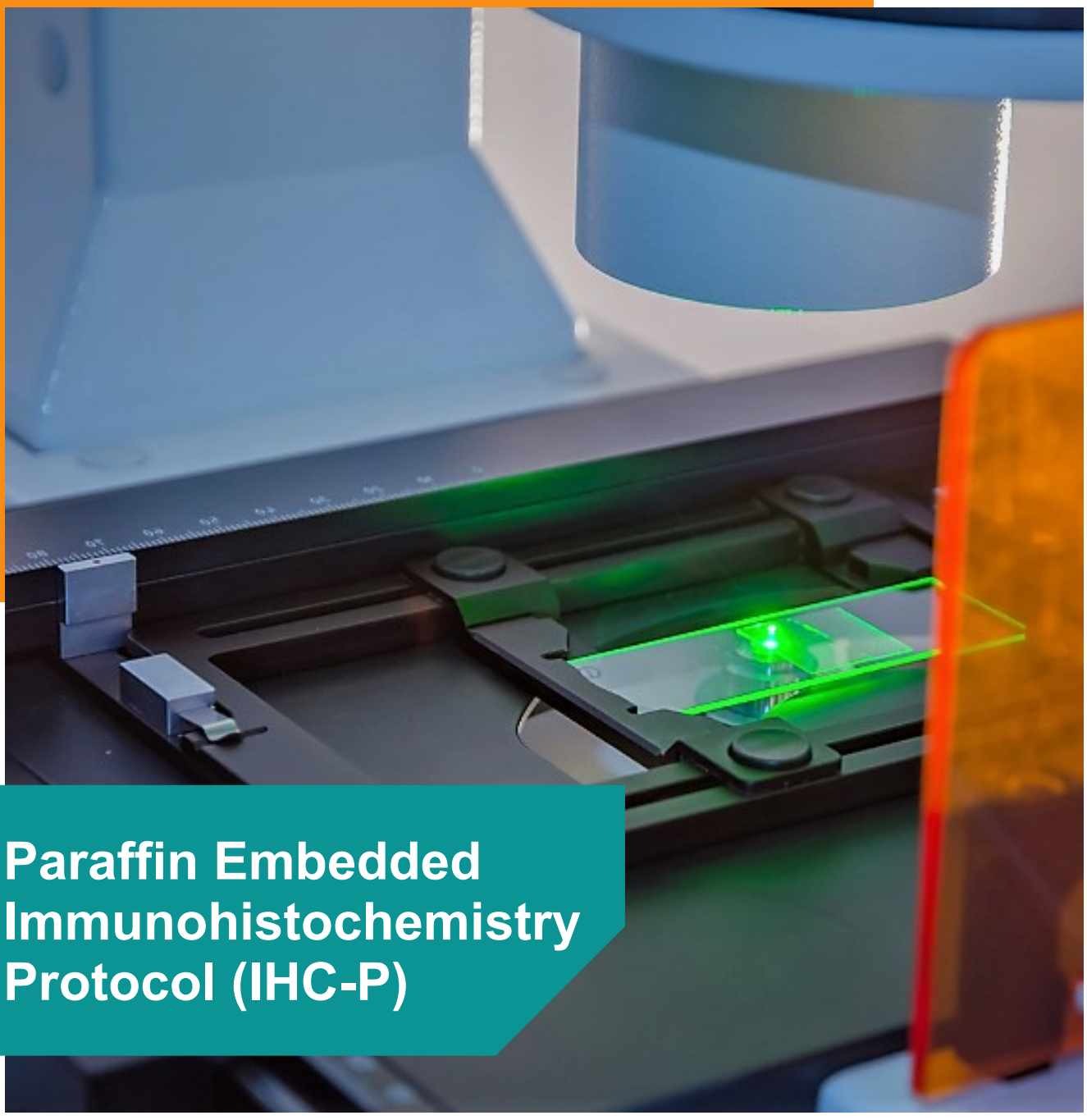

Paraffin Embedded Immunohistochemistry Protocol (IHC-P)

Hello Bio

v1 Aug 2024

CONTENTS

1. Introduction to Paraffin Embedded Immunohistochemistry (IHC-P)

Immunohistochemistry (IHC) is an extremely popular and powerful technique that allows the visualization of protein markers within thin sections of tissue. This can be used to analyze the distribution of receptors, look at the cellular makeup, expression of biomarkers and gross morphology of a tissue amongst a myriad of other applications. Paraffin embedded immunohistochemistry (IHC-P) is a popular variation of IHC where tissue is embedded in paraffin wax following fixation. This better preserves tissue morphology, enables the cutting of extremely thin tissue sections and protects the tissue from degradation (paraffin embedded tissue is stable at room temperature for multiple years). There are two main variations of IHC-P:

- Chromogenic IHC-P: Primary antibodies are visualized by a colorimetric reaction catalyzed by horseradish peroxidase that leads to the deposition of insoluble colored precipitates that can be seen using the naked eye and brightfield microscopy.
- Fluorescent IHC-P: Primary antibodies are visualized by fluorescently conjugated secondary antibodies that can be visualized using fluorescence microscopy.

