RNAscope® Multiplex Fluorescent v2 Assay combined with Immunofluorescence - Integrated Co-Detection Workflow (ICW)

Introduction

This Technical Note provides guidelines for performing *in situ* hybridization (ISH) using an RNAscope® Multiplex Fluorescent Reagent Kit v2 (Catalog No. 323100) combined with immunofluorescence (IF) on formalin-fixed paraffin-embedded (FFPE) tissue sections. ICW protocols for other sample types are available in **Appendix C** and **Appendix D**. To detect fluorescent ISH signals, use the RNAscope® Multiplex Fluorescent Kit v2 with the Opal™ fluorophores or TSA® Plus System (Akoya Biosciences). To detect immunofluorescence (IF), use HRP-conjugated secondary antibody with the Opal™ fluorophores or TSA® Plus System (Akoya Biosciences). 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). 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.**

Required Reagents

The following reagents are required specifically for RNA-protein co-detection. The RNA-Protein Co-Detection Ancillary kit includes Co-Detection Target Retrieval, Co-Detection Antibody Diluent, and Co-Detection Block. For an expanded list of materials and equipment, please refer to *RNAscope® Multiplex Fluorescent Reagent Kit v2 User Manual* (Doc. No. 323100-USM).

ACD Reagents:

RNA-Protein Co-Detection Ancillary kit (Cat. No. 323180)		
Reagent	**Quantity**	**Storage**
Co-Detection Target Retrieval (10X)	70 mL x 4 bottles	Room temp (15–30°C)
Co-Detection Antibody Diluent	120 mL x 1 bottle	2–8°C
Co-Detection Blocker (N/A for fluorescence)	4.5 ml x 1 bottle	2–8°C

Additional ACD Reagents	Ordering Info
RNAscope® H_2O_2 and Protease Reagents	Cat No. 322381
RNAscope® Multiplex Fluorescent Reagent Kit v2	Cat. No. 323100
RNAscope® Multiplex TSA Buffer	Cat. No. 322809
RNAscope® 50X Wash Buffer	Cat. No. 310091
RNAscope® Target and Control Probes	Various

Additional Reagents:

Reagents	Ordering Info
Phosphate Buffered Saline w/0.1% Tween-20 (PBS-T) (1X)	User provided
Primary Antibody	User provided
10% Neutral Buffered Formalin	User provided
Secondary Antibody	User provided
Fluorophore components	See the next section
Prolong Gold Antifade Mountant	Thermo Fisher Scientific Cat. No. P36930

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 immunofluorescence

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

3-plex ISH combined with fluorescent IHC

	Opal™ fluorophore	Akoya Biosciences 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
Immunofluorescence	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. For detailed fluorescent imaging recommendations, please refer to Chapter 5 of *RNAscope® Multiplex Fluorescent Reagent Kit v2 User Manual* (Doc. No. 323100-USM), available at **www.acdbio.com/technical-support/user-manuals**.

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

	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
Immunofluorescence	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 Biosciences 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
Immunofluorescence	Opal™ 520	FP1501001KT: Opal™ Polaris 780 Reagent Pack

IMPORTANT! The Opal™ Polaris 780 is extremely sensitive to cleavage by HRP activity. Following the IF protocol, apply Opal™ Polaris 780 as the last step before DAPI staining and mounting. For detailed steps, see **Appendix A**.

Workflow

Part 1: Prepare and Pretreat Tissues

To prepare 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**.

Prepare Slides

1. Bake slides in a dry air oven for **30 MIN** at **60°C**.
2. In a fume hood, fill 2 Tissue-Tek® clearing agent dishes with ~200 ml fresh xylene and fill 2 Tissue-Tek® staining dishes with ~200 ml fresh 100% ethanol.
3. Place slides in a Tissue-Tek® slide rack in xylene for **5 MIN**. Repeat in second xylene dish for an additional **5 MIN**.
4. Incubate slides in 100% ethanol for **1 MIN**. Repeat in second 100% ethanol dish for **1 MIN**.
5. Remove slides from rack and let air dry for **5 MIN** to **OVERNIGHT** at **RT**.

