Document Number: **SASoM/METHOD/025.v7**

Title: **Immunohistochemistry**

Version: **v7**

Author: **In Hwa Um**

Effective from:	15/02/2021
Valid to:	14/02/2023

SOP History		
Number	Date	Reason for Change
V1	01/02/2013	Original
V2	26/09/2013	Addition detail to Section 4
V3	01/12/2013	Amend and add information in Section 4
V4	15/02/2015	Additional information re retrieval buffers
V5	15/02/2017	Additional info re antigen retrieval method, antibody incubation time & reformatting procedure
V6	15/02/19	Update
V7	15/02/2021	Update

1.0 Purpose –

This SOP describes the current procedure for staining paraffin embedded sections with antibodies use in Laboratory 248 at the St Andrews School of Medicine (SASoM).

2.0 Scope –

This SOP applies to the staff in the SASoM involved with Immunohistochemistry (IHC)

3.0 Responsibilities –

All staff involved in IHC are responsible for ensuring that the methods are followed in accordance with this SOP. All staff must have read and signed the relevant risk assessment documents before performing this procedure.

4.0 Procedure –
Put slide(s) in non-metallic rack

De-waxing & Re-hydrating
Always allow any excess fluid to drain from the slide rack before proceeding to the next solution.

1. Dewax-Xylene 1 — 5 minutes
2. Dewax-Xylene 2 — 5 minutes
3. Dewax-Xylene 3 — 5 minutes

During the 3rd xylene dewax stage prepare the antigen retrieval solution - either (1) EDTA-Tris or (2) Citrate based.

1) *EDTA-Tris 1 L solution:*
10mM Tris Base = 1.21g
1mM EDTA = 0.37g
Tween = 0.5ml
dH20 = 1L
Add the EDTA and Tris Base to a 1L flask and add the dH20 and mix on magnetic stirrer. Ensure that the pH is 9. Add theTween.

2) *Sodium Citrate/Citric Acid solution for Antigen Retrieval by adding 18mls 0.1M Citric Acid to 82mls 0.1M Sodium Citrate (100mls). Make up to 1 litre with Elga Water (pH6).*

Pour the antigen retrieval solution into either the microwave pressure cooker. Screw on the lid and put into the microwave on high power to heat up for 10mins.

*Alternatively pour the antigen retrieval solution into Instant pot electric pressure cooker and set up 1min Manual pressure cooking (**Ref. SASoM-EQUIP/100.v1**)*

4. Rehydration-100% Alcohol — 2 minutes
5. Rehydration-100% Alcohol — 2 minutes
6. Rehydration-80% Alcohol — 2 minutes
7. Rehydration-50% Alcohol — 2 minutes
8. Wash in running water — 2 minutes

Antigen Retrieval using microwave pressure cooker

1. Perform antigen retrieval on slides by putting drained slide holder into pre-heated buffer in pressure cooker. Screw on the lid and place the red weight over the vent. Put back into the microwave and heat on high for 8 mins – there should be an audible hissing sound towards the end of the procedure..
2. Using heat resistant gloves and eye protection carefully lift the pressure cooker from within the microwave and place in sink. Place eye protection on. With the heat resistant gloves release the pressure by removing the weight from the top of the cooker. Stand well back immediately once the weight is removed. Do not stand over the pressure cooker during this step.

3. Remove the lid from the pressure cooker using the heat resistant gloves and ¾ fill with cold water. Allow the slides to cool down for 20mins.

Antigen Retrieval using Instant pot electric pressure cooker –Please read and sign *SASoM-EQUIP/100.v1*

1. Make sure the pressure release handle and float valve are unobstructed and clean, and that the sealing ring is properly inserted.
2. Fill the inner pot with 1 litre of antigen retrieval solution (either Sodium Citrate buffer or Tris EDTA buffer).
3. Close the lid. Make sure that the pressure release handle is pointing to the "Sealing" mark on the lid (Figure 1).

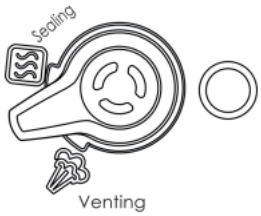

Figure 1 Pressure release handle

4. Press "Manual" button, and press the "+" or "-" to change the time to 1 min. In 10 seconds, the Instant pot will go into preheating cycle (display showing "On")(Figure 2).

