USER MANUAL

ADVANCED CELL DIAGNOSTICS

RNAscope® 2.5 VS Assay

For Ventana DISCOVERY™ XT System

BROWN

Document Number 322200-USM-XT

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Citing RNAscope® Assay 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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ADVANCED CELL DIAGNOSTICS

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1 Chapter 1. Product Information

Before using this product, read and understand the information in **Appendix B. Safety** on page 34 in this document.

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

About this guide

This user manual provides two versions of the RNAscope® 2.5 VS Assay:

- **Chapter 4. Automated RNAscope® 2.5 VS Assay** on page 14.
- **Appendix A. Semi-automated RNAscope® 2.5 VS Assay** on page 24.

Product description

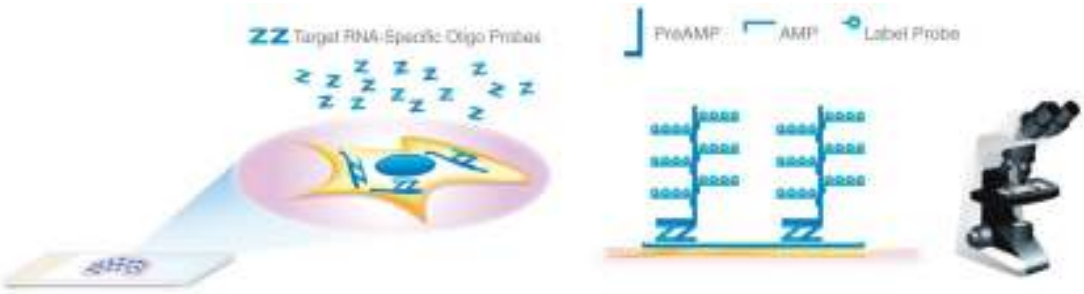
Background

The RNAscope® 2.5 VS Assays use a novel and proprietary method of *in situ* hybridization (ISH) to visualize single RNA molecules per cell in formalin-fixed, paraffin-embedded (FFPE) tissue mounted on slides. The assays are based on Advanced Cell Diagnostic's patented signal amplification and background suppression technology, and can detect RNA molecules in archival samples and partially degraded specimens. The RNAscope® 2.5 VS Assay allows users to automate the highly sensitive RNAscope® Assay using the Ventana DISCOVERY™ XT or ULTRA Systems.

Overview

The RNAscope® 2.5 VS Assay procedure is illustrated in Figure 1 on page 7 and can be completed on the instrument in ~11 hours. Starting with properly prepared samples, sections are first pretreated, and then RNA-specific probes are hybridized to target RNA. The signal is amplified using multiple steps, 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.

Figure 1. Procedure 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 VS Assay requires the RNAscope® 2.5 VS Probes and the RNAscope® 2.5 VS Reagents, available from Advanced Cell Diagnostics.

RNAscope® 2.5 VS Probes

The RNAscope® 2.5 VS 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 **www.acdbio.com/products/target-probes/controls-housekeeping** to order appropriate Control Probes. Each probe is sufficient for staining ~30 standard slides. The probes have a shelf life of two years from the date of manufacturing when stored as indicated in the following table:

Target Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® 2.5 VS Target Probe – *[species]* – *[gene]*	312039	Probe targeting specific RNA	7 mL x 1 bottle	2–8°C
Control Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® 2.5 VS FFPE Reagent Kit — Positive Control Probe – *[Hs]*– PPIB	313909	Probe targeting common housekeeping gene	7 mL x 1 bottle	2–8°C
	RNAscope® 2.5 VS FFPE Reagent Kit — Positive Control Probe – *[Hs]*– TBP	314299	Probe targeting common housekeeping gene	7 mL x 1 bottle	2–8°C
	RNAscope® 2.5 VS FFPE Reagent Kit — Negative Control Probe – DapB	312039	Probe targeting bacterial gene dapB	7 mL x 1 bottle	2–8°C

RNAscope® VS Control Slides

The RNAscope® VS Control Slides (Cat. No. 310045 for Human control slide, HeLa; Cat. No. 310023 for Mouse control slide, 3T3) contain FFPE cell pellets sectioned and mounted on slides. The control slides can be used for assay control with the RNAscope® VS 2.5 Positive Control Probe and RNAscope® VS

2.5Negative Control Probe. The slides have a shelf life of 9 months from the date of manufacturing when stored at 2–8°C with desiccants.

