

RNAscope[®] 2.5 HD Duplex Detection Kit (Chromogenic)

PART 2

Document Number 322500-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® Assay and cite: Wang F, Flanagan J, Su N, Wang L-C, 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 C** on page 26.

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® 2.5 HD Duplex Reagent Kit (Cat. No. 322430), 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
FFPE	Doc. No. 322452-USM
Fresh or flash frozen	Doc. No. 320536-TN
Fixed frozen	Doc. No. 320534-TN

Product description

Background

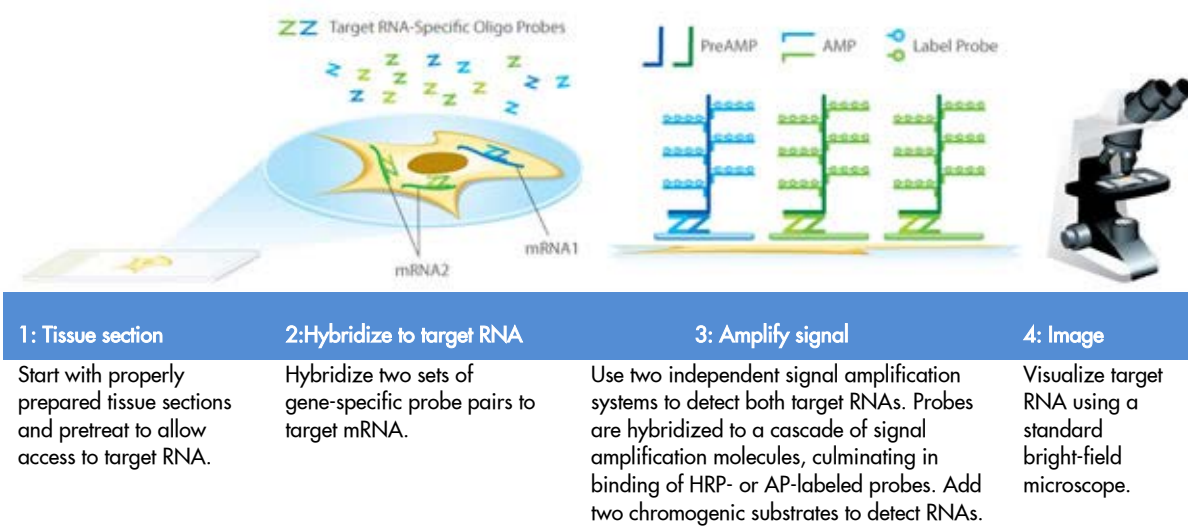
The RNAscope® 2.5 HD Duplex Chromogenic Assay uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize two RNA targets in samples mounted on slides. The assay is based on ACD's patented signal amplification and background suppression technology and incorporates multiplexed signal amplification systems, which enable users to investigate expression as well as positional relationship between two different genes within a cellular context.

Overview

Figure 1 on page 6 illustrates the RNAscope® 2.5 HD Duplex Chromogenic Assay procedure, which can be completed in 12–14 hours or conveniently divided over two days. 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 mounted on glass are first pretreated, and then RNA-specific probes are hybridized to target RNA. The RNAscope® 2.5 HD Duplex Chromogenic Assay employs two independent signal amplification systems, each using a different chromogenic enzyme. Single RNA transcripts for two target genes appear as punctate dots of two distinctly colored chromogen precipitates, visible using a common bright-field microscope at 40–100X magnification.

Figure 1 Procedure overview



Kit contents and storage

The RNAscope® 2.5 HD Duplex Chromogenic Assay requires the RNAscope® Probes and the RNAscope® 2.5 HD Duplex Reagent Kit, 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 two color channels, C1 or C2.

Probe Channel ID	Chromogenic Labels	
	Enzyme	Color
C1*	HRP	GREEN
C2	AP	RED

* Default channel

Channel C1 target probes are Ready-To-Use, while channel C2 probes are shipped as a 50X concentrated stock. To independently detect two target RNAs in a duplex 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 the C2 probe, use the RNAscope® Probe Diluent (Cat. No. 300041) in place of the C1 probe.

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

Target Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® Kit 2.5 HD Duplex Target Probe – [<i>species</i>]- [<i>gene</i>]*	Various	Ready-To-Use (RTU) probe for color channel C1	3 mL x 1 bottle	2–8 °C
	RNAscope® Kit 2.5 HD Duplex Target Probe – [<i>species</i>]- [<i>gene</i>]- C2	Various	50X probe for color channel C2	60 µL x 1 tube	2–8 °C
Control Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® Kit 2.5 HD Duplex Positive Control Probe	Various	RTU mixture of two probes targeting PPIB in channel C1 and POLR2A in color channel C2.	3 mL x 1 bottle	2–8 °C
	RNAscope® Kit Negative Control Probe – <i>dapB</i>	310043	RTU probe targeting a bacterial gene. Each detection channel has its own negative control probe.	3 mL x 1 bottle	2–8 °C
	RNAscope® Probe Diluent	300041	RTU Target Probe diluent	3 mL x 1 bottle	2–8 °C

* No "C1" in label.

