ADVANCED CELL DIAGNOSTICS

RNAscope® 2.5 HD Detection Reagent – BROWN User Manual

PART 2

Document Number 322310-USM

*For **Part 1** Sample Preparation Pretreatment Guide for Formalin-Fixed Paraffin-Embedded (FFPE) For RNAscope® 2.5 Assay, see **Document Number** 322452-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 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 safety information in **Appendix C. Safety** on page 21.

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 Detection Kit – BROWN (Cat. No. 322310). 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 Manual to perform the entire assay.

IMPORTANT! For Part 1, Formalin-Fixed Paraffin-Embedded (FFPE) Sample Preparation and Pretreatment User Manual, see Document No. 322452-UM.

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

Product description

Background

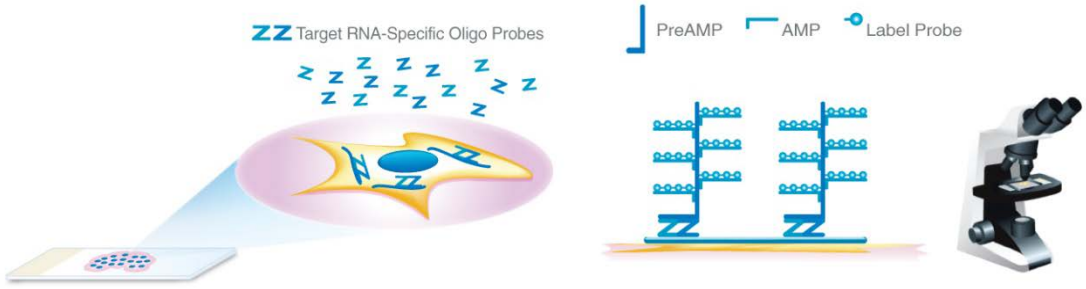
The RNAscope® Assays use a novel and proprietary method of *in situ* hybridization (ISH) to visualize single RNA molecules per cell in samples mounted on slides. RNAscope® Assays do not require the RNA-free environment used for traditional ISH. The assays are based on ACD's patented signal amplification and background suppression technology. Compared with the RNAscope® 2.0 Assay, the 2.5 Assay incorporates an additional signal amplification step, which enhances the signal for low expressing genes and RNA present in archived samples and partially degraded specimens.

Overview

The RNAscope® Assay procedure is illustrated in **Figure 1** on page 6. The procedure can be completed in 7–8 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 tissue samples, sections are first pretreated, and then RNA-specific probes are hybridized to target RNA. The signal is amplified using a multi-step process, followed by hybridization to horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-labeled probes and detection using a chromogenic substrate. Each single RNA transcript appears as a distinct dot of chromogen precipitate visible using a common bright field microscope at 40–100X magnification. The RNAscope® 2.5 Assay has additional amplification steps that allow observable results under 10–20X magnification. RNAscope® 2.5 Assays offer the choice of two Detection Kits: Brown (DAB) and Red (Fast Red), which enable RNA molecules to be visualized as brown or red chromogenic dots, respectively.

Figure 1. Product overview



1: Tissue section	2: Hybridize to target RNA	3: Amplify signal	4: Image
Start with properly prepared sections and pretreat to allow access to target RNA.	Hybridize gene-specific probe pairs to the target mRNA.	Probes are hybridized to a cascade of signal amplification molecules, culminating in binding of HRP- or AP-labeled probes. The 2.5 Assay enhances signal further with additional amplification steps. Add DAB or Fast Red substrate to detect target RNA.	Visualize target RNA using a standard bright field microscope.

Kit contents and storage

The RNAscope® 2.5 Assay requires the RNAscope® Probes and the RNAscope® Detection Kit. Probes and Detection Kits are available separately.

RNAscope® Probes

The RNAscope® Probes consist of the user-specified Target Probe and the Positive and Negative Control Probes Visit **www.acdbio.com/products/target-probes/search-product** to find a gene-specific Target Probe. Visit **http://www.acdbio.com/control-slides-and-probes** to order appropriate Control Probes.

