



**RNAscope™ HiPlex12 Reagent Kit v2 (488,  
550, 650) Assay**  
with Sample Preparation and Pretreatment

**Document Number** UM 324419



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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 HiPlex12 Reagent Kit v2 (488, 550, 650) (Cat. No. 324419) with fluorophores AF488, Dylight550, and Dylight650 on fresh-frozen sections, fixed-frozen sections, FFPE sections, and cultured adherent cells mounted on slides.

Visit [www.acdbio.com/technical-support/user-manuals](http://www.acdbio.com/technical-support/user-manuals) to download a sample preparation user guide or technical note for other sample types.

## Product description

### Background

The RNAscope HiPlex 12 Reagent Kit (488, 550, 650) uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize up to 12 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 multiplexed signal amplification systems, which enable users to investigate expression as well as positional relationship between multiple genes within a cellular context.

### Overview

RNAscope HiPlex Assay v2 reagents are provided in convenient Ready-To-Use dropper bottles allowing a simple, nearly pipette-free workflow. Properly prepared samples are first pretreated, and then RNA-specific probes designed for different detection tails/channels are hybridized to multiple RNAs (up to 12 RNA targets). After a series of highly effective and specific signal amplifications, single RNA transcripts for up to three target genes at a time can be visualized as punctate dots in three distinct fluorescent channels using cleavable versions of the fluorophores AF488, Dylight 550 and Dylight650. These dots are visible with an epifluorescence microscope and the appropriate filters. After imaging, the fluorophores from the first three targets are cleaved off and the next three targets are labeled and imaged. Images from the various rounds can be merged using the RNAscope HiPlex Registration Software v2.0 (refer to the *RNAscope HiPlex Image Registration Software User Manual* Doc. No. 300065-USM). The RNAscope HiPlex 12 Reagent (488, 550, 650; Cat. No. 324419) using fluorophores AF488, Dylight 550, and Dylight650 allows you to perform four rounds of fluorescent target labeling and imaging. The target probe hybridization, signal amplification, and the first round



of signal detection can be completed in 7–8 hours. Each subsequent round of signal detection requires an additional hour.

## Compatible sample types

The RNAscope HiPlex Assay v2 is compatible with fresh-frozen tissue, cultured adherent cells, fixed-frozen tissue, and FFPE tissue.

Use the guide below to determine the appropriate pretreatment reagent from the Universal Pretreatment Reagents (Cat No. 322380) or the RNAscope Protease III & IV Reagents (Cat. No. 322340) and RNAscope Target Retrieval Reagents (Cat. No. 322000).

RNAscope HiPlex Detection Pretreatment Guide		
Tissue Type	Pretreatment Kit	Pretreatment Cat. No.
Fresh frozen	RNAscope Protease IV	322336
Fixed frozen	RNAscope Target Retrieval (10X)	322000
	RNAscope Protease III	322337
FFPE	RNAscope Target Retrieval (10X)	322000
	RNAscope Protease III	322337
Cultured adherent cells	RNAscope Protease III	322337

In terms of relative strength, Protease III < Protease IV.

Please 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 HiPlex Assay v2 requires the RNAscope HiPlex Probes and the RNAscope HiPlex Reagent Kit. Probes and reagent kits are available separately.

## RNAscope HiPlex Probes

The RNAscope HiPlex 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. Visit <http://www.acdbio.com/control-slides-and-probes> to order appropriate control probes. Each target probe contains a mixture of short oligonucleotides designed to bind to a specific target RNA and is detectable in one of four color channels specified in the following table:

Detection (4 rounds)	Probe Tail/Channel	Fluorophore	Emission	Color
Round 1	T1	Alexa Fluor 488	520 +/- 10nm	Green
	T2	Dylight 550	562 +/- 10 nm	Orange
	T3	Dylight 650	652 +/- 10 nm	Far Red
Round 2	T4	Alexa Fluor 488	520 +/- 10nm	Green
	T5	Dylight 550	562 +/- 10 nm	Orange
	T6	Dylight 650	652 +/- 10 nm	Far Red
Round 3	T7	Alexa Fluor 488	520 +/- 10nm	Green



Detection (4 rounds)	Probe Tail/Channel	Fluorophore	Emission	Color
	T8	Dylight 550	562 +/- 10 nm	Orange
	T9	Dylight 650	652 +/- 10 nm	Far Red
Round 4	T10	Alexa Fluor 488	520 +/- 10nm	Green
	T11	Dylight 550	562 +/- 10 nm	Orange
	T12	Dylight 650	652 +/- 10 nm	Far Red

You can select different combinations of targets in the RNAscope HiPlex Assay v2. Each target probe must be assigned to a different probe channel/tail (T1–T12). All RNAscope HiPlex target probes are shipped as 50X concentrated stocks, which need to be diluted in RNAscope HiPlex Probe Diluent (Cat. No. 324301) 1:50.

**IMPORTANT!** Do not use RNAscope HiPlex probes for ANY other RNAscope assays.

Each probe is sufficient for staining ~10 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 Probe					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	Target Probe – [species] – [gene] – T1...T12	Various	50X probe	40 µL x 1 tube	2–8°C
Control Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope HiPlex12 Positive Control Probe - Mm v2	324433	RTU mixture of 12 probes targeting housekeeping gene Polr2a, PPIB, Ubc, Hprt, Actb, Sdha, Tfr, Ldha, Gapdh, Rpl5, Ywhaz, and Rpl28 with T1- T12 tails respectively in each of the 12 channels.	2 mL x 1 bottle	2–8°C





Control Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope HiPlex12 Positive Control Probe-Hs	324311	RTU mixture of 12 probes targeting housekeeping gene Polr2a, PPIB, UBC, HPRT1, TUBB, RPL28, RPL5, B2M, ACTB, LDHA-O1, RPLP0-X-RPLP0P2, and GAPDH with T1- T12 tails respectively in each of the 12 channels.	2 mL x 1 bottle	2–8°C
	RNAscope HiPlex12 Positive Control Probe - Rn v2	324434	RTU mixture of 12 probes targeting housekeeping gene Polr2a, PPIB, UBC, HPRT1, Actb, Sdha, Tfrc, Ldha, Gapdh, Rpl5, Ywhaz, and Rpl28 with T1- T12 tails respectively in each of the 12 channels.	2 mL x 1 bottle	2–8°C
	RNAscope HiPlex12 Negative Control Probe	324341	RTU probe targeting a bacterial gene (dapB), with T1- T12 tails respectively in each of the 12 channels.	2 mL x 1 bottle	2–8°C
	RNAscope HiPlex Probe Diluent	324301	RTU probe diluent	2 mL x 1 bottle	2–8°C

### RNAscope HiPlex Reagent Kit

Each RNAscope HiPlex Detection Kit 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. Please refer to the tables below for the contents of the sub-kits: Pretreatment Kit, Detection Kit, and Wash Buffer Kit etc.

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



Pretreatment Reagents (Cat. No. 322340)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope Protease III	4.5 mL x 1 bottle	2–8°C
	RNAscope Protease IV	4.5 mL x 2 bottles	2–8°C
Detection– RNAscope HiPlex12 Reagents Kit (488, 550, 650) v2 (Cat. No. 324419)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope HiPlex Amp 1	3 mL x 1 bottle	2–8°C
	RNAscope HiPlex Amp 2	3 mL x 1 bottle	2–8°C
	RNAscope HiPlex Amp 3	3 mL x 1 bottle	2–8°C
	RNAscope Fluoro T1–T3 v2	4.5 mL x 1 bottle	2–8°C
	RNAscope Fluoro T4–T6 v2	4.5 mL x 1 bottle	2–8°C
	RNAscope Fluoro T7–T9 v2	4.5 mL x 1 bottle	2–8°C
	RNAscope Fluoro T10–T12 v2	4.5 mL x 1 bottle	2–8°C
	RNAscope FFPE Reagent	200 µl x 1 tube	2–8°C

Wash Buffer Reagents (Cat. No. 310091)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope Wash Buffer Reagents (50X)	60 mL x 4 bottles	Room temperature (15–30°C)
Cleaving Reagents (Cat. No. 324399)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	RNAscope HiPlex Cleaving Stock Solution v2	1.5 mL x 5 ampoules	Room temperature (15–30°C)

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

## Required materials and equipment

The following materials and equipment are needed to perform the RNAscope HiPlex Assay v2.

### HybEZ Hybridization System

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

Use the HybEZ™ II Hybridization System to perform RNAscope HiPlex Assay v2 hybridization and incubation steps. These steps require humid conditions to prevent sections from drying out.



For instructions on how to use the HybEZ II Hybridization System, refer to the *HybEZ II Hybridization System User Manual* (Doc. No. 321710-USM) available at [www.acdbio.com/technical-support/user-manuals](http://www.acdbio.com/technical-support/user-manuals) and view the training video at [www.acdbio.com/technical-support/learn-more](http://www.acdbio.com/technical-support/learn-more). The system contains the following components:

<input checked="" type="checkbox"/>	Component	Quantity	Cat. No.
	HybEZ II Oven (110 or 220 VAC)	1 oven	321710 or 321720
	HybEZ Humidity Control Tray (with lid)	1 tray	310012
	ACD EZ-Batch™ Wash Tray		321717
	ACD EZ-Batch Slide Holder		321716
	HybEZ Humidifying Paper	2 sheets	—

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

### User-supplied materials

<input checked="" type="checkbox"/>	Description	Supplier	Cat. No.
	SuperFrost® Plus Slides (required)	Fisher Scientific	12-550-15
	ImmEdge™ Hydrophobic Barrier Pen (required)	Vector Laboratory	H-4000
	Xylene	Fisher Scientific/MLS	X3P-1GAL
	10% Neutral Buffered Formalin (NBF)/ 4% Paraformaldehyde (PFA)	MLS Sigma	— 252549-1L
	100% alcohol (EtOH)	American Master Tech Scientific/MLS*	ALREAGAL
	10X Phosphate Buffered Saline (PBS)	Fisher Scientific/MLS*	BP3991
	10% Tween	Fisher Scientific/MLS*	PI85115
	20X SSC	Fisher Scientific/MLS*	BP1325
	Tissue-Tek® Vertical 24 Slide Racks (or other slide racks or holders)	StatLab/MLS	LWS2124
	Tissue-Tek Staining Dishes (or similar containers)	StatLab/MLS	LWS20WH
	Paraffin wax	MLS	—
	Microtome	MLS	—
	Oster® Steamer Model 5712, Black and Decker Steamer HS3000, the Braun Multiquick FS 20 Steamer, or the Hamilton Beach Steamer	—	—
	Digital thermometer	MLS	—



☑	Description	Supplier	Cat. No.
	ProLong Gold Antifade Mountant	Fisher Scientific/MLS*	P36930
	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	—
	Distilled water	MLS	—
	Tubes (various sizes)	MLS	—
	Paper towel or absorbent paper	MLS	—
	Fluorescent microscope with filter set: Ex 358 nm/Em 461 nm (DAPI) Ex 501 nm/Em 523 nm (FITC) Ex 554 nm/Em 576 nm (Cy3) Ex 644 nm/Em 669 nm (Cy5) Ex 740 nm/Em 764 nm (Cy7)	MLS	—
	Aluminum foil (Optional)†	MLS	—
	Forceps, large (Optional)†	MLS	—
	Hot plate (Optional)†	MLS	—

\* Major Laboratory Supplier or preferred vendor in North America. For other regions, please check Catalog Numbers with your local lab supplier.

