*We've rebranded some of our products. **Phenoptics**™ is now **PhenoImager**™ and **Vectra**® **Polaris**® is now **PhenoImager**™ **HT**.*

AKOYA BIOSCIENCES®
THE SPATIAL BIOLOGY COMPANY®

AUTOMATED MULTIPLEX IHC

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

PRODUCT INFORMATION

STORAGE

Store dry Opal reagents at -20°C. Upon reconstitution in DMSO, store at 2–8°C. Store remaining kit components at 2–8°C.

STABILITY

See kit label on outside of box for expiration date.

Please see Opal Storage and Stability for more detailed information.

APPLICATION

The MOTiF PD-1/PD-L1 Panel: Auto Melanoma Kit is intended for detection of six protein biomarkers on melanoma FFPE sections. The kit provides ready-to-use (RTU) antibodies for detection of FoxP3, PD-L1, Sox10/ S100 (can be used as a cocktail or individually), PD-1, CD8, and CD68. The MOTiF PD-1/PD-L1 Panel: Auto Melanoma Kit is optimized for use on the Leica BOND™ RX.

SPECIES REACTIVITY

Human

SAFETY NOTE

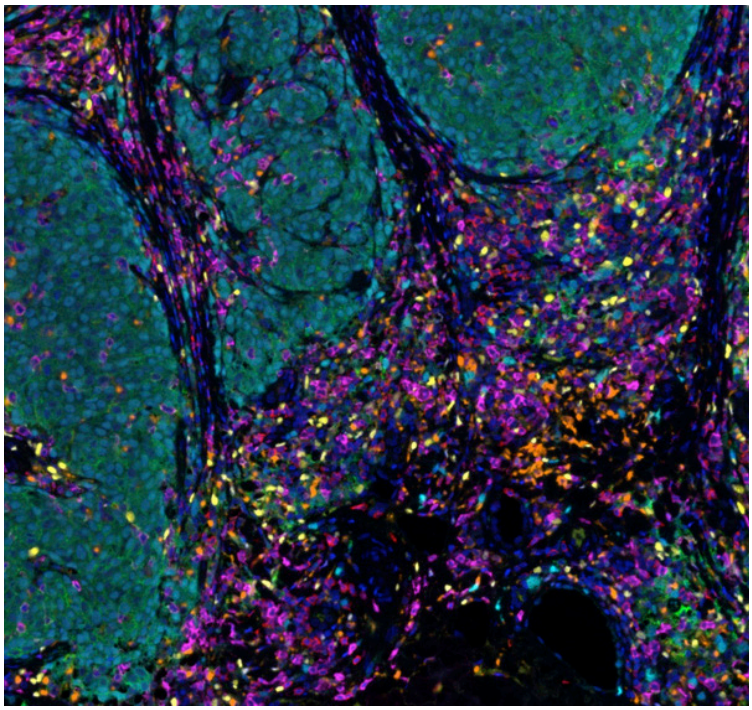
Some reagents in this kit contain DMSO that is classified as hazardous and combustible. Some reagents in this kit contain Proclin® 300 that is classified as corrosive to metals and skin, a skin and eye irritant, and hazardous to the aquatic environment. DAPI is considered corrosive to the skin and an irritant to the eye. All other reagents are classified as nonhazardous. It is strongly recommended to wear disposable gloves and safety glasses while working with the items in this kit. Thorough washing of hands after handling is also recommended.

QUALITY CONTROL

We certify that QC results of these reagents meet our quality release criteria.

PRODUCT NUMBER

OP-000003



Human melanoma section was stained with the MOTiF PD-1/PD-L1 Panel: Auto Melanoma Kit and imaged on the PhenoImager HT™ (formerly Vectra® Polaris™)

Target	Color
FoxP3	Opal 570 in Yellow
PD-L1	Opal 520 in Green
Sox10/S100	Opal 690 in Cyan
PD-1	Opal 620 in Magenta
CD8	Opal Polaris 480 in Red
CD68	Opal Polaris 780 in Orange
Nucleus	DAPI in Blue

What is the Opal™ Method?

The Opal workflow allows for simultaneous detection of multiple biomarkers in tissue. This Opal protocol was written specifically for the MOTiF PD-1/PD-L1 Panel: Auto Melanoma Kit on formalin fixed paraffin embedded (FFPE) tissue using the Leica BOND™ RX to stain and detect FoxP3, PD-L1, Sox10/ S100, PD-1, CD8, and CD68 on melanoma FFPE sections.* The approach involves detection with Opal reactive fluorophores, followed by heat-induced stripping steps for removal of primary and secondary antibodies[1], removal of any

*Please contact us if you would like to work with other types of samples. Akoya Biosciences Inc. provides assistance with assay development and offers multiplex Opal IHC and IF services. Visit: https://www.akoyabio.com/phenopticstm/contract-research-services .

non-specific staining, and reduction of tissue autofluorescence. The Opal signal is largely unaffected by stripping and antibody removal. After stripping, another round of staining can be performed for additional target detection without risk of antibody cross reactivity. Please pay additional attention to Opal Polaris 780. Because this is an antibody-based staining step, stripping can **NOT** be performed after Opal Polaris 780 binds to the tissue. Thus, we assigned Opal Polaris 780 to the last cycle of the staining protocol.

