



USER MANUAL

RNAscope™ Multiplex Fluorescent Reagent Kit v2

With Sample Preparation and Pretreatment

Document Number UM 323100

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Chapter 1. Product Information



Before using this product, read and understand the safety information in **Appendix E. Safety.**

IMPORTANT! We recommend reading the entire user manual before beginning any protocols.

About this guide

This user manual provides guidelines and protocols to use the RNAscope Multiplex Fluorescent Reagent Kit v2 (Cat. No. 323100) on FFPE, fixed frozen, and fresh frozen tissues mounted on slides. Sample preparation and pretreatment methods are described in Chapters 3–5, the RNAscope assay is described in Chapter 6, and a procedure for combining immunofluorescence with RNAscope is described in Chapter 7.

Product description

Background

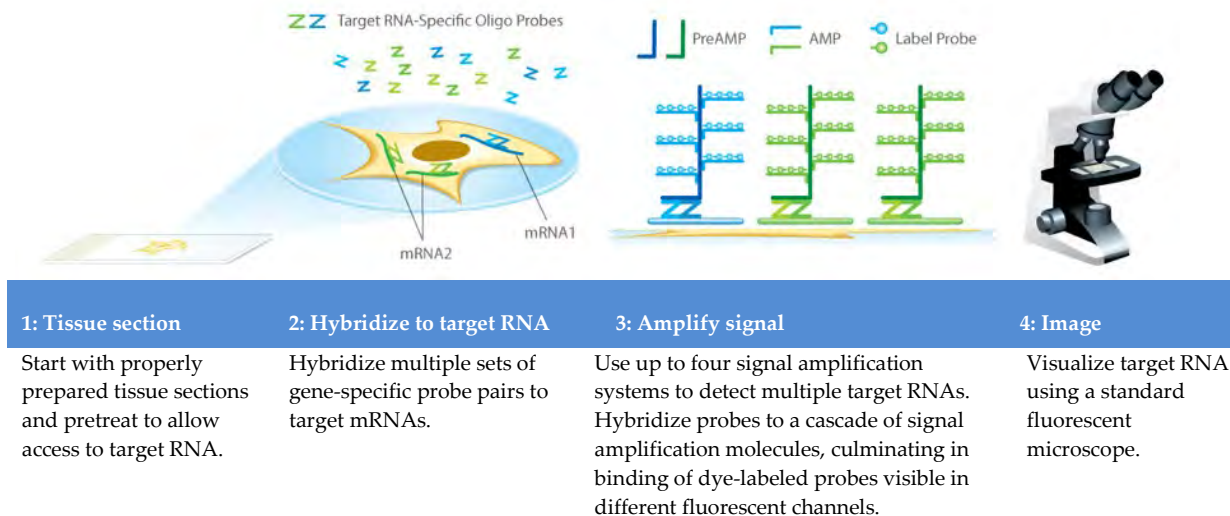
The RNAscope Multiplex Fluorescent v2 Assay uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize at least three and up to four different RNA targets per cell in samples mounted on slides. Simultaneous detection of four different RNA targets requires an additional kit: the RNAscope Ancillary kit for Multiplex v2 (Cat. No. 323120), as well as species-specific 4-plex control probes. The assay is based on ACD's patented signal amplification and background suppression technology and incorporates multiplexed signal amplification systems, which enable users to investigate expression as well as positional relationship between multiple genes within a cellular context.

Overview

Figure 1 on page 7 illustrates the RNAscope Assay procedure. You can complete the procedure in approximately 12 hours. Most of the RNAscope Assay reagents are available in convenient Ready-To-Use (RTU) dropper bottles and provide a simple, nearly pipette-free workflow.

Start with properly prepared samples, pretreat them, and then hybridize RNA-specific probes designed for different fluorescent detection channels to multiple target RNAs. After a series of highly effective and specific signal amplifications, single RNA transcripts for target genes appear as punctate dots in distinct fluorescent channels. These dots are visible using a common fluorescent microscope with the appropriate filters.

Figure 1. Procedure overview



Kit contents and storage

The RNAscope Multiplex Fluorescent v2 Assay requires the RNAscope Probes and the RNAscope Multiplex Fluorescent Reagent Kit v2 (Cat. No. 323100). Probes and reagent kits are available separately. Simultaneous detection of four different RNA targets requires the RNAscope Ancillary kit for Multiplex v2 (Cat. No. 323120), as well as species-specific 4-plex control probes.

RNAscope Probes

The RNAscope Probes consist of user-specified Target Probes and Positive and Negative Control Probes. Visit <https://acdbio.com/products> to find a gene-specific target probe or appropriate control probes. Each target probe contains a mixture of short oligonucleotides designed to bind to a specific target RNA and detected with channel-specific amplification systems, C1, C2, C3, and C4. Different fluorophores are assigned to the C1, C2, C3, or C4 channels depending on the TSA Vivid™ or Opal™ dye fluorophores selected for that channel.

Each bottle contains enough probe to stain ~20 sections, each with an area of approximately 20 mm x 20 mm (0.75" x 0.75"). Larger tissue sections will result in fewer tests. The probes have a shelf life of two years from the manufacturing date when stored as indicated in the following tables:

Target Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	Target Probe – [species] – [gene] – C1	Various	Ready-To-Use (RTU) probe for color channel 1	3 mL x 1 bottle	2–8°C
	Target Probe – [species] – [gene] – C2	Various	50X probe for color channel 2	60 µL x 1 tube	2–8°C
	Target Probe – [species] – [gene] – C3	Various	50X probe for color channel 3	60 µL x 1 tube	2–8°C



	Target Probe – [species] – [gene] – C4	Various	50X probe for color channel 4	60 µL x 1 tube	2–8°C
Control Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	3-Plex Positive Control Probe	Various	RTU mixture of three probes targeting common housekeeping genes <i>POLR2A</i> in channel C1, <i>PPIB</i> in channel C2, and <i>UBC</i> in channel C3.	3 mL x 1 bottle	2–8°C
	3-Plex Negative Control Probe (<i>dapB</i>)	320871	RTU probe targeting a bacterial gene. Each detection channel has its own negative control probe: <i>dapB</i> -C1, <i>dapB</i> -C2, <i>dapB</i> -C3	3 mL x 1 bottle	2–8°C
	4-Plex Positive Control Probe	Various	RTU mixture of four probes targeting common housekeeping genes <i>POLR2A</i> in channel C1, <i>PPIB</i> in channel C2, <i>UBC</i> in channel C3 and <i>HPRT</i> in channel C4.	3 mL x 1 bottle	2–8°C
	4-Plex Negative Control Probe (<i>dapB</i>)	321831	RTU probe targeting a bacterial gene. Each detection channel has its own negative control probe: <i>dapB</i> -C1, <i>dapB</i> -C2, <i>dapB</i> -C3, <i>dapB</i> -C4	3 mL x 1 bottle	2–8°C
	Probe Diluent	300041	RTU probe diluent	3 mL x 1 bottle	2–8°C

RNAscope Multiplex Fluorescent Reagent Kit v2

Each RNAscope Multiplex Fluorescent Reagent Kit v2 (Cat. No. 323100) provides enough reagents to stain ~20 tissue sections, each with an area of approximately 20 mm x 20 mm (0.75" x 0.75"). Larger tissue sections will result in fewer tests. Each kit contains the following components: Pretreatment Reagents including enzyme-free pretreatment, Target Retrieval Reagents, Detection Reagents, Multiplex TSA Buffer and Wash Buffer Reagents. In terms of Protease Reagents relative strength, Protease IV is strongest, Protease III is medium strength, Protease Plus is mild.

The reagents have a shelf life of nine months from the manufacturing date when stored as indicated in the following tables:

RNAscope Multiplex Fluorescent Detection Reagents v2 (Cat. No. 323110)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope Hydrogen Peroxide	4mL x 1 bottle	2–8°C
	RNAscope Manual PretreatPro	3 mL x 2 bottles	2–8°C
	RNAscope Multiplex FL v2 AMP 1	3 mL x 1 bottle	2–8°C
	RNAscope Multiplex FL v2 AMP 2	3 mL x 1 bottle	2–8°C



RNAscope Multiplex Fluorescent Detection Reagents v2 (Cat. No. 323110)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope Multiplex FL v2 AMP 3	4.5 mL x 1 bottle	2–8°C
	RNAscope Multiplex FL v2 HRP-C1	4.5 mL x 1 bottle	2–8°C
	RNAscope Multiplex FL v2 HRP-C2	4.5 mL x 1 bottle	2–8°C
	RNAscope Multiplex FL v2 HRP-C3	4.5 mL x 1 bottle	2–8°C
	RNAscope Multiplex FL v2 HRP blocker	4.5 mL x 3 bottles	2–8°C
	RNAscope Multiplex FL v2 DAPI	4.5 mL x 1 bottle	2–8°C
RNAscope H ₂ O ₂ and Protease Reagents (Cat. No. 322381)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope Hydrogen Peroxide	4 mL x 2 bottles	2–8°C
	RNAscope Protease Plus	4.5 mL x 2 bottles	2–8°C
	RNAscope Protease III	4.5 mL x 2 bottles	2–8°C
	RNAscope Protease IV	4.5 mL x 2 bottles	2–8°C
RNAscope Target Retrieval Reagents (Cat. No. 322000)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope 10X Target Retrieval	70 mL x 4 bottles	Room temp (15–30°C)
RNAscope Multiplex TSA Buffer (Cat. No. 322809)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope TSA Buffer Pack	29 mL x 1 bottle	2–8°C
RNAscope Wash Buffer Reagents (Cat. No. 310091)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope 50X Wash Buffer	60 mL x 4 bottles	Room temp (15–30°C)
RNAscope 4-Plex Ancillary Kit for Multiplex Fluorescent v2 (Cat. No. 323120)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope Multiplex FL v2 HRP-C4	4.5 mL x 1 bottle	2–8°C
	RNAscope Multiplex FL v2 HRP blocker	4.5 mL x 1 bottle	2–8°C

IMPORTANT! To perform a 4-plex assay, you will need both RNAscope Multiplex Fluorescent Detection Reagents v2 (Cat. No. 323110) **and** RNAscope 4-Plex Ancillary Kit for Multiplex Fluorescent v2 (Cat. No. 323120)

IMPORTANT! Do not interchange the reagent components of different RNAscope Reagent Kits, even those having the same name.

Note: To order more TSA Buffer, use Cat. No. 322810 for 29mL x 3 bottles.

Note: The following materials and equipment are needed to perform the RNAscope Assay.



TSA Vivid Dyes

The RNAscope Multiplex Fluorescent v2 assay requires TSA Vivid or Opal (described in table below) dyes. We cannot guarantee assay results if you use other fluorescent dyes. Dilute the dyes in RNAscope Multiplex TSA Buffer provided by the RNAscope Multiplex Fluorescent Reagent Kit v2. Choose a dilution factor for each fluorophore based on recommendations from ACD and your specific experimental conditions such as target expression levels, tissue quality, or microscope setting.

TSA Vivid Fluorophores				
<input checked="" type="checkbox"/>	Reagent*	Quantity	Storage	Recommended dilution range†
	TSA Vivid Fluorophore 520 (Cat. No. 323271)	TSA Vivid Dye x 1 vial DMSO x 1 vial	-20°C	1:750–1:3000
	TSA Vivid Fluorophore 570 (Cat. No. 323272)	TSA Vivid Dye x 1 vial DMSO x 1 vial	-20°C	1:750–1:3000
	TSA Vivid Fluorophore 650 (Cat. No. 323273)	TSA Vivid Dye x 1 vial DMSO x 1 vial	-20°C	1:3000–1:7500

*Reconstitute the TSA Vivid reagent with 100 µL Dimethylsulfoxide (DMSO). Reconstituted TSA Vivid Fluorophores can be stored for three months at –20°C.

† We recommend starting with a dilution of 1:1500 for TSA Vivid 520 and 570, 1:5000 for TSA Vivid 650 and adjusting the dilution based on signal intensity. Optimal fluorophore dilutions may vary based on sample, target expression levels, and imaging system.

IMPORTANT! TSA Vivid Fluorophores are not compatible with RNAscope Manual PretreatPro. Please use RNAscope Protease workflows instead.

Opal Dyes

The RNAscope Multiplex Fluorescent v2 assay is also compatible with Opal dyes from Akoya Biosciences (see the following table). We cannot guarantee assay results with other fluorescent dyes. Dilute the dyes in RNAscope Multiplex TSA Buffer provided by the RNAscope Multiplex Fluorescent Reagent Kit v2. Choose a dilution factor for each fluorophore based on recommendations from ACD and your specific experimental conditions such as target expression levels, tissue quality, or microscope setting.

To perform a 3-plex assay, we recommend Opal 520, 570, and 690. When autofluorescence in FITC channel is a concern and a Cy7 filter is available, use Opal 780 instead of Opal 520. The Opal 780 Reagent Pack contains two reagents: Opal TSA-DIG and Opal 780. We recommend diluting Opal TSA-DIG in TSA buffer, and diluting Opal 780 in Antibody Diluent/Block from Akoya Biosciences (PN: ARD1001EA). We recommend keeping the dilution factors of Opal TSA-DIG and Opal 780 at a constant ratio. For example, when using 1:1500 dilution for Opal TSA-DIG, use 1:375 dilution for Opal 780. When using 1:750 dilution for Opal TSA-DIG, use 1:187.5 dilution for Opal 780.

<input checked="" type="checkbox"/>	Fluorophores	Part number (Akoya Biosciences) *	Recommended dilution range†
	Opal 520 Reagent Pack	FP1487001KT	1:750–1:3000
	Opal 570 Reagent Pack	FP1488001KT	1:750–1:3000



	Opal 690 Reagent Pack	FP1497001KT	1:750–1:3000
	Opal 780 Reagent Pack	FP1501001KT	TSA-DIG: 1:750–1:3000
			Opal 780: 1:187.5–1:750

* Reconstitute all Opals (except Opal 780) and TSA-DIG with 75 μ L Dimethylsulfoxide (DMSO). Reconstitute Opal 780 with 300 μ L double distilled water (ddH₂O).

† We recommend starting with a dilution of 1:1500 for Opal dyes and adjusting the dilution based on signal intensity. Optimal fluorophore dilutions may vary based on sample, target expression levels, and imaging system.

IMPORTANT! Store Opal Dye fluorophore stocks at 2–8°C. Follow manufacturer’s instructions. Akoya Biosciences recommends using reconstituted fluorophores within three months.

HybEZ™ Hybridization System

IMPORTANT! The RNAscope Assay has been validated using this system only.

Use the HybEZ Hybridization System or HybEZ II Hybridization system to perform RNAscope Assay hybridization and incubation steps. These steps require humid conditions to prevent sections from drying out.

For instructions on how to use the HybEZ Hybridization System, refer to the *HybEZ Hybridization System User Manual* available and view the training video at <https://acdbio.com/technical-support/learn-more>. The system contains the following components:

<input checked="" type="checkbox"/>	Component	Quantity	Cat. No.
	HybEZ II Hybridization System (110 or 220V)	1 oven	321711 or 321721
	HybEZ Humidity Control Tray (with lid)	1 tray	310012
	ACD EZ-Batch™ Slide Holder (20 slide capacity)	1 rack	321716
	ACD EZ-Batch Wash Tray	1 tray	321717
	HybEZ Humidifying Paper	2 sheets	—

Note: To order HybEZ Humidifying Paper Pack, 15 sheets, use Cat. No. 310015.

Other materials

<input checked="" type="checkbox"/>	Description	Supplier	Cat. No.
	Superfrost® Plus Slides (required)	Fisher Scientific	12-550-15
	ImmEdge™ Hydrophobic Barrier Pen (required)	Vector Laboratory	H-4000
	Xylene	Fisher Scientific/MLS*	X3P-1GAL
	10% Neutral Buffered Formalin (NBF)/ 4% Paraformaldehyde (PFA)	MLS Sigma	— 252549-1L
	100% alcohol (EtOH)	American Master Tech Scientific/MLS	ALREAGAL
	10X Phosphate Buffered Saline (PBS)	Fisher Scientific/MLS	BP3991
	20X SSC	Fisher Scientific/MLS	BP1325
	Vertical 24 slide racks (or other slide racks or holders)	MLS	—
	Vertical staining dishes (or similar containers)	MLS	—



<input checked="" type="checkbox"/>	Description	Supplier	Cat. No.
	Clearing agent dish, xylene resistant	MLS	—
	Hamilton Beach 5.5 Quart Digital Steamer (Model 3753OZ)	—	—
	Digital thermometer	MLS	—
	ProLong Gold Antifade Mountant	Thermo Fisher Scientific	P36930
	Antibody Diluent/Block (if Opal 780 is used)	Akoya Biosciences	ARD1001EA
	Cover glass 24 x 50 mm	Fisher Scientific/MLS	12-545-F
	Carboy (>3L)	MLS	—
	Water bath or incubator capable of holding temperature at 40 ± 1°C	MLS	—
	Drying oven, capable of holding temperature at 60 ± 1°C (optional)	MLS	—
	Fume hood	MLS	—
	Distilled water	MLS	—
	Tubes (various sizes)	MLS	—
	Paper towel or absorbent paper	MLS	—
	Kimwipe or equivalent	MLS	—
	Recommended fluorescent microscope with filter set (see the following table): Ex Max 358 nm/Em Max 461 nm (DAPI) Ex Max 494 nm/Em Max 517 nm (FITC) Ex Max 550 nm/Em Max 570 nm (Cy3) Ex Max 648 nm/Em Max 667 nm (Cy5) Ex Max 740 nm/Em Max 764nm (Cy7)	MLS	—

* Major Laboratory Supplier in North America. For other regions, please check catalog numbers with your local lab supplier.



Fluorescent imaging recommendations

Microscope	Optics	Image Capture
<ul style="list-style-type: none"> • Leica DM series or equivalent • Zeiss Axio Imager or equivalent • Inverted microscope, if optics and condenser meet requirements • Required excitation/emission filter cube: DAPI/FITC/Cy3/Cy5/Cy7 (if Opal 780 is used) 	<ul style="list-style-type: none"> • 20X (N.A. 0.75) air • 40X (N.A. 0.8) air (recommended) • 40X (N.A. 1.3) oil • 63X (N.A. 1.3) oil – use for low expression targets, if needed 	<ul style="list-style-type: none"> • Microscope with camera for fluorescence capturing. Multispectrum microscope/camera system (e.g. Nuance[®] EX, Mantra[™], Vectra[®] and PhenoImager[™] HT) recommended for imaging tissue with high autofluorescence. • For optimal fluorescence detection, we recommend using a high resolution and high sensitivity cooled CCD camera that is 64 μm pixel size or smaller with > 65% peak quantum efficiency. Common models include Orca-Flash 4.0 (Hamamatsu) and Nuance EX (Perkin Elmer).

Chapter 2. Before You Begin

Prior to running the RNAscope Multiplex Fluorescent v2 Assay on your samples for the first time, we recommend that you view the video demonstrations available at <https://acdbio.com/technical-support/learn-more>.

Important procedural guidelines

- Start with properly fixed and prepared sections. Refer to the sample preparation and pretreatment chapters in this manual.
- Use only samples mounted on Superfrost Plus Slides (Fisher Scientific, Cat. No. 12-550-15).
- It is recommended to always test positive and negative control probes on your sample to assess sample RNA quality and optimal permeabilization before staining with target probes. The positive control will help determine whether the quality of RNA in the tissue specimen is sufficient for detecting your RNA target. The negative control will help determine whether the tissue specimen is appropriately prepared for RNAscope. Refer to **Chapter 8. Evaluate the samples** for further details.
- Do not run more than one probe set per slide.
- Do not substitute required materials and reagents. Assay has been validated with these materials only.
- Follow the protocol exactly for best results.
- Do not let your sections dry out during the procedure except when specified.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix E. Safety** for more information.

Chapter 3. Prepare Samples

This chapter describes preparation methods for formalin-fixed paraffin-embedded (FFPE), fixed-frozen, and fresh-frozen samples. For guidelines to prepare cultured adherent or non-adherent cells, please refer to the following technical notes available at <https://acdbio.com/documents/product-documents>:

- Doc. No. TS 46-004,
- MK-50 010
- MK-50 012

For other sample types and preparation methods, contact support.acd@bio-technie.com for the latest protocols and guidelines.

