

# RNAscope<sup>®</sup> Fluorescent Multiplex Reagent Kit

## PART 2

Document Number 320293-USM

For Research Use Only. Not for diagnostic use.

### **Trademarks**

RNAscope® and HybEZ™ are trademarks of Advanced Cell Diagnostics, Inc. All other trademarks belong to their respective owners.

### **Citing RNAscope® in Publications**

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

### **Disclaimers**

Advanced Cell Diagnostics, Inc. reserves the right to change its products and services at any time to incorporate technological developments. This manual is subject to change without notice.

Although this manual has been prepared with every precaution to ensure accuracy, Advanced Cell Diagnostics, Inc. assumes no liability for any errors, omissions, or for any damages resulting from the use of this information.

### **Copyright**

© 2019. Advanced Cell Diagnostics, Inc. All rights reserved.

# Contents

<b>Chapter 1. Product Information</b> .....	<b>5</b>
About this guide.....	5
Part 1 Sample Preparation.....	5
Product description.....	5
Background.....	5
Overview.....	5
Compatible sample types.....	6
Kit contents and storage.....	7
RNAscope® Probes.....	7
RNAscope® Fluorescent Multiplex Reagent Kit.....	8
Required materials and equipment.....	9
HybEZ™ Hybridization System.....	9
User-supplied materials.....	10
 <b>Chapter 2. Before You Begin</b> .....	 <b>11</b>
Important procedural guidelines.....	11
 <b>Chapter 3. RNAscope® Fluorescent Multiplex Assay</b> .....	 <b>12</b>
Workflow.....	12
Materials required for the assay.....	13
Prepare the materials.....	13
Prepare 1X Wash Buffer.....	13
Prepare probes.....	13
Equilibrate reagents.....	14
Load the slides into the RNAscope® EZ-Batch™ Slide Holder.....	14
Run the assay.....	15
Hybridize probe.....	15
Hybridize Amp 1-FL.....	16
Hybridize Amp 2-FL.....	16
Hybridize Amp 3-FL.....	17
Hybridize Amp 4-FL.....	17
Counterstain and mount the slides.....	17
Evaluate the samples.....	18
Fluorescent Imaging Recommendations.....	18
Control examples.....	19
Troubleshooting.....	19
 <b>Appendix A. Reagent Volume Guidelines</b> .....	 <b>20</b>
Determine reagent volume.....	20

<b>Appendix B. Safety .....</b>	<b>21</b>
Chemical safety .....	21
Biological hazard safety.....	21
In the U.S.: .....	22
In the EU:.....	22
<b>Documentation and support.....</b>	<b>23</b>
Obtaining SDSs .....	23
Obtaining support .....	23
Contact information .....	23
Limited product warranty .....	23

# 1

# Chapter 1. Product Information



Before using this product, read and understand the information in **Appendix B. Safety** on page 21 in this document.

---

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

---

## About this guide

This user manual provides guidelines and protocols to use the RNAscope® Fluorescent Multiplex Reagent Kit (Cat. No. 320850, and only describes RNA detection (**Part 2**). To prepare and pretreat your samples for RNA detection, refer to the following section.

## Part 1 Sample Preparation

Download one of these sample preparation user guides from <https://acdbio.com/technical-support/user-manuals>.

Sample type	Sample preparation and pretreatment guide
Fresh Frozen	Doc. No. 320513-USM
Fixed Frozen	Doc. No. 320535-TN
Cultured Adherent Cells	Doc. No. 320538-TN
Non - Adherent Cells	Doc. No. 46-005-TS

## Product description

### Background

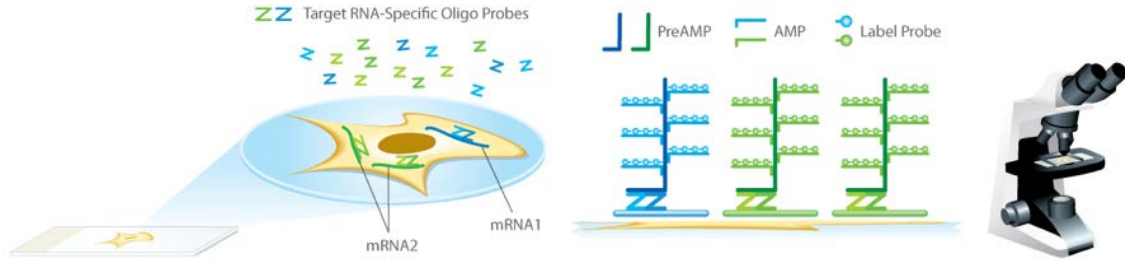
The RNAscope® Fluorescent Multiplex Assay uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize up to three different RNA targets per cell in samples mounted on slides. With the addition of a custom kit, you can also simultaneously detect four different RNA targets. 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