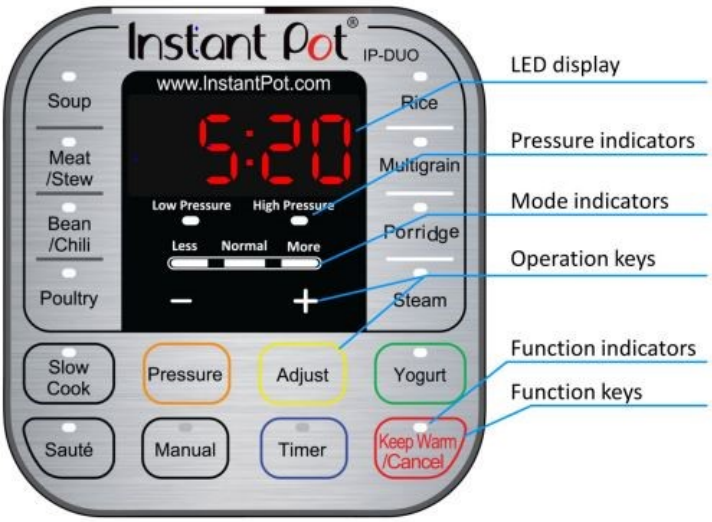

Figure 2 Control panel

5. Once working pressure is reached, which make take a few minutes or up to 10-13mins, the countdown timer will begin. When the countdown is finished, the Instant pot will beep and automatically switch into the "Keep Warm" mode.
6. Turn the pressure release handle to the "Venting" position to let out steam until the float valve drops down (Figure 1). Open the lid with care. **When releasing steam, always wear a pair of thermal gloves and a safety goggles.**
7. Add the rehydrated slides into the inner pot and close the lid. Make sure that the pressure release handle is pointing to the "Sealing" mark on the lid (Figure 3).

8. Press “Manual” button, and press the “+” or “-“ to change the time to 5 min. In 10 seconds, the Instant pot will go into preheating cycle (display showing “On”).
9. Once working pressure is reached, which make take a few minutes or up to 10-13mins, the countdown timer will begin. When the countdown is finished, the Instant pot will beep and automatically switch into the “Keep Warm” mode.
10. Press “Cancel” and Turn the pressure release handle to the “Venting” position to let out steam until the float valve drops down (Figure 1). Open the lid with care.
11. Take the whole inner pot out and add the running tap water and cool the slides down.

Blocking and primary antibody incubation

1. Transfer slide(s) into coplin jar and then wash in PBS/Tween 20 (500mls PBS +500µl Tween 20) for 5 minutes.
2. Treat sections in 3% Hydrogen peroxide (10ml of 30% H_2O_2 stock solution plus 90mL Elga H_2O) for 5 minutes. Store hydrogen peroxide in the fridge.
3. Wash slides in PBS/T for 5 minutes. Blot off excess solution from edge of slides onto tissue. Draw round individual sections on slide with hydrophobic Immedge pen.
4. Block with Dako Total Protein blocking solution (taken straight from fridge or keep on ice) for 10 minutes by dropping solution onto individual sections (as indicated on solution data sheet).
5. Blot off Blocking solution on tissue.
6. Incubate section in primary antibody diluted with Dako antibody diluent as required and place slide in black slide box (humidity chamber) with moist tissue and incubate either 30min at room temperature or at 4^{o}C overnight. Only a very small volume of primary antibody is required - use a Gilson tip to carefully place required antibody onto section. A Negative control section can be included using Antibody Diluent solution alone.

Secondary antibody incubation and primary antibody visualition using DAB

1. Wash with PBS/T for 2 x 5mins
2. Incubate section with appropriate Dako Envision labelled polymer for 30 minutes (use Rabbit Envision if incubating with rabbit primary antibodies; use Mouse Envision if incubating with mouse primary antibodies).
3. Wash slides with PBS/T for 2 x 5 mins
4. Prepare DAB solution by adding 1 drop (20µL) of DAB chromagen to 1mL of DAB substrate buffer (1:50 dilution). Mix and then add a drop or two to cover each section on the slide. Incubate for 10 minutes to visualize until a brown colour develops.
5. Wash sections in Water.