† Required for the alternate target retrieval method in **Appendix B** on page 47.

# 2

## Chapter 2. Before You Begin

Prior to running the RNAscope Assay on your samples for the first time, we recommend that you view the video demonstrations available at [www.acdbio.com/technical-support/learn-more](http://www.acdbio.com/technical-support/learn-more).

### Important procedural guidelines

- Start with properly prepared sections. Refer to our sample preparation and pretreatment user guides available at [www.acdbio.com/technical-support/user-manuals](http://www.acdbio.com/technical-support/user-manuals). Use only samples mounted on SuperFrost Plus® Slides (Fisher Scientific, Cat. No. 12-550-15).
- Follow the recommended pretreatment conditions for your sample. Refer to our sample preparation and pretreatment user guides available at [www.acdbio.com/technical-support/user-manuals](http://www.acdbio.com/technical-support/user-manuals).
- 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 unless otherwise indicated.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix E. Safety** for more information.

# Chapter 3. Prepare and Pretreat Samples

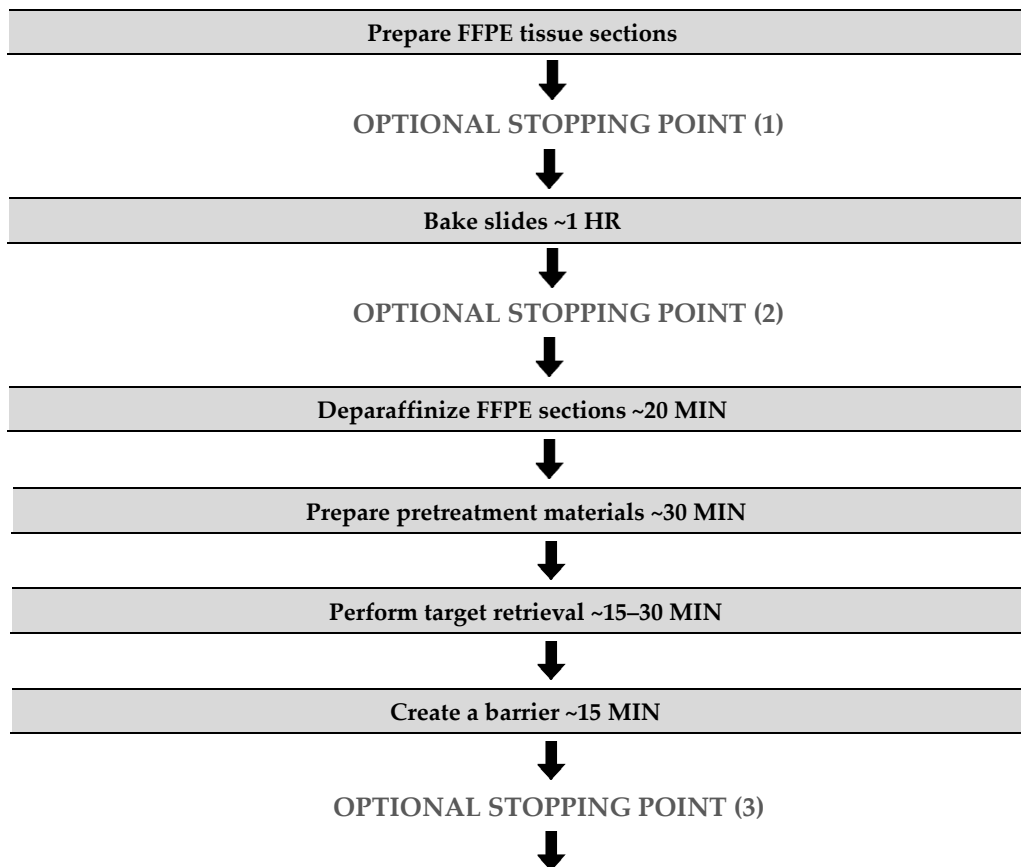
This chapter describes three tissue sample preparation methods: formalin-fixed, paraffin-embedded (FFPE) sample preparation and pretreatment, fixed-frozen sample preparation and pretreatment, and fresh-frozen sample preparation and pretreatment. For other sample types and preparation methods, contact [support.acd@bio-techne.com](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 C. Tissue Pretreatment Recommendation** on page 49 and to <https://acdbio.com/technical-support/solutions>.

### Workflow



Apply RNAscope Protease III ~15–30 MIN



Proceed IMMEDIATELY to the HiPlex Assay

## Materials required

Materials provided by Pretreatment Reagents (Cat. No. 322340 and 322000)	Other Materials and Equipment
<ul style="list-style-type: none"> <li>• RNAscope Target Retrieval Reagents</li> <li>• RNAscope Protease III</li> </ul>	<ul style="list-style-type: none"> <li>• 10% Neutral Buffered Formalin (NBF) or fresh 4% PFA</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.

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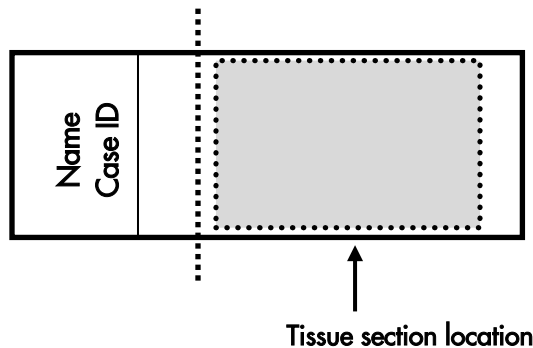
**IMPORTANT!** Use fresh reagents. Embed samples as quickly as possible to preserve RNA quality.

---

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.

---

1. 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 ≤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 (e.g. by adding 180 mL distilled water to 20 mL 10X Target Retrieval buffer). Mix well. Target Retrieval buffer must be heated to  $\geq 99^{\circ}\text{C}$  before use, but not boiled more than ~15min before use (see below “Perform target retrieval using the steamer”)

### Perform target retrieval using the steamer

---

**IMPORTANT!** Before you begin, make sure you know the pretreatment conditions specific to your sample type from **Appendix C. Tissue Pretreatment Recommendation** on page 49.

---

We highly recommend using a steamer for target retrieval. For target retrieval using a hot plate, see **Appendix B. Manual Target Retrieval** on page 47.

**Note:** For each steamer, fill the water to the maximum level before starting and do not refill water during the steaming process. Refilling water during steaming process will drop the temperature and interfere with Target Retrieval.

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

---

**IMPORTANT!** Do not overfill.

---



2. Place two slide holders 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 water.
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 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.
5. Add the slides to the container containing distilled water 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 C. Tissue Pretreatment Recommendation** on page 49.
8. Remove the slides from the steamer and transfer to a separate rinse container with 200 mL of distilled water (RT). 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**.

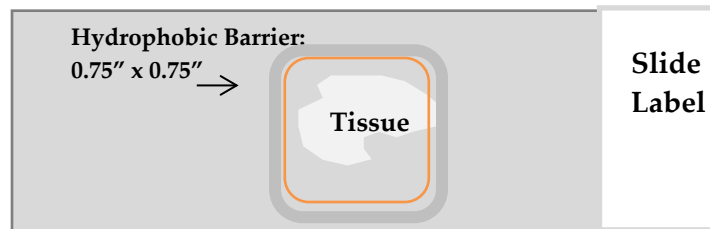
### Create a barrier

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.



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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. Load the dry slides into the ACDEZ-Batch Slide Holder by opening the swing clamp (see page 52 for detailed instructions).
2. Add ~5 drops of RNAscope Protease III to entirely cover each section.
3. Place the ACDEZ-Batch Slide Holder in the pre-warmed HybEZ Humidity Control Tray. Close the lid, seal, and insert the tray back into the oven.
4. Incubate at 40°C for the amount of time specified by the table in **Appendix C. Tissue Pretreatment Recommendation** on page 49.

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

5. Pour at least 200 mL distilled water into the transparent ACDEZ-Batch Wash Tray.
6. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
7. Place the ACDEZ-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.
8. Repeat the wash step with fresh distilled water.

