

Fresh Frozen Tissue Sample Preparation and Pretreatment

For the RNAscope[®] Fluorescent Multiplex Assay

PART 1

Document Number 320513-USM

Compatible with the RNAscope[®] Fluorescent Multiplex Detection Kit User Manual Part 2, see Document Number 320293-USM.



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Citing RNAscope® in Publications

When describing a procedure for publication using this product, please refer to it as the RNAscope® 2.0 Assay and cite: Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, Wu X, Vo H-T, Ma XJ 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 B. Safety** on page 16 in this document.

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

About this guide

This user guide provides guidelines and protocols for the proper preparation and pretreatment of fresh, frozen tissues mounted on slides. The slides can then be assayed using an RNAscope® Reagent Kit.

RNAscope® Reagent Kits come with a separate RNAscope® Assay User Manual. Do not perform an RNAscope® Assay without the correct user manual.

Product description

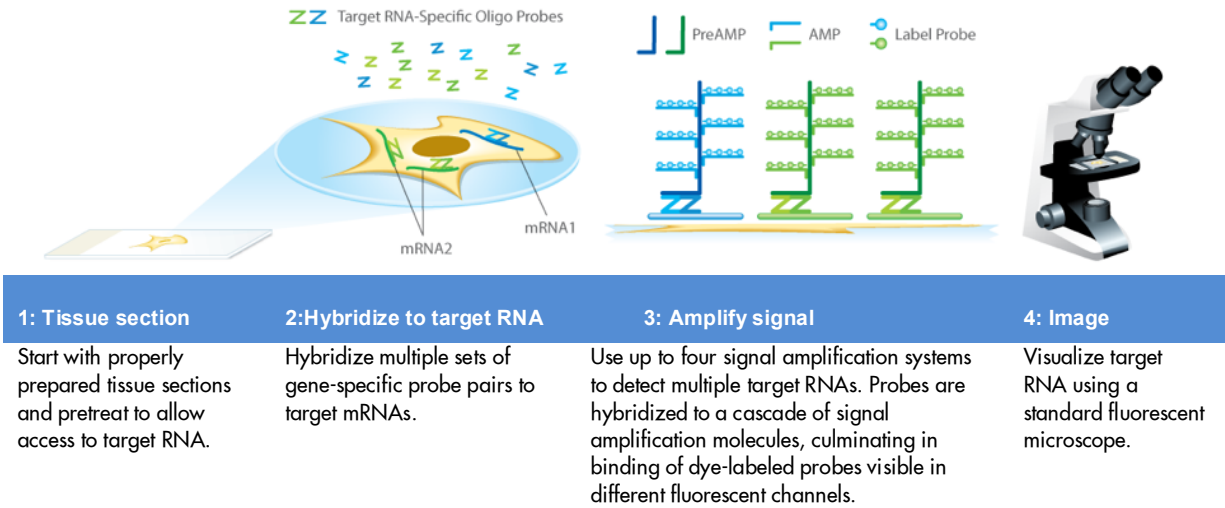
Background

The RNAscope® Assays use a novel and proprietary method of *in situ* hybridization (ISH) to visualize single RNA molecules per cell in samples mounted on slides. The assays are based on ACD's patented signal amplification and background suppression technology. Proprietary RNA-specific probes are hybridized to target RNA, and are then bound to a cascade of signal amplification molecules culminating in signal detection (Figure 1). Single-plex, duplex, multiplex, and automated assays are all available. Figure 1 on page 6 illustrates the RNAscope® Assay, which can be completed in 6–10 hours depending on the assay, or conveniently divided over two days. Most RNAscope® Assay reagents are available in convenient Ready-To-Use (RTU) dropper bottles and provide a simple, nearly pipette-free workflow. Results are observable using standard bright field or fluorescent microscopy.

Sample types

In order to perform the RNAscope® Assays, you must start with properly prepared and pretreated samples. Multiple sample types are now compatible with RNAscope® Assays and include: formalin-fixed, paraffin-embedded (FFPE) tissues, fresh, frozen tissues, fixed, frozen tissues, tissue microarray (TMA), and cell samples. Visit www.acdbio.com and contact support@acdbio.com for more information.