IMPORTANT! We highly recommend following these guidelines. We cannot guarantee assay results with other preparation methods.

FFPE sample preparation

Materials required

-
- 10% neutral buffered formalin (NBF)
 - 1X PBS
 - Paraffin wax
 - 100% alcohol (EtOH)
 - Microtome
 - Water bath
 - Superfrost Plus slides
 - Distilled water
 - Fume hood
 - Paper towel or absorbent paper
 - Digital thermometer
-

Prepare FFPE tissue sections

1. Immediately following dissection, fix tissue in 10% NBF for **16–32 HRS** at **ROOM TEMPERATURE (RT)**. Fixation time will vary depending on tissue type and size.

IMPORTANT! Fixation for **<16 HRS** or **>32 HRS** may impair the performance of the RNAscope Assay.

2. Wash sample with 1X PBS.
3. Dehydrate sample using a standard ethanol series, followed by xylene.

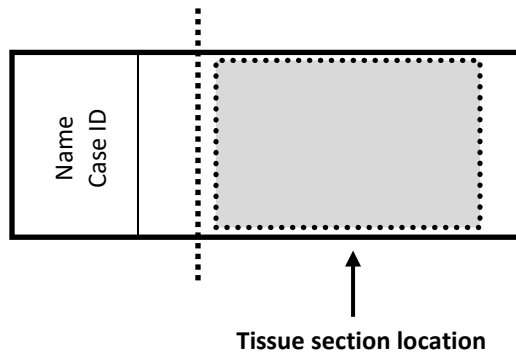
IMPORTANT! Use fresh reagents and embed samples as quickly as possible to preserve RNA quality.

4. Embed sample in paraffin using standard procedures.

Note: Embedded samples may be stored at room temperature with desiccants. To better preserve RNA quality over a long period (>1 yr), storing at 2–8°C with desiccants is recommended.

5. Trim paraffin blocks as needed and cut embedded tissue into $5 \pm 1 \mu\text{m}$ sections using a microtome.

6. Place paraffin ribbon in a 40–45°C water bath, and mount sections on **SUPERFROST PLUS SLIDES**. Place tissue as shown below for optimal staining:



IMPORTANT! Do not mount more than one section per slide. Place sections in the center of the slide.

7. Air dry slides **OVERNIGHT** at RT.

OPTIONAL STOPPING POINT (1). Use sectioned tissue within three months. Store sections at room temperature in a sealed container with desiccants.

Fixed-frozen tissue sample preparation

Materials required

-
- Scalpel
 - Forceps
 - Cryo-embedding medium (OCT)
 - Dry ice, liquid nitrogen, or isopentane
 - Cryostat
 - Slide boxes
 - Superfrost Plus slides
 - Aluminum foil or zip-lock bags
 - 1X PBS
 - 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA)
 - 30% sucrose
-

Fix samples

1. If needed, perfuse the tissue with freshly prepared 4% paraformaldehyde (PFA) in 1X PBS, or go directly to Step 2.

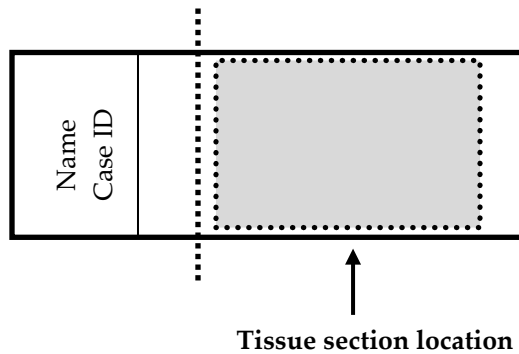
- Dissect the tissue and fix in freshly prepared 4% PFA for **24 HRS** at **4°C**.

Freeze tissues

- Immerse the tissue in 10% sucrose in 1X PBS at **4°C** until the tissue sinks to the bottom of the container (approximately **18 HRS** for brain tissue).
- Repeat this step with 20% sucrose in 1X PBS, followed by 30% sucrose in 1X PBS, each time allowing the tissue to sink to the bottom of the container.
- Freeze the tissue in Optimal Cutting Temperature (OCT) embedding media with dry ice or liquid nitrogen, and store it in an airtight container at **-80°C**.

Prepare sections

- Before tissue sectioning, equilibrate the tissue blocks at **-20°C** for at least **1 HR** in a cryostat.
- Cut 7–15 µm thick sections. Mount the sections on **SUPERFROST PLUS SLIDES**. Place tissue as shown for optimal staining:



IMPORTANT! Do not mount more than one section per slide. Place sections in the center of the slide.

- Air dry the slides for **60 –120 MIN** at **-20°C**.

OPTIONAL STOPPING POINT (1). Use sectioned tissue within three months. Store sections with desiccants at **-80°C**.

Fresh-frozen sample preparation

Materials required

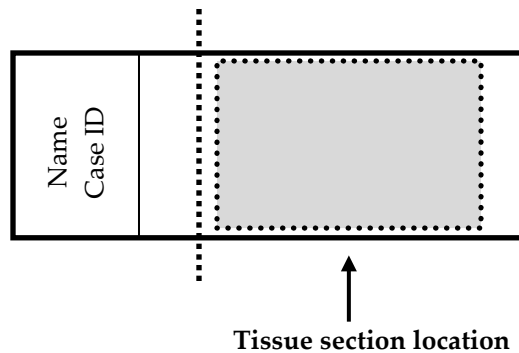
-
- Scalpel
 - Forceps
 - Cryo-embedding medium (OCT)
 - Dry ice, liquid nitrogen, or isopentane
 - Cryostat
 - Slide boxes
 - Superfrost Plus slides
 - Aluminum foil or zip-lock bags
-

Prepare fresh frozen tissue sections

1. Remove tissue and cut to fit into cryomolds.
2. Freeze the specimen within **5 MIN** of tissue harvest.
3. Embed the frozen tissue in cryo-embedding medium (OCT):
 - a. Add two drops of OCT into a cryomold.
 - b. Place the frozen tissue on the OCT in the correct orientation for cutting.
 - c. Add more OCT to fill the cryomold. Do not allow any air bubbles to form.
 - d. Freeze the block by holding the cryomold with forceps on the surface of the liquid nitrogen or isopentane cooled by dry ice or liquid nitrogen or place the cryomold on dry ice.
4. Store the frozen block in an air-tight container at **-80°C** prior to sectioning.

OPTIONAL STOPPING POINT (1). Section tissue within three months.

5. Section the block:
 - a. Equilibrate block to **-20°C** in a cryostat **~1 HR**.
 - b. Cut 10–20 μm thick sections and mount onto **SUPERFROST PLUS SLIDES**.



- c. Dry the sections at **60–120 MIN** at **-20°C** to retain tissue adherence.
6. Store the sections in slide boxes wrapped air-tight with aluminum foil or zip-lock bags at **-80°C** until use.

Note: Sections may be stored for up to three months.

IMPORTANT! Do not fix the slides prior to this step.

OPTIONAL STOPPING POINT (2). Use sectioned tissue within three months.

Chapter 4. Protease-free sample pretreatment

This chapter describes enzyme-free pretreatment method for formalin-fixed paraffin-embedded (FFPE), fixed-frozen, and fresh-frozen sample types. For other cultured cells and other sample types, contact support.acd@bio-techne.com for the latest protocols and guidelines.

IMPORTANT! We highly recommend following these guidelines. We cannot guarantee assay results with other preparation methods.

Determine pretreatment conditions

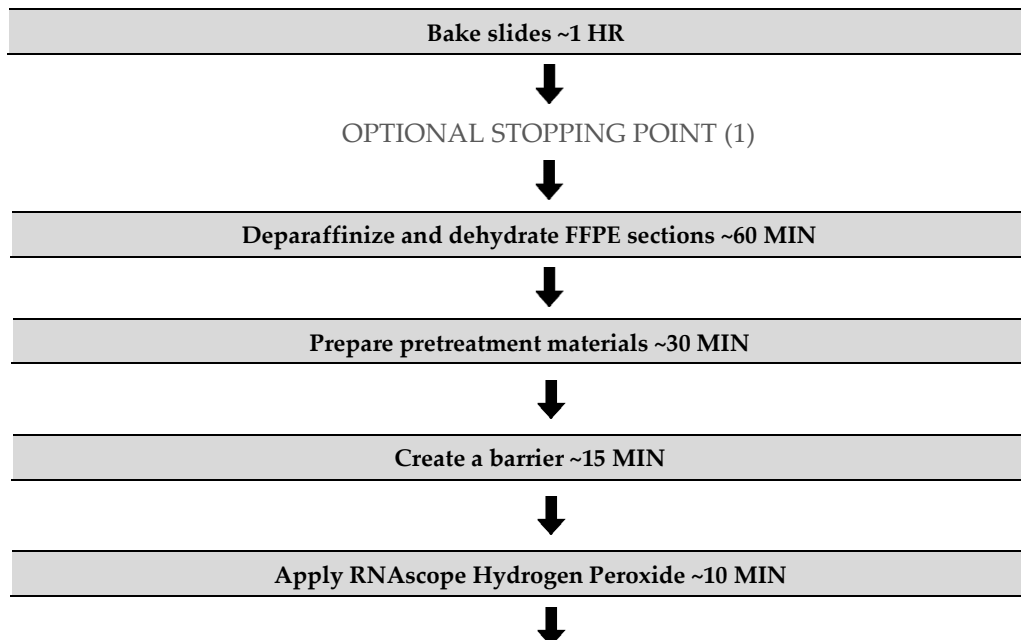
Do one of the following depending on whether you are detecting RNA only or co-detecting RNA and protein:

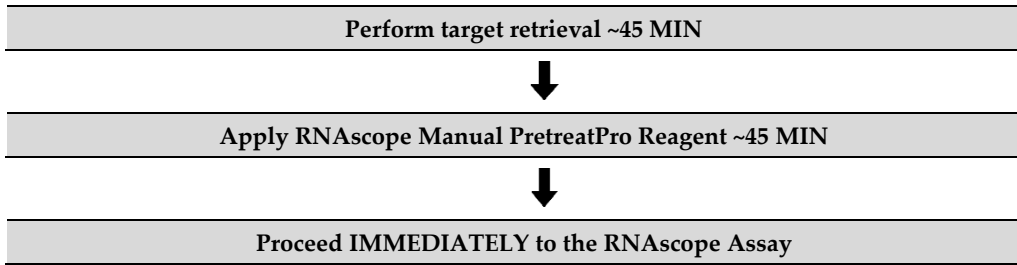
- For experiments requiring RNA-only detection, use the standard, highly cited protease-based workflow.
- For experiments that require RNA and protein co-detection, we recommend using the protease-free workflow now available in this assay kit. This workflow maintains antigen integrity, allowing for co-detection with any pre-qualified antibody, and preserves better tissue morphology in certain samples.

FFPE sample preparation and pretreatment

For sub-optimally prepared samples, you may need to optimize pretreatment conditions. Refer to [Troubleshooting the protease-free assay in Appendix C](#) for optimization tips.

Workflow





Materials required

Materials provided by Pretreatment Reagents (Cat. No. 322381 and 322000) and Detection kit (Cat. No. 323110)	Other Materials and Equipment
<ul style="list-style-type: none">• RNAscope Hydrogen Peroxide• RNAscope Manual PretreatPro• RNAscope 10X Target Retrieval Reagents• RNAscope 50X Wash Buffer	<ul style="list-style-type: none">• Vertical clearing agent dishes• Vertical staining dishes• Vertical slide rack• 100% alcohol (EtOH)• Xylene• ImmEdge Hydrophobic Pen• Drying oven• Distilled water• Fume hood• HybEZ Humidifying System/ACD EZ-Batch Slide Holder and Wash Tray• Paper towel or absorbent paper• Kimwipe• Steamer• Digital thermometer• Timer

Bake slides

IMPORTANT! We recommend using a dry-air oven for slide baking. If a dry oven is not available, you can use the HybEZ II Oven in “Bake Mode”.

1. Bake slides in a dry oven for **1 HR** at **60°C**.

OPTIONAL STOPPING POINT (2). Use immediately, or store at **RT** with desiccants for **≤1 WEEK**. Prolonged storage may degrade sample RNA.

Note: If you continue with the procedure, you can prepare materials for the next steps while the slides are baking.

Prepare 1X Wash Buffer

1. Warm RNAscope 50X Wash Buffer up to **40°C** for **10–20 MIN** before preparation. 1X Wash Buffer may be prepared ahead of time and stored at room temperature for up to one month.



2. Prepare 6 L of 1X Wash Buffer by adding 5.76 L distilled water and 2 bottles (120 mL) of RNAscope Wash Buffer (50X) to a large carboy. Mix well.

Deparaffinize FFPE sections

Reagents may be prepared ahead of time. Ensure that all containers remain covered.

1. In a fume hood:
 - Fill clearing agent dishes with ~200 mL fresh xylene.
 - Fill three staining dishes with ~200 mL fresh 100% ethanol.
 - Fill two staining dishes with ~200mL distilled water.
2. Place slides in a slide rack and submerge in the first xylene-containing dish in the fume hood.
3. Incubate the slides in **xylene** for **5 MIN** at **RT**. Agitate the slides by occasionally lifting the slide rack up and down in the dish.
4. Transfer the slide rack to the second xylene-containing dish in the fume hood. Incubate the slides in **xylene** for **5 MIN** at **RT** with agitation.
5. Transfer the slide rack to a dish containing 100% ethanol. Incubate the slides in **100% ethanol** for **2 MIN** at **RT** with agitation.
6. Transfer the slide rack to the second ethanol-containing dish. Incubate the slides in **100% ethanol** for **2 MIN** at **RT** with agitation.
7. Transfer the slide rack to the third ethanol-containing dish. Incubate the slides in **100% ethanol** for **30 MIN** at **RT**.

Note: You can preheat the steamer following the start of ethanol incubation to ensure that the target retrieval reagents have been heated to at least 99°C by the time the slides are ready for target retrieval.

8. Transfer the slide rack to a dish containing distilled water. Incubate the slides in distilled water for **5 MIN** at **RT** with agitation.

Prepare pretreatment materials

1. Turn on the HybEZ Oven, and set the temperature to 40°C.
2. Place a Humidifying Paper in the Humidity Control Tray and wet completely with distilled water.
3. Insert the covered tray into the oven and close the oven door. Warm the tray for **30 MIN** at 40°C before use. Keep the tray in the oven when not in use.
4. Prepare 1X Target Retrieval Reagent (for example, add 180 mL distilled water to 20 mL 10X Target Retrieval buffer). Mix well.

Note: 1X Target Retrieval Reagent must be heated to $\geq 99^{\circ}\text{C}$ before use (see **Perform target retrieval using the steamer** on page 26).

Create a hydrophobic barrier

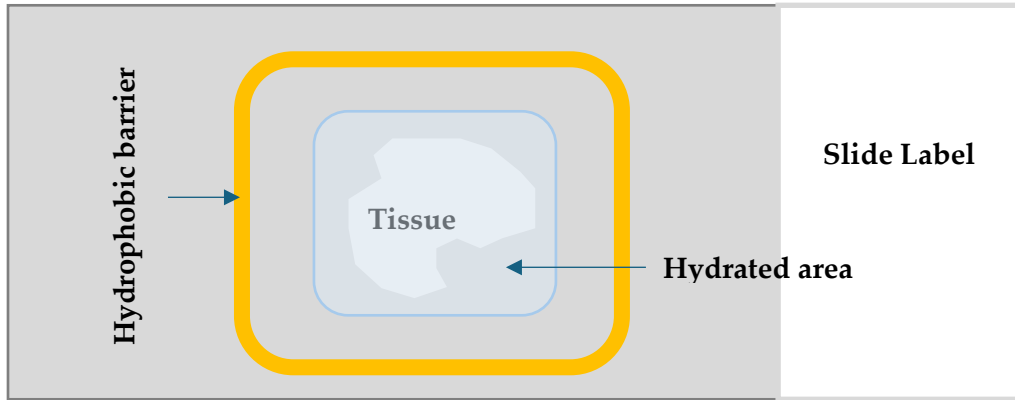
1. One slide at a time, use a tightly braided Kimwipe to thoroughly dry the blank slide area around the tissue while keeping the tissue hydrated.

IMPORTANT! Be very careful not to touch the tissues during this procedure

2. Use the following template to draw a thick barrier 2–4 times around each section with the **ImmEdge hydrophobic barrier pen** while keeping tissues hydrated with distilled water.

IMPORTANT! Do not let the barrier touch the tissue section. The ImmEdge hydrophobic barrier pen is highly recommended. Other pens may cause suboptimal results.

IMPORTANT! Do not let samples dry.



Note: We do not recommend drawing a smaller barrier and using less than the recommended volume amounts, even for smaller sections. Larger barriers will result in fewer tests per kit.

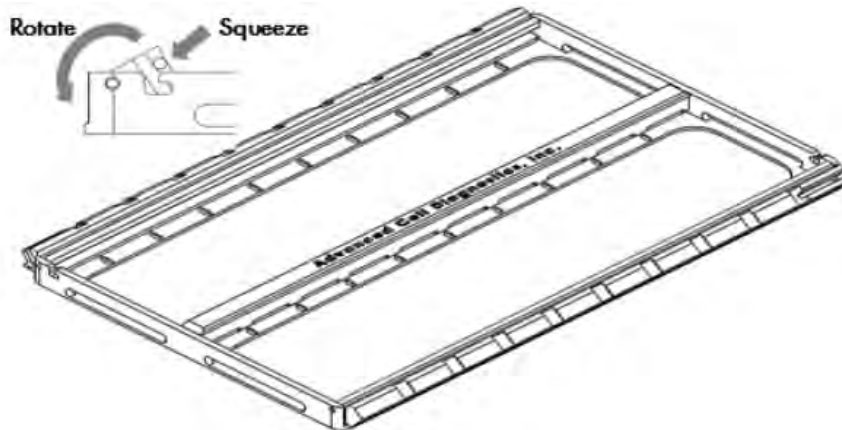
- Let the barrier dry for ~5 MIN while keeping the samples hydrated with distilled water. Immediately move on to the next section.

Note: If you need to reapply the hydrophobic barrier during the following procedures, dry the appropriate area of the slide with a Kimwipe. Do not touch the tissue section.

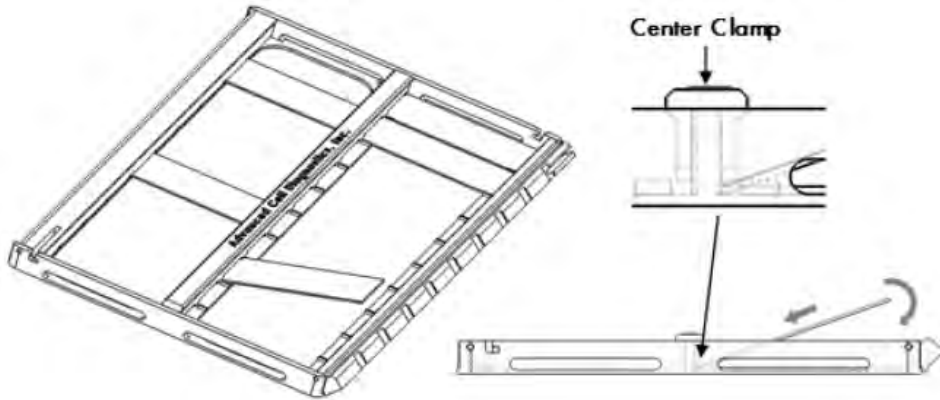
Load the slides into the ACD EZ-Batch Slide Holder

The ACD EZ-Batch Slide Holder can hold up to 20 standard glass slides in secure, lock-down positions arranged in two parallel columns. Lock-down is achieved by two lockable swing clamps, one per column, along both sides of the slide holder. Clamp locking mechanisms are located at the slots found at one end of each clamp.

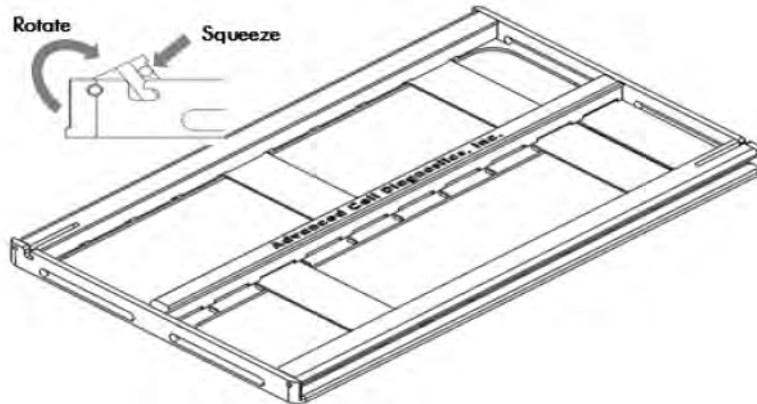
- Open the swing clamps one at a time by simultaneously squeezing (pressing and holding) the slotted portion of each clamp and rotating it up then outwards.



