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## Immunohistochemistry (IHC) SOP

Version 1.0

1. **Analyte(s):** CD3, CD4, CD45RO, CD57 (Leu-7), CD68, CD8, Foxp3, Granzyme B, PD-1, PD-L1

2. **Technical platform(s):**

This procedure describes an automated system for staining paraffin sections using the Bond Max by Leica Biosystems and its Polymer Refine Detection System. The Bond Max 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 Polymer Refine Detection Kit (DAB) (DS9800) (enhance staining quality).
- Horseradish Peroxidase (HRP)
- Secondary antibody conjugated to a polymer
- Post Primary (anti-mouse IgG)
- Polymer (anti-rabbit IgG)
- Bond Dewax Solution (AR9222)
- 100% alcohol
- Wash solution (AR9590)
- Epitope retrieval solution (ER1 Low pH (AR9961) or ER2 High pH (AR9640))
- DAB (diaminobenzidine) and Hematoxylin reagents

MARKER	VENDOR (CAT#)	Clonality	CLONE	Epitope Retrieval	Dilution	Antibody incubation time
CD3	DAKO (cat#A0452)	Rabbit polyclonal	-	ER1 Low pH for 20'	1:100	15 min.
CD4	Novocastra (CD4-368-L-CE)	Mouse monoclonal	4B12	ER2 High pH for 20'	1:80	15 min.
CD45 RO	Leica Microsystems (PA0146)	Mouse monoclonal	UCHL1	ER1 Low pH for 5'	Ready to use	15 min.
CD57 (Leu-7)	BD Biosciences (347390)	Mouse monoclonal	HNK-1	ER1 Low pH for 20'	1:40	15 min.

<b>CD68</b>	DAKO (MO876)	Mouse monoclonal	PG-M1	ER1 Low pH for 20'	1:450	15 min.
<b>CD8</b>	Thermo Scientific (MS-457S)	Mouse monoclonal	C8/144B	ER1 Low pH for 20'	1:25	15 min.
<b>Foxp3</b>	BioLegend (320102)	Mouse monoclonal	206D	ER2 High pH for 20'	1:50	15 min.
<b>Granzyme B</b>	Leica Microsystems (PA0291)	Mouse monoclonal	11F1	ER2 High pH for 20'	RTU	
<b>PD-1</b>	Abcam (ab137132)	Rabbit monoclonal	EPR4877(2)	ER1 Low pH for 20'	1:250	15 min.
<b>PD-L1</b>	Abcam (ab205921)	Rabbit monoclonal	[28-8]	ER2 High pH for 20'	1:100	15 min.
<b>PD-L1</b>	Cell Signaling (13684)	Rabbit monoclonal	E1L3N	ER1 Low pH for 20'	1:100	15 min.
<b>PD-L1</b>	Spring (M4420)	Rabbit monoclonal	SP142	ER1 Low pH for 20'	1:100	15 min.
<b>PD-L1</b>	VENTANA (790-4905)	Rabbit monoclonal	SP263	ER1 Low pH for 20'	RTU	15 min.
<b>PD-L1 22C3</b>	DAKO (M365329-2)	Mouse monoclonal	22C3	ER2 High pH for 20'	1:50	15 min.

**4. Automated steps:** (Deparaffinization, epitope retrieval, immunostaining, and counterstaining):

- a) Bond Dewax Solution (AR9222) x 3 changes at 72o C.
- b) 100% alcohol x 3 changes.
- c) Wash solution (AR9590) x 3 changes.
- d) Epitope retrieval solution (ER1 Low pH (AR9961) or ER2 High pH (AR9640) depending upon antibody protocol for 5, 10, 20 or 30 minutes at 100o C depending upon antibody protocol and cool down to ambient temperature for 12 minutes (See table 1 for specific antibody information).
- e) Wash solution x 6 changes
- f) 3% H2O2 for 10 minutes.
- g) Wash solution x 3 changes
- h) Primary antibody for 15-30 minutes depending upon antibody protocol (See above table for specific antibody information).
- i) Wash solution x 3 changes.
- j) Post primary polymer penetration enhancer reagent for 15-30minutes.
- k) Wash solution x 3 changes at 2 minutes each.
- l) Polymer poly-HRP anti-mouse /rabbit IgG secondary antibody for 15-30 minutes.
- m) Wash solution x 2 changes at 2 minutes each.
- n) DI water x 1 change.
- o) DAB (diaminobenzidine) reagent for 10 minutes.
- p) DI water x 3 changes.
- q) Hematoxylin for 8 minutes.
- r) DI water x 1 change.
- s) Wash solution x 1 change.
- t) DI water x 1 change

**Manual Completion Steps:**

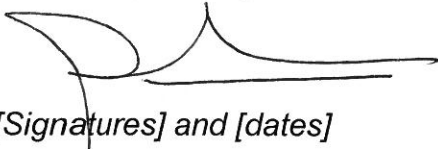
- Unload slides from the instrument and place into DI water.

- Dehydrate and clear sections through 2 changes of 95% ethanol, 2 changes of 100% ethanol and 2 changes of xylene (10 dips each for all solutions).
- Mount sections with cover glass using a xylene based mounting medium

*IHC Assays Laboratory:*

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*[Signatures] and [dates]*



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