RNAscope® 2.5 HD Duplex Reagent Kit

Each RNAscope® 2.5 HD Duplex Reagent Kit (Cat. No. 322430) provides enough reagents to stain ~20 tissue sections, each with an area of approximately 20 mm x 20 mm (0.75" x 0.75"). Larger tissue sections will result in fewer tests. Each kit contains: RNAscope® H₂O₂ & Protease Plus Reagents, RNAscope® Target Retrieval Reagents, RNAscope® Duplex Detection Reagents, and RNAscope® Wash Buffer Reagents.

IMPORTANT! Directions to use RNAscope® H₂O₂ & Protease Plus Reagents and RNAscope® Target Retrieval Reagents are included in separate sample preparation and pretreatment user guides.

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

RNAscope® H ₂ O ₂ & Protease Plus Reagents (Cat. No. 322330) & Target Retrieval (Cat. No. 322000)				
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Quantity	Storage
	RNAscope® Hydrogen Peroxide	322335	3 mL x 2 bottles	2–8°C
	RNAscope® Protease Plus	322331	4.5 mL x 1 bottle	2–8°C
	RNAscope® Target Retrieval Reagents	322000	70 mL x 4 bottles	Room temperature (15–30°C)

RNAscope® 2.5 HD Duplex Detection Kit (Cat. No. 322500)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	2.5 HD Duplex Amp 1	3 mL x 1 bottle	2–8°C
	2.5 HD Duplex Amp 2	4.5 mL x 1 bottle	2–8°C
	2.5 HD Duplex Amp 3	3 mL x 1 bottle	2–8°C
	2.5 HD Duplex Amp 4	4.5 mL x 1 bottle	2–8°C
	2.5 HD Duplex Amp 5	4.5 mL x 1 bottle	2–8°C
	2.5 HD Duplex Amp 6	3 mL x 1 bottle	2–8°C
	2.5 HD Duplex Amp 7	3 mL x 1 bottle	2–8°C
	2.5 HD Duplex Amp 8	4.5 mL x 1 bottle	2–8°C
	2.5 HD Duplex Amp 9	4.5 mL x 1 bottle	2–8°C
	2.5 HD Duplex Amp 10	3 mL x 1 bottle	2–8°C
	Red-A — Fast Red diluent	4 mL x 1 bottle	2–8°C
	Red-B — Fast Red substrate	50 µL x 1 tube	2–8°C
	Green-A — Green diluent	3 mL x 1 bottle	2–8°C
	Green-B — Green substrate	60 µL x 1 vial	2–8°C
Wash Buffer Kit (Cat. No. 310091)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	50X Wash Buffer	60 mL x 4 bottles	Room temperature (15–30°C)

IMPORTANT! RNAscope® Detection Kits share the same Pretreatment Kit and Wash Buffer, but have unique 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 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	—

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

User-supplied materials

IMPORTANT! Do not substitute other materials for the VectaMount listed in the following table.

<input checked="" type="checkbox"/>	Description	Supplier	Cat. No.
	VectaMount Permanent Mounting Medium (required)	Vector Labs	H-5000
	100% alcohol (EtOH)	American Master Tech Scientific/MLS*	ALREAGAL
	Gill's Hematoxylin I	American Master Tech Scientific/MLS	HXGHE1LT
	Xylene	Fisher Scientific/MLS	X3P-1GAL
	20X SSC (final concentration needed 5X)	Sigma-Aldrich/MLS	S6639-1L
	Tissue-Tek® Vertical 24 Slide Racks	American Master Tech Scientific/MLS	LWS2124
	Tissue-Tek® Staining Dishes	American Master Tech Scientific/MLS	LWS20WH
	Tissue-Tek® Clearing Agent Dishes, xylene resistant	American Master Tech Scientific/MLS	LWS20GR
	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	—
	Tubes (various sizes)	MLS	—
	Fume hood	MLS	—
	Graduated cylinder	MLS	—
	Parafilm	MLS	—
	Paper towel or absorbent paper	MLS	—
	Microcentrifuge	MLS	—
	Microscope and accessories	MLS	—
	Drying oven, capable of holding temperature at 60 +/- 1 °C	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

IMPORTANT! Due to the length of this procedure (~14 hours), we recommend you pause the procedure after probe hybridization. Slides should be stored in 5X SSC at RT for overnight.

