Document Number:	**SASoM/METHOD/089.v3**
Title:	**Multiplexing Immunofluorescence for four primary proteins (two mice and two rabbits).**
Version:	**v3**
Author:	**In Hwa Um**

Effective from:	21/03/20
Valid to:	20/03/22

SOP History		
Number	Date	Reason for Change
v1	21/03/2016	Original
v2	21/03/2018	Update
v3	21/03/2020	Biennial Update

1.0 Purpose –
This SOP describes the current procedure for multiplexing immunofluorescence (two mice and two rabbits) 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 multiplexing immunofluorescence (two mice and two rabbits).

3.0 Responsibilities –
All staff involved in multiplexing immunofluorescence (two mice and two rabbits) 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.

List of five different fluorophores which our group has optimised

1. TSA cy5 (PerkinElmer, #NEL745B001KT) should be used for the least abundant antigen detection.
2. TSA fluorescein (PerkinElmer, #NEL741B001KT) should be used for the least abundant antigen detection.
3. Alexa Fluor® 488 (Life tech, #A21428,#A21422, respectively) should be used for the second most abundant antigen detection.
4. Streptavidin conjugated Alexa Flour® 750 (Life tech, #S21384) should be used for the most abundant antigen (like pan cytokeratin) detection.
5. Hoechst (Life tech, #H3570) is used for DNA staining.

Dewaxing and rehydrating

-Dewax sections in xylene 3 x 5min

-Rehydrate sections for 2min each in 100%, 100%, 80%, 50% alcohol and then running tap water

Antigen retrieval and endogenous blocking

-Heat up either Tris-EDTA pH9.0 or NaCitrate pH6.0 buffer in the pressure cooker in the microwave for 12min and then microwave sections to the pressure cooker for 5min

-Cool sections down for 20min and wash sections in 0.05% PBST for 5min

-Block sections in 3% H2O2 (or Dako peroxidase) for 5min

-Wash sections in 0.05% PBST for 5min

-Block sections in Dako serum free protein block for 10min

1st Primary antibody incubation and visualisation *Keep sections in the dark*

-Dilute 1st primary antibody according to the optimal dilution and incubate sections for 1 hour

-Wash sections in 0.05% PBST for 3 x 5min

-Incubate sections in pre-diluted HRP conjugated secondary antibody and incubate for 30min

-Wash sections in 0.05% PBST for 3 x 5min

-Dilute TSA CY5 (1in100) in TSA diluent and incubate sections for 10min

-Wash sections in 0.05% PBST for 3 x 5min

1st microwave stripping and blocking

-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)

-Wash sections in 0.05% PBST for 5min

-Block sections in 3% H2O2 (or Dako peroxidase) for 5min

-Wash sections in 0.05% PBST for 5min

-Block sections in Dako serum free protein block for 10min

2nd Primary antibody incubation and visualisation

-Dilute 2nd primary antibody according to the optimal dilution and incubate sections for 1 hour
-Wash sections in 0.05% PBST for 3 x 5min
-Incubate sections in pre-diluted HRP conjugated secondary antibody and incubate for 30min
-Wash sections in 0.05% PBST for 3 x 5min
-Dilute TSA FITC (1in100) in TSA diluent and incubate sections for 10min
-Wash sections in 0.05% PBST for 5min

2nd Microwave stripping and blocking

-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)
-Wash sections in 0.05% PBST for 5min
(*If staining can't be completed on the same day, then leave sections in 0.05% PBST in the cold room overnight.)
-Block sections in 3% H2O2 (or Dako peroxidase) for 5min
-Wash sections in 0.05% PBST for 5min
-Block sections in Dako serum free protein block for 10min

3rd and 4th primary antibody incubation and visualisation

-Dilute 3rd (rabbit) and 4th (mouse) primary antibody according to the optimal dilution and incubate sections for 1 hour
-Wash sections in 0.05% PBST for 3 x 5min
-Dilute anti-rabbit or mouse alexa555 (1in50) and anti-rabbit (1in250) or mouse (1in25) biotinylated antibody and then incubate sections for 1 hour
-Wash sections in 0.05% PBST for 3 x 5min
-Dilute alexa 750 conjugated streptavidin antibody (1in50) and incubate sections for 30min
-Wash sections in 0.05% PBST for 3 x 5min

Counterstaining and mounting

-Dilute Hoechst 33342 (1in20 in deionised water) and incubate sections for 30min
-Wash sections in 0.05% PBST for 2 x 5min
-Dehydrate sections in 80% alcohol for 1min and air dry sections
-Mount in Prolong anti-fade mounting medium (without DAPI)

5.0 Personal protection -

A Howie laboratory coat and lab gloves must be worn at all times.

6.0 Spillages -

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

7.0 Training -

All staff should undergo training in this technique before performing procedure.

8.0 Related documents –

8.1 Risk assessments –COSHH RA 08
General RA 06

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

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:

QA release by:

Name: Alex MacLellan

Position : QA Manager

Signature : Date:

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