RNAscope® 2.5 VS Reagents

RNAscope® 2.5 VS kits provide enough reagents to stain ~60 standard slides. You will receive three kits when you order the RNAscope® 2.5 VS Reagent Kit (Cat. No. 322000). RNAscope® 2.5 VS Reagents include:

- RNAscope® 2.5 VS Reagent Kit (Cat. No. 322200)
- RNAscope® 2.5 VS Sample Prep Reagents (Cat. No. 322220)
- RNAscope® VS Accessory Kit (Cat. No. 320630)

The reagents are Ready-To-Use (RTU) and have a shelf life of nine months from the date of manufacturing when stored as indicated in the following table:

RNAscope® 2.5 VS Reagent Kit–BROWN (Cat. No. 322200)				
☑	Cat. No.	Reagent	Quantity	Storage
	322211	RNAscope® 2.5 VS AMP 1	14 mL x 1 bottle	2–8°C
	322212	RNAscope® 2.5 VS AMP 2	14 mL x 1 bottle	2–8°C
	322213	RNAscope® 2.5 VS AMP 3	14 mL x 1 bottle	2–8°C
	322214	RNAscope® 2.5 VS AMP 4	14 mL x 1 bottle	2–8°C
	322215	RNAscope® 2.5 VS AMP 5–Brown	14 mL x 1 bottle	2–8°C
	322216	RNAscope® 2.5 VS AMP 6–Brown	14 mL x 1 bottle	2–8°C
	322217	RNAscope® 2.5 VS AMP 7	14 mL x 1 bottle	2–8°C
	322218	RNAscope® 2.5 VS mRNA Pretreat 3–Protease	14 mL x 1 bottle	2–8°C
RNAscope® VS Sample Prep Reagents (Cat. No. 322220)				
	322221	RNAscope® 2.5 VS Target Retrieval	14 mL x 2 bottle	2–8°C
	322222	RNAscope® 2.5 VS Pretreat 2–Dewax	14 mL x 1 bottle	Room Temp (15-30°C)
RNAscope® VS Accessory Kit (Cat. No. 320630)				
☑		Reagent	Quantity	Storage
	320631	RNAscope® VS Hematoxylin — RTU	7 mL x 1 bottle	2–8°C
	320632	RNAscope® VS Bluing Reagent — RTU	7 mL x 1 bottle	2–8°C

IMPORTANT! Dewax must be in solution and at room temperature before use on the instrument. Warm in the hand, or place at 37°C for 15 MIN.

IMPORTANT! Do not substitute the reagent components of the RNAscope® 2.5 VS Reagent Kit with those of other RNAscope® Reagent Kits, even those having the same name. The Target Retrieval solution in the RNAscope® VS 2.5 Kit CANNOT be used for offline boiling. Please use the RNAscope® Target Retrieval Reagents (Cat. No. 322000) to boil samples offline.

Required materials from Roche Diagnostics

The RNAscope® 2.5 VS Assay requires specific materials and equipment available *only* from Roche Diagnostics (Ventana Medical Systems, Inc.). Catalog Numbers are valid in the U.S. only. For other regions, please check Catalog or ordering numbers with your local lab supplier.

Probe Dispensers (Cat. No. 960-761 to 960-780; for Ordering Code, please contact local Roche representative)		
☑	**Component**	**Storage**
	250 Test Probe #1–20 dispensers — fill dispensers with RNAscope® 2.5 VS Probes. Use up to 20 probes at a time.	Room Temp (15–30°C)
mRNA Pretreatment Kit (Cat. No. 760-223; Ordering Code 06614345001)		
☑	**Component**	**Storage**
	mRNA Pretreat A dispenser — fill dispenser with RNAscope® 2.5 VS Pretreat 2–Dewax	Room Temp (15–30°C)
	mRNA Pretreat B dispenser — fill dispenser with RNAscope® 2.5 VS mRNA Pretreat 3–Protease	Room Temp (15–30°C)
mRNA Probe Amplification Kit (Cat. No. 760-222; Ordering Code 06614337001)		
☑	**Component**	**Storage**
	mRNA AMP 1 dispenser — fill dispenser with AMP 1	Room Temp (15–30°C)
	mRNA AMP 2 dispenser — fill dispenser with AMP 2	Room Temp (15–30°C)
	mRNA AMP 3 dispenser — fill dispenser with AMP 3	Room Temp (15–30°C)
	mRNA AMP 4 dispenser — fill dispenser with AMP 4	Room Temp (15–30°C)
	mRNA AMP 5 dispenser — fill dispenser with AMP 5	Room Temp (15–30°C)
	mRNA AMP 6 dispenser — fill dispenser with AMP 6	Room Temp (15–30°C)
	mRNA AMP 7 dispenser — fill dispenser with AMP 7	Room Temp (15–30°C)
mRNA DAB Detection Kit (Cat. No. 760-224; Ordering Code 06614353001)		
☑	**Component**	**Storage**
	mRNA Inhibitor-prefilled	2–8°C
	mRNA DAB dispenser-prefilled	2–8°C
	mRNA H_2O_2 dispenser-prefilled	2–8°C
	mRNA Copper dispenser-prefilled	2–8°C
Generic Dispensers (Cat. No. 771-741; Ordering Code 05271720001, Cat. No. 771-742; Ordering Code 05271738001)		
☑	**Component**	**Storage**
	250 Test Counterstain 1 dispenser — fill dispenser with VS Hematoxylin	Room Temp (15–30°C)
	250 Test Counterstain 2 dispenser — fill dispenser with Bluing Reagent	Room Temp (15–30°C)
Ancillary Dispensers (Cat. No.771-760, Ordering Code 05271932001		
☑	**Component**	**Storage**
	250 Test Option # 10 — fill with VS 2.5 Target Retrieval Reagents	Room Temp (15–30°C)