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>.
- Run the assay on FFPE Control Slides (Cat. No. 310045) for human control slide, HeLa; Catalog No. 310023 for mouse control slide, 3T3) using positive and negative control probes.

Important procedural guidelines

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

- Start with properly fixed and prepared sections. Refer to **Appendix A. Tissue Pretreatment Recommendation** on page 23 and to our sample preparation and pretreatment user guides available at <http://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 optimal results.
- Do not let your sections dry out during the procedure.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix C. Safety** on page 26 for more information.

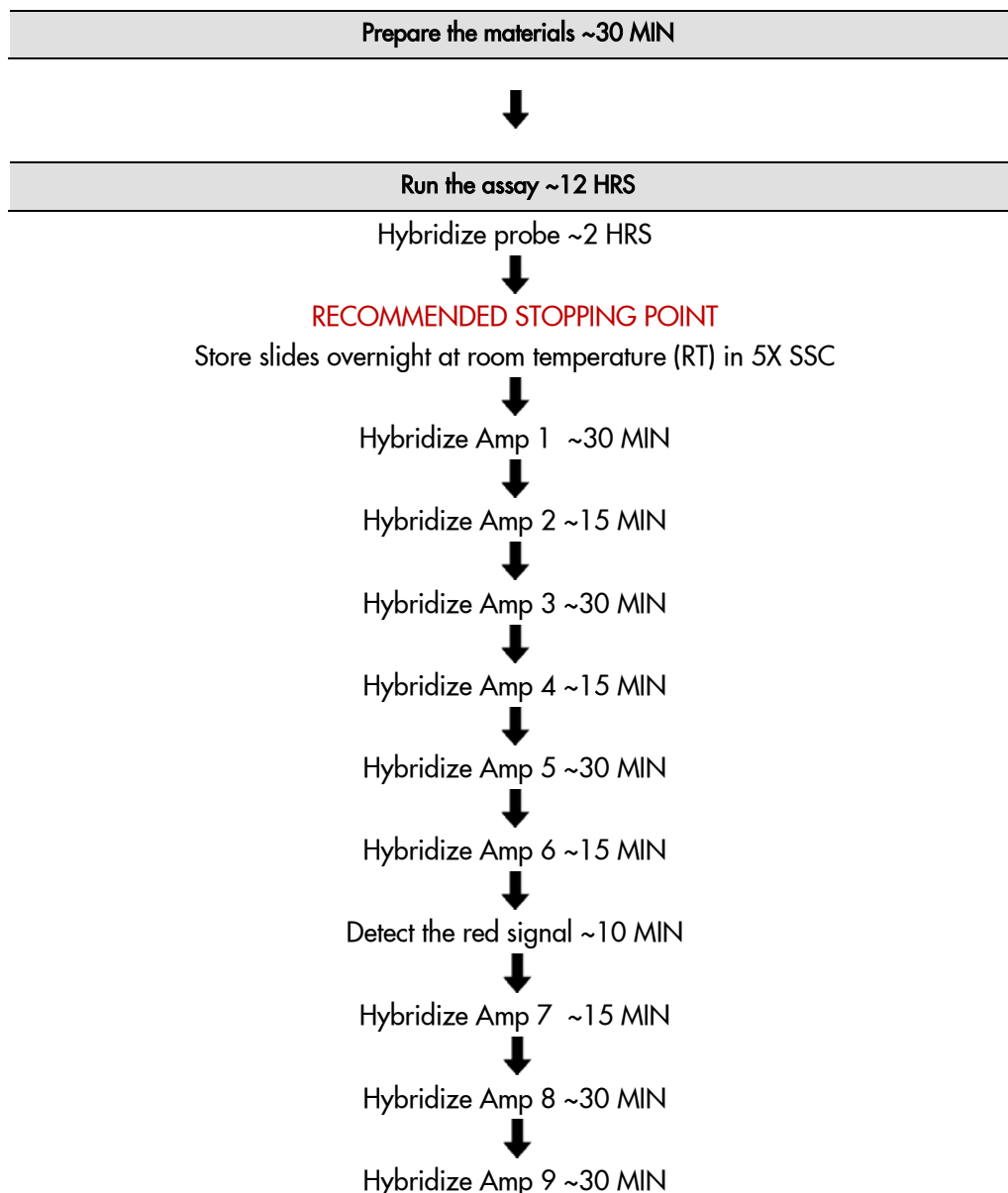
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Chapter 3. RNAscope® 2.5 HD Duplex Assay

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





Run the assay (continued)