The RNAscope® Fluorescent Multiplex Assay procedure is illustrated in Figure 1 on page 6 and can be completed in six hours. Most of the RNAscope® Assay reagents are available in convenient Ready-To-Use

(RTU) dropper bottles and provide a simple, nearly pipette-free workflow. Starting with properly prepared samples, sections are first pretreated, and then RNA-specific probes designed for different fluorescent detection channels are hybridized to multiple target RNAs. After a series of highly effective and specific signal amplifications, single RNA transcripts for two or more target genes appear as punctate dots in two or more distinctly fluorescent channels. These dots are visible using a common fluorescent microscope with the appropriate filters.

**Figure 1. Procedure overview**



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

## Compatible sample types

The RNAscope® Fluorescent Multiplex Assay is compatible with fresh frozen (FF) tissue, cultured adherent cells on chamber slides, fixed frozen tissue, and peripheral blood mononuclear cells (PBMC).

Use the guide below to determine the appropriate pretreatment reagent from the Universal Pretreatment Reagents Kit (Cat No. 322380) or RNAscope® Protease II and IV Reagents (Cat. No. 322340).

Fluorescent Detection Pretreatment Guide		
Tissue Type	Pretreatment Reagent	Pretreatment Cat. No.
Fresh frozen	RNAscope® Protease IV	322336
Fixed frozen	RNAscope® Target Retrieval (10X)*	322001
	RNAscope® Protease III	322337
Non-adherent cells	RNAscope® Protease III	322337
Cultured adherent cells	RNAscope® Protease III	322337

\* RNAscope® Target Retrieval Reagents is not included in the RNAscope® Fluorescent Multiplex Reagent Kit. Please order it separately (Cat. No. 322000, includes quantity of 4, Cat. No. 322001).

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® Fluorescent Multiplex Assay requires the RNAscope® Probes and the RNAscope® Fluorescent Multiplex Reagent Kit. Probes and Reagent Kits are available separately.

## RNAscope® Probes

The RNAscope® Probes consist of user-specified Target Probes and Positive and Negative Control Probes. Visit <https://acdbio.com/products> to find a gene-specific target probe or appropriate control probes. Each target probe contains a mixture of short oligonucleotides designed to bind to a specific target RNA and detectable in one of three color channels, C1, C2, and C3 using the Amp 4 amplification step.

**Note:** Different colors are assigned to the C1, C2, and C3 color channels depending on the particular RNAscope® Assay. The color channels for the RNAscope® Fluorescent Multiplex Assay are shown in the following table:

Probe Channel ID	Amp 4 Alt A Fluorescent Label		
	Excitation	Emission	Color
C1*	Alexa 488 nm	540 ± 10 nm	GREEN
C2	Atto 550 nm	580 ± 10 nm	ORANGE
C3	Atto 647 nm	690 ± 10 nm	FAR RED

\* Default channel

C1 target probes are Ready-To-Use (RTU), while C2 and C3 probes are shipped as a 50X concentrated stock. To independently detect different target RNAs in a multiplex assay, each target probe must be in a different color channel and there must be a C1 probe in the mixture. If you wish to use only the C2 and/ or C3 probe, use the RNAscope® Probe Diluent (Cat. No. 300041) in place of the C1 probe.

---

**IMPORTANT!** C1 and C2 probes can be used for either fluorescent or chromogenic detection. However, C3-probes can only be used with the fluorescent kit.

---

There are three options for alternate fluorescent color modules. Any fluorescent label combinations (Amp 4 Alt A, B, or C) can be selected based on your experiment design.

