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Department of Translational Molecular Pathology (TMP)
Translational Molecular Pathology Immunoprofiling Laboratory (TMP-IL) Moonshot's Platform
Division of Pathology and Lab Medicine
The University of Texas MD Anderson Cancer Center
Director:
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Multiplex Immunofluorescence (mIF) SOP

Version 1.0

1. ***Analyte(s):***
   Panel Vectra 9C (**PD1, CD68, FOXP3, CD45RO, CD34, CD8, PD-L1, CD3**)

2. ***Technical platform(s):***
   This procedure describes an automated system for staining multiplex immunofluorescence paraffin sections using the Bond RX by Leica Biosystems and its Research Detection System 2. The Bond RX instrument enables small volumes of reagent (as little as 150 µl per slide) to be uniformly applied over the tissue sections on a slide and has continuous batch processing, allowing for independent start and finish times for each batch of 10 slides.

3. ***Reagents, controls, and calibrators:***
   Optimized reagents:
   - Bond Research Detection System 2 (Leica Biosystems, DS9777) (enhance staining quality)
   - Detection Buffer (1X TBS)
   - OPAL 7-COLOR AUTOMATION IHC KIT (AKOYA BIOSCIENCES, NEL821001KT):
     - OPAL PKI Blocking Buffer
     - Opal Polymer HRP Mouse + Rabbit
     - 1X Amplification Diluent
     - Opal 520 Reagent
     - Opal 540 Reagent
     - Opal 570 Reagent
     - Opal 620 Reagent
     - Opal 650 Reagent
     - Opal 690 Reagent
     - Spectral DAPI solution 1X
   - OPAL POLARIS 780 REAGENT PACK (AKOYA BIOSCIENCES, FP1501001KT):
     - Opal Polaris 780 Reagent (780R)
     - Opal TSA-DIG 780 (780D)
   - OPAL POLARIS 480 REAGENT PACK (AKOYA BIOSCIENCES, FP1500001KT):
     - Opal Polaris 480 Reagent
   - Bond Dewax Solution (Leica Biosystems, AR9222)
   - 100% alcohol
   - Bond Wash solution (Leica Biosystems, AR9590)
   - Bond Epitope retrieval solution ER1 Low pH (Leica Biosystems, AR9961) or ER2 High pH (Leica Biosystems, AR9640)
   - TBS Buffer (Santa Cruz Biotechnology Inc., SC-362186)

Panel 9C Antibodies

Antibody	PD1	CD68	FOXP3	CD45RO	CD34	CD8	PD-L1	CD3 ε
Clone	EPR4877(2)	PG-M1	D2W8E	UCHL1	EP373Y	C8/144B	E1L3N	D7A6E
Vendor	Abcam	Dako	Cell Signaling Technology	Leica Biosystems	Abcam	Thermo Scientific	Cell Signaling Technology	Cell Signaling Technology
Catalog #	AB137132	M087601-2	98377S	PA0146	AB81289	MS-457s	13684S	85061
(+) Control Tissue	Tonsil							
(-) Control Tissue	Tonsil (only Primary antibody without Opal Polymer-HRP Ms + Rb and TSA-Dye, DAPI) Tonsil (only Opal Polymer-HRP Ms + Rb without Primary antibody and TSA-Dye, DAPI) Tonsil (only TSA-Dye without Primary antibody and Opal Polymer-HRP Ms + Rb, DAPI)							
Retrieval Method	High target	Low target	Low target	Low target	High target	Low target	Low Target	Low Target
Dilution	1:250	1:50	1:50	PURE	1:100	1:25	1:1500	1:100
TSA-Dye	620(1:100)	540(1:100)	650(1:200)	570(1:100)	480 1:100	520(1:100)	690(1:100)	780 R (1:25) 780 D(1:100)
Detection Kit	Opal Polymer HRP Ms + Rb							

4. ***Automated Steps:*** (Deparaffinization, epitope retrieval, immunostaining, and counterstaining): Opal 7 – color (v5.2 plus)

a) Bond Dewax Solution x 3 changes at 72°C.
b) 100% alcohol x 3 changes.
c) Wash solution x 3 changes.
d) Epitope retrieval solution ER1 Low pH or ER2 High pH depending upon antibody protocol for 20 minutes at 95°C and cool down to RT.
e) Wash solution x 3 changes.
f) OPAL PKI Blocking Buffer 10 minutes at RT.
g) Primary antibody (Ab) for 30-60 minutes at RT depending upon antibody protocol.
h) Wash solution x 4 changes.
i) Opal Polymer-HRP Ms + Rb for 10 minutes at RT.
j) Wash solution x 3 changes.
k) TSA-Dye for 10 minutes.
Repeat from step c—step k, staining the next antibody (Ab), totally 8 antibodies (See above tables for Panel 9 / Panel 10).
l) Wash solution x 3 changes or 4 changes.
m) Epitope retrieval solution ER1 Low pH 20 minutes at 95°C and cool down to ambient temperature for 12 minutes.
n) Wash solution x 3 changes.
o) Opal DAPI for 1 minutes
p) Opal DAPI for 5 min
q) Wash solution x 3 changes.

5. **Manual Completion Steps:**

r) Unload slides from the instrument and place into 1X TBS Buffer.
s) Wash and clear sections through 3 changes of TBS Buffer.
t) Mount sections with cover glass using ProLong® Diamond Antifade Mountant (Opal Mount medium)

Multiplex Immunofluorescence and Image Analysis Laboratory:

Research Investigator: Auriole Tamegnon, Translational Molecular Pathology (TMP)

_____ Date 09/29/2022

Laboratory Manager: Mei Jiang, Translational Molecular Pathology (TMP)

_____ Date 09/29/2022

Director: Edwin Parra-Cuentas, MD, PhD, Associate Professor, Translational Molecular Pathology (TMP)

_____ Date 09/29/2022