Figure 1 shows some representative images of both chromogenic and fluorescent paraffin embedded immunohistochemistry that have been created using these protocols. The protocols within this guide are intended for paraffin embedded sections. We have created a whole separate guide for immunohistochemistry in fixed-frozen sections.

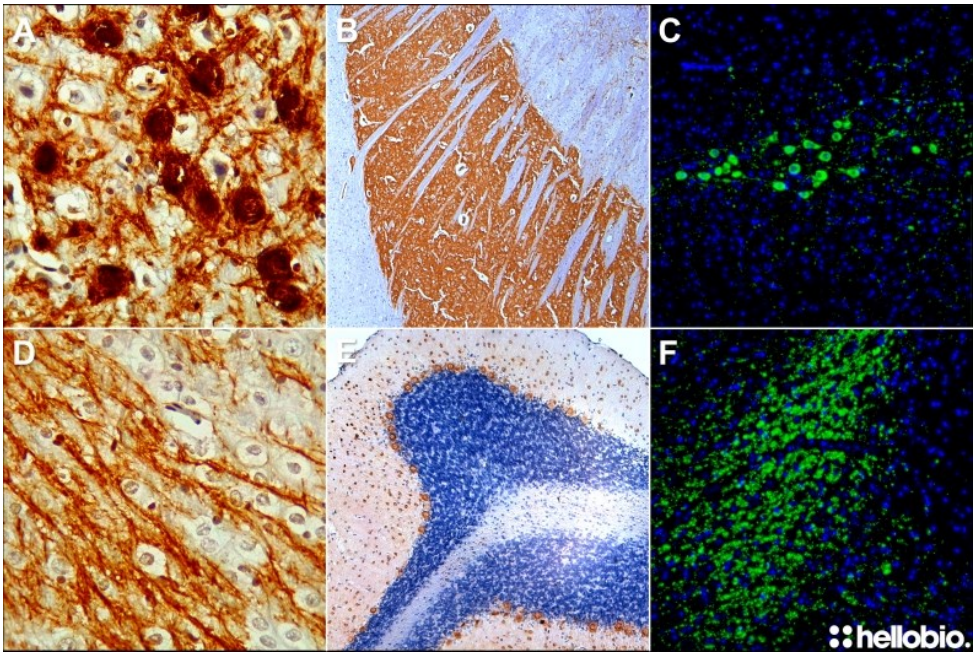

Figure 1. Representative paraffin embedded immunohistochemistry images created using the Hello Bio protocol. (A) Dopaminergic cell bodies in the midbrain stained using HB7167 and HB11345 antibodies via DAB staining. **(B)** Rat caudate putamen stained for tyrosine hydroxylase using HB7167 primary antibody and HB11345 secondary antibody utilizing DAB staining. **(C)** Immunofluorescent detection of dopaminergic midbrain cell bodies using HB6605. **(D)** Dopaminergic fibers projecting to the caudate putamen stained using HB7167 and HB11345 antibodies via DAB staining. **(E)** Parvalbumin neurons in the rat cerebellum stained using HB6457 and HB11345 antibodies via DAB staining. **(F)** Dopaminergic fibers in rat midbrain stained using HB6605 and a fluorescently tagged secondary antibody.

2. Safety

Many of the chemicals used in immunohistochemistry have dangerous properties and can cause serious harm if not handled correctly. Always follow local rules and read the full COSHH document for any chemical that you have not used previously. Always wear appropriate PPE such as a lab coat and gloves. Specifically highlighted hazards:

- Formaldehyde is a highly toxic poison, skin sensitizer and carcinogen and should only ever be used in a fume hood with full PPE and disposed of safely following local regulations.

- Xylene is a volatile carcinogen, CNS depressant, and skin, eye and respiratory system irritant. It should only ever be used in a fume hood with full PPE and disposed of safely following local regulations.

3. Protocol – Fixation, Embedding and Sectioning

For high quality IHC-P results optimization of the fixation, embedding and sectioning process is crucial. A range of different fixatives can be used but the more common ones are 10% neutral buffered formalin (10% NBF), 4% paraformaldehyde and Bouin's solution. There are two main fixation methods: perfusion fixation where fixative is circulated using the animal's intact circulatory system or immersion fixation where dissected tissue is immersed in fixative.

