IHC Staining Method: Manual

IMPORTANT INSTRUCTIONS PRIOR TO INITIAITNG ANY IHC WORK IN THE HISTOLOGY CORE FACILITY

- **Gloves must be worn at all times when working with tissues, slides or reagents used for IHC**
- **A Lab coat must be worn at all times (no exceptions)**
- **IHC can be conducted on the lab bench housing the knife sharpener (opposite the microscopes)**
- **All IHC supplies, reagents can be found in drawers below the embedding station or in the cabinet above microtome#4 (near the eye wash station).**
- **To make specific IHC reagents, refer to section F for recipe**
- **The following chemicals must be disposed as clearly indicated in this SOP:**
  DAB chromogen
  Copper sulphate
  3% hydrogen peroxide mixed with methanol

PLEASE READ THIS SOP THOROUGHLY!!

Tissue sectioning after processing – Tissues embedding in paraffin wax can be sectioned at a thickness of 4-7 micron (thickness can vary depending on tissue) and placed on positively charged frosted slides.

Slide storage

Slides prepared for this method can be stored at 4 °C for up to a week or -20 °C for up to a month and -80 °C for longer term storage.

A. Slide preparation

Always handle the slides with gloves. *Hand lotion and other oils on the skin can cause the tissue to come off of the slides during processing.*

Step 1: Baking the slide

Slides are warmed to room temperature for at least 1 hour to get them to room temperature. After slides are thawed at room temperature, they are baked at 60 °C for at least one hour or overnight prior to the next step i.e. deparaffinization. The slide baking can be conducted in Oven#1 (close to the main lab entrance).

Step 2: Deparaffinization

Epitope retrieval removes the crossbridges formed in formalin fixation so that the antibodies used can bind to the enzymes of interest for staining of slides. Heat induced

epitope retrieval (HIER) is just one method of epitope retrieval. The most commonly used method is the microwave method.
Before HIER the slides need to be hydrated to remove the paraffin.

To deparaffinize slides: Mount slides into holder tray and rinse them through the following washes in the fumehood using the station dedicated only for IHC:

-4 x Xylene (5 minutes per wash)
-3 x 100% ethanol (quick wash)
-2 x 95% ethanol (quick wash)
-1 x 70% ethanol (5 minutes)

It is critical that slides do not dry out at any stage of this process or staining will result in high background or failure.

Step 3: 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 Adi if you have doubts.

B. Heat Induced Epitope Retrieval (HIER)

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).

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). 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.

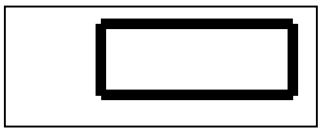
C. Antibody Incubation/Staining

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 the Dako 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.

Ringing with pen 

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 Dako pen. 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.

D. Secondary Antibody Reaction and Chromagen 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.

E. Toning slides, counterstaining and cover slipping:

A counterstaining station is set up in the fumehood 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 Adi). 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 light Hematoxylin 1 for 15 dips (10 dips regular strength) or 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 Adi 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 Entellan (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 of 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 also 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 have to 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. Adi will deal with them.

F. REAGENTS:

Most reagents needed for IHC are available in the Histology Core Facility. Always check with Heather/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 provided at all times:

- Xylene
- absolute alcohol
- 95% alcohol
- Hematoxylin
- Dako envision kit

However, you are responsible to prepare all other reagents needed for running manual IHC or alternatively check with Adi 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 have to log your IHC run in a sheet that is kept on the autostainer. 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 Adi.

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.

Light Hematoxylin 1: In the IHC chemical drawer under the embedding station

Harris Hematoxylin: In the staining chemicals cabinet above the coverslipping station

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 K_2HPO_4 (MW=174.18)
13.2 g $NaH_2PO_4 \cdot H_20$ (MW=137.99)
72 g NaCl
800 mL d H_2O
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 dH_2O. Add 1mL Tween if desired to improve rinsing.

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 dH_2O.
Store in fridge.

Working TRIS:
100 mL stock 0.5M TRIS
Bring to 1L with dH_2O
Store in fridge.

DAB

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

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 SHA IHC protocol previously optimized by HNeufeld and the cIQc for manual IHC work
2. Jan 2019 - Updated for facility by Adi Manek with facility specifics