Equipment and buffers

☑	Component	Cat. No./ Ordering Code
	DISCOVERY Wash (RUO)	950-510 / 07311079001
	LCS (Predilute)	650-010 / 05424534001
	RiboWash (10x)	760-105 / 05266262001
	Reaction Buffer (10x)	950-300 / 05266262001
	DISCOVERY CC1 Predilute	950-500 / 06414575001

IMPORTANT! To run VS 2.5 assay successfully, please be sure to use DISCOVERY Wash (950-510) and not DISCOVERY EZ Prep. Option bulk container can be filled with reaction buffer.

User-supplied materials

IMPORTANT! Do not substitute other materials for the SuperFrost® Plus Slides listed in the following table.

☑	Description	Supplier	Cat. No.
	SuperFrost® Plus Slides (required)	Fisher Scientific	12-550-15
	100% ethanol (EtOH)	American Master Tech Scientific/MLS*	ALREAGAL
	Xylene	Fisher Scientific/MLS	X3P-1GAL
	10% neutral-buffered formalin (NBF)	MLS	—
	Paraffin wax	MLS	—
	1X PBS	MLS	—
	Microtome	MLS	—
	Drying oven, capable of holding temperature at 60 +/– 1°C	MLS	—
	Cytoseal XYL xylene-based mounting medium	Richard-Allen Scientific/MLS	8312-4
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24
	Tissue-Tek® Staining Dish (6 required)	American Master Tech Scientific/MLS	LWT4457EA
	Tissue-Tek® Clearing Agent Dish, xylene resistant (4 required)	American Master Tech Scientific/MLS	LWT4456EA
	Cover Glass 24 x 50 mm	Fisher Scientific/MLS	12--545-F
	Distilled water	MLS	—
	Dawn detergent or similar detergent	MLS	—
	Fume hood	MLS	—
	Glass beaker (1 or 2 L) (Optional)	MLS	—
	Hot plate (Optional)	Fisher Scientific/MLS	11-300-49SHP

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

2 Chapter 2. Before You Begin

Prior to running the RNAscope® 2.5 VS Assay on your samples for the first time, we recommend that you:

- Become familiar with the Ventana DISCOVERY XT system. Refer to the *Ventana System User Manual*.
- Run the assay on FFPE RNAscope® VS Control Slides (Cat. No. 310045 for Human control slide, HeLa; Cat. 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 **Chapter 3. Prepare Samples** on page 12 for preparation of FFPE slides. For preparation of other sample types, contact **support@acdbio.com**.
- Follow the recommended pretreatment conditions for your sample. Refer to **Recommended guidelines** on page 21 for pretreatment conditions and to the manuals available at **http://www.acdbio.com/technical-support/user-manuals**.
- Regularly maintain and clean your automated staining instrument.
- Always run positive and negative control probes on your sample to assess sample RNA quality and optimal permeabilization.
- Do *not* substitute required materials. Assay has been validated with these materials only.
- Follow the protocol exactly for best results.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix B. Safety** on page 34 for more information.

3 Chapter 3. Prepare Samples

Formalin-fixed, paraffin-embedded (FFPE) sample preparation and pretreatment are described in the following protocols.

