Document Number: **SASoM/METHOD/076.v4**

Title: **Amplified Immunofluorescence using MOUSE and RABBIT Primary Antibodies**

Version: **v4**

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Effective from:	16/02/2021
Valid to:	16/02/2023

SOP History		
Number	Date	Reason for Change
V1	16/02/15	Original
V2	16/02/2017	Additional info re antigen retrieval method and secondary antibody dilution
V3	16/02/2019	Update
V4	16/02/2021	Update

1.0 Purpose –

This SOP describes the current procedure for duplexing Mouse and Rabbit Primary Antibody Immunofluorescence 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 Duplex Primary Antibody Immunofluorescence.

3.0 Responsibilities –

All staff involved in Duplex Mouse and Rabbit Primary Antibody Immunofluorescence 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 –
Always put slide(s) in non-metallic rack.

Amplification reagents are expensive so if possible use non-amplified techniques first.

Antibody detection / visualisation can be carried out using HRP-amplified **FITC Tyramide** visualised on the FITC channel, HRP-amplified **Cy5 Tyramide** visualised on the cy5channel and/or HRP-amplified **Cy3 Tyramide** visualised on the cy3 channel. Slides can be viewed on AQUA, the Leica DM5500 B microscope and Zeiss Axio scan z1. *Duplexing is possible if required.*

De-wax & Re-hydrate
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 the microwave pressure cooker. Screw on the lid and put into the microwave on high power to heat up for 10mins.

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 tap water (increasing to hot) — 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).

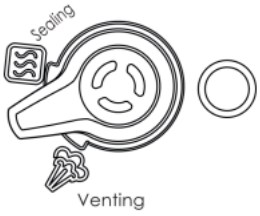

Figure 1 Pressure release handle

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

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.

1st Primary Antibody Incubation and visualisation

1. Add the 1st primary antibody(s) (either mouse or rabbit) diluted at the appropriate concentrations (previously optimised by IHC) in Dako antibody diluent.
2. Incubate either 30min at room temperature or at 4oC overnight according to previously optimised condition by IHC.
3. Wash sections in 0.1% PBST 2 x 5 minutes.
4. Incubate sections in pre-diluted HRP conjugated secondary antibody (either mouse or rabbit, depending on the 1st primary antibody species) for 30min
5. Wash sections in 0.1% PBST 2 x 5 minutes.
6. Dilute TSA either Cy3, FITC or Cy5 (1in50) in TSA diluent and incubate sections for 10min

7. Wash sections in 0.1% PBST for 2 x 5min

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Microwave stripping and blocking

1. Heat up NaCitrate pH6.0 buffer in the pressure cooker (without red button and rubber seal) in the microwave for 12min and then microwave sections with auto defrost function (Meat, 850g- this is 17min defrosting process)
2. Wash sections in 0.1% PBST for 5min
3. Block sections in 3% H2O2 (or Dako peroxidase) for 5min
4. Wash sections in 0.1% PBST for 5min
5. Block sections in Dako serum free protein block for 10min

2nd Primary Antibody Incubation and visualisation

1. Add the 2nd primary antibody(s) (either moues or rabbit) diluted at the appropriate concentrations (previously optimised by IHC) in Dako antibody diluent.
2. Incubate either 30min at room temperature or at 4oC overnight according to previously optimised condition by IHC.
3. Wash sections in 0.1% PBST 2 x 5 minutes.
4. Incubate sections in pre-diluted HRP conjugated secondary antibody (either mouse or rabbit, depending on the 1st primary antibody species) for 30min
5. Wash sections in 0.1% PBST 2 x 5 minutes.
6. Dilute TSA either Cy3, FITC or Cy5 (1in50) in TSA diluent and incubate sections for 10min
7. Wash sections in 0.1% PBST for 2 x 5min

Counterstaining and Coverslipping

1. Blot off excess PBS onto tissue. Apply 40 µl (22x40mm coverslip) Prolong Gold anti-fade reagent with DAPI (Invitrogen, P36931), nuclear visualisation media, onto the sample and gently lower the coverslip over the tissue, ensuring no air bubbles are present. (For the smaller coverslips (22x26mm), apply 20µl).
2. Refer to TSA MSDS sheet for guidance on removal of coverslip to remount, if necessary.
3. Tack down coverslip at 4 corners with small quantity of nail varnish to prevent accidental movement of coverslip.
4. Let the mounted slide dry for at least 24hrs in horizontal position, in the dark, to ‘cure’.
5. After slides are ‘cured’, seal the coverslips with nail polish. Allow to dry in horizontal position and place in slide box for long term storage

5.0 Personal protection –

A Howie coat must be worn at all times. Gloves as specified in the appropriate COSHH RA.

6.0 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 a paper towel, disinfected with 70% ethanol and finally washed with detergent.

7.0 Training –

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

8.0 Related documents –

8.1 Risk assessments COSHH 19

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-EQUIP/100.v1
Use of Instant pot electric pressure cooker for antigen retrieval

9.0 Approval and sign off –

Author:

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Signature: Date:

Management Approval:

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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