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IHC staining for cryopreserved samples, Instructions:

Before you stain for the first time:

- Plan for training with Shreyas prior to using the IHC workstation for the first time
- **Frozen slides need to be thawed at room temperature overnight or at least for 1 H prior to staining**
- **We do not provide readymade 4% paraformaldehyde (PFA) for tissue fixation. If you need to fix your cryo-sections before staining, talk to Shreyas and discuss the process.**
- **We do not provide readymade phosphate buffered saline (PBS). You can procure 1X PBS from the health sciences supply centre (HSSC) prior to initiating staining. Talk to Shreyas if you have questions**
- **You must book a slot for IHC using the booking calendar at least 24 H in advance**
- If back up/replacement of solution/s is needed, talk **to Shreyas about this**
- You can stain up to 24 slides at a time using the slide holder. However, one hydration chamber can house up to 10 slides only. Please consider the logistics of incubation steps and reagent exposure time when planning number of slides and hydration chambers that need to be used for IHC. **We have limited hydration chambers.**
- Please record the number of slides and racks of slides (1-24 slides in 1 rack) that have been stained in the IHC logbook above the weighing scales. This information is needed to invoice and check status of reagents.
- To prevent excessive carryover of reagent from one staining jar to the next, please drain back as much reagent as possible
- **Please leave this staining station in the condition that you'd like others to leave it for you!**
- For IHC staining fees per slide, refer to cost recovery pricelist on the histology core facility website
- **The fume adsorber for the coverslipping station must be switched on before use and left turned on for several hours after. If working after hours or on weekends, leave the fume adsorber turned on overnight or over the weekend.**

Coverslipping Station Instructions:

- First time users must seek training for coverslipping from Shreyas
- Make sure that the fume adsorber is turned on before you start the process of coverslipping
- If you are running low on mounting media, ask Shreyas or alternatively transfer mounting media (Leica Inc.). This can be found in the cabinet above the coverslipping station with a label on the door indicating mounting media
- We provide a variety of sizes of coverslips. These coverslips are placed usually on the fume adsorber for the coverslipping station. **Please use the long coverslips (e.g., 24x50 mm or 24 x 60 mm) only if you have those many sections on a slide. Alternatively use smaller size coverslips**
- **AFTER COVERSLIPPING, SLIDES MUST BE PLACED IN THE FUMEHOOD OVERNIGHT FOR DRYING OUT! THE MOUNTING MEDIA CONTAINS XYLENE AND CAN RELEASE NOXIOUS FUMES - NO EXCEPTIONS!!!!**
- If you run out of coverslips, you can grab a box of coverslips from the drawer labelled coverslip (in the IHC-1 work area, opposite the microwave)

IHC Staining Protocol for Cryo sections:

PLEASE READ THIS SOP THOROUGHLY!!

Tissue sectioning
Frozen tissue sections embedded in OCT can be sectioned at a thickness of 5–20-microns (thickness can be varied depending on the type of tissue and desired result) and placed on positively charged frosted slides by placing the cold sections onto warm slides.

Slide storage
Slides prepared for this method can be stored at -80° C until ready for staining

A. Slide preparation
Always handle the slides with gloves. Hand lotion and other oils on the skin can cause the tissue to come off the slides during processing. Warm slides to room temperature.

If tissue is not previously fixed, you need fixation step first. If already fixed, ignore the 4%PFA Step. Some protocols also use acetone or methanol for tissue fixation, talk to Shreyas if you have queries

1. 1H to overnight- thaw frozen slides at room temperature
2. 5 minutes: 4% PFA 10 minutes: ethanol or methanol (Fixing of sections needed only if tissue is not previously fixed). Using alcohol fixative avoids the subsequent antigen retrieval step as alcohol fixation does not create crosslinks.
3. Rinse in 1X PBS, 2 times, 5 minutes each rinse

Step 1: Blocking of endogenous peroxidase

Many human and animal tissues contain endogenous peroxidase which will cause false positives in the staining methods. The following step blocks endogenous peroxidase in the tissue slides: Mix 20 mL 30% hydrogen peroxide (in the lab fridge) and 180 mL methanol (under the fumehood). Incubate the slides in 3% hydrogen peroxide in methanol for 10 minutes.

Following this, rinse slides with 3 x 5 minutes in dd water to completely hydrate and store in fresh dd water until the next step.

