Document Number:	**SASoM/METHOD/032.v3**
Title:	**Immunofluorescence using a RABBIT Primary Antibody**
Version:	**v3**
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Effective from:	21/11/2013
Valid to:	20/11/2015

SOP History		
Number	Date	Reason for Change
V1	01/02/2013	Original
v2	19/09/2013	Amended to include more detail
v3	21/11/2013	Amended to improve procedure detail

1.0 Purpose –
This SOP describes the current procedure for 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 Rabbit Primary Antibody Immunofluorescence.

3.0 Responsibilities –
All staff involved in 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 –
Put slide(s) in non-metallic rack

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 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) and pour 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 water	2 minutes

Antigen Retrieval

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 until pressure builds up (there should be an audible hissing sound) – time for 5 mins.
2. Using heat resistant gloves, carefully lift the pressure cooker from within the microwave and place in sink. Run cold water over lid ensuring it does not run over valve. Using a pair of heat-resistant gloves and eye protection carefully and gradually remove the pressure relief weight and allow the steam to escape.
3. Remove the lid from the pressure cooker and ¾ fill with cold water. Allow the slides to cool down for 10-15mins.
4. Transfer slide(s) into coplin jar and then wash in PBS/Tween 20 (500mls PBS +500µl Tween 20) for 2 x 5 minutes.
5. Treat sections in 3% Hydrogen peroxide (10ml of 30% H_2O_2 stock solution plus 90mL Elga H_2O) for 10 minutes. Store hydrogen peroxide in the fridge.
6. Wash slides in PBS/T for 2 x 5 minutes. Blot off excess solution from edge of slides onto tissue. Draw round individual sections on slide with hydrophobic Immedge pen.
7. 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).
8. Blot off Blocking solution on tissue.

Primary Antibody and anti-cytokeratin (Tumour mask) incubation

Add the primary antibody diluted at appropriate concentration in Dako antibody diluent and add the second primary antibody Mouse Anti-cytokeratin (Dako, M3515) dilute 1 in 50 in Dako antibody diluent. Incubate overnight at 4°C.

Rinse in 0.05% PBST 3 x 5mins.

Epithelial mask visualisation

Prepare a 1 in 25 dilution of the goat anti-mouse Alexa555 Ab (Invitrogen, #A21422) in the pre-diluted Envision goat-rabbit HRP antibody solution (Dako, #K4003).

Incubate slides in the dark for 1.5 hours at room temperature.

Rinse in 0.05% PBST 3 x 5mins.

Target visualisation

Reconstitute new stock of Cy5 Tyramide (Perkin Elmer SAT705A001EA) which is kept in cold room in black box, with 300µl Elga water to make working stock solution. Dilute this working stock solution of Cy5 Tyramide 1 in 50 in target signal amplification diluent. Vortex to mix thoroughly.

Incubate slides in the dark for 10 mins at room temperature.

Rinse in 0.05% PBST 3 x 5mins.

Rinse x1 PBS for 5 mins.

Counterstaining and coverslipping

Blot off excess PBS onto tissue. Apply 55µl (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 30µl).

Refer to TSA MSDS sheet for guidance on removal of coverslip to remount, if necessary.

Tack down coverslip at 4 corners with small quantity of nail varnish to prevent accidental movement of coverslip.

Let the mounted slide dry for at least 24hrs in horizontal position, in the dark, to 'cure'.

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

9.0 Approval and sign off –

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