Research Use Only. Not for use in diagnostic procedures.

Opal™ 7 Solid Tumor Immunology Kit

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 See kit label on outside of box for expiration date.

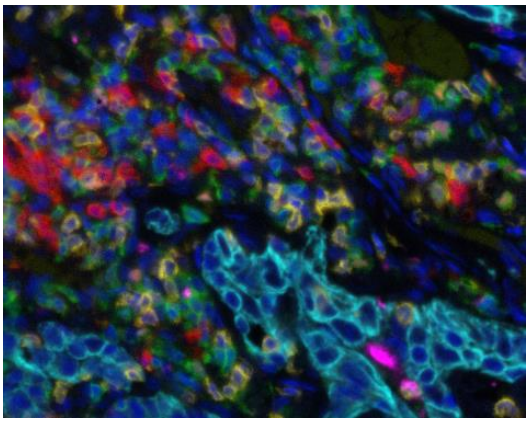
Application The Opal 7 Solid Tumor Immunology Kit is intended for detection of four lymphocyte markers and one macrophage marker in tumor FFPE sections. The Opal 7 Solid Tumor Immunology Kit is optimized for manual use.

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.

What is the Opal Method?



Human lung cancer tissue imaged on the Mantra™ quantitative pathology workstation.

Target	*Color*
CD4	*Green*
CD8	*Yellow*
CD20	*Red*
FoxP3	*Orange*
CD68	*Magenta*
Pan-Cytokeratin	*Cyan*

The Opal workflow allows simultaneous detection of multiple biomarkers in tissue. This Opal protocol was written specifically for 7-Color IHC in formalin fixed paraffin embedded (FFPE) tissue to detect 4 lymphocyte markers and one macrophage marker on tumor FFPE sections.* 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.

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 7 fluorophores at once.**

***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.**

Material Provided

Description	Format*	Catalog #	Kit Components
Opal 7 Solid Tumor Immunology Kit	50 slides	OP7TL4001KT	• 1X Plus Amplification Diluent (1X 50mL) • Opal 520 Fluorophore • Opal 540 Fluorophore • Opal 570 Fluorophore • Opal 620 Fluorophore • Opal 650 Fluorophore • Opal 690 Fluorophore • Spectral DAPI solution (1 X 1.5mL) • DMSO (2 X 500 µL) • 10X AR6 buffer (1 X 250ml) • 10X AR9 buffer (1 X 250ml) • Antibody Diluent (1 X 50ml) • Anti-CD4, anti-CD8, anti-CD20, anti-CD68, anti-FoxP3 and anti-Pan Cytokeratin antibodies (human reactivity) • Opal Polymer HRP Ms + Rb (50 mL)

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

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 1000W or higher with 10 or more power settings.
- Standard staining dishes.
- Opal Slide Processing Jars (STJAR4).
- Slide incubation/humidity tray.
- Hydrophobic barrier pen.
- Orbital platform shaker (optional).
- Glass coverslips.
- Control tissues.
- Charged slides

Required Reagents

- 10% Neutral buffered formalin (NBF)
- TBST wash buffer.
- Peroxidase-free water.
  Note: 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 for fluorescence.

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.

AR9 Buffer Working Solution:
Dilute 10X AR9 buffer at 1:10 with peroxidase-free water.

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

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

Secondary Antibody Working Solution
Opal Polymer HRP Ms + Rb comes as a ready-to-use solution and does not need to be optimized for use with Opal fluorophores.

Opal Fluorophore Working Solution
Dissolve the Opal Reagents in 75µL 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.*

Before each procedure, dilute Opal Fluorophore 1:50 in 1X Amplification Diluent to make Opal Fluorophore Working Solution. Generally, 150 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 each use, spin down the Opal Fluorophore tubes and primary antibody 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 on the Mantra or Vectra systems for all fluorophores should be between 50 msec and 200 msec for good spectral unmixing.
- Spectral 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 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.

