



# **RNAscope™ Plus smRNA-RNA HD Reagent Kit**

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When describing a procedure for publication using this product, please refer to it as the RNAscope Assay and cite: Wang F, Flanagan J, Su N, Wang L-C, Bui S, Nielson A, Wu X, Vo H-T, Ma X-J and Luo Y. RNAscope: A Novel *In Situ* RNA Analysis Platform for Formalin-Fixed Paraffin-Embedded Tissues. *J. Mol. Diagnostics*, 2012, 14:22–29.

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# Chapter 1. Product Information



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

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

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## About this guide

This user manual provides guidelines and protocols to use the RNAscope Plus smRNA-RNA HD Assay (Cat. No. 322785).

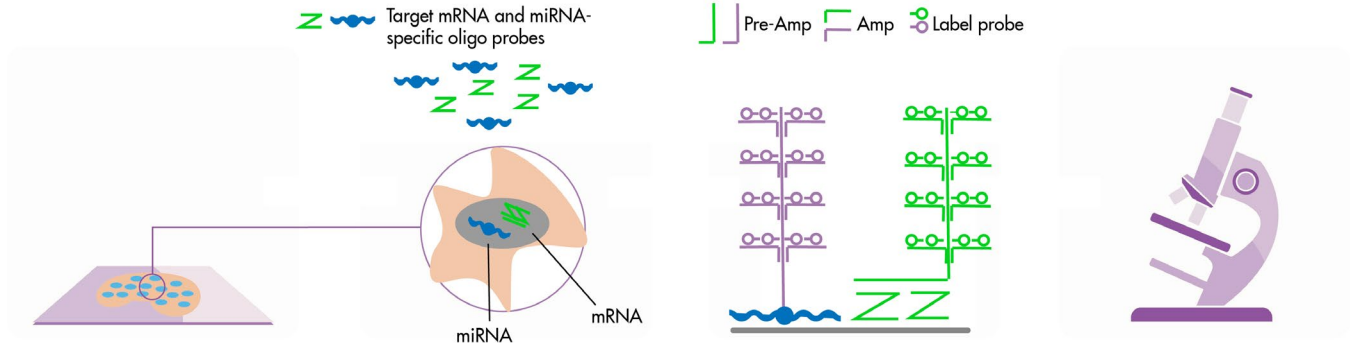
## Background

The RNAscope Plus smRNA-RNA HD Assay uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize up to one smRNA (e.g. miRNA, siRNA or ASO) and up to three different RNA targets per cell in samples mounted on slides. The assay is based on ACD's patented signal amplification and background suppression technology, and incorporates signal amplification systems that 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 Plus smRNA-RNA Fluorescent Assay procedure, which can be completed in ~16 hours. The protocol has several convenient stopping points. Most of the RNAscope Plus Assay reagents are available in convenient Ready-To-Use (RTU) dropper bottles and provide a simple, nearly pipette-free workflow. Starting with properly prepared samples, sections are first pretreated, and then RNA-specific probes designed for different fluorescent detection channels are hybridized to multiple target RNAs. After a series of highly effective and specific signal amplifications, single RNA transcripts for two or more target genes appear as punctate dots in two or more distinctly fluorescent channels. These dots are visible using a common fluorescent microscope with the appropriate filters.

**Figure 1.** Procedure overview



1. Tissue section	2. Hybridize to target RNA	3. Amplify signal	4. Image
Start with properly prepared tissue sections and pretreat to allow access to target RNA.	Hybridize multiple sets of gene-specific probe pairs to target RNAs.	Use up to four signal amplification systems to detect multiple target RNAs. Probes are hybridized to a cascade of signal amplification molecules, culminating in binding of dye-labeled probes visible in different fluorescent channels.	Visualize target RNA using a standard fluorescent microscope.

## Compatible sample types

The RNAscope Plus smRNA-RNA Assay has been validated in formalin-fixed, paraffin-embedded (FFPE) and fresh frozen (FF) tissue. Contact technical support at [support.acd@bio-techne.com](mailto:support.acd@bio-techne.com) if you have any questions.

## Kit contents and storage

The RNAscope Plus smRNA-RNA Fluorescent Assay requires the RNAscope Plus smRNA-RNA Probes and the RNAscope Plus smRNA-RNA Fluorescent Reagent Kit. Probes and Reagent Kits are available separately.

## RNAscope Plus smRNA-RNA 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 smRNA or mRNA sequence and is detectable via channels S1, C2, C3, and C4 using an HRP amplification step. Different colors are assigned to the S1, C2, and C3, and C4 channel tags depending on the TSA Vivid™, Opal™ dye or TSA® plus fluorophore selected for that channel. S1 target probes are Ready-To-Use (RTU) at 1X, while C2, C3, and C4 probes are shipped as a 50X concentrated stock. To independently detect different target RNAs in a multiplex assay, each target probe must be in a different color channel and there must be a S1 probe in the mixture. Probe Diluent (Cat. No. 300041) can be used in place of a S1 probe.



Each probe is sufficient 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] – S1	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
	RNAscope Plus smRNA-RNA 4-plex Positive Control Probe	Various	RTU mixture of four probes <i>RNU6</i> (S1 channel), <i>POLR2A</i> (C2 channel), <i>PPIB</i> (C3 channel), <i>UBC</i> (C4 channel). <i>RNU6</i> is highly expressed and nuclear. <i>UBC</i> has the highest relative expression levels in cytoplasm, followed by <i>PPIB</i> and <i>POLR2A</i> in that order.	3 mL x 1 bottle	2–8°C
	RNAscope Plus smRNA-RNA 4-plex Negative Control Probe	323391	RTU probe targeting a scramble sequence in channel S1 and bacterial gene ( <i>dapB</i> ) in channel C2, C3 and C4. Each detection channel has its own negative control probe.	3 mL x 1 bottle	2–8°C
	Probe Diluent		RTU Target Probe diluent. Used in place of S1 target probe, if desired.	3 mL x 1 bottle	2–8°C





## RNAscope Plus smRNA-RNA Reagent Kit

Each RNAscope Plus smRNA-RNA Reagent Kit (Cat. No.322785) 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 three sub-kits: a Pretreatment Kit, a Detection Kit, and a Wash Buffer Kit.

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

RNAscope H2O2 and Protease Reagents (Cat No. 322381)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope Hydrogen Peroxide	3 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 Reagent (Cat. No. 322000)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope 10X Target Retrieval Reagent	70 mL x 4 bottles	Room temperature (15–30°C)
RNAscope Plus smRNA-RNA Detection Reagents (Cat. No. 322780)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope Plus smRNA-RNA HD AMP 1	4.5 mL x 1 bottle	2–8°C
	RNAscope Plus smRNA-RNA HD AMP 2	4.5 mL x 1 bottle	2–8°C
	RNAscope Plus smRNA-RNA HD AMP 3	4.5 mL x 1 bottle	2–8°C
	RNAscope Plus smRNA-RNA HD HRP-S1	4.5 mL x 1 bottle	2–8°C
	RNAscope Plus smRNA-RNA HD HRP-C2	4.5 mL x 1 bottle	2–8°C
	RNAscope Plus smRNA-RNA HD HRP-C3	4.5 mL x 1 bottle	2–8°C
	RNAscope Plus smRNA-RNA HD HRP-C4	4.5 mL x 1 bottle	2–8°C
	RNAscope Plus smRNA-RNA HD HRP Blocker	4.5 mL x 4 bottle	2–8°C
	RNAscope Plus smRNA-RNA HD DAPI	3 mL x 1 bottle	2–8°C
Wash Buffer Reagents (Cat. No. 310091)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	50X Wash Buffer	60 mL x 4 bottles	Room temperature (15–30°C)
RNAscope Multiplex TSA Buffer (Cat. No. 322809*)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope Multiplex TSA Buffer	29 mL x 1 bottle	2–8°C

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



## Required materials and equipment

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

### Recommended Fluorophores

The RNAscope Plus smRNA-RNA HD Reagent Kit (Cat. No. 322785) requires purchase of TSA Vivid from ACD or Opal dye or TSA plus fluorophores from Akoya Biosciences.

For simplicity, TSA Vivid dyes are recommended when working with a 3-Plex experimental design and Opal dyes are recommended when using a 4-Plex design. Choose a combination of fluorophores that will work best with your imaging system while considering native tissue autofluorescence.

#### 3-Plex assay

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

\* To reconstitute dyes, follow the manufacturer instructions available on the tube labels. Dilute the fluorophores in TSA buffer provided in the RNAscope Plus HD Reagent Kit.

#### 4- Plex assay

Fluorophores	Part number (Akoya Biosciences)*	Recommended dilution range
Opal 520 Reagent Pack	FP1487001KT	1:750–1:3000
Opal 540 Reagent Pack	FP1494001KT	1:750–1:3000
Opal 570 Reagent Pack	FP1488001KT	1:750–1:3000
Opal 620 Reagent Pack	FP1495001KT	1:750–1:3000
Opal 650 Reagent Pack	FP1496001KT	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

\* To reconstitute dyes, follow the manufacturer instructions available on the tube labels. Dilute the fluorophores in TSA buffer provided in the RNAscope Plus HD Reagent Kit. 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)

† We recommend keeping the dilution factors of Opal TSA-DIG and Opal Polaris 780 at a constant ratio. For example, when using 1:1500 dilution for Opal TSA-DIG, use 1:375 dilution for Opal Polaris 780. When using 1:750 dilution for Opal TSA-DIG, use 1:187.5 dilution for Opal Polaris 780.