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 date of bulk manufacturing when stored as indicated in the following table:

Target Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® Singleplex Target Probe – *[species]– [gene]*	Various	Probe targeting specific RNA	3 mL x 1 bottle	2–8°C
Control Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® Positive Control Probe – *[species]– PPIB*	Various	Probe targeting common housekeeping gene	3 mL x 1 bottle	2–8°C
	RNAscope® Negative Control Probe – DapB	310043	Probe targeting bacterial gene dapB	3 mL x 1 bottle	2–8°C

RNAscope® 2.5 HD Reagent Kit – BROWN

Each RNAscope® 2.5 HD Reagent Kit – BROWN (Cat. No. 322300) 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 components: Pretreatment Reagents, Target Retrieval Reagents, Wash Buffer Reagents, and Detection Reagents.

IMPORTANT! Directions on using the Pretreatment Reagents are included in separate sample preparation and pretreatment user manuals.

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

Pretreatment Reagents (Cat. No. 322300 and 322000)			
☑	Reagent	Quantity	Storage
	• RNAscope® Hydrogen Peroxide • RNAscope® Protease Plus	• 3 mL x 2 bottles • 4.5 mL x 1 bottle	2–8°C
	RNAscope® Target Retrieval (10X)	70 mL x 4 bottles	Room temp (15–30°C)
RNAscope® 2.5 HD Detection Reagents – BROWN (Cat. No. 322310)			
☑	Reagent	Quantity	Storage
	RNAscope® 2.5 HD AMP 1	3 mL x 1 bottle	2–8°C
	RNAscope® 2.5 HD AMP 2	4.5 mL x 1 bottle	2–8°C
	RNAscope® 2.5 HD AMP 3	3 mL x 1 bottle	2–8°C
	RNAscope® 2.5 HD AMP 4	4.5 mL x 1 bottle	2–8°C
	RNAscope® 2.5 HD AMP 5–BROWN	4.5 mL x 1 bottle	2–8°C
	RNAscope® 2.5 HD AMP 6–BROWN	3 mL x 1 bottle	2–8°C
	RNAscope® 2.5 HD DAB-A	2 mL x 1 bottle	2–8°C
	RNAscope® 2.5 HD DAB-B	2 mL x 1 bottle	2–8°C
RNAscope® Wash Buffer Reagents (Cat. No. 310091)			
☑	Reagent	Quantity	Storage
	RNAscope® Wash Buffer (50X)	60 mL x 4 bottles	Room temp (15–30°C)

IMPORTANT! RNAscope® 2.5 HD BROWN and RED Reagent Kits share the same Pretreatment Reagents (Hydrogen Peroxide, Target Retrieval, and Protease Plus) and Wash Buffer, but have unique Detection Reagents. 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® 2.5 Assay.

HybEZ™ Hybridization System

IMPORTANT! The RNAscope® 2.5 Assay has been verified 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® 2.5 Assays. Incubation steps in the RNAscope® 2.5 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/technical-support/user-manuals** and view the training video at **www.acdbio.com/technical-support/learning-more**. 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

☑	Description	Supplier	Cat. No.
	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
	95% Ethanol (EtOH)	American Master Tech Scientific ALREACS	—
	Cytoseal XYL xylene-based mounting medium	Richard-Allen Scientific/MLS	8312-4
	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	—
	20% bleach	MLS	—
	Microscope and accessories	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, Formalin-Fixed Paraffin-Embedded (FFPE) Sample Preparation and Pretreatment User Manual, see Document No. 322452-USM.

Prior to running the RNAscope® 2.0 Assay on your samples for the first time, we recommend that you:

- View the video demonstrations available at **http://www.acdbio.com/technical-support/learn-more**.
- Run the assay on FFPE RNAscope® Control Slides (Cat. No. 310045 for Human control slide, HeLa; Catalog No. 310023 for Mouse control slide, 3T3) using the Positive and Negative Control Probes.