2. Insert each slide into the holder (up to 10 slides per column). The non-label end of each slide should be aligned toward the center of the holder and inserted under the fixed clamp. Place the rest of the slide down into the holder.
3. Always keep the slides hydrated. Add the next reagent (see the following procedure) to the slide in the holder before moving on to the next slide.



4. Close and lock the swing clamp of the column by simultaneously squeezing the slotted portion of each clamp and rotating it then downwards in the direction opposite to the direction used to open the clamp.

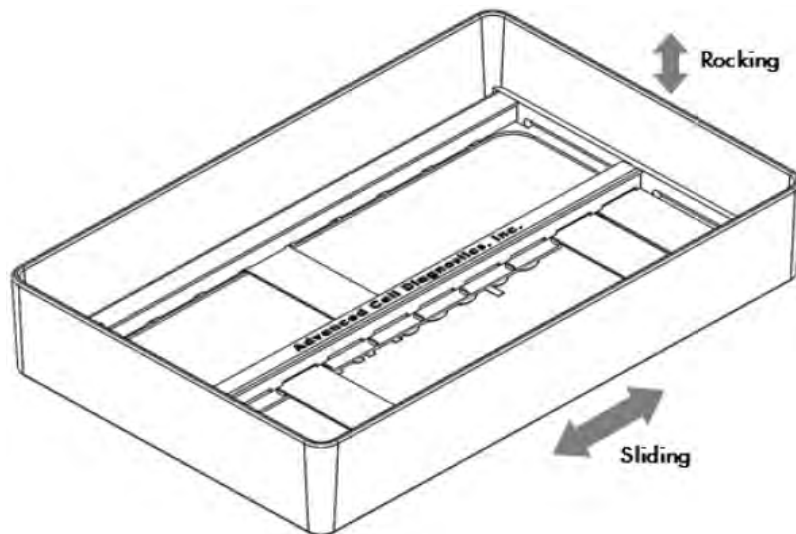


Apply RNAscope Hydrogen Peroxide

1. After loading a slide into the ACD EZ-Batch Slide Holder, immediately add ~5 drops of RNAscope Hydrogen Peroxide to the section. Use enough solution to completely cover the section.

Note: Process only one slide at a time while keeping the rest of the slides hydrated.

2. Incubate slides for **10 MIN** at **RT** in a RT HybEZ Humidity Control Tray on the bench.
3. Pour at least 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.
4. Place the ACD EZ-Batch Slide Holder into the wash tray containing distilled water and wash for **2 MIN**. Make sure all the slides are submerged. If needed, carefully add more water.



5. Repeat the wash step with fresh distilled water.

Perform target retrieval using the steamer

IMPORTANT! Before you begin, make sure you know the pretreatment conditions specific to your sample type.

We highly recommend using a steamer for target retrieval. For target retrieval using a hot plate, see **Appendix B. Manual Target Retrieval**. Always fill steamer water reservoir to the maximum level before starting. Refilling water during the steaming process will drop the temperature and interfere with the target retrieval step.

1. Fill the water reservoir with cold tap water to the MAX fill marking line.

IMPORTANT! Do not overfill.



2. Place the steaming bowl onto the base, and place two staining dishes in the steam bowl.
3. Fill one staining dish with 200 mL of RNAscope 1X Target Retrieval Reagent, and the other staining dish with 200 mL of distilled H₂O.
4. Turn on the steamer and set the heating time to the maximum amount so the steamer does not shut off during the procedure.
5. Insert a digital thermometer through the holes of the lid and into the staining dish containing RNAscope 1X Target Retrieval Reagent. Allow the temperature to rise to at least 99°C.
6. Add the slides to the staining dish containing hot distilled H₂O for **10 SEC** to acclimate the slides.



7. Transfer slides to the staining dish with RNAscope 1X Target Retrieval Reagent. Cover the steamer with the lid.
8. Incubate the slides in RNAscope 1X Target Retrieval reagent for **35 MIN**.

Note: The time needed for target retrieval depends on the sample types. We recommend starting with **35 MIN** for tissues or **15 MIN** for cell samples and reducing the target retrieval time if the result needs to be optimized.

9. Remove the slides from the steamer and transfer to a separate container filled with 200 mL of distilled water.
10. Wash slides with distilled water with continuous agitation for **5 MIN**.
11. Transfer the slides to a new container filled with fresh distilled water. Repeat Step 10 once.
12. Transfer the slides to a separate container filled with 1X Wash Buffer then soak the slides for **5 MIN** at RT.

Apply RNAscope Manual PretreatPro Reagent

1. Follow the procedure in **Load the slides into the ACD EZ-Batch Slide Holder**.
2. Add ~5 drops of RNAscope Manual PretreatPro reagent to entirely cover each section.
3. Place the ACDEZ-Batch Slide Holder in the pre-warmed HybEZ Humidity Control Tray. Close the lid, seal, and insert the tray back into the oven.
4. Incubate the slides at **40°C** for **35 MIN**.

Note: If needed, prepare RNAscope Assay materials during this step.

5. Pour at least 200 mL distilled water into the transparent ACDEZ-Batch Wash Tray.
6. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray and place the tray back into the oven.
7. Place the ACDEZ-Batch Slide Holder into the EZ-Batch Wash Tray. Make sure all the slides are submerged. If needed, carefully add more water. Wash the slides with continuous light agitation for **5 MIN**.
8. Repeat the wash step with fresh distilled water.
9. Soak slides in 1X Wash Buffer for **5 MIN** at RT.

Proceed to the RNAscope Assay

Proceed *immediately* to the target probe hybridization step as described in **Chapter 6. RNAscope Multiplex Fluorescent v2 Assay**.

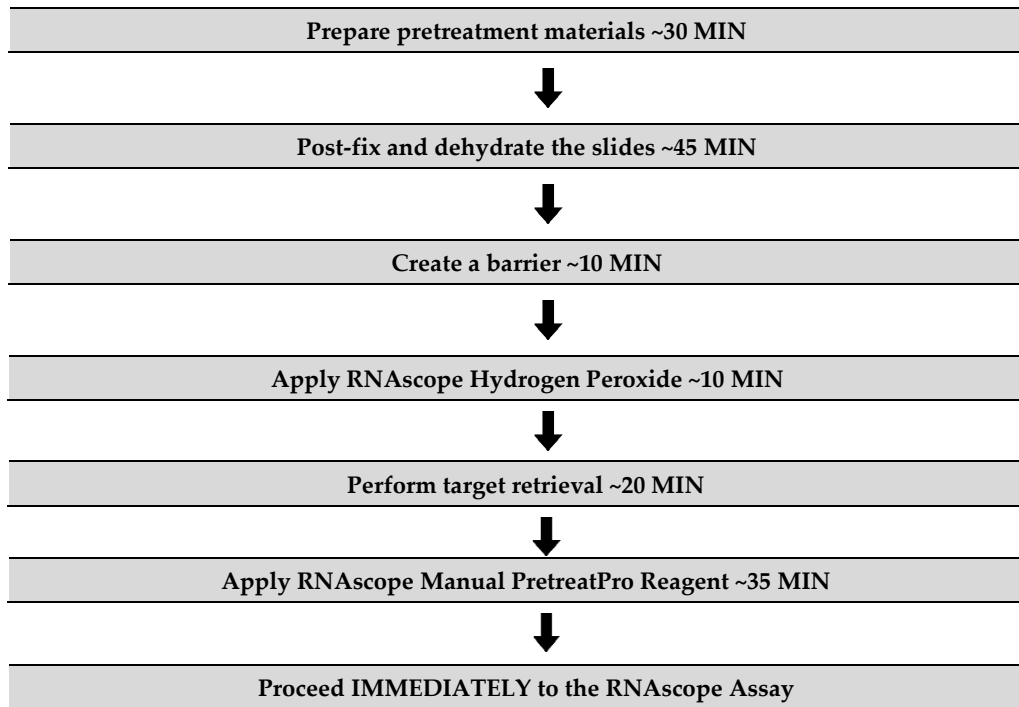
Fixed-frozen tissue sample preparation and pretreatment

For sub-optimally prepared samples, you may need to optimize pretreatment conditions. Contact technical support at support.acd@bio-techne.com.

Workflow

Prepare fixed frozen sections ~30 MIN





Materials required

Materials provided by Pretreatment Reagents (Cat. No. 322381 and 322000) and Detection kit (Cat. No. 323110)	Other Materials and Equipment
<ul style="list-style-type: none"> • RNAscope Hydrogen Peroxide • RNAscope Manual PretreatPro • RNAscope 10X Target Retrieval Reagents • RNAscope 50X Wash Buffer 	<ul style="list-style-type: none"> • 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) • 100% Ethanol (400mL) • 70% Ethanol (200mL) • 50% Ethanol (200mL) • Vertical 24 slide rack • Vertical staining dishes • ImmEdge Hydrophobic Barrier Pen • HybEZ Humidifying System/ ACD EZ-Batch Slide Holder and Wash Tray • Distilled water • Paper towel or absorbent paper • Kimwipe • Steamer • Digital thermometer • Drying oven

Prepare 1X Wash Buffer

1. Warm RNAscope 50X Wash Buffer up to 40°C for 10–20 MIN before preparation. 1X Wash Buffer may be prepared ahead of time and stored at room temperature for up to one month.



2. Prepare 6 L of 1X Wash Buffer by adding 5.76 L distilled water and 2 bottles (120 mL) of RNAscope Wash Buffer (50X) to a large carboy. Mix well.

Fix the frozen sections

1. Chill 10% NBF (fresh 10% NBF or 4% PFA in 1X PBS) to 4°C.
2. Remove slides from -80°C, and bake the slides for **30 MIN** at **60°C**.
3. Wash the slides with 200 mL 1X PBS in a slide rack and staining dish for **5 MIN** while moving the rack up and down to remove OCT.
4. Post-fix the slides by immersing them in prechilled 10% NBF or 4% PFA in 1X PBS for **15 – 60 MIN** at 4°C.

IMPORTANT! For some tissue types you may have to empirically determine the best duration for fixation.

Dehydrate and dry the tissue

1. Prepare 200mL distilled water, 200 mL 50% EtOH, 200 mL 70% EtOH, and 400 mL of 100% EtOH.
2. Remove the slides from the 10% NBF or 4% PFA, and immerse them in 50% EtOH for **5 MIN** at **RT**.
3. Remove the slides from 50% EtOH, and immerse them in 70% EtOH for **5 MIN** at **RT**.
4. Remove the slides from 70% EtOH, and immerse them in 100% EtOH for **5 MIN** at **RT**.
5. Remove the slides from 100% EtOH, and immerse them in fresh 100% EtOH for **5 MIN** at **RT**.
6. Remove the slides from 100% EtOH, and immerse them in fresh distilled water for **5 MIN** at **RT**.

Prepare pretreatment materials

1. Turn on the HybEZ Oven, and set temperature to **40°C**.
2. Place a Humidifying Paper in the Humidity Control Tray and wet completely with distilled water.
3. Insert covered tray into oven and close the oven door. Warm the tray for **30 MIN** at **40°C** before use. Keep the tray in the oven when not in use.
4. Prepare 1X Target Retrieval Reagent (e.g. by adding 180 mL distilled water to 20 mL 10X Target Retrieval reagent stock). Mix well.

Note: 1X Target Retrieval Reagent must be heated to $\geq 99^{\circ}\text{C}$ before use (see **Perform target retrieval using the steamer** later in this section.).

Create a hydrophobic barrier

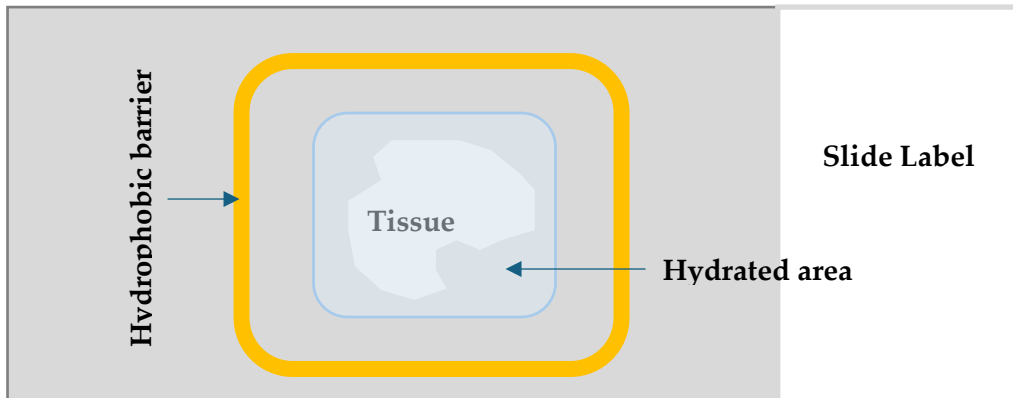
1. One slide at a time, use a tightly braided Kimwipe to thoroughly dry the blank slide area around the tissue while keeping the tissue hydrated.

IMPORTANT! Be very careful not to touch the tissues during this procedure

2. Use the following template to draw a thick barrier **2–4** times around each section with the **ImmEdge hydrophobic barrier pen** while keeping tissues hydrated with distilled water.

IMPORTANT! Do not let the barrier touch the tissue section. The ImmEdge hydrophobic barrier pen is highly recommended. Other pens may cause suboptimal results.

IMPORTANT! Do not let samples dry.



Note: We do not recommend drawing a smaller barrier and using less than the recommended volume amounts, even for smaller sections. Larger barriers will result in fewer tests per kit.

- Let the barrier dry for ~5 **MIN** while keep the samples covered in distilled water. Immediately move on to the next section.

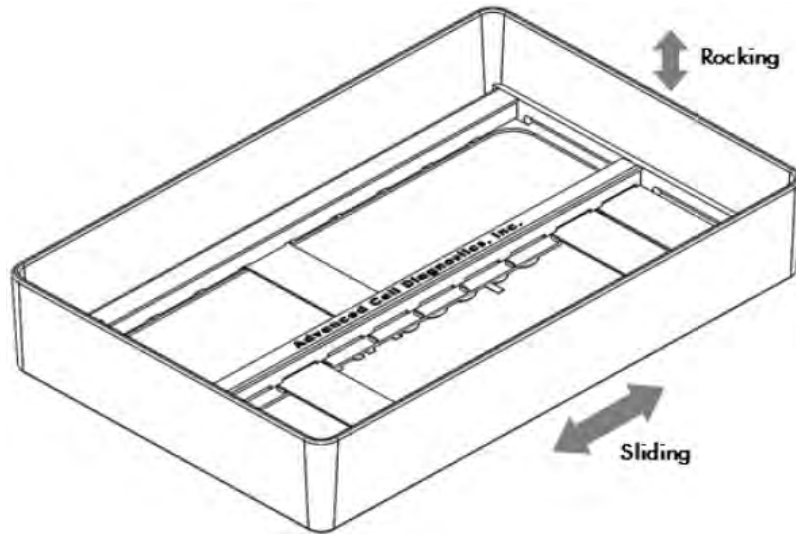
Note: If you need to reapply the hydrophobic barrier during the following procedures, dry the appropriate area of the slide with a Kimwipe. Do not touch the tissue section.

Apply RNAscope Hydrogen Peroxide

- Load the slides into the ACD EZ-Batch Slide Holder (see page 24).
- After loading each slide, add ~5 drops of RNAscope Hydrogen Peroxide to entirely cover the section. Then process the next slide.

Note: Process only one slide at a time while keeping the rest of the slides hydrated.

- Place the ACDEZ-Batch Slide Holder in a room-temperature HybEZ Humidity Control Tray. Close the lid and seal the tray.
- Incubate slides for **10 MIN** on the bench.
- Pour at least 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.
- Place the ACDEZ-Batch Slide Holder into the EZ-Batch Wash Tray. Make sure all the slides are submerged. If needed, carefully add more water. Wash the slides with continuous agitation for **5 MIN**.



7. Repeat the wash step with fresh distilled water.

Perform target retrieval using the steamer

IMPORTANT! Before you begin, make sure you know the pretreatment conditions specific to your sample type.

We highly recommend using a steamer for target retrieval. For target retrieval using a hot plate, see **Appendix B. Manual Target Retrieval**. For each steamer, fill the reservoir with water to the maximum level before starting. Refilling water during the steaming process will drop the temperature and interfere with the target retrieval step.

1. Fill the water reservoir with cold tap water to the MAX fill marking line.

IMPORTANT! Do not overfill.



2. Place the steaming bowl onto the base, and place two staining dishes in the steam bowl.
3. Fill one slide staining dish with 200 mL of RNAscope 1X Target Retrieval Reagent, and the other slide staining dish with 200 mL of distilled H₂O.
4. Turn on the steamer and set the heating time to the maximum amount so the steamer does not shut off during the procedure.
5. Insert a digital thermometer through the holes of the lid and into the staining dish containing RNAscope 1X Target Retrieval Reagent. Allow the temperature to rise to at least 99°C.
6. Add the slides to the staining dish containing hot distilled H₂O for **10 SEC** to acclimate the slides.



7. Transfer the slides to the staining dish containing RNAscope 1X Target Retrieval Reagent. Cover the steamer with the lid.
 8. Start the timer for **10–15 MIN**.
- Note:** The time needed for target retrieval depends on the sample type. We recommend starting with 10–15 minutes and reducing the target retrieval time if the result needs to be optimized.
9. Remove the slides from the steamer and transfer to a separate container filled with 200 mL of distilled water at **RT**.
 10. Rinse slides with distilled water with slight agitation for **2 MIN**.
 11. Transfer the slides to a new container filled with fresh distilled water for **2 MIN**.
 12. Transfer the slides to a separate container filled with 1X Wash Buffer for **5 MIN**.

Apply RNAscope Manual PretreatPro Reagent

1. Load the slides into the ACD EZ-Batch Slide Holder (see page 24).
2. Add ~5 drops of RNAscope Manual PretreatPro Reagent to each section. Use enough solution to completely cover the sections.
3. Remove the HybEZ Humidity Control Tray from the HybEZ Oven and place the ACD EZ-Batch Slide Holder in the tray. Close the lid, seal, and insert the tray back into the oven.
4. Incubate the samples for **30 MIN** at **40°C**.

Note: If needed, prepare RNAscope Assay materials during this step.

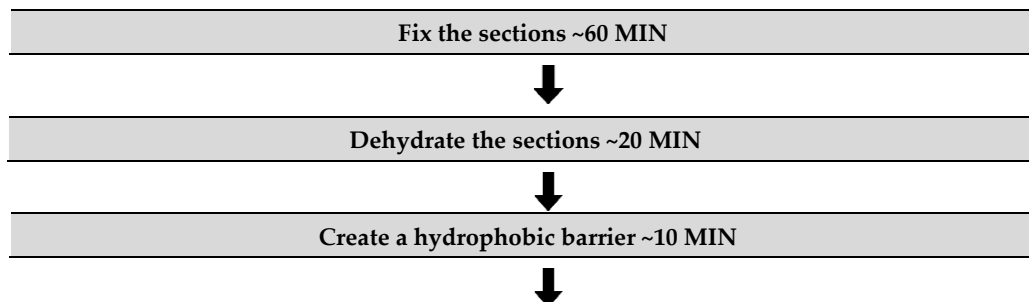
5. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray and place the tray back into the oven.
6. Pour 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.
7. Place the ACD EZ-Batch Slide Holder containing the slides into the wash tray and wash the slides for **2 MIN** at **RT**.
8. Repeat Step 7 with fresh distilled water once.
9. Soak slides in 1X Wash Buffer for **5 MIN** at **RT**.

