USER MANUAL

RNAscope™ Multiplex Fluorescent Reagent Kit v2

With Sample Preparation and Pretreatment

Document Number UM 323100

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1 Chapter 1. Product Information

Before using this product, read and understand the safety information in **Appendix D. 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 & 323270) on FFPE, fixed frozen, and fresh frozen tissues mounted on slides.

Product description

Background

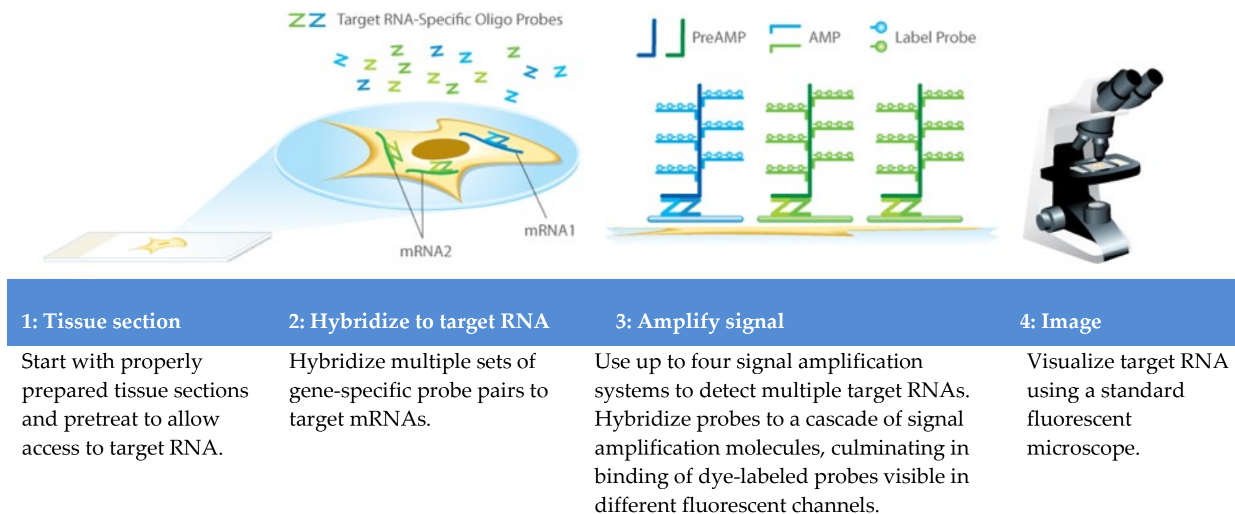
The RNAscope Multiplex Fluorescent v2 Assay uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize up to three different RNA targets per cell in samples mounted on slides. Simultaneous detection of four different RNA targets requires an additional kit: the RNAscope Ancillary kit for Multiplex v2 (Cat. No. 323120), as well as species-specific 4-plex control probes (see the 4-plex Ancillary Tech Note 323120-TN). 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 & 323270). 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, and C3. Different florophores are assigned to the C1, C2, C3 channels depending on the TSA Vivid™, Opal™ dye or TSA® plus fluorophore 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					
☑	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

Control Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	3-Plex Positive Control Probe	Various	RTU mixture of three probes targeting common housekeeping genes *POLR2A* in channel C1, *PPIB* in channel C2, and *UBC* in channel C3.	3 mL x 1 bottle	2–8°C
	3-Plex Negative Control Probe *(dapB)*	320871	RTU probe targeting a bacterial gene. Each detection channel has its own negative control probe: dapB-C1, dapB-C2, dapB-C3	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 and 323270) 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, Target Retrieval Reagents, Detection Reagents, Multiplex TSA Buffer and Wash Buffer Reagents. In terms of Pretreatment 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 H_2O_2 and Protease Reagents (Cat. No. 322381)			
☑	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)			
☑	**Reagent**	**Quantity**	**Storage**
	RNAscope 10X Target Retrieval	70 mL x 4 bottles	Room temp (15–30°C)

RNAscope Multiplex Fluorescent Detection Reagents v2 (Cat. No. 323110)			
☑	Reagent	Quantity	Storage
	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 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	3 mL x 1 bottle	2–8°C
RNAscope Multiplex TSA Buffer (Cat. No. 322809)			
	RNAscope TSA Buffer Pack	29 mL x 1 bottle	2–8°C
RNAscope Wash Buffer Reagents (Cat. No. 310091)			
☑	Reagent	Quantity	Storage
	RNAscope 50X Wash Buffer	60 mL x 4 bottles	Room temp (15–30°C)

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

RNAscope Multiplex Fluorescent Reagent Kit v2 with TSA Vivid Dyes

The RNAscope Multiplex Fluorescent Reagent Kit v2 with TSA Vivid Dyes (Cat No. 323270) contains all the components listed above with the addition of the TSA Vivid fluorophores in the 520, 570 and 650 channels.

TSA Vivid Fluorophores (supplied with Cat. No. 323270)			
☑	Reagent	Quantity	Storage
	TSA Vivid Fluorophore 520 (Cat. No. 323271)	TSA Vivid Dye x 1vial DMSO x 1 vial	-20°C
	TSA Vivid Fluorophore 570 (Cat. No. 323272)	TSA Vivid Dye x 1vial DMSO x 1 vial	-20°C
	TSA Vivid Fluorophore 650 (Cat. No. 323273)	TSA Vivid Dye x 1vial DMSO x 1 vial	-20°C

Required materials and equipment

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

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 at **https://acdbio.com/documents/support-documents** and view the training video at **https://acdbio.com/technical-support/learn-more.** The system contains the following components:

☑	Component	Quantity	Cat. No.
	HybEZ II Hybridization System (110 or 220V)	1 oven	321711 or 321721 (HybEZ II)
	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.

Recommended: TSA Vivid fluorophores

The RNAscope Multiplex Fluorescent Reagent Kit v2 with TSA Vivid Dyes (Cat No. 323270) contains TSA Vivid fluorophores which are very bright dyes that boost signal intensity and detect low-abundance targets in spatial biology applications. They are designed for use in ISH, IHC and ICC experiments where *in situ* detection of target protein or nucleic acid sequences is required. TSA Vivid reagents have been specifically optimized to deliver exceptional signal-to-noise in the RNAScope Multiplex Fluorescent v2 Assay to visualize gene expression at the single cell level. They are suitable for multiplexing and can be combined with DAPI. Dilute the dyes in RNAscope Multiplex TSA Buffer. 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. Materials are qualified using a 1:1500 dilution for all three fluorophores.

To perform a 3-plex assay, use TSA Vivid Fluorophores in the 520, 570, and 650 channels.

Fluorophores	Cat. No.	Recommended dilution range
TSA Vivid Fluorophore 520	323271*	1:750–1:3000
TSA Vivid Fluorophore 570	323272*	1:750–1:3000
TSA Vivid Fluorophore 650	323273*	1:750–1:3000

* 1. Reconstitute the TSA Vivid reagent with 100 µL Dimethylsulfoxide (DMSO). 2. Recommended working dilution range: 1:750 - 1:3000. We recommend starting with a dilution of 1:1500 and adjusting based on signal intensity.

