**USER MANUAL**

# RNAscope® 2-Plex Detection Kit (Chromogenic) User Manual
## PART 2

**Catalog Number** 320494

*For **Part 1** Sample Preparation Pretreatment Guide for Formalin-Fixed Paraffin-Embedded (FFPE), see **Catalog Number** 320511.*

*For Molecular Biology Applications, not intended for diagnosis.*

Rev. Date 20131220

**For Molecular Biology Applications, not intended for diagnosis.**

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**Citing RNAscope® 2.0 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 L-C, Bui S, Nielson A, Wu X, Vo H-T, Ma X-J and Luo Y. RNAscope®: A Novel *In Situ* RNA Analysis Platform for Formalin-Fixed Paraffin-Embedded Tissues. J. Mol. Diagnostics, 2012, 14:22–29.

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# Contents

# 1 Chapter 1. Product Information


Before using this product, read and understand the information in **Appendix C. Safety** on page 25.

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-Plex Detection Kit – Chromogenic (Cat. No. 310035). RNAscope® Assays are compatible with a variety of sample types. You must use both an RNAscope® Detection kit user manual and a sample preparation and pretreatment user guide to perform the entire assay.

IMPORTANT! For *Part 1, Sample Preparation and Pretreatment Guide for FFPE Tissue*, see Catalog No. 320511.

Visit **www.acdbio.com/support/technical-doc** to download a sample preparation user guide.

## Product description

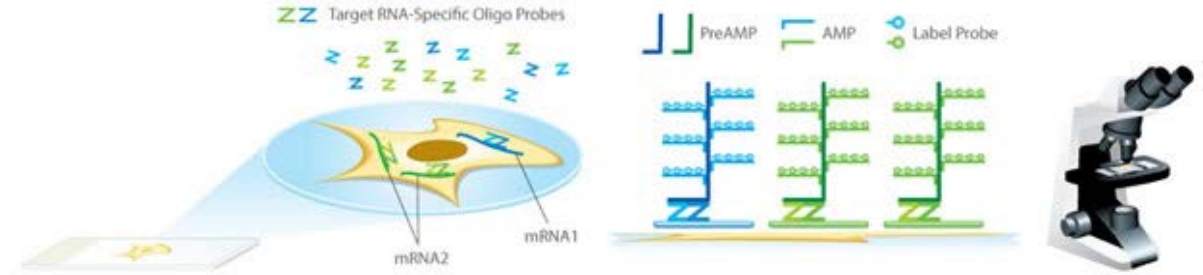
### Background

The RNAscope® 2-Plex 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

The RNAscope® 2-Plex Chromogenic Assay procedure is illustrated in Figure 1 on page **Error! Bookmark not defined.**. The procedure can be completed in 8–10 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 are first pretreated, and then RNA-specific probes are hybridized to target RNA. The RNAscope® 2-Plex 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.




| 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 two sets of gene-specific probe pairs to target mRNA. | Use two independent signal amplification systems to detect both target RNAs. Probes are hybridized to a cascade of signal amplification molecules, culminating in binding of HRP- or AP-labeled probes. Add two chromogenic substrates to detect RNAs. | Visualize target RNA using a standard bright-field microscope. |

**Figure 1** Procedure overview

# Kit contents and storage

The RNAscope® 2-Plex Assay requires the RNAscope® Probes and the RNAscope® 2-Plex Detection Kit (Chromogenic), which are available separately.

## RNAscope® Probes

The RNAscope® Probes consist of user-specified Target Probes and Positive and Negative 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.

**Note:** Different colors are assigned to the C1 and C2 color channels depending on the particular RNAscope® Assay. The color channels for the RNAscope® 2-Plex Detection Kit (Chromogenic) Assay are shown in the following table:

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

* Default channel

Channel C1 target probes are Ready-To-Use (RTU), while channel C2 probes are shipped as a 50X concentrated stock. To independently detect two target RNAs in a 2-Plex assay, each target probe must be in a different color channel and there must be a C1 probe in the mixture. A "Blank Probe – C1" (Cat. No. 300041) can be used in place of a specific target probe.