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 oven unattended and keep the oven chamber clean and clear of debris.

Primary Antibody Optimization
Opal Monoplex IHC assays should be optimized individually before combining for use in multiplexed detection. The optimal primary antibody concentration will result in highest signal to background ratio observed in fluorescence microscopy, with roughly similar signal levels for each target. Vectra and Mantra autoexposure times for optimized assays should fall between 50 and 200 msec for all targets.

Follow the suggested optimization steps below:
- Slide 1: use the suggested dilution rate in table 1.
- Slide 2-3: use half log (3.16-fold) serial dilutions from suggested dilution. Further dilution or less dilution than suggested dilution may be necessary.
- Slide 4: negative control (primary antibody omitted).

Table 1: Suggested dilution for primary antibodies

Antigen retrieval	Antibody	Suggested final concentration (ng/ml)	Incubation time	Opal Fluorophore
AR9	CD4	70	30 min	Opal 520
AR9	CD8	120	30 min	Opal 570
AR6	CD20	100	30 min	Opal 540
AR6	FoxP3	700	30 min	Opal 620
AR6	CD68	15	30 min	Opal 650
AR6	CKPan	400	30 min	Opal 690

If autoexposure times are too long under evaluated conditions, consider extending the incubation time or reducing primary antibody concentration

Slide Definitions
Successful optimization of a multiplex assay requires three different types of slides.
- **Monoplex.** Control tissue slides are labeled for one marker with a single Opal fluorophore and counterstained with DAPI. They are necessary for assessment of staining results and comparison to established standards.
- **Library.** Spectral library slides stained with a single fluorophore and made from relevant control tissue will be necessary for accurate unmixing and analysis of your monoplex and multiplex slides. The following slides will be required.
    - One control tissue slide stained for each antibody-Opal fluorophore combination under optimized conditions, without DAPI. Six single stained slides: CD4-Opal 520, CD20-Opal 540, CD8-Opal 570, FoxP3-Opal 620, CD68-Opal 650 and Pan cytokeratin-Opal 690 stained slides **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.
- **Multiplex.** Tissue slides are labeled for all of the markers in the multiplex panel and counterstained with DAPI. These slides allow assessment of the multiplex assay for interference and crosstalk.

Step by Step Opal IHC Protocol:

Please consult the Opal Assay Development Guide prior to beginning an Opal Mutliplex Protocol: http://www.perkinelmer.com/category/cancer-immunotherapy-immunology

The full Opal 7-color protocol generally takes 2 days to complete. Here are some points where the procedure may be paused.

- After microwave treatment, cooling in AR buffer at room temperature.
- Store in Antibody Diluent or blocking buffer at 4^0C.
- Overnight primary antibody incubation at 4^0C. (Antibody incubation conditions must be validated for the assay.)
- Store slides in TBS or TBST overnight at 4^0C after each staining circle.