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



## HybEZ Hybridization System

**IMPORTANT!** The RNAscope Plus smRNA-RNA Assay has been qualified using this system only.

Use the HybEZ™ 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/technical-support/user-manuals> 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.
	ACD HybEZ II Hybridization System (110 or 220V)	1 oven	321711 or 321721 (HybEZ II)
	ACD HybEZ Humidity Control Tray (with lid)	1 tray	310012
	ACDEZ-Batch Slide Holder (20 slide capacity)	1 rack	321716
	ACDEZ-Batch Wash Tray	1 tray	321717
	ACD HybEZ Humidifying Paper*	2 sheets	—

\* To order HybEZ Humidifying Paper Pack, 15 sheets, use Cat. No. 310015.

## Other materials

**IMPORTANT!** Do not substitute other materials for the SuperFrost® Plus Slides listed in the following table.

<input checked="" type="checkbox"/>	Description	Supplier	Cat. No.
	SuperFrost Plus Slides (required)	Fisher Scientific	12-550-15
	20X SSC	Fisher Scientific/MLS*	BP1325
	ImmEdge™ Hydrophobic Barrier Pen (required)	Vector Laboratories	H-4000
	100% alcohol (EtOH)	American Master Tech Scientific/MLS*	ALREACS
	Xylene	Fisher Scientific/MLS	X3P-1GAL
	Paraffin wax	MLS	—
	1X PBS	MLS	—
	Microtome	MLS	—
	Oster® Steamer Model 5712, Black and Decker Steamer HS3000, or the Hamilton Beach Steamer	—	—



☑	Description	Supplier	Cat. No.
	ProLong Gold Antifade Mountant	Thermo Fisher Scientific/MLS*	P36930
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWS2124
	Tissue-Tek Staining Dishes	American Master Tech Scientific/MLS	LWS20WH
	10% Neutral Buffered Formalin (NBF)/ Freshly prepared 4% Paraformaldehyde (PFA)/ 37% Formaldehyde	MLS  Sigma	—  252549-1L
	Cover glass 24 x 50 mm	Fisher Scientific/MLS	12-545-F
	Carboy (>3L)	MLS	—
	Drying oven, capable of holding temperature at 60 +/- 1°C (optional)	MLS	—
	Water bath or incubator, capable of holding temperature at 40 +/- 1°C	MLS	—
	Distilled water	MLS	—
	Tubes (various sizes)	MLS	—
	Paper towel or absorbent paper	MLS	—
	Fluorescent microscope with filter set appropriate for fluorophores selected for labeling the probes (e.g. Vivid 520/Vivid 570/Vivid 650).	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"> <li>Leica DM series or equivalent</li> <li>Zeiss Axio Imager or equivalent</li> <li>Inverted microscope, if optics and condenser meet requirements</li> <li>Required excitation/emission filter cube: DAPI/FITC/Cy3/Cy5/Cy7 (if Opal Polaris 780 is used)</li> </ul>	<ul style="list-style-type: none"> <li>20X (N.A. 0.75) air</li> <li>40X (N.A. 0.8) air (recommended)</li> <li>40X (N.A. 1.3) oil</li> <li>63X (N.A. 1.3) oil – use for low expression targets, if needed</li> <li>Use 20X and 40X to visualize high expression genes and low expression genes, respectively</li> </ul>	<ul style="list-style-type: none"> <li>Microscope with camera for fluorescence capturing. Multispectrum microscope/camera system (eg. Nuance® EX, Mantra, Vectra and Polaris) recommended for imaging tissue with high autofluorescence.</li> <li>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 &gt; 65% peak quantum efficiency. Common models include Orca-Flash 4.0 (Hamamatsu) and Nuance® EX (Perkin Elmer).</li> </ul>

### Image the slides

For imaging using multiplexed biomarker imaging systems Vectra®, Mantra™ or Polaris, refer to the guidelines from Akoya. The following table lists the corresponding filter settings for each fluorophore.

Opal fluorophore	Filter setting	TSA Vivid fluorophore	Filter setting
Opal 520	FITC	TSA Vivid Fluorophore 520	FITC
Opal 570	Cy3	TSA Vivid Fluorophore 570	Cy3
Opal 620	Texas Red	TSA Vivid Fluorophore 650	Cy5
Opal 690	Cy5.5		
Opal Polaris 780	Cy7		

## Chapter 2. Before You Begin

### Important procedural guidelines

- Start with properly fixed and prepared sections. Refer to Error! Reference source not found..
- Always run positive and negative control probes on your sample to assess sample RNA quality and optimal permeabilization.
- Do *not* substitute required materials. 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 E. Safety** for more information.

# Chapter 3. Prepare and Pretreat Samples

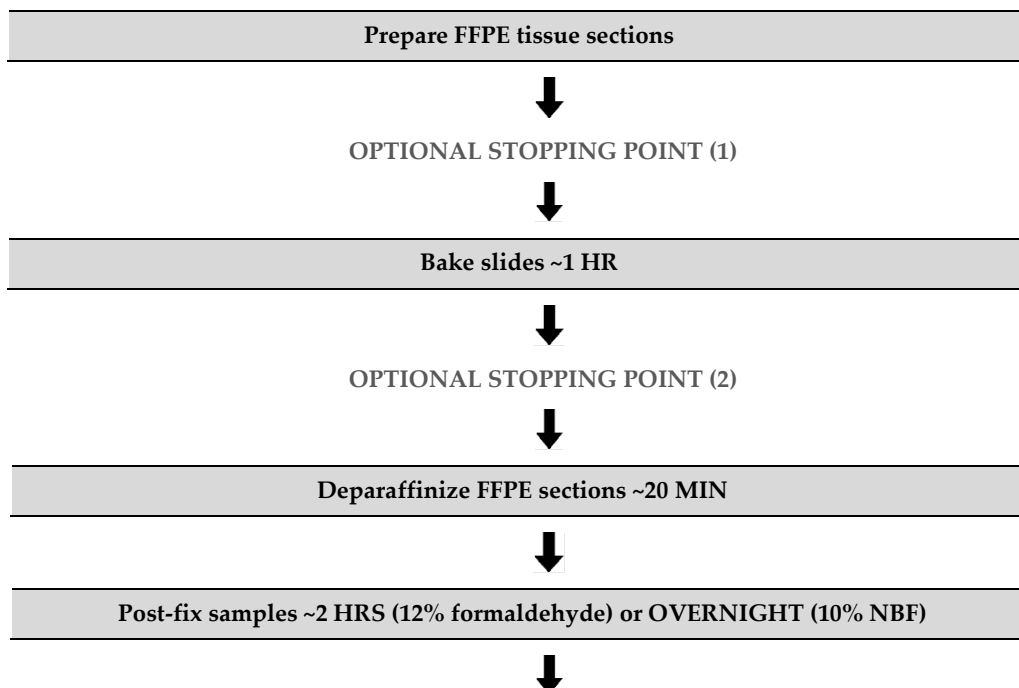
This chapter describes two tissue sample preparation methods: formalin-fixed, paraffin-embedded (FFPE) sample preparation and pretreatment and fresh-frozen sample preparation and pretreatment. For other sample types and preparation methods, contact [support.acd@bio-techne.com](mailto:support.acd@bio-techne.com) for the latest protocols and guidelines.

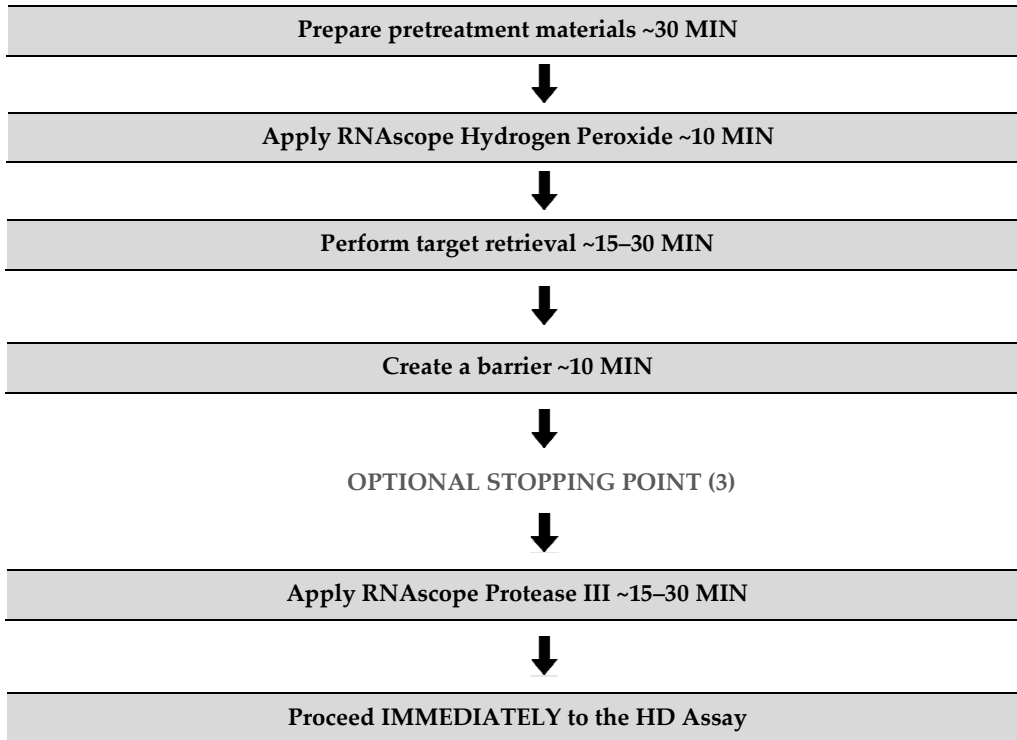
**IMPORTANT!** We highly recommend following these guidelines. We cannot guarantee assay results with other preparation methods.