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 six months from the shipment date when stored as indicated in the following table:

| Target Probes | | | | | |
|---|---|---|---|---|---|
| ☑ | **Reagent** | **Cat. No.** | **Content** | **Quantity** | **Storage** |
| | RNAscope® Kit — 2-Plex Target Probe – *[species]* – *[gene]** | Various | Ready-To-Use (RTU) probe for color channel 1 | 3 mL x 1 bottle | 4°C |
| | RNAscope® Kit — 2-Plex Target Probe – *[species]* – *[gene]* – C2 | Various | 50X probe for color channel 2 | 60 µL x 1 tube | 4°C |
| **Control Probes** | | | | | |
| ☑ | **Reagent** | **Cat. No.** | **Content** | **Quantity** | **Storage** |
| | RNAscope® Kit — Positive Control Probe | Various | RTU probe targeting a common housekeeping gene. Each detection channel has its own positive control probe. | 3 mL x 1 bottle | 4°C |
| | RNAscope® Kit — 2-Plex Positive Control Probe | Various | RTU mixture of two probes targeting POLR2A in channel C1 and PPIB in channel C2. | 3 mL x 1 bottle | 4°C |
| | RNAscope® Kit — Negative Control Probe – *dapB* | 310043 | RTU probe targeting a bacterial gene. Each detection channel has its own negative control probe. | 3 mL x 1 bottle | 4°C |
| | Blank Probe-C1 | 300041 | RTU Target Probe diluent | 3 mL x 1 bottle | 4°C |

* No “C1” in label.

## RNAscope® 2-Plex Detection Kit (Chromogenic)

Each RNAscope® 2-Plex Detection Kit – Chromogenic (Cat. No. 310035) provides enough reagents to stain ~20 tissue sections, each with an area of approximately 20 mm x 20 mm (0.75″ x 0.75″). Larger tissue sections will result in fewer tests. Each kit contains three sub-kits: a Pretreatment Kit, a Detection Kit, and a Wash Buffer Kit.

IMPORTANT! Directions to use the Pretreatment Kit are included in separate sample preparation and pretreatment user guides.

The reagents have a shelf life of six months from the shipment date when stored as indicated in the following table:

| Pretreatment Kit (Cat. No. 310020) | | | |
|---|---|---|---|
| ☑ | **Reagent** | **Quantity** | **Storage** |
| | 1X Pretreat 1 — endogenous enzyme blocker | 4 mL x 2 bottles | 4°C |
| | 10X Pretreat 2* | 70 mL x 4 bottles | Room temperature (20–25°C) |
| | 1X Pretreat 3† — protease | 4.5 mL x 1 bottle | 4°C |
| **2-Plex Detection Kit (Cat. No. 320701)**** | | | |
| ☑ | **Reagent** | **Quantity** | **Storage** |
| | 2-Plex Amp 1 | 3 mL x 1 bottle | 4°C |
| | 2-Plex Amp 2 | 4.5 mL x 1 bottle | 4°C |
| | 2-Plex Amp 3 | 3 mL x 1 bottle | 4°C |

| | | | |
|---|---|---|---|
| | 2-Plex Amp 4A | 4.5 mL x 1 bottle | 4°C |
| | 2-Plex Amp 4B | 30 µL x 1 tube | 4°C |
| | 2-Plex Amp 5 | 4.5 mL x 1 bottle | 4°C |
| | 2-Plex Amp 6 | 3 mL x 1 bottle | 4°C |
| | Red-A — Fast Red diluent | 3 mL x 1 bottle | 4°C |
| | Red-B — Fast Red substrate | 50 µL x 1 tube | 4°C |
| | Green-A — Green diluent | 3 mL x 1 bottle | 4°C |
| | Green-B — Green substrate | 150 µL x 1 vial | 4°C |
| **Wash Buffer Kit (Cat. No. 310091)** | | | |
| ☑ | **Reagent** | **Quantity** | **Storage** |
| | 50X Wash Buffer | 60 mL x 4 bottles | Room temperature (20–25°C) |

* Comes in a separate box.