Opal Monoplex Protocol

1. **Deparaffinization.** Heat slides in a dry oven at 55-60 °C for four hours, positioned to allow drainage of melting paraffin. Wash slides with xylene for 10 minutes, 3 times. Hydrate through an ethanol gradient ending with a distilled water wash.
2. **Slide Fixation.** Fix tissue in NBF for 20 minutes followed by a distilled water wash.
3. **Antigen Retrieval/Microwave treatment (MWT).** Rinse slides with AR6 or AR9 solution as appropriate for the target being detected. Place slides in an Opal slide processing jar and fill with AR6 or AR9 solution immediately to prevent the slides from drying out. Perform MWT under optimized conditions as determined previously and allow slides to cool down to room temperature on the bench for at least 15 minutes.
4. **Blocking.** Rinse slides with TBST. Encircle the area of tissue to be stained with a hydrophobic barrier pen. Incubate the tissue with Antibody Diluent for 10 minutes at room temperature.
5. **Primary Antibody Incubation.** Remove the blocking solution and then apply the primary antibody solution to the tissue (Suggested dilution for each antibody showed in the above table). Antibody concentration and incubation time must be optimized for Opal detection.
6. **Secondary Antibody Incubation.** Apply the secondary antibody solution to the tissue. Opal Polymer HRP should be incubated for 10 minutes at room temperature. Wash slides with TBST 3 times, 2 minutes each.
7. **Opal Fluorophore Incubation.** Apply Opal fluorophore solution to the tissue and incubate for 10 minutes at room temperature. Wash slides with TBST 3 times, 2 minutes each.
8. **Antibody Removal.** Rinse slides twice with AR6 solution . Place slides in an Opal slide processing jar with AR6 solution and fill up to the top. Perform MWT and allow slides to cool down to room temperature on the bench for at least 15 minutes .
9. **Spectral DAPI.** Rinse slides with TBST. Incubate slides in DAPI solution for 5 minutes at room temperature. Wash slides with TBST for 2 minutes, and then with distilled water for 2 minutes. (Skip this step where appropriate for single stain library slides.)
10. **Mount.** Apply mounting medium for fluorescence microscopy and coverslip.

Opal Multiplex Protocol

Optimized Opal Monoplex 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 as outlined below.

A checklist to follow as you perform the protocol is on page 9.

1. Deparaffinization. Heat slides in a dry oven at 55-60 °C for four hours, positioned to allow drainage of melting paraffin. Wash slides with xylene for 10 minutes, 3 times. Hydrate through an ethanol gradient ending with a distilled water wash.

2. Slide Fixation. Fix tissue in NBF for 20 minutes followed by a distilled water wash.

3. Multiplex staining of six markers:

Cycle 1:

C1.1. **Antigen Retrieval/MWT.** Rinse slides with AR9 solution. Place slides in an Opal slide processing jar with AR9 solution and fill up to the top. Perform MWT under optimized conditions as determined previously and allow slides to cool down to room temperature on the bench for at least 15 min.
C1.2. **Blocking.** Rinse slides with TBST. Encircle the area of tissue to be stained with a hydrophobic barrier pen. Incubate the tissue with Antibody Diluent for 10 min at room temperature (RT).
C1.3. **Primary Antibody Incubation.** Remove the blocking solution and then apply the anti-CD4 antibody solution to the tissue using the optimized concentration and incubation time determined previously. Wash slides with 1X TBST for 3 times at 2 min each.
C1.4. **Secondary Antibody Incubation.** Apply Opal Polymer HRP secondary antibody solution to the tissue and incubate for 10 min at RT. Wash slides with 1X TBST for 3 times, 2 min each.
C1.5. **Opal Fluorophore Incubation.** Apply Opal-520 working solution to the tissue and incubate for 10 min at RT . Wash slides with TBST 3 times, 2 minutes each.
C1.6. **Microwave Treatment.** Rinse slides with AR9. Place slides in an Opal slide processing jar with AR9 solution and fill up to the top. Perform MWT as previously and allow slides to cool down to room temperature on the bench for at least 15 minutes.

Cycle 2:

C2.1. **Blocking.** Rinse slides with TBST. Encircle the area of tissue to be stained with a hydrophobic barrier pen. Incubate the tissue with Antibody Diluent for 10 min at room temperature (RT).
C2.2. **Primary Antibody Incubation.** Remove the blocking solution and then apply the anti-CD8 antibody solution to the tissue using the optimized concentration and incubation time determined previously. Wash slides with 1X TBST for 3 times at 2 min each.
C2.3. **Secondary Antibody Incubation.** Apply Opal Polymer HRP secondary antibody solution to the tissue and incubate for 10 min at RT. Wash slides with 1X TBST for 3 times, 2 min each.
C2.4. **Opal Fluorophore Incubation.** Apply Opal-570 working solution to the tissue and incubate for 10 min at RT . Wash slides with TBST 3 times, 2 minutes each.
C2.5. **Microwave Treatment.** Rinse slides with AR6. Place slides in an Opal slide processing jar with AR6 solution and fill up to the top. Perform MWT as previously and allow slides to cool down to room temperature on the bench for at least 15 minutes.