Figure 1. Procedure overview



RNAscope® Assay reagents

RNAscope® Assays require the RNAscope® Probes and an RNAscope® Reagent Kit. If you are performing an automated assay using the Ventana® DISCOVERY XT or ULTRA Systems, you must use RNAscope® VS Probes and the RNAscope® VS Reagent Kit. Probes and Reagent Kits are available separately. Visit <https://acdbio.com/products> to find a gene-specific Target Probe or appropriate Control Probes.

RNAscope® Reagent Kit

Each RNAscope® Reagent Kit provides enough reagents to stain ~20 tissue sections approximately 20 mm x 20 mm large. Larger tissue sections will result in fewer tests. Each kit contains three sub-kits including a Pretreatment Kit, which include Protease III and Protease IV. Protease pretreatment instructions are provided in this guide. For information on the other sub-kits and directions for use, refer to the RNAscope® Fluorescent Multiplex Assay User Manual (320293-USM). The reagents have a shelf life of nine months from the bulk manufacture date when stored as indicated in the following table:

Protease III & Protease IV Reagents (Cat. No. 322340)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	Protease III—1X Ready-To-Use (RTU) protease	4.5 mL x 1 bottle	2–8 °C
	Protease IV—2X Ready-To-Use (RTU) protease	4.5 mL x 2 bottle	

IMPORTANT! RNAscope® 2.5 Reagent Kits share the same Wash Buffer, but have unique pretreatment reagents and Detection Kits. Do not interchange the reagent components of the Detection Kits, even those having the same name.

Required materials and equipment

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

HybEZ™ Hybridization System

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

Use the HybEZ™ Hybridization System to perform BaseScope™ Assay hybridization and incubation steps. These steps require humid conditions to prevent sections from drying out. For instructions on how to use the HybEZ™ Hybridization System, refer to the *HybEZ™ Hybridization System User Manual* available at <https://acdbio.com/documents/support-documents> and view the training video at <https://acdbio.com/technical-support/learn-more>. The system contains the following components:

<input checked="" type="checkbox"/>	Component	Quantity	Cat. No.
	HybEZ™ Oven (110 or 220 VAC) or HybEZ™ II Oven (110 or 220V)	1 oven	310010 or 310013 (HybEZ™) 321710 or 321720 (HybEZ™ II)
	HybEZ™ Humidity Control Tray (with lid)	1 tray	310012
	ACD EZ-Batch™ Slide Rack (20 slide capacity)	1 rack	310017
	HybEZ™ Humidifying Paper	2 sheets	—

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

User-supplied materials

IMPORTANT! Do not substitute other materials for the ImmEdge™ Hydrophobic Barrier Pen and the SuperFrost® Plus Slides listed in the following table.

<input checked="" type="checkbox"/>	Description	Supplier	Cat. No.
	ImmEdge™ Hydrophobic Barrier Pen (required)	Vector Laboratory	H-4000
	SuperFrost® Plus Slides (required)	Fisher Scientific	12-550-15
	100% alcohol (EtOH)	American Master Tech Scientific/MLS*	ALREAGAL
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24
	Tissue-Tek® Staining Dishes	American Master Tech Scientific/MLS	LWT4457EA
	Tissue-Tek® Clearing Agent Dishes, xylene resistant	American Master Tech Scientific/MLS	LWT4456EA
	ProLong® Gold Antifade Mountant	Thermo Fisher	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	—
	Pipettors and tips, 1–1000 µL	MLS	—
	Distilled water	MLS	—

<input checked="" type="checkbox"/>	Description	Supplier	Cat. No.
	Tubes (various sizes)	MLS	—
	Fume hood	MLS	—
	Graduated cylinder	MLS	—
	Parafilm	MLS	—
	Paper towel or absorbent paper	MLS	—
	Glass beaker (1 or 2 L)	MLS	—
	Microscope and accessories	MLS	—

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

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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 <https://acdbio.com/technical-support/learn-more>.

Important procedural guidelines

- Start with properly fixed and prepared sections.
- 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.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix B. Safety** on page 16 for more information.

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Chapter 3. Prepare and Pretreat Samples

Fresh, frozen sample preparation and pretreatment are described in the following protocol. For other sample types and preparation methods, contact support@acdbio.com for the latest protocols and guidelines.

