

MOTiF™ PD-1/PD-L1 Panel: Auto Melanoma Kit

Automated Multiplex IHC Staining

Getting Started

The MOTiF™ PD-1/PD-L1 Panel: Auto Melanoma Kit has been specifically designed for the Leica BOND RX to produce optimal staining of your melanoma FFPE tissue. Stained slides can be imaged using the Vectra Polaris imaging system. Currently, this kit is not compatible with Vectra 3. Additional components are required for imaging on the Mantra Workstation.

Materials

	Slides	Catalog #	Contents
MOTiF PD-1/PD-L1 Panel: Auto Melanoma Kit	50 Slides	OP-000003	<ul style="list-style-type: none"> • 1X Plus Automation Amplification Diluent (2 x 50mL) • Opal Polaris 480 Reagent (2x) • Opal 520 Reagent (2x) • Opal 570 Reagent (2x) • Opal 620 Reagent (2x) • Opal 690 Reagent (2x) • Opal Polaris 780 Reagent <ul style="list-style-type: none"> ◦ Opal TSA-DIG (2x) ◦ Opal Polaris 780 (1x) • Spectral DAPI solution (2 x 1.5mL) • DMSO (2 x 500uL) • Blocking/ Ab Diluent (1 x 100mL) • Opal Polymer HRP Ms+Rb (2 x 50mL) • FoxP3 RTU Antibody (1 x 9.5mL) • PD-L1 RTU Antibody (1 x 9.5mL) • Sox10 RTU Antibody (1 x 4.8mL) • S100 RTU Antibody (1 x 4.8mL) • PD-1 RTU Antibody (1 x 9.5mL) • CD8 RTU Antibody (1 x 9.5mL) • CD68 RTU Antibody (1 x 9.5mL)

***NOTE:** The format of the kit is based on 5 automation runs at 10 slides each with 150µl volume dispenses per slide, with double dispenses for Opals and DAPI.

Additional Required Materials and Reagents

These materials are not included in the kit and must be supplied separately.

Laboratory Materials

- Histological grade ethanol (for rehydration)
- Tris-buffered saline (TBS) wash buffer
 - (25 mM TRIS-HCl; pH 7.5 150 mM NaCl)
- Peroxidase-free water
 - This specification may be met by commercial “cell culture grade” water or ultra-pure (i.e. Milli-Q™) water that has been autoclaved
- Mounting medium
- Glass coverslips

BOND RX Materials

- Titration Kit (OPT9049)
- Open container - 7mL (OP79193)
- Open container - 30mL (OP309700)
- Research Detection System 2 (DS9777)
- Universal Covertiles (S21.4611)
- Slide Tray (S21.0304)
- Reagent Tray (S21.1003)
- Slide Labels and Printer Ribbon (S21.4564)
- Apex Adhesive Slide (3800040)
- Dewax Solution (AR9222)
- Epitope Retrieval Solution 1 (AR9961)
- Epitope Retrieval Solution 2 (AR9640)
- Wash Solution 10X Concentrate (AR9590)
- Aspirating Probe Cleaning System (CS9100)

Solution Preparation

Primary and Secondary Antibody Working Solutions

All primary antibodies and the Opal Polymer HRP Ms+Rb are supplied as ready-to-use (RTU) solutions and do not need to be prepared or optimized for use with the Opal reagents.

Opal Working Solution*

Reconstitute each Opal reagent in 75µL of DMSO, with the exception of Opal Polaris 780 (see below). Before each procedure, dilute Opal reagent in 1X Plus Automation Amplification Diluent to make Opal reagent working solution. We recommend to start diluting the Opal reagent at 1:150.

Opal Polaris 780 Working Solution *

Reconstitute TSA-DIG in 75µL of DMSO, and Opal Polaris 780 in 300µL of deionized water. Before the procedure, dilute TSA-DIG in 1X Plus Amplification Diluent at 1:100 to make TSA-DIG working solution. Dilute Opal Polaris 780 with Antibody Diluent/ Blocking at 1:25 to make the working solution.

DAPI Working Solution *

Add 2-3 drops of DAPI solution into 1mL of TBS. Approximately 300µL of DAPI Working Solution is required per slide. Discard any unused portion of DAPI Working Solution.

***NOTE:** To help assist in your Working Solutions calculations, one dispense equals 150µL of Working Solution. For this assay, two dispenses of each Opal reagent and TSA-DIG are required per slide (with the exception of Opal Polaris 780), plus additional solution for the dead volume of the container. Discard any unused portion of any of the Working Solutions when the run is complete.

BOND RX Wash Solution

Create a working 1X BOND RX Wash Solution by diluting the stock 10X concentrate BOND™ Wash Solution with peroxidase-free water.

Special Considerations and BOND RX Protocol

Opal Polaris 780 Automation Steps

The Opal Polaris 780 reaction is antibody-based. Because of this, there must be additional washing steps to cool down the slide between the TSA-DIG stripping step (with ERI at 95°C) and before the Opal Polaris 780 application step.

NOTE: The Opal Polaris 780 must ALWAYS go last in your multiplex.

BOND RX Wash Solution Steps

In the following protocol, users will see the following step: BOND Wash Solution. This refers to a series of wash steps that are built-in to the Leica protocol. Listed below are a breakdown of those steps:

Post-Primary Antibody, Epitope Retrieval, Opal 780, and DAPI application: 0 min, 1 min, 0 min

Post-Opal Polymer HRP application: 0 min, 1 min, 3x 0 min

Post-Opal application and TSA-DIG (except Opal 780): 0 min, 1 min, 2x 0 min

Sox10 and S100 Antibody Preparation

To use either Sox10 or S100 individually, combine the selected antibody in a 1:1 solution with Blocking/Antibody Diluent to create a Working Solution. Remember to only create enough Working Solution as needed for your staining purposes (300µL total for two dispenses plus extra for BOND RX container dead volume).

