RNAscope® 4-plex Ancillary Kit for Multiplex Fluorescent Reagent Kit v2

Introduction

This Technical Note provides instructions for performing 4-plex *in situ* hybridization (ISH) on pretreated formalin-fixed paraffin-embedded (FFPE) tissue sections, fresh frozen tissues, and other sample types using the RNAscope® Multiplex Fluorescent Kit v2 (Cat. No. 323100) and 4-Plex Ancillary Kit [Cat. No. 323120]. Akoya Biosciences Opal™ fluorophores and multispectral imaging systems (Vectra® or Mantra™) are required for detection of fluorescent signals. For detailed sample preparation procedures 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.**

Workflow

Part 1: Prepare Tissue Samples

Prepare your samples by following the instructions for sample preparation and pretreatment in 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 Materials

1. Warm 50X Wash Buffer at **40°C** for **10–20 MIN** to remove any precipitation.
2. Prepare 3 L of 1X Wash Buffer by adding 2.94 L distilled water and 1 bottle (60 mL) of 50X Wash Buffer to a large carboy. Mix well.

Prepare Probes

1. Warm probes for **10 MIN** at **40°C**, then cool to **RT.**
2. Briefly spin the C2, C3, and C4 probes.
3. Pipette 1 volume of C2, 1 volume of C3, and 1 volume of C4 probes to 50 volumes of C1 probe into a tube. Invert the tube several times to mix.

Note: Do not mix probes of the same channel. Store mixed probes at **2–8°C** for up to **6 MONTHS**.

Prepare Opal™ fluorophores

1. Determine the volume of Opal™ fluorophore needed (150–200 µL per slide).
2. Dilute the Opal™ fluorophore stocks using the RNAscope® Multiplex TSA buffer provided in the RNAscope® Multiplex Fluorescent Kit v2. If using Opal™ 780, note that the reagent pack contains two reagents: Opal™ TSA-DIG and Opal™ Polaris 780. We recommend diluting Polaris TSA-DIG in TSA buffer and diluting Opal™ Polaris 780 in the Antibody Diluent/Block from Akoya Biosciences (PN: ARD1001EA). Follow these recommendations:

Opal™ fluorophore (Option 1)	Akoya Reagent Kit	Recommended dilution range*
Opal™ 520	FP1487001KT: Opal™ 520 Reagent Pack	1:750–1:3000
Opal™ 570	FP1488001KT: Opal™ 570 Reagent Pack	1:750–1:3000
Opal™ 620	FP1495001KT: Opal™ 620 Reagent Pack	1:750–1:3000
Opal™ 690	FP1497001KT: Opal™ 690 Reagent Pack	1:750–1:3000

*Start with a dilution of 1:1500 and adjust based on signal intensity.

Opal™ fluorophore (Option 2)	Akoya Reagent Kit	Recommended dilution range*
Opal™ 520	FP1487001KT: Opal™ 520 Reagent Pack	1:750–1:3000
Opal™ 570	FP1488001KT: Opal™ 570 Reagent Pack	1:750–1:3000
Opal™ 690	FP1497001KT: Opal™ 690 Reagent Pack	1:750–1:3000
Opal™ Polaris 780	FP1501001KT: Opal™ 690 Reagent Pack	TSA-DIG: 1:750–1:3000 Polaris 780: 1:187.5–1:750

Note: Store diluted Opal™ fluorophores up to **1 MONTH** at **2–8°C** in the dark.

Part 3: Run the RNAscope® Assay

Hybridize Probe

1. Remove excess liquid from the slides, place in the HybEZ™ or EZ-Batch™ Slide Rack, and add 4–6 drops of the probe mix to entirely cover each slide.
2. Insert slide rack containing the slides into the HybEZ™ Oven for **2 HRS** at **40°C**.
3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.

OPTIONAL STOPPING POINT. The slides can be stored in 5X SSC (not provided in the kit) overnight at RT.

For the following steps, use reagents from the RNAscope® Multiplex Fluorescent Kit v2 (Cat. No. 323100).

Note: All the three Amp are necessary irrespective of the channels being developed

Hybridize Amp 1

1. Remove excess liquid from slides, place in the HybEZ™ or EZ-Batch™ Slide Rack, and add 4–6 drops RNAscope® Multiplex FL v2 Amp 1 to entirely cover each slide.
2. Insert slides into the HybEZ™ Oven for **30 MIN** at **40°C**.
3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.

Hybridize Amp 2

1. Remove excess liquid from slides, place in the HybEZ™ or EZ-Batch™ Slide Rack, and add 4–6 drops RNAscope® Multiplex FL v2 Amp 2 to entirely cover each slide.
2. Insert slides into the HybEZ™ Oven for **30 MIN** at **40°C**.
3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.