## 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









## Materials required

Materials provided by ACD (Cat. No. 322381 and 322000)	Other Materials and Equipment
<ul style="list-style-type: none"> <li>• RNAscope Hydrogen Peroxide</li> <li>• RNAscope Protease III or Protease IV (depending on sample-specific conditions)</li> <li>• RNAscope 10X Target Retrieval Reagents</li> </ul>	<ul style="list-style-type: none"> <li>• 10% Neutral Buffered Formalin (NBF) or 37% Formaldehyde</li> <li>• 1X PBS</li> <li>• Paraffin wax</li> <li>• Tissue-Tek Clearing Agent Dishes</li> <li>• Tissue-Tek Staining Dishes</li> <li>• Tissue-Tek Vertical 24 Slide Rack</li> <li>• 100% alcohol (EtOH)</li> <li>• Xylene</li> <li>• Microtome</li> <li>• Water bath</li> <li>• SuperFrost Plus slides</li> <li>• ImmEdge™ Hydrophobic Pen</li> <li>• Drying oven</li> <li>• Distilled water</li> <li>• Fume hood</li> <li>• HybEZ Humidifying System/ACD EZ-Batch Slide Holder and Wash Tray</li> <li>• Paper towel or absorbent paper</li> <li>• Steamer</li> <li>• Digital thermometer</li> </ul>

## 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 assay.

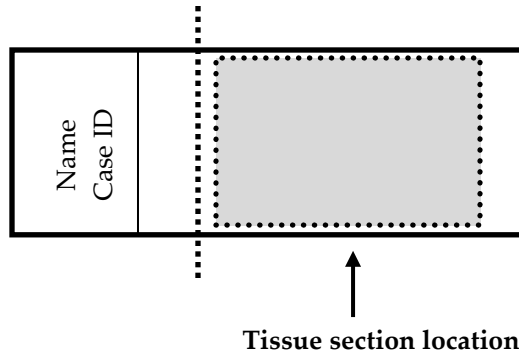
2. Wash sample with 1X PBS.
3. Dehydrate sample using a standard ethanol series, followed by xylene.

**IMPORTANT!** Use fresh reagents.

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).** You can store sections with desiccants at room temperature. Use sectioned tissue within three months.

---

## Bake slides

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  $\leq 1$  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).

## Post-fix samples

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**IMPORTANT!** For optimal performance of the RNAscope Plus smRNA-RNA Assay, you must post-fix your samples. Choose one of the following procedures:

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### Post-fixation with NBF

1. In a fume hood, fill a Tissue-Tek Clearing Agent dish with ~200 mL fresh 10% NBF.
2. Place slides in a Tissue-Tek Slide Rack and submerge them in the 10% NBF.
3. Incubate slides **OVERNIGHT (16–18 HRS)** at **RT**.
4. Remove slides from the 10% NBF and wash them for **2 MIN** in distilled water.
5. Dry slides for **5 MIN** at **60°C** or until completely dry.

### Post-fixation with formaldehyde

1. In a fume hood, prepare 12% formaldehyde in a Tissue-Tek Clearing Agent dish by combining 65 mL fresh 37% formaldehyde to 135 mL of 1X PBS.
2. Place slides in a Tissue-Tek Slide Rack and submerge them in the 12% formaldehyde.
3. Incubate slides for **2 HRS** at **RT**.
4. Remove slides from the 12% formaldehyde and wash them for **2 MIN** in distilled water.
5. Dry slides 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 200 mL of fresh RNAscope 1X Target Retrieval Reagents by adding 180 mL distilled water to 20 mL 10X Target Retrieval Reagents. Mix well.

## 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 an alternate method, see **Appendix B. Manual Target Retrieval**. For each steamer, fill the water to the maximum level before starting and do not refill water during the steaming process.

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

---

**IMPORTANT!** Do not overfill.

---



2. Place two slide holders (we recommend Tissue-Tek containers) in the steam bowl. Fill one slide holder with 200 mL of RNAscope 1X Target Retrieval Reagent. Fill the other slide holder with 200 mL of distilled H<sub>2</sub>O.
3. Turn on the steamer. Set the heating time to the maximum so the steamer does not shut off during Target Retrieval.
4. Insert a digital thermometer through one of 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.

**Note:** To achieve a consistent temperature, you may need to seal the rest of the holes with tape.

5. Add the slides to the container containing distilled H<sub>2</sub>O for **10 SEC** to acclimate the slides.
6. Remove the slides and move them to the container containing RNAscope 1X Target Retrieval Reagent. Cover the steamer with the lid.
7. 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**.
8. Immediately remove the slides from the steamer and transfer to a separate rinse container with 200 mL of room temperature distilled water. Allow the slides to rinse for **15 SEC**.
9. Transfer the slides to 100% ethanol for **3 MIN**.
10. Dry the slides in a **60°C** incubator (or at RT) for **5 MIN**.

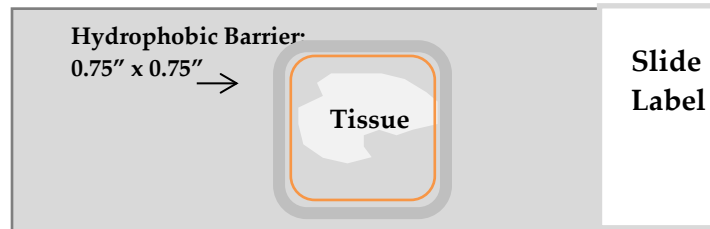
## Create a barrier

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 **~10 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 III

1. The following procedure describes standard pretreatment conditions. You may need to optimize conditions based on tissue type and sample preparation.
2. Load the dry slides into the ACD EZ-Batch Slide Holder by opening the swing clamp (see **Appendix D** for details) then lock slides in place. Add ~5 drops or enough RNAscope Protease III to entirely cover each section.
3. 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.
4. 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.