Compatible: Opal dyes or TSA Plus fluorophores

The RNAscope Multiplex Fluorescent Reagent Kit v2 without the TSA Vivid reagents (Cat. No. 323100) requires purchase of Opal dyes or TSA Plus fluorophores from Akoya Biosciences (see the following table). We cannot guarantee assay results if you use other fluorescent dyes. Dilute the dyes in RNAscope Multiplex TSA Buffer provided by the RNAscope Multiplex Fuorescent 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. Materials are qualified using a 1:1500 dilution for all three fluorophores.

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 Polaris 780 instead of Opal 520. The Opal Polaris 780 Reagent Pack contains two reagents: Opal TSA-DIG and Opal Polaris 780. We recommend diluting Polaris TSA-DIG in TSA buffer, and diluting Opal Polaris 780 in Antibody Diluent/Block from Akoya Biosciences (PN: ARD1001EA).

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 Polaris 780 Reagent Pack	FP1501001KT	TSA-DIG: 1:750–1:3000
		Polaris 780: 1:187.5–1:750

* Reconstitute all Opals (except Opal Polaris 780) with 75 μL Dimethylsulfoxide (DMSO). Reconstitute Opal Polaris 780 with 300 μL double distilled water (ddH2O).

Other materials

☑	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
	Tissue-Tek® Vertical 24 Slide Racks (or other slide racks or holders)	American Master Tech Scientific/MLS	LWS2124
	Tissue-Tek Staining Dishes (or similar containers)	American Master Tech Scientific/MLS	LWS20WH
	Tissue-Tek Clearing Agent Dish, xylene resistant	American Master Tech Scientific/MLS	LWT4456EA
	Oster® Steamer Model 5712, Black and Decker Steamer HS3000, the Braun Multiquick FS 20 Steamer, or the Hamilton Beach Steamer	—	—
	Digital thermometer	MLS	—
	ProLong Gold Antifade Mountant	Thermo Fisher Scientific	P36930
	Antibody Diluent/Block (if Opal Polaris 780 is used)	Akoya Bioscieces	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	—

☑	Description	Supplier	Cat. No.
	Tubes (various sizes)	MLS	—
	Paper towel or absorbent paper	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)	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
• 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 Polaris 780 is used)	• 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	• Microscope with camera for fluorescence capturing. Multispectrum microscope/camera system (eg. Nuance® EX, Mantra™, Vectra® and Polaris) 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).

2 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**.
- Consult the RNAscope Multiplex Fluorescent Assay v2 checklist.

Important procedural guidelines

- Start with properly fixed and prepared sections. Refer to **Chapter 3. Prepare and Pretreat Samples**, **Appendix A. Tissue Pretreatment Recommendation**, and to our sample preparation and pretreatment user guides available at **https:// acdbio.com/technical-support/user-manuals**.
- Use only samples mounted on SuperFrost Plus Slides (Fisher Scientific, Cat. No. 12-550-15).
- Always run positive and negative control probes on your sample to assess sample RNA quality and optimal permeabilization.
- 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.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix D. Safety** for more information.

3

Chapter 3. Prepare and Pretreat Samples

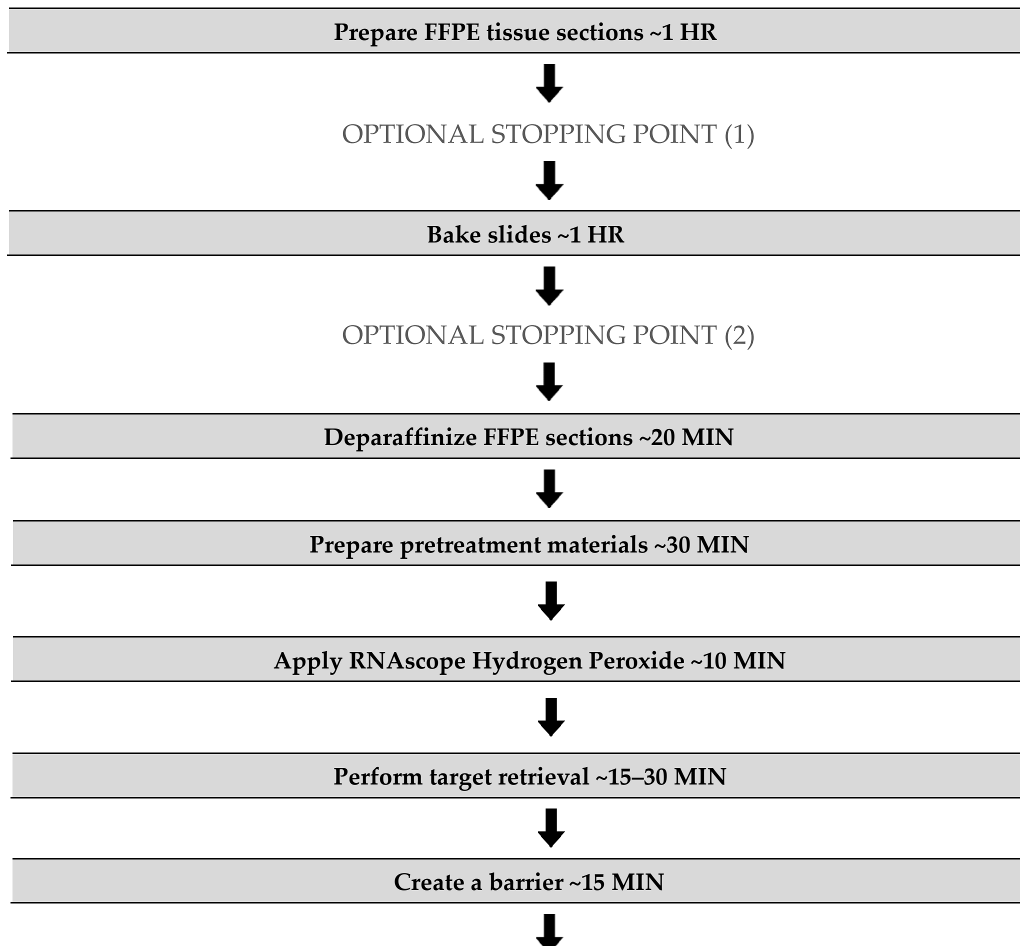
This chapter describes three tissue sample preparation methods: formalin-fixed, paraffin-embedded (FFPE) sample preparation and pretreatment, fixed-frozen sample preparation and pretreatment, and fresh-frozen sample preparation and pretreatment. For other sample types and preparation methods, contact **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.

FFPE sample preparation and pretreatment

For suboptimally prepared samples, you may need to optimize pretreatment conditions. Refer to **Appendix A. Tissue Pretreatment Recommendation** and to **https://acdbio.com/technical-support/solutions**.

Workflow



OPTIONAL STOPPING POINT (3)

Apply RNAscope Protease Plus ~15–30 MIN

Proceed IMMEDIATELY to the RNAscope Assay

Materials required

Materials provided by Pretreatment Reagents (Cat. No. 322381 and 322000)	Other Materials and Equipment
• RNAscope Hydrogen Peroxide • RNAscope Protease Plus • RNAscope 10X Target Retrieval Reagents	• 10% neutral buffered formalin (NBF) • 1X PBS • Paraffin wax • Tissue-Tek Clearing Agent Dishes • Tissue-Tek Staining Dishes • Tissue-Tek Vertical 24 Slide Rack • 100% alcohol (EtOH) • Xylene • Microtome • Water bath • SuperFrost Plus slides • 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 • Steamer • 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.