† Comes in two boxes.

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.

The HybEZ™ Hybridization System (110 VAC, Cat. No. 310010; 220 VAC, Cat. No. 310013) is designed for the hybridization and incubation steps in the RNAscope® Assays. Incubation steps in the RNAscope® Assay 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 **www.acdbio.com/support/technical-doc** and view the training video at **www.acdbio.com/support/online-training-videos**. The system contains the following components:

| ☑ | Component | Quantity | Cat. No. |
|---|---|---|---|
| | HybEZ™ Oven (110 or 220 VAC) | 1 oven | 310010 or 310013 |
| | HybEZ™ Humidity Control Tray (with lid) | 1 tray | 310012 |
| | HybEZ™ Slide Rack (20 slide capacity) | 1 rack | 310014 |
| | HybEZ™ Humidifying Paper | 2 sheets | — |
| | HybEZ™ Humidifying Paper Pack | 15 sheets | 310015 |

## User-supplied materials

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

| ☑ | Description | Supplier | Cat. No. |
|---|---|---|---|
| | EcoMount (required) | Biocare | EM897L |
| | 100% ethanol (EtOH) | American Master Tech Scientific/MLS* | ALREAGAL |
| | Gill's Hematoxylin I | American Master Tech Scientific/MLS | HXGHE1LT |
| | Xylene | Fisher Scientific/MLS | X3P-1GAL |
| | Tissue-Tek® Vertical 24 Slide Rack | American Master Tech Scientific/MLS | LWSRA24 |
| | Tissue-Tek® Staining Dish (3 required) | American Master Tech Scientific/MLS | LWT4457EA |
| | Tissue-Tek® Clearing Agent Dish, xylene resistant (1 required) | American Master Tech Scientific/MLS | LWT4456EA |
| | Cover Glass 24 x 50 mm | Fisher Scientific/MLS | 12--545-F |
| | Ammonium hydroxide, 28–30% | Sigma-Aldrich/MLS | 320145-500mL |
| | 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.

# 2 Chapter 2. Before You Begin

IMPORTANT! For *Part 1, Sample Preparation and Pretreatment Guide for FFPE Tissue*, see Catalog No. 320511.

Prior to running the RNAscope® Assay on your samples for the first time, we recommend that you:

- View the video demonstrations available at **www.acdbio.com/support/online-training-videos**.
- Run the assay on FFPE RNAscope® Control Slides (Cat. No. 310045) using the Positive and Negative Control Probes.