3.1 Perfusion Fixation

Generally, the best quality tissue sections are achieved when the animal has been perfusion fixed. This process involved replacing the blood and it's high autofluorescence with first buffer then fixative. Before attempting, make sure this is covered under your regulatory regime and obtain training from an experienced practitioner. A general protocol for rodents is:

1. Overdose with anesthetic by an appropriate route until the animal has no toe pinch and blink reflexes but the heart is still beating.
2. Open the ribcage to reveal the heart, snip the right atrium. Place a needle into the left ventricle and perfuse through ice-cold PBS (≈200ml for a rat, ≈15ml for a mouse) followed by a similar volume of 4% paraformaldehyde in PBS.
3. Remove the brain / other organs and place into a vial of 4% paraformaldehyde for 24hrs at 4°C.
4. Move the tissue into 70% ethanol before embedding

Please see Gage et al., 2012 J. Vis. Exp. (65), e3564 for a detailed protocol with accompanying video.

3.2 Immersion Fixation

Where perfusion fixation is not possible or practicable (e.g. for patient biopsy samples) then immersion fixation is a good alternative. The presence of blood within the tissue can however cause issues with background staining.

1. Dissect the tissue with clean tools on ice.
2. Wash briefly with ice-cold PBS
3. Place tissue into 4% paraformaldehyde in PBS for 24hrs at 4°C. The length of incubation in fixative often needs optimizing as under-fixation can result in edge staining while over-fixation can result in a low signal intensity or no signal at all. A good range of incubation times is between 18 and 24 hours for most tissue samples.
4. Move the tissue into 70% ethanol before embedding

3.3 Embedding and Sectioning

The final stage of tissue preparation is embedding and sectioning where the tissue is embedded into paraffin wax and then cut into sections of appropriate thickness.

1. Perform paraffin embedding in an automated tissue processing system. Where this is not available then please follow this protocol for specific steps and incubation times.
2. Following embedding, trim the tissue blocks and orientate in the desired direction on a microtome.
3. Cut sections at a thickness between 3µm and 10µm (5µm is the most commonly cut thickness).
4. Place cut sections into a water bath set at 45°C before then mounting onto microscope slides.
5. Air dry sections for 30 minutes before incubating at 45°C overnight.
6. Sections can now be stored at room temperature before further processing.

4. Protocol - Colorimetric Detection using Streptavidin HRP

Chromogen based immunohistochemistry utilizes enzyme conjugated streptavidin to catalyze a chemical reaction which results in the deposition of a colored precipitate. This is an extremely popular technique due to its high sensitivity, the high stability of the stained sections and the ease of imaging with simple widefield microscope systems. There are multiple chromogens available dependent upon experimental needs (see table 2 for a summary) in a range of different colors allowing a degree of multiplexing. This protocol has been optimized to use DAB staining however will work with other chromogens with minimal modification. Many of the wash and incubation stages in this protocol can be carried out in staining jars with slide racks to save time and allow more consistent staining across a batch. Following the successful completion of this protocol imaging can be carried out using a standard brightfield microscope using visible light.

Enzyme	Chromogen	Color	Recommended nuclear counterstain	Mounting Media	Description
Horseradish Peroxidase (HRP)	AEC	Red	Hematoxylin	Aqueous (e.g. glycerol jelly)	Intense color but not the best sensitivity
	DAB	Brown	Hematoxylin	Organic (e.g. DPX)	Intense permanent color with medium sensitivity
	DAB with Ni^{2+} enhancer	Dark blue to black	Neutral Red	Organic	High sensitivity with intense permanent color
	DAB with Co^{2+} enhancer	Dark blue	Neutral Red	Organic	High sensitivity with intense permanent color
	TMB	Blue	Hematoxylin	Aqueous	High sensitivity and intense permanent color
	4-CN	Dark blue to purple	Neutral Red	Aqueous	Low sensitivity with less permanent color
Alkaline Phosphatase (AP)	BCIP/NBT	Blue	Neutral Red	Organic	Intense color with good sensitivity
	BCP/TNBT	Purple	Hematoxylin	Organic	Intense color with good sensitivity
	Naphthol ASMXP + Fast Red	Orange to red	Hematoxylin	Aqueous	Medium intensity color that can fade easily
	Naphthol ASMXP + Fast Blue	Blue	Hematoxylin	Aqueous	Medium intensity color that can fade easily.

Figure 2. Chromogen options for paraffin embedded immunohistochemistry.

4.1 Deparaffinization

Before any antibody staining can happen, it is crucial to remove the paraffin wax that the samples are embedded into to allow the penetration of buffers and antibodies.