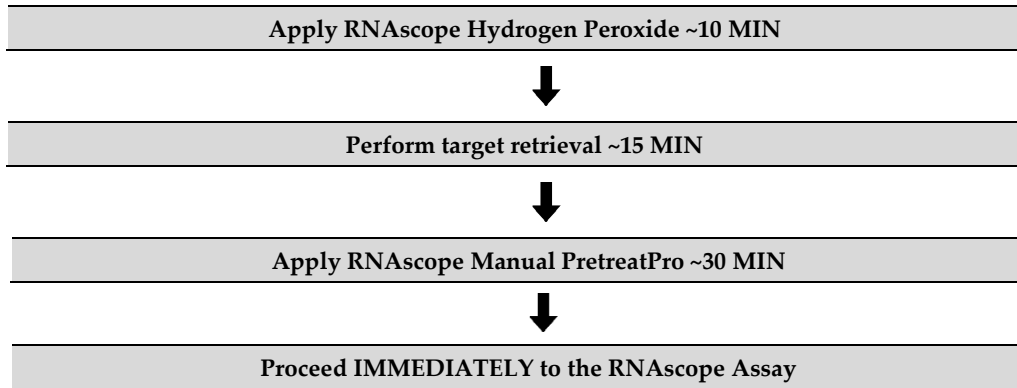
Proceed to the RNAscope Assay

Proceed *immediately* to the target probe hybridization step as described in **Chapter 6. RNAscope Multiplex Fluorescent v2 Assay**.

Fresh-frozen sample preparation and pretreatment

Workflow





Materials required

Materials provided by Pretreatment Reagents (Cat. No. 322381) and Detection kit (Cat. No. 323110)	Other Materials and Equipment
<ul style="list-style-type: none">• RNAscope Hydrogen Peroxide• RNAscope Manual PretreatPro• RNAscope 10X Target Retrieval ReagentsRNAscope 50X Wash Buffer	<ul style="list-style-type: none">• 1X PBS• 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA)• 100% alcohol (EtOH) (400mL)• 70% alcohol (EtOH) (200mL)• 50% alcohol (EtOH) (200mL)• Vertical 24 slide rack• Vertical staining dishes• ImmEdge Hydrophobic Barrier Pen• HybEZ Humidifying System/ ACD EZ-Batch Slide Holder and Wash Tray• Steamer• Distilled water• Paper towel or absorbent paper• Kimwipe• Timer

Prepare 1X Wash Buffer

1. Warm RNAscope 50X Wash Buffer up to 40°C for 10–20 MIN before preparation. 1X Wash Buffer may be prepared ahead of time and stored at room temperature for up to one month.
2. Prepare 6 L of 1X Wash Buffer by adding 5.76 L distilled water and 2 bottles (120 mL) of RNAscope Wash Buffer (50X) to a large carboy. Mix well.

Fix the sections

1. Chill fresh 10% NBF (or 4% PFA in 1X PBS) to 4°C.
2. Remove frozen slides from –80°C, and place in the pre-chilled fixative. Fix for 1 HR at 4°C.
3. Rinse the slides 2 times with 1X PBS to remove excess fixative.

Note: Formalin that has been stored for more than six months, exposed to air for more than a week, or used repeatedly may result in suboptimal tissue fixation.

IMPORTANT! For some tissue types you may have to empirically determine the best duration for fixation.

Dehydrate the sections

Reagents may be prepared ahead of time. Ensure all containers remain covered.

1. Prepare 200mL distilled water, 200 mL 50% ethanol, 200 mL 70% ethanol, and 400 mL 100% ethanol.
2. Place the slides in 50% ethanol for **5 MIN** at **ROOM TEMPERATURE (RT)**.
3. Place the slides in 70% ethanol for **5 MIN** at **RT**.
4. Place the slides in 100% ethanol for **5 MIN** at **RT**.
5. Place slides in fresh 100% ethanol for **5 MIN** at **RT**.
6. Wash slides in distilled water for **5MIN** at **RT**.

Create a hydrophobic barrier

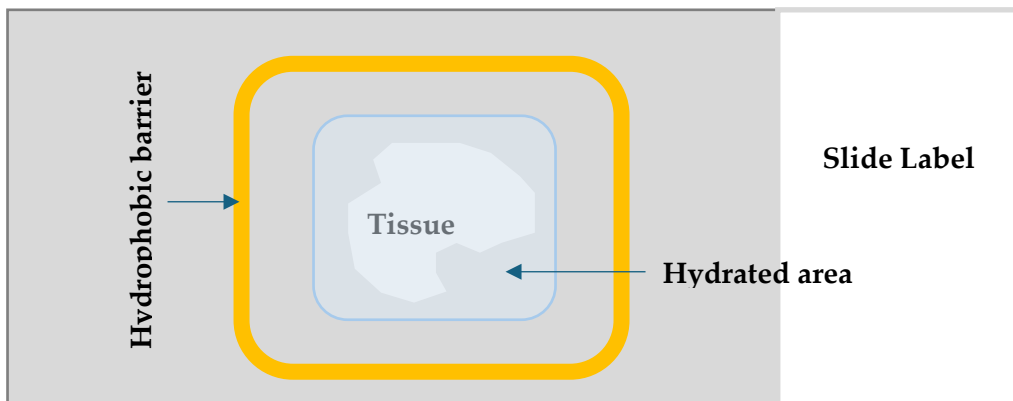
1. One slide at a time, use a tightly braided Kimwipe to thoroughly dry the blank slide area around the tissue while keeping the tissue hydrated.

IMPORTANT! Be very careful not to touch the tissues during this procedure

2. Use the following template to draw a thick barrier **2–4** times around each section with the **ImmEdge hydrophobic barrier pen** while keeping tissues hydrated with distilled water.

IMPORTANT! Do not let the barrier touch the tissue section. The ImmEdge hydrophobic barrier pen is highly recommended. Other pens may cause suboptimal results.

IMPORTANT! Do not let samples dry.



Note: We do not recommend drawing a smaller barrier and using less than the recommended volume amounts, even for smaller sections. Larger barriers will result in fewer tests per kit.

3. Let the barrier dry for **~5 MIN** while keep the samples covered in distilled water. Immediately move on to the next section.

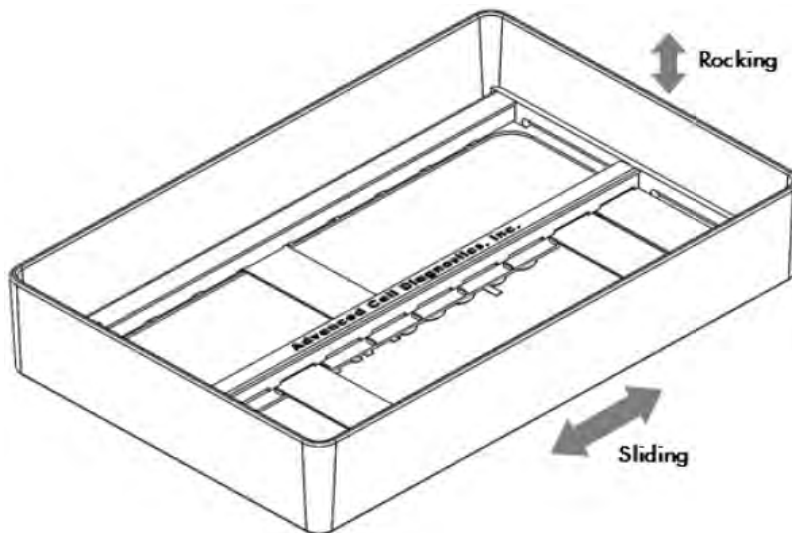
Note: If you need to reapply the hydrophobic barrier during the following procedures, dry the appropriate area of the slide with a Kimwipe. Do not touch the tissue section.

Apply RNAscope Hydrogen Peroxide

1. Load the slides into the ACD EZ-Batch Slide Holder (see page 24).
2. After loading each slide, add ~5 drops of RNAscope Hydrogen Peroxide to entirely cover the section. Then process the next slide.

Note: Process only one slide at a time while keeping the rest of the slides hydrated.

3. Place the ACDEZ-Batch Slide Holder in a RT HybEZ Humidity Control Tray. Close the lid and seal the tray.
4. Incubate slides for **10 MIN** at **RT**.
5. Pour at least 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.
6. Place the ACDEZ-Batch Slide Holder into the EZ-Batch Wash Tray. Make sure all the slides are submerged. If needed, carefully add more water. Wash the slides with continuous agitation for **5 MIN**.



7. Repeat the wash step with fresh distilled water.

Perform target retrieval using the steamer

IMPORTANT! Before you begin, make sure you know the pretreatment conditions specific to your sample type.

We highly recommend using a steamer for target retrieval. For target retrieval using a hot plate, see **Appendix B. Manual Target Retrieval**. For each steamer, fill the water to the maximum level before starting. Refilling water during the steaming process will drop the temperature and interfere with the target retrieval step.

1. Fill the water reservoir with cold tap water to the MAX fill marking line.

IMPORTANT! Do not overfill.



2. Place the steaming bowl onto the base, and place two staining dishes in the steam bowl.
3. Fill one slide staining dish with 200 mL of RNAscope 1X Target Retrieval Reagent, and the other slide staining dish with 200 mL of distilled H₂O.
4. Turn on the steamer and set the heating time to the maximum amount so the steamer does not shut off during the procedure.
5. Insert a digital thermometer through the holes of the lid and into the staining dish containing RNAscope 1X Target Retrieval Reagent. Allow the temperature to rise to at least 99°C.
6. Add the slides to the staining dish containing hot distilled H₂O for **10 SEC** to acclimate the slides.
7. Transfer the slides to the staining dish containing RNAscope 1X Target Retrieval Reagent. Cover the steamer with the lid.
8. Start the timer for **5-10 MIN**.

Note: The time needed for target retrieval depends on the sample types. We recommend starting with **5-10 MIN** and reducing the target retrieval time if the result needs to be optimized.

9. Remove the slides from the steamer and transfer to a separate container filled with 200 mL of distilled water.
10. Rinse slides with distilled water with slight agitation for **2 MIN**.
11. Transfer the slides to a new container filled with fresh distilled water for **2 MIN**.
12. Transfer the slides to a separate container filled with 1X Wash Buffer for **5 MIN**.

Apply RNAscope Manual PretreatPro Reagent

IMPORTANT! View the wash step video at <https://acdbio.com/technical-support/learn-more> before proceeding.

1. Follow the procedure in **Load the slides into the ACD EZ-Batch Slide Holder** on page 24.
2. Add ~5 drops of RNAscope Manual PretreatPro Reagent to each section. Use enough solution to completely cover the sections.
3. Remove the HybEZ Humidity Control Tray from the HybEZ Oven and place the ACD EZ-Batch Slide Holder in the tray. Close the lid, seal, and insert the tray back into the oven.
4. Incubate the samples for **30 MIN** at **40°C**.

Note: If needed, prepare RNAscope Assay materials during this step.

5. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray and place the tray back into the oven.
6. Pour 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.
7. Place the ACD EZ-Batch Slide Holder containing the slides into the wash tray and wash the slides for **2 MIN** at RT (see page x for details).
8. Repeat Step 7 with fresh distilled water once.



9. Soak slides in 1X Wash Buffer for **5 MIN** at **RT**.

Proceed to the RNAscope Assay

Proceed *immediately* to the target probe hybridization step as described in **Chapter 6. RNAscope Multiplex Fluorescent v2 Assay**.

Chapter 5. Protease-Based Sample Pretreatment

This chapter describes protease-based pretreatment method for formalin-fixed paraffin-embedded (FFPE), fixed-frozen, and fresh-frozen sample types. For other cultured cells and other sample types, contact support.acd@bio-techne.com for the latest protocols and guidelines.

Determine pretreatment conditions

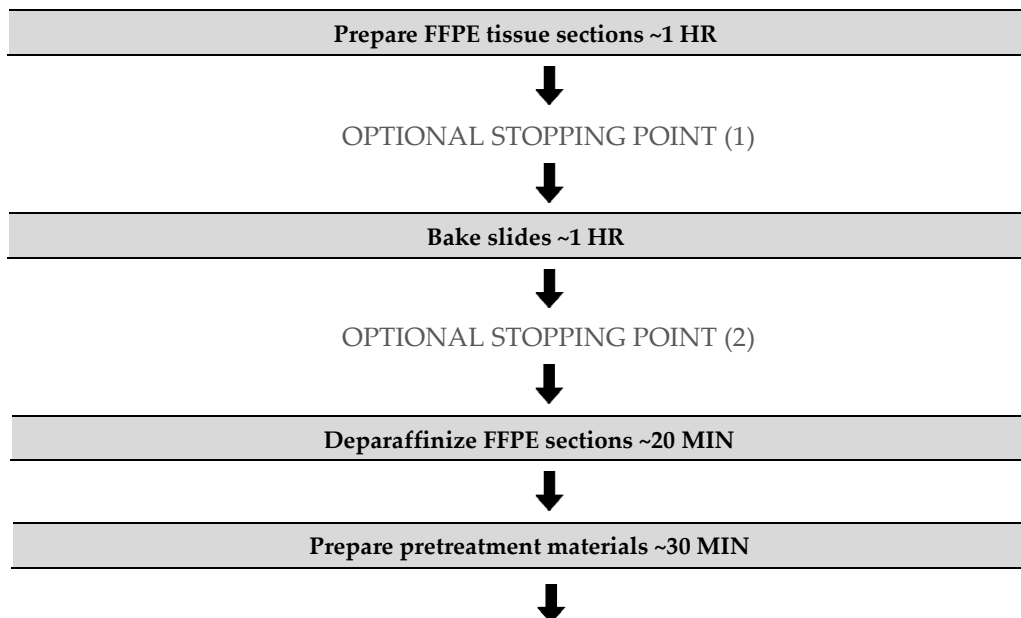
Do one of the following depending on whether you are detecting RNA only or co-detecting RNA and protein:

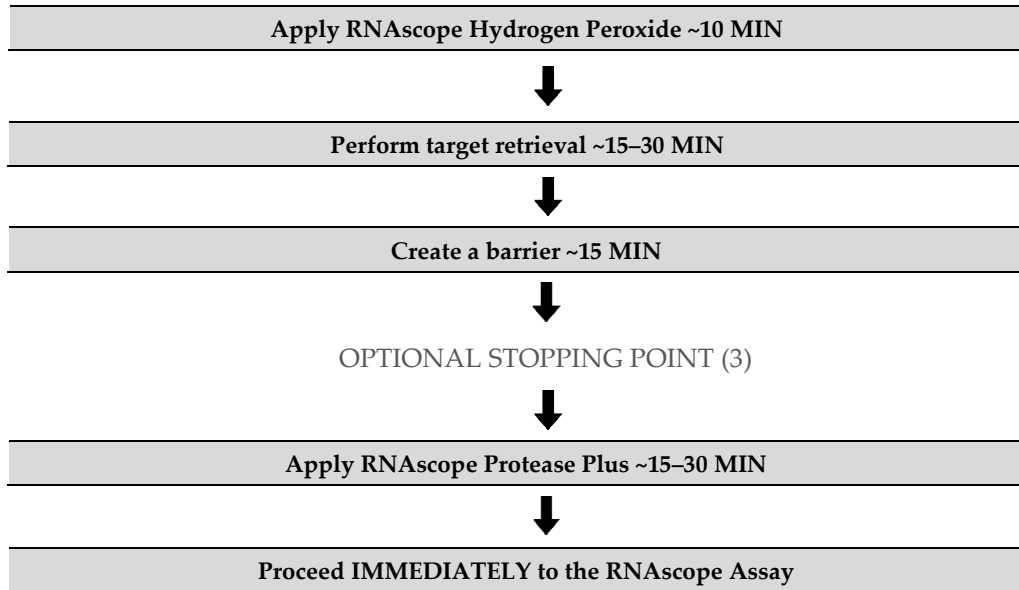
- For experiments requiring RNA-only detection, use the standard, highly cited protease-based workflow.
- For experiments that require RNA and protein co-detection, we recommend using the protease-free workflow now available in this assay kit. This workflow maintains antigen integrity, allowing for co-detection with any pre-qualified antibody, and preserves better tissue morphology in certain samples.

FFPE sample pretreatment

For sub-optimally prepared samples, you may need to optimize pretreatment conditions. Refer to **Appendix A** and to <https://acdbio.com/technical-support/solutions>.

Workflow





Materials required

Materials provided by Pretreatment Reagents (Cat. No. 322381 and 322000) and Detection kit (Cat. No. 323110)	Other Materials and Equipment
<ul style="list-style-type: none"> • RNAscope Hydrogen Peroxide • RNAscope Protease Plus • RNAscope 10X Target Retrieval Reagents 	<ul style="list-style-type: none"> • Vertical clearing agent dishes • Vertical staining dishes • Vertical slide rack • 100% alcohol (EtOH) • Xylene • ImmEdge Hydrophobic Pen • Drying oven • Distilled water • Fume hood • HybEZ Humidifying System/ACD EZ-Batch Slide Holder and Wash Tray • Paper towel or absorbent paper • Kimwipe • Steamer • Digital thermometer • Timer

Bake slides

IMPORTANT! We recommend using a dry-air oven for baking. If a dry oven is not available, you can use the HybEZ II Oven in “Bake Mode”.

1. Bake slides in a dry oven for **1 HR** at **60°C**.

OPTIONAL STOPPING POINT (2). Use immediately, or store at **RT** with desiccants for **≤1 WEEK**. Prolonged storage may degrade sample RNA.



Note: If you continue with the procedure, you can prepare materials for the next steps while the slides are baking.

Deparaffinize FFPE sections

Reagents may be prepared ahead of time. Ensure that all containers remain covered.

1. In a fume hood:
 - Fill two clearing agent dishes with ~200 mL fresh xylene.
 - Fill two staining dishes with ~200 mL fresh 100% ethanol.
2. Place slides in a slide rack and submerge in the first xylene-containing dish in the fume hood.
3. Incubate the slides in **xylene** for **5 MIN** at **RT**. Agitate the slides by occasionally lifting the slide rack up and down in the dish.
4. Transfer the slide rack to the second xylene-containing dish in the fume hood. Incubate the slides in **xylene** for **5 MIN** at **RT** with agitation.
5. Transfer the slide rack to a dish containing 100% ethanol. Incubate the slides in **100% ethanol** for **2 MIN** at **RT** with agitation.
6. Transfer the slide rack to the second ethanol-containing dish. Incubate the slides in **100% ethanol** for **2 MIN** at **RT** with agitation.
7. Remove the slides from the rack, and place on absorbent paper with the section face-up. Dry slides in a drying oven for **5 MIN** at **60°C** (or until completely dry).

Prepare pretreatment materials

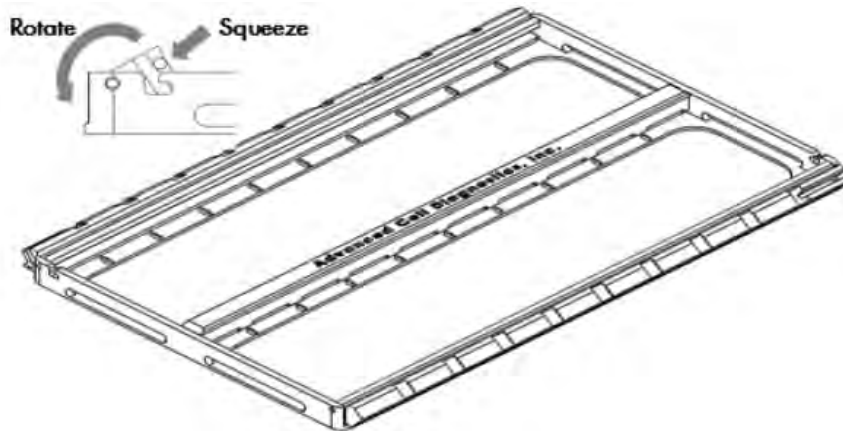
1. Turn on the HybEZ Oven, and set the temperature to **40°C**.
2. Place a Humidifying Paper in the Humidity Control Tray and wet completely with distilled water.
3. Insert the covered tray into the oven and close the oven door. Warm the tray for **30 MIN** at **40°C** before use. Keep the tray in the oven when not in use.
4. Prepare 1X Target Retrieval Reagents (for example, add 180 mL distilled water to 20 mL 10X Target Retrieval buffer). Mix well.

Note: Target Retrieval buffer must be heated to $\geq 99^{\circ}\text{C}$ before use (see **Perform target retrieval using the steamer**).

