone from one animal, resulting in a homogenous population of immunoglobin directed against a single epitope. Polyclonal antibodies are derived from multiple B-cell clones resulting in a heterogenous mixture of antibodies directed against various epitopes of the same antigen.

Monoclonal antibodies are favoured, as batches produced from an established hybridoma cell line allow for standardization, as the population is identical. Furthermore, the use of monoclonal antibodies has been shown to limit background staining and result in reduced cross-reactivity with other proteins. Using a polyclonal antibody can help increase the detection signal of the antigen and is the preferred choice when dealing with denatured protein. As polyclonal antibodies can recognise multiple epitopes they are also more tolerant of minor changes during fixation and processing when compared to monoclonal antibodies.

Species choice should be taken into consideration when choosing antibodies for IHC. In order to ensure low background the secondary antibody used should be against the host species of the primary antibody used.

Different fixative agents may be used depending on the antigen under investigation. The most widely used fixative for IHC is 10% neutral buffered formalin. Other fixatives include methanol, ethanol and acetone which may be used for fixation and permeabilization of the membrane. Below is a table outlining recommended fixation solutions for selected antigens in tissue.

ANTIGEN	FIXATIVE
Blood forming organs/connective tissue	Zenker's or Helly Solution
Delicate Tissue	Bouin's Fixative
Immunoglobulins	Ice-cold Acetone or Methanol (100%)
Nuclear Morphology	Zinc Formalin
Nucleic Acids	Carnoy's Solution
Proteins, peptides and enzymes	10% Neutral Buffered Formalin
Small Molecule	4% Formaldehyde

Table 2: Fixative solution recommend for specific antigen

3. EMBEDDING

Embedding is an essential component of sample preparation to preserve morphology and provide tissue support during processing and sectioning. Like fixation various embedding compounds can be used depending of the specific requirements of the antibody. Frozen and paraffin embedding are the most widely used, each offer distinct advantages and disadvantages depending on the antibody used.

3.1 FROZEN TISSUE

Frozen tissue sections can be processed in a shorter amount of time when compared to paraffin embedded sections. Frozen tissue samples are prepared by immersing the tissue in liquid nitrogen, isopentane or by snap freezing in dry ice. Frozen samples require a short fixation after freezing and sectioning which is normally done with alcohol. Alcohol, unlike formaldehyde, does not mask epitopes and therefore antigen retrieval does not need to be done on tissue fixed with alcohol.

Long term storage of frozen tissue sections is not recommended, as the formation of ice crystals within cells may negatively affect subcellular details. The recommended maximum storage of frozen sections is 1 year at -80°C.

It should be noted that frozen sections are often thicker than paraffin sections which can result in a lower microscopic resolution and poor images of tissue morphology. As frozen tissue often retains enzymatic activities, the activity of the endogenous enzymes may affect the IHC detection method

3.2 PARAFFIN EMBEDDED TISSUE

Paraffin embedding is the best option for long term preservation of a tissue sample. Paraffin sections are generally cut between 3-5 microns using a microtome.

Prior to being embedded in paraffin the sample must be fixed, this can be done by perfusion or immersion directly after dissection generally requiring 4-24 hours. Do not fix your sample for longer than 24 hours as over-fixation may mask the antigen. The duration of fixation may require optimisation and can vary depending on tissue and antigen.

As paraffin is immiscible with water the tissue sample must be dehydrated before the addition of paraffin wax. The sample can be dehydrated by immersion in increasing concentrations of alcohol. The gradual increase in alcohol concentration minimizes cell damage. Once dehydration in alcohol is complete the sample is placed in xylene to remove any remaining ethanol.

The paraffin is heated to 60°C for embedding and allowed to harden overnight. Following this, the tissue can be cut into thin sections using a microtome. These tissue sections can be stored at room temperature until rehydration and initiation of the IHC protocol.

*Some antigens, e.g. those with post-translationally modified residues, will not survive even mild aldehyde fixation, and in such cases, the tissue should be snap-frozen and sectioned in a cryostat and stored at -80°C until fixing in alcohol or cold acetone.

