RNAscope® Multiplex Fluorescent v2 Assay combined with Immunofluorescence

Introduction

This Technical Note provides guidelines for performing in situ hybridization (ISH) using an RNAscope® Multiplex Fluorescent Reagent Kit v2 (Cat. No. 323100) combined with immunofluorescence (IF) on formalin-fixed paraffin-embedded (FFPE) tissue sections. To detect fluorescent ISH signals, use the RNAscope® Multiplex Fluorescent Kit v2 with the Akoya Biosciences Opal™ fluorophores or TSA® Plus System. To detect fluorescent immunohistochemistry (IHC), use HRP-conjugated secondary antibody with the Akoya Biosciences Opal™ fluorophores or TSA® Plus System. For detailed RNAscope® in situ hybridization on FFPE tissue sections and safety guidelines, refer to the RNAscope® Multiplex Fluorescent Reagent Kit v2 User Manual (Doc. No. 323100-USM). Consult our Technical Notes available at www.acdbio.com/technical-support/user-manuals to prepare other sample types. For every chemical, read the Safety Data Sheet (SDS) and follow handling instructions. Wear appropriate protective eyewear, clothing, and gloves. For the latest services and support information, go to www.acdbio.com/support.

Recommended Fluorophore Combinations

Use the Opal™ fluorophores or TSA® Plus System from Akoya Biosciences to develop the fluorescent IHC signal following the RNAscope® assay. The following combinations are recommended:

### 2-plex ISH combined with fluorescent IHC

<table>
<thead>
<tr>
<th>RNAscope® Multiplex Assay –C1</th>
<th>Opal™ fluorophore</th>
<th>Akoya Bioscience Reagent Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA® Plus fluorochrome</td>
<td>Opal™ 620</td>
<td>FP1495001KT: Opal™ 620 Reagent Pack</td>
</tr>
<tr>
<td>Fluorescent IHC</td>
<td>Opal™ 690</td>
<td>FP1497001KT: Opal™ 690 Reagent Pack</td>
</tr>
</tbody>
</table>

### 3-plex ISH combined with fluorescent IHC

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Opal™ 520</td>
<td>FP1487001KT: Opal™ 520 Reagent Pack</td>
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<tr>
<td>Opal™ 570</td>
<td>FP1488001KT: Opal™ 570 Reagent Pack</td>
<td></td>
</tr>
<tr>
<td>Opal™ 690</td>
<td>FP1497001KT: Opal™ 690 Reagent Pack</td>
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</tbody>
</table>

**IMPORTANT!** You can mix and match channels and fluorophores. Do not assign the same fluorophore to more than one channel.

If the Cy7 filter is available, you can use Opal™ Polaris 780 as the fourth color.

<table>
<thead>
<tr>
<th>RNAscope® Multiplex Assay –C1</th>
<th>Opal™ fluorophore</th>
<th>Akoya Bioscience Reagent Kit</th>
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<tbody>
<tr>
<td>Opal™ 520</td>
<td>FP1487001KT: Opal™ 520 Reagent Pack</td>
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<tr>
<td>Opal™ 570</td>
<td>FP1488001KT: Opal™ 570 Reagent Pack</td>
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<tr>
<td>Opal™ 690</td>
<td>FP1497001KT: Opal™ 690 Reagent Pack</td>
<td></td>
</tr>
</tbody>
</table>
**Opal™ fluorophore**

**Akoya Bioscience Reagent Kit**

**Fluorescent IHC**
- Opal™ Polaris 780
  - FP1501001KT: Opal™ Polaris 780 Reagent Pack

Many users prefer to use fluorescein or Opal™ 520 for immunofluorescent staining. You may use Opal™ Polaris 780 for ISH staining in any of the three channels. The following table displays one workflow example.

<table>
<thead>
<tr>
<th>RNAsecope® Multiplex Assay –C1</th>
<th>Opal™ fluorophore</th>
<th>Akoya Bioscience Reagent Kit</th>
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</thead>
<tbody>
<tr>
<td>FP1488001KT: Opal™ 570 Reagent Pack</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>RNAsecope® Multiplex Assay –C2</th>
<th>Opal™ fluorophore</th>
<th>Akoya Bioscience Reagent Kit</th>
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</thead>
<tbody>
<tr>
<td>FP1497001KT: Opal™ 690 Reagent Pack</td>
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</table>

<table>
<thead>
<tr>
<th>RNAsecope® Multiplex Assay –C3</th>
<th>Opal™ fluorophore</th>
<th>Akoya Bioscience Reagent Kit</th>
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</thead>
<tbody>
<tr>
<td>FP1487001KT: Opal™ 520 Reagent Pack</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fluorescent IHC</th>
<th>Opal™ fluorophore</th>
<th>Akoya Bioscience Reagent Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP1501001KT: Opal™ Polaris 780 Reagent Pack</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**IMPORTANT!** If Opal™ Polaris 780 is assigned to an ISH marker, do not follow it with any other ISH markers. The 780 fluorophore is extremely sensitive to cleavage by HRP activity and must be developed last. Following the IHC protocol, apply Polaris 780 as the last step before counter staining and mounting. For detailed steps, see Appendix A on page 4.

