Document Number:	**SASoM/METHOD/072.v5**
Title:	**Duplex Immunofluorescence combining Amplified and Non-Amplified MOUSE and RABBIT Primary Antibodies.**
Version:	**v5**
Author:	**In Hwa Um**

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Valid to:	01/03/2023

SOP History		
Number	Date	Reason for Change
V1	30/9/2014	Original
V2	16/02/2015	Minor amendments
V3	01/03/2017	Additional info re antigen retrieval method and secondary antibody dilution
V4	01/03/2019	Update
V5	01/03/2021	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 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.

Non-amplified antibody detection / visualisation can be carried out using, amongst others, the following secondary antibodies: (i) Alexa Fluor® 488 (FITC), (ii) Alexa Fluor® 555 (Cy3) or (iii) Alexa Fluor 568 (Texas Red).

Amplified antibody detection / visualisation is usually carried out using amplified 488 (FITC) Tyramide or amplified 647 (Cy5) Tyramide.

The optimal antibody combination would be Alexa Fluor® 488 (FITC) combined with 647 (Cy5) Tyramide (this gives best separation of wavelength and allows visualisation on both the Leica DM5500B miciroscope and 'AQUA').

The choice of which primary antibody is allocated to FITC / Cy5 is chosen by the end user - as a general rule the antibody with the lowest expression would be assigned to the (HRP-amplified) Cy5 channel. Other antibody combinations can be used as per the appendix.

<u>De-wax & Re-hydrate</u>
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).

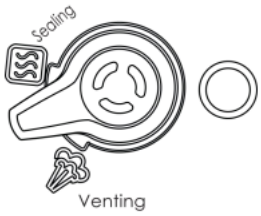

Figure 1 Pressure release handle

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).

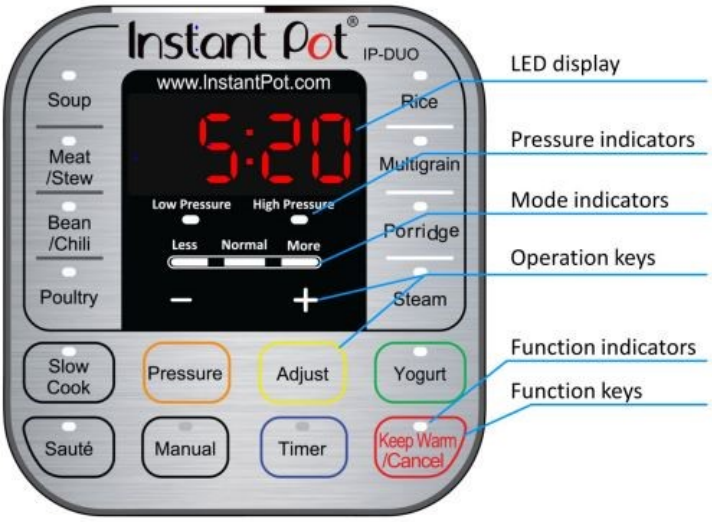

Figure 2 Control panel

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.

Primary Antibody Incubation

1. Add the two primary antibodies diluted at appropriate concentrations in Dako antibody diluent (previously optimised via IHC). It is important that the two primary antibodies are from a different host species.
2. Incubate either 30min at room temperature or at 4°C overnight and then rinse in 0.1% PBST 2 x 5 minutes.

Non-Amplified Antibody Visualisation

1. *The Alexa Fluor® 488 (FITC) or Alexa Fluor® 555 (Cy3) Ab should be used for the 'non-amplified' antibody. Ensure both the Alexa 488 / 555 Ab secondary antibody (and the HRP antibody solution) is from the same host species as the primary antibody.*

2. Prepare a 1 in 100 dilution of the appropriate (anti-mouse or anti-rabbit) Alexa 488 / 555 Ab in the appropriate (anti-mouse or anti-rabbit) Dako Envision HRP antibody solution.

3. Incubate slides in the dark for 30min at room temperature. If there are problems with visualisation, the slides can alternatively be incubated in the HybEZ oven (37°C) for 45 minutes.

4. Rinse in 0.05% PBST 2 x5 minutes

Amplified Antibody visualisation

1. If required, reconstitute new stock of Cy5 Tyramide (Perkin Elmer SAT705A001EA) according to manufacturer's instruction. Usually reconstitute in 150µl DMSO

2. Dilute this working stock solution of Cy5 Tyramide 1 in 50 in target signal amplification diluent. Invert to mix thoroughly.

3. Incubate slides in the dark for 10 minutes at room temperature, be careful not to move slides as this produces a 'fuzzy' signal.

4. Rinse in 0.1% PBST 2 x5 minutes

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 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: 22/03/2021
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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