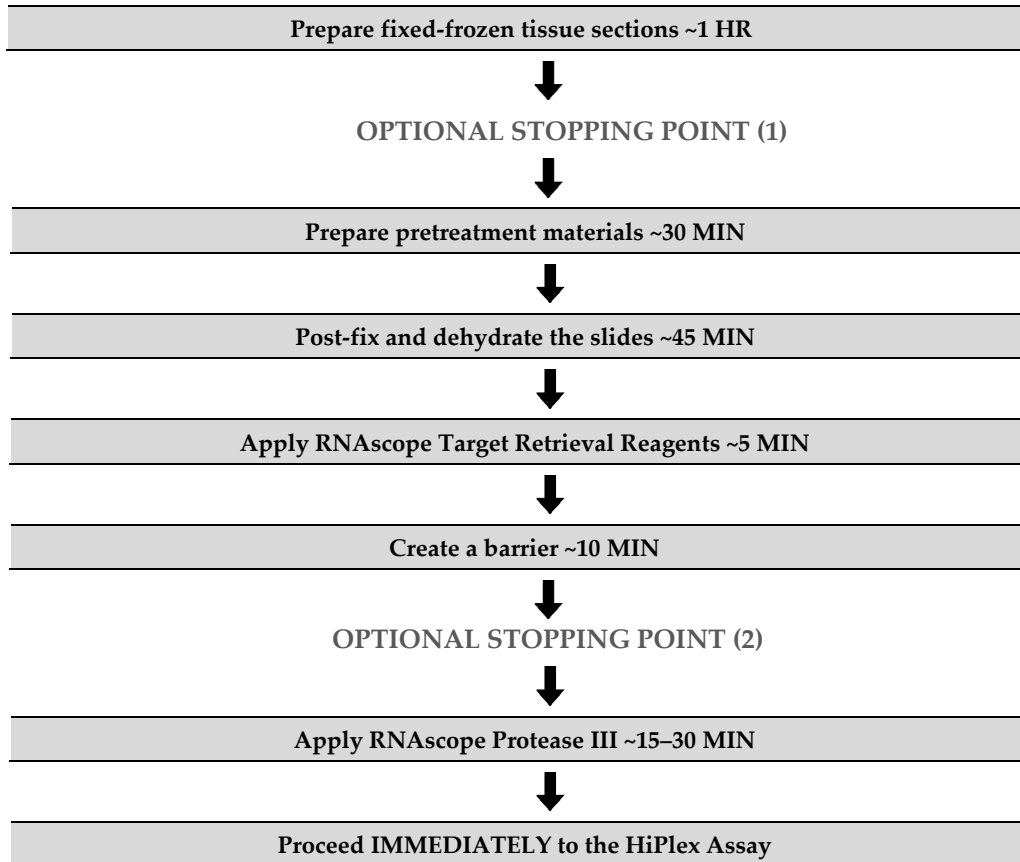
### Proceed to the RNAscope Assay

Proceed *immediately* to **Chapter 4. RNAscope HiPlex Assay v2** on page 29.



## Fixed-frozen tissue sample preparation and pretreatment

### Workflow





## Materials required

Materials provided by Pretreatment Reagents (Cat. No. 322340 and 322000)	Other Materials and Equipment
<ul style="list-style-type: none"> <li>• RNAscope Protease III</li> <li>• RNAscope 10X Target Retrieval Reagents</li> </ul>	<ul style="list-style-type: none"> <li>• Scalpel</li> <li>• Forceps</li> <li>• Cryo-embedding medium (OCT) or Tissue Freezing Media (TFM)</li> <li>• Dry ice, liquid nitrogen, or isopentane, or 2-methyl butane</li> <li>• Cryostat</li> <li>• Slide boxes</li> <li>• SuperFrost Plus slides</li> <li>• Aluminum foil or zip-lock bags</li> <li>• 1X PBS</li> <li>• 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA)</li> <li>• 30% sucrose</li> <li>• Tissue-Tek Vertical 24 Slide Rack (or similar)</li> <li>• Tissue-Tek Staining Dishes (or similar)</li> <li>• ImmEdge™ Hydrophobic Barrier Pen</li> <li>• HybEZ Humidifying System/ACD EZ-Batch Slide Holder and Tray</li> <li>• Distilled water</li> <li>• Paper towel or absorbent paper</li> <li>• Steamer (Hamilton Beach or Oster)</li> <li>• Digital thermometer</li> </ul>

## Fix samples

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

**Note:** We recommend perfusing tissues with 1X PBS followed by freshly prepared 4% paraformaldehyde (PFA) in 1X PBS. For suboptimally prepared samples, you may need to adjust pretreatment conditions.

2. Dissect tissue and fix in freshly prepared 10% NBF or 4% PFA for **24 HRS** at **4°C**.

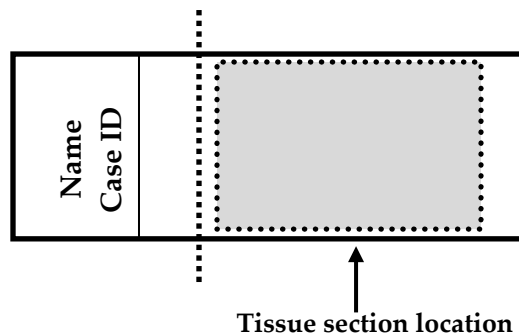
## Freeze tissues

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.

- Freeze the tissue in Optimal Cutting Temperature (OCT) embedding media with dry ice, liquid nitrogen, or 2-methyl butane, and store it in an airtight container at  $-80^{\circ}\text{C}$ .

### Prepare sections

- Before tissue sectioning, equilibrate the tissue blocks at  $-20^{\circ}\text{C}$  for at least **1 HR** in a cryostat.
- Section the blocks by cutting 7–15  $\mu\text{m}$  thick sections. Mount the sections on **SUPERFROST PLUS SLIDES**. Place tissue as shown for optimal staining:




---

**IMPORTANT!** Do not place sections too close to the edges of the slide.

---

- Air dry the slides for **60 –120 MIN** at  $-20^{\circ}\text{C}$ .

---

**OPTIONAL STOPPING POINT (1).** Use sectioned tissue within three months. Store sections with desiccants at  $-80^{\circ}\text{C}$ .

---

- Place slides in a slide rack or holder and wash with 200 mL 1X PBS in a staining dish or similar container for **5 MIN** while moving the rack up and down to remove the embedding media.
- Bake the slides for **30 MIN** at  $60^{\circ}\text{C}$ .
- Post-fix the slides by immersing them in prechilled 10% NBF or 4% PFA in 1X PBS for **15 MIN** at  $4^{\circ}\text{C}$ .

### Dehydrate the tissue

- Prepare 200mL 50% EtOH, 200 mL 70% EtOH, and 400 mL of 100% EtOH (enough to fill staining dishes).
- Remove the slides from the 10% NBF or 4% PFA, and immerse them in 50% EtOH for **5 MIN** at **RT**.
- Remove the slides from 50% EtOH, and immerse them in 70% EtOH for **5 MIN** at **RT**.
- Remove the slides from 70% EtOH, and immerse them in 100% EtOH for **5 MIN** at **RT**.
- Repeat step 4 with fresh 100% ethanol.

### Dry the slides

- Remove the slides from 100% EtOH, and let them air dry for **5 MIN** at **RT**.

### Prepare pretreatment materials

- Turn on the HybEZ Ovendr and set temperature to  $40^{\circ}\text{C}$ .

2. Place a Humidifying Paper in the Humidity Control Tray and wet completely with distilled water.
3. Insert 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 RNAscope Target Retrieval buffer and mix well).

**Note:** Target Retrieval buffer must be heated to  $\geq 99^{\circ}\text{C}$  before use, but do not boil for more than ~15 minutes before use. See the following procedure.

### Perform target retrieval using the steamer

We highly recommend using a steamer for target retrieval. For target retrieval using a hot plate, see **Appendix B. Manual Target Retrieval** on page 47.

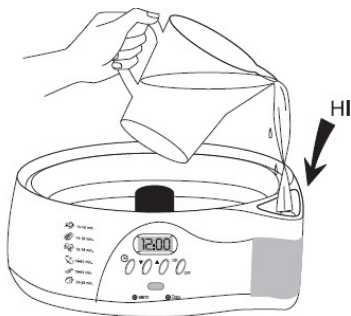
**Note:** For each steamer, fill the water to the maximum level before starting and do not refill water during the steaming process. Refilling water during the steaming process will drop the temperature and interfere with target retrieval.

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

---

**IMPORTANT!** Do not overfill.

---



2. Place two slide holders 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 water.
3. Turn on the steamer. Set the heating time to the maximum so steamer does not shut off during Target Retrieval.
4. 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**.
5. Add the slides to the container containing distilled water 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 **5 MIN**.
8. Remove the slides from the steamer and immediately transfer to a separate rinse container with 200 mL of distilled water (**RT**). 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**.

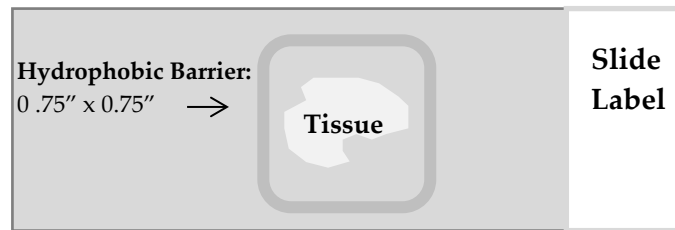
## Create a barrier

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 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 amounts, even for smaller sections. Larger barriers will result in fewer tests per kit.

2. 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.

---

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

---

## Apply RNAscope Protease III

**Note:** The following procedure describes standard pretreatment conditions. You may need to optimize conditions based on tissue type and sample preparation.

1. Load the dry slides into the ACD EZ-Batch Slide Holder by opening the swing clamp (see page 52 for details) then lock slides in place.
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 HybEZ oven.
3. Incubate the samples for **15–30 MIN** at **40°C**.

**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 wash tray containing water. 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.
8. Proceed *immediately* to **Chapter 4. RNAscope HiPlex Assay v2**.

