For Research Use Only. Not for use in diagnostic procedures.

Opal Polaris™ 7-Color Manual IHC Kit (NEL861001KT)

Product Information

Storage Store kits in the dark at 4 ºC.

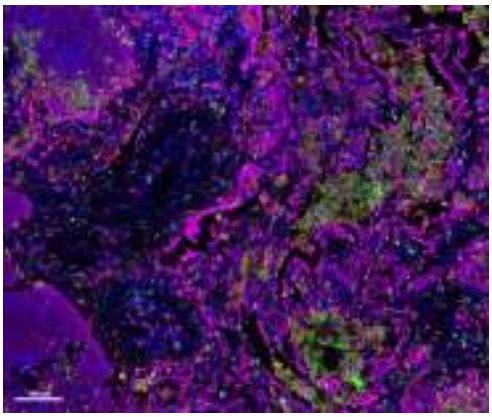
Stability See kit label on outside of box for expiration date

Application The Opal Polaris 7-Color kit is intended for multiplex fluorescent IHC.

Safety Note DMSO 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.

What is the Opal Method?



Human Tonsil section was stained with Opal Polaris 7-Color Manual IHC Kit and imaged on the Vectra Polaris

Target	*Color*
CD8	*Opal Polaris 480*
PD-L1	*Opal 520*
FoxP3	*Opal 570*
CD68	*Opal 620*
PD-1	*Opal 690*
PanCK	*Opal Polaris 780*

The Opal workflow allows simultaneous detection of multiple biomarkers in tissue. This Opal protocol was written specifically for Opal Polaris 7-Color Manual IHC on formalin fixed paraffin embedded (FFPE) tissue*. The approach involves detection with Opal reactive fluorophores, followed by microwave treatment (MWT) for: removal of primary and secondary antibodies[1]; removal of any non-specific staining; and reduction of tissue auto-fluorescence. The Opal signal is largely unaffected by MWT and antibody removal. After MWT, 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: since it's an antibody based staining step, MWT can **NOT** be performed after Opal Polaris 780 binds to the tissue. Thus, please assign Opal Polaris 780 staining to the last cycle of your staining protocol.

Opal allows staining of multiple IHC targets using unlabeled primary antibodies raised in the same species[2]. Combining Opal with multispectral imaging and analysis enables simultaneous, quantitative results for up to 6 biomarkers in fluorescence, even with co-localized markers, plus nuclear counterstain (DAPI). Opal Polaris 7-Color assay is specially designed to work with Vectra Polaris imaging system. **It is not compatible with Vectra® 3 and may need additional components for imaging on Mantra.**

***Please contact us if you would like to work with other types of samples. PerkinElmer provides assistance with assay development and offers multiplex Opal IHC and IF services. Visit: www.perkinelmer.com/Opal.**

Materials Provided

	Format*	Catalog #	Kit Components
Opal Polaris 7-Color Manual IHC Kit	50 slides	NEL861001KT	• 1X Plus Amplification Diluent (50 mL X2) • Opal Polaris 480 Fluorophore (X1) • Opal 520 Fluorophore (X1) • Opal 570 Fluorophore (X1) • Opal 620 Fluorophore (X1) • Opal 690 Fluorophore (X1) • Opal Polaris 780 Reagent Pack (X1) a. Opal TSA-DIG (X1) b. Opal Polaris 780 (X1) • Spectral DAPI solution (1.5 mL X1) • DMSO (1 x 500 µL) • Opal Polymer HRP Ms+Rb (50 ml X2) • Blocking/Ab Diluent (100 mL X2) • 10X AR6 buffer (250 mL X4)

*The format of the kit is based on ~150 µL of Opal Working Solution (see page 4) per slide.

Reagents and Materials

Required Materials

- Baths and solvents for deparaffinization and rehydration of FFPE tissue. Xylene is recommended for deparaffinization. Histological grade ethanol is required for rehydration.
- Standard microwave oven with carousel, rated at 1,000 W or higher with 10 or more power settings
- Standard staining dishes
- Opal slide processing jars (PerkinElmer catalogue number STJAR4) or equivalent
- Slide incubation/humidity tray
- Hydrophobic barrier pen
- Glass coverslips (No. 1.5)
- Control tissues
- Charged slides