Opal allows for staining of multiple IHC targets using unlabeled primary antibodies raised in the same species2. Combining Opal with multispectral imaging and analysis enables simultaneous, quantitative results for up to 6 biomarkers using immunofluorescence, even with co-localized markers, plus nuclear counterstain (DAPI). The MOTiF PD-1/PD-L1 Panel: Auto Melanoma Kit is specfically designed to work with Leica BOND™ RX and stained slides will be imaged by PhenoImager Fusion and PhenoImager HT (formerly Vectra Polaris) imaging systems. **Currently, this kit is not compatible with Vectra 3*. Additional components are required for imaging on the Mantra Workstation.**

MATERIAL PROVIDED

Description	Format*	Product #	Kit Components

MOTiF PD1/PD-L1 Panel: Auto Melanoma Kit	50 Slides	OP-000003	• 1 X Plus Automation Amplification Diluent (2 x 50 mL)
			• 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
			  - a. Opal TSA-DIG (2x)
			  - b. Opal Polaris 780 (1x)
			• DMSO (2 x 500 µL)
			• Spectral DAPI solution (2 x 1.5 mL)
			• Blocking/Ab Diluent (1 x 100 mL)
			• Opal Polymer HRP Ms + Rb (2 x 50 mL)
			• FoxP3 (D608R) RTU (1 x 9.5 mL)
			• PD-L1 (E1L3N®) XP® RTU (1 x 9.5 mL)
			• Sox10 (EP268-1) RTU (1 x 4.8 mL)
			• S100 (4C4.9) RTU (1 x 4.8 mL)
			• PD-1 (EPR4877(2)) RTU (1 x 9.5 mL)
			• CD8 (4B11) RTU (1 x 9.5 mL)
			• CD68 (PG-M1) RTU (1 x 9.5 mL)

* The format of the kit is based on 5 automation runs at 10 slides each with 150 µl per slide dispenses (double dispense for Opals and DAPI)

ADDITIONAL REQUIRED REAGENTS AND MATERIALS

Laboratory Materials

- Histological grade ethanol (for rehydration)
- Tris-buffered saline (TBS) wash buffer
  - (25 mM TRIS-HCL; pH7.5 150 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
- Follow all appropriate PPE and biological specimen handling protocols established by your laboratory when performing this assay

*Please contact us if you would like to work with other sample types. Akoya Biosciences Inc. provides assistance with assay panel development and offers a full line of services offerings covering multiplex Opal IHC staining and tissue analysis. Visit us at: **www.akoyabio.com/phenopticstm/contract-research-services** to learn more.

REQUIRED INSTRUMENTS	
Component	**Product #**
Leica Biosystems BOND RX Automated Stainer	21.2821
PhenoImager HT, PhenoImager Fusion and Mantra™ Quantitative Pathology Workstation	---

REQUIRED BOND RX MATERIALS		
Component	**Storage**	**Product #**
Titration Kit	RT	OPT9049
Open container - 30 mL	RT	OP309700
Open container - 7 mL	RT	OP79193
Research Detection System 2	RT	DS9777
Universal Covertiles	RT	S21.4611
Slide Tray	RT	S21.0304
Reagent Tray	RT	S21.1003
Slide Labels and Printer Ribbon	RT	S21.4564
Apex Adhesive Slide	RT	3800040
Dewax Solution	2–26°C	AR9222
Epitope Retrieval Solution 1	2–8°C	AR9961
Epitope Retrieval Solution 2	2–8°C	AR9640
BOND Wash Solution 10X Concentrate	2–8°C	AR9590
BOND Aspirating Probe Cleaning System	2–8°C	CS9100
RT: Room Temperature, 20-25°C		

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.

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 Automation 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 ER1 at 95°C) and before the Opal Polaris 780 application step.

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

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 Sox10/ S100, combine the RTU 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.

Please find the BOND RX Protocol including antibody and Opal pairings for MOTiF PD-1/PD-L1 Panel: Auto Melanoma Kit here:

https://www.akoyabio.com/products/motiftm-pd1pd-l1-panel-auto-melanoma-kit-50-slides

Library Slides

Spectral library slides stained with a single fluorophore and made from relevant control tissue may improve unmixing quality for your multiplex slides. To generate library slides, the following slides will be required.

- One control tissue slide stained for each antibody-Opal fluorophore combination under optimized conditions, without DAPI.
- One control tissue slide stained with DAPI alone.
- One unstained control tissue slide, for assessment of autofluorescence. The unstained slides should be processed in the same way as the other slides, omitting both the Opal fluorophore and DAPI.