---

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

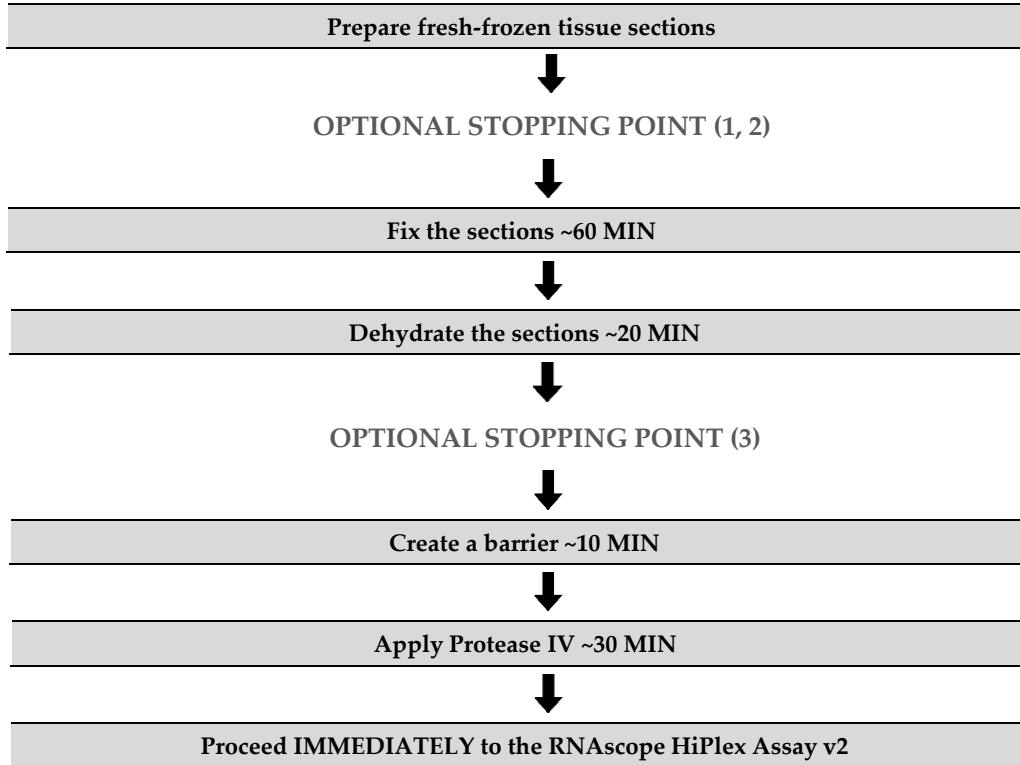
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## Fresh-frozen sample preparation and pretreatment

### Workflow





## Materials required

Materials provided by Pretreatment Reagents (Cat. No. 322380)	Other Materials and Equipment
<ul style="list-style-type: none"> <li>• RNAscope Protease III or IV (depending on sample-specific conditions)</li> </ul>	<ul style="list-style-type: none"> <li>• Scalpel</li> <li>• Forceps</li> <li>• Cryo-embedding medium (OCT) or Tissue Freezing Media (TFM)</li> <li>• Dry ice, liquid nitrogen, or isopentane, or 2-methyl butane</li> <li>• Cryostat</li> <li>• Slide boxes</li> <li>• SuperFrost Plus slides</li> <li>• Aluminum foil or zip-lock bags</li> <li>• 1X PBS</li> <li>• 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA)</li> <li>• 100% alcohol (EtOH)</li> <li>• Tissue-Tek Vertical 24 Slide Rack (or similar)</li> <li>• Tissue-Tek Staining Dishes (or similar)</li> <li>• ImmEdge Hydrophobic Barrier Pen</li> <li>• HybEZ Humidifying System/ ACD EZ-Batch Slide Holder and Wash Tray</li> <li>• Distilled water</li> <li>• Paper towel or absorbent paper</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.

---

5. Section the block:
  - e. Equilibrate block to **-20°C** in a cryostat **~1 HR.**
  - f. Cut 10–20 µm 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.

---

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

---

### Fix the sections

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

---

**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 **60 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.

**Note:** 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 of 50% ethanol, 200 mL of 70% ethanol, and 400 mL of 100% ethanol (enough to fill 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. Repeat step 4 with fresh 100% ethanol.

---

**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

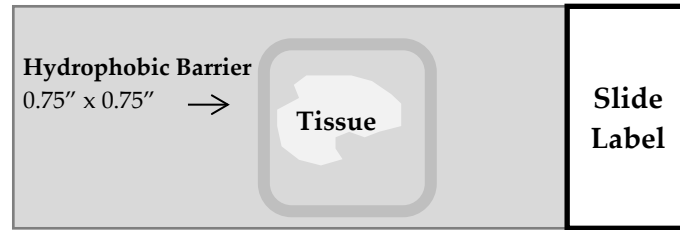
1. Take the slides out of 100% ethanol and place on absorbent paper with the section face-up. Air dry for **5 MIN** at **RT**.
2. Use the following template to draw a barrier 2–4 times around each section with the ImmEdge hydrophobic barrier pen.

**Note:** Refer to **Appendix A. Reagent Volume Guidelines** on page 46 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.

2. 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.

## Prepare the equipment

Place a Humidifying Paper in the Humidity Control Tray and wet completely with distilled water.

## Apply Protease IV

1. Load the dried slides into the ACD EZ-Batch Slide Holder (see page 52 for details) and add ~5 drops of Protease IV or enough to entirely cover each section.
2. Incubate for **30 MIN** at **RT**.
3. 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.
4. Wash slides in 1X PBS with slight agitation and repeat with fresh 1X PBS.
5. Proceed *immediately* to the next chapter **Chapter 4. RNAscope HiPlex Assay v2**.

---

**IMPORTANT!** Slides should not stay in 1X PBS for longer than **5 MIN**.

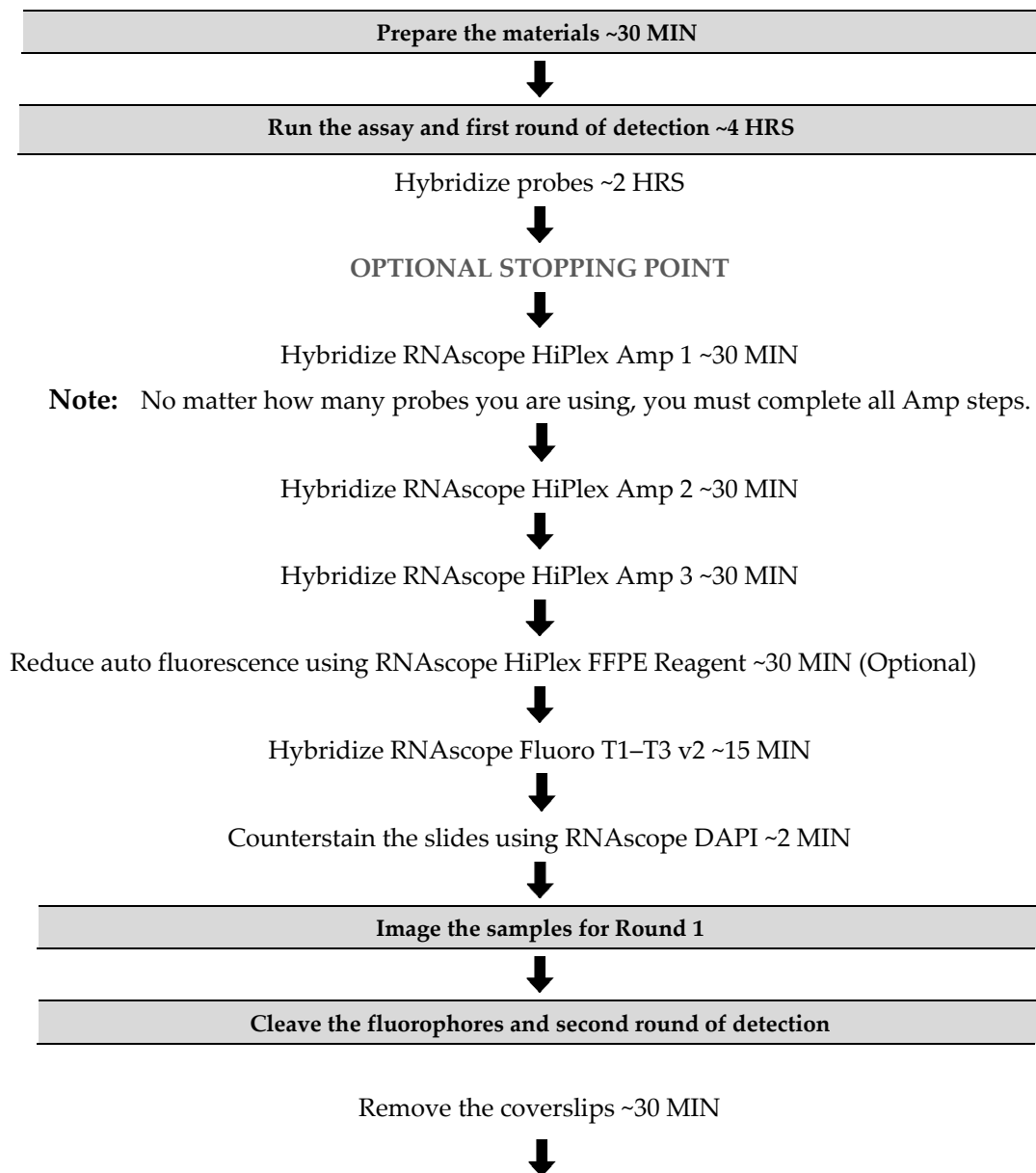
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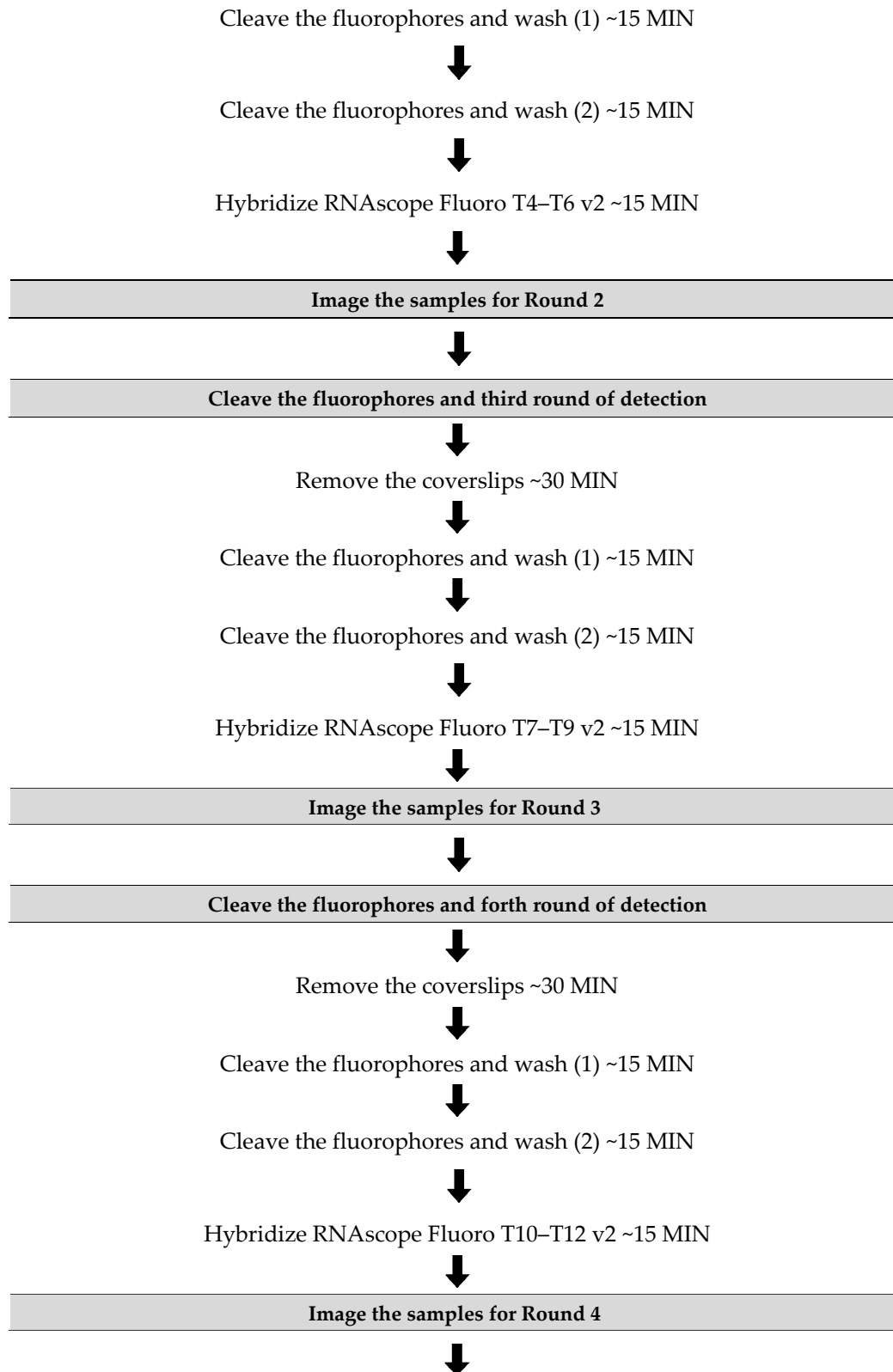
# 4