Part 2: Prepare the Materials

Prepare Reagents

1. Prepare 2–3 L 1X Phosphate Buffered Saline with 0.1% Tween-20 (PBS-T) and check that the pH is 7.2–7.4.

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 immunofluorescence.

Note: If using Opal™ Polaris 780, dilute Polaris TSA-DIG in TSA buffer and dilute Opal™ Polaris 780 in Antibody Diluent/Block from Akoya Biosciences (Part No. ARD1001EA). Recommended dilution range is 1:750 for TSA-DIG and 1:187.5-1:750 for Polaris 780.

Part 3: Tissue block and target retrieval

Prepare Materials – Day 1

1. Prepare 700 mL fresh 1X Co-Detection Target Retrieval in a beaker. Cover with foil and bring temperature to **98–102°C** using a hotplate. Maintain this temperature.
2. Place a wet humidifying paper in the Humidity Control Tray, leaving the HybEZ™ Slide Rack on bench.
3. Prepare working dilution of primary antibody using Co-Detection Antibody Diluent.

IMPORTANT! Use antibody diluted in Co-Detection Antibody Diluent (Cat. No. 323160). Standard antibody diluents negatively impact RNA signal and are not recommended for use with this workflow. Co-Detection Antibody Diluent is formulated for maximum compatibility with the RNAscope® assay and will best preserve RNA signal.

Note: For optimal protein detection, you may want to use a higher primary antibody concentration than you normally use for immunofluorescence alone.

Apply Hydrogen Peroxide

1. Add 2–4 drops of RNAscope® Hydrogen Peroxide to each section for **10 MIN** at **RT**. Use enough solution to completely cover the sections.
2. Place slides in a Tissue-Tek® Slide Rack and wash twice with distilled water.

Apply Target Retrieval

1. With a pair of forceps *very slowly* submerge the slide rack into the hot 1X Co-Detection Target Retrieval solution for **15 MIN**.

Note: Depending on tissue type, you may need to adjust the boiling time.

Note: Maintain temperature at **98–102°C** for the duration of target retrieval.

Note: Alternatively, use the steamer method for target retrieval. For details on target retrieval methods, please refer to 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**.

2. *Immediately* transfer the hot slide rack to a staining dish containing distilled water.
3. Wash slides in the distilled water by moving the rack up and down 3–5 times and repeat with fresh distilled water.
4. Wash slides in 1X PBS-T by moving the rack up and down 3–5 times.

Create Barrier

1. Working quickly to prevent the sample from drying out, use a tissue to carefully dry the glass surrounding your sample. Then, draw 2–4 times around section using the ImmEdge™ hydrophobic barrier pen. Let the barrier dry ~**30 SEC.**

IMPORTANT! Do not allow the sample to dry out completely as this may negatively impact IF signal.

Part 4: Perform Immunofluorescence Part A

Apply Primary Antibody

1. Place slides in the HybEZ™ Slide Rack and add 150–200 µl of primary antibody diluted in Co-Detection Antibody Diluent to each section. Use enough solution to cover each tissue section completely.
2. Place the HybEZ™ Slide Rack in the Humidity Control Tray lined with damp humidifying paper and incubate **OVERNIGHT** at **4°C**.

Prepare Materials – Day 2

1. Bring HybEZ™ Oven to **40°C**.
2. Place a damp humidifying paper in the Humidity Control Tray, leaving the HybEZ™ Slide Rack on bench. Insert the covered tray into the oven and close the oven door. The tray should be pre-warmed for at least **30 MIN** before use. Keep tray warm during the assay.