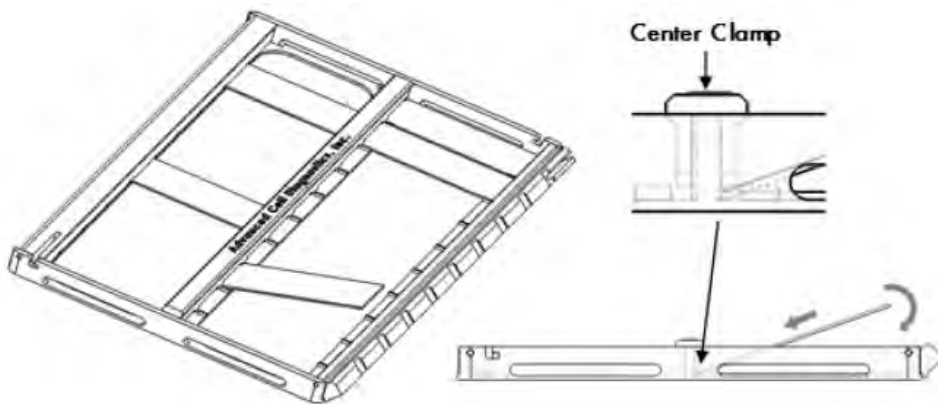
Load the slides into the ACD EZ-Batch Slide Holder

The ACD EZ-Batch Slide Holder can hold up to 20 standard glass slides in secure, lock-down positions arranged in two parallel columns. Lock-down is achieved by two lockable swing clamps, one per column, along both sides of the slide holder. Clamp locking mechanisms are located at the slots found at one end of each clamp.

1. Open the swing clamps one at a time by simultaneously squeezing (pressing and holding) the slotted portion of each clamp and rotating it up then outwards.



2. Insert slides one at a time into the holder (up to 10 slides per column). The non-label end of each slide should be aligned toward the center of the holder and inserted under the fixed clamp. Place the rest of the slide down into the holder.



3. Close and lock the swing clamp of the column by simultaneously squeezing the slotted portion of each clamp and rotating it in then downwards in the direction opposite to the direction used to open the clamp.

Apply RNAscope Hydrogen Peroxide

1. Add ~5 drops of RNAscope Hydrogen Peroxide to each section. Use enough solution to completely cover the sections.
2. Incubate slides for **10 MIN** at **RT** in the HybEZ Humidity Control Tray on the bench.
3. Pour at least 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.
4. Place the ACD EZ-Batch Slide Holder into the wash tray and wash with agitation for **2 MIN**. Make sure all the slides are submerged. If needed, carefully add more water.
5. Repeat the wash step with fresh water.

Perform target retrieval using the steamer

IMPORTANT! Before you begin, make sure you know the pretreatment conditions specific to your sample type from **Appendix A**.

We highly recommend using a steamer for target retrieval. For target retrieval using a hot plate, see **Appendix B. Manual Target Retrieval**. Always fill steamer water reservoir to the maximum level before starting. Refilling water during the steaming process will drop the temperature and interfere with the target retrieval step.

1. Fill the water reservoir with cold tap water to the MAX fill marking line.

IMPORTANT! Do not overfill.

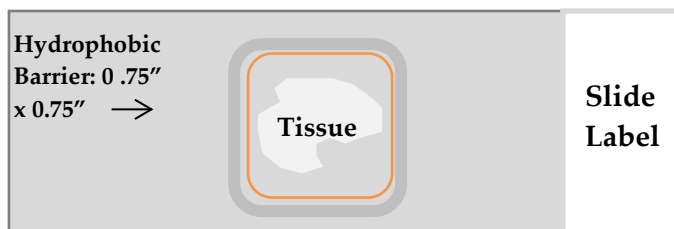


2. Place the steaming bowl onto the base, and place two slide holders in the steam bowl.
3. Fill one slide holder with 200 mL of RNAscope 1X Target Retrieval Reagent, and the other slide holder with 200 mL of distilled H₂O.
4. Turn on the steamer and set the heating time to the maximum amount so the steamer does not shut off during the procedure.
5. Insert a digital thermometer through the holes of the lid and into the container containing RNAscope 1X Target Retrieval Reagent. Allow the temperature to rise to at least 99°C.
6. Add the slides to the container containing distilled H₂O for **10 SEC** to acclimate the slides.
7. Transfer slides to the container with RNAscope 1X Target Retrieval Reagent. Cover the steamer with the lid.
8. Start the timer for **15 MIN** for mild and standard conditions, and **30 MIN** for extended pretreatment. For recommended pretreatment times, consult **Appendix A**.
9. Remove the slides from the steamer and transfer to a separate rinse container with 200 mL of distilled water. Allow the slides to rinse for **15 SEC**.
10. Transfer the slides to 100% ethanol for **3 MIN**.
11. Dry the slides in a **60°C** incubator (or at **RT**) for **5 MIN**.

Create a hydrophobic barrier

1. Use the following template to draw a barrier 2–4 times around each section with the **ImmEdge hydrophobic barrier pen**.

IMPORTANT! Do not let the barrier touch the tissue section. An ImmEdge hydrophobic barrier pen is highly recommended. Other pens may cause suboptimal results.

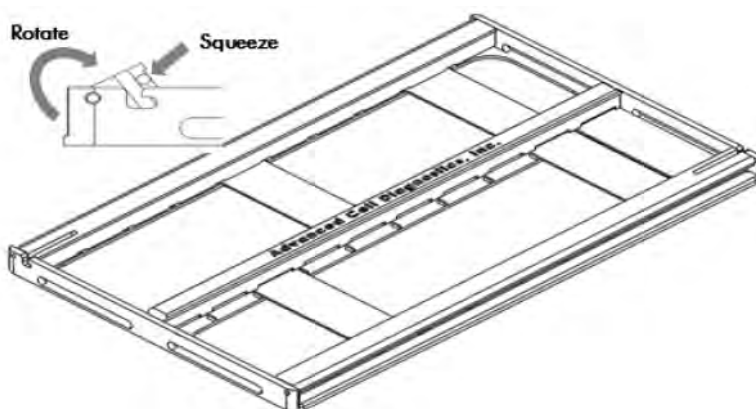


Note: We do not recommend drawing a smaller barrier and using less than the recommended volume amounts, even for smaller sections. Larger barriers will result in fewer tests per kit.

2. Let the barrier dry completely ~5 MIN or **OVERNIGHT** at RT.

Note: If you need to reapply the hydrophobic barrier during the following procedures, dry the appropriate area of the slide with a Kimwipe. Do not touch the tissue section.

OPTIONAL STOPPING POINT (3). Dry slides overnight at room temperature for use the following day or proceed directly to the next section.



Apply RNAscope Protease Plus

1. Add ~5 drops of RNAscope Protease Plus to entirely cover each section.
2. Place the ACDEZ-Batch Slide Holder in the pre-warmed HybEZ Humidity Control Tray. Close the lid, seal, and insert the tray back into the oven.
3. Incubate at 40°C for the time specified by the table in **Appendix A**.

Note: If needed, prepare RNAscope Assay materials during this step.

4. Pour at least 200 mL distilled water into the transparent ACDEZ-Batch Wash Tray.
5. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray and place the tray back into the oven.
6. Place the ACDEZ-Batch Slide Holder into the EZ-Batch Wash Tray. Make sure all the slides are submerged. If needed, carefully add more water. Wash the slides with slight agitation for **2 MIN**.
7. Repeat the wash step with fresh distilled water.

Proceed to the RNAscope Assay

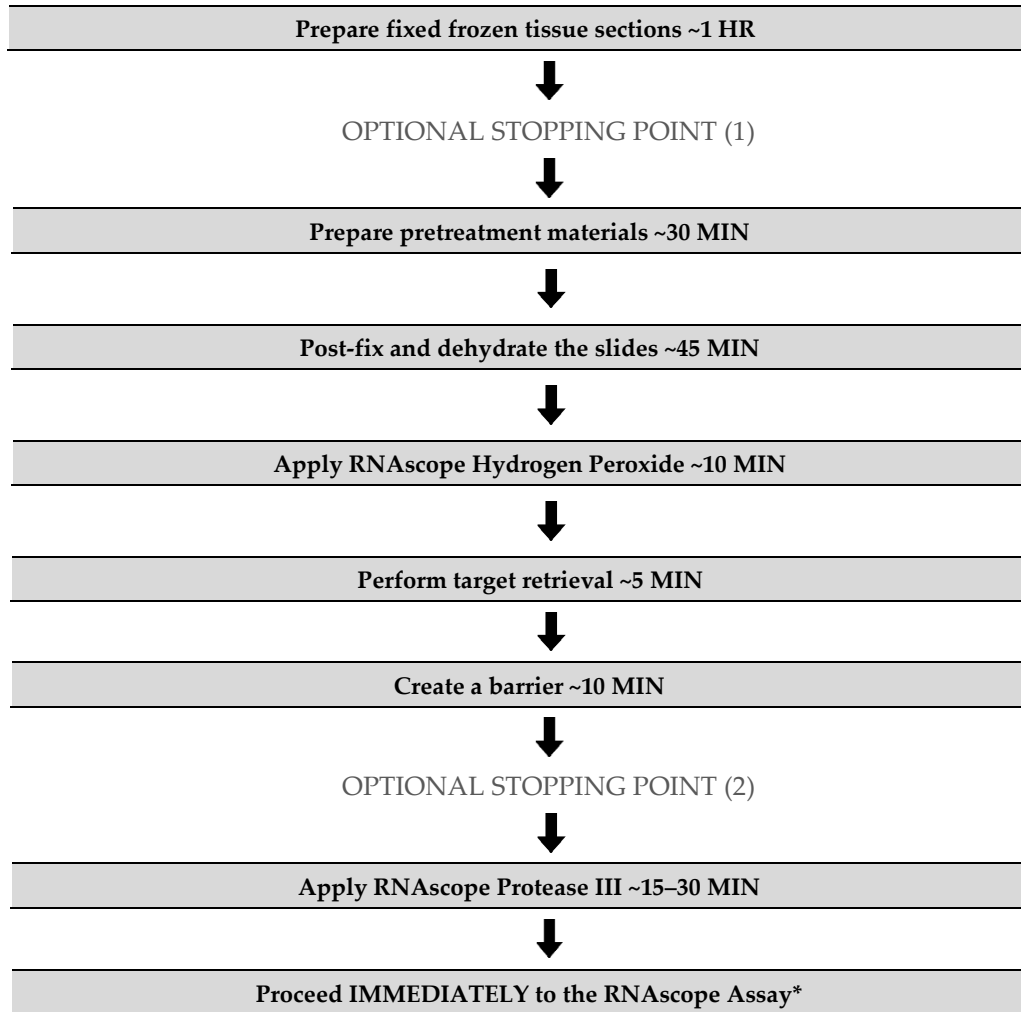
Proceed *immediately* to **Chapter 6. RNAscope Multiplex Fluorescent v2 Assay**.



Fixed-frozen tissue sample pretreatment

For sub-optimally prepared samples, you may need to optimize pretreatment conditions. Contact technical support at support.acd@bio-techne.com.

Workflow





Materials required

Materials provided by Pretreatment Reagents (Cat. No. 322381 and 322000) and Detection kit (Cat. No. 323110)	Other Materials and Equipment
<ul style="list-style-type: none">• RNAscope Hydrogen Peroxide• RNAscope Protease III• RNAscope 10X Target Retrieval Reagents	<ul style="list-style-type: none">• 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA)• 100% Ethanol (400mL)• 70% Ethanol (200mL)• 50% Ethanol (200mL)• Vertical 24 slide rack• Vertical staining dishes• ImmEdge Hydrophobic Barrier Pen• HybEZ Humidifying System/ ACD EZ-Batch Slide Holder and Wash Tray• Distilled water• Paper towel or absorbent paper• Kimwipe• Steamer• Digital thermometer• Drying oven

Fix the frozen sections

1. Chill 10% NBF (fresh 10% NBF or 4% PFA in 1X PBS) to 4°C.
2. Remove slides from -80°C, and bake the slides for **30 MIN** at 60°C.
3. Wash the slides with 200 mL 1X PBS in a slide rack and staining dish for **5 MIN** while moving the rack up and down to remove OCT.
4. Post-fix the slides by immersing them in prechilled 10% NBF or 4% PFA in 1X PBS for **15 MIN** at 4°C.

Dehydrate and dry the tissue

1. Prepare 200 mL 50% EtOH, 200 mL 70% EtOH, and 400 mL of 100% EtOH.
2. Remove the slides from the 10% NBF or 4% PFA, and immerse them in 50% EtOH for **5 MIN** at **RT**.
3. Remove the slides from 50% EtOH, and immerse them in 70% EtOH for **5 MIN** at **RT**.
4. Remove the slides from 70% EtOH, and immerse them in 100% EtOH for **5 MIN** at **RT**.
5. Remove the slides from 100% EtOH, and immerse them in fresh 100% EtOH for **5 MIN** at **RT**.
6. Remove the slides from 100% EtOH, and let them air dry for **5 MIN** at **RT**.

Prepare pretreatment materials

1. Turn on the HybEZ Oven, and set temperature to 40°C.
2. Place a Humidifying Paper in the Humidity Control Tray and wet completely with distilled water.
3. Insert covered tray into oven and close the oven door. Warm the tray for **30 MIN** at 40°C before use. Keep the tray in the oven when not in use.

4. Prepare 1X Target Retrieval Reagents (e.g. by adding 180 mL distilled water to 20 mL 10X Target Retrieval buffer). Mix well.

Note: Target Retrieval buffer must be heated to $\geq 99^{\circ}\text{C}$ before use (see **Perform target retrieval using the steamer** on page 24.).

Apply RNAscope Hydrogen Peroxide

1. Load the slides into the ACD EZ-Batch Slide Holder (see page 40).
2. Add ~5 drops of RNAscope Hydrogen Peroxide to each section. Use enough solution to completely cover the sections.
3. Incubate slides for **10 MIN** at **RT** in the HybEZ Humidity Control Tray on the bench.
4. Pour at least 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.
5. Place the ACD EZ-Batch Slide Holder into the wash tray containing water and wash with agitation for **2 MIN**. Make sure all the slides are submerged. If needed, carefully add more water.
6. Repeat the wash step with fresh water.

Perform target retrieval using the steamer

IMPORTANT! Before you begin, make sure you know the pretreatment conditions specific to your sample type from **Appendix A.**

We highly recommend using a steamer for target retrieval. For target retrieval using a hot plate, see **Appendix B. Manual Target Retrieval.** For each steamer, fill the water to the maximum level before starting. Refilling water during the steaming process will drop the temperature and interfere with the target retrieval step.

1. Fill the water reservoir with cold tap water to the MAX fill marking line.

IMPORTANT! Do not overfill.

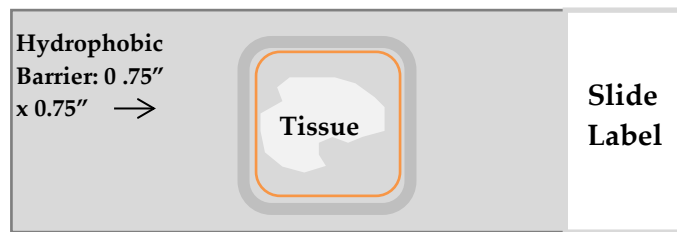


2. Place the steaming bowl onto the base, and place two slide holders in the steam bowl.
3. Fill one slide holder with 200 mL of RNAscope 1X Target Retrieval Reagent, and the other slide holder with 200 mL of distilled H₂O.
4. Turn on the steamer and set the heating time to the maximum amount so the steamer does not shut off during the procedure.
5. Insert a digital thermometer through the holes of the lid and into the container containing RNAscope 1X Target Retrieval Reagent. Allow the temperature to rise to at least **99°C**.
6. Add the slides to the container containing distilled H₂O for **10 SEC** to acclimate the slides.

7. Transfer the slides to the container containing RNAscope 1X Target Retrieval Reagent. Cover the steamer with the lid.
8. Start the timer for **5 MIN**.
9. Remove the slides from the steamer and transfer to a separate rinse container with 200 mL of distilled water. Allow the slides to rinse for **15 SEC**.
10. Transfer the slides to 100% ethanol for **3 MIN**.
11. Dry the slides in a **60°C** incubator (or at **RT**) for **5 MIN**.

Create a hydrophobic barrier

1. Use the following template to draw a barrier **2–4** times around each section with the **ImmEdge hydrophobic barrier pen**.



IMPORTANT! Do not let the barrier touch the tissue section. An ImmEdge hydrophobic barrier pen is highly recommended. Other pens may cause suboptimal results.

Note: We do not recommend drawing a smaller barrier and using less than the recommended volume amounts, even for smaller sections. Larger barriers will result in fewer tests per kit.

2. Let the barrier dry completely **~5 MIN** or **OVERNIGHT** at **RT**.
3. If you need to reapply the hydrophobic barrier during the following procedures, dry the appropriate area of the slide with a Kimwipe. Do not touch the tissue section.

OPTIONAL STOPPING POINT (2). Dry slides overnight for use the following day or proceed directly to the next section.

Apply RNAscope Protease III

1. Follow the procedure in Load the slides into the ACD EZ-Batch Slide Holder. Add ~5 drops of Protease III to each section. Use enough solution to completely cover the sections.
2. Remove the HybEZ Humidity Control Tray from the HybEZ Oven and place the ACD EZ-Batch Slide Holder in the tray. Close the lid, seal, and insert the tray back into the oven.
3. Incubate the samples for **30 MIN** at **40°C**.

Note: If needed, prepare RNAscope Assay materials during this step.

4. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray and place the tray back into the oven.
5. Pour 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.
6. Place the ACD EZ-Batch Slide Holder containing the slides into the wash tray and wash the slides with agitation for **2 MIN**. Repeat the wash step with fresh water.

IMPORTANT! If over-digestion is observed, first reduce the protease digestion time. In cases of severe over-digestion, use RNAscope Protease Plus instead of RNAscope Protease III.



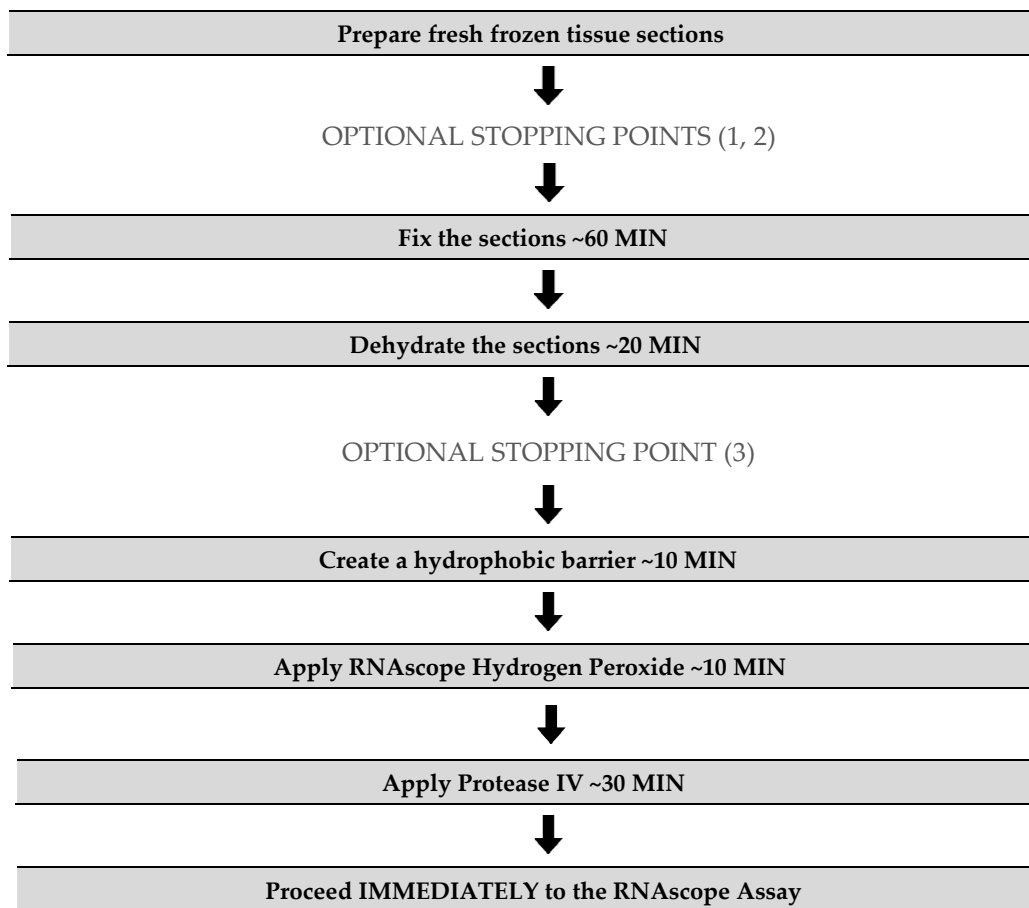
Proceed to the RNAscope Assay

Proceed *immediately* to **Chapter 6. RNAscope Multiplex Fluorescent v2 Assay.**

Fresh-frozen sample pretreatment

IMPORTANT! Do not apply RNAscope Target Retrieval to fresh-frozen sections when using the RNAscope Multiplex Fluorescent v2 Kit with Protease pretreatment. Other sample types may require target retrieval.