Hybridize Amp 10 ~15 MIN



Detect the green signal ~10 MIN



Counterstain the slides ~2 MIN



Bake slides ~15 MIN



Mount samples ~5 MIN



Evaluate the samples

Materials required for the assay

Materials provided by the RNAscope® 2.5 HD Duplex Reagent Kit	Materials provided by RNAscope® Probes	Other materials and equipment
<ul style="list-style-type: none"> • 50X Wash Buffer • 2.5 HD Duplex Amp 1 • 2.5 HD Duplex Amp 2 • 2.5 HD Duplex Amp 3 • 2.5 HD Duplex Amp 4 • 2.5 HD Duplex Amp 5 • 2.5 HD Duplex Amp 6 • 2.5 HD Duplex Amp 7 • 2.5 HD Duplex Amp 8 • 2.5 HD Duplex Amp 9 • 2.5 HD Duplex Amp 10 • Fast Red-A • Fast Red-B • Green-A • Green-B 	<ul style="list-style-type: none"> • C1 Target Probe • 50X C2 Target Probe • 2.5 HD Duplex Positive Control Probe • 2-Plex Negative Control Probe 	<ul style="list-style-type: none"> • Prepared sections • Distilled water • Carboy (>3L) • Fume hood • Xylene • 100% alcohol (EtOH) • Tissue-Tek® Staining Dishes • Tissue-Tek® Clearing Agent Dishes, xylene-resistant • Gill's Hematoxylin I • Ammonium hydroxide, 28–30% • 20X SSC (dilute to 5X SSC) • Graduated cylinder • Parafilm • HybEZ™ Humidifying System/ RNAscope® EZ-Batch™ Slide Holder and Wash Tray • Water bath or incubator • Tissue-Tek® Vertical 24 Slide Rack • Tubes (various sizes) • Paper towel or absorbent paper • Pipettors and tips, 1–1000 µL • Microcentrifuge • Dry oven • VectaMount • Cover Glass, 24 mm x 50 mm

Prepare the materials

You may prepare the reagents at the same time you prepare pretreatment reagents. Refer to a sample preparation and pretreatment user 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 the materials for day one

Prepare 1X Wash Buffer

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

Note: Warm 50X Wash Buffer up to **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 (**RT**) for up to one month.

Prepare 20X SSC

1. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800ml of distilled H₂O.
2. Adjust the pH to 7.0 with a few drops of 1M HCl.
3. Adjust the volume to 1 L with additional distilled H₂O.
4. Sterilize by autoclaving.

Prepare 5X SSC

- Prepare 200 mL of 5X SSC by diluting 50 mL of 20X SSC with 150 mL distilled water. Mix well.

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

Prepare probes

1. Warm probes for at least **10 MIN** at **40°C** in a water bath or incubator.
2. Briefly spin the C2 probe to collect the liquid at the bottom of the tubes.
3. Mix 1:50 ratio of C2 probe to C1 probe by pipetting 1 volume of C2 probe to 50 volumes of C1 probe into a tube. Invert the tube several times.

Note: Do not mix probes of the same color channel. The mixed target probes can be stored at **4°C** for up to six months.

Equilibrate reagents

- Remove Amp 1–10 reagents from refrigerator.
- Ensure HybEZ™ Oven and prepared Humidity Control Tray are at **40°C**.
- Before each use, warm the Target and/or Control Probes for **10 MIN** at **40°C** in a water bath or incubator.

Prepare the materials for day two

Prepare counterstaining reagents

1. In the fume hood, prepare 50% Hematoxylin staining solution by adding 100 mL Gill's Hematoxylin I to 100 mL distilled water in a staining dish.

Note: 50% Hematoxylin staining solution can be reused for up to one week.

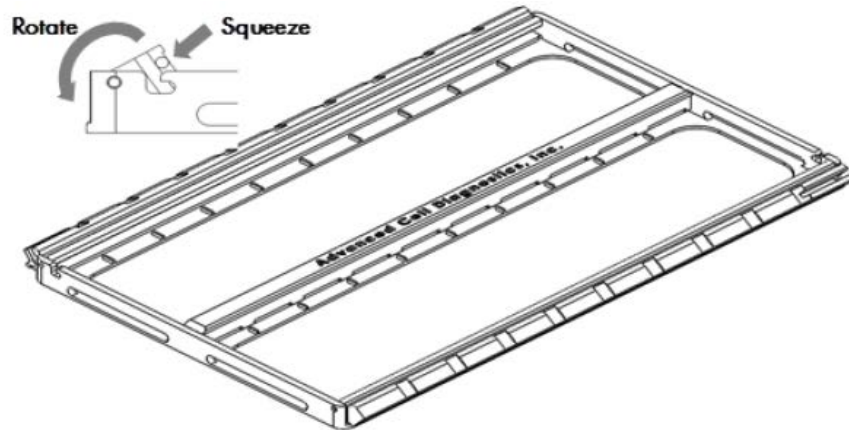
2. In the fume hood, prepare 0.02% (w/v) Ammonia water (bluing reagent) by diluting the 28% Ammonium Hydroxide with distilled water in a graduated cylinder or other container.
3. Seal the cylinder with parafilm. Mix well 3–5 times.