Probe Channel ID	Color Module Options		
	Amp 4 Alt A-FL	Amp 4 Alt B-FL	Amp 4 Alt C-FL
C1	Alexa 488	Atto 550	Atto 550
C2	Atto 550	Alexa 488	Atto 647
C3	Atto 647	Atto 647	Alexa 488

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

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

## RNAscope® Fluorescent Multiplex Reagent Kit

Each RNAscope® Fluorescent Multiplex Reagent Kit (Cat. No. 320850) provides enough reagents to stain ~20 tissue sections ~20 sections, each with an area of approximately 20 mm x 20 mm (0.75" x 0.75"). Larger tissue sections will result in fewer tests. Each kit contains three sub-kits: a Pretreatment Kit, a Detection Kit, and a Wash Buffer Kit.

**IMPORTANT!** Directions to use the Pretreatment Kit are included in separate sample preparation and pretreatment user guides on page # 5.

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



Fluorescent Multiplex Detection Reagents (Cat. No. 320851)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	Amp 1-FL	3 mL x 1 bottle	2–8°C
	Amp 2-FL	4.5 mL x 1 bottle	2–8°C
	Amp 3-FL	3 mL x 1 bottle	2–8°C
	Amp 4-FL-Alt A Display module	4.5 mL x 1 bottle	2–8°C
	Amp 4-FL-Alt B Display module	4.5 mL x 1 bottle	2–8°C
	Amp 4-FL-Alt C Display module	4.5 mL x 1 bottle	2–8°C
	DAPI	3 mL x 1 bottle	2–8°C
Wash Buffer Reagents (Cat. No. 310091)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	50X Wash Buffer	60 mL x 4 bottles	Room temperature (15–30°C)

**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® Assay.

### HybEZ™ Hybridization System

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

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

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

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

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

## User-supplied materials

<input checked="" type="checkbox"/>	Description	Supplier	Cat. No.
	ProLong Gold Antifade Mountant	Thermo Fisher Scientific/MLS*	P36930
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWS2124
	Tissue-Tek® Staining Dishes	American Master Tech Scientific/MLS	LWS20WH
	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)	MLS	—

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

# 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 <https://acdbio.com/technical-support/learn-more>.
- Use an RNAscope® Fluorescent Multiplex Channel Assessment Slide (Cat. No. 310022) to ensure that the fluorescent microscope is properly equipped with the correct excitation and emission filter set.

### Important procedural guidelines

---

**IMPORTANT!** For **Part 1 Sample Preparation and Pretreatment**, download the relevant user guide. See page 5.

---

- Start with properly prepared sections. Refer to our sample preparation and pretreatment user guides available at <https://acdbio.com/technical-support/user-manuals>. Use only samples mounted on SuperFrost Plus® Slides (Fisher Scientific; Cat. No. 12-550-15).
- Follow the recommended pretreatment conditions for your sample. Refer to our sample preparation and pretreatment user guides available at <https://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.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix B. Safety** on page 21 for more information.