Hydrogen peroxide mixed with methanol cannot be dumped in the sink!!! A waste bottle for 3% hydrogen peroxide + methanol can be found under sink # 3 (near the cryostat). Ask Shreyas if you have doubts.

Step 2: Heat Induced Epitope Retrieval (HIER) (Not needed if alcohol fixed)

Antigen retrieval methods can be too harsh on cryostat sections, use caution and decide if you want to perform this step or use alternative fixatives.

For most HIER we use 1mM EDTA, pH 9.0.
(Note: for some tests/antibodies, other buffers may be better, such as Citrate buffer pH6.0, PBS/EDTA pH 10).
Many antigen retrieval methods can be too harsh on cryostat cut tissue sections, choose this option wisely.

Antigen Retrieval
Prepare 1mM EDTA buffer pH 9.0 - dilute 10 mM stock EDTA 1/10 and pH to 9. Make fresh weekly to ensure proper pH. For consistency, always microwave 2 FULL slide containers (insert blank slides if necessary to ensure the consistency of the same number of slides per tray every time). You can use distilled water to fill up the jar housing ONLY blank slides. Cover slides completely in 1mM EDTA buffer and place the lids tightly on

the jars. Microwave for 13 minutes at power level 6 as follows: Microwave for 10 minutes and top up the volume of EDTA buffer before microwaving for remaining 3 minutes. It is critical that there is enough buffer in the jar submerging all sections on the slides for the entire duration. After the 13-minute microwave exposure, slides MUST sit in the hot buffer for 20 minutes either in microwave or on lab bench before proceeding. (Microwave settings and duration of exposure are optimized for the current microwave in the histology core facility and may need to be adjusted for up to 20 minutes boiling time for less powerful/new microwaves). After the 20-minute incubation, place slides under slowly running distilled water in sink #2 or 3 only to rinse off EDTA and cool off the slides. Once rinsed and cooled, let the slides sit for a minimum of 5 minutes in distilled water. Wash the slides with PBS/tween buffer - 2 x 5 minutes and store in buffer until the next step.

The jars used for HIER must not be mixed with other jars in the fumehood or in the IHC jar drawer (under the embedding station). After use, wash and leave near the microwave only.

Step 3: Blocking non-specific binding:

Block non-specific binding by incubating the tissue sections with 5% serum (NGS, BSA) in 0.4% Triton X-100 in PBS (PBS-T) for 30 minutes at room temperature.

Note: The species of the animal serum used in permeabilization and blocking buffers is dependent on the host of your secondary antibody. (e.g., when using a goat anti-mouse secondary, use goat serum).

B. Primary antibody Incubation

Antibody preparation: Prepare the primary antibody dilutions for the day immediately prior to use in room temperature antibody diluent with background reducing reagents (Dako Cat# S3022). You require ~100-200 µL diluted antibody solution per slide for manual staining, depending on the size of tissue sections. Fill the hydration chamber wells with distilled water prior to use to allow the chamber to acclimatize to whatever temperature it is used in.

Primary Antibody Incubation:
To prepare the slides, you can label and ring slides with a hydrophobic marker (as shown below) to keep the antibody solution on the tissue section and prevent it from running off the slide and the tissue drying out. The hydrophobic marker MUST be stored in the fridge after use with the cap facing down. If no marker is available, you can wipe around the tissue on the slides with a kimwipe to create a “well” for the solutions you are adding to the slide instead.

Doing one slide at a time: Either wipe the slide (back and edges) with a kimwipe (better as it is faster, giving the slide less chance to dry out) or shake the buffer off the slide and ring it with the hydrophobic marker. The kimwipe method is preferred unless the sections are unreasonably large.

Place the slide perfectly flat into the filled hydration chamber and add primary antibody solution to the slide IMMEDIATELY. Ensure that all tissue sections on the slide are covered. **Do NOT** move the chamber once the slides are loaded with primary buffer or the movement can spill water onto the slides from the chamber wells.

The primary antibody concentration will need to be optimized for each tissue and antibody. Incubation times are usually 1-2 hours at room temperature but may require overnight incubation at 4°C in precooled hydration chambers. Plan accordingly.

C. Secondary Antibody and Chromogen Incubation

NOTE: this method is specific for the Envision Dako Kit. Other HRP labelled secondary antibodies can be used, but their dilution and incubation time should be adjusted accordingly.

Remove slides from the humidity chamber one at a time, gently rinsing slides with PBS (not directly on tissue). Place the slides in a PBS rinse for 3 x 5 minutes –for specificity a separate jar is preferred for each primary antibody rinse treatment.