Note: For assay quantitation, you must use Ammonium Hydroxide.

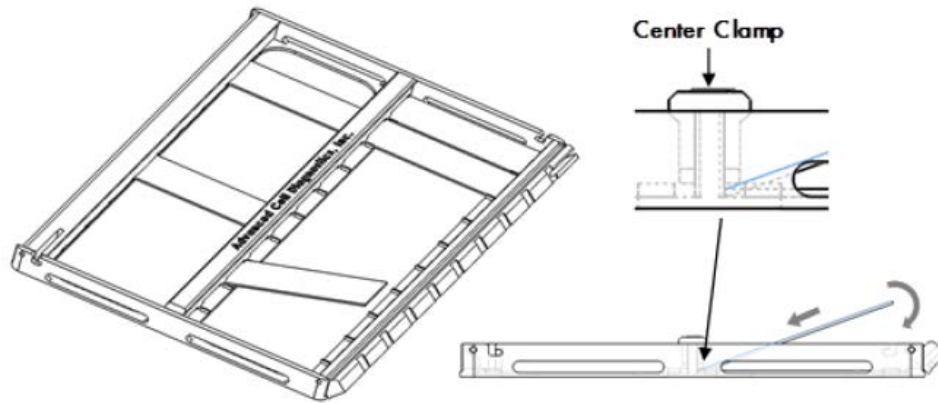
Load the slides into the RNAscope® EZ-Batch™ Slide Holder

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.

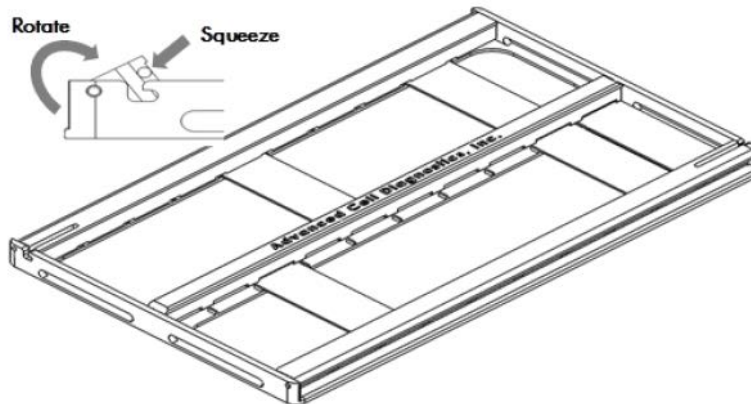
1. Open the swing clamps one at a time by simultaneously squeezing (pressing and holding) the slotted portion of each clamp and rotating it up then outwards.



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

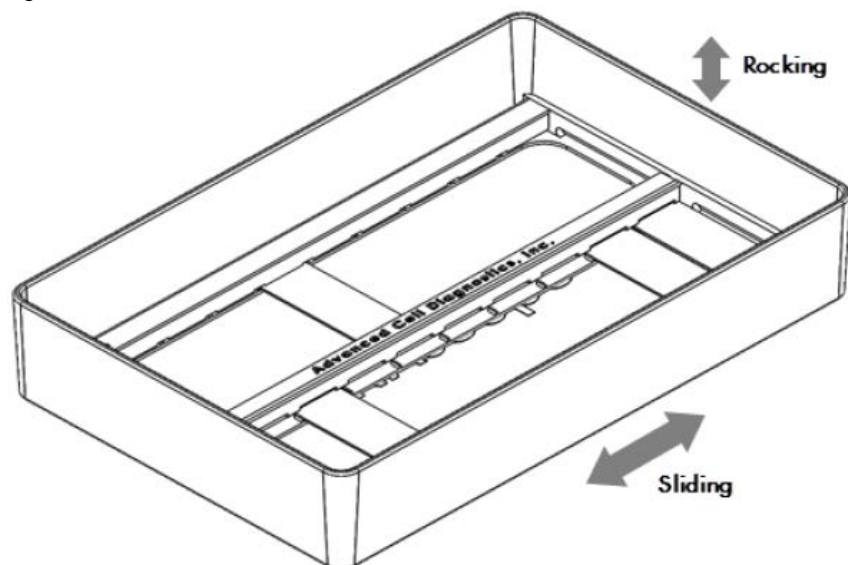
IMPORTANT! Do **NOT** let sections dry out between incubation steps. Work *quickly* and fill barrier with solutions.