## Chapter 4. RNAscope HiPlex Assay (12-Plex) v2

This procedure flows directly from sample preparation and pretreatment. Refer to **Chapter 3. Prepare and Pretreat Samples** on page 14 or the appropriate sample preparation and pretreatment user manual or technical note for your specific sample type.

### Workflow





**OPTIONAL: Cleave the fluorophores and acquire blank images for use with *in silico* background subtraction**



Remove the coverslips ~30 MIN



Cleave the fluorophores and wash (1) ~15 MIN



Cleave the fluorophores and wash (2) ~15 MIN



**Image the cleaved samples (Round 5)**



**Image registration for all four (or four plus blank) rounds of imaging**



## Materials required for the assay

Materials provided by the RNAscope HiPlex Standard Detection Kits	Materials provided by the RNAscope HiPlex Cleaving Kit	Materials provided by RNAscope HiPlex Probes	Other materials and equipment
<ul style="list-style-type: none"> <li>• 50X Wash Buffer</li> <li>• RNAscope HiPlex Amp 1</li> <li>• RNAscope HiPlex Amp 2</li> <li>• RNAscope HiPlex Amp 3</li> <li>• RNAscope Fluoro T1–T3 v2</li> <li>• RNAscope Fluoro T4–T6 v2</li> <li>• RNAscope Fluoro T7–T9 v2</li> <li>• RNAscope Fluoro T10–T12 v2</li> <li>• RNAscope FFPE Reagent</li> <li>• RNAscope DAPI</li> </ul>	<ul style="list-style-type: none"> <li>• Cleaving stock solution v2</li> </ul>	<ul style="list-style-type: none"> <li>• 50X RNAscope HiPlex Target Probes</li> <li>• RNAscope HiPlex Probe Diluent</li> <li>• RNAscope HiPlex12 Positive Control Probe (RTU)</li> <li>• RNAscope HiPlex12 Negative Control Probe (RTU)</li> </ul>	<ul style="list-style-type: none"> <li>• Prepared sections</li> <li>• Distilled water</li> <li>• 10X PBS</li> <li>• 20X SSC</li> <li>• 10% Tween</li> <li>• Carboy (&gt;3L)</li> <li>• Tissue-Tek Staining Dish</li> <li>• HybEZ Humidifying System/ACD EZ-Batch Slide Holder and 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. Refer to a sample preparation and pretreatment user guide available at [www.acdbio.com/technical-support/user-manuals](http://www.acdbio.com/technical-support/user-manuals).

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 of 50X Wash Buffer (60 mL) in a large carboy. Mix well.

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

### Prepare 4X SSC

1. To prepare 4X SSC, dilute 20X SSC with distilled water by pipetting one volume of 20X SSC with four volumes of distilled water.
2. Mix thoroughly by inverting the container at least ten times.





**Note:** Prepare 20X SSC by dissolving 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of distilled water and adjusting the pH to 7.0 with a few drops of 1M HCl. Use water to adjust the volume to 1 liter. Sterilize by autoclaving or filtering under vacuum.

### Prepare PBST (0.5% Tween)

1. To make 1 liter PBST (0.5% Tween), add 100 mL 10X PBS, 850 mL distilled water, and 50 mL of 10% Tween in a container. Scale up or down as needed.
2. Mix thoroughly by inverting the container at least ten times.

### Prepare probes

1. Warm RNAscope HiPlex probe stocks and control probes at **40°C** in a water bath or incubator for about **10 MIN**.
2. Warm RNAscope HiPlex diluent at **40°C** in a water bath or incubator for about **10 MIN**.
3. Briefly spin down all 50X probe stocks to collect the liquid at the bottom of the tubes.
4. Mix each unique target probe set by diluting 50X probe stocks with RNAscope HiPlex probe diluent. Dilute probes to 1X by pipetting 1 volume of each stock to 50 volumes of probe diluent.
  - For example, to make 2 mL of solution containing all 12 probes, use 40  $\mu$ L of each probe stock and add 1520  $\mu$ L of RNAscope HiPlex Probe Diluent. . Mix well.
5. Mix well by vortexing or invert the tube several times.

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

### Equilibrate reagents

- Place RNAscope HiPlex Amp 1–3 and RNAscope HiPlex Fluoro T1–T4 v2 reagents at **RT**.
- Ensure that the HyBEZ Oven and prepared Humidity Control Tray are at **40°C**.

### Equilibrate and prepare RNAscope HiPlex FFPE Reagent (optional for fixed-frozen and fresh-frozen samples)

**Note:** Apply RNAscope HiPlex FFPE Reagent to tissues with high autofluorescence. The reagent can also be used on fixed-frozen and fresh-frozen samples. Apply the RNAscope HiPlex FFPE Reagent before Fluoro Fluoro T1-3 V2, T4-6 v2, T7-9 v2, and/or T10-12 v2.

1. Bring the RNAscope HiPlex FFPE Reagent to **RT**.
3. Briefly spin down the contents of the FFPE reagent tube to be sure content is at the bottom of the tube before opening the cap.
4. Depending on the size of your hydrophobic barrier, prepare sufficient 2.5%–5% FFPE reagent to cover each section by using a 1:40–1:20 ratio of FFPE reagent to 4X SSC. Mix well.

---

**IMPORTANT!** You must use only 4X SSC to dilute the RNAscope HiPlex FFPE Reagent. Due to oxidation, use freshly diluted FFPE reagent to maximize results. During the application of FFPE reagent, the color will turn from light brown to a darker color over time and precipitation could occur. However, the color change and precipitate will not affect the autofluorescence reducing activity.

---



## 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 [www.acdbio.com/technical-support/learn-more](http://www.acdbio.com/technical-support/learn-more) before proceeding.

---

**Note:** We recommend running control probes on your sample and optimizing the protocol before running any target probes.

## Hybridize probe

---

**IMPORTANT!** Ensure that the probes are prewarmed to **40°C** and cooled to **RT** prior to use.

---

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

**Note:** Refer to **Appendix A. Reagent Volume Guidelines** on page 46 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 tray 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 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 wash tray (see page 52 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** at **RT**.

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## Hybridize RNAscope HiPlex Amp 1

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 HybEZ Humidity Control Tray.
2. Add enough RNAscope HiPlex Amp 1 to entirely cover each section.
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 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, and wash the slides for **2 MIN** at **RT**.
7. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.



## Hybridize RNAscope HiPlex Amp 2

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 HybEZ Humidity Control Tray.
2. Add enough RNAscope HiPlex Amp 2 to entirely cover each section.
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 at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
6. Place the ACDEZ-Batch Slide Holder into the wash tray, and wash the slides for **2 MIN** at **RT**.
7. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.

## Hybridize RNAscope HiPlex Amp 3

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 HybEZ Humidity Control Tray.
2. Add enough RNAscope HiPlex Amp 3 to entirely cover each section.
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 or keep it at RT if you are using FFPE Reagent.
5. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
6. Place the ACDEZ-Batch Slide Holder into the wash tray, and wash the slides for **2 MIN** at **RT**.
7. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.

## Apply RNAscope HiPlex FFPE Reagent to reduce autofluorescence (optional for fresh-frozen and fixed-frozen samples)

**Note:** FFPE reagent is optional for tissues with low or no autofluorescence. You may skip this procedure and go directly to the RNAscope HiPlex Fluoro T1–T3 v2 hybridization step.