Important procedural guidelines

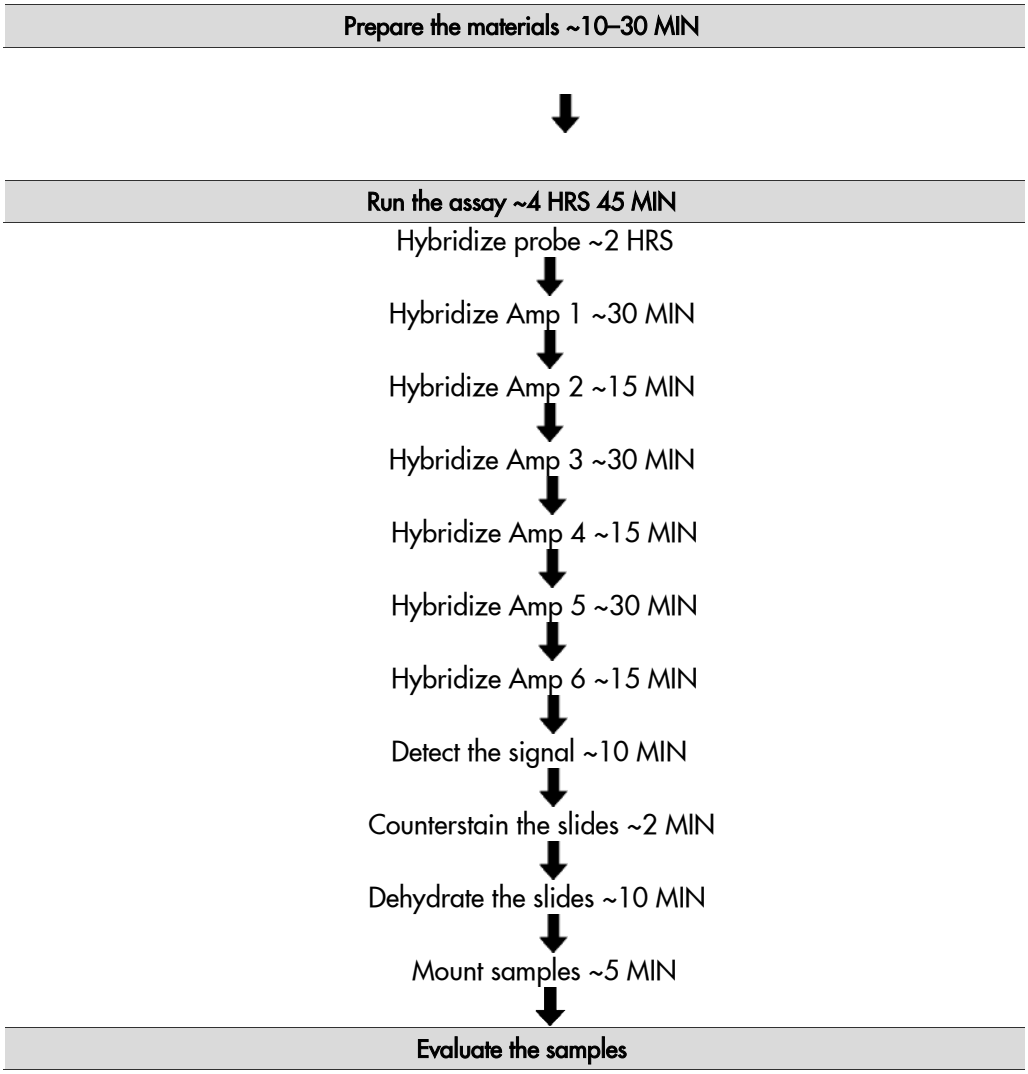
- Start with properly fixed and prepared sections. Refer to **Appendix A. Tissue Pretreatment Recommendation** on page 18 and to our sample preparation and pretreatment user guides available at **http://www.acdbio.com/technical-support/user-manuals**.
- Use only samples mounted on SuperFrost Plus® Slides (Fisher Scientific; Cat. No. 12-550-15).
- Follow the recommended pretreatment guidelines for your sample. Refer to our sample preparation and pretreatment user guides available at **http://www.acdbio.com/technical-support/user-manuals**.
- Always run positive and negative control probes on your sample to assess sample RNA quality and optimal permeabilization.
- Do *not* substitute required materials. Assay has been validated with these materials only.
- Follow the protocol exactly for best results.
- Do not let your sections dry out during the procedure.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix C. Safety** on page 21 for more information.

3 Chapter 3. RNAscope® 2.5 Assay

IMPORTANT! For Part 1, Formalin-Fixed Paraffin-Embedded (FFPE) Sample Preparation and Pretreatment User Manual, see Document No. 322452-USM.