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

For samples treated differently from the following protocol, please see the sample pretreatment optimization procedure described in **Recommended guidelines** on page 21 and user manuals available at **http://www.acdbio.com/technical-support/user-manuals**

Prepare FFPE sections

Materials required

- 10% neutral buffered formalin (NBF)
- 1X PBS
- Paraffin wax
- 100% ethanol (EtOH)
- Xylene
- Microtome
- Water bath
- SuperFrost® Plus slides

Fix the sample

1. Immediately following dissection, fix tissue in 10% NBF for **16–32 HRS** at **ROOM TEMPERATURE (RT)**. Fixation time will vary depending on tissue type and size.

⚠ CAUTION! Handle biological specimens appropriately.

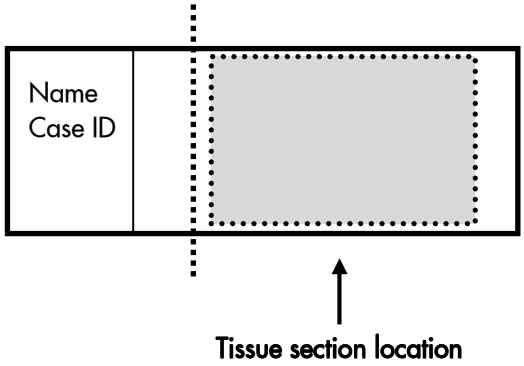
IMPORTANT! Fixation for **<16 HRS** or **>32 HRS** will impair the performance of the RNAscope® 2.5 VS Assay.

Dehydrate, embed, and cut the sample

IMPORTANT! Use fresh reagents.

1. Wash sample with 1X PBS.
2. Dehydrate sample using a standard ethanol series, followed by xylene.
3. Embed sample in paraffin using standard procedures.

   Note: Embedded samples may be stored at 15-25C with desiccation. To better preserve RNA quality over long period (>1 yr), storing at 2-8C with desiccation is recommended.
4. Trim paraffin blocks as needed, and cut embedded tissue into 5 +/– 1 µm sections using a microtome.
5. Place paraffin ribbon in a **40–45°C** water bath, and mount sections on **SUPERFROST® PLUS SLIDES**. Place tissue as shown for optimal staining:



Do not mount more than one section per slide. Place sections in the center of the slide.

6. Air dry slides **OVERNIGHT** at **RT**. Do NOT bake slides unless they will be used for RNAscope® within 1 week.

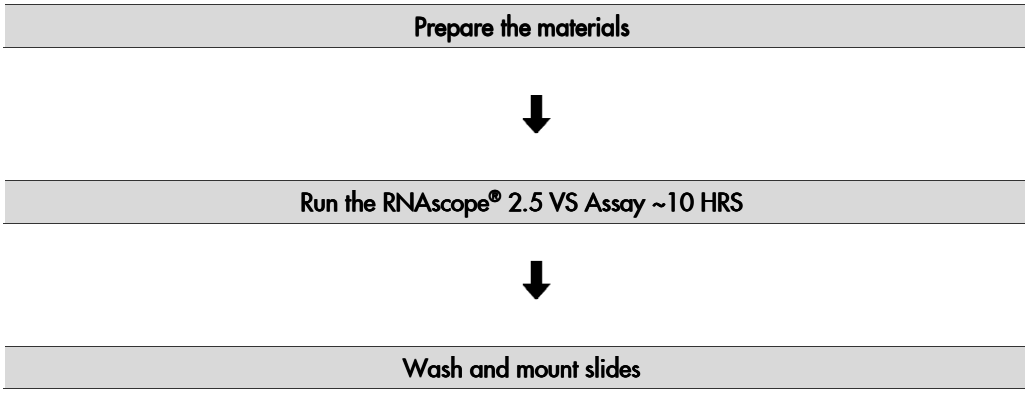
OPTIONAL STOPPING POINT. Use sectioned tissue within 3 months. Store sections with dessicants at **RT**.

4 Chapter 4. Automated RNAscope® 2.5 VS Assay

IMPORTANT! We strongly recommend you run the RNAscope® Control Slides (Cat. No. 310045 or 310023) using the positive and negative control probes along with your samples in every run.

Note: Appendix A. Semi-automated RNAscope® 2.5 VS Assay describes an offline boiling procedure for use with Cat. No. 322000.

Workflow



Prepare the materials

Materials can be prepared ahead of time, unless otherwise stated.