3.3 ADVANTAGES AND DISADVANTAGES OF PARAFFIN VS FROZEN EMBEDDING

	PARAFFIN EMBEDDED	FROZEN TISSUE
Advantages	Preserves tissue morphology	Preserves enzyme and antigen function
Disadvantages	Over-fixation can mask the epitope	Formation of ice crystals can affect tissue structure
Downstream Assays	PCR amplification	DNA, RNA, FISH and cell cycle analysis

Fixation	Pre-embedding	Pre/Post Sectioning
Precautions	Duration and intensity of tissue heating can be harmful to antigens	Freeze quickly. Don't cut directly from frozen
Sectioning	Microtome	Cryostat
Storage	Years at room temperature	1 year at - 80°C

Table 3: Advantages and disadvantages of paraffin vs frozen embedding

4. ANTIGEN RETRIEVAL

The process of sample fixation although necessary for tissue preservation can mask the epitope of interest preventing binding of the primary antibody. Antigen retrieval therefore refers to any technique in which the masking of an epitope is reversed and epitope-antigen binding restored. Antigen retrieval acts to break the cross-linkage induced by fixation and expose the antigen.

On paraffin embedded tissues it is necessary to perform a deparaffinisation step with xylene and alcohol prior to antigen retrieval. Deparaffinisation generally consists of 2 washes in xylene followed by three minutes washes in 100%, 95% and 70% ethanol. To complete the rehydration process the sections should be washed twice in ddH_2O for 5 minutes each.

There are 2 common processes for antigen retrieval:

1. Heat mediated (Heat induced epitope retrieval – HIER)
2. Enzymatic digestion (Proteolytic-induced epitope retrieval - PIER)

4.1 HEAT-INDUCED EPITOPE RETRIEVAL – HIER

HIER uses heat to unmask the epitope. The source of which can be a microwave, pressure cooker, steamer, water bath or autoclave.

4.2 PROTEOLYTIC-INDUCED EPITOPE RETRIEVAL – PIER

This method uses enzymes such as Proteinase K, Trypsin and Pepsin to restore the binding of an antibody to its epitope. When using enzymatic digestion reagents it is important not to over or under digest – the digestion time should be optimized. Caution should be exerted when using PIER as it has been shown in some cases to have a low success rate for restoring immunoreactivity and can destroy both tissue morphology and the antigen of interest.

*The technique used for antigen retrieval ultimately depends on the tissue, fixation method and primary antibody. Antigen retrieval is not required for frozen tissues as antigen masking is the result of cross-linking formed during formalin fixation.

4.3 HIER VS PIER

	HIER	PIER
Advantages	Maintains epitope	Commonly used for difficult to retrieve epitopes
Buffer Composition	Sodium citrate buffer, 1mM EDTA pH 8.0, Tris-EDTA.	Neutral buffer solutions are used of enzymes such as pepsin, proteinase K or trypsin
Incubation Time	10-20 minutes	5-30 minutes
pH	pH 6 most widely used, optimum pH may vary	pH 7.4
Precautions	Heating can lead to uneven antigen retrieval	Enzymatic activity can damage the sample
Recommended Antigens	No specific antigen	Immunoglobulins, cytokeratins
Temperature	95°C	37°C

Table 4: Comprison of HIER and PIER antigen retrieval techniques

5. PERMEABILIZATION

Permeabilization may be required for certain samples whereby the antibody needs to gain access to the inside of the cell to detect the protein. Permeabilization is also required in order to detect transmembrane proteins if the epitope is located in the cytoplasmic region.

Permeabilization is achieved using solvents or detergents. Solvents such as acetone and methanol can be used after fixation with a crosslinking agent e.g. Paraformaldehyde. Solvents are recommended for cytoskeletal, viral and enzyme antigens. Detergents are generally much milder on the sample and will not dissolve the plasma membrane. Detergents such as Triton or Tween 20 are used for the permeabilization of antigens in the cytoplasm, plasma membrane or for soluble nuclear antigens.