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough freshly prepared 2.5%–5% FFPE reagent to entirely cover each section.
3. Close the tray and incubate for **30 MIN** at **RT**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
5. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for **2 MIN** at **RT**.
6. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.

## Hybridize RNAscope HiPlex Fluoro T1–T3 v2

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough RNAscope HiPlex Fluoro T1–T3 to entirely cover each section.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Remove the tray from the oven, and remove the slide holder.



5. Pour at least 200 mL 1X Wash Buffer into the transparent ACDEZ-BatchWash Tray.
6. Place the ACDEZ-BatchSlide Holder into the wash tray, and wash the slides for **2 MIN** at **RT**.
7. Repeat the wash step with fresh 1X Wash 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 the slides, and add ~4 drops of DAPI to each section.
2. Incubate for **30 SEC** at **RT**.
3. Remove DAPI from slides and *immediately* place 1–2 drops of the ProLong Gold Antifade Mountant onto each section.
4. Carefully place a coverslip over each tissue section. Avoid trapping air bubbles. Store slides in the dark at **2–8°C**.

---

**IMPORTANT!** Use ProLong Gold Antifade Mountant as the mounting medium to best preserve the RNAscope signals.

---

**IMPORTANT!** Store slides in the dark at **2–8°C** before and after imaging. To the extent possible, it is ideal to keep slides cool while imaging to slow mountant curing process. As mountant cures, autofluorescence may increase and subsequent removal of coverslips will take longer. Preventing the slides from completely curing can shorten the assay running time and help to preserve tissue morphology throughout different rounds. When possible, imaging slides within 30 MIN of mounting is ideal.

---

## Image the slides for Round 1

1. Image the slides under a fluorescent microscope or fluorescent slide scanner.

**Note:** To make it easier to locate the same region of interest during the second round of image acquisition, use a microscope with a precise stage recorder. You will need filters for DAPI as well as the AF488, Dylight 550, and Dylight 650 fluorophores.

5. As there will be four rounds of detection and imaging, we recommend including the initials R1 (round 1), R2 (round 2), R3 (round 3), R4 (round 4) and the target names when saving image files. Implementing a naming convention will help you to identify each group of images during the image registration process.
6. Store the slides in the dark at **2–8°C** for up to three days or proceed immediately to the fluorophore cleaving step.

## Equilibrate reagents

- Place RNAscope HiPlex Fluoro T4–T6 reagent v2 at **RT**.
- Ensure that the HybEZ Oven is at **40°C**.

## Cleave the fluorophores

1. To remove the coverslips:



---

**IMPORTANT!** Use only 4X SSC buffer. To reduce tissue damage, do not remove the coverslips by force. Soak the slides in 4X SSC until the coverslips can be moved easily. If the slides have been dried completely, you may need to soak the slides in 4X SSC overnight.

---

- a. Soak the slides in 4X SSC at **RT** for at least **30 MIN** or until the coverslips fall off the slides easily.
  - b. Gently remove each coverslip.
  2. Once the coverslips have been removed, briefly wash the slides once in 4X SSC.
  3. Break open a **FRESH** glass ampoule of provided Cleaving Stock Solution v2.
  4. Prepare a 10% cleaving solution v2 by diluting with 4X SSC.
- 

**IMPORTANT!** Due to oxidation, do not use the Cleaving Stock Solution v2 more than once. Use only 4X SSC to make 10% cleaving solution v2.

---

5. Load the slides in the ACD EZ-Batch Slide Holder.
  6. 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.
  7. Apply enough freshly prepared 10% cleaving solution v2 to entirely cover each section.
  8. Close the tray and incubate for **15 MIN** at **RT**.
  9. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.
  10. Remove the slide holder from the tray.
  11. Place the slide holder into the wash tray and wash the slides for **2 MIN** at **RT**.
  12. Repeat the wash step one more time with fresh PBST (0.5% Tween) for **2 MIN** at **RT**.
- 

**IMPORTANT!** Use only PBST (0.5% Tween) for this step.

---

13. Repeat a second cleaving reaction starting with the following step.
  14. 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.
  15. Apply enough 10% cleaving solution v2 to entirely cover each section.
  16. Close the tray and incubate for **15 MIN** at **RT**.
  17. Remove the slide holder from the tray, and **place the tray into the HybEZ Oven to warm for the next step**.
  18. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.
  19. Place the slide holder into the wash tray and wash the slides for **2 MIN** at **RT**.
  20. Repeat the wash step one more time with fresh PBST (0.5% Tween) for **2 MIN** at **RT**.
- 

## Hybridize RNAscope HiPlex Fluoro T4–T6 v2

**Note:** If necessary, reapplication of diluted RNAscope HiPlex FFPE Reagent to tissues with high autofluorescence is recommended for improvement in signal to noise ratio. See the procedure on page 35.

1. The ACD EZ-Batch Slide Holder should be warm to begin this step.
2. 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.
3. Add enough RNAscope HiPlex Fluoro T4–T6 v2 to entirely cover each section.
4. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.



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

## Mount the slides

---

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

---

1. Remove access liquid from the slides, and *immediately* place 1–2 drops of the ProLong Gold Antifade Mountant onto each section.
2. Carefully place a coverslip over each tissue section. Avoid trapping air bubbles. Store the slides in the dark at **2–8°C**.

## Image the slides for Round 2

1. Image the slides under a fluorescent microscope or fluorescent slide scanner.

**Note:** To make it easier to locate the same region of interest during each round of image acquisition, use a microscope with a precise stage recorder. You will need filters for DAPI, as well as the AF488, Dylight 550, and Dylight 650 fluorophores.

---

**IMPORTANT!** Round 1 and Round 2 images require at least 70% overlap to be successfully registered using RNAscope HiPlex Image Registration Software v2.0. Image registration uses nuclear staining, most commonly DAPI staining, as a reference. Adjust the exposure times to make sure that the nuclear signal matches between Round 1 and Round 2 imaging.

---

7. As there will be four rounds of detection and imaging, we recommend including the initials R1 (round 1), R2 (round 2), R3 (round 3), R4 (round 4) and the target names when saving image files. Implementing a naming convention will help you identify each group of images during the image registration process.
8. Store the slides in the dark at **2–8°C** for up to three days or proceed immediately to fluorophore cleaving.

## Equilibrate reagents

- Place RNAscope HiPlex Fluoro T7–T9 reagent v2 at **RT**.
- Ensure that the HybEZ Oven is at **40°C**.

## Cleave the fluorophores

1. To remove the coverslips:

---

**IMPORTANT!** Use only 4X SSC buffer. To reduce tissue damage, do not remove the coverslips by force. Soak the slides in 4X SSC until the coverslips can be moved easily. If the slides have been dried completely, you may need to soak the slides in 4X SSC overnight.

---



- a. Soak the slides in 4X SSC at **RT** for at least **30 MIN** or until the coverslips fall off the slides easily.
- b. Gently remove each coverslip.
2. Once the coverslips have been removed, briefly wash the decoverslipped slides once in 4X SSC.
3. Break open a **FRESH** glass ampoule of provided Cleaving Stock Solution v2.
4. Prepare a 10% cleaving solution v2 by diluting with 4X SSC.

---

**IMPORTANT!** Due to oxidation, do not use the Cleaving Stock Solution v2 more than once. Use only 4X SSC to make 10% cleaving solution v2.

---

5. Load the slides in the ACD EZ-Batch Slide Holder.
6. 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.
7. Apply enough freshly prepared 10% cleaving solution v2 to entirely cover each section.
8. Close the tray and incubate for **15 MIN** at **RT**.
9. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.
10. Remove the slide holder from the tray.
11. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT**.
12. Repeat the wash step one more time with fresh PBST (0.5% Tween) for **2 MIN** at **RT**.

---

**IMPORTANT!** Use only PBST (0.5% Tween) for this step.

---

13. Repeat a second cleaving reaction starting with Step 14.
14. 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.
15. Apply enough 10% cleaving solution v2 to entirely cover each section.
16. Close the tray and incubate for **15 MIN** at **RT**.
17. Remove the slide holder from the tray, and **place the tray into the HybEZ Oven to warm for the next step**.
18. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.
19. Place the slide holder into the wash tray and wash the slides for **2 MIN** at **RT**.
20. Repeat the wash step one more time with fresh PBST (0.5% Tween) for **2 MIN** at **RT**.

## Hybridize RNAscope HiPlex Fluoro T7–T9 v2

**Note:** If necessary, reapplication of diluted RNAscope HiPlex FFPE Reagent to tissues with high autofluorescence is recommend for improvement in signal to noise ratio. See the procedure on page **Error! Bookmark not defined.**

1. The ACD EZ-Batch Slide Holder should be warm to begin this step.
2. 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.
3. Add ~4 drops of RNAscope HiPlex Fluoro T7–T9 v2 to entirely cover each section.
4. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
5. Remove the tray from the oven, and remove the slide holder from the tray.
6. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.



7. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for **2 MIN** at **RT**.
8. Repeat the wash step with fresh 1X Wash 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 access liquid from the slides, and *immediately* place 1–2 drops of the ProLong Gold Antifade Mountant onto each section.
2. Carefully place a coverslip over each tissue section. Avoid trapping air bubbles. Store the slides in the dark at **2–8°C**.

## Image the slides for Round 3

1. Image the slides under a fluorescent microscope or fluorescent slide scanner.

**Note:** To make it easier to locate the same region of interest during each round of image acquisition, use a microscope with a precise stage recorder. You will need filters for DAPI, as well as the AF488, Dylight 550 and Dylight 650 fluorophores.

---

**IMPORTANT!** Round 1, Round 2, Round 3 and Round 4 images require at least 70% overlap to be successfully registered using RNAscope HiPlex Image Registration Software v2.0. Image registration uses nuclear staining, most commonly DAPI staining, as a reference. Adjust the exposure times to make sure that the nuclear signal matches between Round 1 and Round 2 imaging.