1. Incubate slides in either Histo-Clear or xylene for 20 minutes at room temperature
2. Incubate slides in 100% ethanol for 3 minutes
3. Incubate slides in 90% ethanol for 3 minutes
4. Incubate slides in 70% ethanol for 3 minutes
5. Leave sections in cold water until ready to proceed with staining.

4.2 Antigen Retrieval

Unlike immunohistochemistry using fixed frozen sections (see our frozen IHC protocol) antigen retrieval is essential for success using paraffin embedded sections. Two main buffers are used for IHC-P antigen retrieval: Citrate and Tris-EDTA. These can either be made in-house (see recipes below for citrate and Tris-EDTA buffers) or purchased in a convenient concentrated format (see: HB7943 100X Tris-EDTA Buffer, pH9 and HB8687 10X Citrate buffer pH6). We carry out our antigen retrieval in a vegetable steamer although multiple other options are available including using a microwave, pressure cooker or autoclave (see Taylor et al., 1996 for a comparative study). There are a range of different antigen retrieval protocols available (see Krenacs et al., 2010 for a review) however this protocol forms a good foundation and can be then optimized if required:

1. Set up the vegetable steamer so that there is a suitably sized heatproof container of citrate retrieval buffer contained within for the slides to antigen retrieve in. Preheat the steamer for 10-15 minutes (this can be carried out during the deparaffinization process)
2. Place the slides in the citrate buffer and leave in the steamer for 20 minutes
3. Remove the slides and run under cold tap water for 10 minutes.

4.3 Blocking and Staining

1. Place slides in a humidified chamber and aspirate excess liquid. It is easy to make a homemade humidified chamber by soaking paper towels in water then placing them in an enclosed slide box with the slides.
2. Draw around each tissue section with a PAP pen
3. Wash slides twice for 5 minutes each with TBST
4. Add 100µl of blocking buffer to each section (the volume can vary depending on the size of the tissue section) and incubate for 2 hours at room temperature
5. Aspirate the blocking buffer and add primary antibody diluted (100µl per section) in antibody/streptavidin diluent then incubate overnight at 4°C. The concentration of primary antibody will need optimizing with manufacturers providing recommended dilutions. In the absence of any information a 1:500 dilution is a good starting point.
6. Wash slides three times for 5 minutes each with TBST
7. Incubate sections in 0.3% hydrogen peroxide in TBS for 15 minutes to block endogenous peroxidases.
8. Wash slides twice for 5 minutes each with TBST
9. Incubate sections (100µl / section) with biotinylated secondary antibody to the primary antibody species diluted in antibody/streptavidin diluent for 1 hour at room temperature with gentle agitation. A dilution of 1:300 works well for this stage.
10. Wash slides three times for 5 minutes each with TBST
11. Incubate sections (100µl / section) with HRP conjugated streptavidin diluted in antibody/streptavidin diluent for 30 minutes at room temperature with gentle agitation. A dilution of 1:500 works well for this stage.
12. Wash slides three times for 5 minutes each with TBST. Meanwhile make the DAB Staining Solution.
13. Add 100µl of DAB Staining Solution to each section and incubate for 10 minutes. This development time can be extended or shortened dependent upon the progression of the staining reaction.
14. Rinse slides in running tap water for 5 minutes.

4.4 Counterstaining, Dehydration and Mounting

1. Incubate in hematoxylin solution for 5 minutes (available preformulated as HB6189 - Modified Mayer's Hematoxylin)
2. Rinse slides in running tap water for 5 minutes
3. Dip sections quickly 8-12 times in acid alcohol
4. Incubate sections for 2 minutes in tap water
5. Incubate sections for 2 minutes in Scott's water
6. Dip and quickly swish sections in 70% ethanol, then 90% ethanol then leave the sections in 100% ethanol for 3 minutes
7. Incubate sections in three changes of xylene in the fume hood. You can keep and reuse these xylene washes but make sure to label them from clean to dirty and always use them in the same order.
8. Add a couple of drops of a mounting media like DPX to each slide then gently place a coverslip being careful to remove all bubbles. This can be done by gently pressing bubbles with a pipette tip until they leave the slide.
9. Leave slides in the fume hood overnight for the mounting media to cure.

5. Protocol - Fluorescent Detection

Fluorescence based immunohistochemistry utilizes fluorophore conjugated secondary antibodies (or streptavidin) for visualization of target proteins. Immunofluorescence is ideal for multiplexing due to the wide variety of fluorophores available (see our guide on choosing a secondary antibody) and additionally is a shorter protocol than colorimetric IHC-P. Due to the ability to easily multiplex fluorescent detection is ideal where there is a need to colocalize two proteins as the different fluorophores provide a clean signal within their channel and minimal overlap. Many of the wash and incubation stages in this protocol can be carried out in staining jars with slide racks to save time and allow more consistent staining across a batch. Following the successful completion of this protocol imaging can be carried out using either a standard widefield fluorescence microscope or a confocal laser scanning microscope.