Materials required

Materials Provided by Advanced Cell Diagnostics	Materials Provided by Ventana™ Medical Systems	Other Materials and Equipment
• RNAscope® VS Target Probe • RNAscope® VS Positive Control Probe • RNAscope® VS Negative Control Probe • RNAscope® 2.5 VS Pretreat 2–Dewax • RNAscope® 2.5 VS Pretreat 3-Protease • RNAscope® 2.5 VS Target Retrieval (Option 10) • RNAscope® 2.5 VS AMP 1 • RNAscope® 2.5 VS AMP 2 • RNAscope® 2.5 VS AMP 3 • RNAscope® 2.5 VS AMP 4 • RNAscope® 2.5 VS AMP 5–Brown • RNAscope® 2.5 VS AMP 6–Brown • RNAscope® 2.5 VS AMP 7 • RNAscope® VS Hematoxylin • RNAscope® VS Bluing Reagent	• DISCOVERY™ XT — automated slide stainer • DISCOVERY Wash • LCS • RiboWash Buffer • DISCOVERY CC1 • Reaction Buffer • Probe dispensers • mRNA Pretreatment Kit • mRNA Probe Amplification Kit • mRNA DAB Detection Kit • User-fillable dispensers • Option 10 dispenser	• Distilled water • Dawn detergent or similar detergent • Fume hood • Xylene • Tissue-Tek® Staining Dish (1) • Tissue-Tek® Clearing Agent Dish, xylene-resistant (1) • Tissue-Tek® Vertical 24 Slide Rack • Cytoseal XYL xylene-based • Cover Glass, 24 mm x 50 mm

Prepare the instrument

- Most sample types can be fully automated using the DISCOVERY XT Kits. Manual pretreatment may give a better result in some cases (see **Appendix A. Semi-automated RNAscope® 2.5 VS Assay** on page 24). Use the semi-automated procedure for tissues that do not have a satisfactory result when using the fully automated procedure.
- If your instrument has been used recently, run the Prime-XT protocol two times to clear the fluid lines before setting up the experiment. Refer to the *Ventana™ System User Manual*.

Dilute bulk reagents

Prepare DISCOVERY Wash, RiboWash, and Reaction Buffer by diluting them 1:10 with distilled water.

Register new reagents

Reagent dispensers come with appropriate barcode labels and registration buttons for dispensing RNAscope® VS Reagents. Refer to the *Ventana™ DISCOVERY ULTRA System User Manual* for details. To register reagents:

1. Log all ACD reagents and probes into the software as "log user-fillable reagents" or "'log user-fillable probes".
2. Use the wand that comes with the instrument to register *new* reagent kits.

Prepare instrument reagents

Refer to the table on page 9 to determine the proper dispenser for each reagent.

1. For RNAscope® 2.5 VS Amp 1 to Amp 7, transfer the entire volume of each RNAscope® 2.5 VS Reagent Kit component into the correspondingly labeled dispenser.

2. Transfer the rest of the RNAscope® VS Reagents (VS Target Probe, VS Positive Control Probe, VS Negative Control Probe, 2.5 VS Pretreat 2–Dewax, 2.5 VS Protease, both bottles of 2.5 VS Target Retrieval, VS Hematoxylin, and VS Bluing Reagent) to the correspondingly labeled dispensers.

IMPORTANT! Avoid cross contamination between reagents. Dewax must be warmed to room temperature and be completely in solution before transfer.

3. Properly prime and handle the dispensers.
4. Press the dispenser caps down tightly.

Note: Store tightly capped dispensers at **4°C** (except the Dewax dispenser) when not in use.