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

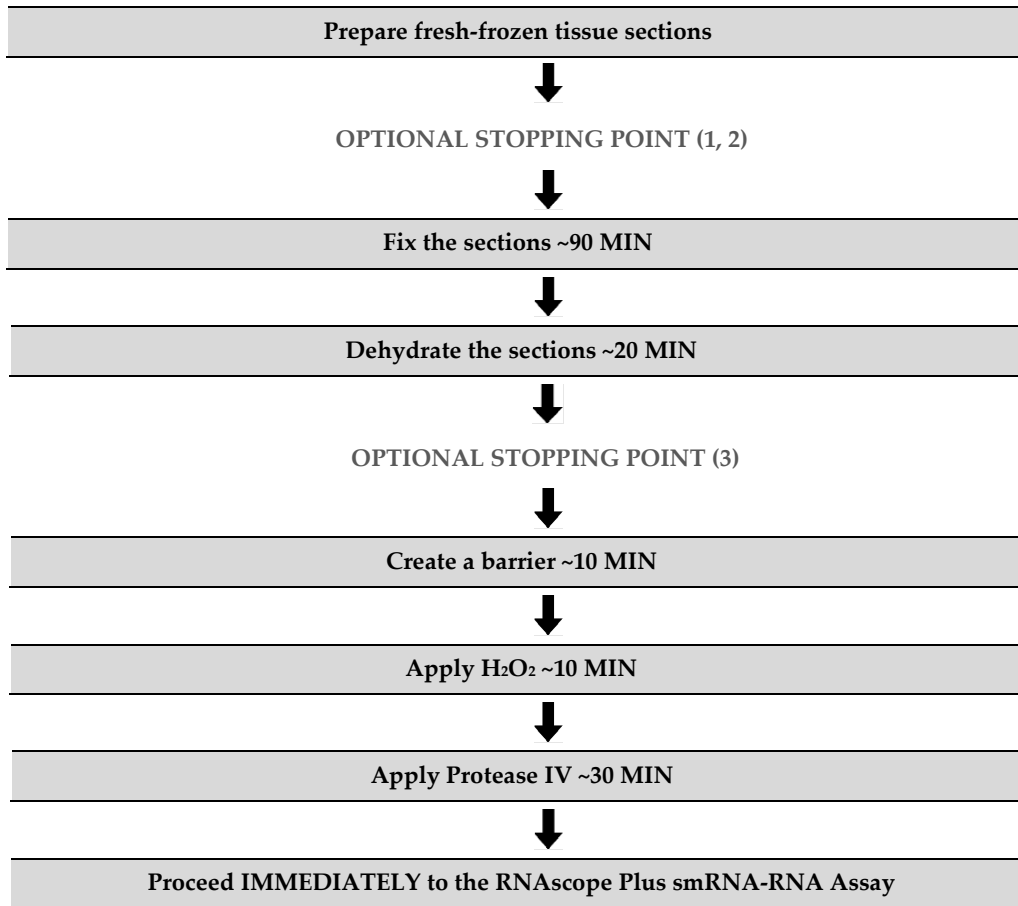
## Proceed to the RNAscope Assay

Proceed *immediately* to the **Chapter 4. RNAscope Plus smRNA-RNA HD Assay.**



## Fresh-frozen sample preparation and pretreatment

### Workflow



### Materials required

Materials provided by ACD (Cat. No. 322381)	Other Materials and Equipment
<ul style="list-style-type: none"><li>• RNAscope Hydrogen Peroxide</li><li>• RNAscope Protease III or Protease IV (depending on sample-specific conditions)</li></ul>	<ul style="list-style-type: none"><li>• 10% Neutral Buffered Formalin (NBF) or freshly-prepared 4% Paraformaldehyde (PFA)</li><li>• 1X PBS</li><li>• Tissue-Tek Staining Dishes</li><li>• Tissue-Tek Vertical 24 Slide Rack</li><li>• 100% alcohol (EtOH)</li></ul>



## Prepare fresh-frozen tissue sections

1. Remove tissue and cut to fit into cryomolds.
2. Freeze the specimen on dry ice or in liquid nitrogen, isopentane, or 2-methyl butane within **5 MIN** of tissue harvest.
3. Embed the frozen tissue in cryo-embedding medium (OCT) or Tissue Freezing Medium (TFM):
  - 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).** Embedded tissue may be stored for up to three months.

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5. Section the block:
  - e. Equilibrate block to **-20°C** in a cryostat **~1 HR**.
  - f. Cut 10–20  $\mu\text{m}$  thick sections and mount onto **SUPERFROST PLUS SLIDES**.
  - g. 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.

---

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

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**OPTIONAL STOPPING POINT (2).** Sections may be stored for up to three months.

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## Fix the sections

1. Prepare 10% NBF (10% NBF or freshly made 4% PFA in 1X PBS).

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**IMPORTANT!** Use **FRESH** fixatives. Do **NOT** reuse.

---

2. Remove the slides from **-80°C** and place in a slide rack or holder.
3. *Immediately* immerse slides in fresh 10% NBF or 4% PFA in 1X PBS fixative. Fix for **~90 MIN** at **RT**.
4. Wash slides with 1X PBS by moving the rack up and down 3–5 times and repeat with fresh 1X PBS.

---

**IMPORTANT!** Do **NOT** use 10% NBF that has been stored for more than six months, exposed to air for more than a week, or used repeatedly. This can result in suboptimal tissue 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 2X 200 mL 100% ethanol in Tissue Tek Staining Dishes.
2. Place the slides in 50% ethanol for **5 MIN** at **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. Remove slides from ethanol, and let them dry for **5 MIN** at **RT**.

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**OPTIONAL STOPPING POINT (2).** Slides may be stored in 100% ethanol at **-20°C** for up to **1 WEEK**. Prolonged storage may degrade sample RNA.

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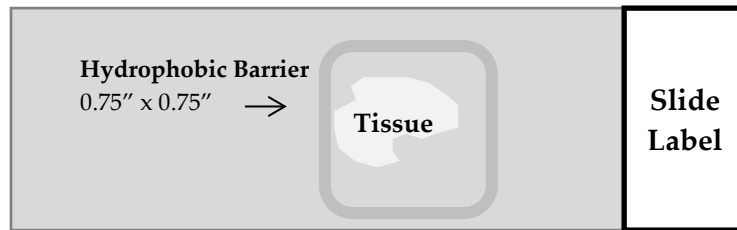
## Create a hydrophobic barrier

1. Take the 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 A. 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 result in suboptimal results.

---



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

3. Let the barrier dry completely **~10 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

0. Load the dry slides into the ACD EZ-Batch Slide Holder by opening the swing clamp (see **Appendix D** for details) then lock slides in place.
2. Add ~5–8 drops of RNAscope Hydrogen Peroxide to cover each section and incubate slides for **10 MIN** at **RT**.
3. Pour at least 200 mL distilled water into the transparent ACD EZ-Batch Wash Tray.
4. Place the ACDEZ-Batch Slide Holder into the ACD EZ-Batch Wash Tray. Make sure all the slides are submerged. If needed, carefully add more water.
5. Wash the slides with slight agitation.
6. Repeat the wash step with fresh distilled water.

## Apply Protease IV

1. Remove excess distilled water from the slides by decanting and shaking the locked slides in the ACD EZ-Batch Slide Holder.





2. Add ~5 drops of Protease IV or enough to entirely cover each section.
3. Incubate for **30 MIN** at **RT** on the bench.
4. Remove excess liquid from the slides by decanting and shaking the locked slides in the ACD EZ-Batch Slide Holder. Immediately place the slide holder in the transparent EZ-Batch Wash Tray filled with 1X PBS.
5. Wash slides in 1X PBS with slight agitation and repeat with fresh 1X PBS.

## **Proceed to the RNAscope Assay**

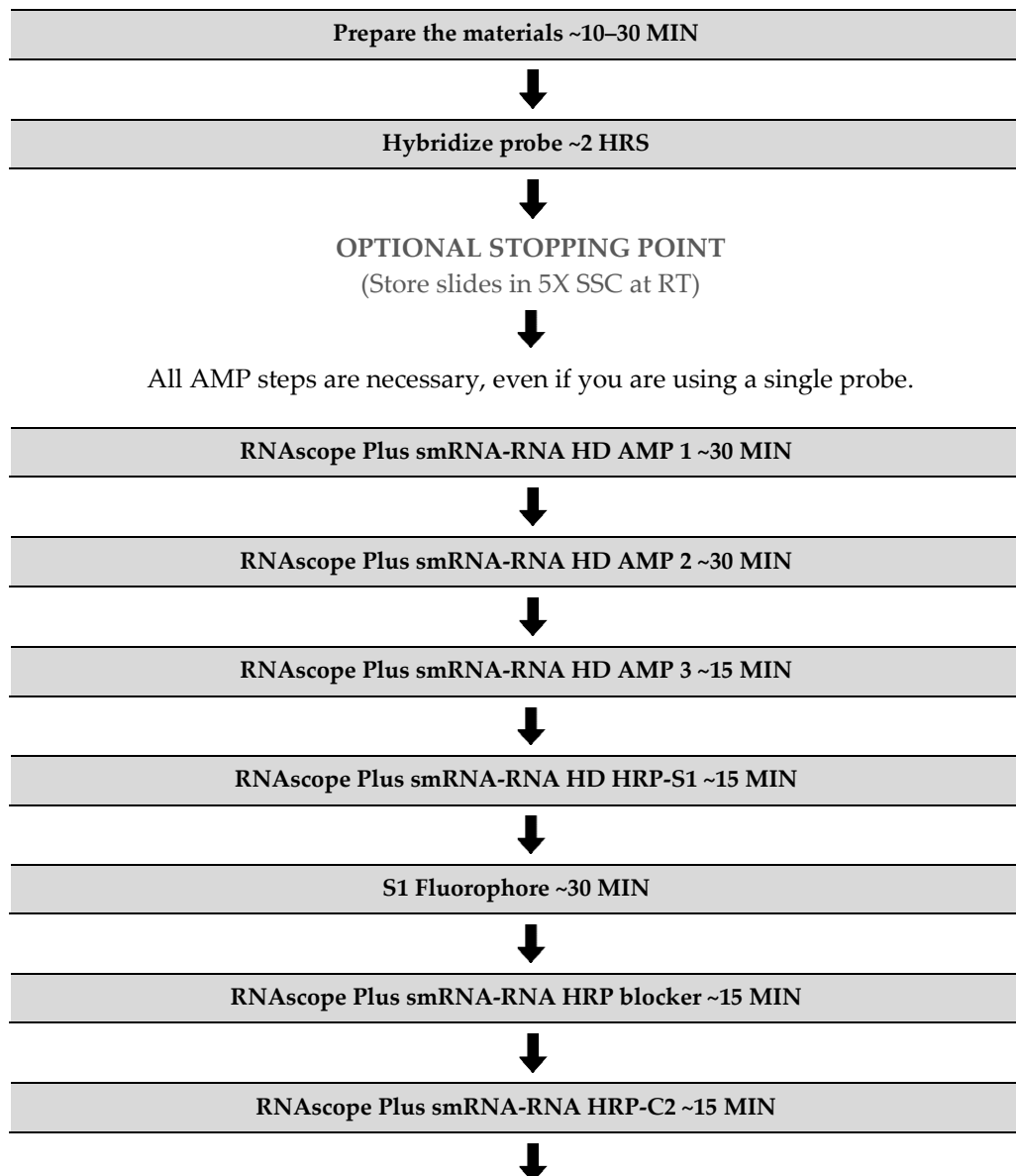
Proceed *immediately* to the **Chapter 4. RNAscope Plus smRNA-RNA HD Assay**.