5.1 Deparaffinization

Before any antibody staining can happen, it is crucial to remove the paraffin wax that the samples are embedded into to allow the penetration of buffers and antibodies.

6. Incubate slides in either Histo-Clear or xylene for 20 minutes at room temperature
7. Incubate slides in 100% ethanol for 3 minutes
8. Incubate slides in 90% ethanol for 3 minutes
9. Incubate slides in 70% ethanol for 3 minutes
10. Leave sections in cold water until ready to proceed with staining.

5.2 Antigen Retrieval

Unlike immunohistochemistry using fixed frozen sections (see our frozen IHC protocol) antigen retrieval is essential for success using paraffin embedded sections. Two main buffers are used for IHC-P antigen retrieval: Citrate and Tris-EDTA. These can either be made in-house (see recipes for citrate and Tris-EDTA buffers below) or purchased in a convenient concentrated format (see: HB7943 100X Tris-EDTA Buffer, pH9 and HB8687 10X Citrate buffer pH6). We carry out our antigen retrieval in a vegetable steamer although multiple other options are available including using a microwave, pressure cooker or autoclave (see Taylor et al., 1996 for a comparative study). There are a range of different antigen retrieval protocols available (see Krenacs et al., 2010 for a review) however this protocol forms a good foundation and can be then optimized if required:

1. Set up the vegetable steamer so that there is a suitably sized heatproof container of citrate retrieval buffer contained within for the slides to antigen retrieve in. Preheat the steamer for 10-15 minutes (this can be carried out during the deparaffinization process)
2. Place the slides in the citrate buffer and leave in the steamer for 20 minutes
3. Remove the slides and run under cold tap water for 10 minutes.

5.3 Blocking and Staining

1. Place slides in a humidified chamber and aspirate excess liquid. It is easy to make a homemade humidified chamber by soaking paper towels in water then placing them in an enclosed slide box with the slides.
2. Draw around each tissue section with a PAP pen
3. Wash slides twice for 5 minutes each with TBST
4. Add 100µl of blocking buffer to each section (the volume can vary depending on the size of the tissue section) and incubate for 2 hours at room temperature
5. Aspirate the blocking buffer and add primary antibody diluted (100µl per section) in antibody/streptavidin diluent then incubate overnight at 4°C. The concentration of primary antibody will need optimizing with manufacturers providing recommended dilutions. In the absence of any information a 1:500 dilution is a good starting point.
6. Wash slides three times for 5 minutes each with TBST

From now onwards protect the sections from light

7. Incubate sections (100µl / section) with fluorophore conjugated secondary antibody to the primary antibody species diluted in antibody/streptavidin diluent for 90 minutes at room temperature with gentle agitation. A dilution of 1:300 works well for this stage.
8. Wash slides two times for 5 minutes each with TBST
9. Incubate sections with 1µg/ml DAPI for 10 minutes at room temperature (100µl / section)
10. Wash slides for 5 minutes with TBST
11. Add 100µl / section of 0.1% Sudan black and incubate for 5 minutes
12. Wash slides three times for 5 minutes each with TBS
13. Aspirate all liquid from the slide then add a drop of mounting media before gently adding a coverslip. If using an aqueous mounting media then application of clear nail varnish round the edge of the slide will be required to seal the section.

6. Solutions and Recipes

10x PBS

Note: store at room temperature

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
NaCl	40g	80g	160g	1.37M
KCl	1g	2g	4g	27mM
Na_2HPO_4	7.2g	14.4g	28.8g	100mM
KH_2PO_4	1.2g	2.4g	4.8g	20mM
dH_2O	≈400ml	≈800ml	≈1600ml	-
Conc HCl	Adjust to pH 7.4			-
dH_2O	Make up to final volume required			-

10x TBS

Note: store at room temperature

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
NaCl	40g	80g	160g	1.37M
Tris-base	12.1g	24.2g	48.5g	200mM
dH_2O	≈400ml	≈800ml	≈1600ml	-
Conc HCl	Adjust to pH 7.4			-
dH_2O	Make up to final volume required			-

1x PBST / TBST

Notes:

- Tween20 is extremely viscous therefore it is often helpful to cut the end off the pipette tip using scissors to made pipetting easier.
- The solution will need a good mixing with a stirring bar before being ready to use
- Generally, make up fresh and don't keep for longer than a few days.