Cycle 3:

C3.1. **Blocking.** Rinse slides with TBST. Encircle the area of tissue to be stained with a hydrophobic barrier pen. Incubate the tissue with Antibody Diluent for 10 min at room temperature (RT).
C3.2. **Primary Antibody Incubation.** Remove the blocking solution and then apply the anti-CD20 antibody solution to the tissue using the optimized concentration and incubation time determined previously. Wash slides with 1X TBST for 3 times at 2 min each.
C3.3. **Secondary Antibody Incubation.** Apply Opal Polymer HRP secondary antibody solution to the tissue and incubate for 10 min at RT. Wash slides with 1X TBST for 3 times, 2 min each.
C3.4. **Opal Fluorophore Incubation.** Apply Opal-540 working solution to the tissue and incubate for 10 min at RT . Wash slides with TBST 3 times, 2 minutes each.
C3.5. **Microwave Treatment.** Rinse slides with AR6. Place slides in an Opal slide processing jar with AR6 solution and fill up to the top. Perform MWT as previously and allow slides to cool down to room temperature on the bench for at least 15 minutes.

Cycle 4:

C4.1. **Blocking.** Rinse slides with TBST. Encircle the area of tissue to be stained with a hydrophobic barrier pen. Incubate the tissue with Antibody Diluent for 10 min at room temperature (RT).
C4.2. **Primary Antibody Incubation.** Remove the blocking solution and then apply the anti-FoxP3 antibody solution to the tissue using the optimized concentration and incubation time determined previously .Wash slides with 1X TBST for 3 times at 2 min each.
C4.3. **Secondary Antibody Incubation.** Apply Opal Polymer HRP secondary antibody solution to the tissue and incubate for 10 min at RT. Wash slides with 1X TBST for 3 times, 2 min each.

C4.4. **Opal Fluorophore Incubation.** Apply Opal-620 working solution to the tissue and incubate for 10 min at RT . Wash slides with TBST 3 times, 2 minutes each.
C4.5. **Microwave Treatment.** Rinse slides with AR6. Place slides in an Opal slide processing jar with AR6 solution and fill up to the top. Perform MWT as previously and allow slides to cool down to room temperature on the bench for at least 15 minutes.

Cycle 5:

C5.1. **Blocking.** Rinse slides with TBST. Encircle the area of tissue to be stained with a hydrophobic barrier pen. Incubate the tissue with Antibody Diluent for 10 min at room temperature (RT).
C5.2. **Primary Antibody Incubation.** Remove the blocking solution and then apply the anti-CD68 antibody solution to the tissue using the optimized concentration and incubation time determined previously. Wash slides with 1X TBST for 3 times at 2 min each.
C5.3. **Secondary Antibody Incubation.** Apply Opal Polymer HRP secondary antibody solution to the tissue and incubate for 10 min at RT. Wash slides with 1X TBST for 3 times, 2 min each.
C5.4. **Opal Fluorophore Incubation.** Apply Opal-650 working solution to the tissue and incubate for 10 min at RT . Wash slides with TBST 3 times, 2 minutes each.
C5.5. **Microwave Treatment.** Rinse slides with AR6. Place slides in an Opal slide processing jar with AR6 solution and fill up to the top. Perform MWT as previously and allow slides to cool down to room temperature on the bench for at least 15 minutes.