IMPORTANT! View the wash step video at <https://acdbio.com/technical-support/learn-more> before proceeding.

Hybridize probe

IMPORTANT! Ensure probes are prewarmed to dissolve any precipitation 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 C. Reagent Volume Guidelines** on page 25 to determine the recommended number of drops needed per slide. For example, add 4 drops of the appropriate probe to a 0.75" x 0.75" barrier.
3. Close the tray and insert into the HybEZ™ Oven for **2 HRS** at **40°C**.
4. 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.
6. 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**.



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

RECOMMENDED STOPPING POINT. Keep slides overnight in 5X SSC at room temperature (**RT**).

Hybridize Amp 1

1. Remove the slides from 5X SSC, and wash them in 1X Wash Buffer 1–2 times.
Note: You can keep the slides in the RNAscope® EZ-Batch™ Slide Holder.
2. 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.

3. Add ~4 drops of AMP 1 to entirely cover each slide.
4. Close the tray and insert into the HybEZ™ Oven for **30 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. Place the tray back into the oven.
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.

Hybridize Amp 2

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 AMP 2 to entirely cover each slide.
3. Close the tray and insert into the HybEZ™ Oven for **15 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent 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.
6. 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

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 AMP 3 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.
6. 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

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 AMP 4 to entirely cover each slide.
3. Close the tray and insert into the HybEZ™ Oven for **15 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent RNAscope® EZ-Batch™ Wash Tray.
5. Remove the HybEZ™ Humidity Control Tray from the oven. Remove the slide holder from the tray.

IMPORTANT! Do not insert the tray into the HybEZ™ Oven for the next two AMP hybridization steps

6. 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 5

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 AMP 5 to entirely cover each slide.

3. Close the tray and incubate for **30 MIN** at **RT**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent RNAscope® EZ-Batch™ Wash Tray.
5. Remove the HybEZ™ Slide Rack from the HybEZ™ Humidity Control Tray.
6. 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 6

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 AMP 6 to entirely cover each slide.
3. Close the tray and incubate for **15 MIN** at **RT**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent RNAscope® EZ-Batch™ Wash Tray.
5. Remove the HybEZ™ Slide Rack from the HybEZ™ Humidity Control Tray.
6. 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.

Detect the red signal

1. Briefly spin down the contents of the Red-B tube to be sure that the contents are at the bottom of the tube before opening the cap.
2. Depending on the size of your hydrophobic barrier, make RED working solution per section by using a 1:60 ratio of Red-B to Red -A. For example, for a 0.75" x 0.75" barrier, add 2.5 µL of Red-B to 150 µL of Red-A into a tube. Mix well.

IMPORTANT! Use the RED solution within **5 MIN**. Do not expose to direct sunlight or UV light.

3. Remove excess liquid from the slides while keeping them locked in the EZ-Batch™ Slide holder. Insert the slide holder into the HybEZ™ Humidity Control Tray.
4. Pipette ~150 µL RED solution onto each tissue section. Ensure that the sections are covered.
5. Close the tray and incubate for **10 MIN** at **RT**.
6. Pour at least 200 mL 1X Wash Buffer into the transparent RNAscope® EZ-Batch™ Wash Tray.
7. Remove the HybEZ™ Slide Rack from the HybEZ™ Humidity Control 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.

Hybridize Amp 7

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 AMP 7 to entirely cover each slide.
3. Close the tray and insert into the HybEZ™ Oven for **15 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent 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.
6. 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 8

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 AMP 8 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.

IMPORTANT! Do not insert the tray into the HybEZ™ Oven for the rest of the procedure.

6. 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 9

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 AMP 9 to entirely cover each slide.
3. Close the tray and insert into the HybEZ™ Oven for **30 MIN** at **RT**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent RNAscope® EZ-Batch™ Wash Tray.
5. Remove the HybEZ™ Slide Rack from the HybEZ™ Humidity Control Tray.
6. 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 10

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 AMP 10 to entirely cover each slide.
3. Close the tray and insert into the HybEZ™ Oven for **15 MIN** at **RT**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent RNAscope® EZ-Batch™ Wash Tray.
5. Remove the HybEZ™ Slide Rack from the HybEZ™ Humidity Control Tray.
6. 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.

Detect the green signal

1. Briefly spin down the contents of the Green-B tube to be sure that the contents are at the bottom of the tube before opening the cap.
2. Depending on the size of your hydrophobic barrier, make GREEN working solution per section by using a 1:50 ratio of Green-B to Green-A. For example, for a 0.75" x 0.75" barrier, add 3 µL of Green-B to 150 µL of Green-A into a tube. Mix well.