CAUTION! Handle biological specimens appropriately.

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

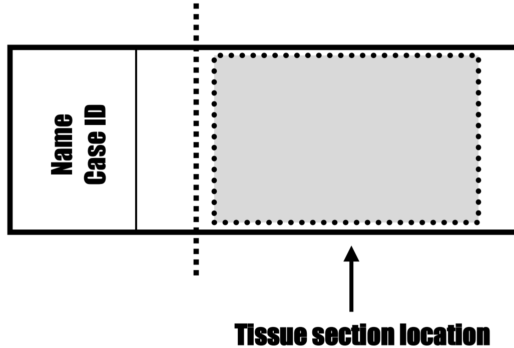
2. Wash sample with 1X PBS.
3. Dehydrate sample using a standard ethanol series, followed by xylene.

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 +/– 1 µm 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 with desiccants at room temperature.

Bake slides

IMPORTANT! Please note that we recommend using a dry-air oven for baking. If it 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 Tissue-Tek Clearing Agent dishes with ~200 mL fresh xylene.
   - Fill two Tissue-Tek Staining dishes with ~200 mL fresh 100% ethanol.
2. Place slides in a Tissue-Tek 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. Remove the slide rack from the first xylene-containing dish, and *immediately* place in the second xylene-containing dish in the fume hood.
5. Incubate the slides in **xylene** for **5 MIN** at **RT** with agitation.
6. Remove the slide rack from the second xylene-containing dish, and *immediately* place in a dish containing 100% ethanol.
7. Incubate the slides in 100% ethanol for **2 MIN** at **RT** with agitation.
8. Remove the slide rack from the first ethanol -containing dish, and *immediately* place in the second ethanol -containing dish.
9. Incubate the slides in 100% ethanol for **2 MIN** at **RT** with agitation.
10. 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 **≥99°C** before use (see **Perform target retrieval using the steamer**).

Apply RNAscope Hydrogen Peroxide

1. Lay the deparaffinized slides on the bench, and add ~5–8 drops of RNAscope Hydrogen Peroxide to cover each section.
2. Incubate slides for **10 MIN** at **RT**.
3. Remove RNAscope Hydrogen Peroxide solution from one slide at a time by tapping and/or flicking the slide on absorbent paper. Immediately insert the slide into a Tissue-Tek Slide Rack submerged in a Tissue-Tek Staining Dish filled with distilled water.
4. Wash slides 3–5 times by moving the Tissue-Tek Slide Rack up and down in the distilled water.
5. Repeat Step 4 with fresh distilled water.

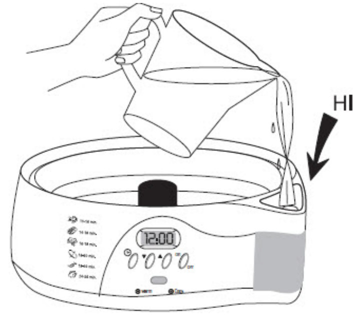
Perform target retrieval using the steamer

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

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 steaming process will drop the temperature and interfere with the Target Retrieval step.

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

IMPORTANT! Do not overfill.

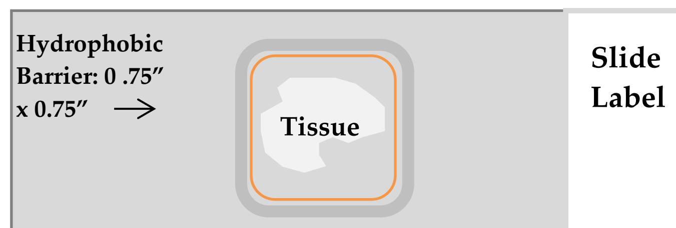


7. Place the steaming bowl onto the base, and place two Tissue Tek slide holders in the steam bowl.
8. Fill one slide holder with 200 mL of RNAscope 1X Target Retrieval Reagent, and the other slide holder with 200 mL of distilled H_2O.
9. Turn on the steamer, and set the heating time to the maximum amount so the steamer does not shut off during the procedure.
10. 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**.
11. Add the slides to the container containing distilled H_2O for **10 SEC** to acclimate the slides.
12. Remove the slides and move them to the container containing RNAscope 1X Target Retrieval Reagent. Cover the steamer with the lid.
13. Start the timer for **15 MIN** for mild and standard conditions, and **30 MIN** for extended pretreatment. For pretreatment times, consult **Appendix A. Tissue Pretreatment Recommendation**.
14. 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**.
15. Transfer the slides to 100% ethanol for **3 MIN**.
16. Dry the slides in a **60°C** incubator (or at **RT**) for **5 MIN**.

Create a 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**.

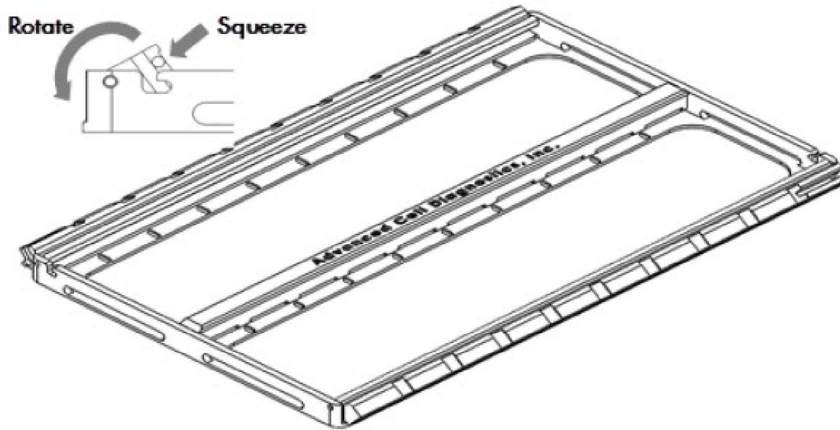
Note: If you need to reapply the hydrophobic barrier during the following procedures, dry the appropriate area of the slide with a Kimwipe®. Do not touch the tissue section.

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

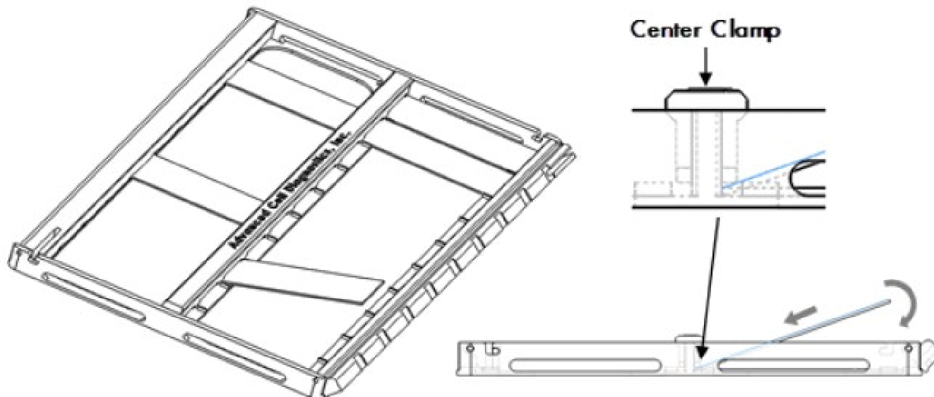
Load the slides into the ACD EZ-Batch Slide Holder

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.