Hybridize Amp 3

1. Remove excess liquid from slides, place in the HybEZ™ or EZ-Batch™ Slide Rack, and add 4–6 drops RNAscope® Multiplex FL v2 Amp 3 to entirely cover each slide.
2. Insert slides into the HybEZ™ Oven for **15 MIN** at **40°C**.

Note: Prepare TSA® Plus or Opal™ fluorophores or during this step. See the following section.

3. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.

Develop HRP-C1 Signal

1. Remove excess liquid from slides, place in the HybEZ™ or EZ-Batch™ Slide Rack, and add 4–6 drops RNAscope® Multiplex FL v2 HRP-C1 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 1X Wash Buffer.
4. Remove excess liquid from slides, place in the HybEZ™ or EZ-Batch™ Slide Rack, and add 150–200 µL diluted Opal™ 520 to each slide, and incubate for **30 MIN** at **40°C**.

Note: You can mix and match channels and fluorophores. For example, you can assign Opal™ 570 to the C1 channel instead of Opal™ 520. Do not assign the same fluorophore to more than one channel.

5. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash 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 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 1X Wash Buffer.

Develop HRP- C2 Signal

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack or EZ-Batch™ Slide Rack, and add 4–6 drops RNAscope® Multiplex FL v2 HRP-C2 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 1X Wash Buffer.
4. Remove excess liquid from slides, place in the HybEZ™ or EZ-Batch™ Slide Rack, and add 150–200 µL diluted Opal™ 570 to each slide.

Note: You can mix and match channels and fluorophores. For example, you can assign Opal™ 620 to the C2 channel instead of Opal™ 570. Do not assign the same fluorophore to more than one channel.

5. Incubate for **30 MIN** at **40°C**.
6. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.
7. 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 to entirely cover each slide.
8. Insert slides into the HybEZ™ Oven for **15 MIN** at **40°C**.
9. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.

Develop HRP-C3 Signal

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack or EZ-Batch™ Slide Rack, and 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 1X Wash Buffer.
4. Remove excess liquid from slides, place in the HybEZ™ or EZ-Batch™ Slide Rack, and add 150–200 µL diluted Opal™ 620 to each slide, and incubate for **30 MIN** at **40°C**.

Note: You can mix and match channels and fluorophores. For example, if you are using Opal™ Polaris 780 for C4, we recommend using Opal™ 690 for C3, as both fluorophores can be imaged using conventional filters. Do not assign the same fluorophore to more than one channel.

5. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash 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 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 1X Wash Buffer.

For the following steps, use reagents from 4-Plex Ancillary Kit [Cat. No. 323120].

Develop HRP-C4 Signal

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack or EZ-Batch™ Slide Rack, and add 4–6 drops RNAscope® Multiplex FL v2 HRP-C4 from 4-Plex Ancillary Kit For Fluorescent Multiplex v2 (Cat. No. 323120) 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 1X Wash Buffer.
4. Remove excess liquid from slides, place in the HybEZ™ or EZ-Batch™ Slide Rack, and add 150–200 µL diluted Opal™ 690 to each slide.

Note: You can mix and match channels and fluorophores. Do not assign the same fluorophore to more than one channel.

5. Incubate for **30 MIN** at **40°C**.
6. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.
7. 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.
8. Insert slides into the HybEZ™ Oven for **15 MIN** at **40°C**.
9. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.

Develop HRP-C4 signal using Opal™ Polaris 780

Note: The following procedure assigns Opal™ Polaris 780 to the C4 channel. Opal™ Polaris 780 cannot be followed by any other fluorophore.

1. Remove excess liquid from slides, place in the HybEZ™ Slide Rack or EZ-Batch™ Slide Rack, and add 4–6 drops RNAscope® Multiplex FL v2 HRP-C4 from 4-Plex Ancillary Kit For Fluorescent Multiplex v2 (Cat. No. 323120) 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 1X Wash Buffer.
4. Remove excess liquid from slides, place in the HybEZ™ or EZ-Batch™ Slide Rack and add 150–200 µL diluted TSA-DIG to each slide.

Incubate for **30 MIN** at **RT**.

5. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash 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 1X Wash Buffer.
9. Remove excess liquid from the slides, and insert the slide holder back into the humidity control tray.
10. Add 150–200 µL diluted Polaris 780 to each slide, and incubate for **30 MIN** at **RT**.
12. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Repeat with fresh 1X Wash Buffer.

Counterstain and Mount the Slides

Note: Do this procedure with no more than five slides at a time.

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

Note: Image the slides after **8 HRS** or within **2 WKS**.

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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7707 Gateway Blvd Suite 200, Newark, CA 94560 Phone 1-510-576-8800 Toll Free 1-877-576-3636
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