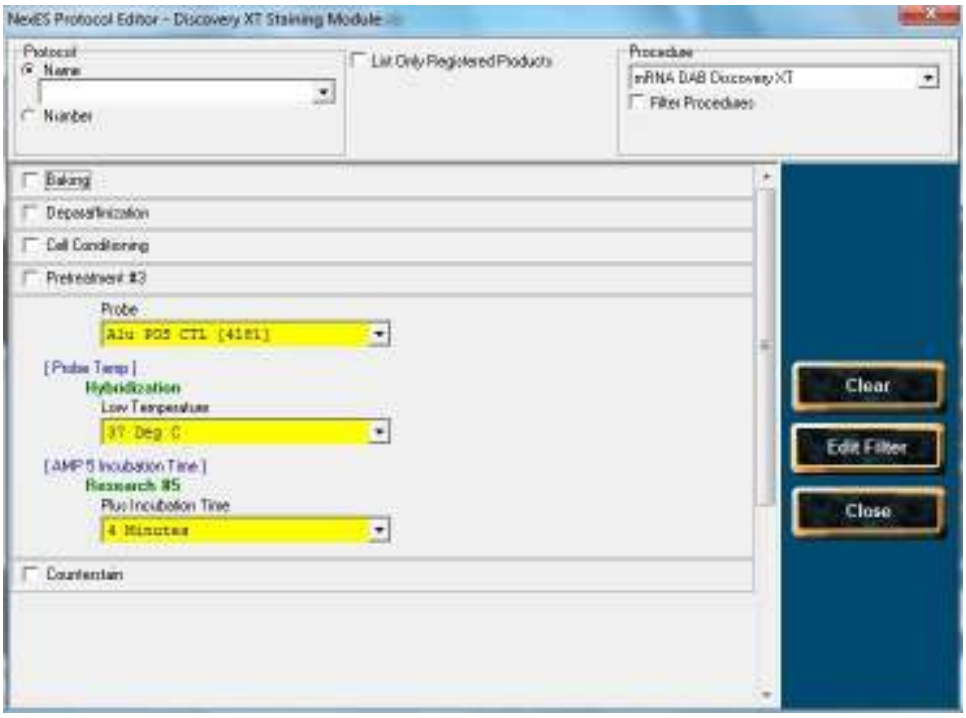
5. Check bulk solution levels: DISCOVERY Wash, RiboWash, Reaction Buffer, and LCS. Refill if they are less than half full. To run a full tray of 30 slides, fully fill the RiboWash Buffer container.
6. For CC1, the optimal volume is approximately 25% of the container capacity. Refill to this level if the volume in the container falls below 10% capacity.

IMPORTANT! Use reagents that have not expired.

7. Empty the waste carboy if needed.

Create an instrument protocol

1. Open the NexES software and click on the **Protocol** button.
2. Click on **Create/Edit Protocols**, go to the Procedure drop down menu, and select **mRNA DAB Discovery XT**.
3. This window will appear as shown:



4. By selecting the main protocol steps, drop down menus become available. Select the appropriate protocol steps by clicking on the associated check boxes as shown:

5. Select the appropriate assay conditions from the drop down menus according to the following table:

Tissue Type	Cell Conditioning Temperature	Time
Brain	97°C	16 MIN
Cell pellet	97°C	16 MIN
Colon	97°C	24 MIN
Heart	97°C	24 MIN
Intestine	97°C	24 MIN
Kidney	97°C	24 MIN
Liver	97°C	24 MIN
Lung	97°C	24 MIN

Tissue Type	Cell Conditioning Temperature	Time
Prostate	97°C	24 MIN
Spleen	97°C	24 MIN
Tonsil	97°C	24 MIN

Suggested Temperatures/Times	
Protease	Protease: 37°C
Suggested probe temperatures	Single Probes 43°C
	Pooled Probes 50°C
AMP 5 incubation time*	32 MIN

* Staining intensity can be modified by adjusting Amp 5 incubation times.

6. Click **Save As**, then select a protocol number from the drop down menu and choose a protocol name for each probe. Click **Save**.
7. Click **Close** to go back to the main screen.
8. Assign a probe number from the list to each probe of interest. For each probe selected, assign a protocol.

Print the labels

1. Select the **Print Label** icon from the bottom of the home page screen.
2. Select your preferred template or create a new template. To create a new template, refer to the *Ventana™ DISCOVERY XY System User Manual* for details.
3. Select the protocol you created for the RNAscope® 2.5 VS Assay.
4. Click on **Protocol** to add and print the label.
5. Proceed to **Load the slides** in the next section.

Run the RNAscope® 2.5 VS Assay

Materials required

- Prepared Slides
- Prepared instrument reagents
- Distilled water
- Prepared detergent
- Fume hood
- Xylene
- Tissue-Tek® Staining Dish (1)
- Tissue-Tek® Clearing Agent Dish, xylene-resistant (1)
- Tissue-Tek® Vertical 24 Slide Rack
- Cytoseal XYL xylene-based
- Cover Glass, 24 mm x 50 mm

Load the slides

IMPORTANT! Prior to loading the slides, ensure heater pads are completely dry. Wipe off any solution using laboratory tissue paper.

1. Load each slide onto a heater pad with the label facing away from you. Ensure that the slides sit securely on the pads.

Load the reagents

1. Remove the nozzle caps of the filled dispensers and place each cap in their holders (found on the post located on the back of the dispenser).
2. If needed, remove any air bubbles at the nozzle tips by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle.

IMPORTANT! Do not dispense any drops as this could compromise your drop inventory.

