IHC Protocol - Frozen Tissue

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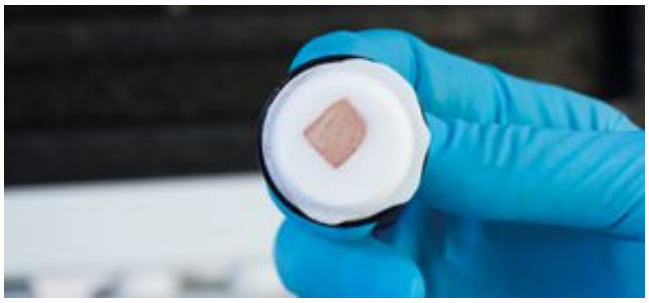
Immunohistochemistry on Frozen tissues

IHC Protocol - Frozen Tissue: An introduction

This is the second post in a series on immunohistochemistry (IHC). The first post looked at one of the two main ways of carrying out IHC – paraffin embedded sections – and in this post we'll take a look at preparing frozen sections.

Which do you choose, frozen or paraffin?



Frozen tissue section

Depending on what you want to achieve, and what's available in your lab will affect whether you choose paraffin embedded or frozen tissue sections. For example, paraffin embedded sections are typically better at preserving tissue morphology and allow larger pieces of tissue to be used. However, the use of fixatives like paraformaldehyde can cross link antigens reducing their immune reactivity. Getting them back requires antigen retrieval procedures to 'unmask' them, and some antigens can even be irrecoverably destroyed by the preparation of paraffin sections.

The alternative is using frozen sections. Generally, the snap freezing process behind preparing frozen sections is better at preserving antigens, but this may come at the cost of a loss of tissue morphology.

Preparing frozen sections is probably the easier of the two methods.

There are entire textbooks written about IHC, so what follows below is only a guide to help get you started. Fortunately, nearly all antibody manufacturers who have validated their antibodies for use in IHC will have a recommended protocol for you to follow, so whether you are using one from American Research Products or another, please always check the data sheet.

So, without further preamble, let's get started.

Reagents for IHC-Fr:

- PBS (Ice cold)
- Isopentane (cooled in dry ice)
- Optimal Cutting Temperature Medium (OCT) – available from many manufacturers
- Fixation medium (e.g. acetone at -20°C)
- Blocking buffer (5% BSA in PBS)

The basic steps of the IHC-Fr protocol

Step one: Preparing the tissue

1. **Wash you tissue using ice cold PBS**

Make sure you tissue is clean. You'll probably need to change the buffer a couple of times.

2. **Cut the tissue into slices of about 3mm**
3. **Snap freeze the tissue in the dry ice cooled isopentane and keep at -70°C until ready**

Freezing very quickly, rather than slowly letting the sample cool, means only small ice crystals are formed in the sample, as opposed to slowly growing large ice crystals, helping to preserve the tissue and cell morphology.

Step two: preparing for cryostat sectioning

1. **Prepare a mold**

This will make it easier to handle your tissue when sectioning. You can make a mold out of tinfoil if you don't have anything specifically designed for it.

2. **Place your tissue in the mold and cover with Optimal Cutting Temperature (OCT) medium**

Tip: Add a little OCT to your mold first – a few mm worth – then carefully place your tissue section on top, then cover with OCT, making sure the tissue is reasonably central to it.

3. **Carefully, using forceps around the bottom of the mold, place in liquid nitrogen for one to two minutes**

The OCT should turn white

Step three: Cryostat sectioning

Again, this step may be slightly different depending on what equipment you have, so not all of this may apply to you – always follow the manufacturer's instructions where possible.

1. **Pre-cool the cryostat according to the manufacturer's instructions**
2. **Place the mold in the slicer**

Tip: Allow the tissue section and mold to equilibrate to -20°C for about 15 minutes before use. This can help prevent the section from fracturing.

