Research Use Only. Not for use in diagnostic procedures.

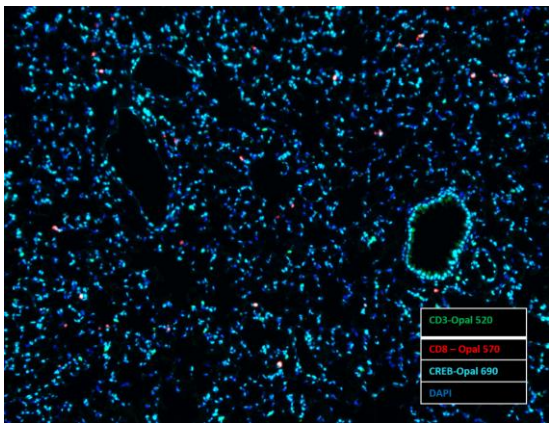
Opal™ 4-Color anti-Rabbit Manual IHC Kit 50 slides

Product Information

Storage	Store dry Opal reagent at -20 ºC. Upon reconstituting in DMSO, store at 2–8ºC. Store remaining kit components at 2–8ºC
Stability	This product is stable for a minimum of 3 months when stored at 2–8 °C
Application	The Opal 4-Color anti-Rabbit Manual IHC 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.
Slide Number	When using this kit's recommended Opal dilution of 1:100, it will enable a 4-color assay on 50 slides.

What is the Opal Method?

The Opal workflow allows simultaneous detection of multiple biomarkers in tissue. This Opal protocol was written specifically for a 4 or 7-Color IHC in formalin-fixed paraffin-embedded (FFPE) tissue. The approach involves detection with Opal 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.



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). **Fluorescent multispectral imaging (usually with the Mantra™ or Vectra® systems) is required for successful analysis of more than 3 Opal fluorophores at once.**

PerkinElmer provides assistance with assay development and offers multiplex Opal IHC and IF services. Visit: www.perkinelmer.com/Opal.

Material Provided

	Format*	Catalog #	Kit Components
Opal 4-Color anti-Rabbit Manual IHC Kit	50 slides	NEL840001KT	• 1X Plus Amplification Diluent (1 X 50 mL) • Opal 520 Fluorophore • Opal 570 Fluorophore • Opal 690 Fluorophore • DMSO (1 X 500 µL) • Spectral DAPI solution (1 X 1.5 mL) • Opal Polymer anti-Rabbit HRP (1 X 10mL) • Opal Polymer anti-Rabbit HRP Diluent (1x 40mL) • Blocking/Ab Diluent (1 X 100 mL) • 10X AR6 buffer (2 X 250ml)

*The format of the kit is based on ~150 µL per slide of Opal Working Solution.

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 (Perkin Elmer catalogue number STJAR4) or equivalent
- Slide incubation/humidity tray
- Hydrophobic barrier pen
- Glass coverslips (No. 1.5)
- Control tissues
- Charged slides

Required Reagents

- 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
  - Antibody Diluent / Block (PerkinElmer catalog number ARD1001EA) is recommended
  - Mouse tissue often requires more robust blocking methods, such as goat or horse serum.
  - Other options should be validated independently
- Primary antibodies for targets of interest
- Mounting medium for fluorescence (i.e. ProLong® Diamond Antifade Mountant (Thermofisher)).
- AR9 Buffer (PerkinElmer catalog number AR900250ML) may be required for certain antigens requiring a higher pH antigen retrieval buffer

Solutions to prepare

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.

Antibody Diluent
Antibody Diluent / Block from PerkinElmer is supplied as a ready-to-use solution.

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 anti-Rabbit HRP is supplied as a concentrate. Working dilution recommendation for manual assays is 1:5 in the supplied Opal Polymer anti-Rabbit HRP Diluent.

Opal Reagent Stock Solution
Dissolve the Opal Reagents in 75 uL of DMSO. Carefully dispense DMSO along the sides of the vial several times to dissolve any Opal Reagent that might coat the sides of the vial. (Minimize bubbles while mixing.)

Opal Fluorophore Working Solution
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 on Opal Assay Development Guide. Generally, 100-300 µL of Opal Working Solution is required per slide. Discard any unused portion of Opal Working Solution.

DAPI working solution
Add two drops of DAPI solution into 1ml of TBST. 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.
- Before the first use, 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.
- 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.
- Optimize your monoplex staining according to the Opal Assay Development Guide, with all MWT steps incorporated.
- Spectral DAPI in this kit is formulated for optimal separation from other fluorophores. Exposure time may be somewhat longer than other DAPI formulations.
- Before attempting multiplexed staining, assay conditions for each analyte should be optimized singly with Opal detection (refer to Opal Assay Development Guide for more details).
- 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.