---

2. As there will be four rounds of detection and imaging, we recommend including the initials R1 (round 1), R2 (round 2), R3 (round 3), R4 (round 4) and the target names when saving image files. Implementing a naming convention will help you identify each group of images during the image registration process.
3. Store the slides in the dark at **2–8°C**.

## Equilibrate reagents

- Place RNAscope HiPlex Fluoro T10–T12 reagent v2 at **RT**.
- Ensure that the HybEZ Oven is at **40°C**.

## Cleave the fluorophores

1. To remove the coverslips:

---

**IMPORTANT!** Use only 4X SSC buffer. To reduce tissue damage, do not remove the coverslips by force. Soak the slides in 4X SSC until the coverslips can be moved easily. If the slides have been dried completely, you may need to soak the slides in 4X SSC overnight.

---

- a. Soak the slides in 4X SSC at **RT** for at least **30 MIN** or until the coverslips fall off the slides easily.
  - b. Gently remove each coverslip.
2. Once the coverslips have been removed, briefly wash the slides once in 4X SSC.
  3. Break open a **FRESH** glass ampoule of provided Cleaving Stock Solution v2.





4. Prepare a 10% cleaving solution v2 by diluting with 4X SSC.

---

**IMPORTANT!** Due to oxidation, do not use the Cleaving Stock Solution v2 more than once. Use only 4X SSC to make 10% cleaving solution v2.

---

5. Load the slides in the ACD EZ-Batch Slide Holder.
6. 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.
7. Apply enough freshly prepared 10% cleaving solution v2 to entirely cover each section.
8. Close the tray and incubate for **15 MIN** at **RT**.
9. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.
10. Remove the slide holder from the tray.
11. Place the slide holder into the wash tray and wash the slides for **2 MIN** at **RT**.
12. Repeat the wash step one more time with fresh PBST (0.5% Tween) for **2 MIN** at **RT**.

---

**IMPORTANT!** Use only PBST (0.5% Tween) for this step.

---

13. Repeat a third cleaving reaction starting with Step 14.
14. 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.
15. Apply enough 10% cleaving solution v2 to entirely cover each section.
16. Close the tray and incubate for **15 MIN** at **RT**.
17. Remove the slide holder from the tray, and **place the tray into the HybEZ Oven to warm for the next step.**
18. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.
19. Place the slide holder into the wash tray and wash the slides for **2 MIN** at **RT**.
20. Repeat the wash step one more time with fresh PBST (0.5% Tween) for **2 MIN** at **RT**.

## Hybridize RNAscope HiPlex Fluoro T10–T12 v2

**Note:** If necessary, reapplication of diluted RNAscope HiPlex FFPE Reagent to tissues with high autofluorescence is recommend for improvement in siggal to noise ratio. . See the procedure on page 35.

1. The ACD EZ-Batch Slide Holder should be warm to begin this step.
2. 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.
3. Add enough RNAscope HiPlex Fluoro T10–T12 v2 to entirely cover each section.
4. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
5. Remove the tray from the oven, and remove the slide holder from the tray.
6. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
7. Place the ACD EZ-Batch Slide Holder into the wash tray, and wash the slides for **2 MIN** at **RT**.
8. Repeat the wash step with fresh 1X Wash Buffer for **2 MIN** at **RT**.

## Mount the slides

---

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

---



1. Remove excess liquid from the slides, and immediately place 1–2 drops of the ProLong Gold Antifade Mountant onto each section.
2. Carefully place a coverslip over each tissue section. Avoid trapping air bubbles. Store the slides in the dark at 2–8°C.

## Image the slides for Round 4

1. Image the slides under a fluorescent microscope or fluorescent slide scanner.

NOTE: To make it easier to locate the same region of interest during each round of image acquisition, use a microscope with a precise stage recorder. You will need filters for DAPI, as well as the AF488, Dylight 550 and Dylight 650 fluorophores.

---

**IMPORTANT!** Round 1, Round 2, Round 3 and Round 4 images require at least 70% overlap to be successfully registered using RNAscope HiPlex Image Registration Software v2.0. Image registration uses nuclear staining, most commonly DAPI staining, as a reference. Adjust the exposure times to make sure that the nuclear signal matches between Round 1 and Round 2 imaging.

---

2. As there will be four rounds of detection and imaging, we recommend including the initials R1 (round 1), R2 (round 2), R3 (round 3), R4 (round 4) and the target names when saving image files. Implementing a naming convention will help you identify each group of images during the image registration process.
3. Store the slides in the dark at 2–8°C for up to three days or proceed immediately to fluorophore cleaving.

## Image the slides for Blank Image Reference – OPTIONAL

---

**IMPORTANT!** This round of blank slide imaging is recommended if the tissue specimen exhibited high autofluorescence background that interfered with the detection of positive fluorescent signals.

---

1. After round-4 imaging, remove the coverslips:

---

**IMPORTANT!** Use only 4X SSC buffer. To reduce tissue damage, do not remove the coverslips by force. Soak the slides in 4X SSC until the coverslips can be moved easily. If the slides have been dried completely, you may need to soak the slides in 4X SSC overnight.

---

2. Soak the slides in 4X SSC at RT for at least **30 MIN** or until the coverslips fall off the slides easily.
3. Gently remove each coverslip.
4. Once the coverslips have been removed, briefly wash the decovered slides once in 4X SSC.
5. Break open a **FRESH** glass ampoule of provided Cleaving Stock Solution v2.
6. Prepare a 10% cleaving solution v2 by diluting with 4X SSC.

---

**IMPORTANT!** Due to oxidation, do not use the Cleaving Stock Solution v2 more than once. Use only 4X SSC to make 10% cleaving solution v2.

---

7. Load the slides in the ACD EZ-Batch Slide Holder.



8. 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.
9. Apply enough freshly prepared 10% cleaving solution v2 to entirely cover each section.
10. Close the tray and incubate for **15 MIN** at **RT**.
11. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.
12. Remove the slide holder from the tray.
13. Place the slide holder into the wash tray, and wash the slides for **2 MIN** at **RT**.
14. Repeat the wash step one more time with fresh PBST (0.5% Tween) for **2 MIN** at **RT**.

---

**IMPORTANT!** Use only PBST (0.5% Tween) for this step.

---

15. Repeat Steps 6–12 but warm the HybEZ Humidity Control Tray back up to **40°C**:
16. 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.
17. Apply enough 10% cleaving solution v2 to entirely cover each section.
18. Close the tray and incubate for **15 MIN** at **RT**.
19. Remove the slide holder from the tray, and place the tray into the HybEZ Oven.
20. Pour at least 200 mL PBST (0.5% Tween) into the transparent ACD EZ-Batch Wash Tray.
21. Place the slide holder into the wash tray and wash the slides for **2 MIN** at **RT**.
22. Repeat the wash step one more time with fresh PBST (0.5% Tween) 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 the slides, and *immediately* place 1–2 drops of the ProLong Gold Antifade Mountant onto each section.
2. Carefully place a coverslip over each tissue section. Avoid trapping air bubbles. Store the slides in the dark at **2–8°C**.

## Image the slides to use with background subtraction function in HiPlex Registration Software v2.0

1. Image the slides under a fluorescent microscope or fluorescent slide scanner in all fluorescent channels employed in previous imaging rounds.

To make it easier to locate the same region of interest during each round of image acquisition, use a microscope with a precise stage recorder. You will need filters for DAPI, as well as the AF488, Dylight550, and Dylight650 fluorophores.

---

**IMPORTANT!** Round 1, Round 2, Round 3, Round 4 and Round 5 images require at least 70% overlap to be successfully registered using RNAscope HiPlex Image Registration Software v2.0. Image registration uses nuclear staining, most commonly DAPI staining, as a reference. Adjust the exposure times to make sure that the nuclear signal matches between Round 1, Round 2, Round 3, Round 4, and Round 5 imaging. For Hiplex v2 assay with more than 12 gene targets, blanking imaging may be performed after the completion of all target gene rounds.

---

2. Store the slides in the dark at 2–8°C.

## Image registration using RNAscope HiPlex Registration Software v2.0

- Register the DAPI, AF488, Dylight550 fluorescent images generated from all rounds.
- To ensure accuracy, make sure that the DAPI channel images are similarly exposed.
- Refer to the *RNAscope HiPlex Registration Software User Manual* (Doc. No. 300065-USM). A step by step guide for how to use the software is also available in the installer package of the software. If you have any questions, contact ACD technical support at [support.acd@bio-techne.com](mailto:support.acd@bio-techne.com).

## Evaluate the samples

For an example of successful staining, see **Figure 2** on page 44. Examine tissue sections under a standard fluorescent microscope at 20–40X magnification. You can also use a confocal microscope.

- Assess tissue and cell morphology.
- Assess the positive control signal strength. Positive control signal should be visible as punctuate dots within a cell at 20X magnification.
- Assess the negative control background. Five dots in every 10 cells displaying background staining per microscope field is acceptable at 20X magnification.