Required Reagents

- Tris Buffered Saline with and Tris Buffered Saline with Tween 20(TBST) wash buffer
- Neutral buffered formalin (NBF)
- Peroxidase-free water. Note: This specification may be met by commercial “cell culture grade” water or ultra-pure (i.e. Milli-Q™) water.
- Antibody diluent & blocking reagent
  o Antibody Diluent / Block (PerkinElmer catalog number ARD1001EA) is recommended

o Other options should be validated independently

- Primary antibodies for targets of interest
- Mounting medium for fluorescence (i.e. ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific).
- AR9 Buffer (PerkinElmer catalog number AR900250ML) may be required for certain antigens requiring a higher pH antigen retrieval buffer

Solutions to prepare

TBS Wash Buffer
25 mM TRIS-HCl, pH 7.5
150 mM NaCl

TBST Wash Buffer
25 mM TRIS-HCl, pH 7.5
150 mM NaCl
0.05% Tween®20 (v/v)

AR6 Buffer Working Solution:
Dilute 10X AR6 buffer at 1:10 with peroxidase-free water.

Primary Antibody Working Solution
Dilute primary antibody in PerkinElmer Antibody Diluent / Block at optimal concentration for Opal detection as determined below.

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 fluorophores.

Opal Working Solution
Reconstitute each Opal Fluorophore (**with the exception of Opal Polaris 780)** in 75 µL of DMSO. Before each procedure, dilute Opal Fluorophore in 1X Amplification Diluent to make Opal Fluorophore Working Solution. Recommend to start diluting the Opal Fluorophore at 1:100 . Optimize your assay according to the Opal Assay Development Guide. Generally, 100-300 µL of Opal Working Solution is required per slide. Discard any unused portion of Opal Working Solution.

Opal Polaris 780 Working Solution
Reconstitute Opal TSA-DIG in 75 µL of DMSO, and Opal Polaris 780 in 300 µl of water. Before the procedure, dilute Opal TSA-DIG in 1X Amplification Diluent at 1:100 to make Opal TSA-DIG Working Solution. Dilute Opal Polaris 780 with Ab diluent/blocking at 1:25 to make a working solution.

DAPI working solution
Add three drops of DAPI solutions into 1ml of TBS. Approximately 150 µL of DAPI Working Solution is required per slide. Discard any unused portion of DAPI Working Solution.

Recommendations

- Use xylene for removal of paraffin from FFPE tissue sections. Do not let slides dry out between steps.
- After dissolving with DMSO, before each use, mix, then spin down the Opal Fluorophore tubes with a standard microcentrifuge to make sure that all of the solution is at the bottom of the tubes.
- A humidified chamber is recommended for all incubation steps (i.e., a damp paper towel in a covered box).
- Drain off as much of the incubation solutions as possible before addition of the next solution, to prevent reagent dilution and uneven staining. Blot area around, but not on, tissue section using absorbent paper.
- Be sure to use enough volume of each reagent to completely cover sections or cells.
- Optimized exposure times for all fluorophores should be between 10 to 30 counts for good spectral unmixing.
- DAPI in this kit is formulated for optimal separation from other fluorophores. Exposure time may be somewhat longer than other DAPI formulations.
- If there is too much signal, dilute the primary antibody or HRP conjugated secondary antibody further.
- Before attempting multiplexed staining, assay conditions for each analyte should be optimized singly with Opal detection.
- Microwave treatment (MWT) as outlined in this protocol performs antigen retrieval, quenches endogenous peroxidases, and removes antibodies from earlier staining procedures.
- This protocol was developed with specified reagents. Other options should be independently validated.

- If the antigen requires higher pH retrieval, it is recommended to purchase PerkinElmer's AR9 Buffer.

Opal Optimization Strategies

Microwave Optimization
Microwave treatment (MWT) is used in the Opal method to quench endogenous peroxidase activity, for antigen retrieval, and to remove antibodies after a target has been detected. Timing for each step in the procedure may have to be modified for the microwave oven that you are using. Slides are placed vertically in an Opal Slide Processing Jar which is then filled to the top (~140 mL) with AR6 or AR9 buffer and covered loosely. One jar is placed in the microwave at a time, near the edge of the carousel to ensure even distribution of energy. The microwave procedure consists of two steps:

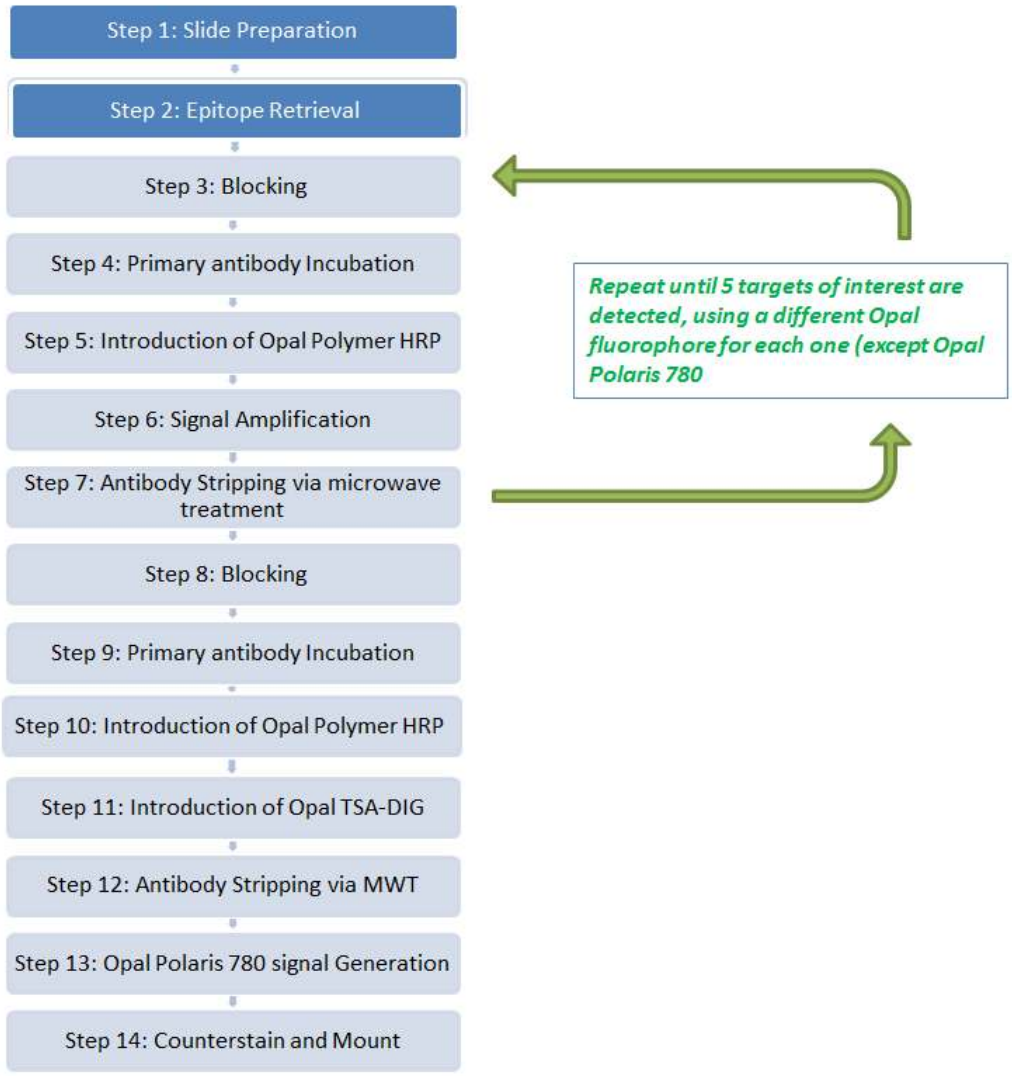
1. 100% power until the boiling point is reached. The time for this step may have to be increased or decreased depending upon the performance of the microwave in your lab. This will usually take 45-90 seconds.
2. 20% power for 15 minutes. Do not operate the microwave unattended and keep the oven chamber clean and clear of debris. Any liquid spilled should be promptly cleaned off with water and paper towel.

Opal Multiplexed IHC

Single analyte Opal IHC methods may be combined for multiplexed detection within a single tissue section. After signal amplification, MWT is performed to strip away detection antibodies. The Opal Fluorophore is largely unaffected by MWT because it is covalently bound. Then the process is repeated using another Opal Fluorophore.

Importantly, a different Opal Fluorophore should be used for each target.

Opal Workflow Schematic



Step by Step Opal IHC Protocol (Single Analyte)

Single analyte Opal IHC assays should be optimized before combining for use in multiplexed detection. Concentration for each primary antibody should be optimized with the selected Opal Fluorophore to yield 10 to 30 brightness counts in inForm. Optimized single fluorophore images (without DAPI counterstain) will subsequently be used for spectral library development.

The following protocol details the workflow for a single analyte and can subsequently be employed for multiplexed IHC. In multiplexed IHC, the order of target/fluorophore detection may be a point of optimization and must be independently validated.