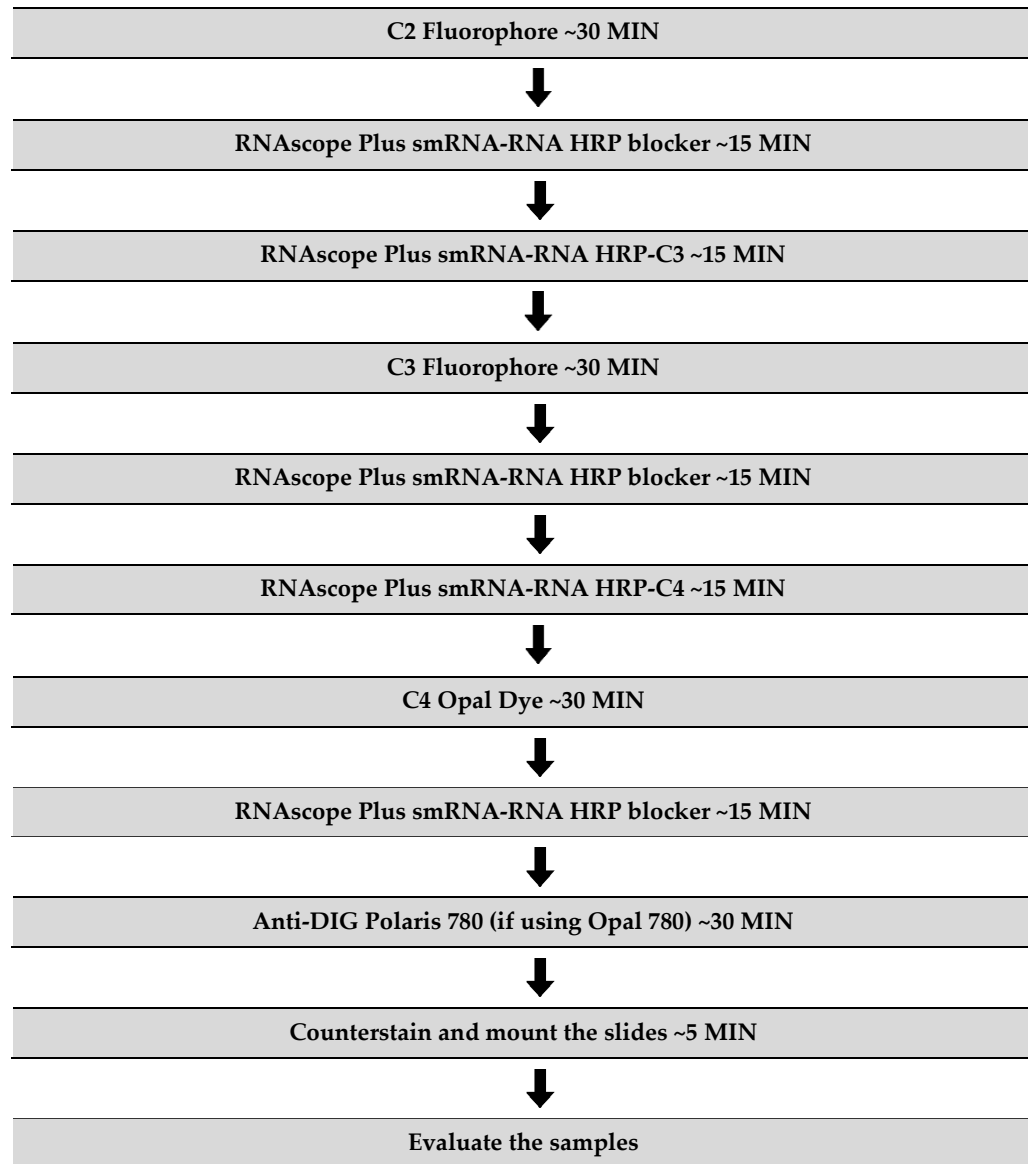
# 4

## Chapter 4. RNAscope Plus smRNA-RNA HD 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 or technical note for your specific sample type.

### Workflow







## Materials required for the assay

Materials provided by the RNAscope Plus smRNA-RNA HD Kit	Materials provided by RNAscope Plus smRNA-RNA Probes	Other materials and equipment
<ul style="list-style-type: none"> <li>• 50X Wash Buffer</li> <li>• RNAscope Plus smRNA-RNA HD AMP 1</li> <li>• RNAscope Plus smRNA-RNA HD AMP 2</li> <li>• RNAscope Plus smRNA-RNA HD AMP 3</li> <li>• RNAscope Plus smRNA-RNA HD HRP-S1</li> <li>• RNAscope Plus smRNA-RNA HD HRP-C2</li> <li>• RNAscope Plus smRNA-RNA HD HRP-C3</li> <li>• RNAscope Plus smRNA-RNA HD HRP-C4</li> <li>• RNAscope Plus smRNA-RNA Blocker</li> <li>• DAPI</li> </ul>	<ul style="list-style-type: none"> <li>• S1 Ready to Use Target Probe</li> <li>• 50X C2 Target Probe</li> <li>• 50X C3 Target Probe</li> <li>• 50X C4 Target Probe</li> </ul>	<ul style="list-style-type: none"> <li>• Prepared sections</li> <li>• Distilled water</li> <li>• Carboy (&gt;3L)</li> <li>• TSA Vivid/Opal or TSA Plus fluorophores</li> <li>• Antibody Diluent/Block (only for Opal 780)</li> <li>• Tissue-Tek Staining Dish</li> <li>• HybEZ Humidifying System/ RNAscope EZ-Batch Slide Holder and Wash Tray</li> <li>• Water bath or incubator</li> <li>• Tissue-Tek Vertical 24 Slide Rack</li> <li>• Tubes (various sizes)</li> <li>• Paper towel or absorbent paper</li> <li>• ProLong Gold Antifade Mountant</li> <li>• Cover Glass, 24 mm x 50 mm</li> </ul>

## Prepare the materials

You may prepare the reagents at the same time you prepare pretreatment reagents. Some of the materials may be prepared in advance and stored at room temperature.

### Prepare 1X Wash Buffer

1. Prepare 3 L of **1X Wash Buffer** by adding 2.94 L distilled water to 1 bottle (60 mL) 50X Wash Buffer in a large carboy. Mix well.

**Note:** If precipitation occurs in 50X Wash Buffer, warm it up at 40°C for 10–20 MIN before making 1X Wash Buffer. 1X Wash Buffer may be prepared ahead of time and stored at room temperature for up to one month.

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

0. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800ml of distilled H<sub>2</sub>O.
1. Adjust the pH to 7.0 with a few drops of 1M HCl.
2. Adjust the volume to 1 L with additional distilled H<sub>2</sub>O.
3. Sterilize by autoclaving.

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



## Prepare probes

1. Briefly spin the C2, C3 and C4 probes to collect the liquid at the bottom of the tubes.
2. Mix 1:1:1:50 ratios of C2, C3, and C4 probes by pipetting 1 volume of C2 and 1 volume of C3 probes to 50 volumes of S1 probe into a tube. Invert the tube several times.
3. If using only the C2 or C3 probe, dilute the probe with probe diluent). Probe diluent can be purchased separately.

**Note:** Do not mix probes of the same channel. The mixed Target Probes can be stored at 2–8°C for up to six months.

## Equilibrate reagents

- Remove smRNA-RNA HD AMP1, AMP2, AMP3, HRP-S1, HRP-C2, HRP-C3, HRP-C4 and HRP-blockers from the refrigerator and place at RT to equilibrate before use.
- 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.

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

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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
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



Recommended Fluorophore	Compatible Fluorophore	Recommended dilution range*	Dilute in
	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	Akoya Biosciences 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

## Run the assay

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

**IMPORTANT!** We recommend running control probe on your sample before running any of your specific target probes to optimize the protocol.

**Note:** View the wash step video at [www.acdbio.com/technical-support/learn-more](http://www.acdbio.com/technical-support/learn-more) before proceeding.

## Hybridize probe

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

**Note:** Refer to **Appendix A. Reagent Volume Guidelines** determine the recommended number of drops needed per slide. For example, add 4 drops of the appropriate probe for a 0.75" x 0.75" barrier.

3. Close the metal tray with its lid and insert into the oven for **2 HRS** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
5. Remove the metal HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
6. Place the slide holder into the wash tray (see **Appendix D** for details), and wash the slides for **2 MIN** at **RT**.
7. Repeat the wash step with fresh 1X Wash 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 twice with 1X Wash Buffer for **2 MIN**.