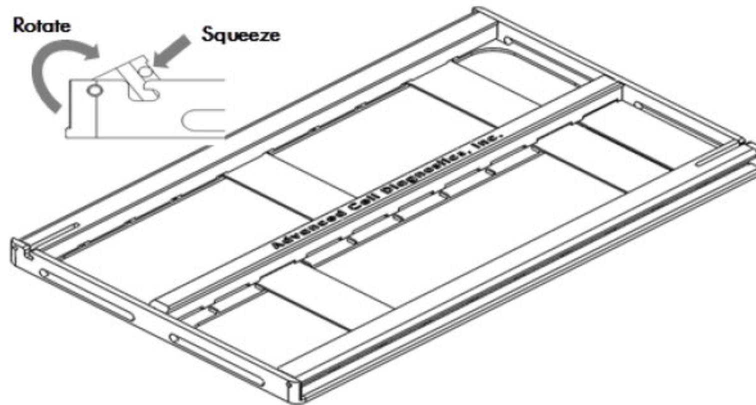
1. Open the swing clamps one at a time by simultaneously squeezing (pressing and holding) the slotted portion of each clamp and rotating it up then outwards.



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.

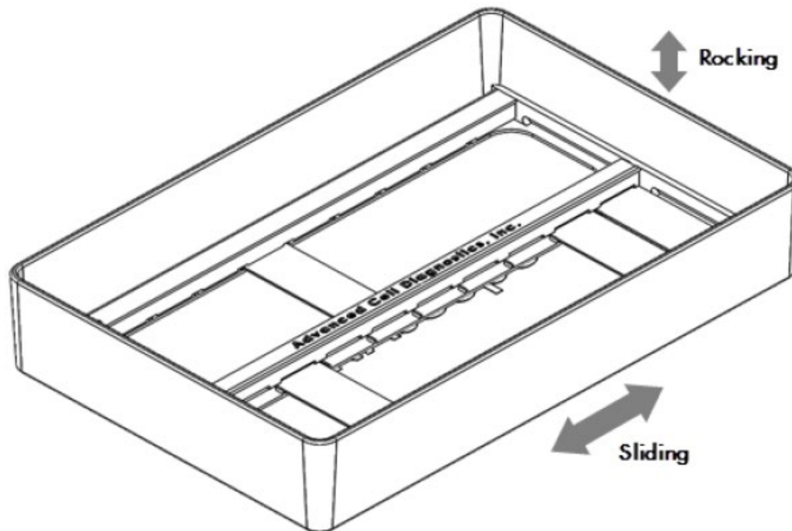


Apply RNAscope Protease Plus

1. Add ~5 drops of RNAscope Protease Plus to entirely cover each section.
2. Place the ACD EZ-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 amount of time specified by the table in **Appendix A. Tissue Pretreatment Recommendation**.

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

4. Pour at least 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.
5. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
6. Place the ACD EZ-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.



7. Repeat the wash step with fresh distilled water.

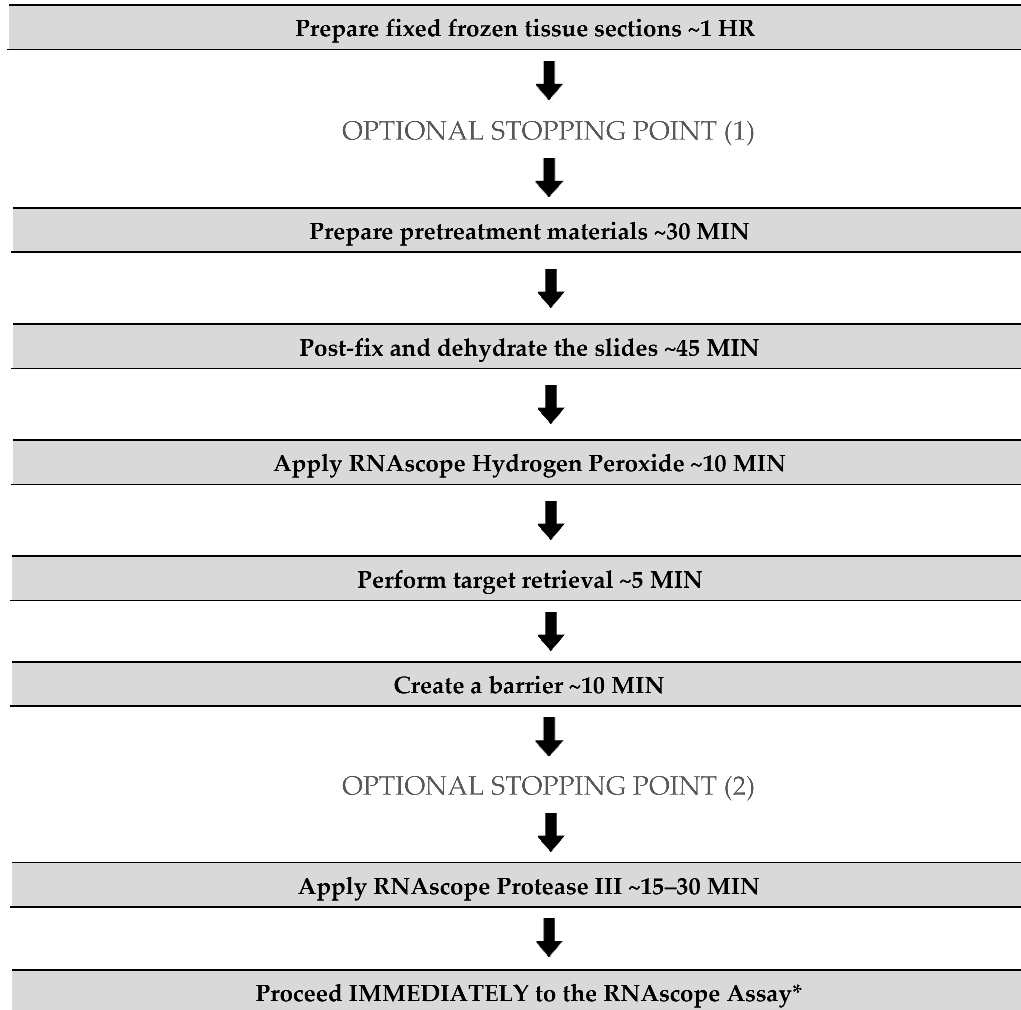
Proceed to the RNAscope Assay

Proceed *immediately* to **Chapter 4. RNAscope Multiplex Fluorescent v2 Assay**.