## Important procedural guidelines

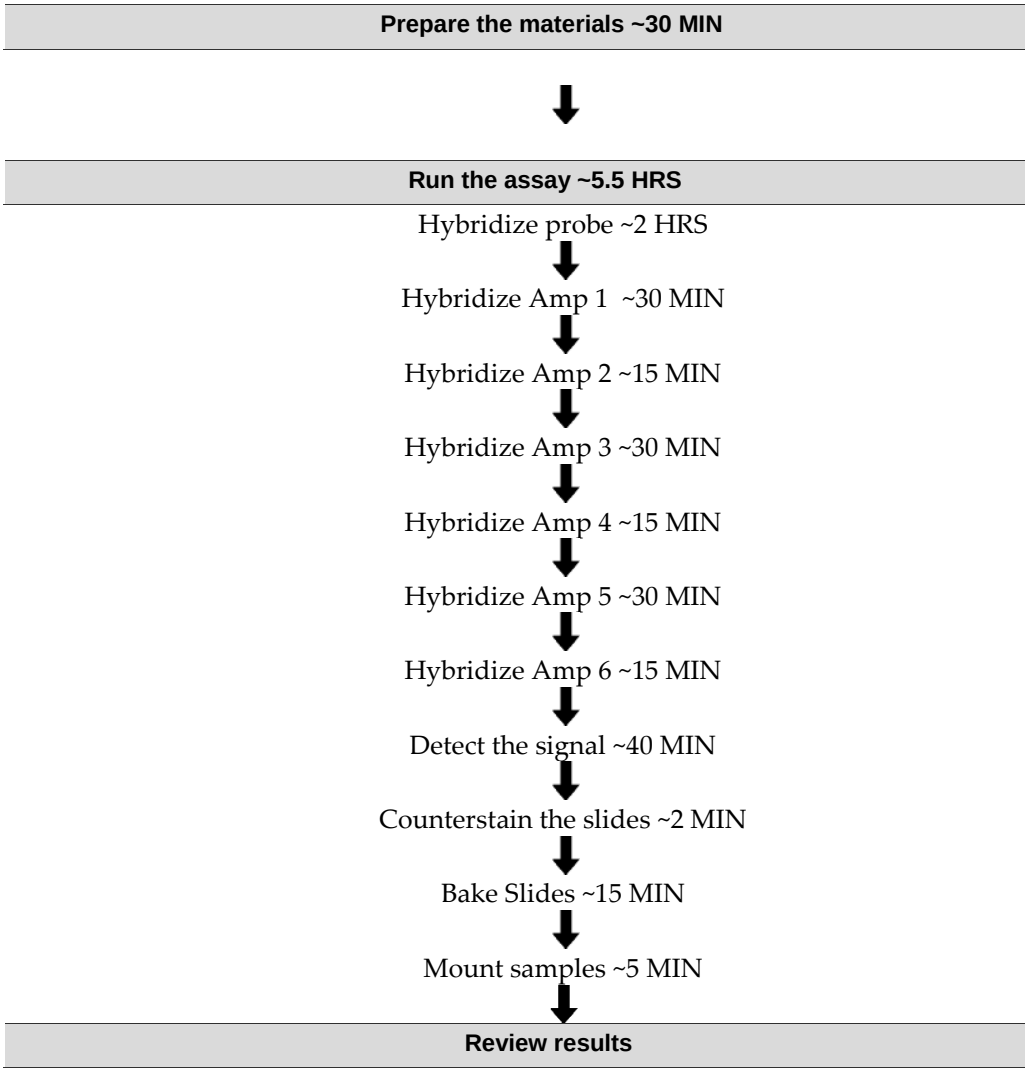
- Start with properly fixed and prepared sections. Refer to **Appendix A. Tissue Pretreatment Recommendation** on page 21 and to our sample preparation and pretreatment user guides available at **www.acdbio.com/support/technical-doc**.
- Use only samples mounted on SuperFrost Plus® Slides (Fisher Scientific; Cat. No. 12-550-15).
- Follow the recommended pretreatment conditions for your sample. Refer to our sample preparation and pretreatment user guides available at **www.acdbio.com/support/technical-doc**.
- 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 C. Safety** on page 25 for more information.

# 3 Chapter 3. RNAscope® 2.0 Assay

IMPORTANT! For *Part 1, Sample Preparation and Pretreatment Guide for FFPE Tissue,* see Catalog No. 320511.