**Workflow**

**Part 1: Prepare and Pretreat Tissues**

To prepare and pretreat formalin-fixed paraffin-embedded (FFPE) samples, follow the instructions in Chapter 3 of the RNAsecope® Multiplex Fluorescent Reagent Kit v2 User Manual (Doc. No. 323100-USM), available at www.acdbio.com/technical-support/user-manuals.

**Part 2: Prepare the Materials**

**Prepare Reagents**

1. Prepare 1X TBS: Add 6.057 g Tris Base and 8.766 g NaCl to 1 L distilled water. Mix until dissolved, and adjust pH to 7.6.
2. Prepare TBST Wash Buffer: Add 500 µl 10% Tween® 20 to 1 L 1X TBS buffer.
3. Prepare TBS-0.1% BSA: Add 1 g BSA to 1 L 1XTBS

**Note:** Use the incubation time recommended by the manufacturer of the primary antibody.

**Prepare TSA® Plus Fluorophores or Opal™ Reagents**

1. Determine the volume of fluorophore needed (approximately 150–200 µL per slide).
2. Dilute the TSA® Plus fluorophore (fluorescein, Cy3 or Cy5) stocks or Opal™ reagent stocks using Multiplex TSA buffer provided in the RNAsecope® Multiplex Fluorescent Kit v2. Recommended dilution range is 1:300–1:1500 for fluorescent IHC.

**Note:** If using Opal™ Polaris 780, dilute Polaris TSA-DIG in TSA buffer and dilute Opal™ Polaris 780 in Antibody Diluent/Block from Akoya (PN: ARD1001EAng).

**Part 3: Run the RNAsecope® Multiplex Fluorescent v2 Assay**

To run the fluorescent ISH assay, follow the instructions in Chapter 4 of the RNAsecope® Multiplex Fluorescent Reagent Kit v2 User Manual (Doc. No. 323100-USM), available at www.acdbio.com/technical-support/user-manuals.

**IMPORTANT!** You must stop after the HRP blocker step. Do not counterstain the slides with DAPI until the IHC assay is finished.

**Part 4: Perform Immunofluorescence**

**IMPORTANT!** Keep the slides covered by using a HybEZ™ Humidity Control Tray, or any other light proof humidity tray, during the IHC assay. Avoid exposing the slides to light as much as possible.

**Block Tissue**

1. Wash the slides 2 x 2 MIN in TBST Wash Buffer with gentle agitation.
2. Incubate tissue in 10% normal serum in TBS-0.1% BSA for 30 MIN at RT, or OVERNIGHT at 4°C. Keep slides covered in HybEZ™ tray to avoid drying.

**Note:** Use serum from the species the secondary antibody was raised in.

**Primary Antibody Staining**

1. Remove the blocking reagents from the slides. DO NOT rinse.
2. Add primary antibody diluted in TBS-0.1% BSA to completely cover the sections. Incubate 45 MIN – 2 HRS at RT.

**Note:** Use the incubation time recommended by the manufacturer of the primary antibody.
3. Rinse slides with TBST wash buffer for **5 MIN at RT**. Gently agitate the slides.
4. Repeat the rinse step twice.

**Secondary Antibody Staining**

1. Add HRP-conjugated secondary antibody diluted in TBS-0.1% BSA to completely cover the sections.
2. Incubate the slides for **30 MIN at RT**.
3. Rinse the slides with gentle agitation in TBST Wash Buffer for **5 MIN at RT**.
4. Repeat the rinse step twice.
5. Add 150–300 µL diluted TSA® Plus fluorophore or Opal™ reagents to completely cover the sections.
6. Incubate the slides in the HybEZ™ Tray for **10 MIN at RT**.
7. Rinse the slides with gentle agitation in TBST Wash Buffer for **2 MIN at RT**.
8. Repeat the rinse step twice.

**Secondary Antibody Staining using Opal™ Polaris 780**

*Note:* The following steps only describe how to use Opal™ Polaris 780 for IHC staining. If Opal™ Polaris 780 is used for an ISH staining prior to IHC, refer to Appendix A for detailed instructions.

1. Add HRP-conjugated secondary antibody diluted in TBS-0.1% BSA to completely cover the sections.
2. Incubate the slides for **30 MIN at RT**.
3. Rinse the slides with gentle agitation in TBST Wash Buffer for **5 MIN at RT**.
4. Repeat the rinse step twice.
5. Add 150–300 µL diluted TSA-DIG reagents to completely cover the sections.
6. Incubate the slides in the HybEZ™ Tray for **10 MIN at RT**.
7. Rinse the slides with gentle agitation in TBST Wash Buffer for **2 MIN at RT**. Repeat with fresh buffer.