Fixed-frozen tissue sample preparation and pretreatment

For suboptimally 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)	Other Materials and Equipment
• RNAscope Hydrogen Peroxide • RNAscope Protease III or Protease Plus • RNAscope 10X Target Retrieval Reagents	• 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 • Tissue-Tek Vertical 24 Slide Rack • Tissue-Tek Staining Dishes • ImmEdge Hydrophobic Barrier Pen • HybEZ Humidifying System/ ACD EZ-Batch Slide Holder and Wash Tray • Distilled water • Paper towel or absorbent paper • Steamer • Digital thermometer • Drying oven

Fix samples

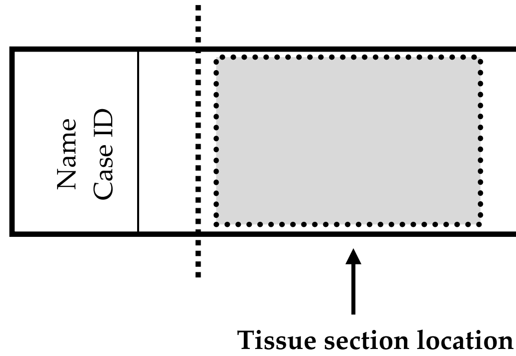
1. If needed, perfuse the tissue with freshly prepared 4% paraformaldehyde (PFA) in 1X PBS, or go directly to Step 2.
2. Dissect the tissue and fix in freshly prepared 4% PFA for **24 HRS** at **4°C**.

Freeze tissues

1. 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).
2. 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.
3. 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

1. Before tissue sectioning, equilibrate the tissue blocks at **–20°C** for at least **1 HR** in a cryostat.
2. Section the blocks by cutting 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.

3. 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**.

4. Wash the slides with 200 mL 1X PBS in a Tissue-Tek slide rack for **5 MIN** while moving the rack up and down to remove OCT.
5. Bake the slides for **30 MIN** at **60°C**.
6. 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 200mL 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 **≥99°C** before use (see **Perform target retrieval using the steamer** on page **24**.).

Apply RNAscope Hydrogen Peroxide

1. Take the dehydrated slides and lay them on the bench, and add ~5–8 drops of RNAscope Hydrogen Peroxide to cover the entire section.
2. Incubate the slides for **10 MIN** at **RT**.
3. Remove RNAscope Hydrogen Peroxide solution from one slide at a time by tapping and/or flicking the slide on absorbent paper. Immediately insert the slide into a Tissue-Tek Slide Rack submerged in a Tissue-Tek Staining Dish filled with distilled water.
4. Wash the slides 3–5 times by moving the Tissue-Tek Slide Rack up and down in the distilled water.
5. Repeat Step 4 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 from **Appendix A. Tissue Pretreatment Recommendation**.

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 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.

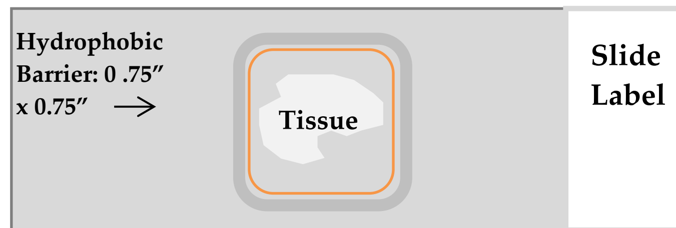
2. Place the steaming bowl onto the base, and place two Tissue Tek slide holders in the steam bowl.
3. Fill one slide holder with 200 mL of RNAscope 1X Target Retrieval Reagent, and the other slide holder with 200 mL of distilled H_2O.
4. Turn on the steamer, and set the heating time to the maximum amount 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 H_2O for **10 SEC** to acclimate the slides.
7. Remove the slides and move them to the container containing 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 pretreatment times, consult **Appendix A. Tissue Pretreatment Recommendation**.
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 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**.

- 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. Load the dry slides into the ACD EZ-Batch Slide Holder by opening the swing clamp (see page 19 for details).
2. Add ~5 drops of Protease III 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. 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 (see page 20 for details). Repeat the wash step with fresh water.

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

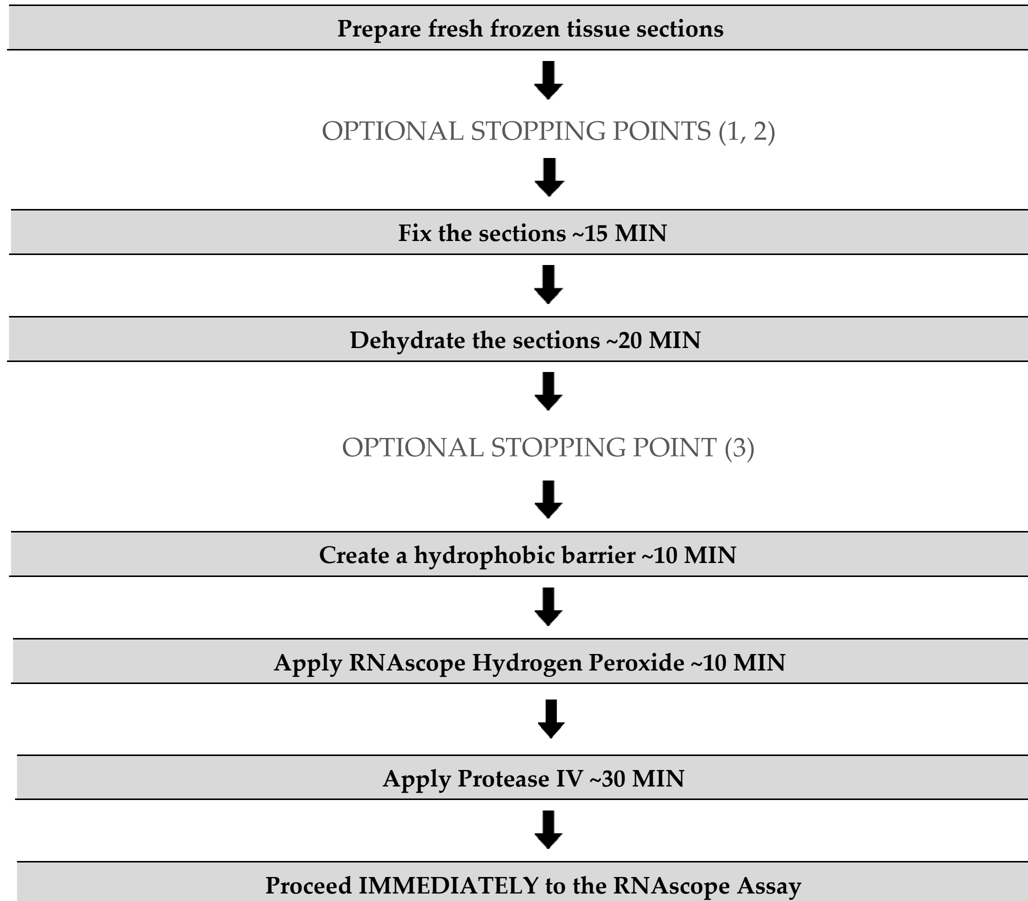
Proceed to the RNAscope Assay

Proceed *immediately* to **Chapter 4. RNAscope Multiplex Fluorescent v2 Assay**.