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® 2-Plex Detection Kit (Chromogenic) | Materials provided by RNAscope® Probes | Other materials and equipment |
|---|---|---|
| • 50X Wash Buffer<br>• 2-Plex Amp 1<br>• 2-Plex Amp 2<br>• 2-Plex Amp 3<br>• 2-Plex Amp 4A<br>• 2-Plex Amp 4B<br>• 2-Plex Amp 5<br>• 2-Plex Amp 6<br>• Red-A<br>• Red-B<br>• Green-A<br>• Green-B | • C1 Target Probe<br>• 50X C2 Target Probe<br>• 2-Plex Positive Control Probe<br>• Negative Control Probe | • Prepared sections<br>• Distilled water<br>• Carboy (>3L)<br>• Fume hood<br>• Xylene<br>• 100% ethanol<br>• Tissue-Tek® Staining Dish (3)<br>• Tissue-Tek® Clearing Agent Dish, xylene-resistant (1)<br>• Gill's Hematoxylin I<br>• Ammonium hydroxide, 28–30%<br>• Graduated cylinder<br>• Parafilm<br>• HybEZ™ Humidifying System<br>• Water bath or incubator<br>• Tissue-Tek® Vertical 24 Slide Rack<br>• Tubes (various sizes)<br>• Paper towel or absorbent paper<br>• Pipettors and tips, 1–1000 µL<br>• Microcentrifuge<br>• Dry oven<br>• EcoMount<br>• 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 **www.acdbio.com/support/technical-doc**.

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 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. The mixed Target Probes can be stored at **4°C** for up to **6 MONTHS**

### Prepare counterstaining reagents

- 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 1 week.

- In the fume hood, prepare **0.02% (w/v) AMMONIA WATER** (bluing reagent) by adding 1.43 mL of 1N ammonium hydroxide to 250 mL distilled water in a graduated cylinder or other container.
- Seal the cylinder with parafilm. Mix well **3–5 TIMES**.

**Note:** For assay quantitation, it is critical to use Ammonium Hydroxide.

### Prepare mounting reagents

IMPORTANT! Do not reuse deparaffinization reagents for dehydration of the slides after the assay.

- In the fume hood, add **~200 mL XYLENE** to a Clearing Agent Dish.

**Note:** Reagents may be prepared ahead of time. Ensure all containers remain covered**.**

### Equilibrate reagents

- Place **AMP 1–6** reagents at **RT**.
- Ensure HybEZ™ **OVEN** and prepared Humidity Control **TRAY** are at **40°C**.

## 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/support/online-training-videos/wash-slides** before proceeding.

**Note:** We recommend running controls before running any of your samples to optimize the protocol.

### Hybridize probe

IMPORTANT! Prior to this step, ensure you have pretreated your samples. See Catalog No. 320511 for FFPE Tissue.

IMPORTANT! Ensure probes are prewarmed to dissolve any precipitation prior to use.

1. Tap and/or flick to remove excess liquid from slides and place in the HybEZ™ Slide Rack. Add **~4 DROPS** of the appropriate **PROBE** to entirely cover each section.

   **Note:** Refer to **Appendix B. Reagent Volume Guidelines** on page **Error! Bookmark not defined.** 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.

2. Place the HybEZ™ Slide Rack in the HybEZ™ Humidity Control Tray removed from the HybEZ™ Oven. Close tray and insert back into the oven for **2 HRS** at **40°C**.

IMPORTANT! To prevent evaporation, make sure the turn nob is completely turned to lock position.

3. Remove the HybEZ™ Control Tray from the oven and remove HybEZ™ Slide Rack.
4. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with **1X WASH BUFFER**.
5. Wash slides in 1X Wash Buffer for **2 MIN** at **RT.** Agitate slides by moving the Slide Rack up and down in the dish.
6. Repeat Step 5 with fresh 1X Wash Buffer.

## Hybridize Amp 1

1. Take each slide one at a time from the Tissue-Tek® Slide Rack and tap/and or flick to remove the excess liquid before placing in the HybEZ™ Slide Rack. Add **~4 DROPS** of **AMP 1** to entirely cover each section.
2. Place the HybEZ™ Slide Rack in the HybEZ™ Humidity Control Tray. Close tray and insert into the oven for **30 MIN** at **40°C**.
3. Remove the HybEZ™ Control Tray from the oven and remove HybEZ™ Slide Rack.
4. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with **1X WASH BUFFER**.
5. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
6. Repeat Step 5 with fresh 1X Wash Buffer.