Sox10/ S100 Cocktail Preparation

To generate a cocktail of the Sox10/ S100, combine the ready-to-use Sox10 and S100 antibodies in a 1:1 solution. No additional preparations are required.

Sox10/ S100 Stripping Step

Removal of the Sox10/ S100 (cocktailed or individual antibodies) requires a two-step stripping process, where the tissue is to be incubated twice at 98°C for 20 minutes with ER1. This two-step process is solely for Sox10/ S100, all other antibodies can be removed in one step.

Directions

Follow the steps listed below to perform the automated multiplex immunofluorescence staining assay.

#Differs from standard Opal BOND RX protocol.

Step 0: BOND RX Protocol Creation

- Use the associated kit BOND RX protocol available for download from the Leica website.
- If this is unavailable, create a copy of the *Opal 7-color (v5.2 plus) IHC protocol and alter the copied version to reflect the protocol beginning at Step 2.

Step 1: Slide Preparation

- Bake the FFPE slides for 3 hours at 65°C in a laboratory oven.
- When setting up the BOND RX Study Protocol, select the following listed settings for Preparation and HIER (see Figure 1).
- Label the slides with their corresponding BOND RX barcode.
- Load the slides into the BOND RX trays, and prepare and load the reagents.
- Press "Start" to begin the BOND RX protocol.

Step 2: Research Detection System

- TBS wash, 0 min

Step 3: Blocking

- Akoya blocking buffer, 5 min

Step 4: Primary Antibody Incubation

- Antibody incubation, 30 min
 - Use the chart below to determine antibody staining order information and Opal dilution factor.

Order	Antibody	Clone	Opals	Opal Dilution Factor
1	FoxP3	D608R	Opal 570	1:150
2	PD-L1	E1L3N	Opal 520	1:150
3	Sox10/ S100#	EP268-1/ 4C4.9	Opal 690	1:150
4	PD-1	EPR4877(2)	Opal 620	1:150
5	CD8	4B11	Opal 480	1:150
6	CD68	PG-M1	TSA-DIG, Opal 780	1:100, 1:25

#NOTE: See Step 7 for stripping requirements of Sox10/ S100.

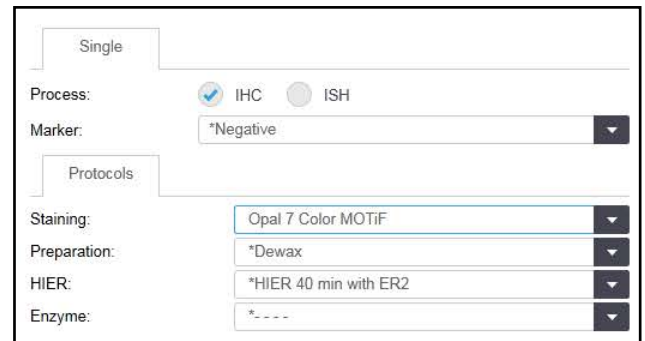


Figure 1. Protocol settings on BOND RX.

Step 5: Opal Polymer HRP

- Bond Wash Solution
- Opal Polymer HRP, 10 min

Step 6: Signal Amplification

- a. Bond Wash Solution
- b. Double dispense the Opal fluorophore (Use the chart to determine Opal pairing and dilution factor)
 - i. First dispense, 0 min
 - ii. Second dispense, 10 min

Step 7: Antibody Stripping

- a. Bond Wash Solution
- b. Bond ER solution 1
 - i. For all antibodies except Sox10/ S100:
 1. Temperature at 95°C, incubate at 20 min
 - ii. For Sox10/ S100 antibody:#
 1. Temperature at 98°C, incubate at 20 min
 2. Temperature at 98°C, incubate at 20 min
- c. Bond Wash Solution

Step 8: Repeat Steps 3-7

- a. Repeat Steps 3-7 for the next four antibodies.
- b. Go on to Step 9 for Opal Polaris 780 staining.

Step 9: Blocking

- a. Akoya blocking buffer, 5 min

Step 10: Primary Antibody Incubation

- a. Antibody 6 (CD68) incubation, 30 min
 - i. Use the chart for antibody information and dilution factor.

Step 11: Opal Polymer HRP

- a. Bond Wash Solution
- b. Opal Polymer HRP, 10 min

Step 12: Introduction of Opal TSA-DIG

- a. Bond Wash Solution
- b. Opal TSA-DIG [1:100]#
 - i. First dispense, 0 min
 - ii. Second dispense, 10 min

Step 13: Antibody Stripping

- a. Bond Wash Solution
- b. Bond ER Solution 1
 - i. 20 min, 95°C
- c. Bond Wash Solution[#]
 - i. Wash
 - ii. 2X Wash, 10 min incubations each
 - iii. Wash

Step 14: Opal Polaris 780 Signal Generation

- a. Opal Polaris 780 [1:25], 60 min[#]
- b. Bond Wash Solution

Step 15: DAPI Counterstain and Mount

- a. Spectral DAPI[#]
 - i. Open dispense, 0 min
 - ii. Open dispense, 5 min
- b. Bond Wash Solution

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