Post-primary fixation

1. Following primary antibody incubation, wash slides in PBS-T for **2 MIN**. Repeat twice with fresh PBS-T each time.
2. In a fume hood, place slides in a Tissue-Tek® Slide Rack and submerge in 10% Neutral Buffered Formalin (NBF) for **30 MIN** at **RT**.
3. In a fume hood, wash slides in PBS-T for **2 MIN**. Repeat wash with fresh PBS-T buffer.
4. Wash slides in PBST for an additional **2 MIN**. Repeat wash with fresh PBS-T buffer.

Apply Protease Plus

1. Place slides in the HybEZ™ Slide Rack and add 2–4 drops of RNAscope® Protease Plus to each section. Use enough solution to completely cover each tissue section.
2. Place the HybEZ™ Slide Rack in the pre-warmed HybEZ™ Humidity Control Tray. Seal tray and insert back into the HybEZ™ Oven. Incubate at **40°C** for **30 MIN**.

Note: Prepare RNAscope® Multiplex Fluorescent v2 assay materials during this incubation.

3. Place slides in a Tissue-Tek® Slide Rack submerged in distilled water.
4. Wash the slides by moving the rack up and down 3–5 times. Repeat with fresh distilled water.
5. Proceed to fluorescent ISH staining.

Part 5: 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! After the final ISH HRP blocker step, proceed directly to **Immunofluorescence Part B**. Do not counterstain slides with DAPI until the IF assay is finished.

Part 6: Perform Immunofluorescence Part B

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

Note: Use one of the following three procedures to perform standard secondary antibody staining. If Opal™ Polaris 780 is used for an ISH staining prior to IF, refer to **Appendix A** for detailed instructions.

Secondary Antibody Staining using TSA-based detection (Option 1)

1. Add HRP-conjugated secondary antibody diluted in Co-Detection Antibody Diluent to completely cover the sections.
2. Incubate the slides for **30 MIN** at **RT**.
3. Wash the slides with gentle agitation in PBS-T for **2 MIN** at **RT**. Repeat wash with fresh PBS-T.
4. Add 150–300 µL diluted Opal™ reagents to completely cover the sections.
5. Incubate the slides in the HybEZ™ Tray for **10 MIN** at **RT**.
6. Wash the slides with gentle agitation in PBS-T for **2 MIN** at **RT**. Repeat wash with fresh PBS-T.
7. Proceed to **Mount the Slides**.

Secondary Antibody Staining using Fluorophore-Conjugated Secondary (Option 2)

1. Add fluorophore-conjugated secondary antibody diluted in Co-Detection Antibody Diluent to completely cover the sections.
2. Incubate the slides for **30 MIN** at **RT.**
3. Wash the slides with gentle agitation in PBS-T for **2 MIN** at **RT**. Repeat wash with fresh PBS-T.
4. Proceed to **Mount the Slides**.

Secondary Antibody Staining using Opal™ Polaris 780 (Option 3)

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

1. Add HRP-conjugated secondary antibody diluted in Co-Detection Antibody Diluent to completely cover the sections.
2. Incubate the slides for **30 MIN** at **RT**.
3. Wash the slides with gentle agitation in PBS-T for **2 MIN** at **RT**. Repeat wash with fresh PBS-T.
4. Add 150–300 µL diluted TSA-DIG reagents to completely cover the sections.
5. Incubate the slides in the HybEZ™ Tray for **10 MIN** at **RT**
6. Wash the slides with gentle agitation in PBST for **2 MIN** at **RT**. Repeat wash with fresh PBST.
7. Remove excess liquid from slides, 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 the slides with gentle agitation in PBST for **2 MIN** at **RT**. Repeat wash with fresh PBS-T.
10. Remove excess liquid from the slides and add 150–200 µL diluted Polaris 780 to each slide.
11. Incubate for **10 MIN** at **RT**.
12. Rinse the slides with gentle agitation in PBS-T Wash Buffer for **2 MIN** at **RT**. Repeat wash with fresh PBS-T.
13. Proceed to **Mount the Slides**.

