Automated / Manual

Multiplex IHC

Research Use Only. Not for use in diagnostic procedures.

Opal™ Polaris 480 Reagent Pack (FP1500001KT)

Materials Provided

- Opal Polaris 480 Reagent, 1 vial
- Dimethyl sulfoxide, DMSO, 1 x 100 uL

Please note that Opal Polaris 480 Reagent is provided dry. DMSO has been included to reconstitute this dye. Please follow the protocol provided below.

Product Information

Protocol

1. Dissolve the Opal Polaris 480 Reagent in 75 uL of DMSO

2. Carefully dispense DMSO along the sides of the vial and mix several times to dissolve any Opal Reagent that might coat the sides of the vial. Wait at least 20 minutes before use to allow the entire solution to dissolve.
   Minimize bubbles while mixing.

Storage Store dry reagent in the dark at -20 ºC. Upon reconstituting in DMSO, store in the dark at 4ºC.

Stability See label on outside of box for expiration date.

Safety Note DMSO is classified as hazardous and combustible. 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.

Opal Fluorophore Working Solution
Before each procedure, dilute Opal Fluorophore in 1X Plus Amplification Diluent for manual staining or 1X Plus Automation Amplification Diluent for automation to make Opal Fluorophore Working Solution. The following dilution ratios are recommended:

- 1:100 for manual kits
- 1:150 for automated kits
  Generally, 100-300 µL of Opal Working Solution is required per slide. Discard any unused portion of Opal Working Solution.

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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 an exposure time of 50 – 250ms. or have an intensity between 5 to 30. 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 at65^{0}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 for 20min. Longer times of fixation in NBF (neutral balanced formalin) 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 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.*

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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 slides stained with Opal Polaris 480 can be performed using Vectra Polaris or Mantra (with additional filter cube). 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.

Troubleshooting

Technical Support Resources

- **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**

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