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

Workflow



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Materials required for the assay

Materials provided by RNAscope® 2.0 HD Detection Kit – BROWN	Materials provided by RNAscope® Probes	Other Materials and Equipment
• RNAscope® Wash Buffer (50X) • RNAscope® 2.5 AMP 1 • RNAscope® 2.5 AMP 2 • RNAscope® 2.5 AMP 3 • RNAscope® 2.5 AMP 4 • RNAscope® 2.5 AMP 5 – BROWN • RNAscope® 2.5 AMP 6 – BROWN • RNAscope® 2.5 DAB-A • RNAscope® 2.5 DAB-B	• Target Probe • Positive Control Probe • Negative Control Probe	• Prepared sections • Distilled water • Carboy (>3L) • Fume hood • Xylene • 95% Ethanol (EtOH) • Tissue-Tek® Staining Dish (3) • Tissue-Tek® Clearing Agent Dish, xylene-resistant (1) • Gill's Hematoxylin I • Ammonium hydroxide, 28–30% • Graduated cylinder • Parafilm • HybEZ™ Humidifying System • Water bath or incubator • Tissue-Tek® Vertical 24 Slide Rack • Tubes (various sizes) • Paper towel or absorbent paper • Pipettors and tips, 1–1000 µL • Cytoseal XYL xylene-based • 20% bleach • 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 manual available at **http://www.acdbio.com/technical-support/user-manuals**.

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

Prepare 1X Wash Buffer

1. Prepare 3 L of 1X Wash Buffer by adding 2.94 L distilled water and 1 bottle (60 mL) of RNAscope® Wash Buffer (50X) to a large carboy. Mix well.
   Note: Warm RNAscope® 50X Wash Buffer up to **40°C** for **10–20 MIN** before preparation. 1X Wash Buffer may be prepared ahead of time and stored at room temperature for up to one month.

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 1 week.
2. 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.
3. Seal the cylinder with parafilm. Mix well 3–5 times.
   Note: For assay quantitation, it is critical to use Ammonium Hydroxide.

Prepare dehydrating reagents

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

1. In the fume hood, add ~200 mL xylene to a clearing agent dish.
2. In the fume hood, fill two staining dishes with ~200 mL 95% EtOH.
3. Prepare 70% EtOH by adding 140 mL 95% EtOH to 60 mL distilled water in a staining dish. Seal the dish with parafilm, mix well, and place in fume hood.
   Note: Reagents may be prepared ahead of time. Ensure all containers remain covered.

Equilibrate reagents

1. Remove AMP 1–6 reagents from refrigerator and place at **RT**.
2. Ensure HybEZ™ Oven and prepared Humidity Control Tray are at **40°C**.
3. Before each use, warm the Target and/or Control Probes for at least **10 MIN** at **40°C** in a water bath or incubator.

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 **http://www.acdbio.com/technical-support/learn-more** before proceeding.

Hybridize probe

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 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.
2. Cover the HybEZ™ Humidity Control Tray with lid and insert 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 from slides. Place slides in the HybEZ™ Slide Rack in the HybEZ™ Humidity Control Tray. Add ~4 drops of AMP 1 to entirely cover each section.
2. 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 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 from slides. Place slides in the HybEZ™ Slide Rack in the HybEZ™ Humidity Control Tray. Add ~4 drops of AMP 2 to entirely cover each section.
2. 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 from slides. Place slides in the HybEZ™ Slide Rack in the HybEZ™ Humidity Control Tray. Add ~4 drops of AMP 3 to entirely cover each section.
2. 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 4

1. Take each slide one at a time from the Tissue-Tek® Slide Rack and tap and/or flick to remove the excess liquid from slides. Place slides in the HybEZ™ Slide Rack in the HybEZ™ Humidity Control Tray. Add ~4 drops of AMP 4 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.

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

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 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.

IMPORTANT! Staining intensity can be modified by adjusting the AMP 5 incubation time.

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, cover with lid, 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, *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.

Detect the signal

1. Mix equal volumes of DAB-A and DAB-B in an appropriately sized tube by dispensing the same number of drops of each solution. Make ~120 µL DAB substrate per section (~2 drops of each reagent/total of 4). Mix well 3–5 times.

   ⚠ **CAUTION!** DAB is toxic. Follow appropriate precautions and safety guidelines when disposing of and handling this chemical.

2. 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.
3. Pipette ~120 µL of DAB onto each tissue section. Ensure sections are covered, and incubate for **10 MIN** at **RT**.
4. Dispose the remaining DAB according to local regulation and insert the slide into a Tissue-Tek® Slide Rack submerged in a Tissue-Tek® Staining Dish filled with tap water.