Part 7: Counterstain and Mount the Slides

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.
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.Note: Image slides after 8 hours and within 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 immunofluorescence (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:

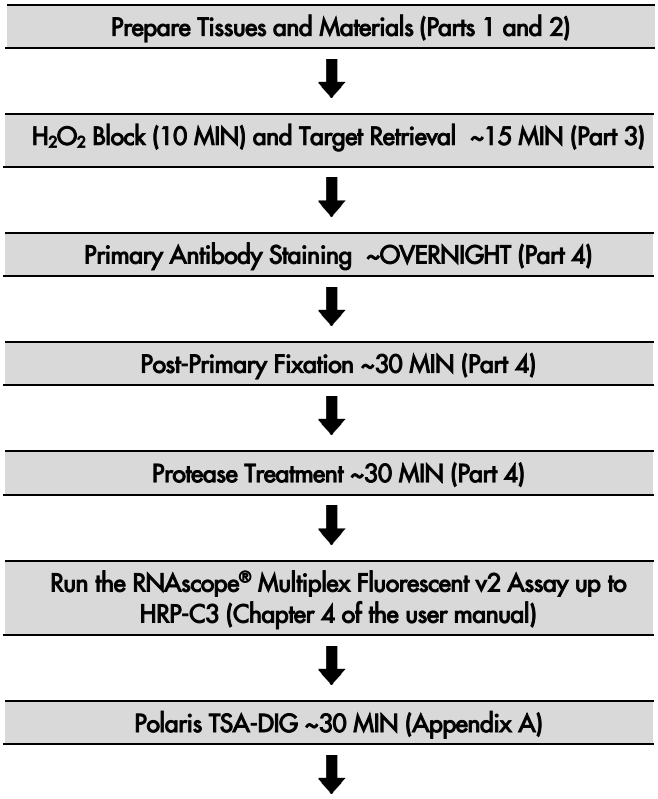
- Access telephone and fax numbers to contact Technical Support and Sales.
- Search through FAQs.
- Submit a question directly to Technical Support.

Appendix A. Co-Detection Using Opal™ Polaris 780 for ISH detection

IMPORTANT! The Opal™ Polaris 780 is extremely sensitive to cleavage by HRP activity. If the 780 fluorophore 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 IF protocol as the final step before counter staining and mounting.

The following workflow uses Opal™ Polaris 780 in the C3 channel followed by IF staining. Follow Parts 1–4 of this technical note, then , follow the instructions in Chapter 4 of the *RNAscope® Multiplex Fluorescent Reagent Kit v2 User Manual* (Doc. No. 323100-USM)to develop the C1 and C2 channels. Opal™ Polaris 780 steps are described in this Appendix.

Workflow



RNAscope® Multiplex FL v2 HRP blocker ~15 MIN (Appendix A)

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Secondary Antibody Staining ~30 MIN (Appendix A)

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RNAscope® Multiplex FL v2 HRP blocker ~15 MIN (Appendix A)

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Opal™ Polaris 780 ~30 MIN (Appendix A)

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Counterstain and mount the slides ~10 MIN (Part 7)

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Evaluate the samples (Part 7)

Opal™ Polaris 780 staining: Part A

1. Remove excess liquid from slides 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 wash with fresh wash buffer.
4. Remove excess liquid from slides 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 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**.
9. Perform a second wash using fresh PBS-T instead of 1X Wash Buffer for **2 MIN** at **RT**.

Secondary antibody staining (Option 1 or 2)

Follow the steps for secondary antibody staining on page 4. After the final PBS-T wash, proceed with Part B of Opal™ Polaris 780 staining.

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 PBS-T for **2 MIN** at **RT**. Repeat with fresh PBS-T.
4. Continue to **Mount the Slides**.

Appendix B. Multiplex v2 Co-Detection Troubleshooting Guide

To optimize protein detection, we recommend titrating antibody concentration within the Co-Detection workflow.