3. Load dispensers onto the reagent racks.
4. Remove the yellow locking ring from the dispensers in the prefilled RNAscope® 2.5 VS BROWN Detection Kit (Cat. No. 322200). Refer to the instructions provided by Ventana™ Medical Systems.
5. Load the reagent racks onto the reagent carousel.

Start the run

1. Click the **Run** button.

2. Select both checkboxes as shown below from the Pre-Run Checklist window.

3. Ensure that the slides sit securely on the pads.
4. Close slide drawer.
5. Click the **Run** button. Automated assay will finish in **~10 HRS**.

IMPORTANT! Before leaving the instrument unattended, ensure all reagents and slides are successfully registered and the instrument is running.

Prepare detergent

1. Prepare 200 mL of diluted detergent by adding 1 to 2 drops detergent to 200 mL distilled water in a container with a cap.
2. Mix well by inverting the container 4–5 times.
3. Add diluted detergent to a Tissue-Tek® Staining Dish.

Note: Store diluted detergent at **RT**.

Prepare dehydrating reagents

IMPORTANT! Do not reuse deparaffinization reagents.

1. In a fume hood, fill two clearing agent dishes with ~200 mL fresh xylene.
2. In a fume hood, fill three staining dishes with ~200 mL fresh 100% EtOH.

Note: Ensure all containers remain covered when not in use.

Complete the run

1. After the run is complete, remove the Dewax (Pretreatment A) reagent, place nozzle cap on the dispenser, and store at room temperature.
2. For the remaining reagents, place nozzle caps back on the dispensers and place racks onto magnet locking tray.

IMPORTANT! Store reagent racks at **4°C** until next use. Store the Dewax dispenser at room temperature.

Wash the slides

1. Submerge a Tissue-Tek® Slide Rack into the Tissue-Tek® Staining Dish containing 200 mL diluted detergent.
2. Open the instrument slide tray drawers and unload slides.
3. Decant solution on the slides into the slide tray drawer, then *immediately* load slides into the Tissue-Tek® Slide Rack submerged in detergent.
4. Rinse oil off the slides by moving the slide rack up and down in the dish 10 times.
5. Replace the detergent with distilled water and rinse slides by moving the slide rack up and down a minimum of 10 times.
6. Repeat Step 5, 3–5 times.

Dehydrate the slides

1. Move the Tissue-Tek® Slide Rack into the first staining dish containing 100% EtOH in the fume hood for **2 MIN**. Agitate the slides by occasionally lifting the slide rack up and down.
2. Move the Tissue-Tek® Slide rack into the second Staining Dish containing 100% EtOH for **2 MIN** with occasional agitation.
3. Move the Tissue-Tek® Slide rack into the third Staining Dish containing 100% EtOH for **2 MIN** with occasional agitation.
4. Move the Tissue-Tek® Slide rack into the Staining Dish containing xylene for **1 MIN** with occasional agitation.
5. Move the Tissue-Tek® Slide rack into the Staining Dish containing xylene for **1 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 1–2 drops 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 at least **5 MIN**.
4. Repeat steps 2 and 3 for each slide.
5. Proceed to **Chapter 5. Evaluate the Results** on page 22.

Recommended guidelines

We highly recommend following the guidelines for Cell Conditioning (Target Retrieval), and Pretreatment #3 (Protease) conditions for:

- Any new or previously untested FFPE tissue types.
- Samples prepared differently than the protocol in **Chapter 3. Prepare Samples** on page 12.

1. Stain six representative slides using the positive and negative control probes according to the following table:

Slide No.	Probe	Target Retrieval	Protease
1	Positive control	16 MIN	24 MIN
2	Negative control	16 MIN	24 MIN
3	Positive control	16 MIN	16 MIN
4	Negative control	16 MIN	16 MIN
5	Positive control	24 MIN	16 MIN
6	Negative control	24 MIN	16 MIN

2. Evaluate staining and tissue morphology as **Chapter 5. Evaluate the Results** on page 22 and determine which pretreatment condition yielded the highest positive control signal and lowest negative control signal. Positive control signal should have a staining score of 3 or higher, and the negative control signal should be 0.
3. Use the optimized pretreatment conditions to run the assay with the target probe.
4. If none of the conditions are satisfactory, contact technical support at **support@acdbio.com**.

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5 Chapter 5. Evaluate the Results

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 punctate dots within the cytoplasm at 20–40X magnification.
- Assess negative control background. One dot to every 10 cells displaying background staining per 40X microscope field is acceptable.
- Evaluate target probe signal using the scoring guidelines in the next section.