Counterstaining

1. Place the slides into haematoxylin* for 10 secs to 1 min maximum. Remove and wash in running water for 2 minutes. Slides can be dipped in 1% Acid

Alcohol (1ml Conc. Hydrochloric Acid + 99mls 70% Ethanol) at this point to reduce Haematoxylin colour if required – see trouble shooting.
2. Transfer to a dish of *Scott's tap water substitute for 1 minute until the tissue sections turn blue (can be anything from 30secs to maximum 10 minutes). *(3.5g sodium bicarbonate, 20g magnesium sulphate, dissolve and make up to 1 litre with Elga water)
3. Remove and wash in running water for 2 minutes.

The Haematoxylin used is ready made Harris Haematoxylin. This **should be filtered before use and discarded after every 200 slides have been stained. Although slides should always be quality controlled by eye, solutions can be changed sooner if there is a problem.*

De-hydrating, Clearing and Mounting

1. Allow any excess fluid to drain from the slide rack before proceeding to the next solution
2. Dehydration-50% Alcohol 30 seconds
3. Dehydration-80% Alcohol 30 seconds
4. Dehydration-100% Alcohol 2 minutes
5. Dehydration-100% Alcohol 2 minutes
6. Clearing-Xylene 16 5 minutes
7. Clearing-Xylene 17 5 minutes
8. Clearing-Xylene 18 5 minutes
9. Mount in DPX as follows:

Mounting Slides

1. Slide mounting should always be performed under the fume extraction unit within the main laboratory.
2. Unless otherwise stated, slides should be mounted directly from xylene using an appropriate mountant (eg DPX).
3. Nitrile gloves are available for use when mounting sections.
4. Place appropriately sized coverslips onto the blotting paper under the fume extraction unit.
5. Using a pastette, place a drop of mountant onto each coverslip.
6. Remove a slide from the xylene and align the long edge of the slide with the coverslip, ensuring that the section is facing towards the coverslip.
7. Tilt the slide towards the coverslip until it touches the mountant. Gently release the slide allowing the mountant to spread between the coverslip and the slide.
8. Turn the slide over and gently press on the cover slip with a pair of forceps to push any remaining air bubbles out towards the edge of the slide.
9. Leave in the fume cupboard overnight to dry.

5.0 Troubleshooting

If at any time during the staining run, the staining does not look right there are steps that can be taken to correct this:

- If after staining the haematoxylin looks too dark microscopically, the slides can be washed in water and placed into a dish of 1% Acid Alcohol either:
  - For 5-10seconds to remove some of the haematoxylin and then 'blued' again in Scott's Tap Water substitute.
  - Or the slides can be left in 1% Acid Alcohol until all the haematoxylin has been removed and the slides can be washed and placed back into haematoxylin for a reduced amount of time.
- If during dehydrating steps the eosin leaches out of the sections the slides can be taken back through the alcohols to water to re-hydrate the sections and placed back into eosin for a further 5 minutes before completing the staining protocol.
- If after completing the staining protocol, and the slides have been mounted a problem is found, the slides can be placed into a dish of remounting xylene overnight to remove the coverslip and then re-hydrated and re-stained as required.

If after completing the staining run, the slide does have bubbles between the slide and the coverslip, the slide can be placed into a dish of remounting xylene overnight to remove the coverslip and then re-mount it.

6.0 Personal protection –

A Howie coat must be worn at all times and nitrile gloves where COSHH recommends.

7.0 Spillages –

Always clean up any spills immediately after use, only you know what you have spilt and are aware of its hazard.

Spillages should be mopped up with white tissue and disinfected with 70% ethanol and

8.0 Training –

All staff should under go training in this technique before performing procedure

9.0 Related documents –

8.1 Risk assessments COSHH 08, 19 & 21
General RA 06, 47

8.2 SOP SASoM/METHOD/024
Dewaxing and Rehydration of Paraffin Embedded Sections

8.3 SOP SASoM/EQUIP/015
Use of the Pressure Cooker for Antigen Retrieval

8.4 SOP SASoM/METHOD/041
Haematoxylin and Eosin (H&E) Staining

9.0 Approval and sign off –

Author:

Name: In Hwa Um

Position: Post Doc

Signature: Date:

Management Approval:

Name: Peter Mullen

Position: Research Fellow

Signature:  Date: 22/03/2021

QA release by:

Name: Alex MacLellan

Position: QA Manager

Signature:  Date: 22/03/2021

STANDARD OPERATING PROCEDURE

Please sign below to indicate you have read this S.O.P and understand the procedures involved.

NAME	POSITION HELD	SIGNATURE	DATE