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THE UNIVERSITY OF TEXAS
MD Anderson
Cancer Center
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Department of Translational Molecular Pathology (TMP)
Immune Profiling Laboratory
Cancer Immune Monitoring and Analysis Center
Division of Pathology and Lab Medicine
The University of Texas MD Anderson Cancer Center

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Multiplex ImmunoFluorescence (mIF) SOP

Version 1.1

- ***Pre-analytic variation(s):*** In our experience, a good pre-analytic standardization is an important step to handing samples, and it is a modifiable factor to a proper assessment of the different biomarkers studies with mIF.
    a. Handing Samples Recommendations:
        - Rapid fixation of the tissue as quickly as possible after resection in Buffered Formalin 10% (less than 20 minutes is a prudent guideline to follow),
        - Recommended overall sample dimensions (1.5 x 1.5 x 0.4 cm as maximum for a good fixation),
        - Adequate volume of fixative (should be 10-20 times the volume of the tissues for immersion fixation),
        - Adequate time of fixation (6-18 hours for biopsy specimens and 24-72 hours for standard samples), can be the difference between higher or poorly quality of mIF staining.
    b. Storage Conditions Recommendations:
        - Avoid oxidation process of the tissue through paraffin coating blocks before and after their use to avoid any type of tissue antigenicity degradation,
        - Storage slides using vacuum sealed desiccator, paraffin coating, colder conditions (-4/−18°), as well as complete removal of water (presence of water both endogenously and exogenously plays a central role in loss of antigenicity) from those is highly recommended.
    c. Freshly Cutting Sections:
        - Making freshly cutting sections or sections stored for less than two months will be ideal to use to for mIF.
    d. Different Sample Collections Used as a Controls:
        - Fresh tissue controls tumors from the same type of cancer staining together with the batch of study samples is highly commended to detect any pre-analytic interference during the staining.

- ***mIF Panel (s):***
  Panel Vectra 1 (**Cytokeratin AE1/AE3, CD3, CD8, CD68, PD1, PD-L1**)
  Panel Vectra 2 (**Cytokeratin AE1/AE3, CD3, CD8, GZB, CD45RO, Foxp3**)

- ***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.

- ***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 (Perkin Elmer, NEL821001KT):
    - OPAL PKI Blocking Buffer
    - Opal Polymer HRP Mouse + Rabbit
    - 1X Amplification Diluent
    - Opal 520 Fluorophore
    - Opal 540 Fluorophore
    - Opal 570 Fluorophore
    - Opal 620 Fluorophore
    - Opal 650 Fluorophore
    - Opal 690 Fluorophore
    - Spectral DAPI solution 1X

  - 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 1 Antibodies

Antibody	Cytokeratin	CD3	CD8	CD68	PD-1	PD-L1
Clone	AE1/AE3	-	C8/144B	PG-M1	EPR4877(2)	E1L3N
Vendor	Dako	Dako	Thermo Scientific	Dako	Abcam	Cell Signaling Technology
Catalog #	M351501-2	A045201-2	MS-457S	M087601-2	AB137132	13684S
(+)Control Tissue	Tonsil/sample case					
(-)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	Low target	Low target	Low target	Low target	High target	Low target
Dilution	1:100	1:200	1:25	1:50	1:3000	1:1500
TSA-Dye	620(1:100)	690(1:150)	540(1:100)	520(1:100)	650(1:200)	570(1:100)
Detection Kit	Opal Polymer HRP Ms + Rb					

Panel 2 Antibodies

Antibody	Cytokeratin	CD3	CD8	GZB	CD45RO	FoxP3
Clone	AE1/AE3	-	C8/144B	11F1	UCHL1	D2W8E
Vendor	Dako	Dako	Thermo Scientific	Leica Biosystems	Leica Biosystems	Cell Signaling Technology
Catalog #	M351501-2	A045201-2	MS-457s	PA0291	PA0146	98377S
(+)Control Tissue	Tonsil/sample case					
(-)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	Low target	High target	Low target	Low target	High target	Low target
Dilution	1:100	1:200	1:25	Pure	Pure	1:50
TSA-Dye	520(1:100)	690(1:100)	570(1:100)	540(1:300)	620(1:100)	650(1:150)
Detection Kit	Opal Polymer HRP Ms + Rb					

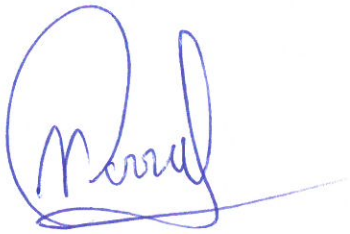
- ***Automated Steps:*** (Deparaffinization, epitope retrieval, immunostaining, and counterstaining): Opal 7 – color (v5.2 plus)

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 for 12 minutes.
e) Wash solution x 3 changes.
f) OPAL PKI Blocking Buffer 5 minutes at RT.
g) Primary antibody (Ab) for 30-60 minutes at RT depending upon antibody protocol.
h) Wash solution x 3 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 6 antibodies (See above tables for Panel 1 / Panel 2).
l) Wash solution x 3 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 5 minutes
p) Wash solution x 3 changes.

- **Manual Completion Steps:**

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

Multiplex Assays Laboratory:



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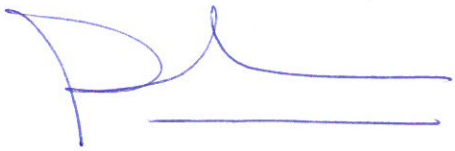

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