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
10x TBS / PBS	50ml	100ml	200ml	1x
dH_2O	450ml	900ml	1800ml	-
Tween20	0.5ml	1ml	2ml	0.1%

Sodium Citrate Antigen Retrieval Buffer

Notes:

- Tween20 is extremely viscous therefore it is often helpful to cut the end off the pipette tip using scissors to made pipetting easier.
- The solution will need a good mixing with a stirring bar before being ready to use
- Store at room temperature

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
Tris-sodium citrate dihydrate	1.47g	2.94g	5.88g	10mM
dH_2O	≈450ml	≈900ml	≈1800ml	-
Tween20	0.25ml	0.5ml	1ml	0.05%
Conc. HCl	Adjust to pH6			-
dH_2O	Make up to final volume required			-

Tris-EDTA Antigen Retrieval Buffer

Notes:

- Tween20 is extremely viscous therefore it is often helpful to cut the end off the pipette tip using scissors to made pipetting easier.
- The solution will need a good mixing with a stirring bar before being ready to use
- Store at room temperature

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
Tris base	0.61g	1.21g	2.42g	10mM
EDTA	0.19g	0.37g	0.74g	1mM
dH_2O	≈450ml	≈900ml	≈1800ml	-
Tween20	0.25ml	0.5ml	1ml	0.05%
Conc. HCl	Adjust to pH9.0			-
dH_2O	Make up to final volume required			-

Blocking Buffer

Notes:

- Make up fresh
- For best results use serum from the same animal as the secondary antibody host.

Reagent	Amount to add			Final concentration
	5ml	10ml	50ml	
Normal serum (e.g. goat)	0.5ml	1ml	5ml	10%
Bovine serum albumin (BSA)	0.05g	0.1g	0.5g	1%
TBST / PBST	5ml	10ml	50ml	-

Antibody/Streptavidin Diluent

Notes:

- Make up fresh
- Also available to purchase as HB8223 - Antibody Diluent

Reagent	Amount to add			Final concentration
	5ml	10ml	50ml	
Bovine Serum Albumin (BSA)	0.05g	0.1g	0.5g	1%
TBST / PBST	5ml	10ml	50ml	-

70% Ethanol

Note: Store at room temperature

Reagent	Amount to add			Final concentration
	100ml	250ml	1000ml	
Ethanol	70ml	175ml	700ml	70%
dH_2O	30ml	75ml	300ml	-

90% Ethanol

Note: Store at room temperature

Reagent	Amount to add			Final concentration
	100ml	250ml	1000ml	
Ethanol	90ml	225ml	900ml	90%
dH_2O	10ml	25ml	100ml	-

Acid Alcohol

Note: Store at room temperature

Reagent	Amount to add			Final concentration
	50ml	250ml	1000ml	
Ethanol	35ml	175ml	700ml	70%
dH_2O	15ml	75ml	300ml	30%
10M HCl	125µl	625µl	2.5ml	25mM

Scott's Water

Note: Store at room temperature

Reagent	Amount to add			Final concentration
	50ml	250ml	1000ml	
$MgSO_4$	0.5g	2.5g	10g	83mM
$NaHCO_3$	0.033g	0.165g	0.66g	7.85mM
dH_2O	50ml	250ml	1000ml	-

1% DAB

Notes:

- Ensure that the solution changes color to a light brown before then mixing for a further 10 minutes to ensure the DAB is fully dissolved.
- Aliquot and store at -20°C

Reagent	Amount to add			Final concentration
	10ml	50ml	100ml	
3,3-Diaminobenzidine (DAB) tetrahydrochloride (HB0687)	0.1g	0.5g	1g	1%
dH_2O	10ml	50ml	100ml	-
10M HCl	3-5 drops	15 - 25 drops	30 - 50 drops	-

0.3% Hydrogen Peroxide

Notes:

- Make fresh as dilute hydrogen peroxide is not very stable.
- Use distilled water for making the DAB working solution and TBS for quenching endogenous peroxidases.

Reagent	Amount to add			Final concentration
	10ml	50ml	100ml	
30% Hydrogen Peroxide	0.1ml	0.5ml	1ml	0.3%
dH_2O / TBS	10ml	50ml	100ml	-

DAB Staining Solution

Note: Make up fresh just before use

Reagent	Amount to add			Final concentration
	5ml	10ml	50ml	
PBS / TBS	4.5ml	9ml	45ml	-
1% DAB	0.25ml	0.5ml	2.5ml	0.05%
0.3% Hydrogen Peroxide	0.25ml	0.5ml	2.5ml	0.015%

0.1% Sudan Black

Note: Store at room temperature

Reagent	Amount to add			Final concentration
	10ml	50ml	100ml	
Sudan Black	10mg	50mg	100mg	0.1%
Ethanol	7ml	35ml	70ml	70%
Distilled water	3ml	15ml	30ml	30%

DAPI Staining Solution (1µg/ml)

Note: Aliquot and store at -20°C. Protect from light.