Peroxidase Labeled Secondary Antibody Incubation: Remove excess buffer and place slides in a moistened humidity chamber one at a time, ringed/dried as before, with 3-4 drops of the Peroxidase labeled secondary antibody solution. For the Dako Envision kit incubate at room temperature, in the hydration chamber, for 30 minutes. Incubation for other antibodies will vary with time and dilution.

Following this, rinse slides with PBS and rinse in 3 x 5-minute PBS buffer washes.

Substrate Labeled Chromogen Incubation: (*Remember: handle DAB with care!****)***

Mix 1 drop of DAB chromogen with 1 mL Chromogen buffer immediately prior to use and mix well. As before, place the slides in the hydration chamber, ringed/dried, and incubate the tissue sections with ~200 µL of Chromogen substrate for 10 minutes. Rinse slides with PBS into a special waste bottle (found under sink#3) and then incubate slides 3 x 5 minutes in PBS**. DAB is a carcinogenic environmental toxicant** – collect all DAB rinse into the DAB waste bottle found under sink#3 appropriately. Place slides in distilled water and rinse well before continuing as soon as possible to the next step.

Note: If there are many slides it may be helpful to stagger the exposure duration so that there is consistency in incubation times for all slides for this step.

D. Toning slides, counterstaining and cover slipping:

A counterstaining station is set up in the fume hood only for IHC samples. Use this station for toning, counterstaining, and hydrating your slides

Tone slides by incubating in 2% copper sulphate solution for 5 minutes (make fresh weekly-monthly depending on use, if unsure check with Shreyas). Rinse well with distilled water (quick rinse and then wash with running tap water for 4-5 minutes). Copper sulphate is also an environmental toxicant at very low concentrations. **The copper sulphate waste needs to be disposed of in a waste bottle found under sink#3 appropriately.**

Counter stain slides with Harris Hematoxylin for 5 dips (1dip=1 second). Rinse immediately and thoroughly with distilled water until slides are completely clear. “Blue” the slides in tap water (alkaline pH) for 30 seconds. Blueing water can also be made or purchased if needed. Then rinse slides well with distilled water and proceed immediately to the next step.

Dehydrate slides for cover slipping with the following rinses (~10 seconds per rinse, 10 times rinsing up/down – quick rinses):

- 3 x 95% ethanol
- 3 x 100% ethanol
- 4 x xylene

Store slides in xylene-4 jar. The xylene jar-4 housing these slides must be carefully transferred to the coverslipping station. The fume adsorber of the coverslipping station MUST BE TURNED ON prior to use. A variety of sizes for coverslips are available in the histology core facility. Check with Shreyas if unsure or requiring specific types. After selecting the appropriate size of coverslip (found in a drawer labelled coverslips along the IHC working lab bench), add 2-3 drops of Surgipath MM24 (mounting medium) on coverslip and

slide. Gently transfer slide on the coverslip ensuring that the entire tissue is covered. Once coverslipping is done and you have got rid of air bubbles, wipe off excess mounting media from beneath the slide. Once you have finished coverslipping all slides, transfer them on a metal plate and let them dry in the fumehood for at least 24 hours in the fumehood. Once completely dried, the slides must be transferred to a slide box for long term storage. **DO NOT transfer a slide which has not dried out completely on to the stage of a microscope since the mounting media can corrode the stage and cause the slide to stick to the stage of the microscope.**

The coverslipping station fume adsorber must be left on for several hours after use. If working in the evenings, you can leave it on overnight or throughout the weekend if working on a Friday evening. You must replace paper towels exposed to xylene or mounting media in the coverslipping station with new ones and leave the exposed ones in the fumehood to dry. Shreyas will deal with them.

E. REAGENTS:

Most reagents needed for IHC are available in the Histology Core Facility. Always check with Shreyas/Adi prior to planning an IHC manual run in the lab. We will set up deparaffinization and staining stations for you on the day of IHC in the fume hood. In addition, the coverslipping station will be set up as well. The following reagents will be available to use:

- Xylene
- absolute alcohol
- 95% alcohol
- 70% alcohol
- Hydrogen Peroxide
- Methanol
- Copper sulphate
- Hematoxylin
- Dako envision kit
- DAB chromogen kit
- DAPI mounting media

However, you are responsible to prepare all other reagents needed for running manual IHC or alternatively check with Shreyas if they have been recently made (within a week) or readily available in the histology core facility.