IMPORTANT! Use the GREEN solution within **5 MIN**. Do not expose to direct sunlight or UV light.

3. Remove excess liquid from the slides while keeping them locked in the EZ-Batch™ Slide holder. Insert the slide holder into the HybEZ™ Humidity Control Tray.
4. Pipette ~150 µL GREEN solution onto each tissue section. Ensure that the sections are covered.
5. Close the tray and incubate for **10 MIN** at **RT**.
6. Pour at least 200 mL 1X Wash Buffer into the transparent RNAscope® EZ-Batch™ Wash Tray.
7. Remove the HybEZ™ Slide Rack from the HybEZ™ Humidity Control Tray.

8. Place the RNAscope® EZ-Batch™ Slide Holder into the wash tray, and wash the slides for **5 MIN** at **RT** with slight agitation.
9. Rinse the slides quickly in water to remove excess wash buffer.

IMPORTANT! Proceed quickly to the next step. Green substrate may fade if stored in water for too long.

Counterstain the slides

1. Place the slides into a Tissue-Tek® Slide Rack, then move the rack into the staining dish containing 50% Hematoxylin staining solution for **30 SEC** at **RT**. Slides turn purple.

IMPORTANT! Proceed quickly to the next step. Green substrate may fade if in Hematoxylin for longer than 30 seconds.

2. *Immediately* transfer the slide rack into a staining dish filled with tap water. Do not let the slides remain in the water for more than 30 seconds. Repeat wash step once or twice.
3. Replace tap water in the staining dish with 0.02% ammonia water. Move the rack up and down five times. Sections should turn blue.
4. Replace ammonia water with tap water, and wash the slides 3–5 times.

IMPORTANT! Use *only* 0.02% ammonia water for bluing step. Commercial bluing solutions may degrade the green signal.

IMPORTANT! Follow these instructions closely. Extended exposure to water and ammonia water can degrade the green signal.

Mount the samples

1. Remove the slide rack from the staining dish, and dry the slides in a **60°C** dry oven for **15–30 MIN**.

IMPORTANT! The GREEN and RED substrates are alcohol sensitive. Do not dehydrate the slides in alcohol.

2. Cool the slides for **5 MIN** at **RT**.

IMPORTANT! Use the VectaMount mounting medium only.

3. Briefly dip the slide into FRESH pure xylene and *immediately* place 1–2 drops of VectaMount Mounting Medium on the slide before the xylene dries.
4. Carefully place a 24 mm x 50 mm coverslip over the section. Avoid trapping air bubbles.
5. Repeat steps 3 and 4 for each slide.
6. Air dry slides for **≥5 MIN**.

Evaluate the samples

Examine tissue sections under a standard bright field microscope at 20–40X magnification:

- Assess tissue and cell morphology.
- Assess positive control signal strength. Positive control signal should be visible as punctuate dots within the cell cytoplasm at 20–40X magnification.
- Assess negative control background. One dot to every 10 cells displaying background staining per 20X microscope field is acceptable.
- Evaluate the target probe signal using the scoring guidelines in the next section.

Scoring guidelines

The RNAscope® Assay enables a semi-quantitative scoring guideline by using the estimated number of punctate dots present within each cell boundary. An example of how to develop such a guideline for semi-quantitative assessment of RNAscope® staining intensity is presented below for a gene with expression level varying between 1 to >10 copies per cell. If your gene expression level is higher or lower than this range, you may need to scale the criteria accordingly.