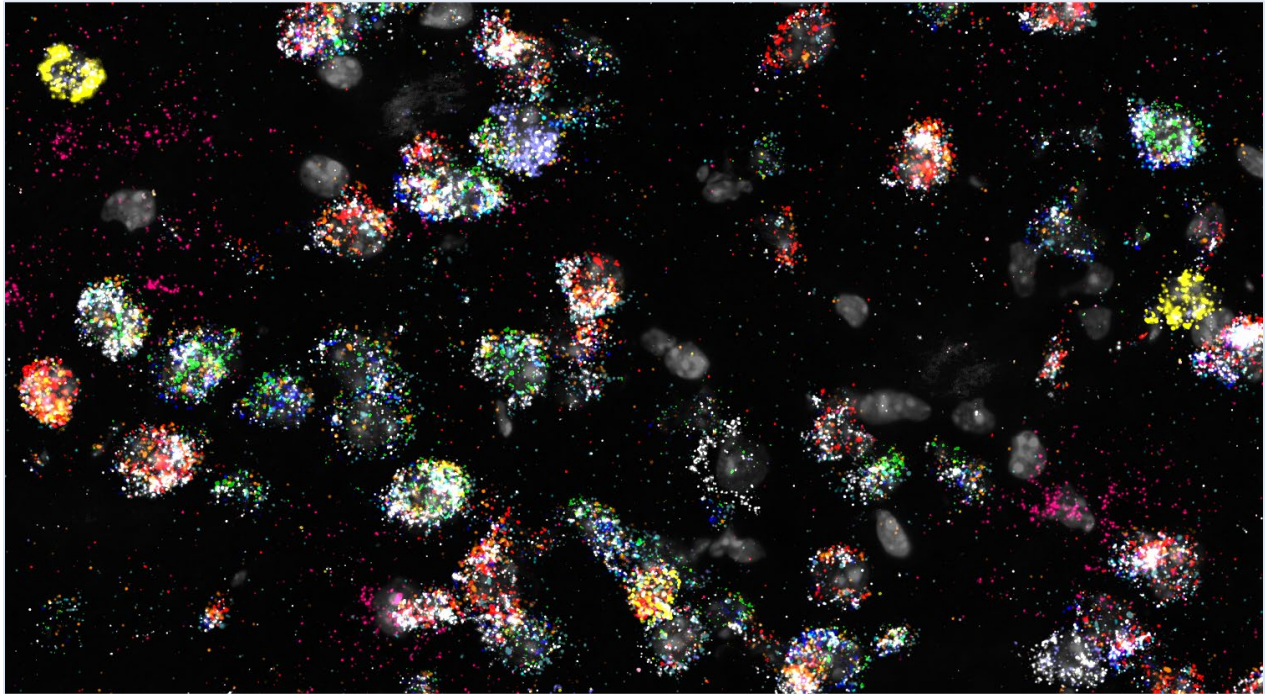
## Fluorescent Imaging Recommendations

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 μm 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 is okay 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>

## Example image

Figure 2 is an example of RNA expression in the striatum region of a normal mouse brain.



**Figure 2.** Visualizing striatal *Drd1a* and *Drd2* Medium Spiny Neurons (MSNs) using the RNAscope HiPlex Assay v2 for 12 targets in fresh-frozen mouse brain sections. Targets are *Drd1a*, *Drd2*, *Foxp1*, *Pcdh8*, *Synpr* (in white), *Htr7*, *Meis2*, *Calb1*, *Crym*, *Cnr1*, *Wfs1*, *Th* (in yellow).

## Troubleshooting

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

# Appendix A. 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 ~10 sections. Larger tissue sections will result in fewer tests.

# 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 C. 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 HiPlex Assay v2.





# Appendix C. 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 this user manual.

## Tissue pretreatment recommendation

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 III. Refer to the following section.

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

Some sample types, such as certain xenografts and cell pellets, could require less time. For these tissue types, vary the RNAscope Target Retrieval Reagents time to **8 MIN** and RNAscope Protease III time to **15 MIN**. For the ACD Cell Pellet sample, we recommend a **10 MIN** treatment with Target Retrieval Reagents, and a **30 MIN** treatment with RNAscope Protease III. If you have a tissue type not listed, contact support at [support@acdbio.com](mailto:support@acdbio.com).

## Tissue-specific pretreatment conditions

For suboptimally treated samples, you may need to optimize pretreatment conditions. Refer this document and information provided at <http://acdbio.com/technical-support/solutions>.

If your sample fixation is successful in fresh 10% NBF (see Step 2 from the preceding protocol), 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@acdbio.com](mailto:support@acdbio.com).

Species	Tissue Type	Pathology	Pretreatment Condition
Mouse/Rat	Intestine	Normal	Standard
	Intestine	Tumor	Standard
	Embryo	Normal	Standard
	Brain	Normal	Standard
	Spleen	Normal	Mild



Species	Tissue Type	Pathology	Pretreatment Condition
Mouse/Rat	Eye/Retina	Normal	Standard
	Liver	Normal	Extended
	Kidney	Normal	Standard
Human	Breast	Tumor	Standard
	Colon	Tumor/Normal	Standard
	Lung	Tumor	Standard
	Lung	Normal	Standard
	Prostate	Tumor	Standard
	Prostate	Normal	Standard
	Lymph node	Tumor	Standard
	Lymph node	Normal	Standard
	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	



Species	Tissue Type	Pathology	Pretreatment Condition
Human	Cell pellets, fixed with 10% NBF	—	Mild
	HeLa cells, fixed with 10% Formaldehyde/PBS/ACD Control	—	10 MIN Target Retrieval; 30 MIN Protease III

\* Tissue Microarray

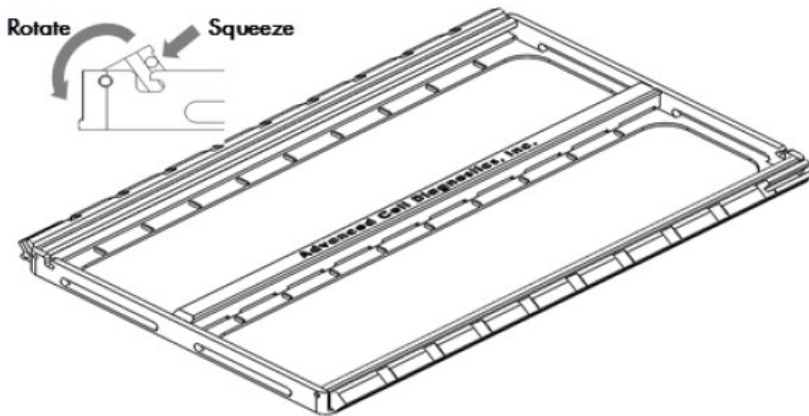
# D

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

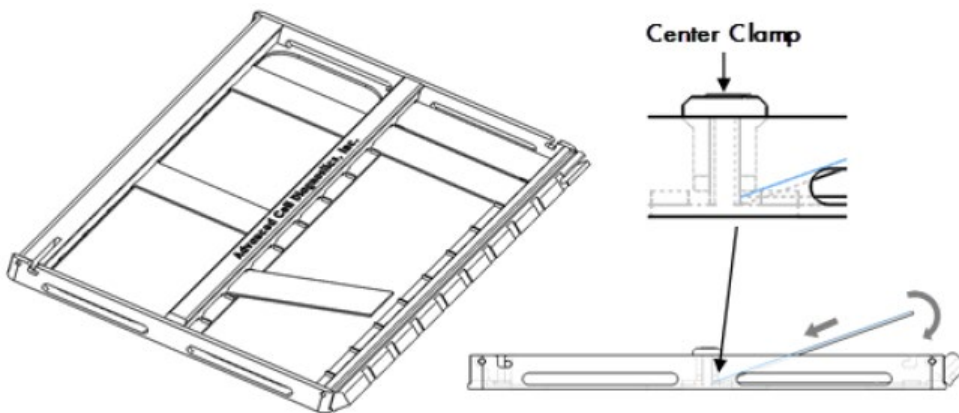
### Load the slides in the ACD EZ-Batch Slide Holder

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.

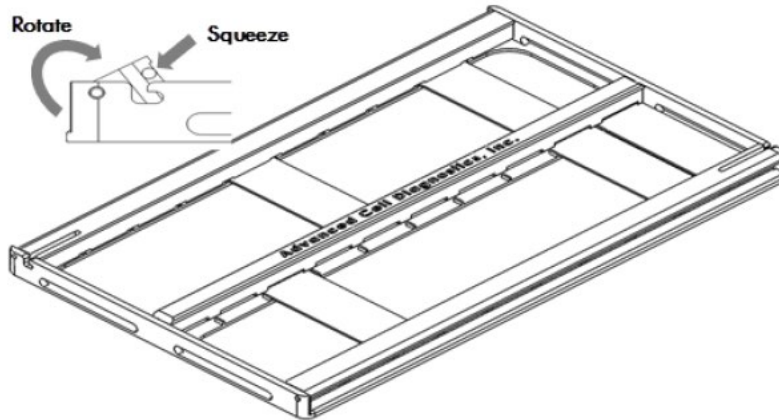
1. Open the swing clamps one at a time by simultaneously squeezing (pressing and holding) the slotted portion of each clamp and rotating it up then outwards, as shown.



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.

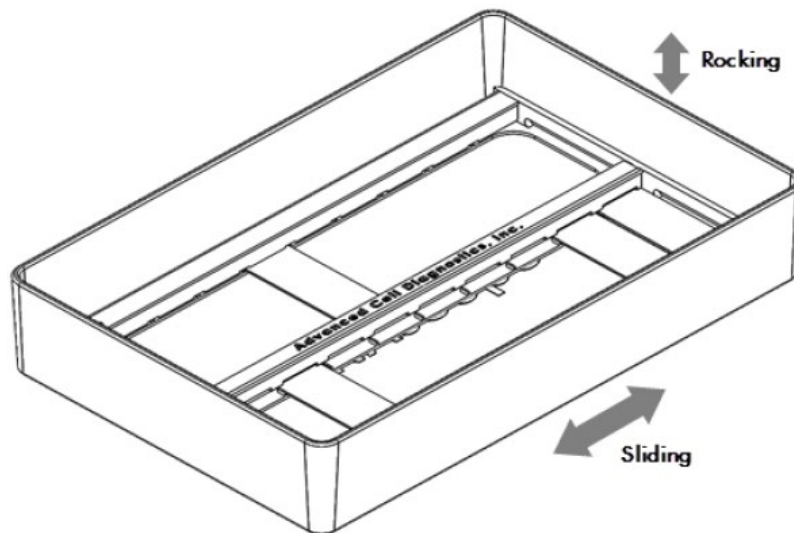


3. Close and lock the swing clamp of the column by simultaneously squeezing the slotted portion of each clamp and rotating it in then downwards in the direction opposite to the direction used to open the clamp, as shown.



## 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.



## E

## Appendix E. 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](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: [www.acdbio.com/technical-support/user-manuals](http://www.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: [www.acdbio.com/technical-support/support-overview](http://www.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 Boulevard

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)

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 ADC website at [www.acdbio.com/store/terms](http://www.acdbio.com/store/terms). If you have any questions, please contact Advanced Cell Diagnostics at [www.acdbio.com/about/contact](http://www.acdbio.com/about/contact).



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