Note: For optimal protein detection, you may want to use a higher primary antibody concentration than you normally use for immunofluorescence alone.

The post-primary fixation and pretreatment conditions in this Tech Note provide optimal RNA and protein detection across most tissue samples. If you need to optimize the protocol for a specific sample or target of interest, you can adjust the following parameters:

Reagent	Incubation Temperature	Recommended Incubation Time	Optimization Range
Target Retrieval	40°C	15 MIN	15-30 MIN
Primary Antibody	4°C	OVERNIGHT	60–120 MIN at RT or OVERNIGHT at 4°C
10% NBF	Ambient	30 MIN	15–60 MIN
Protease Plus	40°C	30 MIN	15–30 MIN
Secondary Antibody	Ambient	30 MIN	15–60 MIN

Appendix C. Integrated Co-Detection for Fresh Frozen Tissue

Prepare Tissue

To prepare fresh frozen tissue sections, follow the instructions in Parts 1 and 2 of *Preparing Fresh Frozen Tissue for RNAscope® and BaseScope™ Assays* (Doc. No. MK 50-013) available at **www.acdbio.com/technical-support/user-manuals**. After preparing tissue sections, fix your samples using the following procedure.

1. In a fume hood, pre-chill 200 mL of 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) in 1X PBS to **4°C**.

2. Remove fresh frozen tissue slides from **–80°C**. In a fume hood, immediately immerse the slides in the pre-chilled 10% NBF or 4% PFA.
3. Incubate the slides for at least **15 MIN** at **4°C**.

Dehydrate the Tissue

1. Prepare 200 mL 50% EtOH, 200 mL 70% EtOH, and 400 mL 100% EtOH.
2. Remove the slides from NBF or PFA. Immerse in 50% EtOH. Incubate for **5 MIN** at **RT**.
3. Remove the slides from 50% EtOH. Immerse in 70% EtOH. Incubate for **5 MIN** at **RT.**
4. Remove the slides from 70% EtOH. Immerse in 100% EtOH. Incubate for **5 MIN** at **RT.**
5. Remove the slides from 100% EtOH. Immerse in fresh 100% EtOH. Incubate for **5 MIN** at **RT.**

Create Barrier

1. Remove slides from 100% EtOH. Leave slides for **5 MIN** at **RT.**
2. Draw 2–4 times around tissue using the ImmEdge™ hydrophobic barrier pen. Let the barrier dry ~**30 SEC.**

ICW Pretreatment and Immunofluorescence

Prepare Materials

1. Prepare working dilution of primary antibody using Co-Detection Antibody Diluent.

IMPORTANT! Use antibodies diluted in Co-Detection Antibody Diluent (Cat. No. 323160). Standard antibody diluents negatively impact RNA signal and are not recommended for use with this workflow. Co-Detection Antibody Diluent is formulated for maximum compatibility with the RNAscope® assay and will best preserve RNA signal.

Note: For optimal protein detection, you may want to use a higher primary antibody concentration than you would normally use for immunofluorescence alone.

Apply Hydrogen Peroxide

1. Add 2–4 drops of RNAscope® Hydrogen Peroxide to each section for **10 MIN** at **RT**. Use enough solution to completely cover the sections.
2. Place slides in a Tissue-Tek® Slide Rack and wash twice with distilled water.
3. Wash slides in 1X PBS-T.

Apply Primary Antibody

1. Place slides in the HybEZ™ Slide Rack and add 150–200 µl of primary antibody diluted in Co-detection Antibody Diluent to each section. Use enough solution to cover each tissue section completely.
2. Place the HybEZ™ Slide Rack in the Humidity Control Tray and incubate at **4°C OVERNIGHT**.