Imaging and Analysis

Visualization of 7-color Opal slides can be performed using PhenoImager HT and PhenoImager Fusion Imaging System. This system utilizes patented multispectral imaging for quantitative unmixing of multiple fluorophores and tissue autofluorescence, enabling advanced analysis including in situ cellular phenotyping.

A PhenoImager scanning protocol compatible with this kit can be found on the web at **https://www.akoyabio.com/products/motiftm-pd1pd-l1-panel-auto-melanoma-kit-50-slides**. To use, the PhenoImager software requires version 1.0.10 or above. Akoya's scanning protocol provides an ideal starting point to optimize your own scanning protocol, as exposure times should be similar to the levels required for your image acquisition. Since staining intensity may vary between tissues, adjustments to the exposure times are recommended to ensure that they are high enough to detect each marker, while also not saturating. Akoya recommends checking the exposure times for each study in the live view before using the protocol for scanning.

Imaging acquisition and analysis protocols can be found:

https://www.akoyabio.com/products/motiftm-pd1pd-l1-panel-auto-melanoma-kit-50-slides

For additional assistance, please see the **Opal Assay Development Guide** for more detail on imaging and analysis.

References

[1] Toth, Zsuzsanna E., and Eva Mezey. "Simultaneous visualization of multiple antigens with tyramide signal amplification using antibodies from the same species." Journal of Histochemistry & Cytochemistry 55.6 (2007): 545-554

[2] Stack, E.C., Wang, C., Roman, K., and Hoyt, C.C. "Multiplexed immunohistochemistry, imaging, and quantitation: a review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis." Methods: (2014) doi:10.1016/j.ymeth.2014.08.016.

OPAL FLUOROPHORE EXCITATION AND EMISSION SPECTRA			
Fluorophore	**Excitation**	**Emission**	**Cap Color**
Spectral DAPI	368 nm	461 nm	Blue
Opal Polaris 480	450 nm	500 nm	Violet
Opal 520	494 nm	525 nm	Green
Opal 570	550 nm	570 nm	Red
Opal 620	588 nm	616 nm	Amber
Opal 690	676 nm	694 nm	Clear
Opal Polaris 780	750 nm	770 nm	Pink
Opal™ fluorophores included with each of the three IHC kits, as well as their excitation peak, emission peak, and corresponding cap color.			

Related Products

OPAL MULTIPLEX IHC DETECTION KITS		
Component	**Sizes**	**Product #**
Opal 4-Color Automation IHC Kit*	50 slides	NEL820001KT
Opal 7-Color Automation IHC Kit*	50 slides	NEL821001KT
Opal 7-Color Automation IHC Kit*	50 slides	NEL871001KT
Opal 4-Color anti-Rabbit Automation IHC Kit*	50 Slides	NEL830001KT
Opal 4-Color Manual IHC Kit	50 slides	NEL810001KT
Opal 7-Color Manual IHC Kit	50 slides	NEL811001KT
Opal 7-Color Manual IHC Kit	50 slides	NEL861001KT
Opal 4-Color anti-Rabbit Manual IHC Kit	50 Slides	NEL840001KT
Opal 4 Lymphocyte Kit	50 slides	OP4LY2001KT
Opal 7 Immunology Discovery Kit	50 slides	OP7DS2001KT
Opal 7 Tumor Infiltrating Lymphocyte Kit	50 slides	OP7TL3001KT
Opal 7 Solid Tumor Immunology Kit	50 slides	OP7TL4001KT
*Optimized for Leica Biosystems BOND RX System		

OPAL REAGENTS		
Component	**Sizes**	**Product #**
Opal Polaris 480	50 slides	FP1500001KT
Opal 520 Reagent	50 slides	FP1487001KT
Opal 540 Reagent	50 slides	FP1494001KT
Opal 570 Reagent	50 slides	FP1488001KT
Opal 620 Reagent	50 slides	FP1495001KT
Opal 650 Reagent	50 slides	FP1496001KT
Opal 690 Reagent	50 slides	FP1497001KT
Opal Polaris 780	50 slides	FP150001KT
Spectra DAPI	50 slides	FP1501001KT

Related Products (continued)

ANCILLARY	
Component	**Product #**
1X Plus Automation Amplification Diluent 1 X 50 mL	FP1609
AR6 buffer (10X) 4 x 250 mL	AR6001KT
AR6 buffer (10X) 250 mL	AR600250ML
AR9 buffer (10X) 4 x 250 mL	AR9001KT
AR9 buffer (10X) 250 mL	AR900250ML
Antibody Diluent / Block 100 mL	ARD1001A
Opal Polymer HRP Ms + Rb 50 mL	ARH1001A
Opal Polymer anti-Rb HRP Kit	ARR1001KT

Troubleshooting	https://www.akoyabio.com/support/ | support@akoyabio.com

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*Vectra® 3 has been discontinued and replaced by the new PhenoImager™ Fusion Instrument.
Contact us for details or visit akoyabio.com/phenoimager.

To learn more visit AKOYABIO.COM or email us at INFO@AKOYABIO.COM

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