Fresh-frozen sample preparation and pretreatment

IMPORTANT! Do not apply RNAscope Target Retrieval to fresh-frozen sections when using the RNAscope Multiplex Fluorescent v2 Kit. Other sample types may need these treatments.

Workflow



Materials required

Materials provided by Pretreatment Reagents (Cat. No. 322381)	Other Materials and Equipment
• RNAscope Hydrogen Peroxide • RNAscope Protease IV	• 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) • 100% alcohol (EtOH) • Tissue-Tek Vertical 24 Slide Rack • Tissue-Tek Staining Dishes • ImmEdge Hydrophobic Barrier Pen • HybEZ Humidifying System/ ACD EZ-Batch Slide Holder and Wash Tray • Distilled water • Paper towel or absorbent paper

Prepare fresh frozen tissue sections

1. Remove tissue and cut to fit into cryomolds.

CAUTION! Handle biological specimens appropriately.

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. Hold the block 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.

Fix the sections

1. Chill 10% NBF (fresh 10% NBF or 4% PFA in 1X PBS) to **4°C**.
2. Remove 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

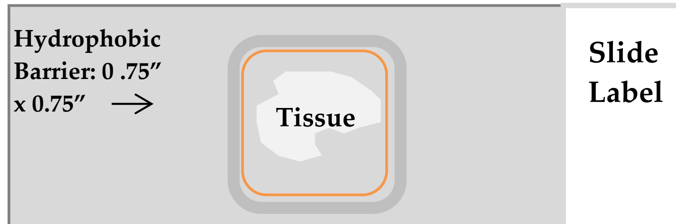
Reagents may be prepared ahead of time. Ensure all containers remain covered.

1. Prepare **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.**
2. 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.

3. 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

1. Place a Humidifying Paper in the HybEZ Humidity Control Tray and wet completely with distilled water.
2. Load the dry slides into the EZ-Batch Slide Holder (see page 19 for details). 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 (see page 20 for details). Make sure all the slides are submerged. If needed, carefully add more water.
6. 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.

1. 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.
2. Incubate for **30 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 containing the slides into the wash tray and wash the slides (see page 19 for details). Repeat the wash step with fresh distilled water.

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

Proceed to the RNAscope Assay

Proceed *immediately* to **Chapter 4. RNAscope Multiplex Fluorescent v2 Assay**.

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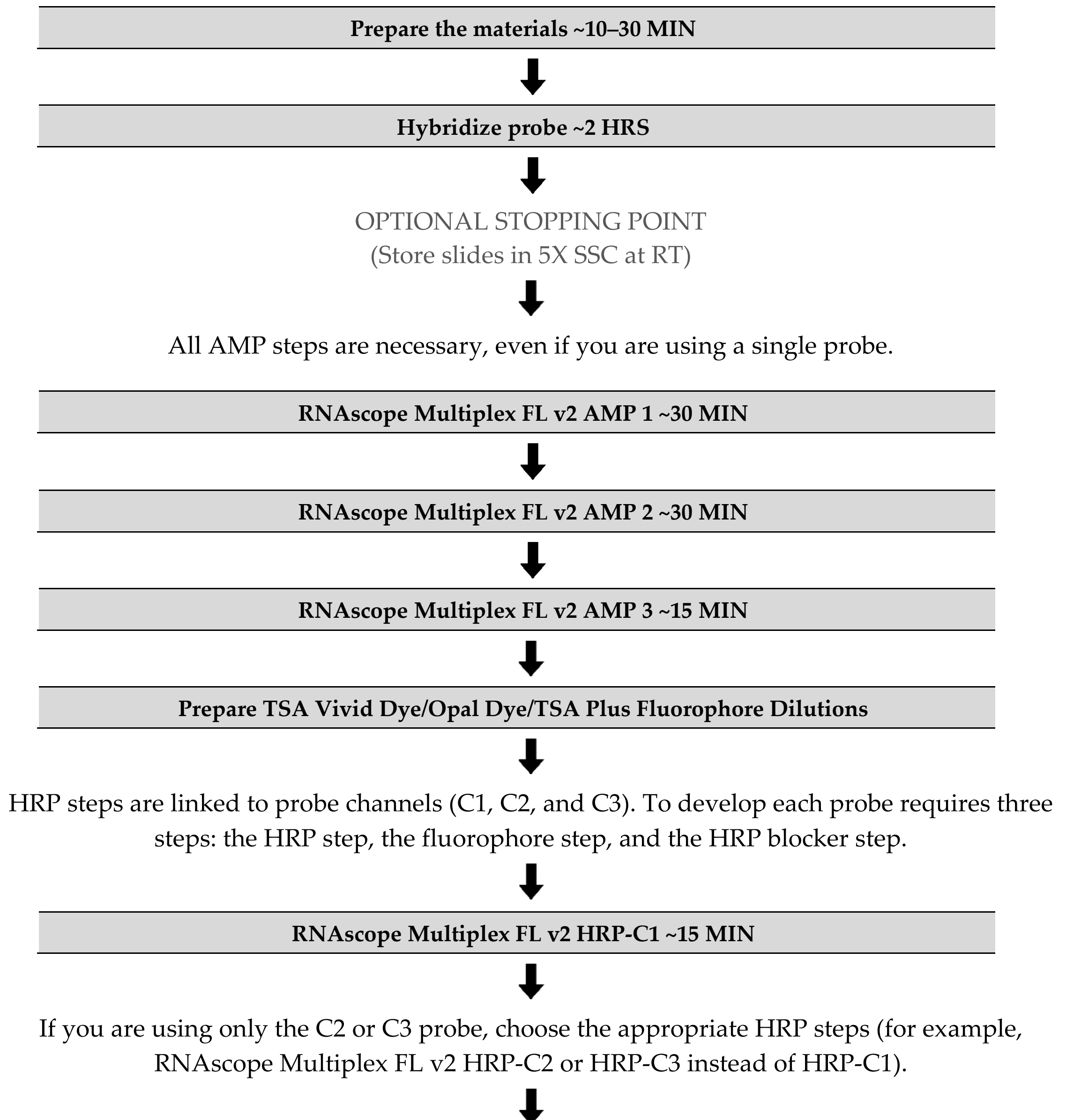
4 Chapter 4. RNAscope Multiplex Fluorescent v2 Assay

This procedure flows directly from sample preparation and pretreatment. Refer to **Chapter 3. Prepare and Pretreat Samples** or the appropriate sample preparation and pretreatment user manual for your specific sample type.

Workflow



1st TSA Vivid Dye/Opal Dye/TSA Plus fluorophore ~30 MIN

⬇

RNAscope Multiplex FL v2 HRP blocker ~15 MIN

⬇

IF USING JUST THE C1 PROBE, STOP HERE TO COUNTERSTAIN AND MOUNT YOUR SLIDES.

⬇

RNAscope Multiplex FL v2 HRP-C2 ~15 MIN

⬇

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

⬇

2nd TSA Vivid Dye/Opal Dye/TSA Plus fluorophore ~30 MIN

⬇

RNAscope Multiplex FL v2 HRP blocker ~15 MIN

⬇

IF USING JUST C1 AND C2 PROBES, STOP HERE TO COUNTERSTAIN AND MOUNT YOUR SLIDES.