6. BLOCKING

Blocking of the IHC sample is essential to achieve a good IHC signal and reduce the signal-to-noise ratio. Inadequate blocking can result in a high level of background noise whilst over-blocking can mask your signal.

Blocking takes place after the sample has been fixed, embedded, antigen retrieved and permeabilized if necessary, it is the last step performed before incubation with the primary antibody. The duration of blocking depends on the sample ranging from 30 minutes to overnight. Blocking can be performed at 4°C or at room temperature. Blocking is performed using a protein that does not specifically bind the epitope of interest or to the antibodies in the IHC assay.

The most commonly used blocking methods are outlined below.

6.1 BLOCKING SERA

The use of normal sera is considered one for the most effective blocking agents and is carried out using normal (unchallenged) sera from the same species that the secondary antibody was raised in. The antibodies in the normal sera will bind any non-specific epitopes in your sample and in turn prevent unconjugated antibodies from doing the same. Normal sera is an ideal blocking agent when using polyclonal antibodies.

6.2 PROTEIN BUFFERS

Protein buffers can be used instead of sera to block your sample. Protein buffers compete with your antibody to bind non-specific epitopes therefore, the use of high concentrations of protein competitors can out-compete your antibody and ultimately lower background noise. Commonly used protein blocking buffers are: 0.1 to 0.5% bovine serum albumin (BSA), gelatin or non-fat dry milk.

6.3 COMMERCIAL MIXES

Commercial blocking buffers are also available.

6.4 BIOTIN BLOCKING

If an Avidin-biotin detection system is used it is recommended to block for endogenous biotin before the addition of the secondary antibody.

6.5 PEROXIDASE BLOCKING

If using a HRP conjugated antibody non-specific staining may occur due to the activity of endogenous peroxidases already in the tissue. The presence of peroxidases in your sample can be identified by incubating the sample with DAB substrate which will turn brown on peroxidase identification. To block peroxidases H_2O_2 is commonly used.

6.6 ALKALINE PHOSPHATE (AP) BLOCKING

Endogenous alkaline phosphate (AP) can produce a high background when using AP chromogen substrates. Endogenous AP is typically found in kidney, intestine, osteoblasts, lymphoid and placenta tissue. Tissue can be tested for endogenous AP through incubation with BCIP/NBT, if a blue colour is observed endogenous AP is present and blocking is necessary. Endogenous AP can be blocked by including levamisole in the chromogen substrate

* Not all blocking agents and buffers are compatible with all detection methods. For example, alkaline phosphatase conjugates are not compatible with any sodium azide preservative in buffers and avidin-biotin complex system is not compatible with non-fat dry milk.

7. IMMUNOSTAINING

Following successful sample preparation the tissue can now be incubated with selected antibodies for the detection of a targeted antigen.

There are multiple labelling techniques available such as direct, indirect and indirect with signal amplification.

Direct labelling uses a primary antibody directly conjugated to a signalling source, while indirect labelling has a signal generating secondary antibody that will attach to the primary antibody making contact with the antigen. Direct detection is quicker and simpler than indirect detection, as the label is attached via a covalent bond directly to the primary antibody. This means only one incubation step and one round of washes is needed.

It is important to note that a primary antibody produced from a mouse would be detected using a secondary antibody that is anti-mouse and conjugated to a detecting probe. Finally, the indirect with signal amplification technique is accomplished using a biotinylated secondary antibody and an amplification reagent such as streptavidin.

7.1 DIRECT VS INDIRECT IMMUNOSTAINING

DIRECT	INDIRECT
Limited amount of directly conjugated antibodies available	Only a small number of standard conjugated antibodies required
Low signal amplification	Strong signal amplification
No species cross reactivity	Commonly used technique
No non-specific binding	Non-specific binding may occur
Short protocol	Extra Incubation and wash steps required
Straightforward Dual staining	Dual staining is difficult to achieve

Table 5: Comparison of direct and indirect immunostaining

7.2 PRIMARY ANTIBODY

When choosing a primary antibody consider its specificity for the antigen of choice and its suitability for IHC. Antibodies that are designed for western blot or ELISA assays may not always work for a target antigen which has been crosslinked in fixed tissue. Therefore, it is paramount to identify examples of IHC on the commercial antibody data sheet.