3. **Use the cryostat to make slices of about 5-10 µm**

Collect the slices on a cover slip

4. **Mount slices on a cover slip (e.g. gelatin-coated histology slides)**

Note: if the specimen is too cool, sections may curl. If too warm, they may stick to the knife.

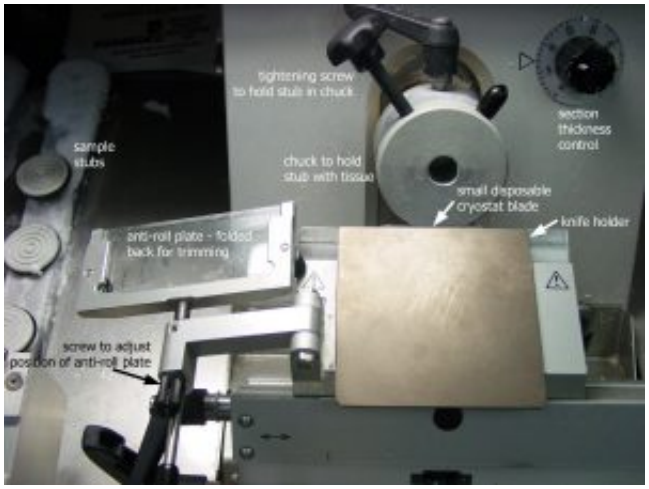
5. **Air dry at room temp for ~30 min**

This helps to fix them to the slides, preventing them falling off during antibody incubations.

6. **Store slides at -70°C**

You can store slides like this, un-fixed, in a slide box for several months, but if analysis is to be

much later, it’s better to store the whole un-sectioned sample until ready.



Cryostat Sectioning

Step four: Fixing

There are many possible fixing methods, below is an example

1. **Immerse slide in pre-cooled acetone (-20°C) for 10 minutes**
2. **Pour off fixative and allow to evaporate for ~20 minutes at room temp**
3. **Immerse slide in PBS (pH 7) to wash for about 5 minutes. Repeat twice.**
4. **Incubate slides in 0.3% H2O2 in PBS at room temp for 10 minutes**

This helps block endogenous peroxidase activity

5. **Immerse slide in PBS (pH 7) to wash for about 5 minutes. Repeat twice.**

NOTE: it may be necessary to carry out an antigen retrieval step at this point depending on how you fixed your sections.

Step five: Staining

As always, if your antibody has an included protocol for IHC staining, follow that first. If not, the below is a guide to get you started, but you will likely have to optimise it for your antigen, species or tissue type, etc.

1. **Blocking**
    - Add 5% BSA in PBS to your antigen retrieved samples, ensuring you add enough to

cover them
    - Incubate for 30 min to 2 hours at +37°C, with gentle agitation
    - Discard the excess – no need to wash
2. **Primary antibody incubation**
    - Dilute primary antibody to manufacturer's instructions to suggested concentration
    - Add to sample and incubate overnight at +4°C
    - Rinse twice with PBS, 20 min each time with gentle agitation
3. **Secondary antibody incubation (biotinylated)**
    - Dilute secondary antibody to manufacturer's instructions to suggested concentration
    - Incubate for 1 hour at room temperature
    - Rinse twice with PBS, 20 min each time with gentle agitation
4. **Staining** – this will depend on your chosen detection method, we're assuming strep-HRP. Again, follow manufacturer's instructions if given
    - Add the strep-HRP and incubate for 30 min at room temp
    - Rinse twice with PBS, 20 min each time with gentle agitation
    - Add detection reagent (e.g. DAM, TMB) incubate in the dark at room temp for 10 to 30 minutes.
    - Rinse in running tap water for 5 minutes
    - Counter stain (e.g. with haematoxylin) if needed

We hope you'll have found this protocol a useful start to preparing your frozen IHC sections. As we noted in our previous post on IHC, with so many steps and possible variations in reagents, tissues and antigens of interest, IHC can be as much art as science.

In the future we'll be offering up more posts on trouble shooting IHC, to help you get the best result possible.