Cycle 6:

C6.1. **Blocking.** Rinse slides with TBST. Encircle the area of tissue to be stained with a hydrophobic barrier pen. Incubate the tissue with Antibody Diluent for 10 min at room temperature (RT).
C6.2. **Primary Antibody Incubation.** Remove the blocking solution and then apply the anti-Pan Cytokeratin antibody solution to the tissue using the optimized concentration and incubation time determined previously. Wash slides with 1X TBST for 3 times at 2 min each.
C6.3. **Secondary Antibody Incubation.** Apply Opal Polymer HRP secondary antibody solution to the tissue and incubate for 10 min at RT. Wash slides with 1X TBST for 3 times, 2 min each.
C6.4. **Opal Fluorophore Incubation.** Apply Opal-690 working solution to the tissue and incubate for 10 min at RT . Wash slides with TBST 3 times, 2 minutes each.
C6.5. **Microwave Treatment.** Rinse slides with AR6. Place slides in an Opal slide processing jar with AR6 solution and fill up to the top. Perform MWT as previously and allow slides to cool down to room temperature on the bench for at least 15 minutes.

4. **Spectral DAPI.** Rinse slides in distilled water and then in TBST. Incubate slides in DAPI solution for five minutes at room temperature. Wash slides with TBST for two minutes, and then with distilled water for two minutes.
5. **Mount.** Apply mounting medium for fluorescence microscopy and coverslip.

Imaging and Analysis

Visualization of 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.

Please see the Phenoptics Assay Development Guide for more detail on imaging and analysis.

Important Notes:

1. All standard Vectra or Mantra epi-fluorescent cubes should be used for imaging Opal slides: DAPI, FITC, CY3, Texas Red, 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**

Related Products

Opal Fluorophore Excitation and Emission Maxima

Fluorophore	Wavelength		Cap color
	Excitation	Emission	
Spectral DAPI	358nm	461 nm	Blue
Opal 520	494 nm	525nm	Green
Opal 540	523nm	536nm	Yellow
Opal 570	550 nm	570 nm	Red
Opal 620	588 nm	616 nm	Amber
Opal 650	627 nm	650 nm	Orange
Opal 690	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 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

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 Amplification Diluent 1 X 50 mL	FP1498A
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	ARD1001EA
Opal Polymer HRP Ms + Rb 50 mL	ARH1001EA
Spectra DAPI	FP1490A

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

Opal 7 Solid Tumor Immunology Kit

o Multiplex slides

	√	Marker 1 **CD4**	√	Marker 2 **CD8**	√	Marker 3 **CD20**	√	Marker 4 **FoxP3**	√	Marker 5 **CD68**	√	Marker 6 **PanCK**	√	Counter Stain **DAPI**
Deparaffinization & Fixation														
AR/MWT		AR9		AR9		AR6		AR6		AR6		AR6		AR6
Wash		TBST		TBST		TBST		TBST		TBST		TBST		TBST
Blocking		10min @RT		10min @RT		10min @RT		10min @RT		10min @RT		10min @RT		
Antibody / Counter Stain		**Anti-CD4**		**Anti-CD8**		**Anti-CD20**		**Anti-FoxP3**		**Anti-CD68**		**Anti-PanCK**		**DAPI**
Dilution														
Inc.Time														5min @ RT
Inc.Temp.														
Wash		TBST		TBST		TBST		TBST		TBST		TBST		
Opal HRP Polymer Ms+ Rb		10min @ RT		10min @ RT		10min @ RT		10min @ RT		10min @ RT		10min @ RT		
Wash		TBST		TBST		TBST		TBST		TBST		TBST		
Opal fluor		Opal 520		Opal 570		Opal 540		Opal 620		Opal 650		Opal 690		
Incubation		10min @ RT		10min @ RT		10min @ RT		10min @ RT		10min @ RT		10min @ RT		
Wash		TBST		TBST		TBST		TBST		TBST		TBST		

Spectral library slides

o Six single stained slides: CD4-Opal 520, CD20-Opal 540, CD8-Opal 570, FoxP3-Opal 620, CD68-Opal 650 and Pan cytokeratin-Opal 690 stained slides **without DAPI.**

o One control tissue slide stained with DAPI alone.

o One AF slide processed in the same way as the multiplex slide, omitting both the Opal fluorophore and DAPI.