Step 1: Slide Preparation
Prepare tissues or cells for detection with Opal kits using standard fixation and embedding techniques. We recommend running an isotype control slide with all steps replacing the primary antibody with corresponding isotype control for each experiment. For each slide, baked in the oven at 65 C for 1 hour; dewax with xylene (3 x 10 min) and rehydrate through a graded series of ethanol solutions: (100% 1 x 10 min; 95% 1 x 10 min; and rinse in 70%). After rehydration, briefly rinse slides in distilled water and fix in 10% neutral buffered formalin (NBF) for 20 min. Longer times of fixation in NBF may be needed for certain tissues such as skin.

Rinse slides in distilled water and then in the appropriate AR buffer

Step 2: Microwave treatment
Place slides in an Opal Slide Processing Jar and fill it completely with the appropriate AR buffer. Loosely cover the jar with lid, place it in microwave for 45 secs at 100% power; (may require optimization as described). Microwave for an additional 15 min at 20% power. Allow slides to cool down at room temperature before proceeding (15 – 30 min). Importantly, do not let slides dry out. Rinse slides in distilled water followed by TBST.

Step 3: Blocking
Use a hydrophobic barrier pen to completely surround the tissue section on the slide. Cover tissue sections with blocking buffer and incubate slides in a humidified chamber for 10 min at room temperature.

- *Note: This protocol was developed with PerkinElmer Antibody Diluent / Block for blocking. Other options should be independently validated.*

Step 4: Primary Antibody Incubation
Drain off the blocking buffer and apply Primary Antibody Working Solution. Incubate according to the manufacturer's instructions regarding incubation time and temperature requirements or conditions optimized within your lab. Use enough volume to completely cover the tissue section (generally 100-300 µL per slide).

Rinse slides in TBST. Wash the slides 3 x 2 min in TBST at room temperature preferably with agitation.

Step 5: Introduction of Opal Polymer HRP
Incubate slides in Opal Polymer HRP Ms+Rb for 10 min at room temperature. Use adequate reagent volume to cover the tissue section, generally 100-300 µL per slide.

- *Note: Opal Polymer HRP Ms + Rb is recommended for experiments with human tissue and mouse or rabbit primary antibodies. Other options should be independently validated.*

Rinse slides in TBST. Wash the slides 3 x 2 min in TBST at room temperature preferably with agitation.

Step 6: Opal Signal Generation
Drain off excess wash buffer and pipette 100-300 µL of Opal Working Solution onto each slide. Incubate the slides at room temperature for 10 min.

Wash the slides 3 x 2 min each in TBST Buffer at room temperature with agitation.
Rinse slides in the appropriate AR buffer.

Step 7: Microwave treatment
Place slides in an Opal Slide Processing Jar and fill it completely with the appropriate AR buffer. Loosely cover the jar with lid, place it in microwave for 45 sec at 100% power; (may require optimization as described). Microwave for an additional 15 min at 20% power. Allow slides to cool down at room temperature before proceeding (15 – 30 min). Importantly, do not let slides dry out. Rinse slides in distilled water followed by TBST.

This microwave step strips the primary-secondary- HRP complex allowing introduction of the next primary antibody. For detection of the next target with Opal fluorophores, restart the protocol at Step 3: Blocking.

Once all 5 targets have been labeled, continue to Step 8 to complete ***Opal Polaris 780 labeling****.*

Step 8: Blocking
Cover tissue sections with blocking buffer and incubate slides in a humidified chamber for 10 min at room temperature.

Step 9: Primary Antibody Incubation
Drain off the blocking buffer and apply Primary Antibody Working Solution. Incubate according to the manufacturer's instructions regarding incubation time and temperature requirements or conditions optimized within your lab. Use enough volume to completely cover the tissue section (generally 100-300 µL per slide).

Rinse slides in TBST. Wash the slides 3 x 2 min in TBST at room temperature preferably with agitation.

Step 10: Introduction of Opal Polymer HRP
Incubate slides in Opal Polymer HRP Ms+Rb for 10 min at room temperature. Use adequate reagent volume to cover the tissue section, generally 100-300 µL per slide.

Rinse slides in TBST. Wash the slides 3 x 2 min in TBST at room temperature preferably with agitation.

Step 11: Introduction of Opal TSA-DIG
Drain off excess wash buffer and pipette 100-300 µL of Opal TSA-DIG Working Solution onto each slide. Incubate the slides at room temperature for 10 mins.
Wash the slides 3 x 2 min each in TBST Buffer at room temperature with agitation.