Post-primary fixation

1. Following primary antibody incubation, wash slides in PBS-T for **2 MIN**. Repeat twice with fresh PBS-T each time.
2. In a fume hood, place slides in a Tissue-Tek® Slide Rack and submerge in 10% Neutral Buffered Formalin (NBF) for **30 MIN** at **RT**.
3. In a fume hood, wash slides in PBS-T for **2 MIN**. Repeat with fresh PBS-T.
4. Wash slides in PBST for an additional **2 MIN**. Repeat with fresh PBS-T.

IMPORTANT! Formalin that has been stored for more than six months or opened for more than one month can result in suboptimal fixation.

Apply Protease

1. Place slides in the HybEZ™ Slide Rack. Add 2–4 drops of RNAscope® Protease IV to each section. Use enough solution to cover each tissue section completely. Incubate for **30 MIN** at **RT**.

IMPORTANT! Be sure to use the correct protease for you assay of choice. Using the incorrect protease may result in suboptimal RNA or protein detection.

Note: Depending on tissue type and IF target, you may need to adjust protease treatment strength and/or time. If stronger protease treatment is required, perform two 30 minute incubations. Fresh protease must be applied prior to the second incubation.

Note: Prepare RNAscope® Multiplex Fluorescent v2 assay materials during this incubation.

2. Place slides in a Tissue-Tek® Slide Rack submerged in distilled water
3. Wash the slides by moving the rack up and down 3–5 times. Repeat with fresh distilled water.
4. Complete Parts 5–7 of this technical note.

Appendix D. Integrated Co-Detection for Fixed Frozen Tissue

Prepare Tissue

To prepare fixed frozen tissue sections, follow the instructions for *"Fixed frozen tissue sample preparation"* in Chapter 3 of the *RNAscope®*

Multiplex Fluorescent Reagent Kit v2 Assay (Doc. No. 323100-USM) available at **www.acdbio.com/technical-support/user-manuals**. After preparing tissue sections, fix your samples using the following procedure.

Sample Fixation

1. In a fume hood, pre-chill 200 mL of 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) in 1X PBS to **4°C**.
2. Remove fresh frozen tissue slides from **–80°C**. Wash the slides with 200 mL 1X PBS in a Tissue Tek® slide rack for **5 MIN** while moving the rack up and down to remove Optimal Cutting Temperature (OCT) embedding media.
3. Bake the slides for **30 MIN** at **60°C**.
4. In a fume hood, post-fix the slides by immersing them in the pre-chilled 10% NBF or 4% PFA in 1X PBS for **15 MIN** at **4°C**.

Dehydrate the Tissue

1. Prepare 200 mL 50% EtOH, 200 mL 70% EtOH, and 400 mL 100% EtOH.
2. Remove the slides from NBF or PFA. Immerse in 50% EtOH. Incubate for **5 MIN** at **RT**.
3. Remove the slides from 50% EtOH. Immerse in 70% EtOH. Incubate for **5 MIN** at **RT.**
4. Remove the slides from 70% EtOH. Immerse in 100% EtOH. Incubate for **5 MIN** at **RT.**
5. Remove the slides from 100% EtOH. Immerse in fresh 100% EtOH. Incubate for **5 MIN** at **RT.**
6. Remove slides from EtOH and let air dry for **5 MIN** to **OVERNIGHT** at **RT**.

ICW Pretreatment and Immunofluorescence

Prepare Materials – Day 1

1. Prepare 700 mL fresh 1X Co-Detection Target Retrieval in a beaker. Cover with foil and bring temperature to **98–102°C** using a hotplate. Maintain this temperature.
2. Place a wet humidifying paper in the Humidity Control Tray, leaving the HybEZ™ Slide Rack on bench.
3. Prepare working dilution of primary antibody using Co-Detection Antibody Diluent.

IMPORTANT! Use antibodies diluted in Co-Detection Antibody Diluent (Cat. No. 323160). Standard antibody diluents negatively impact RNA signal and are not recommended for use with this workflow. Co-Detection Antibody Diluent is formulated for maximum compatibility with the RNAscope® assay and will best preserve RNA signal.