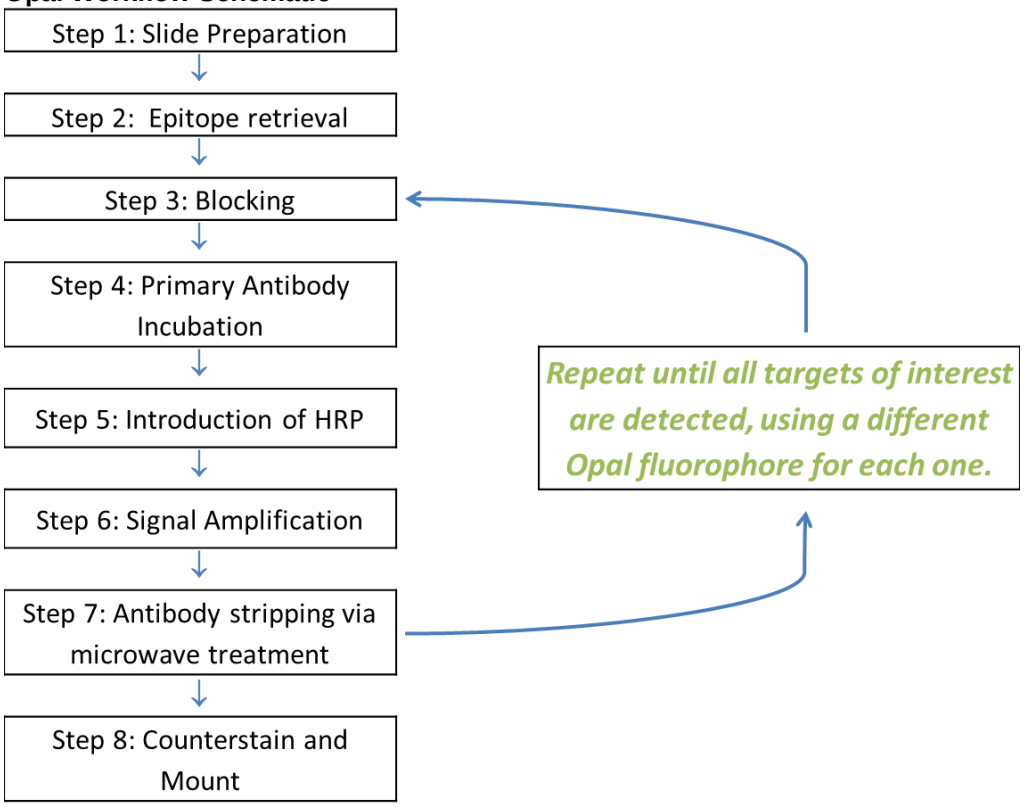
Do not operate the microwave unattended and keep the oven chamber clean and clear of debris.

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) Refer to Opal Assay Development Guide for more detailed instruction:

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 intensity between 10 to 30 counts. 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 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 65^0C 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 for 20min. 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 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 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 Secondary-HRP
Incubate slides in the working solution Opal Polymer anti-Rabbit HRP for 10 min at room temperature. Use adequate reagent volume to cover the tissue section, generally 100-300 µL per slide.

- *Note: Opal Polymer anti-Rabbit HRP working concentration is recommended at 1:5 with the provided Opal Polymer Diluent. Other concentrations need to be 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 Fluorophore Working Solution onto each slide. Incubate the slides at room temperature for 10 mins.

Rinse slides in TBST. Wash the slides 3 x 2 min in TBST at room temperature preferably 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 mins 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, restart the protocol at Step 3: Blocking.

If all targets have been detected, continue to Step 8.

Step 8: 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 4- or 7-color Opal slides can be performed using Mantra or Vectra 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.

Important Notes:

1. All standard Vectra or Mantra epi-fluorescent cubes should be used for imaging Opal slides: DAPI, FITC, CY3, Texas Red, and CY5.
2. If the Opal fluorophores are not found in the Stain Store Manager in your version of inForm, please visit http://www.perkinelmer.com/resources/software-downloads.xhtml to download the latest inForm update that contains the novel Opal stains. If you believe that you have the latest version of inForm and you still cannot find the stains in the store manager, please contact your Field Application Specialist.

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

- **Email:** **global.techsupport@perkinelmer.com**
- **Telephone**
  - **USA toll-free** **800-762-4000**
  - **Worldwide** **+1 203-925-4602**
  - **Fax** **+1 203-944-4904**
  - **Local contact numbers:** **http://www.perkinelmer.com/corporate/locations**

IHC Troubleshooting

PROBLEM	REMEDY
Low Signal	• Titer primary and Opal dye to determine optimum concentration for Opal detection. • Increase primary antibody and Opal Working Solution incubation time. (Suggested range is 3-10 minutes.) • Use additional rounds of microwave treatment to unmask the target.
Excess Signal	• Decrease concentration of primary antibody. • Decrease concentration of Opal dyes • Decrease Opal Working Solution incubation time. (Suggested range is 3-10 minutes.)
High Background	• Confirm that microwave treatment step has fully quenched endogenous peroxidases. • Titer primary and/or Opal dyes to determine optimum concentration for Opal detection. • Freshly prepare buffers. • Evaluate laboratory water source for contamination with HRP. • Check for endogenous biotin (if using streptavidin conjugates) • Increase number and/or length of washes.

Opal Fluorophore Excitation and Emission Maxima

Fluorophore	Opal Multicolor IHC Kits		Excitation	Emission	Cap color
	4-color	7-color			
Spectral DAPI	✓	✓	358nm	461 nm	Blue
Opal520	✓	✓	494 nm	525nm	Green
Opal540		✓	523nm	536nm	Yellow
Opal570	✓	✓	550 nm	570 nm	Red
Opal620		✓	588 nm	616 nm	Amber
Opal650		✓	627 nm	650 nm	Orange
Opal690	✓	✓	676nm	694 nm	Clear

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 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.

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