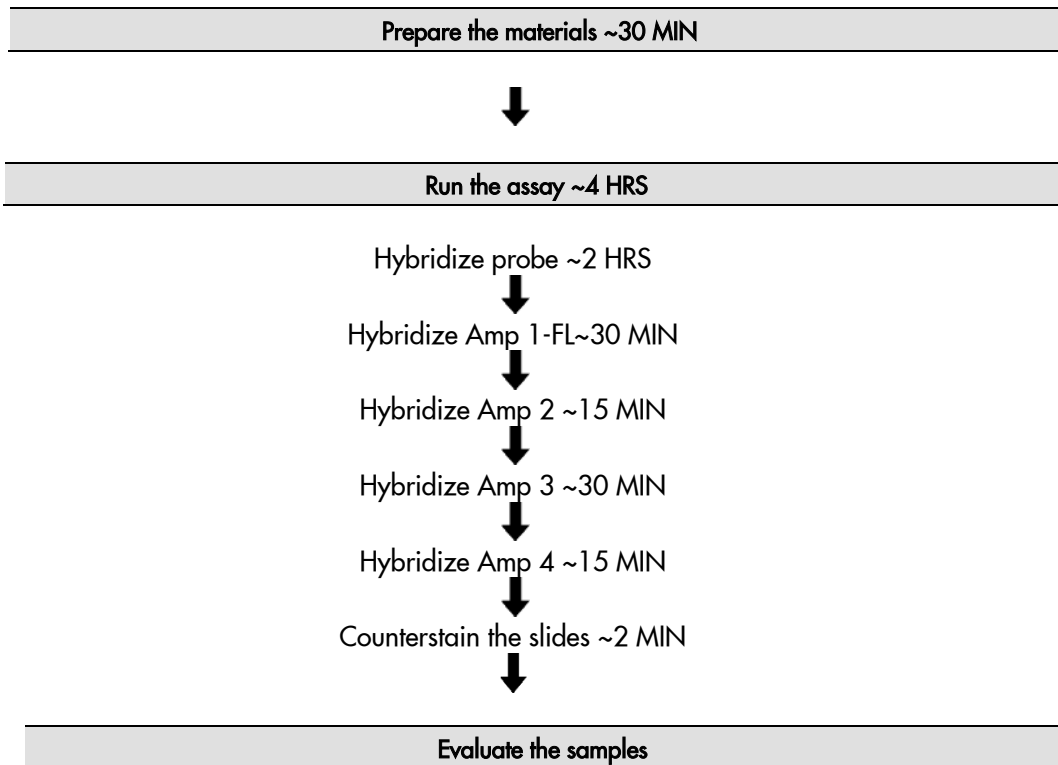
# 3

## Chapter 3. RNAscope® Fluorescent Multiplex Assay

**IMPORTANT!** For **Part 1 Sample Preparation and Pretreatment**, download the relevant user guide. See page 5.

This procedure flows directly from sample preparation and pretreatment. Refer to the appropriate sample preparation and pretreatment user guide for your specific sample type.

### Workflow



## Materials required for the assay

Materials provided by the RNAscope® Fluorescent Multiplex Kit	Materials provided by RNAscope® Probes	Other materials and equipment
<ul style="list-style-type: none"> <li>• 50X Wash Buffer</li> <li>• Amp 1-FL</li> <li>• Amp 2-FL</li> <li>• Amp 3-FL</li> <li>• Amp 4-FL-Alt A, Amp 4-FL-Alt B, or Amp 4-FL-Alt C</li> <li>• DAPI</li> </ul>	<ul style="list-style-type: none"> <li>• C1 Target Probe</li> <li>• 50X C2 Target Probe</li> <li>• 50X C3 Target Probe</li> <li>• 3-Plex Positive Control Probe</li> <li>• 3-Plex Negative Control Probe</li> </ul>	<ul style="list-style-type: none"> <li>• Prepared sections</li> <li>• Distilled water</li> <li>• Carboy (&gt;3L)</li> <li>• Tissue-Tek® Staining Dish</li> <li>• HybEZ™ Humidifying System/ RNAscope® EZ-Batch™ Slide Holder and Wash Tray</li> <li>• Water bath or incubator</li> <li>• Tissue-Tek® Vertical 24 Slide Rack</li> <li>• Tubes (various sizes)</li> <li>• Paper towel or absorbent paper</li> <li>• ProLong Gold Antifade Mountant</li> <li>• Cover Glass, 24 mm x 50 mm</li> </ul>

## Prepare the materials

You may prepare the reagents at the same time you prepare pretreatment reagents. Refer to a sample preparation and pretreatment user guide available at <https://acdbio.com/technical-support/user-manuals>.

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

### Prepare 1X Wash Buffer

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

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

### Prepare probes

1. Warm probes for **10 MIN** at **40°C** in a water bath or incubator, then cool to **ROOM TEMPERATURE (RT)**.
2. Briefly spin the C2 and C3 probes to collect the liquid at the bottom of the tubes.
3. Mix 1:1:50 ratios of C2, C3, and C1 probes by pipetting 1 volume of C2 and 1 volume of C3 probes to 50 volumes of C1 probe into a tube. Invert the tube several times.
4. If using only the C2 or C3 probe, dilute the probe with probe diluent (Cat. No. 300041). Probe diluent can be purchased separately.