After you are done conducting IHC, it is your responsibility to clean up and wash all the glassware, hydration chamber/s, you may have used. Please include clean up time in your plan for the day when you run IHC in the Histology Core Facility. You can leave the deparaffinization and staining stations as it is. Also, you must log your IHC run in a sheet that is kept above the weighing machine. The following chemicals must be disposed as indicated in this SOP

DAB chromogen

Copper sulphate

3% hydrogen peroxide mixed with methanol

If unsure about anything, please contact Shreyas.

Dako Envision Kit (peroxidase block, labeled antibody and chromagen):
Bring to room temperature before use. Dilute DAB + as instructed below. Kits are VERY expensive so be as frugal as possible.

Harris Hematoxylin: In the drawer labelled IHC reagents in front of the fumehood

70%, 95% and 100% ethanol: In the IHC staining/deparrafinizing chemicals cabinet above microtome#4 (near emergency shower)

Xylene: In the IHC staining/deparrafinizing chemicals cabinet above microtome#4 (near emergency shower)

Mounting medium: In an amber bottle within the coverslipping station

Blocking Reagent for endogenous Peroxidase: 3% hydrogen peroxide in absolute methanol (20 ml 30% hydrogen peroxide + 180 ml methanol)

30% hydrogen peroxide: In the fridge

Methanol: In cabinet below the fumehood

Secondary Antibody and Detection

Use appropriately prepared kit of choice. Envision works best on the Dako machine for most staining. Other antibodies are not prediluted and they will need to be optimized for time and dilution.

2% Copper Sulphate: prepared solution is stored in the IHC chemicals drawer under the embedding station.

2.5 g copper sulphate
250 mL distilled water

Make fresh every week. Caution – POISON!

Phosphate Buffered Saline (PBS) (if not using Dako buffer solution)

10X Stock solution:

75.2 g K2HPO4 (MW=174.18)
13.2 g NaH2PO4·H20 (MW=137.99)
72 g NaCl
800 mL d H2O
Dissolve and bring to 1 L.
Autoclave if not using immediately.

1 X Working PBS Solution:
200 mL 10X stock solution
Bring to 2L in large bottle with dH2O. Add 1mL Tween if desired to improve rinsing.

PBS-T- 0.4% Triton X-100 in PBS

5% serum- Normal Goat Serum or Bovine Serum Albumin

TRIS

Stock 0.5M Tris:

Dissolve 30.3 g TRIS (THAM) in 250 mL dH2O
Adjust pH to 7.6 with HCl
Bring to 500 mL with dH2O.
Store in fridge.

Working TRIS:
100 mL stock 0.5M TRIS
Bring to 1L with dH2O
Store in fridge.

DAB

Dilute 1 drop in 1mL as needed.
Reagent must be mixed fresh prior to use. Remember it is light sensitive.

DAPI antifade mounting medium

Take the volume needed in an Eppendorf tube and cover it with aluminum foil to prevent exposure to light. Remember it is light sensitive. It is also very expensive, please take only as much needed (usually 50-100 µL/sample) and enter in the chemical purchase folder.

10 mM EDTA Stock Solution:

3.7224g disodium EDTA diluted to 1 L

1mM EDTA HIER working solution:
Dilute stock solution 1/10 and pH to 9.0
Make fresh every run (500mL/run) if not using daily to ensure proper pH.

For the Dako Autostainer, the 10X Dako Wash buffer solution is Cat#: S3006 – at 1X dilution it is 0.05 mol/L TBST (Tris Buffered Saline with Tween20)

For manual staining the kit suggests either 0.05 mol/L TBS or 0.02mol/L PBS, with the option of adding a bit of Tween20 (0.5-1mL of Tween 20 to 2L) but this is not strictly necessary unless background issues are occurring. Manual washing is more thorough than on the instrument, so I have done it both with and without and had good results.

Revisions:

1. Based on IHC staining method: Manual and H&E STAINING PROTOCOL-FROZEN SECTIONS Version 1.0 September 2021 by Adi Manek and H Neufeld
2. June 2022- Updated for facility by Shreyas Jois and Adi Manek with facility specifics

Other references:

3. https://www.novusbio.com/sample-preparation-for-ihc-experiments
4. https://www.novusbio.com/support/support-by-application/immunohistochemistry-frozen/protocol.html
5. Sample preparation for cryosection- https://www.youtube.com/watch?v=MYlSj-gCx6g