

# SOP 15 Multiplex-Immunofluorescence

## SOP 15.1 Panel: SOX10, PD-L1, PD-1, CD8 and DAPI

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### Materials

Name	Source/Vendor	Catalog#	LOT# (optional)
AR6 solution	PerkinElmer	AR600125ML	
Dako Envision Kit	Agilent Technologies	K4010 and K4011	
Opal 7-Color Automation IHC Kit	Perkin Elmer	NEL801001KT	
Protein Block serum free	Dako	XO909	
Bond Polymer refine detection	Leica Biosystems	DS9800	

### Buffer and Media

Da Vinci Green diluent, Biocare Medical, cat # PD900L  
 Discovery Ab Diluent, Ventana, cat # 760-108  
 1x Plus Amplification Diluent, PerkinElmer, cat # EP1498

### Protocol

**PD-1 (clone EH33); Cell Signaling Technologies, Cat # 43248**  
**PD-L1 (clone 405.9A11); Cell Signaling Technologies, Cat # 29122**  
**SOX10 (clone EP268); Cell Marque, Cat # 383R-16**  
**CD8 (clone C8/144B); DAKO; Cat # MA5-13473**

The staining is performed using an automated Bond RX automated IHC and ISH stainer (Leica)

1. Overnight baked FFPE slides are de-paraffinized and then placed in AR6 solution (PerkinElmer) for 15 minutes using a microwave.

- a. Note: AR6 solution was developed for use in the multicolor immunohistochemistry protocol in microwave treatment, and provides antigen retrieval for FFPE tissues, quenches endogenous peroxidase, and removes antibodies after epitopes have been labeled with Opal dyes.
2. After the antigen retrieval step, the slides are cooled in room temperature for 20 minutes and then washed with distilled water and then with Tris buffer.
3. Afterwards, the first antibody for 'Protein 1' (example: PD1) is applied for 40 minutes.
4. HRP-linked secondary antibody (Dako Envision kit) is applied for 30 minutes.
5. After a few washes in Tris buffer (3 x 2 minutes) the 1st Opal Fluorophore is applied (1:50, diluted in Amplification Diluent) for 5 minutes.
6. The slides are washed in Tris buffer and placed in pre-heated AR6 solution for 15 minutes using a microwave.
7. After this, the first antibody for 'Protein 2' (example: CD30) is applied and Steps 2-6 are repeated.
  - a. Note: Currently, using the Opal-7 Color Immunohistochemistry Kit, it is possible to stain for 6 different proteins and Dapi.

Information on the order of antibody staining and which fluorophore is used (Order of staining: Fluorophore)

First protein target: **Sox10- 520**

Second protein target: **PD-1 – 540**

Third protein target: **CD8 – 620**

Fourth protein target: **PD-L1- 690**