Primary antibodies can be used for multiple targets in one incubation solution once they are of different host origins and detected with different secondary antibodies.

7.3 SECONDARY ANTIBODIES

A secondary antibody should be chosen based on its specificity for the primary antibody. For example, a primary antibody raised in rabbit requires a secondary antibody against rabbit, anti-rabbit. Multiple antigens can be assessed through the use of secondary antibodies linked to contrasting fluorophores that distinguish between primary antibodies based on host origin. Secondary antibody incubation should be carried out in the dark to protect the fluorophore.

It is also important that the isotype of the secondary antibody matches the primary antibody. Affinity purified antibodies are widely used as they provide the lowest amount of non-specific binding. It should be noted though that IgG fractions can potentially contain very high affinity antibodies and may be of use when an antigen is poorly expressed or in low abundance.

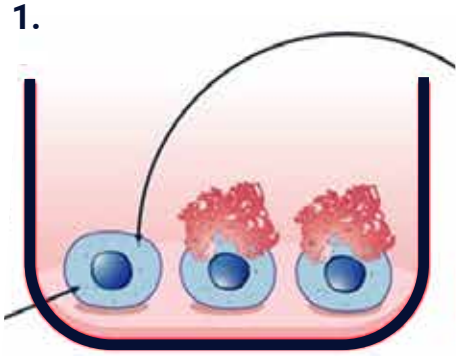
IMMUNOSTAINING SCHEMATIC

IHC Paraffin

DEPARAFFINIZATION & REHYDRATION
Xylene, 100% ethanol, 100% ethanol down to 50% ethanol

ANTIGEN RETRIEVAL
Hier or Pier

1.



IHC • Frozen

FIX SLIDES
4% PFA/ Methanol / Acetone

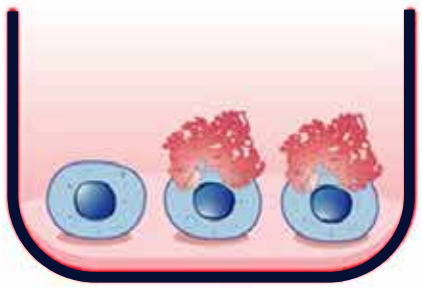
Block with serum or BSA for 30 min to 1hr

Wash in PBS Tween 4 times for 5 minutes

PERMEABILIZE THE CELLS
if detecting an intracellular target

0.2% Triton for 10 min (not necessary if fixed in Acetone or Methanol)

2.



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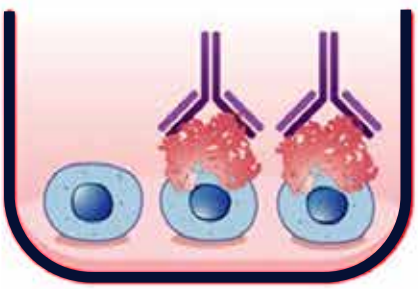
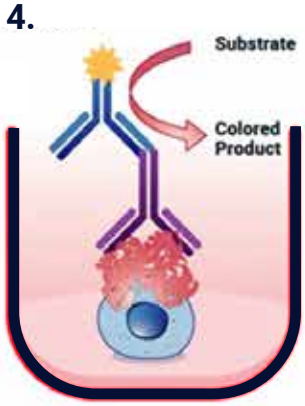


INCUBATE WITH PRIMARY ANTIBODY

Wash in PBS Tween 4 times for 5 minutes

ENZIMATYIC DETECTION

4.