## Hybridize Amp 2

1. Take each slide one at a time from the Tissue-Tek® Slide Rack and tap and/or flick to remove the excess liquid before placing in the HybEZ™ Slide Rack. Add **~4 DROPS** of **AMP 2** to entirely cover each section.
2. Place the HybEZ™ Slide Rack in the HybEZ™ Humidity Control Tray. Close tray and insert into the oven for **15 MIN** at **40°C**.
3. Remove the HybEZ™ Control Tray from the oven and remove HybEZ™ Slide Rack.
4. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with **1X WASH BUFFER**.
5. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
6. Repeat Step 5 with fresh 1X Wash Buffer.

## Hybridize Amp 3

1. Take each slide one at a time from the Tissue-Tek® Slide Rack and tap/and or flick to remove the excess liquid before placing in the HybEZ™ Slide Rack. Add **~4 DROPS** of **AMP 3** to entirely cover each section.
2. Place the HybEZ™ Slide Rack in the HybEZ™ Humidity Control Tray. Close tray and insert into the oven for **30 MIN** at **40°C**.
3. Remove the HybEZ™ Control Tray from the oven and remove HybEZ™ Slide Rack.
4. One slide at a time, remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with **1X WASH BUFFER**.
5. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
6. Repeat Step 5 with fresh 1X Wash Buffer.

## Hybridize Amp 4

1. Briefly spin down the contents of **AMP 4B** to be sure content is at the bottom of the tube before opening cap.
2. Depending on the size of your hydrophobic barrier, make **AMP4 WORKING SOLUTION** per section by using a **1:50** ratio of Amp 4B to Amp 4A. For example, for a 0.75" x 0.75" barrier, add **2.4 µL** of **AMP 4B** to **120 µL** of **AMP 4A** into a tube. Mix well.
3. Take each slide one at a time from the Tissue-Tek® Slide Rack and tap/and or flick to remove the excess liquid before placing in the HybEZ™ Slide Rack.
4. Pipette **~120 µL** of **AMP 4** onto each section. Ensure sections are covered.
5. Place the HybEZ™ Slide Rack in the HybEZ™ Humidity Control Tray. Close tray and insert into the oven for **15 MIN** at **40°C**.
6. Remove the HybEZ™ Control Tray from the oven and remove HybEZ™ Slide Rack.

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

7. One slide at a time, remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with **1X WASH BUFFER**.
8. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
9. Repeat Step 8 with fresh 1X Wash Buffer.

## Hybridize Amp 5

1. Take each slide one at a time from the Tissue-Tek® Slide Rack and tap/and or flick to remove the excess liquid before placing in the HybEZ™ Slide Rack. Add **~4 DROPS** of **AMP 5** to entirely cover each section.
2. Place the HybEZ™ Slide Rack in the HybEZ™ Humidity Control Tray. Seal tray and incubate for **30 MIN** at **RT**.
3. Remove the HybEZ™ Slide Rack from the HybEZ™ Humidity Control Tray.
4. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with **1X WASH BUFFER**.
5. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
6. Repeat Step 5 with fresh 1X Wash Buffer.

## Hybridize Amp 6

1. Take each slide one at a time from the Tissue-Tek® Slide Rack and tap/and or flick to remove the excess liquid before placing in the HybEZ™ Slide Rack. Add **~4 DROPS** of **AMP 6** to entirely cover each section.
2. Place the HybEZ™ Slide Rack with the slides in the HybEZ™ Humidity Control Tray. Close tray and incubate for **15 MIN** at **RT**.
3. Remove the HybEZ™ Slide Rack from the HybEZ™ Humidity Control Tray.
4. One slide at a time, remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with **1X WASH BUFFER**.
5. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
6. Repeat Step 5 with fresh 1X Wash Buffer.