⬇

RNAscope Multiplex FL v2 HRP-C3 ~15 MIN

⬇

3rd TSA Vivid Dye/Opal Dye/TSA Plus fluorophore ~30 MIN

⬇

RNAscope Multiplex FL v2 HRP blocker ~15 MIN

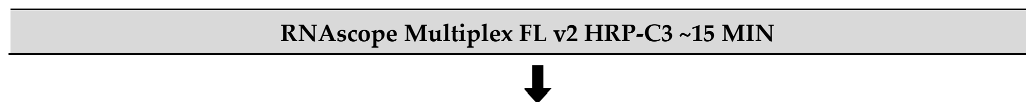
⬇

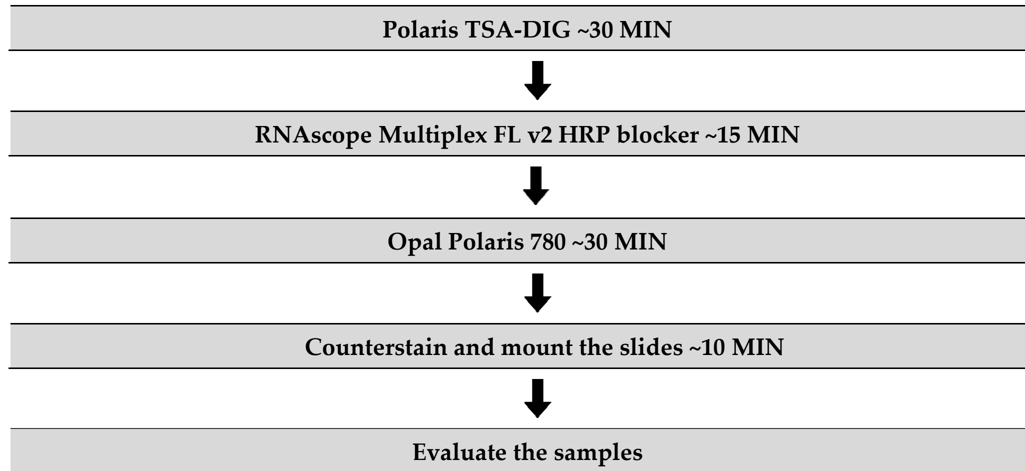
Counterstain and mount the slides ~10 MIN

⬇

Evaluate the samples

IF USING OPAL POLARIS 780, USE THE FOLLOWING WORKFLOW AS AN EXAMPLE.
You can use Opal Polaris 780 in the C1 or C2 channel, but do not follow it with any other fluorophore or a complete loss of Opal Polaris 780 signal occurs. The 780 fluorophore is easily cleaved off by HRP activity. Opal Polaris 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
• 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 blocker • RNAscope Multiplex FL v2 DAPI • RNAscope Multiplex TSA Buffer	• Target Probes • Positive Control Probe • Negative Control Probe	• Prepared sections • Distilled water • Carboy (>3L) • TSA Vivid/Opal or TSA Plus fluorophores • Antibody Diluent/Block • Tissue-Tek Staining Dishes • HybEZ Humidifying System/ACD EZ-Batch Slide Holder and Wash Tray • Water bath or incubator • Tissue-Tek 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/technical-support/user-manuals**.

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

Prepare 1X Wash Buffer

- 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.
- Prepare 6 L of 1X Wash Buffer by adding 5.88 L distilled water and 2 bottles (120 mL) of RNAscope Wash Buffer (50X) to a large carboy. Mix well.

Prepare probes

1. 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 and C3 probes come as 50X stocks and need to be diluted with the C1 probe.
2. If using only the C2 or C3 probe, dilute the probe with Probe Diluent (Cat. No. 300041) instead of C1 probe. Probe Diluent can be purchased separately.
3. Warm probes for **10 MIN** at **40°C** in a water bath or incubator, then cool to **ROOM TEMPERATURE (RT)**.
4. Briefly spin the C2 and C3 probes to collect the liquid at the bottom of the tubes.
5. Pipette 1 volume of C2 and 1 volume of C3 probes to 50 volumes of C1 probe into a tube. Invert the tube several times to mix.

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

Equilibrate reagents

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

Prepare TSA Vivid Dyes/Opal Dyes or TSA Plus fluorophores

Prepare only the fluorophores that you will be using. For example, if you only using one probe, there is no need to prepare more than one fluorophore. You can mix and match channels and fluorophores. For example, you may assign TSA Vivid 570 or TSA Vivid 650 to the C1 channel instead of TSA Vivid 520. If Opal 690 is assigned to the C2 channel, you may need to increase the concentration of Opal 690. Do not assign the same fluorophore to more than one channel.

1. Reconstitute the TSA Vivid Dye/Opal Dye or TSA Plus fluorophore using the instructions from the corresponding manufacturers.

IMPORTANT! Store TSA Vivid Dye/Opal Dye or TSA Plus fluorophore stocks at **2–8°C**. Follow manufacturer's instructions.

2. Determine the volume of TSA Vivid Dye/Opal Dye or TSA Plus fluorophore needed (150–200 µL per slide).
3. Dilute the TSA Vivid Dye /Opal Dye or TSA Plus fluorophore stocks in appropriate buffer. Follow these recommendations:

Recommended Fluorophore	Compatible Fluorophore	Recommended dilution range*	Dilute in
TSA Vivid Fluorophore 520 (323271)	Opal 520 Reagent Pack (FP1487001KT)	1:750–1:3000	TSA buffer

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

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

IMPORTANT! Store diluted TSA Vivid Dyes/Opal Dyes up to one month at **2–8°C** in the dark.

Note: You may replace Opal Dyes with certain TSA Plus fluorophores from Akoya Biosciences.

Opal Dye	TSA Plus fluorophore	PerkinElmer Reagent Kit
Opal 520	TSA Plus fluorescein	NEL741E001KT or NEL741001KT
Opal 570	TSA Plus Cyanine 3	NEL744E001KT or NEL744001KT
Opal 690	TSA Plus Cyanine 5	NEL744E001KT or NEL744001KT

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

1. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800ml of distilled H_2O.
2. Adjust the pH to 7.0 with a few drops of 1M HCl.
3. Adjust the volume to 1 L with additional distilled H_2O.
4. 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 A. 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 20 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**.

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 theslides 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 Plus 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 µL 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 38.

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-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 µL diluted fluorophore for labeling the C2 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 C1 & C2 PROBES. Continue to **Counterstain and mount the slides** on page 38.

Develop HRP-C3 signal

If you are using Opal Polaris 780 instead of Opal 690 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-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 µL diluted fluorophore for labeling the C3 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 theslides, 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 insertinto 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-C3 signal with Opal Polaris 780

If you are using a fluorophore instead of Opal Polaris 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-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 µL diluted TSA-DIG 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 insertinto 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 Polaris 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.

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 **30 MIN** 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 5. Evaluate the samples**.

Chapter 5. Evaluate the samples

5

Refer to **Fluorescent Imaging Recommendations** on page 12.