**Note:** Do not mix probes of the same channel. The mixed Target Probes can be stored at **2–8°C** for up to 6 months.

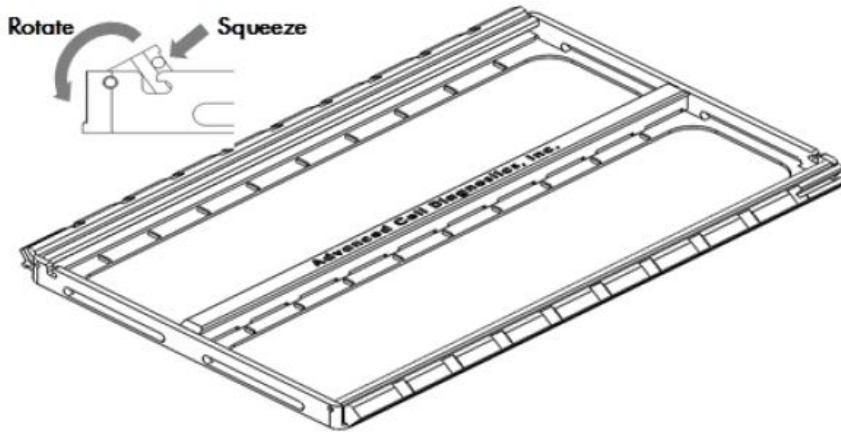
## Equilibrate reagents

- Place Amp 1–4 FL reagents at **RT**.
- Ensure HybEZ™ Oven and prepared Humidity Control Tray are at **40°C**.

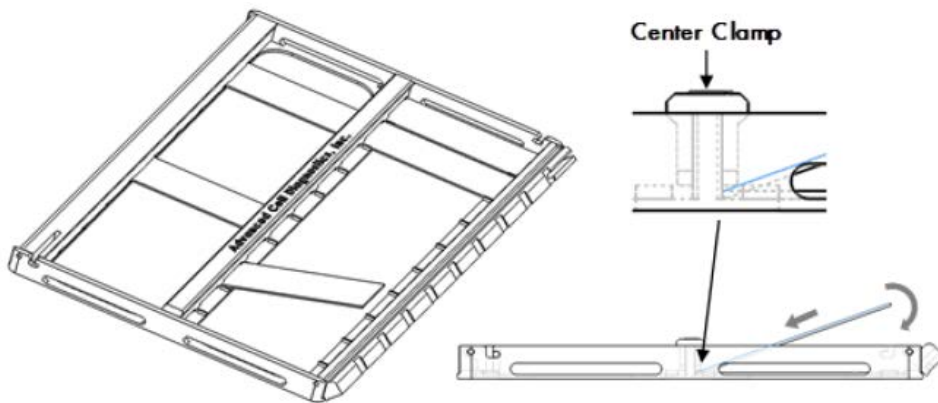
## Load the slides into the RNAscope® EZ-Batch™ Slide Holder

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

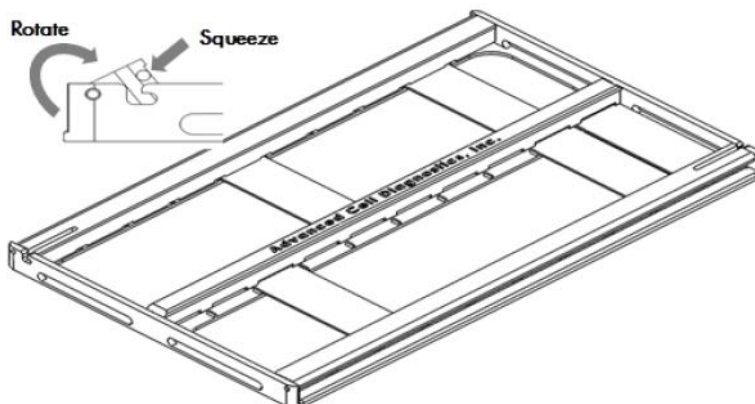
1. Open the swing clamps one at a time by simultaneously squeezing (pressing and holding) the slotted portion of each clamp and rotating it up then outwards.



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



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



## 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 probe on your sample before running any of your specific target probes to optimize the protocol.

## Hybridize probe

---

**IMPORTANT!** Prior to this step, ensure you have pretreated your samples according to the relevant user guide. See page 5.