Reagent	Amount to add			Final concentration
	5ml	10ml	50ml	
DAPI (1mg/ml) - HB8199	5µl	10µl	50µl	1µg/ml
TBS	5ml	10ml	50ml	-

7. Troubleshooting

Immunohistochemistry is a long multi-step process with many things that can go wrong and numerous factors that should be considered for a successful experiment. At some point it is inevitable that something will go wrong or not be optimal. Below are compiled some of the most common pitfalls that can cause sub-optimal immunohistochemistry results.

Problem	Potential cause	Suggested solutions
Weak or no staining	Too low antibody concentration	• Try increasing the concentration of primary antibody • Consider using an amplification system using biotinylated secondary antibodies
	Antibody incompatibility	• Ensure that the secondary antibody is compatible with the species and antibody subclass of the primary antibody
	High background is obscuring signal	• Try following suggestions detailed under the “High background or non-specific staining” section of this troubleshooting guide below.
	Antibody is not suitable for IHC-P	• Some antibodies are not suitable for the way the antigen is presented in IHC-P. Check the datasheet to see if it has been previously tested in IHC-P and if not consider using a different antibody. Many antibodies that work in standard IHC do not work in IHC-P. • Try using a different fixation method which will present the antigen in a different way therefore potentially allow the antibody to bind.
	Low abundance of target protein	• Try increasing the primary antibody concentration • Try using an amplification method such as biotin / streptavidin
	Fixation may be obscuring the target epitope	• Try a different fixative • Try reducing the fixation time
	Poor antibody penetration into tissue section	• Try incubating the antibody for longer or at higher concentration • Try using thinner tissue sections
	Incompatible secondary fluorophores used with detection system (Fluorescent IHC-P only)	• Double check that the microscope system being used has the correct excitation and emission filters for the fluorophores being used. • Consider using different fluorophore conjugated secondary antibodies. At a minimum nearly all fluorescent microscopes have filter sets enabling imaging of DAPI and FITC / Alexa Fluor 488 / Dylight 488.

Problem	Potential cause	Suggested solutions
Weak or no staining	Degradation of fluorophore following mounting (Fluorescent IHC-P only)	• Image sections within 2 days of mounting in immunofluorescent sections.
	Degraded buffers	• Check the pH of buffers before use • Be vigilant for signs of bacterial growth and throw out if observed. • Make fresh where necessary.
	Degraded primary antibody	• Ensure that the antibody is within the best before date supplied by the manufacturer. • Consider aliquoting antibodies, snap freezing then storing at -80°C in the future to avoid the risk of antibodies degrading.
	Damaged fluorophore conjugated secondaries due to light bleaching	• Ensure that all steps carried out when using fluorophore conjugated secondaries are carried out either in dim light or the dark • When imaging try to use the minimum illumination possible to capture the best image. This is particularly important on confocal microscopes where high laser power settings can bleach some fluorophores extremely quickly. • Consider using fluorophores that are more resilient to bleaching compared to older compounds such as FITC or TRITC.
	Incompatible buffers	• Some antibodies perform better in TBS based buffers while others prefer PBS. Trying changing buffer to see if this increases staining.
	Insufficient chromogen development (Colorimetric IHC-P only)	• Increase the incubation time with chromogen and allow the reaction to develop for longer before quenching
	Insufficient sensitivity of chromogen (Colorimetric IHC-P only)	• Try a different chromogen from the table with higher sensitivity (e.g. try using a Ni^{2+} enhancer with DAB staining).
	Over-fixation	• Try reducing the amount of time that the tissue is incubated with fixative. • Consider trying an alternative fixative solution