## Hybridize RNAscope Plus smRNA-RNA HD AMP 1

1. Remove excess liquid from the slides while keeping the slides locked in the RNAscope EZ-Batch Slide holder. Insert the slide holder into the metal HybEZ Humidity Control Tray.
2. Add enough RNAscope Plus smRNA-RNA HD **AMP 1** to entirely cover the tissue on each slide (e.g. ~4 drops for a 0.75" x 0.75" barrier).
3. Close the metal tray with its lid and insert into the HybEZ Oven for **30 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent RNAscope EZ-Batch Wash Tray.
5. Remove the metal HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
6. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
7. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.

## Hybridize RNAscope Plus smRNA-RNA HD AMP 2

1. Remove excess liquid from the slides while keeping the slides locked in the slide holder. Insert the slide holder into the metal tray.
3. Add enough RNAscope Plus smRNA-RNA HD **AMP 2** to entirely cover each tissue section.
2. Close the tray and insert into the HybEZ Oven for **30 MIN** at **40°C**.
3. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
4. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
4. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
5. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.

## Hybridize RNAscope Plus smRNA-RNA HD AMP 3

1. Remove excess liquid from the slides while keeping the slides locked in the slide holder. Insert the slide holder into the metal tray.
2. Add enough RNAscope Plus smRNA-RNA HD **AMP 3** to entirely cover each tissue section.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
5. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
6. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
7. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.

## Develop RNAscope Plus smRNA-RNA HD HRP-S1

1. Remove excess liquid from the slides while keeping the slides locked in the slide holder. Insert the slide holder into the metal tray.
2. Add enough RNAscope Plus smRNA-RNA **HRP-S1** to entirely cover each slide.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.



4. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
5. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
6. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
7. Repeat the wash step with fresh 1X Wash 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  $\mu\text{L}$  of diluted fluorophore of your choice to cover the tissue on each slide and incubate for **30 MIN** at **40°C**.

**Note:** Whichever fluorescent dye you apply here labels the -S1 probe with that color. We recommend far red dye (e.g. Opal 690) for the -S1 channel, but you can mix and match channels and fluorophores. Do not assign the same fluorophore to more than one channel. To label the -S1 probe with Opal 780, apply TSA-DIG at this step instead.

10. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
11. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
12. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
13. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.
14. Remove excess liquid from the slides, and insert the slide holder back into the humidity control tray.
15. Add enough RNAscope Plus smRNA-RNA **HRP Blocker** to entirely cover each slide.
16. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
17. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
18. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
19. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
20. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.

## Develop RNAscope Plus smRNA-RNA HD HRP-C2

**Note:** Make sure that you match color channels with HRP molecules. For example, if you are developing the C2 channel, use HRP-C2.

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 RNAscope Plus smRNA-RNA **HRP-C2** to entirely cover each slide.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
5. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
6. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.





7. Repeat the wash step with fresh 1X Wash 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  $\mu\text{L}$  of diluted fluorophore of choice to cover the tissue on each slide and incubate for **30 MIN** at **40°C**.

**Note:** You can mix and match channels and fluorophores. For example, you can assign Opal 690, Opal 620, Opal 570, or Opal 520 to the C2 channel. Do not assign the same fluorophore to more than one channel. To label the –C2 probe with Opal 780, apply TSA-DIG at this step instead.

10. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
11. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
12. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
13. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.
14. Remove excess liquid from the slides, and insert the slide holder back into the humidity control tray.
15. Add enough RNAscope Plus smRNA-RNA **HRP Blocker** to entirely cover each slide.
16. Close the tray and insert the slides into the HybEZ Oven for **15 MIN** at **40°C**.
17. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
18. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
19. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
20. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.

## Develop RNAscope Plus smRNA-RNA HD 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 RNAscope Plus smRNA-RNA **HRP-C3** to entirely cover each slide.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
5. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
6. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
7. Repeat the wash step with fresh 1X Wash 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  $\mu\text{L}$  of diluted Opal of choice to cover the tissue on each slide and incubate for **30 MIN** at **40°C**.

**Note:** You can mix and match channels and fluorophores. Do not assign the same fluorophore to more than one channel. To label the –C3 probe with Opal 780, apply TSA-DIG at this step instead.



10. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
11. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
12. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
13. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.
14. Remove excess liquid from the slides, and insert the slide holder back into the humidity control tray.
15. Add enough RNAscope Plus smRNA-RNA **HRP Blocker** to entirely cover each slide.
16. Close the tray and insert the slides into the HybEZ Oven for **15 MIN** at **40°C**.
17. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
18. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
19. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.

## Develop RNAscope Plus smRNA-RNA HD HRP-C4

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 RNAscope Plus smRNA-RNA **HRP-C4** to entirely cover each slide.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
5. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
6. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
7. Repeat the wash step with fresh 1X Wash 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  $\mu\text{L}$  of diluted fluorophore of choice to cover the tissue on each slide and incubate for **30 MIN** at **40°C**.

**Note:** You can mix and match channels and fluorophores. Do not assign the same fluorophore to more than one channel. To label the -C4 probe with Opal 780, apply TSA-DIG at this step instead.

9. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
10. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
11. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
12. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.
13. Remove excess liquid from the slides, and insert the slide holder back into the humidity control tray.
14. Add enough RNAscope Plus smRNA-RNA HRP Blocker to entirely cover each slide.
15. Close the tray and insert the slides into the HybEZ Oven for **15 MIN** at **40°C**.



16. Remove the metal tray from the oven. Remove the slide holder from the tray. Place the metal tray back into the oven.
17. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
18. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.

### Labeling with Opal 780 (optional)

1. Remove excess liquid from the slides, and insert the slide holder back into the humidity control tray.
2. Add enough  $\mu\text{L}$  of Anti-DIG Opal Polaris 780 diluted in double distilled  $\text{H}_2\text{O}$  to cover the tissue on each slide.

**Note:** 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.

3. Close the tray and insert into the HybEZ Oven for **30 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent wash tray.
5. Remove the metal tray from the oven. Remove the slide holder from the tray.
6. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation.
7. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.

### Counterstain and mount the slides

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

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1. Remove excess liquid from the slides, and add enough **DAPI** to each section to cover the tissue.
2. Incubate for **30 SEC** at **RT**.
3. Remove excess DAPI from the slides, and *immediately* place 1–2 drops of the ProLong Gold Antifade Mountant onto each section.
4. Carefully place a 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Let slides sit at RT, protected from light, to allow coverslips to dry (minimum 30 minutes; we recommend overnight).
6. Store slides in the dark at **2–8°C**.

---

**IMPORTANT!** Image the slides after eight hours or within a few days before fluorescence starts to fade.

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## Evaluate the samples

For an example of successful staining, see Figure 4 on page 40. Examine tissue sections under a standard fluorescent microscope at 20–40X magnification. A confocal microscope may also be used:

- Assess tissue and cell morphology.
- Assess positive control signal strength. Positive control signal should be visible as punctuate dots within cell with 20X magnification.
- Assess negative control background. Five dots in every 10 cells displaying background staining per microscope field is acceptable with 20X magnification.
- Evaluate target probe signal using the scoring guidelines in the next section.
  - 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.

## 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.

<b>TSA Vivid fluorophore</b>	<b>Filter setting</b>
TSA Vivid Fluorophore 520	FITC
TSA Vivid Fluorophore 570	Cy3
TSA Vivid Fluorophore 650	Cy5

<b>Opal fluorophore</b>	<b>Filter setting</b>
Opal 520	FITC
Opal 570	Cy3
Opal 690	Cy5.5
Opal Polaris 780	Cy7



## Fluorescent Imaging Recommendations

Here are a few fluorescent imaging recommendations:

Viewing	Detection	Microscope	Optics
<ul style="list-style-type: none"> <li>Image capture is the recommended digital capturing option</li> <li>Fluorescence viewing is the recommended viewing option</li> </ul>	<ul style="list-style-type: none"> <li>Microscope with camera and fluorescence options. Multispectrum microscope/camera system recommended (eg. Nuance FX)</li> <li>Fluorescence detection requires a high resolution and high sensitivity cooled CCD camera that is 64 <math>\mu\text{m}</math> pixel size or smaller with &gt; 65% peak quantum efficiency</li> <li>Common models include: Orca-Flash 4.0 (Hamamatsu), and Nuance FX (Nuance)</li> </ul>	<ul style="list-style-type: none"> <li>Leica DM series or equivalent</li> <li>Zeiss Axio Imager or equivalent</li> <li>Inverted microscope can be used if optics and condenser meet requirements</li> </ul>	<ul style="list-style-type: none"> <li>20X (N.A. 0.75) air, 40X (N.A. 0.8) air, 40X (N.A. 1.3) oil, 63X (N.A. 1.3) oil, and 100X (N.A. 1.4) oil</li> <li>20X and 40X objective can be used for visualization of high expression genes and low expression genes, respectively</li> </ul>