Workflow





Materials required

Materials provided by Pretreatment Reagents (Cat. No. 322381) and Detection kit (Cat. No. 323110)	Other Materials and Equipment
<ul style="list-style-type: none">• RNAscope Hydrogen Peroxide• RNAscope Protease IV• RNAscope 10X Target Retrieval Reagents	<ul style="list-style-type: none">• 1X PBS• 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA)• 100% alcohol (EtOH) (400mL)• 70% alcohol (EtOH) (200mL)• 50% alcohol (EtOH) (200mL)• Vertical 24 slide rack• Vertical staining dishes• ImmEdge Hydrophobic Barrier Pen• HybEZ Humidifying System/ ACD EZ-Batch Slide Holder and Wash Tray• Steamer• Distilled water• Paper towel or absorbent paper• Kimwipe• Timer

Fix the sections

7. Chill fresh 10% NBF (or 4% PFA in 1X PBS) to 4°C.
8. Remove slides from -80°C, and place in the pre-chilled fixative. Fix for **1 HR** at 4°C.
9. Rinse the slides 2 times with 1X PBS to remove excess fixative.

Note: Formalin that has been stored for more than six months, exposed to air for more than a week, or used repeatedly may result in suboptimal tissue fixation.

IMPORTANT! For some tissue types you may have to empirically determine the best duration for fixation.

Dehydrate the sections

Reagents may be prepared ahead of time. Ensure all containers remain covered.

1. Prepare **200 mL** 50% ethanol, **200 mL** 70% ethanol, and **400 mL** 100% ethanol.
2. Place the slides in 50% ethanol for **5 MIN** at **ROOM TEMPERATURE (RT)**.
3. Place the slides in 70% ethanol for **5 MIN** at **RT**.
4. Place the slides in 100% ethanol for **5 MIN** at **RT**.
5. Place slides in fresh 100% ethanol for **5 MIN** at **RT**.

OPTIONAL STOPPING POINT (3). Slides may be stored in 100% ethanol at -20°C for up to one week. Prolonged storage may degrade sample RNA.

Create a hydrophobic barrier

1. Take slides out of 100% ethanol and place on absorbent paper with the section face-up. Air dry for **5 MIN** at **RT**.

- Use the following template to draw a barrier 2–4 times around each section with the **ImmEdge hydrophobic barrier pen**.



Note: Refer to **Appendix C. Reagent Volume Guidelines** to determine the recommended number of drops needed per slide.

IMPORTANT! Do not let the barrier touch the section. The ImmEdge hydrophobic barrier pen is highly recommended. Other pens may cause suboptimal results.

Note: We do not recommend drawing a smaller barrier and using less than the recommended volume amounts, even for smaller sections. Larger barriers will result in fewer tests per kit.

- Let the barrier dry completely ~5 MIN.

Note: If you need to reapply the hydrophobic barrier during the following procedures, dry the appropriate area of the slide with a Kimwipe. Do not touch the tissue section.

Apply RNAscope Hydrogen Peroxide

- Place a Humidifying Paper in the HybEZ Humidity Control Tray and wet completely with distilled water.
- Load the slides into the ACD EZ-Batch Slide Holder (see page 40).
- Add ~5 drops of RNAscope Hydrogen Peroxide to each section. Use enough solution to completely cover the sections.
- Incubate slides for **10 MIN** at **RT** in the HybEZ Humidity Control Tray on the bench.
- Pour at least 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.
- Place the ACD EZ-Batch Slide Holder into the wash tray containing water and wash with agitation for **2 MIN**. Make sure all the slides are submerged. If needed, carefully add more water.
- Repeat the wash step with fresh water.

Apply RNAscope Protease IV

IMPORTANT! View the wash step video at <https://acdbio.com/technical-support/learn-more> before proceeding.

- Place the washed slides in the EZ-Batch Slide Holder and add ~5 drops of Protease IV to each section. Use enough solution to completely cover the sections.
- Incubate for **30 MIN** at **RT** in the HybEZ Humidity Control Tray on the bench.
- Pour at least 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.
- Place the ACD EZ-Batch Slide Holder containing the slides into the wash tray and wash the slides with agitation for **2 MIN**. Repeat the wash step with fresh distilled water.

IMPORTANT! If over-digestion is observed, first reduce the protease digestion time. In cases of severe over-digestion, use RNAscope Protease III instead of RNAscope Protease IV.



Proceed to the RNAscope Assay

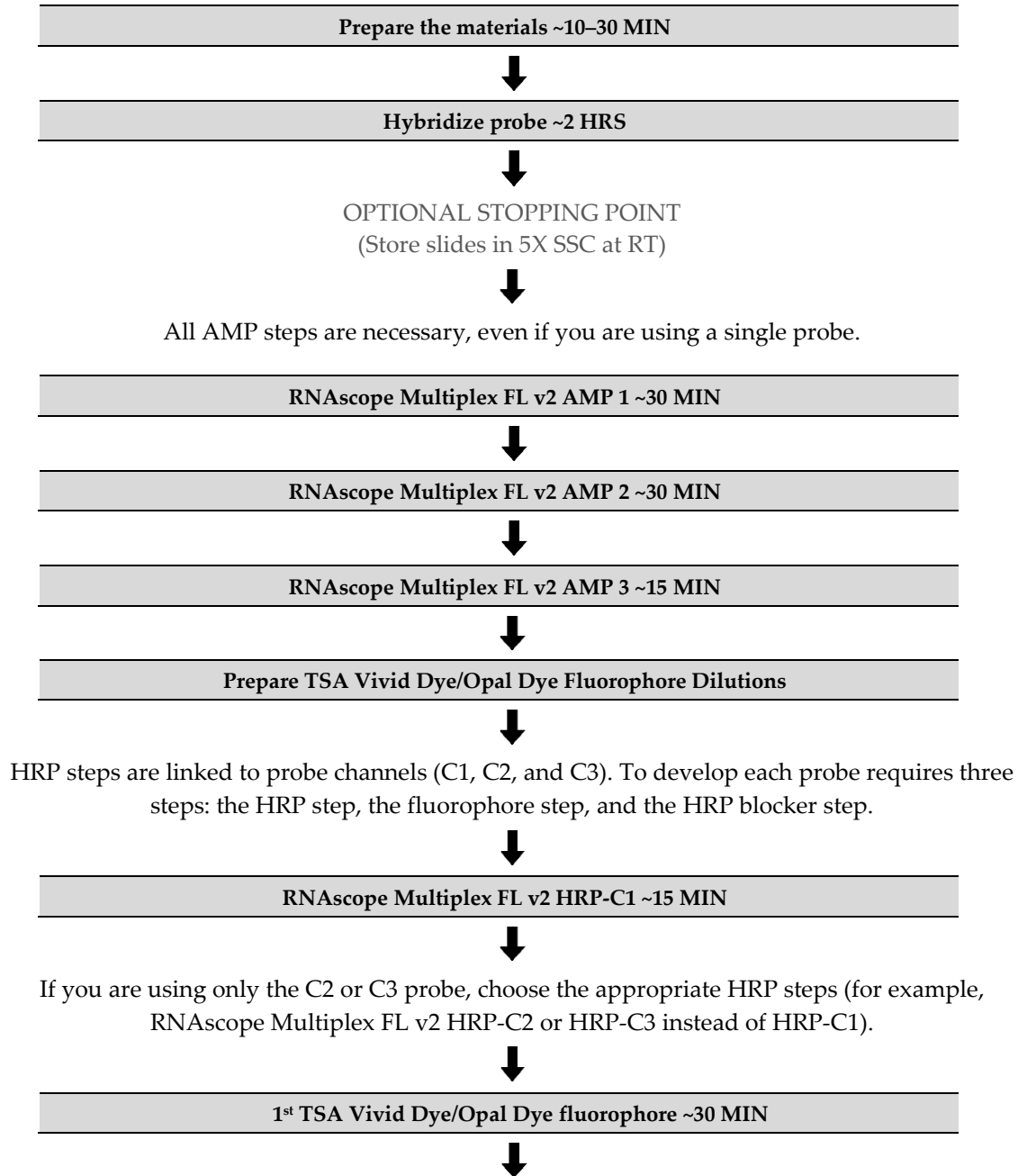
Proceed *immediately* to **Chapter 6. RNAscope Multiplex Fluorescent v2 Assay.**

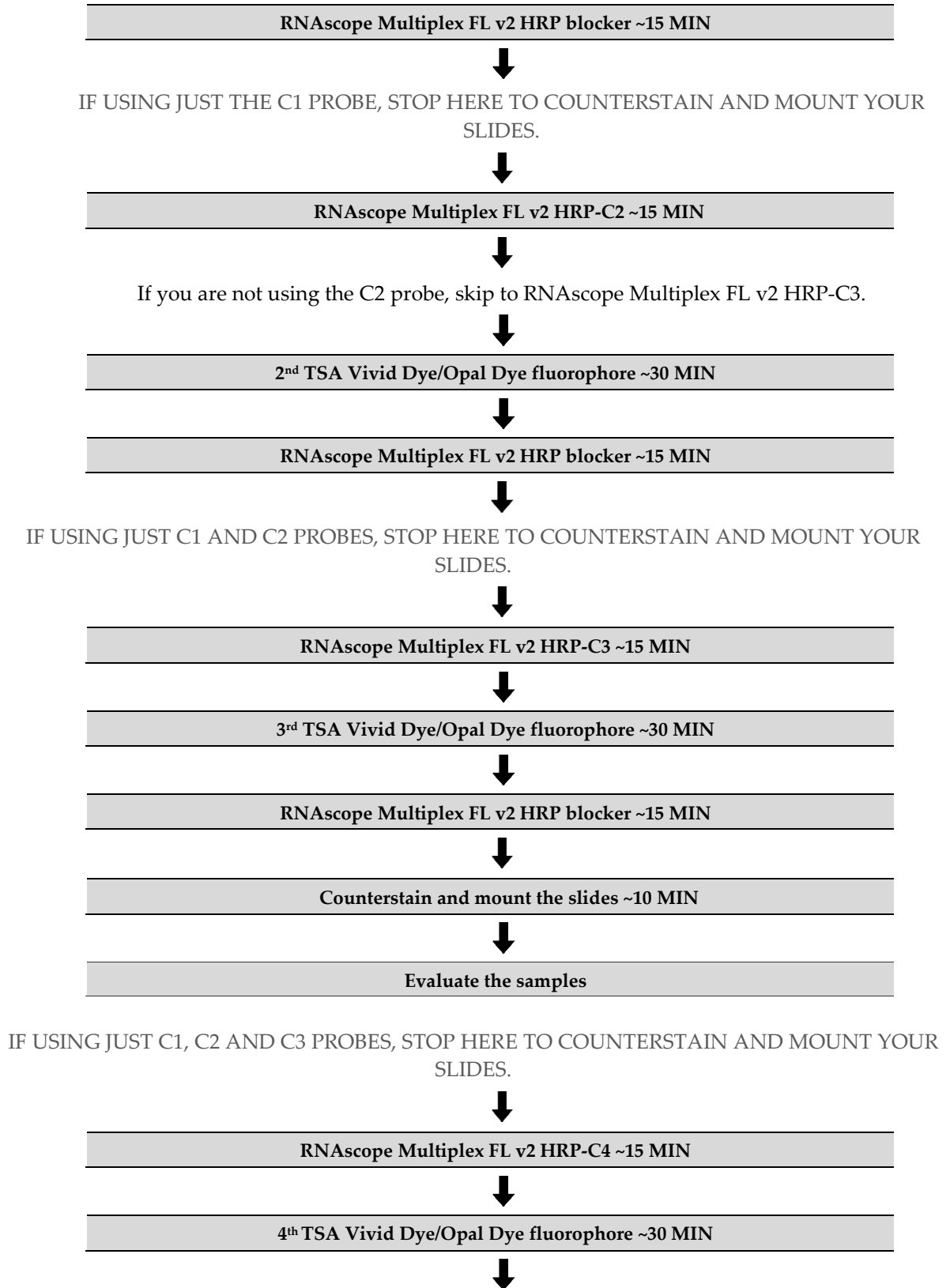
6

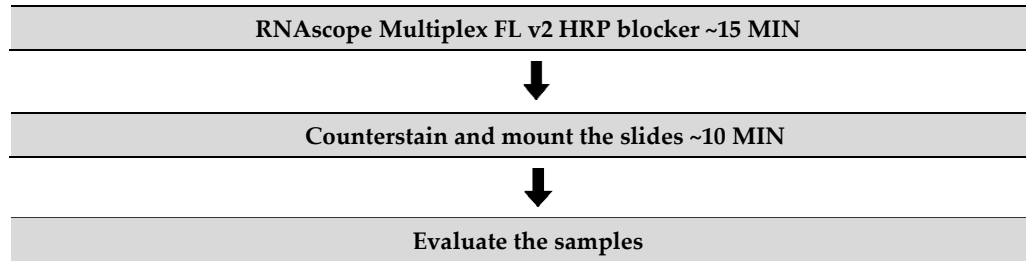
Chapter 6. RNAscope Multiplex Fluorescent v2 Assay

This procedure flows directly from sample preparation and pretreatment. Refer to **Chapters 4 and 5** or the appropriate sample preparation and pretreatment user manual for your specific sample type.

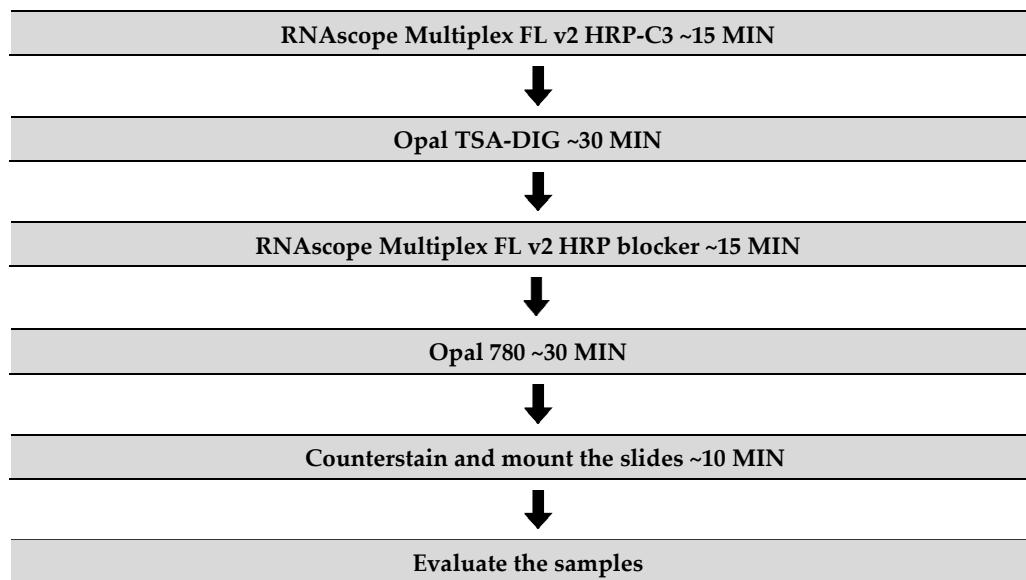
Workflow







IF USING OPAL 780, USE THE FOLLOWING WORKFLOW AS AN EXAMPLE.
 You can use Opal 780 in the C1, C2, or C3 channel, but do not follow it with any other fluorophore or a complete loss of Opal 780 signal occurs. The 780 fluorophore is easily cleaved off by HRP activity.
 Opal 780 must be applied to the slide just before counterstaining and mounting.





Materials required for the assay

Materials provided by RNAscope Multiplex Fluorescent Reagent Kit v2	Materials provided by RNAscope Probes	Other Materials and Equipment
<ul style="list-style-type: none"> • RNAscope 50X Wash Buffer • RNAscope Multiplex FL v2 AMP 1 • RNAscope Multiplex FL v2 AMP 2 • RNAscope Multiplex FL v2 AMP 3 • RNAscope Multiplex FL v2 HRP-C1 • RNAscope Multiplex FL v2 HRP-C2 • RNAscope Multiplex FL v2 HRP-C3 • RNAscope Multiplex FL v2 HRP-C4 • RNAscope Multiplex FL v2 HRP blocker • RNAscope Multiplex FL v2 DAPI • RNAscope Multiplex TSA Buffer 	<ul style="list-style-type: none"> • Target Probes • Positive Control Probe • Negative Control Probe 	<ul style="list-style-type: none"> • Prepared sections • Distilled water • Carboy (>3L) • Opal dyes or TSA Vivid dyes • Antibody Diluent/Block • Vertical Staining Dishes • HybEZ Humidifying System/ACD EZ-Batch Slide Holder and Wash Tray • Water bath or incubator • Vertical 24 Slide Rack • Tubes (various sizes) • Paper towel or absorbent paper • ProLong Gold Antifade Mountant • Cover Glass, 24 mm x 50 mm

Prepare the materials

You may prepare the reagents at the same time you prepare pretreatment reagents. Refer to a sample preparation and pretreatment user manual available at <https://acdbio.com/documents/product-documents>.

Some of the materials may be prepared in advance and stored at room temperature.

Prepare 1X Wash Buffer

1. Always warm RNAscope 50X Wash Buffer up to 40°C for 10–20 MIN before preparation. 1X Wash Buffer may be prepared ahead of time and stored at room temperature for up to one month.
2. Prepare 6 L of 1X Wash Buffer by adding 5.76 L distilled water and 2 bottles (120 mL) of RNAscope Wash Buffer (50X) to a large carboy. Mix well.

Prepare probes

C1 probes are 1X, ready-to-use. If you are using only the C1 probe, you will only need to follow the first step. C2, C3, and C4 probes come as 50X stocks and must be diluted with the C1 probe.

1. Warm probe(s) for 10 MIN at 40°C in a water bath or incubator, then cool to **ROOM TEMPERATURE (RT)**.
2. Briefly spin the C2, C3, and/or C4 probes to collect the liquid at the bottom of the tubes.
3. Pipette 1 volume of C2, 1 volume of C3 and/or 1 volume of C4 probes to 50 volumes of C1 probe into a tube. Invert the tube several times to mix.



Note: If you are not using a C1 probe, dilute C2, C3 and/or C4 probes with Probe Diluent (Cat. No. 300041). Do not mix probes of the same channel. Store mixed probes at 2–8°C for up to six months.

Control probes

RNAscope 3-plex or 4-plex control probes are species-specific ready-to-use (RTU) probe mixtures that do not require dilution. They can be applied directly to your samples when the probes are at room temperature.

The 3-plex positive control is a RTU mixture of three probes targeting common housekeeping genes *POLR2A* in channel C1, *PPIB* in channel C2, and *UBC* in channel C3.

The 4-plex positive control is a RTU mixture of four probes targeting common housekeeping genes *POLR2A* in channel C1, *PPIB* in channel C2, *UBC* in channel C3, and *HPRT* in channel 4.

The 3-plex/4-plex negative control probes are RTU mixture of 3/4 probes targeting *dapB* which is a soil bacterium gene. Each detection channel has its own negative control probe: *dapB-C1*, *dapB-C2*, *dapB-C3* and/or *dapB-C4*

The 3-plex/4-plex positive control probes will have the following expression pattern in most tissues: *Polr2a-C1/Hprt-C4* (low-medium), *Ppib* (medium-high) and *Ubc* (high).

Prepare TSA Vivid Dyes or Opal Dyes

Prepare only the fluorophores that you will need for each assay. For example, if you only using one probe, there is no need to prepare more than one fluorophore. Do not assign the same fluorophore to more than one channel.