## Detect the red signal

1. Briefly spin down the contents of the **RED-B** tube to be sure content is 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 µL** of **RED-B** to **120 µL** of **RED-A** into a tube. Mix well.

IMPORTANT! Use the **RED** solution within **15 MIN**. Do not expose to direct sunlight or UV light.

3. Take each slide one at a time from the Tissue-Tek® Slide Rack and tap and/or flick to remove the excess liquid before placing in the HybEZ™ Slide Rack.
4. Pipette **~120 µL RED** solution onto each tissue section. Ensure sections are covered.
5. Place the HybEZ™ Slide Rack with the slides in the HybEZ™ Humidity Control Tray. Seal tray and incubate for **30 MIN** at **RT**.
6. Remove the HybEZ™ Slide Rack from the HybEZ™ Humidity Control Tray.
7. To remove the **RED** solution from the slides, tilt each slide one at a time over a waste container and tap the corner on the edge of the container. *Immediately* insert the slide into a Tissue-Tek® Slide Rack submerged in a Tissue-Tek® Staining Dish filled with **1X WASH BUFFER**.
8. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
9. Repeat Step 8 with fresh 1X Wash Buffer.

## Detect the green signal

1. Briefly spin down the contents of the **GREEN-B** tube to be sure content is 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 **2.4 µL** of **GREEN-B** to **120 µL** of **GREEN-A** into a tube. Mix well.

IMPORTANT! Use the **GREEN** solution within **15 MIN**. Do not expose to direct sunlight or UV light.

3. Take each slide one at a time from the Tissue-Tek® Slide Rack and tap and/or flick to remove the excess liquid before placing in the HybEZ™ Slide Rack.
4. Pipette **~120 µL GREEN** solution onto each tissue section. Ensure sections are covered.
5. Place the HybEZ™ Slide Rack with the slides in the HybEZ™ Humidity Control Tray. Seal tray and incubate **10 MIN** at **RT**.
6. Remove the HybEZ™ Slide Rack from the HybEZ™ Humidity Control Tray.
7. To remove the **GREEN WORKING SOLUTION** from the slides, tilt each slide one at a time over a waste container and tap the corner on the edge of the container. *Immediately* insert the slide into a Tissue-Tek® Slide Rack submerged in a Tissue-Tek® Staining Dish filled with **DISTILLED WATER**. Rinse again with fresh distilled water.

### Counterstain the slides

1. Move the Tissue-Tek® Slide Rack into the Staining Dish containing the **50% HEMATOXYLIN I** staining solution for **30 SEC** at **RT**. Slides will be purple.
2. *Immediately* transfer the Slide Rack back into the Staining Dish containing distilled water, and **WASH** slides **3–5 TIMES** by moving the rack up and down. **Keep repeating** with fresh distilled water until the slides are clear, while sections remain purple.
3. Replace distilled water in the Staining Dish with **0.02% AMMONIA WATER**. Move rack up and down **2–3 TIMES.** Section should turn blue.
4. Replace ammonia water with **DISTILLED WATER**. Wash slides **3–5 TIMES**.

### Mount the samples

1. Remove the Slide Rack from the Staining Dish and dry slides in a **60°C** dry oven for **15 MIN**.

IMPORTANT! The **GREEN AND RED SUBSTRATES** are alcohol sensitive. Do not dehydrate the slides in alcohol.

2. Briefly dip one slide into fresh pure xylene and *immediately* place **1–2 DROPS** of EcoMount on the slide before the xylene dries.

IMPORTANT! Use the EcoMount mounting medium only.

3. Carefully place a 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
4. Repeat steps 2 and 3 for each slide.
5. 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 cell nuclei at 20–40X magnification.
- Assess negative control background. One dot to every 10 cells displaying background DAB staining per 20X microscope field is acceptable.

- Evaluate target probe signal using the scoring guidelines in the next section.

