

# 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](http://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](http://www.acdbio.com/support).

## Recommended Fluorophore Combinations

Use the Opal™ fluorophores or TSA® Plus System from Akoya Biosciences to develop RNAscope® Multiplex Fluorescent v2 Assay combined with fluorescent IHC signal. Use the following recommended combinations:

### 2-plex ISH combined with fluorescent IHC

	TSA® Plus fluorophore	Akoya Bioscience PartNo.
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

	Opal™ fluorophore	Akoya Bioscience Reagent Kit
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.

## 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](http://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](http://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 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.

### 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

### 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.

### 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 1:300–1:1500 for fluorescent IHC.

### Secondary Antibody Staining

1. Add HRP-conjugated secondary antibody diluted in TBS-0.1% 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 HybEZ™ 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](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's guidelines for imaging.

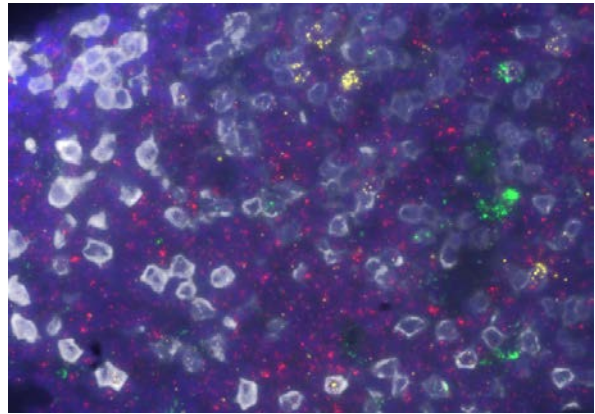
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- Access telephone and fax numbers to contact Technical Support and Sales.
- Search through FAQs.
- Submit a question directly to Technical Support.



**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 with fluorescent IHC of CD3 (Opal 690-White) in FFPE human tonsil. DAPI staining is shown in blue.

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