1. Use these guidelines when choosing which fluorophore to assign to each probe channel for control or target probes.
 - You can mix and match channels and fluorophores. Any of the TSA Vivid or Opal dyes may be assigned to any of the probe channels (i.e. C1, C2, C3 or C4)
 - For optimal signal/noise, pair the target or control probe with highest expression with either Opal 520 or TSA Vivid 520
 - The target or control probe with lowest expression should be paired with Opal 690 or Vivid 650
 - For a 3-plex assay, you should assign the highest expressing target to Opal/TSA Vivid 520 and the lowest to Opal 690/TSA Vivid 650
 - For a 4-plex assay you should assign the Opal dyes according to target expression levels as follows: [Opal 520] > [Opal 780] > [Opal 570] > [Opal 690]
 - Using the 4-plex positive control probe as an example, you would assign the highest target (*Ubc-C3*) to Opal 520, the medium-high expressing target (*Ppib-C2*) to Opal 780 and the lower expressing targets (*Polr2a-C1/Hprt-C4*) to Opal 570/Opal 690. Use the same pattern for your target probe assignments
2. Reconstitute the TSA Vivid Dye/Opal Dye fluorophores using the instructions from the corresponding manufacturers.

IMPORTANT! Store TSA Vivid dye stocks at -20°C. Opal dye stocks should be stored at 2–8°C per manufacturer's instructions.

3. Determine the volume of TSA Vivid or Opal fluorophore needed (150–200 µL per slide).



- Dilute the TSA Vivid or Opal fluorophore stocks in appropriate buffer. Follow these recommendations:

Compatible Fluorophore*	Compatible Fluorophore	Recommended dilution range†	Dilute in
TSA Vivid Fluorophore 520 (323271)	Opal 520 Reagent Pack (FP1487001KT)	1:750–1:3000	TSA buffer
TSA Vivid Fluorophore 570 (323272)	Opal 570 Reagent Pack (FP1488001KT)	1:750–1:3000*	TSA buffer
TSA Vivid Fluorophore 650 (323273)	N/A	1:3000-1:7500	TSA buffer
	Opal 690 Reagent Pack (FP1497001KT)	1:750–1:3000	TSA Buffer
	Opal TSA-DIG (FP1501001KT)	1:750–1:3000	TSA buffer
	Opal 780 (FP1501001KT)	1:187.5–1:750	Antibody/Block Diluent

*Reconstitute the TSA Vivid reagent with 100 μ L Dimethylsulfoxide (DMSO). Reconstituted TSA Vivid Fluorophores can be stored for three months at -20°C .

† We recommend starting with a dilution of 1:1500 for TSA Vivid 520 and 570 and 1:5000 for TSA Vivid 650 and adjusting the dilution based on signal intensity (this also applies to the positive and negative control probes). Optimal fluorophore dilutions may vary based on sample, target expression levels, and imaging system.

IMPORTANT! Diluted Opal dyes may be stored for up to one month at $2-8^{\circ}\text{C}$ in the dark. Diluted TSA Vivid Dyes fluorophores should be freshly made for each assay. Do not store or reuse TSA Vivid working solutions.

IMPORTANT! TSA Vivid Fluorophores are not compatible with RNAscope Manual PreTreatPro. Please use RNAscope Protease workflows instead.

Equilibrate reagents

- Remove AMP1, AMP2, AMP3, HRP-C1, HRP-C2, HRP-C3, HRP-C4 and HRP blockers from the refrigerator. Place at **RT**.
- Ensure the HyBEZ II Oven and prepared Humidity Control Tray are at **40°C**.

Prepare 20X (SSC) Saline Sodium Citrate (optional)

- Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800ml of distilled H₂O.
- Adjust the pH to 7.0 with a few drops of 1M HCl.
- Adjust the volume to 1 L with additional distilled H₂O.
- Sterilize by autoclaving.

Note: 5X SSC may be prepared ahead of time and stored at room temperature for up to two months.



Run the assay

IMPORTANT! Do not let sections dry out between incubation steps. Work quickly and fill barrier with solutions.

IMPORTANT! View the wash step video at <https://acdbio.com/technical-support/learn-more> before proceeding.

Hybridize probe

1. Remove excess liquid from the slides while keeping them locked in the ACD EZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough drops of the appropriate probe mix to entirely cover each slide.

Note: Refer to **Appendix C. Reagent Volume Guidelines** to determine the recommended number of drops needed per slide. For example, add four drops of the appropriate probe to a 0.75" x 0.75" barrier.

3. Close the tray and insert into the HybEZ Oven for **2 HRS** at **40°C**.
4. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
5. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
6. Place the ACD EZ-Batch Slide Holder into the wash tray (see page 27 for details) and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.

OPTIONAL STOPPING POINT. You can store the slides in 5X SSC (not provided in the kit) **OVERNIGHT** at **RT**. Before continuing with the assay, wash the slides once with 1X Wash Buffer for **2 MIN** at **RT**.

IMPORTANT! All the three Amp steps are required irrespective of the channels (probes) being developed

Hybridize AMP 1

1. Remove excess liquid from the slides while keeping them locked in the EZ-Batch Slide holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough drops RNAscope Multiplex FL v2 Amp 1 to entirely cover each slide.
3. Close the tray and insert into the HybEZ Oven for **30 MIN** at **40°C**.
4. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
5. Pour 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
6. Place the ACD EZ-Batch Slide Holder into the wash tray and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.

Hybridize AMP 2

1. Remove excess liquid from the slides while keeping them locked in the EZ-Batch Slide holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough drops RNAscope Multiplex FL v2 Amp 2 to entirely cover each slide.
3. Close the tray and insert into the HybEZ Oven for **30 MIN** at **40°C**.



4. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
1. Pour 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
2. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.

Hybridize AMP 3

1. Remove excess liquid from the slides while keeping them locked in the EZ-Batch Slide holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough drops RNAscope Multiplex FL v2 Amp 3 to entirely cover each slide.
3. Close the tray and insert slides into the HybEZ Oven for **15 MIN** at **40°C**.

IMPORTANT! Prepare TSA Vivid, Opal, or fluorophores during this step. See the following section.

4. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
5. Pour 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
6. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.

Develop HRP-C1 signal

If you are using only the C2 or C3 probe, choose the appropriate HRP channel (for example, skip to RNAscope Multiplex FL v2 HRP-C2 or HRP-C3 instead of proceeding with HRP-C1).

1. Remove excess liquid from the slides while keeping them locked in the EZ-Batch Slide holder. Insert the slide holder into the HybEZ Humidity Control Tray
2. Add enough drops RNAscope Multiplex FL v2 HRP-C1 to entirely cover each slide.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Place the ACD EZ-Batch Slide Holder into the wash tray containing 200 mL 1X Wash Buffer, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.
5. Remove excess liquid from the slides and insert the slide holder back into the humidity control tray.
6. Add 150–200 μ L diluted fluorophore for labeling the C1 probe to each slide and incubate for **30 MIN** at **40°C**.
7. Place the ACD EZ-Batch Slide Holder into the wash tray containing 200 mL 1X Wash Buffer and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.
8. Remove excess liquid from the slides and insert the slide holder back into the humidity control tray.
9. Add enough drops RNAscope Multiplex FL v2 HRP blocker to entirely cover each slide.
10. Insert the slides into the HybEZ Oven for **15 MIN** at **40°C**.
11. Place the ACD EZ-Batch Slide Holder into the wash tray containing 200 mL 1X Wash Buffer, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.

STOP HERE IF YOU ARE USING JUST ONE C1 PROBE. Continue to **Counterstain and mount the slides** on page 62.



Develop HRP- C2 signal

If you are not using the C2 probe, skip to RNAscope Multiplex FL v2 HRP-C3.

1. Remove excess liquid from the slides while keeping them locked in the EZ-Batch Slide holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough drops RNAscope Multiplex FL v2 HRP-C2 to entirely cover each slide.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Place the ACD EZ-BatchSlide Holder into the wash tray containing 200 mL 1X Wash Buffer and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.
5. Remove excess liquid from the slides and insert the slide holder back into the humidity control tray.
6. Add 150–200 μ L diluted fluorophore for labeling the C2 probe to each slide, and incubate for **30 MIN** at **40°C**.
7. Place the ACD EZ-BatchSlide Holder into the wash tray containing 200 mL 1X Wash Buffer and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.
8. Remove excess liquid from the slides and insert the slide holder back into the humidity control tray.
9. Add enough drops RNAscope Multiplex FL v2 HRP blocker to entirely cover each slide.
10. Insert the slides into the HybEZ Oven for **15 MIN** at **40°C**.
11. Place the ACD EZ-BatchSlide Holder into the wash tray containing 200 mL 1X Wash Buffer and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.

STOP HERE IF YOU ARE USING JUST C1 & C2 PROBES. Continue to **Counterstain and mount the slides** later in this chapter.

Develop HRP-C3 signal

If you are using Opal 780 to develop the HRP-C3 signal, skip this section and go to the next section.

1. Remove excess liquid from the slides while keeping them locked in the EZ-Batch Slide holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough drops RNAscope Multiplex FL v2 HRP-C3 to entirely cover each slide.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Place the ACD EZ-BatchSlide Holder into the wash tray containing 200 mL 1X Wash Buffer, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.
5. Remove excess liquid from the slides and insert the slide holder back into the humidity control tray.
6. Add 150–200 μ L diluted fluorophore for labeling the C3 probe to each slide, and incubate for **30 MIN** at **40°C**.
7. Place the ACD EZ-BatchSlide Holder into the wash tray containing 200 mL 1X Wash Buffer and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer.
8. Remove excess liquid from the slides and insert the slide holder back into the humidity control tray.
9. Add enough drops RNAscope Multiplex FL v2 HRP blocker to entirely cover each slide.
10. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.



11. Place the ACD EZ-BatchSlide Holder into the wash tray containing 200 mL 1X Wash Buffer and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.

Develop HRP-C3 signal with Opal 780

If you are using a fluorophore instead of Opal 780 to develop the HRP-C3 signal, go to the previous section.

1. Remove excess liquid from the slides while keeping them locked in the EZ-Batch Slide holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough drops RNAscope Multiplex FL v2 HRP-C3 to entirely cover each slide.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Place the ACD EZ-BatchSlide Holder into the wash tray containing 200 mL 1X Wash Buffer, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.
5. Remove excess liquid from the slides
6. Add 150–200 μ L diluted TSA-DIG to each slide, and incubate for **30 MIN** at **RT**.
7. Place the ACD EZ-BatchSlide Holder into the wash tray containing 200 mL 1X Wash Buffer and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.
8. Remove excess liquid from the slides and insert the slide holder back into the humidity control tray.
9. Add enough drops RNAscope Multiplex FL v2 HRP blocker to entirely cover each slide.
10. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
11. Place the ACD EZ-BatchSlide Holder into the wash tray containing 200 mL 1X Wash Buffer, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.
12. Remove excess liquid from the slides and insert the slide holder back into the humidity control tray.
13. Add 150–200 μ L diluted 780 to each slide, and incubate for **30 MIN** at **40°C**
14. Place the ACD EZ-BatchSlide Holder into the wash tray containing 200 mL 1X Wash Buffer, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.

STOP HERE IF YOU ARE USING JUST C1, C2 & C3 PROBES. Continue to **Counterstain and mount the slides** later in this chapter.

Develop HRP-C4 signal

Note: For the following steps, you will need reagents from Cat No. 323120, 4-Plex Ancillary Kit.

If you are using Opal 780 to develop the HRP-C4 signal, skip this section and go to the next section.

1. Remove excess liquid from the slides while keeping them locked in the EZ-Batch Slide holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough drops RNAscope Multiplex FL v2 HRP-C4 to entirely cover each slide.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Place the ACD EZ-BatchSlide Holder into the wash tray containing 200 mL 1X Wash Buffer, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.
5. Remove excess liquid from the slides and insert the slide holder back into the humidity control tray.



6. Add 150–200 μL diluted fluorophore for labeling the C4 probe to each slide, and incubate for **30 MIN** at **40°C**.
7. Place the ACD EZ-Batch Slide Holder into the wash tray containing 200 mL 1X Wash Buffer and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer.
8. Remove excess liquid from the slides and insert the slide holder back into the humidity control tray.
9. Add enough drops RNAscope Multiplex FL v2 HRP blocker to entirely cover each slide.
10. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
11. Place the ACD EZ-Batch Slide Holder into the wash tray containing 200 mL 1X Wash Buffer and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.

Develop HRP-C4 signal using Opal 780

Note: The following steps assigns Opal 780 to the C4 channel. Due to extreme sensitivity to cleavage by HRP activity, Opal 780 cannot be followed by any other fluorophore

1. Remove excess liquid from the slides while keeping them locked in the EZ-Batch Slide holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough drops RNAscope Multiplex FL v2 HRP-C4 to entirely cover each slide.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Place the ACD EZ-Batch Slide Holder into the wash tray containing 200 mL 1X Wash Buffer, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.
5. Remove excess liquid from the slides.
6. Add 150–200 μL of diluted TSA-DIG for labeling the C4 probe to each slide, and incubate for **30 MIN** at **RT**.
7. Place the ACD EZ-Batch Slide Holder into the wash tray containing 200 mL 1X Wash Buffer, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.
8. Remove excess liquid from the slides and insert the slide holder back into the humidity control tray.
9. Add enough drops RNAscope Multiplex FL v2 HRP blocker to entirely cover each slide.
10. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
11. Place the ACD EZ-Batch Slide Holder into the wash tray containing 200 mL 1X Wash Buffer, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.
12. Remove excess liquid from the slides and insert the slide holder back into the humidity control tray.
13. Add 150–200 μL diluted Opal 780 to each slide, and incubate for **30 MIN** at **40°C**.
14. Place the ACD EZ-Batch Slide Holder into the wash tray containing 200 mL 1X Wash Buffer, and wash the slides for **2 MIN** at **RT**. Repeat the wash step with fresh buffer for **2 MIN** at **RT**.

Counterstain and mount the slides

IMPORTANT! Do this procedure with no more than five slides at a time.

IMPORTANT! If you are planning to combine the RNAscope Multiplex Fluorescent assay with protein detection, you must stop after the HRP blocker step. Do not counterstain the slides with DAPI until the IHC assay is finished. See **Chapter 7** for more details



1. Remove excess liquid from slides and add enough DAPI to cover each section.
2. Incubate for **30 SEC** at **RT**.
3. Remove DAPI by tapping or flicking the slides, and *immediately* place 1–2 drops of ProLong Gold Antifade Mountant on each 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 **8 HRS** to **OVERNIGHT** in the dark.
6. Store slides in the dark at **2–8°C**.

Note: Image the slides after eight hours and within two weeks. See **Chapter 8. Evaluate the samples**.

7

Chapter 7. RNAscope Multiplex Fluorescent v2 Assay combined with Immunofluorescence

Follow the procedures in this chapter directly after completing the steps in **Chapters 5 or 6**. To detect immunofluorescence (IF), use HRP-conjugated secondary antibody with Opal fluorophores or a fluorophore-conjugated secondary antibody. The following fluorophore combinations are recommended.

2-plex ISH combined with immunofluorescence		
	Opal fluorophore	Akoya Biosciences Part No.
RNAscope Multiplex Assay –C1	Opal 520 Reagent Pack	FP1487001KT
RNAscope Multiplex Assay –C2	Opal 570 Reagent Pack	FP1488001KT
Immunofluorescence	Opal 690 Reagent Pack	FP1497001KT

3-plex ISH combined immunofluorescence		
	Opal fluorophore	Akoya Biosciences Part No.
RNAscope Multiplex Assay –C1	Opal 520 Reagent Pack	FP1487001KT
RNAscope Multiplex Assay –C2	Opal 570 Reagent Pack	FP1488001KT
RNAscope Multiplex Assay –C3	Opal 690 Reagent Pack	FP1497001KT
Immunofluorescence	Opal 780 Reagent Pack	FP1501001KT

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

Note: If you prefer to assign Opal 520 for protein staining, you may use Opal 780 for ISH staining in any of the three channels. The following table displays one workflow example.

3-plex ISH with IF	Opal fluorophore	Akoya Biosciences Part No.
RNAscope Multiplex Assay –C1	Opal 570 Reagent Pack	FP1488001KT
RNAscope Multiplex Assay –C2	Opal 690 Reagent Pack	FP1497001KT
RNAscope Multiplex Assay –C3	Opal 780 Reagent Pack	FP1501001KT
Immunofluorescence	Opal 520 Reagent Pack	FP1487001KT

IMPORTANT! If Opal 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 immunofluorescence protocol, apply Opal 780 as the last step before counter staining and mounting.



Prepare 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 μ L 10% Tween[®] 20 to 1 L 1X TBS buffer.
3. Prepare TBS-0.1% BSA: Add 1 g BSA to 1 L 1XTBS
4. Prepare 10% normal serum in TBS-0.1% BSA: Add 10g normal serum to 1 L 1X TBS-0.1% BSA.

Prepare fluorophores

1. Determine the volume of fluorophore needed (approximately 150–200 μ L per slide).
2. Dilute the Opal fluorophore 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 780, dilute Opal TSA-DIG in TSA buffer and dilute Opal 780 in Antibody Diluent/Block from Akoya Bioscience (PN: ARD1001EA).

Perform immunofluorescence

IMPORTANT! Keep the slides covered by using a HybEZ Humidity Control Tray during the ISH 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 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 μ L diluted Opal fluorophore reagent 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 780

IMPORTANT! The following steps only describe how to use Opal 780 for IF staining. If Opal 780 is used for an ISH staining prior to IHC, you must follow the modified protocol described in the **Opal 780 for ISH detection followed by IF staining** workflow.

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 μ L 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 μ L diluted Opal 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.

Secondary antibody staining using fluorophore-conjugated secondary

1. Add fluorophore-conjugated secondary antibody diluted in 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.

Mount the slides

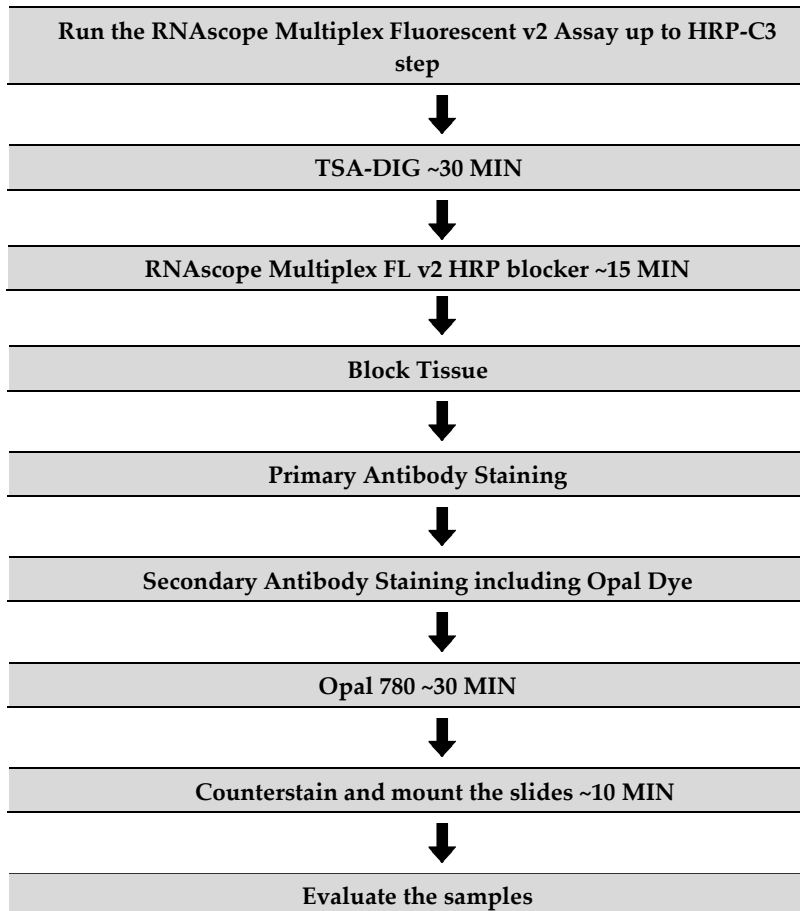
1. Remove excess liquid from the slides and add ~4 drops of DAPI to each slide. Incubate for **30 SEC** at **RT**.
2. Remove DAPI and *immediately* place 1–2 drops of ProLong Gold antifade mounting medium on the slide
3. Carefully place a 24 mm x 50 mm glass coverslip over the tissue section. Avoid trapping 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.