Store the reagents

1. Place all reagents back in refrigerator for storage.
2. Wash slides in tap water by moving the Tissue-Tek® Slide Rack up and down 3–5 times. Replace with fresh tap water.

Counterstain the slides

1. Move the Tissue-Tek® Slide Rack into the staining dish containing 50% Hematoxylin staining solution for **2 MIN** at **RT**. Slides will be purple.
2. *Immediately* transfer the slide rack back into the staining dish containing tap water, and wash slides 3–5 times by moving the rack up and down. Keep repeating with fresh tap water until the slides are clear, while sections remain purple.
3. Replace tap 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 tap water. Wash slides 3–5 times.

Dehydrate the slides

1. Move the Tissue-Tek® Slide Rack into the staining dish containing 70% EtOH in the fume hood for **2 MIN** with occasional agitation.
2. Move the Tissue-Tek® Slide Rack into the first staining dish containing 95% EtOH for **2 MIN** with occasional agitation.
3. Move the Tissue-Tek® Slide Rack into the second staining dish containing 95% EtOH for **2 MIN** with occasional agitation.
4. Move the Tissue-Tek® Slide Rack into the staining dish containing Xylene for **5 MIN** with occasional agitation.

Mount the samples

1. Remove the slides from the Tissue-Tek® Slide Rack and lay flat with the sections facing up in the fume hood.
2. Mount one slide at a time by adding 1 drop of Cytoseal or other xylene-based mounting medium to each slide and carefully placing a 24 mm x 50 mm coverslip over the section. Avoid trapping air bubbles.
3. 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 on 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 to every 10 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.

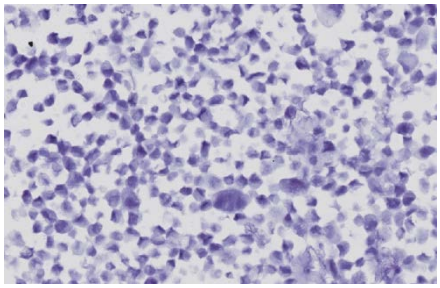
Quantitative image analysis

RNAscope® Spot Studio Software is designed for pathologists with no prior training in image analysis. This intuitive software allows users to obtain 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**.

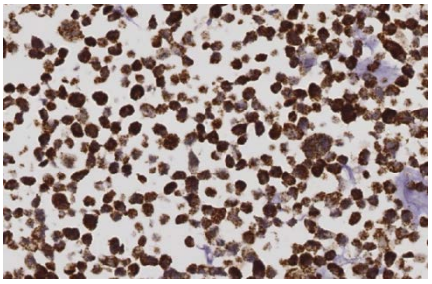
Control examples

Figure 2 is an example of human liver cancer sections using dapB Negative Control Probe and PPIB Positive Control Probe at 40X magnification.

Figure 2. RNAscope® 2.5 HD Detection Kit – BROWN performed on human liver cancer sections using the dapB Negative Control Probe (Cat. No. 310043) and PPIB Positive Control Probe (Cat. No. 313901), 40X magnification.



2 (a). dapB



2 (b). Hs-PPIB

Troubleshooting

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

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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, Formalin-Fixed Paraffin-Embedded (FFPE) Sample Preparation and Pretreatment User Manual* (Document No. 322452-USM).

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 amount of time for the RNAscope® Target Retrieval Reagents and/or RNAscope® Protease Plus.

Reagent	Mild	Standard	Extended
RNAscope® Target Retrieval Reagents	15 MIN	15 MIN	15–30 MIN
RNAscope® Protease Plus	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 RNAscope® Target Retrieval Reagents time to **8 MIN** and RNAscope® Protease Plus 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/Mild
	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
	Cell pellets, fixed with 10% NBF	—	Mild
	HeLa cells, fixed with 10% Formaldehyde/PBS/ACD Control	—	Standard

* Tissue Microarray

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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.

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

Biological hazard safety

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

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- 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: **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: **http://echa.europa.eu/regulations/reach**

Documentation and Support

Obtaining SDSs

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

Obtaining support

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

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.
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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/store/terms**. If you have any questions, please contact Advanced Cell Diagnostics at **www.acdbio.com/about/contact**.

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