---

**IMPORTANT!** Ensure probes are prewarmed and cooled to RT prior to use.

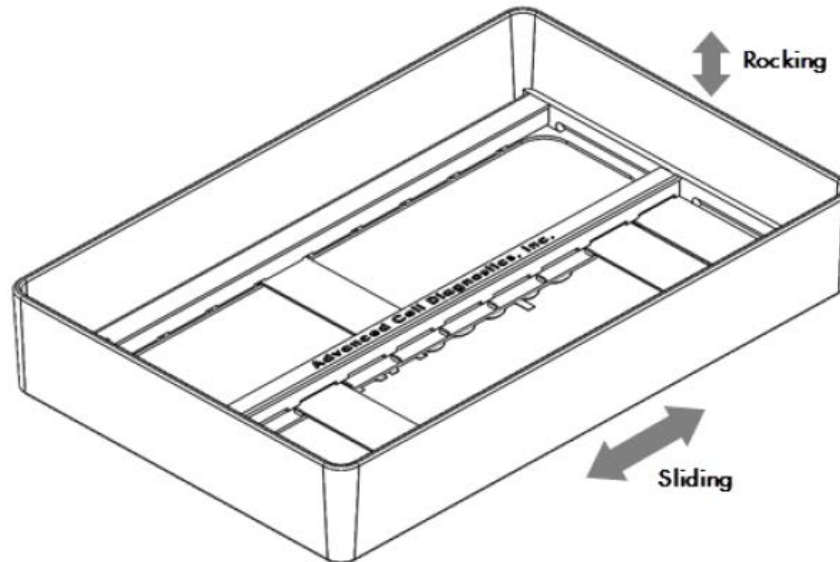
---

1. Remove excess liquid from the slides while keeping the slides locked in the RNAscope® EZ-Batch™ Slide Holder. Insert the slide holder into the HybEZ™ Humidity Control Tray.
2. Add ~4 drops of the appropriate probe mix to entirely cover each slide.

**Note:** Refer to **Appendix A. Reagent Volume Guidelines** on page 20 to determine the recommended number of drops needed per slide. For example, for a 0.75" x 0.75" barrier add 4 drops of the appropriate probe.

3. Close the tray and insert into the HybEZ™ Oven for **2 HRS** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent RNAscope® 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.

- Place the RNAscope® EZ-Batch™ Slide Holder into the wash tray containing 1X Wash Buffer. Make sure all the slides are submerged. If needed, carefully add more buffer. Wash the slides with slight agitation for **2 MIN** at **RT**.



- Repeat the wash step with fresh 1X Wash Buffer.

## Hybridize Amp 1-FL

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

## Hybridize Amp 2-FL

- Remove excess liquid from the slides while keeping the slides locked in the RNAscope® EZ-Batch™ Slide holder. Insert the slide holder into the HybEZ™ Humidity Control Tray.
- Add ~4 drops of Amp 2-FL to entirely cover each slide.
- Close the tray and insert into the HybEZ™ Oven for **15 MIN** at **40°C**.
- Pour at least 200 mL 1X Wash Buffer into the transparent RNAscope® EZ-Batch™ Wash Tray.
- Remove the HybEZ™ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
- Place the RNAscope® EZ-Batch™ Slide Holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.



## Hybridize Amp 3-FL

1. Remove excess liquid from the slides while keeping the slides locked in the RNAscope® EZ-Batch™ Slide holder. Insert the slide holder into the HybEZ™ Humidity Control Tray.
2. Add ~4 drops of Amp3-FL to entirely cover each slide.
3. Close the tray and insert into the HybEZ™ Oven for **30 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent RNAscope® 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.
9. Place the RNAscope® EZ-Batch™ Slide Holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.

## Hybridize Amp 4-FL

1. Remove excess liquid from the slides while keeping the slides locked in the RNAscope® EZ-Batch™ Slide holder. Insert the slide holder into the HybEZ™ Humidity Control Tray.

**Note:** There are three options for alternate fluorescent color modules. Choose one of the following fluorescent labels (Amp 4-FL-Alt A, B, or C).

2. Add ~4 drops of Amp 4-FL (A, B, or C) to entirely cover each slide.
3. Place the HybEZ™ Slide Rack in the HybEZ™ Humidity Control Tray. Close tray and insert into the oven for **15 MIN** at **40°C**.
4. Close the tray and insert into the HybEZ™ Oven for **15 MIN** at **40°C**.
5. Pour at least 200 mL 1X Wash Buffer into the transparent RNAscope® EZ-Batch™ Wash Tray
6. Remove the HybEZ™ Humidity Control Tray from the oven. Remove the slide holder from the tray.
7. Place the RNAscope® EZ-Batch™ Slide Holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.