Opal 780 for ISH detection followed by IF staining

The following workflow uses Opal 780 in the C3 channel followed by IF staining. To develop the C1 and C2 channels, follow the instructions in **Chapter 6**.

IMPORTANT! If Opal 780 is assigned to an ISH marker, you must follow the following modified protocol in which the steps for developing 780 for ISH must stop after TSA-DIG is applied. Apply Opal 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.

Workflow



Opal 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 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** in the Perform immunofluorescence section found earlier in this chapter. Before staining the slides with DAPI, perform the following procedure.

Opal 780 staining: Part B

1. Remove excess liquid from the slides and add 150–200 μ L diluted Opal 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.

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.

8

Chapter 8. Evaluate the samples

Imaging the slides

For imaging using multiplexed biomarker imaging systems Vectra, Mantra or PhenoImager HT™, refer to the guidelines from Akoya Biosciences. The following tables list the corresponding filter settings for each fluorophore.

TSA Vivid fluorophore	Filter setting
TSA Vivid Fluorophore 520	FITC
TSA Vivid Fluorophore 570	Cy3
TSA Vivid Fluorophore 650	Cy5

Opal fluorophore	Filter setting
Opal 520	FITC
Opal 570	Cy3
Opal 690	Cy5.5
Opal 780	Cy7

Evaluate the sample

Examine tissue sections under a standard fluorescent or confocal microscope at 20–40X magnification. Refer to **Fluorescent imaging recommendations** in **Chapter 1**.

- Assess tissue and cell morphology.
- Assess negative control background first. One dot to every 10 cells displaying background staining at 20X magnification is acceptable. Adjust the light source intensity, gain, and exposure time for each channel until background levels are acceptable.
- Use 20X and 40X to visualize high expression targets and low expression targets, respectively.
- Assess positive control signal strength. Positive control signal should be visible as punctate dots within a cell at 20X magnification.

The RNAscope assay should produce clear, intense, punctate dots. Single dots may merge into a cluster when highly abundant targets are detected.

Scoring guidelines

When used for RNA detection, the assay enables a semi-quantitative scoring guideline utilizing the estimated number of punctate dots present within each cell boundary. An example of how to develop such a guideline for semi-quantitative assessment of mRNA staining intensity is presented below for a gene with expression level varying between 1 to > 10 dots per cell.



Note: If your gene expression level is higher or lower than this range, you may need to scale the criteria accordingly.

mRNA scoring is divided into five categories:

Staining Score	Microscope Objective Scoring
0	No staining or less than 1 dot per 10 cells
1	1–3 dots per cell
2	4–9 dots per cell, no or very few dot clusters
3	10–15 dots per cell and/or <10% dots are in clusters
4	>15 dots per cell and/or >10% dots are in clusters

Control probe evaluation

- Use the table above to review positive and negative control probe staining in your samples
- The negative control probe (dapB) staining should produce results with a score of 0, showing either no staining or less than one dot for every 10 cells. A negative control slide with a score of 1 or more would indicate high background. **Refer to Appendix C** for tips on addressing non-specific signal
- Positive control probe (PPIB and/or UBC, this will depend on tissue/species type) staining with a score of 2 or more, which corresponds to at least 4–9 dots per cell indicates that RNA quality is sufficient such that you may proceed with target probe staining. Samples with score of 0–1 would not pass quality control suggesting that RNA integrity in the sample is compromised or that pretreatment conditions may require optimization

Staining examples

If the assay is successful, the staining should look like the following images.

Figure 1. RNAscope Multiplex Fluorescent Assay detection of POLR2A (green), PPIB (red), and UBC (white), mRNAs, and DAPI nuclear stain (blue), in HeLa FFPE cell pellet using **protease workflow**, captured at 40X using the PhenoImager HT Imaging System. POLR2A= Opal 520 (1:1000), PPIB= Opal 570 (1:1000), UBC= Opal 690 (1:1000)

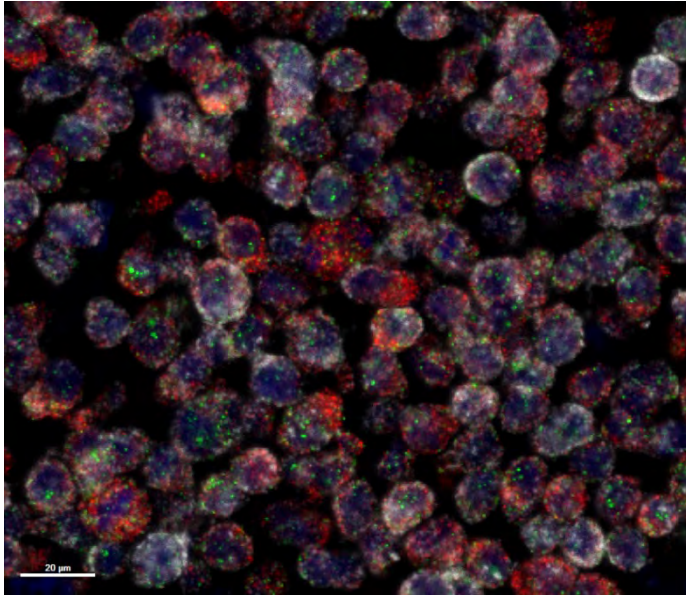


Figure 2. RNAscope Multiplex Fluorescent Assay detection of POLR2A (green), PPIB (red), UBC (white) mRNAs, and DAPI nuclear stain (blue), in HeLa FFPE cell pellet using **RNAscope Manual**

PretreatPro, captured at 40X using the PhenoImager HT Imaging System. POLR2A= Opal 520 (1:1000), PPIB= Opal 570 (1:1000), UBC= Opal 690.

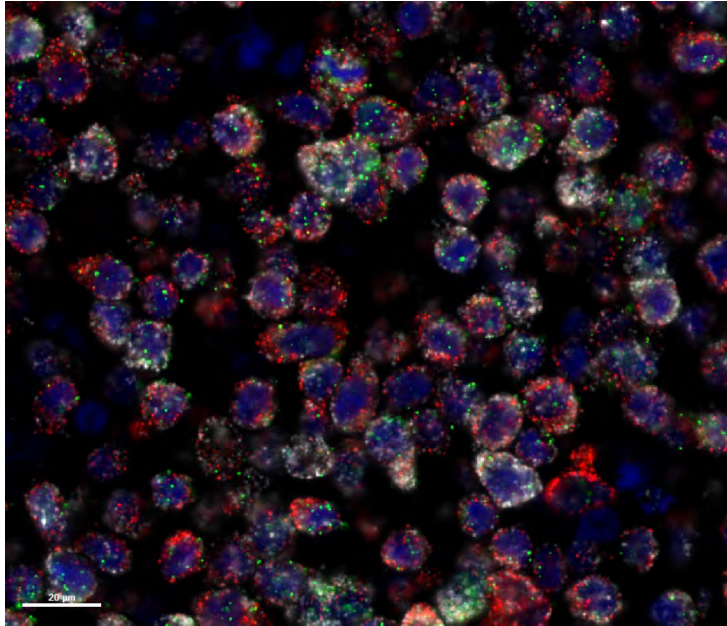
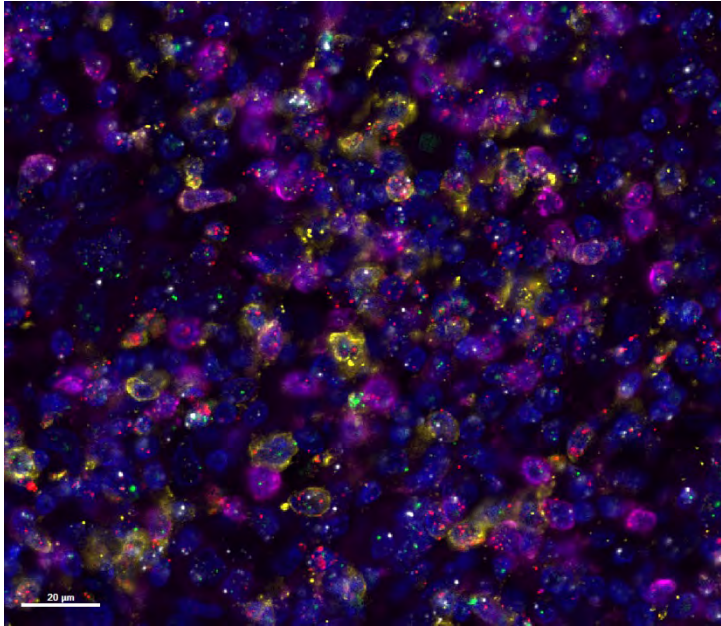


Figure 3. RNAscope Multiplex Fluorescent Assay detection of TNFA (green), TCF7 (red), and IFNG (white) mRNAs with sequential immunofluorescent staining against PD1 (yellow) and CD8A (Magenta), and DAPI nuclear stain (blue), in FFPE human breast cancer tissue using **RNAscope Manual PretreatPro**, captured at 40X using the PhenoImager HT Imaging System.

POLR2A= Opal 520 (1:1000), PPIB= Opal570 (1:1000), UBC= Opal 690; sequential IF fluorophores:
CD8A: secondary antibody with AF750, PD-1: secondary antibody with AF594.





Appendix A. Protease Pretreatment Recommendations for FFPE Samples

Follow the recommended protease pretreatment conditions based on your tissue type for:

- Any new or previously untested FFPE tissue types
- Samples prepared differently than the sample preparation protocol found in **Chapter 3**.

Protease pretreatment recommendations for FFPE samples

1. Stain representative samples using the positive and negative control probes.
2. Fix sample in fresh 10% NBF for **16–32 HRS** at **RT**.

Note: Perform tissue fixation step using the recommended amount of time. Over or under-fixation will result in significant signal loss when performing the RNAscope Assay.

3. Depending on your tissue type, vary the amount of time for the Target Retrieval Reagents and/or Protease Plus (see the following section).

Reagent	Mild	Standard	Extended
RNAscope Target Retrieval Reagents	15 MIN	15 MIN	30 MIN
RNAscope Protease Plus	15 MIN	30 MIN	30 MIN

Note: Sample types, such as certain xenografts and cell pellets, may require shorter incubation time. For these tissue types, vary the RNAscope Target Retrieval Reagents time to **8 MIN** and RNAscope Protease Plus time to **15 MIN**. For the ACD Cell Pellet sample, we recommend using the mild conditions listed in the table.



Tissue-specific protease pretreatment conditions

If your sample fixation is successful in fresh 10% NBF (Step 2 above), then refer to the following table for tissue-specific pretreatment conditions. For information about species or tissue type not listed here, contact support at support.acd@bio-techne.com.

Species	Tissue Type	Pathology	Pretreatment Condition
Mouse/Rat	Intestine	Normal	Standard
	Intestine	Tumor	Standard
	Embryo	Normal	Standard/Mild
	Brain	Normal	Standard
	Spleen	Normal	Mild
	Eye/Retina	Normal	Standard/Mild
	Liver	Normal	Extended
	Kidney	Normal	Standard
Human	Breast	Tumor	Standard
	Colon	Tumor	Standard
	Colon	Normal	Standard
	Lung	Tumor	Standard
	Lung	Normal	Standard
	Prostate	Tumor	Standard
	Prostate	Normal	Standard
	Lymph node	Tumor	Standard
	Lymph node	Normal	Mild
	Tonsil	Normal	Standard
	Pancreas	Normal	Standard
	Cervical	Cancer	Standard
	Cervical	Normal	Standard
	Cervical dysplasia	Abnormal	Standard
	Brain	Tumor	Standard
	Brain	Normal	Standard
	Head	Cancer	Standard
	Neck	Cancer	Standard
	Liver	Cancer	Standard
	Kidney	Normal	Standard
Skin	Normal	Standard	



Species	Tissue Type	Pathology	Pretreatment Condition
	Melanoma	Tumor	Standard
	Nevus	Benign	Standard
	Placenta	Normal	Standard
	Skin (TMA*)	Normal	Standard
	Breast (TMA)	Normal	Standard
	Melanoma (TMA)	Normal	Standard
	Nevus (TMA)	Benign	Standard
	Stomach (TMA)	Normal	Standard
	Stomach (TMA)	Tumor	Standard
	Cell pellets, fixed with 10% NBF	—	Mild
	HeLa or 3T3 cells, fixed with 10% Formaldehyde/PBS/ACD Control	—	Mild

* Tissue Microarray

B

Appendix B. Manual Target Retrieval

Materials required

Materials provided by the Universal Pretreatment Kit	Other Materials and Equipment
<ul style="list-style-type: none"> • RNAscope 10X Target Retrieval Reagents 	<ul style="list-style-type: none"> • Prepared slides • Distilled water • 1 L glass beakers • Paper towel or absorbent paper • Hot plate, isotemp brand • Aluminum foil • Thermometer • Forceps, large • Vertical Slide Rack • Vertical Staining Dish • ImmEdge Hydrophobic Barrier Pen

Prepare 1X RNAscope Target Retrieval Reagents

IMPORTANT! Do not boil the 1X RNAscope Target Retrieval Reagents for more than **15 MIN** before use.

1. Prepare 700 mL of fresh RNAscope 1X Target Retrieval Reagents by adding 630 mL distilled water to 1 bottle (70 mL) 10X Target Retrieval Reagents in the beaker. Mix well.
2. Place the beaker containing RNAscope 1X Target Retrieval Reagents on the hot plate. Cover the beaker with foil, and turn the hot plate on high for **10–15 MIN**.
3. Heat a separate 1L beaker containing distilled H₂O to a mild boil (**98–102°C**).
4. Once 1X RNAscope Target Retrieval Reagents reaches a mild boil (**98–102°C**), turn the hot plate to a lower setting to maintain the correct temperature. Check the temperature with a thermometer.

Apply RNAscope Target Retrieval Reagents

1. With a pair of forceps *very slowly* submerge the slide rack containing the slides into the mildly boiling distilled H₂O for **10 SEC** to acclimate the slides.
2. Remove the slides and move them to the container containing RNAscope 1X Target Retrieval Reagents solution. Cover the beaker with foil and boil the slides for time specified (for fixed-frozen tissue boil for **5 MIN**; for FFPE refer to **Appendix A. Tissue Pretreatment Recommendation**).
3. Use the forceps to *immediately* transfer the hot slide rack from the RNAscope 1X Target Retrieval Reagents to a separate staining dish containing distilled water. Do not let the slides cool in the Target Retrieval Reagents solution.
4. Rinse the slides 3–5 times by moving the Slide Rack up and down in the distilled water.
5. Transfer the slides to 100% ethanol for **3 MIN**.



6. Dry the slides in a **60°C** incubator (or at **RT**) for **5 MIN**.
7. Draw the hydrophobic barrier and continue with the RNAscope Assay.



Appendix C. Troubleshooting

Troubleshooting the protease-free assay

Tissue detachment

Tissue detachment can occur after protease-free or pretreatment. The following tips can be used to minimize the detachment.

- To enhance tissue attachment to the Superfrost glass slide, we recommend baking the FFPE slides for 60 min at 60°C after removing the slides from their storage and before starting the pretreatment procedure.
- For fixed-frozen tissue, we recommend baking the slides for up to 60 min at 60°C after washing the slides in 1X PBS.
- For fresh-frozen tissue slides, please follow the guidelines in **Chapter 3. Prepare Samples**.

Weak fluorescent signal

- In some cases, protease-free pretreatment can lead to relatively weaker fluorescent signals when compared with the protease pretreatment. Follow the guidelines below to improve the assay results. Ensure the tissue sections are sufficiently hydrated during the entire pretreatment process.
- Strictly follow the guidance for PretreatPro workflow, especially the ethanol incubation and washing steps. Do not use the methods described in the standard protease workflow when running these steps.
- Increase the target retrieval time up to 60 min for dense or over-fixed tissues.
- Make sure the target retrieval temperature is maintained at >99°C.
- Maintain the oven temperature for PretreatPro reagent incubation at 40°C throughout the entire step.
- Increase the Opal dye dilution from 1:1000 to 1:750.
- Increase the exposure time or digital gain of the camera.
- The stopping point after target retrieval in the protease pretreatment workflow is **not** applicable to the protease-free pretreatment workflow. The first stopping point of the protease-free assay is after probe hybridization.

Non-specific background dots

- Ensure the tissue sections are sufficiently hydrated during the entire pretreatment process.
- Strictly follow the PretreatPro workflow protocol, especially the ethanol incubation and washing steps. Do not use the methods described in the standard protease workflow when running these steps.
- Increase the target retrieval time to 60 min while maintaining the temperature at >99°C throughout the incubation period.



- Extend the length of the wash steps in the protease-free pretreatment workflow (up to **5 min** per wash).
- During each wash step in the protease-free pretreatment workflow, vigorously agitate the slides using both sliding and rocking motions. Wash the tissue for at least 40 complete sliding/rocking cycles.
- Before each imaging session, first set up the microscope settings (e.g. light intensity, exposure time, gain, binning, histogram etc.) using the negative control slides until the negative slides show an acceptable number of background dots. Use the same microscope settings to image all the slides from one single experiment. We recommend imaging the RNAscope Multiplex v2 slides with a 20X air objective.

Troubleshooting for Protease-based Assay

Tissue Detachment

Tissue detachment can occur after protease-free pretreatment. The following tips can be used to minimize the detachment.

- To enhance tissue attachment to the Superfrost glass slide, we recommend baking the FFPE slides for 60 min at 60°C after removing the slides from their storage and before starting the pretreatment procedure.
- For fixed-frozen tissue, we recommend baking the slides for up to 60 min at 60°C after washing the slides in 1X PBS.
- For fresh-frozen tissue slides, please follow the guidelines in **Chapter 3. Prepare Samples**.

If you obtain less than satisfactory results, troubleshoot your assay following these simple guidelines:

- If the signal intensity is too low for imaging, increase the fluorophore concentration.
- Always run positive and negative control probes to assess signal-to-noise ratio.
- Use appropriate fluorescence filter sets for each fluorophore to minimize fluorescence bleed-through. If you observe fluorescence bleed-through, reduce the fluorophore concentration of the channel that is bleeding through, and/or reduce the exposure time during image acquisition to avoid overexposure.
- If your ISH signal cannot be distinguished from autofluorescence in tissues with high autofluorescence, increase the fluorophore concentration.
- If you observe the presence of background staining, limit the sensitivity of image acquisition or reduce the corresponding fluorophore concentration. Always acquire images using the setting in which background is minimal.
- Use only the reagents listed in this document. Do not replace any components with components from any other RNAscope reagent kits.

For additional troubleshooting guidance, please contact technical support at support.acd@bio-technne.com.

Appendix D. Reagent Volume Guidelines

Determine reagent volume

Before starting your experiment, measure the inner edge of the hydrophobic barrier to determine the recommended number of drops needed per slide.

Size of Hydrophobic Barrier* (in)	Recommended Number of Drops per Slide	Recommended Volume per Slide (μL)	Relative Template Size
0.75" x 0.75" †	4	120	
0.75" x 1.0"	5	150	
0.75" x 1.25"	6	180	

* Hydrophobic barrier measured at inner edge. References in this user manual are for the 0.75" x 0.75" hydrophobic barrier size.

† Recommended hydrophobic barrier size is 0.75" x 0.75". With this barrier size, each probe is sufficient for staining ~20 sections. Larger tissue sections will result in fewer tests.

Chemical safety



WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, visit <http://www.acdbio.com/technical-support/user-manuals>.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety



WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:



In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at www.cdc.gov/biosafety
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030)
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials
- Additional information about biohazard guidelines is available at www.cdc.gov/

In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition
- Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)



Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available at: <https://acdbio.com/documents/product-documents>. For the SDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

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 facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Search for user documents, SDSs, application notes, citations, training videos, and other product support documents.
- Find out information about customer training events.

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Orders: orders.acd@bio-techne.com
Support Email: support.acd@bio-techne.com

Limited product warranty

Advanced Cell Diagnostics, Inc. and/or its affiliate(s) warrant their products as set forth in the ACD General Terms and Conditions of Sale found on the ACD website. If you have any questions, please contact Advanced Cell Diagnostics at <https://acdbio.com/about/contact>.

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