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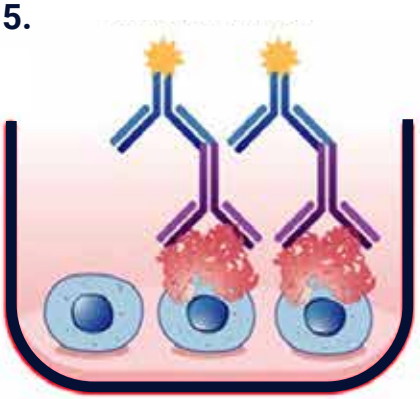


INCUBATE WITH CONJUGATED SECONDARY ANTIBODY

Table 6: Sample immunostaining protocol

10. IHC BUFFER RECIPES

Sample Fixation Buffers

Zenker's Solution

REAGENT	AMOUNT
Mercuric Chloride	5 g
Potassium Dihromate	2.5 g
ddH_20	100 mL

*Heat and cool. Add 5 ml of glacial acetic acid just before use. Never use metal forceps when handling tissues fixed in Zenker Solution. Ideally used for bloody samples such as spleen and connective tissue.

Helly Solution

REAGENT	AMOUNT
Mercuric Chloride	5 g
Potassium Dihromate	2.5 g
ddH_20	100 mL

*Heat and cool. Add 5 ml of formaldehyde just before use. Use for blood forming organs such as bone marrow, liver and spleen.

Bouin's Fixative

REAGENT	AMOUNT
Picric Acid (Saturated)	75 mL
Formaldehyde (37-40%)	25 mL
Glacial Acetic Acid	5 mL

*Ideal for preserving soft tissue structures.

Carnoy's Solution

REAGENT	AMOUNT
Ethanol (100%)	60 mL
Chloroform	30 mL
Glacial Acetic Acid	10 mL

*Used for fixation of DNA and RNA.

Zinc Formalin

1. Make a 0.1M Tris Buffer, pH 7.4

REAGENT	AMOUNT
Trizma® Tris-Base	12.1 g
1N HCL	81.5 mL
Distilled Water	900 mL

2. Prepare Zinc Fixative

REAGENT	AMOUNT
Calcium Acetate	0.5 g
Zinc Acetate	5.0 g
Zinc Chloride	5.0 g
0.1M Tris Buffer (see above)	1000 mL

* Mix to dissolve. Adjust the final pH to 6.5-7.0. Store the fixative reagent at room temperature
4% Formaldehyde Fixative Solution

1. Make a 0.2M Phosphate Buffer (PBS) , pH 7.4

REAGENT	AMOUNT
Na_2HPO_4	21.8 g
$NaH_2 PO_4$	6.4 g
ddH_20	1000 mL

2. From this make a 0.1M Phosphate Buffer, pH 7.4

REAGENT	AMOUNT
0.2M PB	500 mL
ddH_20	500 mL

3. Proceed to make 4% Paraformaldehyde in 0.1M Phosphate Buffer

REAGENT	AMOUNT
Paraformaldehyde	40 g
0.1M Phosphate Buffer	1000 mL

* Heat to 60-65 ºC while stirring. Add a few drops of 1N NaOH until solution clear. Continue stirring until the solution is dissolved. Cool and filter.

10% Neutral Buffered Formalin

REAGENT	AMOUNT
Formalin (37-40% stock solution)	100mL
ddH_20	900mL
NaH_2PO_4 (monobasic)	4g/L
Na_2HPO_4 (dibasic/anhydrous)	6.5g/L

* 10% formalin actually represents10% of the 37-40% stock solution. The actual amount of dissolved formaldehyde in the 10% formalin is therefore only 3.7-4.0%.

Buffers for heat-induced epitope retrieval (HIER)

Sodium Citrate Buffer (10 mM Sodiem Citrate, 0.05% Tween 20, pH 6.0)

REAGENT	AMOUNT
Tri-sodium citrate (dihydrate)	2.94 g
ddH_20	1000 mL
TWEEN®20	0.5 mL

*Adjust pH to 6.0 with 1N HCL. Store at room temeperature for 3 months, for longer storage store at 4º C

1 mM EDTA, pH 8.0

REAGENT	AMOUNT
EDTA	0.37 g
ddH_20	1 L

*Adjust pH to 8.0 with NaOH, store at room temperature for 3 months.

