**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 PerkinElmer TSA® Plus System or Opal™ fluorophores. To detect fluorescent immunohistochemistry (IHC), use HRP-conjugated secondary antibody with the PerkinElmer TSA® Plus System or Opal™ fluorophores. 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 TSA® Plus System or Opal™ dyes from Perkin Elmer to develop RNAscope® Multiplex Fluorescent v2 Assay combined with fluorescent IHC signal. Use the following recommended combinations:

**2-plex ISH combined with fluorescent IHC**

<table>
<thead>
<tr>
<th></th>
<th>Opal™ fluorophore</th>
<th>PerkinElmer Reagent Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAscope® Multiplex Assay – C1</td>
<td>TSA® Plus fluorescein</td>
<td>NEL741001KT</td>
</tr>
<tr>
<td>RNAscope® Multiplex Assay – C2</td>
<td>TSA® Plus Cyanine 3</td>
<td>NEL744001KT</td>
</tr>
</tbody>
</table>

**3-plex ISH combined with fluorescent IHC**

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<thead>
<tr>
<th></th>
<th>Opal™ fluorophore</th>
<th>PerkinElmer Reagent Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAscope® Multiplex Assay – C1</td>
<td>Opal 520</td>
<td>FP1487001KT: Opal 520 Reagent Pack</td>
</tr>
<tr>
<td>RNAscope® Multiplex Assay – C2</td>
<td>Opal 570</td>
<td>FP1488001KT: Opal 570 Reagent Pack</td>
</tr>
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</table>

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

**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 RNAscope® Multiplex Fluorescent Reagent Kit v2 User Manual (Doc. No. 323100-USM), available at www.acdbio.com/technical-support/user-manuals.
Part 2: Run the RNAscope® Multiplex Fluorescent v2 Assay

To run the fluorescent ISH assay, 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.

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

Part 3: Perform Immunofluorescence

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

**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-1% BSA: Add 1 g BSA to 1 L 1XTBS

**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-1% BSA for 30 MIN at RT, or OVERNIGHT at 4°C. Keep slides covered in HybeEZ™ 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-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.

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

1. Determine the volume of TSA® Plus 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 RNAscope® Multiplex Fluorescent Kit v2. Recommended dilution range is 1:300–1:1.500 for fluorescent IHC.

**Secondary Antibody Staining**

1. Add HRP-conjugated secondary antibody diluted in TBS-1% BSA to completely cover the sections. Incubate the slides for 30 MIN at RT.
2. Rinse the slides with TBST Wash Buffer for 5 MIN at RT. Gently agitate the slides.
3. Repeat the rinse step twice.
4. Add 150–300 µl diluted TSA® fluorophore or Opal™ reagents to completely cover the sections. Incubate the slides in the HybeEZ™ Tray for 10 MIN at RT
5. Rinse the slides with TBST Wash Buffer for 2 MIN at RT. Gently agitate the slides.
6. Repeat the rinse step twice.

**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. 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's guidelines for imaging.

**Obtaining Support**

For the latest services and support information, go to:
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 CD4 (Opal 520-Green), CD8A (Opal 570-Yellow), and CD19 (Opal 620-Red) using the RNAscope® Multiplex Fluorescent v2 Assay, combined with fluorescent IHC of CD3 (Opal 690-White) in FFPE human tonsil. DAPI staining is shown in blue.

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