Step 12: Microwave treatment
Place slides in an Opal Slide Processing Jar and fill it completely with the appropriate AR buffer. Loosely cover the jar with lid, place it in microwave for 45 sec at 100% power; (may require optimization as described). Microwave for an additional 15 min at 20% power. Allow slides to cool down at room temperature before proceeding (15 – 30 min). Importantly, do not let slides dry out. Rinse slides in distilled water followed by TBST.

Step 13: Opal Polaris 780 Signal Generation
Drain off excess wash buffer and pipette 100-300 µL of Opal Polaris 780 Working Solution onto each slide. Incubate the slides at room temperature for 1 hour.

Wash the slides 3 x 2 min each in TBST Buffer at room temperature with agitation.

DO NOT perform microwave treatment after this step.

Step 14: Counterstain and Mount
Apply DAPI Working Solution for 5 min at room temperature in a humidity chamber. Wash the slides for 2 min in TBST buffer and then for 2 min in water. Coverslip slides with mounting medium (i.e. ProLong® Diamond Antifade Mountant (Thermofisher)). (Note: do not counterstain monoplex slides to be used for spectral library development.)

Imaging and Analysis

Visualization of 7-color Opal slides can be performed using Vectra Polaris Quantitative Pathology Imaging Systems. The systems use multispectral imaging for quantitative unmixing of many fluorophores and tissue autofluorescence, enabling advanced analysis including *in situ* cellular phenotyping. For more information, please see: http://www.perkinelmer.com/quantitative-pathology.

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.

Troubleshooting

Technical Support Resources

- **Assay Support Knowledge Base:** **www.perkinelmer.com/askTSA**
- **Email:** **global.techsupport@perkinelmer.com**
- **Telephone**
  - **USA toll-free** **800-762-4000**
  - **EU toll-free** **00800 33 29 0000**
  - **China toll-free** **800 820 5046**
  - **Local contact numbers:** **http://www.perkinelmer.com/corporate/locations**

IHC Troubleshooting

PROBLEM	REMEDY
Low Signal	• Titer primary and/or secondary antibodies to determine optimum concentration for Opal detection. • Increase Opal Working Solution incubation time. (Suggested range is 3-10 minutes.) • Use microwave treatment to unmask the target.
Excess Signal	• Decrease concentration of primary antibody. • Decrease concentration of secondary antibody HRP conjugates. • Decrease Opal Working Solution incubation time. (Suggested range is 3-10 minutes.)
High Background	• Confirm that microwave treatment step has fully quenched endogenous peroxidases. (See AR – Microwave Optimization procedure, page 3.) • Titer primary and/or secondary antibodies to determine optimum concentration for Opal detection. • Filter buffers. • Evaluate laboratory water source for contamination. • Check for endogenous biotin (if using streptavidin conjugates) • Increase number and/or length of washes. • Evaluate other blocking reagents.

Opal Fluorophore Excitation and Emission Maxima

Fluorophore	Excitation	Emission	Cap color
Spectral DAPI	368 nm	461 nm	Blue
Opal Polaris 480	450 nm	500 nm	Violet
Opal520	494 nm	525 nm	Green
Opal570	550 nm	570 nm	Red
Opal620	588 nm	616 nm	Amber
Opal690	676 nm	694 nm	Clear
Opal Polaris 780	750 nm	770 nm	Pink

Related Products

Opal Multiplex IHC Detection Kits

	SIZES	PRODUCT NUMBER
Opal 4-Color Automation IHC Kit*	50 slides	NEL820001KT
Opal 7-Color Automation IHC Kit*	50 slides	NEL821001KT
Opal 4-Color Manual IHC Kit	50 slides	NEL810001KT
Opal 7-Color Manual IHC Kit	50 slides	NEL811001KT
Opal 4-Color anti-Rabbit Automation IHC Kit*	50 Slides	NEL830001KT
Opal 4-Color anti-Rabbit Manual IHC Kit	50 Slides	NEL840001KT
Opal 4-Color 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 Reagent Packs

	PRODUCT NUMBER
Opal 520 Reagent Pack	FP1487001KT
Opal 540 Reagent Pack	FP1494001KT
Opal 570 Reagent Pack	FP1488001KT
Opal 620 Reagent Pack	FP1495001KT
Opal 650 Reagent Pack	FP1496001KT
Opal 690 Reagent Pack	FP1497001KT

Ancillary

	PRODUCT NUMBER
1X Plus Automation Amplification Diluent 1 X 50 mL	FP1609
1X Plus Amplification Diluent 1 x 50 mL	FP1498
Opal Polymer anti-Rabbit HRP Kit	ARR1001KT
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

For the latest product listings, please go to www.perkinelmer.com/opal.