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

Prepare fresh frozen sections

Materials required

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

Prepare the block

1. Remove tissue and cut to fit into cryomolds.

 **CAUTION!** Handle biological specimens appropriately.

2. Freeze the specimen on dry ice or in liquid Nitrogen within **5 MIN** of tissue harvest.
3. Embed frozen tissue in cryo-embedding medium (OCT):
 - a. Add two drops of OCT into a cryomold.
 - b. Place the frozen tissue on the OCT in the correct orientation for cutting.
 - c. Add more OCT to fill the cryomold. Do not allow any air bubbles to form.
 - d. Hold the block with forceps on the surface of 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.

Note: Embedded tissue may be stored for up to three months.

Section the block

1. Equilibrate block to **-20°C** in a cryostat **~1 HR**.
2. Cut 10–20 µm sections and mount onto **SUPERFROST® PLUS SLIDES**.
3. Keep the sections at **-20°C** to dry for **~1 HR**.
4. Store the sections in slide boxes wrapped air-tight with aluminum foil or zip-lock bags at **-80°C** until use.

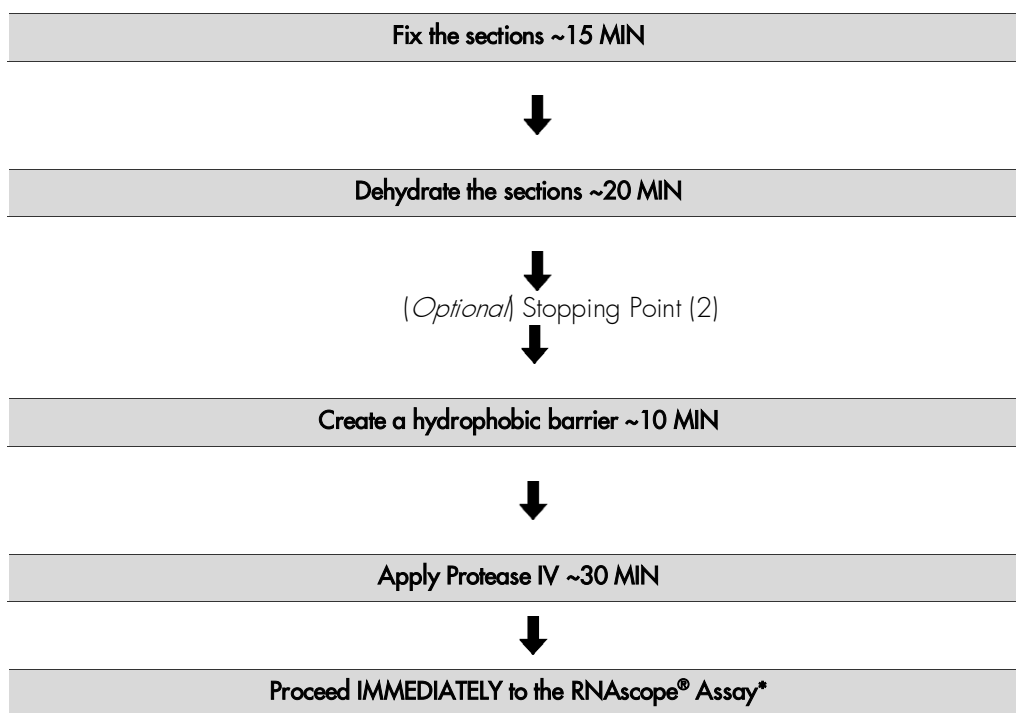
Note: Sections may be stored for up to three months.

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

OPTIONAL STOPPING POINT (1). Use sectioned tissue within three months.

Prepare the slides for the RNAscope® Assay

Workflow



* *Immediately* proceed to the RNAscope® Fluorescent Multiplex Detection Kit Part 2 User Manual, Document No. 320293-USM.

Materials required

- 1X PBS
 - 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA)
 - 100% ethanol
 - Tissue-Tek® Vertical 24 Slide Rack
 - Tissue-Tek® Staining Dishes (5 required)
 - ImmEdge™ Hydrophobic Barrier Pen
-

Fix the sections

1. Chill 200 mL 10% NBF (fresh 10% NBF or 4% PFA in 1X PBS) to **4°C** in a Tissue Tek® Staining Dish.
2. Remove slides from **-80°C**, and place in a Tissue Tek® Slide Rack.
3. *Immediately* immerse slides in the pre-chilled fixative. Fix for **15 MIN** at **4°C**.
4. Wash slides with 1X PBS by moving the rack up and down 3–5 times and repeat with 1X PBS.

