6-Plex, 7-Color MOTiF™ Checkpoint Proliferation Protocol

GETTING STARTED

The following protocol has been optimized for use with the Leica BOND RX and the Opal Polaris 7 Color Automation IHC Detection Kit. 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

Opal Polaris 7 Color Automation IHC Detection Kit	50 Slides	NEL871001KT	· 1X Plus Automation Amplification Diluent (2 x 50mL)
			· Opal Polaris 480 Reagent
			· Opal 520 Reagent
			· Opal 570 Reagent
			· Opal 620 Reagent
			· Opal 690 Reagent
			· Opal Polaris 780 Reagent
			  - Opal TSA-DIG
			  - Opal Polaris 780
			· Spectral DAPI solution (1.5mL)
			· DMSO (500uL)
			· Blocking/ Ab Diluent (2 x 100mL)
			· Opal Polymer HRP Ms+Rb (2 x 50mL)

***NOTE:** All dispensing volumes will be at 150µL 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	BOND RX 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
	· 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 Antibody Working Solution
Dilute primary antibody in the Antibody Diluent/Block at optimal concentration for Opal detection as recommended in the chart on page 4.

Secondary Antibody Working Solution
Opal Polymer HRP Ms+Rb is supplied as a ready-to-use solution and does not need to be optimized for use with 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. Optimize your assay according to the Opal Assay Development Guide.

Opal Polaris 780 Working Solution*
Reconstitute Opal TSA-DIG in 75µL of DMSO, and Opal Polaris 780 in 300µL of deionized water. Before the procedure, dilute Opal TSA-DIG in 1X Plus Automation Amplification Diluent at 1:100 to make Opal 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 Opal and TSA-DIG are required per slide (with the exception of Opal 780 fluorophore), 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 BONDT™ 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 ER1 at 95oC) 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

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

a. Create a copy of the *Opal 7-color (v5.2 plus) IHC protocol.

b. Alter the copied version to reflect the protocol beginning at Step 2.

STEP 1 Slide Preparation

a. Bake the FFPE slides for 3 hours at 65°C in a laboratory oven, rotating the slides every 30 min until all the wax is removed.

b. In the BOND RX Study Protocol, select the following listed settings (see Figure 1).

i. Akoya recommends an additional bake step on the BOND RX prior to dewaxing, which can be selected under the Preparation Step.

c. Label the slides with the corresponding BOND RX barcode.

d. Load the slides into the BOND RX trays and prepare and load the reagents.

e. Press "Start" to begin the BOND RX protocol.

STEP 2 Research Detection System

a. TBS wash, 0 min

STEP 3 Blocking

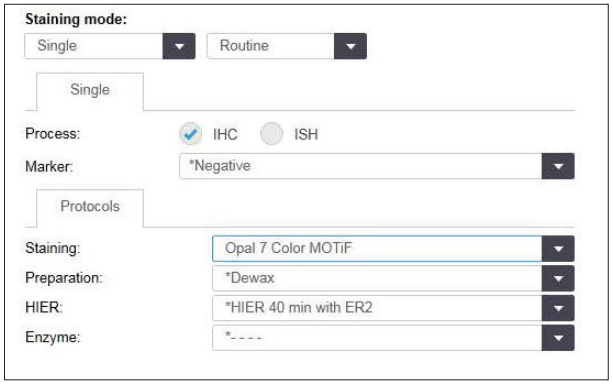
a. Akoya blocking buffer, 5 min

STEP 4 Primary Antibody Incubation

a. Antibody 1 incubation, 30 min

i. Use the chart below to determine antibody information, staining order, and dilution factor.

b. Ki67 primary antibody incubation, 60 min#



Order	Antibody	Clone	Vendor	Species	Antibody Dilution Factor	Opal Pairing	Opal Dilution Factor
1	PD-L1	E1L3N	Cell Signaling	rabbit mAB	1:500	Opal 520	1:100
2	CD68	PG-M1	Agilent	mouse mAB	1:200	Opal 620	1:100
3	CD8	4B11	Leica	mouse mAB	1:200	Opal Polaris 480	1:300
4	PanCK	AE1/AE3	Biocare	mouse mAB	RTU	Opal 690	1:300
5	Ki67#	MIB1	Agilent	mouse mAB	1:100	Opal 570	1:100
6	PD-1	EPR4877(2)	Abcam	rabbit mAB	1:300	TSA-DIG, Opal Polaris 780	1:100, 1:25

STEP 5 Opal Polymer HRP

a. Bond Wash Solution
b. 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. Temperature at 95°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 incubation, 30 min

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

MULTIPLEX ASSAY PANEL TABLE

Use the table provided below to keep track of your assay panel development.

Project Name: _____

Date: _____ Tissue(s): _____

Researcher: _____

Order	Antibody	Supplier	Clone/ Lot	Category #	Dilution Factor	Opal Pairing	AR
1							
2							
3							
4							
5							
6							
7							
8							

Notes: _____

_____

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