Note: For optimal protein detection, you may want to use a higher primary antibody concentration than you would normally use for immunofluorescence alone.

Apply Hydrogen Peroxide

4. Add 2–4 drops of RNAscope® Hydrogen Peroxide to each section for **10 MIN** at **RT**. Use enough solution to completely cover the sections.
5. Place slides in a Tissue-Tek® Slide Rack and wash twice with distilled water.

Apply Target Retrieval

1. With a pair of forceps *very slowly* submerge the slide rack into the hot 1X Co-Detection Target Retrieval solution for **5 MIN**.

Note: Depending on tissue type, boiling time may need to be adjusted.

Note: Maintain temperature at **98–102°C** for the duration of target retrieval.

Note: Alternatively, use the steamer method for target retrieval. For details on target retrieval methods, please refer to 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**. Make sure that you use 1X Co-Detection Target Retrieval.

2. *Immediately* transfer the hot slide rack to a staining dish containing distilled water.
4. Wash slides in the distilled water by moving the rack up and down 3–5 times and repeat with fresh distilled water.
5. Wash slides in 1X PBS-T by moving the rack up and down 3–5 times.

Create Barrier

1. Working quickly to prevent the sample from drying out, use a tissue to carefully dry the glass surrounding your sample. Then, draw 2–4 times around section using the ImmEdge™ hydrophobic barrier pen. Let the barrier dry **~30 SEC.**

IMPORTANT! Do not allow the sample to dry out completely as this may negatively impact IF signal.

Apply Primary Antibody

1. Place slides in the HybEZ™ Slide Rack and add 150-200 µl of primary antibody diluted in Co-detection Antibody Diluent to each section. Use enough solution to cover each tissue section completely.
2. Place the HybEZ™ Slide Rack in the Humidity Control Tray lined with damp humidifying paper and incubate at **4°C OVERNIGHT**.

Prepare Materials – Day 2

1. Bring HybEZ™ Oven to **40°C**.
2. Place a wet humidifying paper in the Humidity Control Tray, leaving the HybEZ™ Slide Rack on bench. Insert the covered tray into the oven and close the oven door. The tray should be pre-warmed for at least **30 MIN** before use. Keep tray warm during the assay.

Post-primary fixation

1. Following primary antibody incubation, wash slides in PBS-T for **2 MIN**. Repeat twice with fresh PBS-T each time.
2. In a fume hood, place slides in a Tissue-Tek® Slide Rack and submerge in 10% Neutral Buffered Formalin (NBF) for **30 MIN** at **RT**.
3. In a fume hood, wash slides in PBS-T for **2 MIN**. Repeat with fresh PBS-T.
4. Wash slides in PBS-T for an additional **2 MIN**. Repeat with fresh PBS-T.

IMPORTANT! Formalin that has been stored for more than six months or opened for more than one month can result in suboptimal fixation.

Apply Protease

1. Place slides in the HybEZ™ Slide Rack. If doing RNAscope®, add 2–4 drops of RNAscope® Protease Plus to each section. Alternatively, if doing BaseScope™, add 2–4 drops of RNAscope® Protease IV to each section. Use enough solution to completely cover each tissue section.

IMPORTANT! Be sure to use the correct protease for each assay. Using the incorrect protease can result in suboptimal RNA or protein detection.

2. Place the HybEZ™ Slide Rack in the pre-warmed HybEZ™ Humidity Control Tray. Seal tray and insert back into the HybEZ™ Oven. Incubate at **40°C** for **30 MIN**.

NOTE: Depending on tissue type and IF target, you may need to adjust protease treatment strength and/or time.

NOTE: Prepare RNAscope® Multiplex Fluorescent v2 assay materials during this incubation.

3. Place slides in a Tissue-Tek® Slide Rack submerged in distilled water
4. Wash the slides by moving the rack up and down 3–5 times. Repeat with fresh distilled water.
5. Complete Parts 5–7 of this technical note..

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