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| **Document Number:** | **SASoM/METHOD/091.v3** |
| **Title:** | **Multiplexing Immunofluorescence for three different target proteins** |
| **Version:** | **v3** |
| **Author:** | **In Hwa Um** |

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| Effective from: | 21/03/2020 |
| Valid to: | 20/03/2022 |

| **SOP History** | | |
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| Number | Date | Reason for Change |
| v1 | 21/03/2016 | Original |
| v2 | 21/03/2018 | Update |
| v3 | 21/03/2020 | Biennial Update |
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**1.0 Purpose –**
This SOP describes the current procedure for immunofluorescence for two same species' antibodies using TSA 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 immunofluorescence for two same species antigen detection using two different TSA fluorophores.

**3.0 Responsibilities –**
All staff involved in immunofluorescence for two same species antigen detection using two different TSA fluorophores 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 three different TSA 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® 555 should be for the most abundant antigen (like pan-cytokeratin) detection.

**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 with pan-cytokeratin incubation and visualisation**

-Dilute 2nd primary antibody (rabbit or mouse) and pan-cytokeratin (mouse or rabbit) according to the optimal dilution and incubate sections for 1 hour

-Wash sections in 0.05% PBST for 3 x 5min
-Dilute anti-mouse or anti-rabbit alexa 555 in anti-rabbit or anti-mouse pre-diluted HRP conjugated secondary antibody with 1 in 50 dilution
-Incubate sections for 1 hour at room temperature
-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

**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 |
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