Trsi-EDTA buffer (10mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0)

REAGENT	AMOUNT
Tris	1.21 g
EDTA	0.37 g
ddH_20	1000 mL
TWEEN®20	0.5 mL

*Adjust pH to 9.0. Store at room temperature for 3 months for longer store at 4º C.

Buffers for enzymatic antigen retrieval

Trypsin Buffer

1. Make a trypsin stock solution (0.05%)

REAGENT	AMOUNT
Trypsin	50 mg
ddH_20	1000mL

*Store at -20°C

2. Make 1% Calcium Chloride Stock Solution

REAGENT	AMOUNT
Calcuim Chloride	0.1 g
ddH_20	1000 mL

*Store at 4° C

3. Trypsin working solution (0.05%)

REAGENT	AMOUNT
Trypsin Stock Solution (0.05%)	1 mL
Calcium Cholride stock Solution (1%)	1 mL
Distilled Water	8 mL

*Adjust the pH to 7.8 with NaOH, store at 4° C for one month for longer store at -20° C.

Permeabilization Buffers

Triton or NP-40
Use 0.1 – 0.2 % in PBS for10 minutes only
*Partially dissolves the nuclear membrane for nuclear antigen staining.
Tween 20, Saponin, Digitonin and Leucoperm
Use 0.2 – 0.5% for 10-30 minutes.
*Suitable for cytoplasmic antigens and soluble nuclear antigens.

Blocking Buffers

Peroxidase Blocking Solution (3% H202 in PBS)

REAGENT	AMOUNT
30% H_2O_2	10 mL
1X PBS	90 mL

*Store at 4° C for up to 3 months. Recommended solution for paraffin sections.
Peroxidase Blocking Soluiton (0.3% H_2O_2 in Methanol)

REAGENT	AMOUNT
30% H_2O_2	1 mL
Methanol	99 mL

*Store at 4º C. Recommended for Frozen sections.

Biotin blocking buffer

Biotin 0.001% in PBS.

*Store at 4 ºC

Blocking Sera Sample Buffer Recipes

Normal Rabbit Sera blocking buffer

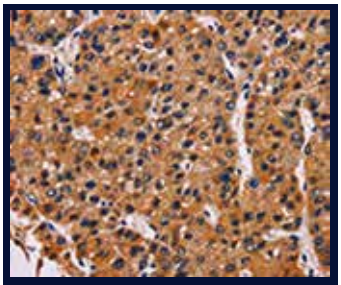
REAGENT	AMOUNT
Rabbit serum	2%
BSA	1%
Cold fish skin gelatin	0.1%
Triton X-100	0.1%
TWEEN®20	0.05%
Sodium azide	0.1%
1M PBS, pH 7.2	

*Store at 4 ºC

Universal Blocking Buffer

REAGENT	AMOUNT
BSA	1%
Cold fish skin gelatin	0.1%
Triton X-100	0.5%
Sodium azide	0.05%
0.01M PBS, pH 7.2-7.4	

