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

	TSA® Plus fluorophore	Akoya Bioscience Part No.
RNAscope® Multiplex Assay –C1	TSA® Plus fluorescein	NEL741001KT
RNAscope® Multiplex Assay –C2	TSA® Plus Cyanine 3	NEL744001KT
Fluorescent IHC	TSA® Plus Cyanine 5	NEL745001KT

3-plex ISH combined with fluorescent IHC

	Opal™ fluorophore	Akoya Bioscience Reagent Kit
RNAscope® Multiplex Assay –C1	Opal™ 520	FP1487001KT: Opal™ 520 Reagent Pack
RNAscope® Multiplex Assay –C2	Opal™ 570	FP1488001KT: Opal™ 570 Reagent Pack
RNAscope® Multiplex Assay –C3	Opal™ 620	FP1495001KT: Opal™ 620 Reagent Pack
Fluorescent IHC	Opal™ 690	FP1497001KT: Opal™ 690 Reagent Pack

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.

	Opal™ fluorophore	Akoya Bioscience Reagent Kit
RNAscope® Multiplex Assay –C1	Opal™ 520	FP1487001KT: Opal™ 520 Reagent Pack
RNAscope® Multiplex Assay –C2	Opal™ 570	FP1488001KT: Opal™ 570 Reagent Pack
RNAscope® Multiplex Assay –C3	Opal™ 690	FP1497001KT: Opal™ 690 Reagent Pack

	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.

	Opal™ fluorophore	Akoya Bioscience Reagent Kit
RNAscope® Multiplex Assay –C1	Opal™ 570	FP1488001KT: Opal™ 570 Reagent Pack
RNAscope® Multiplex Assay –C2	Opal™ 690	FP1497001KT: Opal™ 690 Reagent Pack
RNAscope® Multiplex Assay –C3	Opal™ Polaris 780	FP1487001KT: Opal™ 520 Reagent Pack
Fluorescent IHC	Opal™ 520	FP1501001KT: Opal™ Polaris 780 Reagent Pack

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 *RNAscope® 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

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 RNAscope® 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 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 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.
8. Remove excess liquid from slides, add 4–6 drops RNAscope® Multiplex FL v2 HRP blocker to entirely cover each slide.
9. Insert slides into the HybEZ™ Oven for **15 MIN** at **40°C**.
10. Rinse the slides with gentle agitation in TBST Wash Buffer for **2 MIN** at **RT**. Repeat with fresh buffer.
11. Remove excess liquid from the slides, and add 150–200 µL diluted Polaris 780 to each slide.
12. Incubate for **10 MIN** at **RT**.
13. 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**.

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

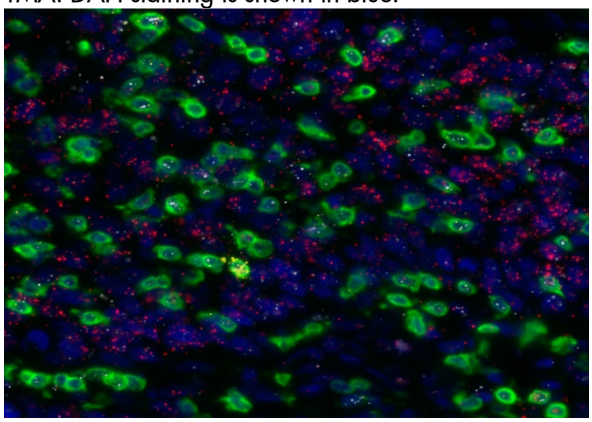
For the latest services and support information, go to:

https://acdbio.com/technical-support/support-overview.

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.

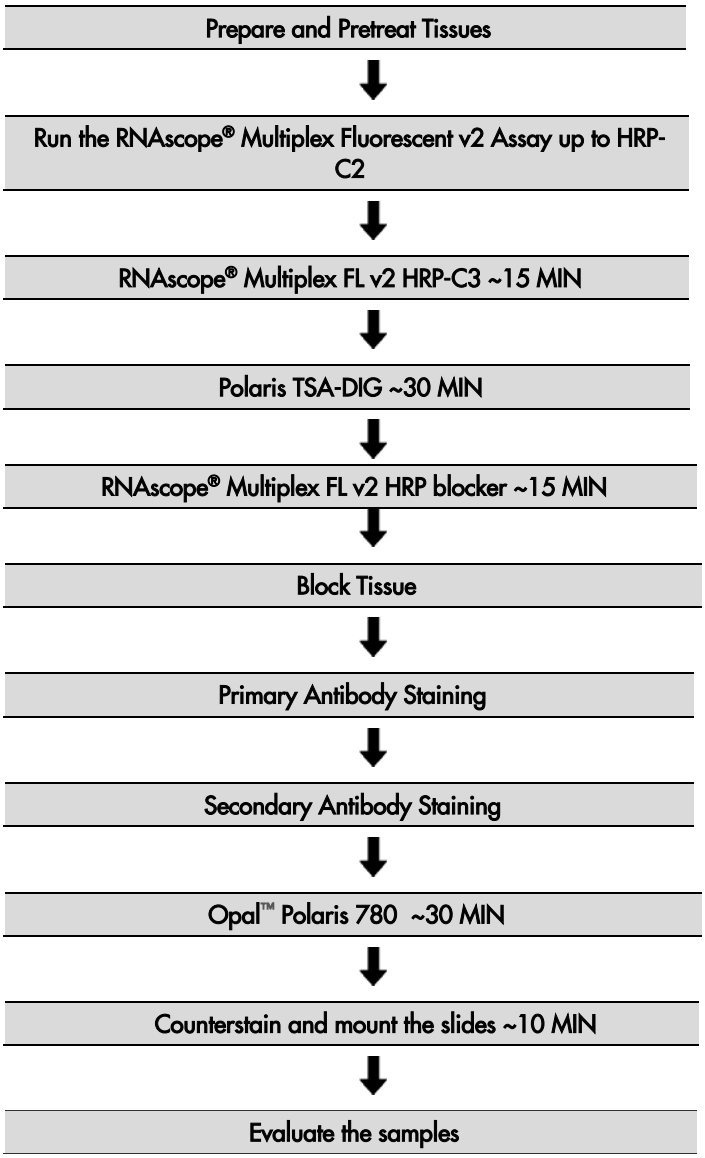
Figure 1. Detection of CD279 (Opal 520-White), CD274 (Opal 570-Red), and IFNg (Opal 620-Yellow) using the RNAscope® Multiplex Fluorescent v2 Assay, combined with fluorescent IHC of CD3 (Opal 690-Green) in FFPE human TMA. DAPI staining is shown in blue.



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



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:

Opal™ fluorophore	Filter setting
Opal™ 520	FITC
Opal™ 570	Cy3
Opal™ 620	Texas Red
Opal™ 690	Cy5.5
Opal™ Polaris 780	Cy7

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