Evaluate the sample

Examine tissue sections under a standard fluorescent or confocal microscope at 20–40X magnification.

- Assess tissue and cell morphology.
- Assess negative control background first. One dot to every 10 cells displaying background staining at 20X magnification is acceptable. Set the light source and exposure time of image acquisition to acceptable background levels.
- Assess positive control signal strength. Positive control signal should be visible as punctate dots within a cell at 20X magnification.

Imaging the slides

For imaging using multiplexed biomarker imaging systems Vectra, Mantra or Polaris, refer to the guidelines from Akoya. 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	Cy 5

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

Control examples

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

Figure 2. RNAscope Multiplex Fluorescent Assay detection of TBP (green), PPIB (orange) and POLR2A (white) mRNA in HeLa FFPE tissues, captured at 40X using the Nuance Fx Multiplex Biomarker Imaging System

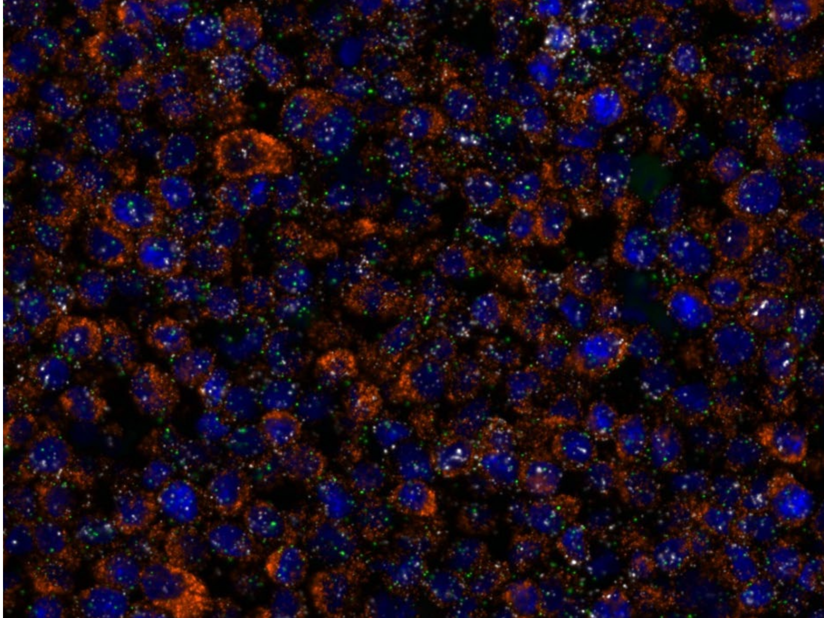
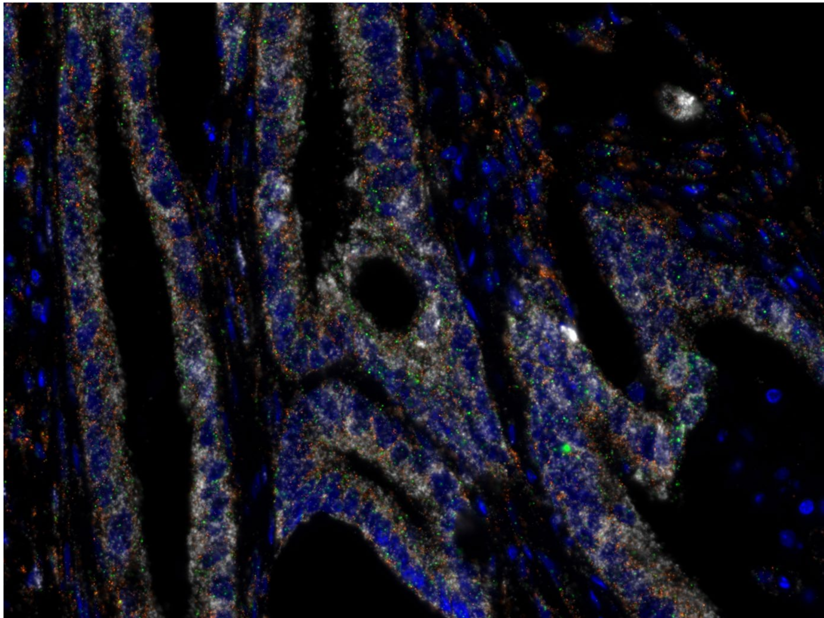


Figure 3. RNAscope Multiplex Fluorescent Assay detection of POLR2A (green), PPIB (orange) and UBC (white) mRNA in human colon cancer FFPE tissue, captured at 20X using the Nuance Fx Multiplex Biomarker Imaging System

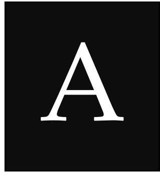


Troubleshooting

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

- If signal intensity is too low for imaging tools, increase the fluorophore concentration.
- Always run positive and negative control probes to assess signal-to-noise ratio.
- Use optimaized fluorescence filters sets 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 over-exposure.
- If your RNA ISH signal cannot be distinguished from autofluorescence in tissues with high autofluorescence, increase the flurophore 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 under-detected.
- Use only the reagents listed in this document. Do not replace any components with components from any other RNAscope reagent kits.

For troubleshooting information, please contact technical support at **support.acd@bio-techne.com**.



A Appendix A. Tissue Pretreatment Recommendations for FFPE Samples

Follow the recommended 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**.

Tissue 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.** If you have a tissue type not listed, contact support at **support.acd@bio-techne.com.**

Tissue-specific 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
	Brain	Normal	Standard
	Spleen	Normal	Mild
	Eye/Retina	Normal	Standard
	Liver	Normal	Extended
	Kidney	Normal	Standard

Species	Tissue Type	Pathology	Pretreatment Condition
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
	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 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
• RNAscope 10X Target Retrieval Reagents	• Prepared slides • Distilled water • 1 L glass beakers • Paper towel or absorbent paper • Hot plate, isotemp brand • Aluminum foil • Thermometer • Forceps, large • Tissue Tek Slide Rack • Tissue Tek Staining Dish • ImmEdge Hydrophobic Barrier Pen

Prepare 1X RNAscope Target Retrieval Reagents

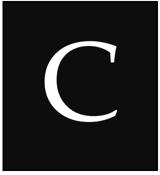
IMPORTANT! Do not boil the 1X RNAscope Target Retrieval Reagents more than **15 MIN** before use.

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 H_2O 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 H_2O 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 the amount of time specified (for fixed-frozen tissue boil for **5 MIN**; for FFPE refer to table in **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. Wash the slides 3–5 times by moving the Tissue-Tek 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.



C

Appendix C. 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 (see table below).

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.

D

Appendix D. Safety

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/technical-support/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.

Contact information

Advanced Cell Diagnostics, Inc.
7707 Gateway Blvd Suite 200
Newark, CA 94560
Toll Free: 1-877-576-3636
Direct: 1-510-576-8800
Fax: 1-510-576-8801
Information: **info.acd@bio-techne.com**
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**.

Headquarters
7707 Gateway Blvd Suite 200, Newark, CA 94560 Phone 1-510-576-8800 Toll Free 1-877-576-3636
For support, email **support.acd@bio-techne.com**
www.acdbio.com