## Scoring Guidelines

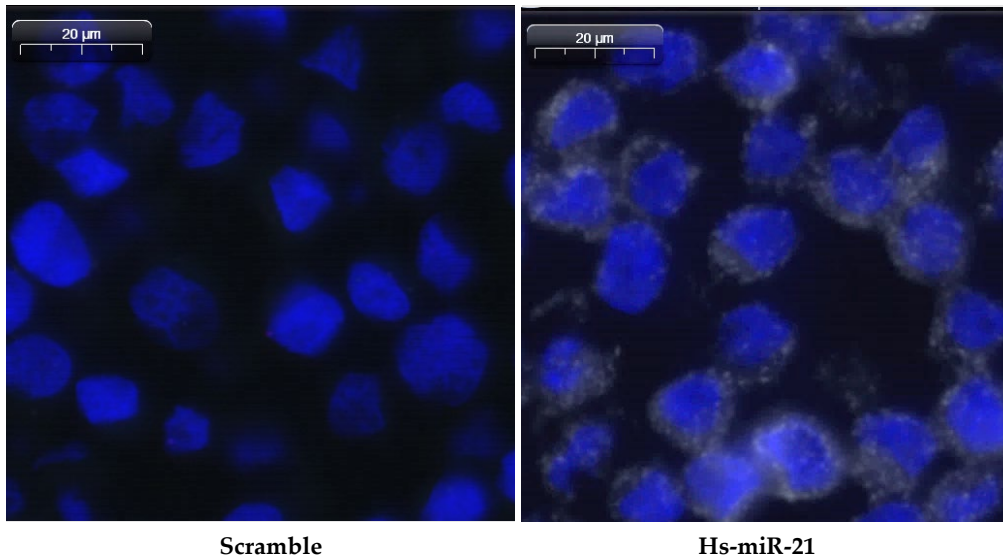
RNAscope Plus smRNA-RNA HD Assay enables a semi-quantitative scoring guideline utilizing the estimated number of punctate dots present within each cell boundary. miRNA and mRNA targets follow different scoring guidelines. An example of how to develop such a guideline for semi-quantitative assessment of miRNA and mRNA staining intensity is presented below for a gene with expression level varying between 1 to > 10 dots per cell.

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

**smRNA (e.g. siRNA, miRNA, etc.) scoring is divided into four categories:**

Staining Score	Microscope Objective Scoring*
0	No staining or less than 1 dot per cell
1	2–10 dots per cell, no or very few cell clusters
2	11–20 dots per cell and/or <25% dots are in clusters
3	>20 dots per cell and/or >25% dots are in clusters

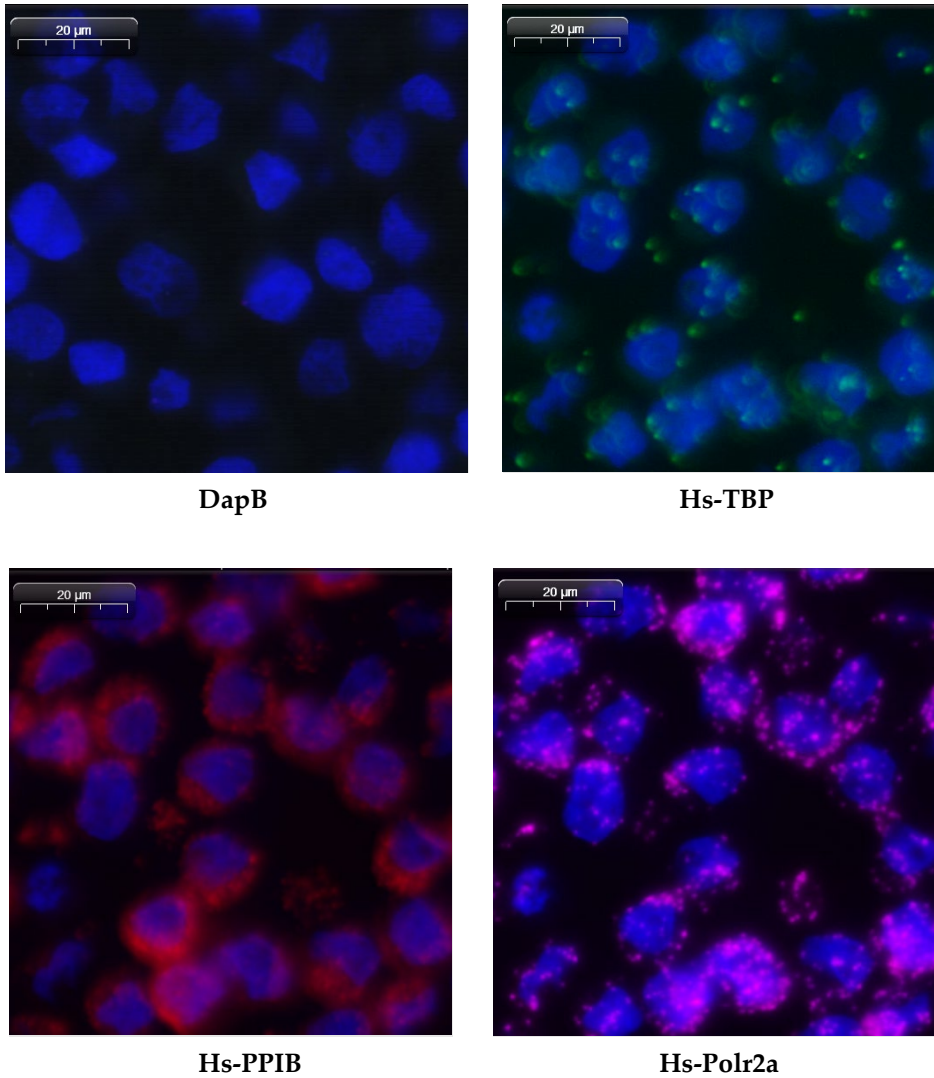
**Figure 2.** miRNA staining examples



**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

**Figure 3.** mRNA staining examples



## Troubleshooting

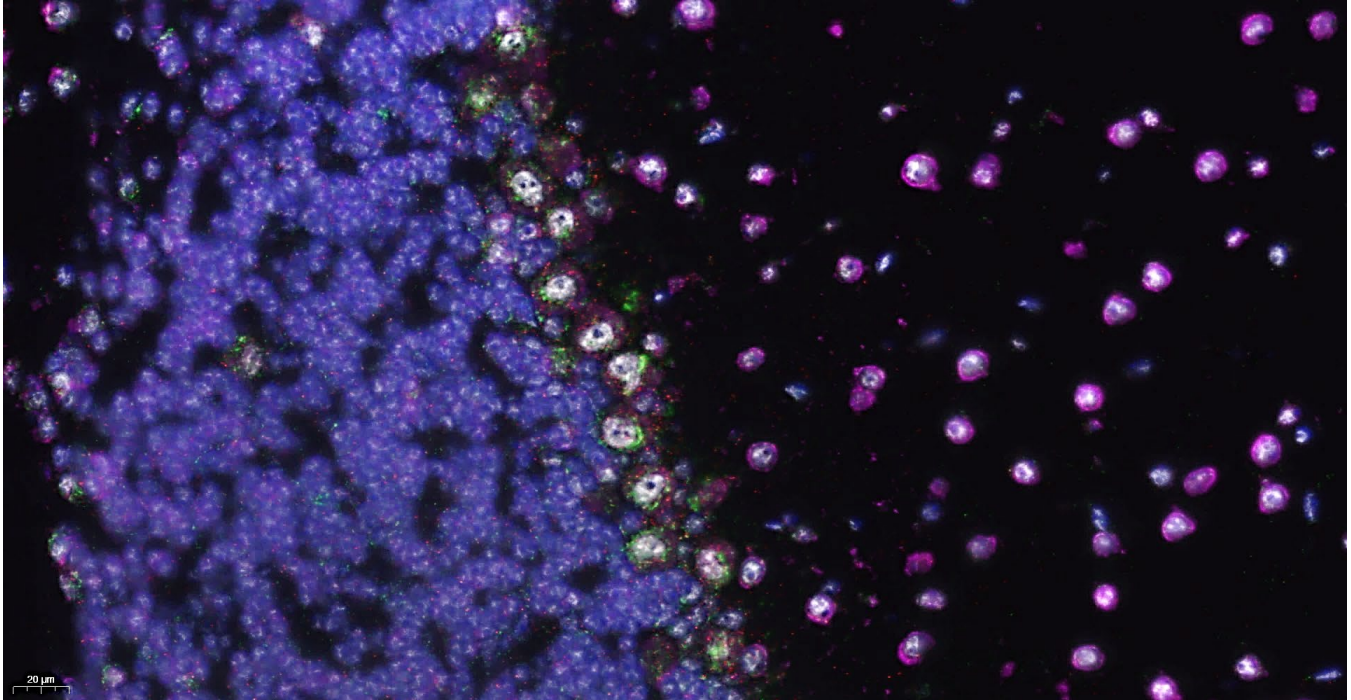
For troubleshooting information, please contact technical support at [support.acd@bio-technie.com](mailto:support.acd@bio-technie.com).



## Control example

If the assay is successful, the staining should look like the following image:

**Figure 4.** Mouse Brain at 40x with RNU6-S1(white), PPIB-C2(green), Polr2a-C3(red), and GAPDH-C4(pink) signal using RNAscope Plus smRNA-RNA HD assay.