*Store at 4 ºC. Do not use to dilute HRP conjugated antibody as the sodium azide is inhibitor of HRP.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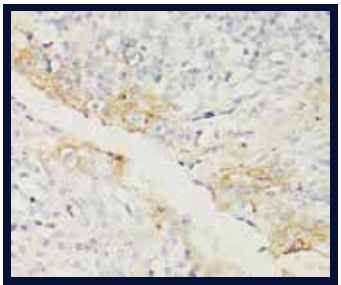
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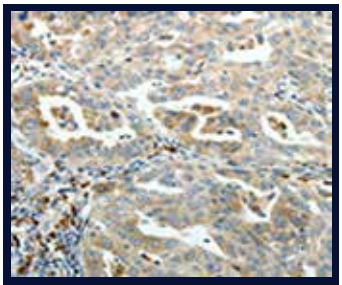
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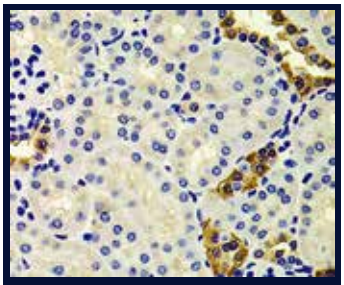
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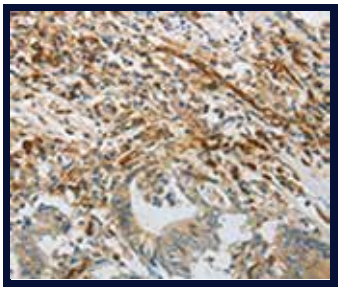
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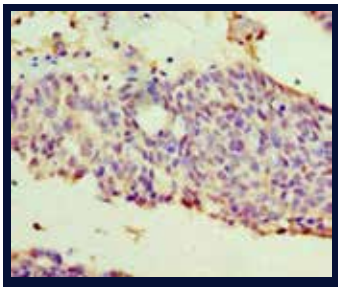
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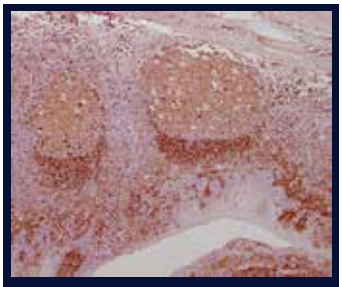
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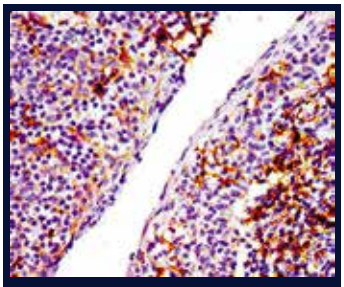
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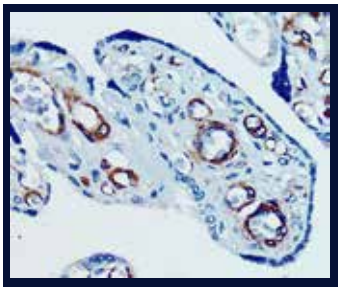
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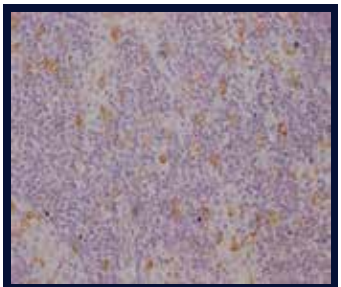
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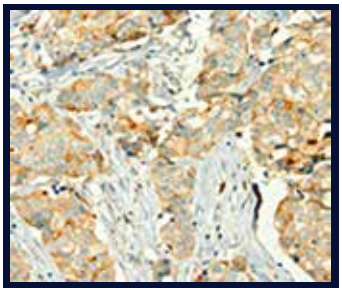
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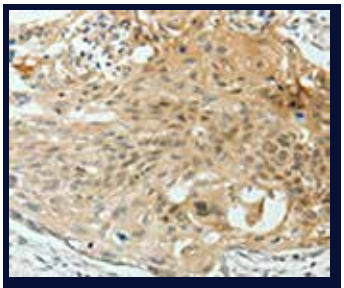
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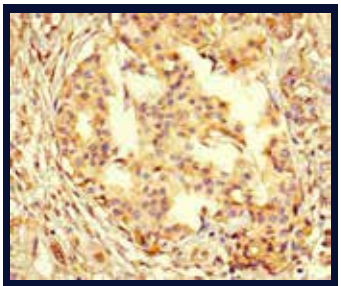
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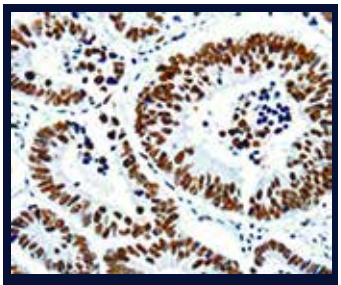
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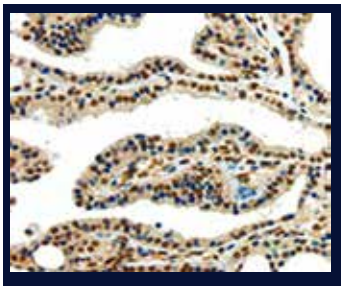
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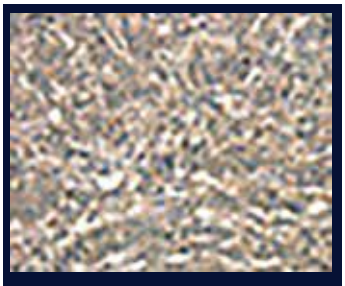
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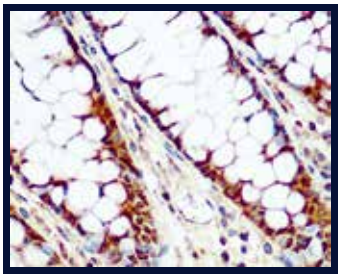
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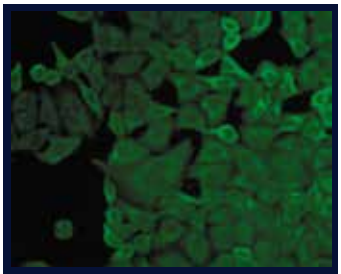
P53