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

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 600 mL 100% ethanol in Tissue Tek® Staining Dishes.
2. Place the slides in 50% ethanol for **5 MIN** at **ROOM TEMPERATURE (RT)**.
3. Place the slides in 70% ethanol for **5 MIN** at **RT**.
4. Place the slides in 100% ethanol for **5 MIN** at **RT**.
5. 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.

Create a hydrophobic barrier

1. Take slides out of 100% ethanol and place on absorbent paper with the section face-up. Air dry for **5 MIN** at **RT**.
2. Use the following template to draw a barrier 2–4 times around each section with the ImmEdge™ hydrophobic barrier pen. See example below (size of this hydrophobic barrier is 0.75" x 0.75").

Note: Refer to **Appendix A. Reagent Volume Guidelines** on page 15 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 amounts, even for smaller sections. Larger barriers will result in fewer tests per kit.

3. Let the barrier dry completely **~1 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.

Pretreat samples

IMPORTANT! Do not apply RNAscope® Hydrogen Peroxide or RNAscope® Target Retrieval to fresh, frozen sections when using the RNAscope® Fluorescent Multiplex Kit. Other sample types may need these treatments.

Materials required

Materials provided by the RNAscope® Reagent Kit	Other materials and equipment
<ul style="list-style-type: none"> Protease III & IV 	<ul style="list-style-type: none"> Prepared slides Distilled water Paper towel or absorbent paper HybEZ™ Humidifying System Tissue Tek® Slide Rack Tissue Tek® Staining Dish

Prepare the equipment

- Turn on HybEZ™ Oven and set temperature to **40°C**.
- Place a Humidifying Paper in the Humidity Control Tray and wet completely with distilled water.

Apply Protease IV

IMPORTANT! View the wash step video at www.acdbio.com/technical-support/learn-more before proceeding.

- Place the dried slides on the HybEZ™ Slide Rack, and add ~5 drops of Protease IV to entirely cover each section.
- Incubate for **30 MIN** at **RT**.

3. Take each slide one at a time from the HybEZ™ Slide Rack and tap and/or flick to remove the excess liquid. *Immediately* place each slide in a Tissue-Tek® Slide Rack submerged in a Tissue-Tek® Staining Dish filled with 1X PBS.
4. Wash slides with 1X PBS by moving the rack up and down 3–5 times and repeat with 1X PBS.

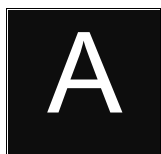
IMPORTANT! Slides should not stay in 1X PBS for longer than **15 MIN.**

Proceed to the RNAscope® Assay

Proceed *immediately* to the RNAscope® Multiplex Fluorescent Detection using the RNAscope® Multiplex Fluorescent Detection Kit User Manual PART 2 (Document No. 320293-USM). Ensure you have the appropriate user manual. User manuals are available at www.acdbio.com/technical-support/user-manuals. For any questions, please contact support@acdbio.com.

Troubleshooting



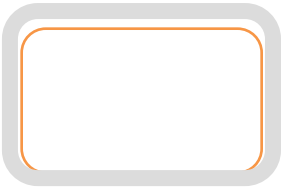
For troubleshooting information, please contact technical support at support@acdbio.com.



Appendix A. Reagent Volume Guidelines

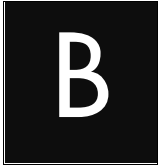
Determine reagent volume

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 ~20 sections. Larger tissue sections will result in fewer tests.



Appendix B. 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) before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see <https://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: : <https://www.cdc.gov/biosafety/>

- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§ 1910.1030), found at:
https://www.osha.gov/pls/oshaweb/owadisp.show_document?p_id=10051&p_table=STANDARDS
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at:
<https://www.cdc.gov/biosafety/>

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, found at:
http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/
- Information about the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) can be found at: **<https://echa.europa.eu/regulations/reach>**

Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available at: [https:// acdbio.com/technical-support/user-manuals](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

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Information: info@acdbio.com
Orders: orders@acdbio.com
Support Email: support@acdbio.com

Limited product warranty

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

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For support, email support@acdbio.com

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