### Scoring guidelines

The RNAscope® Assay can enhance the value of *in situ* hybridization results by enabling a semi-quantitative scoring guideline utilizing 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 | 4–10 dots/cell. Very few dot clusters (visible at 20–40X magnification) |
| 3 | >10 dots/cell. Less than 10% positive cells have dot clusters (visible at 20X magnification) |
| 4 | >10 dots/cell. More than 10% positive cells have dot clusters (visible at 20X magnification) |

* Discount cells with artificially high nuclear background staining.

### Quantitative Image Analysis

RNAscope® Spot Studio Software is designed for pathologists with no prior training in image analysis. This intuitive software allows users to get statistical results with complete information of cell-count/region and number of spots/cell. Simply load any image, select a region of interest, define settings and run analysis, followed by a quality control review before results are exported. Further information is available on our website at **www.acdbio.com**.

### Control examples

Figure 2 is an example of the detection of PECAM1 and EGFR in lung FFPE tissue at 40 X magnification.

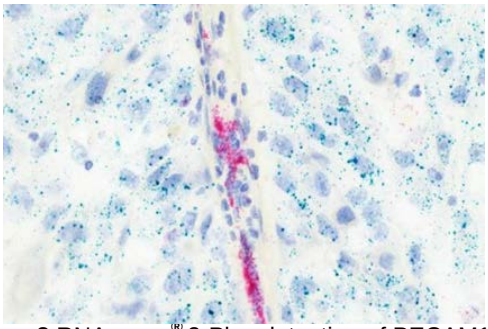


**Figure 2** RNAscope® 2-Plex detection of PECAM1 and EGFR mRNA in lung FFPE tissue.

## Troubleshooting

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

# A 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* (Cat. No. 320511).

## Tissue pretreatment recommendation

1. Stain representative samples using the positive and negative control probes.
2. Fix sample in fresh 10% NBF for **16–32 HRS** at **RT**.

**Note:** Perform tissue fixation step using the recommended amount of time. Over or under-fixation will result in significant signal loss when performing the RNAscope® Assay.

3. Depending on your tissue type (see section below), vary the **PRETREAT 2** and/or **PRETREAT 3 TIME**.

| Reagent | Mild | Standard | Extended |
|---|---|---|---|
| Pretreat 2 | 15 MIN | 15 MIN | 30 MIN |
| Pretreat 3 | 15 MIN | 30 MIN | 30 MIN |

**Note:** Sample types such as certain Xenografts and Cell Pellets, require less time. For these tissue types, vary the **PRETREAT 2 TIME** to 8 min and **PRETREAT 3 TIME** to 15 min**.** If you have a tissue type not listed, contact support at **support@acdbio.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@acdbio.com.**

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

| Species | Tissue type | Pathology | Pretreatment Condition |
|---|---|---|---|
| Human | Breast | Tumor | Standard |
| | lon | 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 |
| | Cell pellets, fixed with 10% NBF | — | Mild |
| | HeLa cells, fixed with 10% Formaldehyde/PBS (ACD control) | — | Standard |

* 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 hyrophobic 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.

# C 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 Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain MSDSs, see **Documentation and support** in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

## Biological hazard safety

**WARNING! BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

### In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/%2029cfr1910a_01.html**

- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **www.cdc.gov**

**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: **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: **eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:133:0001:0043:EN:PDF**

# Documentation and support

## Obtaining MSDSs

Material Safety Data Sheets (MSDSs) are available at: **www.acdbio.com/support/technical-doc/category/msds**. For the MSDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

## Obtaining support

For the latest services and support information, go to: **www.acdbio.com/support**

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, MSDSs, application notes, citations, training videos, and other product support documents.
- Find out information about customer training events.

## Contact information

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Hayward, CA 94545
Toll Free: 1-877-576-3636
Direct: 1-510-576-8800
Fax: 1-510-576-8801
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 at **www.acdbio.com/tos/terms-and-conditions-of-sale**. If you have any questions, please contact Advanced Cell Diagnostics at **www.acdbio.com/support**.

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