## 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 the slides, and *immediately* place 1–2 drops of the ProLong Gold Antifade Mountant onto each section.
4. Carefully place a 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles. Store slides in the dark at **2–8°C**.

---

**IMPORTANT!** Image the slides after eight hours or within a few days.

---

## Evaluate the samples

For an example of successful staining, see Figure 2 on page 18. Examine tissue sections under a standard fluorescent microscope at 20–40X magnification. A confocal microscope may also be used:

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

## Fluorescent Imaging Recommendations

Here are a few fluorescent imaging recommendations:

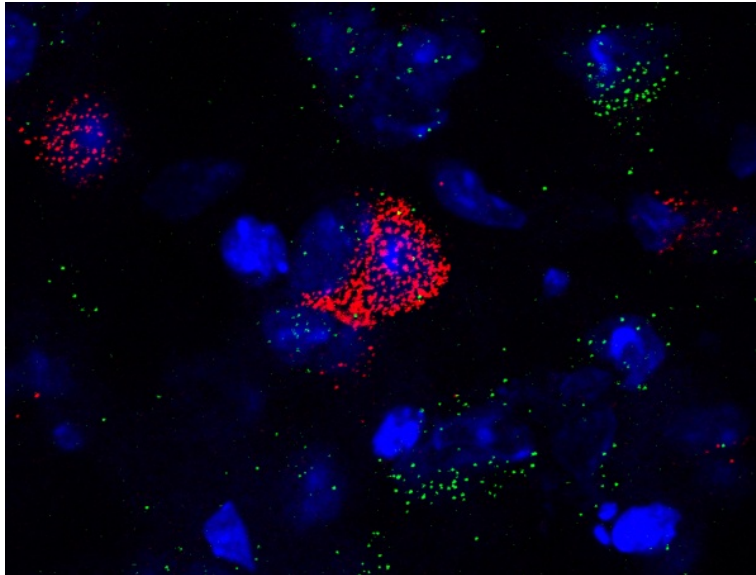
Viewing	Detection	Microscope	Optics
<ul style="list-style-type: none"> <li>• Image capture is the recommended digital capturing option</li> <li>• Fluorescence viewing is the recommended viewing option</li> </ul>	<ul style="list-style-type: none"> <li>• Microscope with camera and fluorescence options. 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>

**IMPORTANT!** The RNAscope® Fluorescent kits are primarily targeted for fresh frozen and cultured cells. This is mainly due to imaging and analysis challenges with interference from tissue autofluorescence. You can run the RNAscope® Fluorescent kit on solid tumor FFPE tissues if you have access to a multi-spectral imaging system, such as Nuance FX (Nuance). Solid tumors such as breast, colon, kidney, and liver have been successfully tested.

## Control example

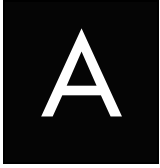
Figure 2 is an example of expression in the cerebral cortex of normal mouse brain.

**Figure 2.** *Npy* (red) and *Fezf2* (green) expression in the cerebral cortex of normal mouse brain stained using the RNAscope® Fluorescent Multiplex Kit; 63X oil lens, confocal image.



## Troubleshooting




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

# B

## 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) provided before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, <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](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/](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>. For the SDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

## Obtaining support

For the latest services and support information, go to: <https://acdbio.com/technical-support/support-overview>.

At the website, you can:

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

## Contact information

Advanced Cell Diagnostics, Inc.  
7707 Gateway Blvd Suite 200  
Newark, CA 94545  
Toll Free: 1-877-576-3636  
Direct: 1-510-576-8800  
Fax: 1-510-576-8801  
Information: [info.acd@bio-techne.com](mailto:info.acd@bio-techne.com)  
Orders: [orders.acd@bio-techne.com](mailto:orders.acd@bio-techne.com)  
Support Email: [support.acd@bio-techne.com](mailto:support.acd@bio-techne.com)

## Limited product warranty

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

**Headquarters**

7707 Gateway Blvd Suite 200, Newark, CA 94545

Phone 1-510-576-8800

Toll Free 1-877-576-3636

For support, email [support.acd@bio-technie.com](mailto:support.acd@bio-technie.com)

[www.acdbio.com](http://www.acdbio.com)