# Appendix A. Tissue Pretreatment Recommendation

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

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 assay.

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

Reagent	Light	Mild	Standard	Extended
RNAscope Target Retrieval Reagents	15 MIN	15 MIN	15 MIN	15–30 MIN
RNAscope Protease III	15 MIN at RT	15 MIN at 40°C	30 MIN at 40°C	30 MIN at 40°C

**Note:** Some sample types, including certain xenografts and cell pellets, require less pretreatment time. For these tissue types, change the target retrieval time to **8 MIN** and use RNAscope Protease III for **15 MIN** at **RT**. For the ACD Cell Pellet sample, we recommend using the mild conditions listed in the table. If you have a tissue type not listed, contact support at [support.acd@bio-techne.com](mailto: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-technie.com](mailto:support.acd@bio-technie.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/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	Mild
	Lymph node	Normal	Mild
	Tonsil	Normal	Mild
	Pancreas	Normal	Standard
	Cervical	Cancer	Standard
	Cervical	Normal	Standard
	Cervical dysplasia	Abnormal	Standard
	Brain	Tumor	Standard
	Brain	Normal	Standard
	Head	Cancer	Standard
	Human	Neck	Cancer
Liver		Cancer	Standard
Kidney		Normal	Standard
Skin		Normal	Standard
Melanoma		Tumor	Standard



Species	Tissue Type	Pathology	Pretreatment Condition
	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	—	Light†
	HeLa cells fixed with 10% formaldehyde (ACD controls)	—	Mild

\* Tissue Microarray

† If Protease III does not give you good results, try Protease Plus instead.

# 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"> <li>RNAscope 10X Target Retrieval Reagents</li> </ul>	<ul style="list-style-type: none"> <li>Prepared slides</li> <li>Distilled water</li> <li>Glass beaker (1 or 2 L)</li> <li>Paper towel or absorbent paper</li> <li>Hot plate, isotemp brand</li> <li>Aluminum foil</li> <li>Thermometer</li> <li>Forceps, large</li> <li>Tissue Tek Slide Rack</li> <li>Tissue Tek Staining Dish</li> <li>ImmEdge Hydrophobic Barrier Pen</li> </ul>

### 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. Once the 1X RNAscope Target Retrieval Reagents reach 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 RNAscope 1X Target Retrieval Reagents solution. Cover the beaker with foil, and boil the slides for the amount of time specified by the table in **Appendix A. Tissue Pretreatment Recommendation**.






2. Use the forceps to *immediately* transfer the hot slide rack from the RNAscope 1X Target Retrieval Reagents to the staining dish containing distilled water. Do not let the slides cool in the Target Retrieval Reagents solution.
3. Wash slides 3–5 times by moving the Tissue-Tek Slide Rack up and down in the distilled water.
4. Wash slides in fresh 100% alcohol, and allow the slides to dry completely at **60°C** for **5 MIN**.
5. Draw the hydrophobic barrier, and continue with RNAscope Plus smRNA-RNA HD Assay.

# 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.

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.

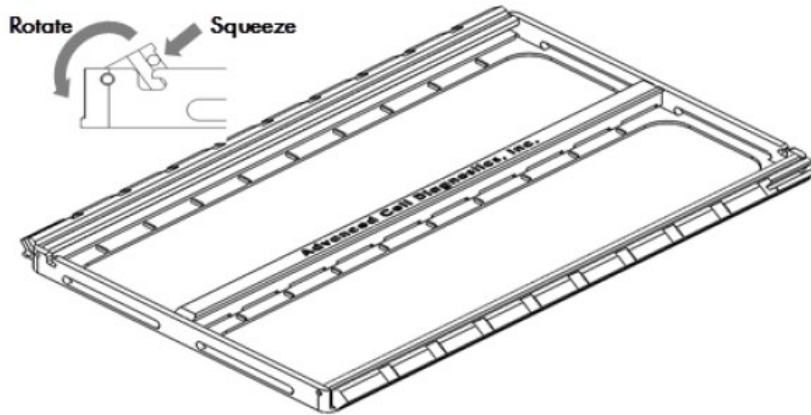
# D

## Appendix D. Using the EZ-Batch Slide Holder and Wash Tray

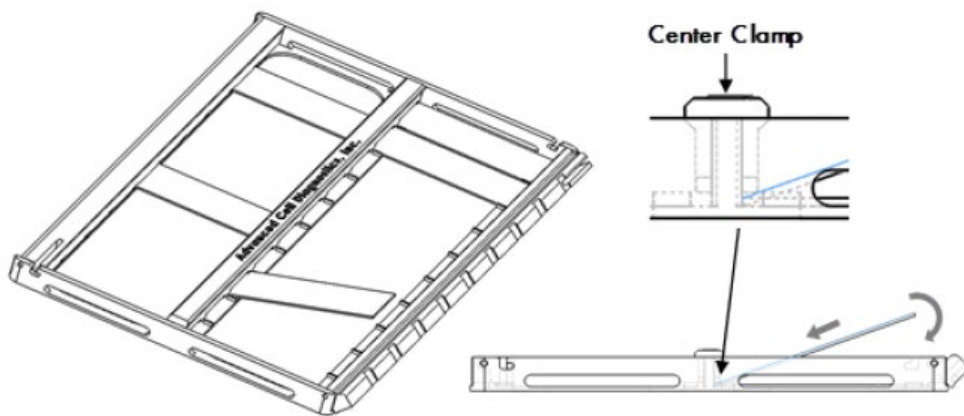
### Load the slides in the ACD EZ-Batch Slide Holder

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, as shown.

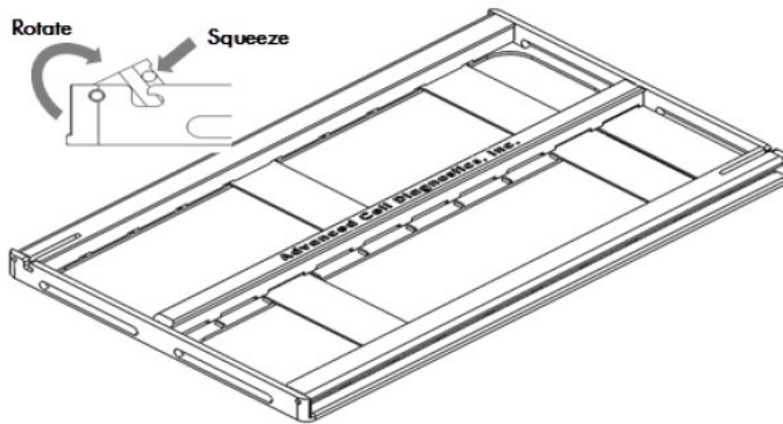


2. Insert the slides one at a time into the holder (up to 10 slides per column). The non-label end of each slide should be aligned toward the center of the holder and inserted under the fixed clamp, as shown. Place the rest of the slide down into the holder.



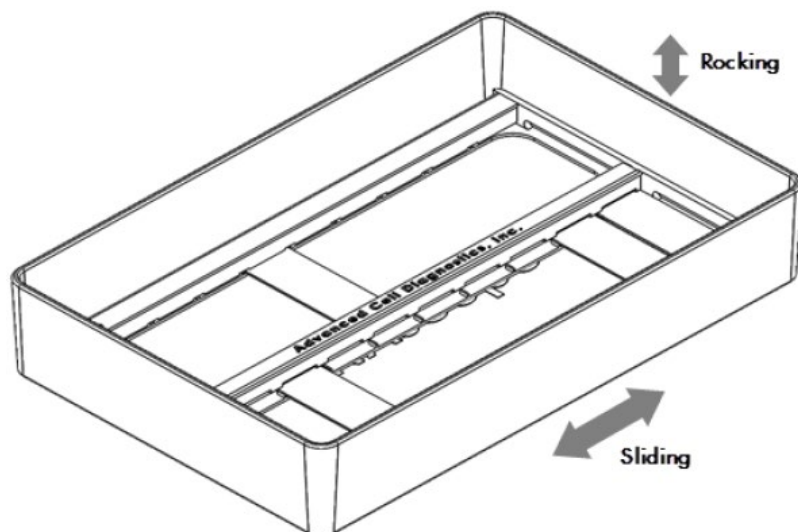


3. Close and lock the swing clamp of the column by simultaneously squeezing the slotted portion of each clamp and rotating it in then downwards in the direction opposite to the direction used to open the clamp, as shown.



## Wash slides in the Wash Tray

1. Place the ACD EZ-Batch Slide Holder into the clear plastic wash tray containing water. Make sure all the slides are submerged. If needed, carefully add more water. Wash the slides with slight agitation.





## 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](http://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/](http://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/user-manuals>. 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](mailto:info.acd@bio-techne.com)  
Orders: [orders.acd@bio-techne.com](mailto:orders.acd@bio-techne.com)  
Support Email: [support.acd@bio-techne.com](mailto: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](mailto:support.acd@bio-techne.com)  
[www.acdbio.com](http://www.acdbio.com)