**Mount the Slides**

1. Remove excess liquid from the slides, and add ~4 drops of DAPI to each slide. Incubate for **30 SEC at RT**.
2. Remove DAPI and immediately place 1–2 drops of Prolong Gold antifade mounting medium on the slide (not provided).
3. Carefully place a 24 mm x 50 mm glass coverslip over the tissue section. Avoid trapping air bubbles.
4. Dry slides for at least **30 MIN** in the dark before imaging.
5. Store slides at **2–8°C** in the dark for up to two weeks.

**Evaluate the Results**

To image the slides, refer to Chapter 5 of the RNAscope® Multiplex Fluorescent Reagent Kit v2 User Manual (Doc. No. 323100-USM), available at [www.acdbio.com/technical-support/user-manuals](http://www.acdbio.com/technical-support/user-manuals). The RNAscope® assay should produce clear, intense, punctate dots. Single dots may merge into a cluster when highly abundant targets are detected.

**IMPORTANT!** To image 3-plex ISH combined with fluorescent IHC (4-plex fluorescent staining), use a multiplex biomarker imaging system such as the Nuance® EX, Mantra™, or Vectra® System. Please refer to the Perkin Elmer guidelines for imaging.

**Obtaining Support**

For the latest services and support information, go to: [https://acdbio.com/technical-support/support-overview](https://acdbio.com/technical-support/support-overview).

At the website, you can:

- Access telephone and fax numbers to contact Technical Support and Sales.
- Search through FAQs.
- Submit a question directly to Technical Support.

**Figure 1.** Detection of CD279 (Opal 520-White), CD274 (Opal 570-Red), and IFNg (Opal 620-Yellow) using the RNAscope® Multiplex Fluorescent v2 Assay, combined with fluorescent IHC of CD3 (Opal 690-Green) in FFPE human TMA. DAPI staining is shown in blue.
Appendix A. Opal™ Polaris 780 followed by IHC staining

The following workflow uses Opal™ Polaris 780 in the C3 channel followed by IHC staining. To develop the C1 and C2 channels, follow the instructions in Chapter 4 of the RNAscope® Multiplex Fluorescent Reagent Kit v2 User Manual (Doc. No. 323100-USM), available at www.acdbio.com/technical-support/user-manuals.

Workflow

**Prepare and Pretreat Tissues**

**Run the RNAscope® Multiplex Fluorescent v2 Assay up to HRP-C2**

**RNAscope® Multiplex FL v2 HRP-C3 ~15 MIN**

**Polaris TSA-DIG ~30 MIN**

**RNAscope® Multiplex FL v2 HRP blocker ~15 MIN**

**Block Tissue**

**Primary Antibody Staining**

**Secondary Antibody Staining**

**Opal™ Polaris 780 ~30 MIN**

**Counterstain and mount the slides ~10 MIN**

**Evaluate the samples**

**IMPORTANT!** If Opal™ Polaris 780 is assigned to an ISH marker, you must follow a modified protocol in which the steps for developing 780 for ISH must stop after TSA-DIG is applied. Apply Polaris 780 following the IHC protocol, as the last step before counter staining and mounting. The 780 fluorophore is extremely sensitive to cleavage by HRP activity.

**Opal™ Polaris 780 staining: Part A**

1. Remove excess liquid from slides, add 4–6 drops RNAscope® Multiplex FL v2 HRP-C3 to entirely cover each slide.
2. Insert slides into the HybEZ™ Oven for **15 MIN** at **40°C**
3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.
4. Remove excess liquid from slides, and add 150–200 µL diluted TSA-DIG to each slide, and incubate for **30 MIN** at **RT**.
5. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.
6. Remove excess liquid from slides, place in the HybEZ™ or EZ-Batch™ Slide Rack, and add 4–6 drops RNAscope® Multiplex FL v2 HRP blocker from 4-Plex Ancillary Kit For Fluorescent Multiplex v2 (Cat. No. 323120) to entirely cover each slide.
7. Insert slides into the HybEZ™ Oven for **15 MIN** at **40°C**
8. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.

**IHC staining**

Follow the steps for Block Tissue, Primary Antibody Staining, and Secondary Antibody Staining on pages 2–3. Before staining the slides with DAPI, perform the following procedure.

**Opal™ Polaris 780 staining: Part B**

1. Remove excess liquid from the slides, and add 150–200 µL diluted Polaris 780 to each slide.
2. Incubate for **30 MIN** at **RT**.
3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.

Continue with the rest of the procedure, starting with Mount the Slides on page 3.
Image the Slides

For imaging using multiplexed biomarker imaging systems Vectra®, Mantra™, or Polaris, refer to the guidelines from Akoya. The following table lists the corresponding filter settings for each fluorophore:

<table>
<thead>
<tr>
<th>Opal™ fluorophore</th>
<th>Filter setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opal™ 520</td>
<td>FITC</td>
</tr>
<tr>
<td>Opal™ 570</td>
<td>Cy3</td>
</tr>
<tr>
<td>Opal™ 620</td>
<td>Texas Red</td>
</tr>
<tr>
<td>Opal™ 690</td>
<td>Cy5.5</td>
</tr>
<tr>
<td>Opal™ Polaris 780</td>
<td>Cy7</td>
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</tbody>
</table>

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