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1.1 IHC ASSAY OPTIMIZATION AND VARIABLES

Below is a table of key components of an IHC assay and considerations which must be taking into account before beginning IHC assay.

IHC ASSAY COMPONENT	CONSIDERATIONS
Antigen	Species antigen expression levels
Antigen retrieval	PIER or HIER
Blocking reagent	Normal serum, protein, commercial buffer, other
Detection method	Direct/Indirect
Fixative	Formaldehyde, alcohol, acetone
Label	Fluorochrome or Chromogen
Primary antibody	Monoclonal or polyclonal
Secondary antibody	Species and detection label
Visualisation and analysis	Florescence/Light microscope. Software analysis

Table 1: IHC Assay Components & Considerations

2. SAMPLE PREPARATION

Correct sample preparation is an essential component of a high quality IHC stain and involves several key steps outlined below.

2.1 SAMPLE FIXATION

The first step of an IHC assay is sample collection. Correct sample collection is critical in order avoid autolysis and necrosis of the excised tissue. Factors that will affect the quality of sample for IHC analysis will include time until fixation and the temperature of the samples. It is therefore advised to place the sample in a fixative solution soon as possible.

Fixation ensures sample preservation, the immobilization of antigens and the maintenance of subcellular structures through cross-linking methylene bridges and Schiff bases between basic amino acid residues of proteins. An optimized fixation protocol is advised as an inappropriately fixed antigen may not be detected further on.

8. COUNTERSTAINING AND MOUNTING

Counterstaining can be used to identify all the cells in your sample that do not express the antigen of interest. Counterstaining can be performed using haematoxylin or DAPI.

To mount coverslips to slides, use mounting medium designated for fluorescence microscopy to help preserve the fluorescent signal in your sample. Apply the coverslip with enough mounting medium, take care to eliminate bubbles which can distort or obscure your images.

9. IHC CONTROLS

IHC like all other assays requires controls to confirm that the staining pattern is accurate and reliable. For IHC both an antigen control and reagent control is advised.

Antigen Control - There should be both a positive and negative antigen control. The positive control may be a tissue known to express the protein under investigation. The negative control a tissue known not to express the protein the protein of interest. Using a negative control will help identify non-specific bonding and false positives.

Reagent Control - A reagent control is used to ensure that staining is produced from the primary antibody staining the antigen and not from the detection system or the specimen. This can be determined using the detection system with diluent alone and no primary antibody.

For each experiment, it is also recommended to include a tissue section incubated with a non-specific isotype control antibody that matches the class and type of the primary antibody (if the primary antibody is a monoclonal antibody), but does not recognize the target epitope. Isotype controls help distinguish non-specific background fluorescence from specific fluorescent labelling of your target antigen. If the primary antibody is a polyclonal antibody, include a tissue section incubated with a non-specific, species-matched polyclonal antibody.