Problem	Potential cause	Suggested solutions
Bleed through between channels when multiplexing (Fluorescent IHC-P only)	Overlapping fluorophore excitation/emission spectra (Fluorescent IHC-P only)	• Try using well known pairs of fluorophores with limited overlap (e.g. DAPI, Alexa Fluor 488 and Alexa Fluor 594) • Use a spectral analysis tool (eg. FPbase Spectraviewer) to make sure there is limited overlap between fluorophore pairs. • Try single antibody controls to see if signal in one channel is real or just due to bleed through. • Some advanced image analysis software has functions that can correct (to a degree) for bleed through.
	Unsuitable excitation and emission filters on imaging system (Fluorescent IHC-P only)	• Check compatibility between fluorophores and filters to ensure that each filter only captures on fluorophore. • For confocal microscopes consider tightening the window of light wavelengths allowed into the detector and use sequencing so only one laser is active for each fluorophore.
Altered tissue morphology	Incomplete fixation	• Incomplete fixation can cause tissue to degrade rapidly. Try increasing the amount of time tissue is incubated with fixative or consider increasing the concentration of fixative.
High background or non-specific staining	Auto fluorescent molecules in tissue section (Fluorescent IHC-P only)	• If tissue hasn't been perfused, consider this for the next experiment due to the strong autofluorescence caused by remaining red blood cells. • Autofluorescence can be caused by the fixative. Trying using a different fixative. • Ensure that the Sudan Black incubation step has not been skipped • Try using fluorophores that are in a different wavelength from the autofluorescence (e.g. red shifted dyes such as Alexa Fluor 680)
	Non-specific secondary binding	• Run a no primary control to assess if the secondary is binding to the tissue section. • If the primary antibody is from the same species as the tissue this can cause major problems. Consider using a different species for the primary antibody.

Problem	Potential cause	Suggested solutions
High background or non-specific staining	Too high primary antibody concentration	• Try reducing the concentration of primary antibody
	Too high secondary antibody concentration	• Try reducing the concentration of secondary antibody. We find a 1:300 to 1:500 dilution a good place to start.
	Too high streptavidin HRP concentration (Colorimetric IHC-P only)	• Try reducing the concentration of streptavidin HRP as this will reduce the level of background staining
	Too long chromogen development (Colorimetric IHC-P only)	• Try reducing the chromogen development time. The reaction can be monitored by eye and stopped when a suitable signal has been developed.
	Insufficient purification of antibodies	• Un-purified polyclonal antibodies can contain antibodies that are cross-reactive with a range of non-target proteins. Check the datasheet; polyclonal antibodies should ideally be purified by affinity chromatography using the immunogen as bait. • While issues with monoclonal antibodies are rarer they should still be purified at least using Protein A or G affinity chromatography. • Where other alternatives are available consider switching to a better purified antibody or where alternatives are not possible consider purifying the antibody in-house.
	Insufficient blocking	• Ensure that the blocking serum is from the same species as the secondaries were raised in. • Try using a different blocking solution. • Try increasing the length of the blocking step or increasing the concentration of serum
	Insufficient washing	• Try increasing the length and/or number of washes. • Ensure that there is sufficient agitation during washes to aide unbound antibody diffusion out of tissue
	Primary antibody is from the same species as the tissue sections.	• Run a no primary control to assess if the secondary is binding to the tissue section. • If the primary antibody is from the same species as the tissue this can cause major problems. Consider using a different species for the primary antibody.

Problem	Potential cause	Suggested solutions
High background or non-specific staining	Endogenous enzyme activity (Colorimetric IHC-P only)	• Attempt to block endogenous enzyme activity with specific inhibitors. • For HRP conjugated secondaries use 0.3% H_2O_2 for 10-15 minutes. If blocking is not successful at this concentration consider increasing to 3%. • For AP conjugated secondaries use 2mM levamisole.
	Sections have dried out	• Ensure that sections always are kept moist and in a humidifying chamber for long incubations.
	Thickness of tissue section	• The thicker the tissue section the more scattered light in a widefield microscope. Try either using thinner sections of switch to a confocal microscope.

8. Further Reading

- Muniz Partida C, Walters E. A novel immunohistochemical protocol for paraffin embedded tissue sections using free-floating techniques. Front Neuroanat. 2023
- Zaqout S, Becker LL, Kaindl AM. Immunofluorescence Staining of Paraffin Sections Step by Step. Front Neuroanat. 2020
- Shi SR, Liu C, Taylor CR. Standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections based on the antigen-retrieval technique: from experiments to hypothesis. J Histochem Cytochem. 2007
- Syrbu SI, Cohen MB. An enhanced antigen-retrieval protocol for immunohistochemical staining of formalin-fixed, paraffin-embedded tissues. Methods Mol Biol. 2011
- Grillo F, Bruzzone M, Pigozzi S, Prosapio S, Migliora P, Fiocca R, Mastracci L. Immunohistochemistry on old archival paraffin blocks: is there an expiry date? J Clin Pathol. 2017
- Kim SW, Roh J, Park CS. Immunohistochemistry for Pathologists: Protocols, Pitfalls, and Tips. J Pathol Transl Med. 2016
- Magaki S, Hojat SA, Wei B, So A, Yong WH. An Introduction to the Performance of Immunohistochemistry. Methods Mol Biol. 2019