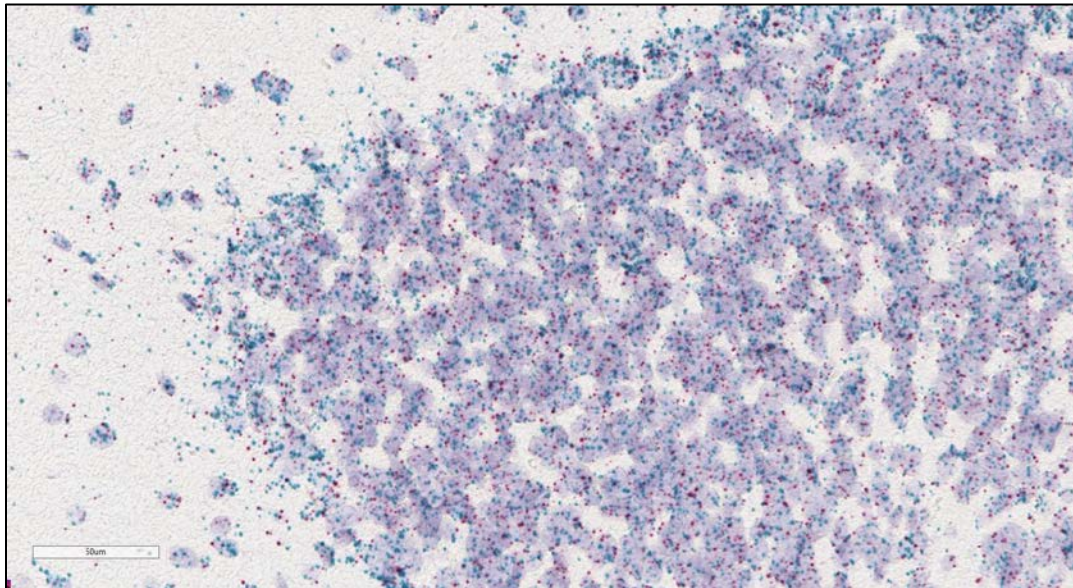
Categorize staining into five grades: **0**, **1+**, **2+**, **3+**, and **4+** according to the following table:

Staining score	Microscope objective scoring*
0	No staining or less than 1 dot in every ten cells (40X magnification)
1	1–3 dots/cell (visible at 20–40X magnification)
2	4–9 dots/cell. Very few dot clusters (visible at 20–40X magnification)
3	10–15 dots/cell and / or <10% positive cells have dots in clusters (visible at 20X magnification)
4	>15 dots/cell and / or >10% positive cells have dots in clusters (visible at 20X magnification)

* Discount cells with artificially high nuclear background staining.

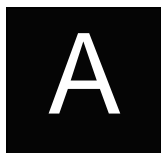
Control example

Figure 2. RNAscope® 2.5 Duplex detection of PPIB (green) and POLR2A (red) mRNA in Brain FFPE tissue at 40X magnification.



Troubleshooting

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



Appendix A. Tissue Pretreatment Recommendation

Follow the recommended pretreatment conditions based on your tissue type for:

- Any new or previously untested FFPE tissue types
- Samples prepared differently than the sample preparation protocol found in *Part 1, Sample Preparation and Pretreatment Guide for FFPE Tissue* (Doc. No. 322452-USM).

Pretreatment recommendations for FFPE tissues

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 Target Retrieval and/or Protease Plus times (see the following table).

Reagent	Mild	Standard	Extended
Target Retrieval	15 MIN	15 MIN	30 MIN
Protease Plus	15 MIN	30 MIN	30 MIN

Note: Sample types such as certain Xenografts and Cell Pellet, require less time. For these tissue types, vary the Hydrogen Peroxide time to **8 MIN** and Protease Plus time to **15 MIN**. For the ACD Cell Pellet sample, we recommend a target retrieval time of **15 MIN** and an RNAscope® Protease Plus time of **15 MIN**. If you have a tissue type not listed, contact support at support.acd@bio-techne.com.

Tissue-specific pretreatment conditions

If your sample fixation is successful in fresh 10% NBF (Step 2 above), then refer to the following table for tissue-specific pretreatment conditions. For information about species or tissue type not listed here, contact support at support.acd@bio-techne.com.

Species	Tissue Type	Pathology	Pretreatment Condition
Mouse/Rat	Intestine	Normal	Standard
	Intestine	Tumor	Standard
	Embryo	Normal	Standard
	Brain	Normal	Standard
	Spleen	Normal	Mild
	Eye/Retina	Normal	Standard/Mild
Mouse/Rat	Liver	Normal	Extended
	Kidney	Normal	Standard

Species	Tissue Type	Pathology	Pretreatment Condition
Human	Breast	Tumor	Standard
	Colon	Tumor	Standard
	Colon	Normal	Standard
	Lung	Tumor	Standard
	Lung	Normal	Standard
	Prostate	Tumor	Standard
	Prostate	Normal	Standard
	Lymph node	Tumor	Mild
	Lymph node	Normal	Mild
	Tonsil	Normal	Mild
	Pancreas	Normal	Standard
	Cervical	Cancer	Standard
	Cervical	Normal	Standard
	Cervical dysplasia	Abnormal	Standard
	Brain	Tumor	Standard
	Brain	Normal	Standard
	Head	Cancer	Standard
	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
HeLa (ACD controls) or Cell pellets, fixed with 10% NBF or 10% Formaldehyde	—	Mild	




* Tissue Microarray

B

Appendix B. 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 C. 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, 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>. For the SDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

Obtaining support

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

At the website, you can:

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

Contact information

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

Limited product warranty

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

Headquarters

7707 Gateway Blvd Suite 200, Newark, CA 94560

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