Scoring guidelines

The RNAscope® 2.5 VS Assay enables 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.

Note: 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/10 cells (40X magnification)
1	1–3 dots/cell (visible at 20–40X magnification)
2	4–10 dots/cell. No or 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**.

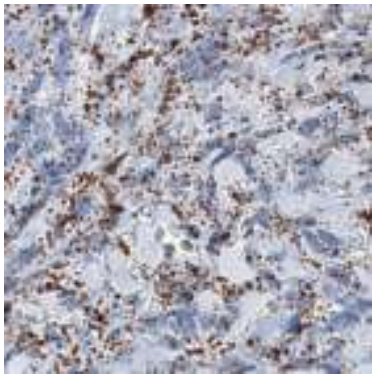
Troubleshooting

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

Example

If the assay is successful, the staining should look like the following images:

Figure 2. RNAscope® 2.5 VS Assay detection of Hs-PPIB larynx FFPE tissue



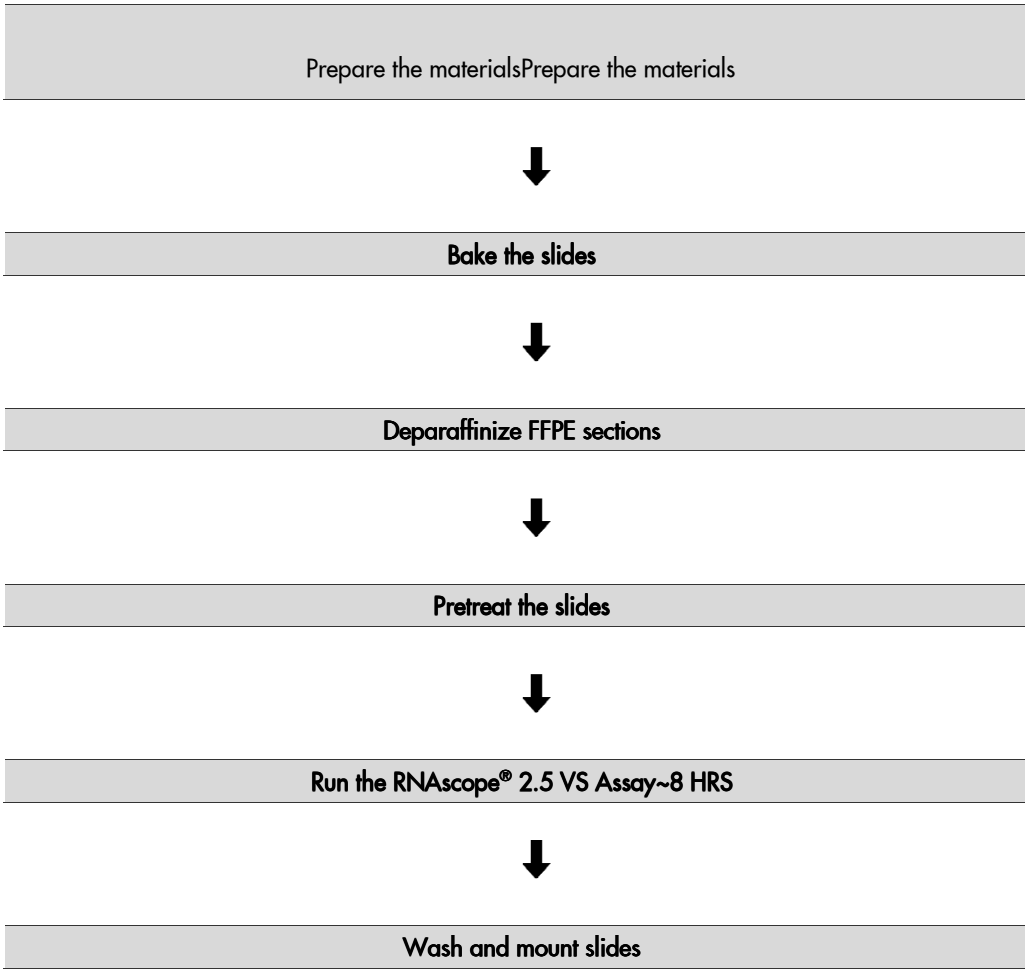
Hs-PPIB (Positive Control)

DapB (Negative Control)

A Appendix A. Semi-automated RNAscope® 2.5 VS Assay

Most sample types can be fully automated on the Discovery XT (**see Chapter 4. Automated RNAscope® 2.5 VS Assay** on page 14). Manual pretreatment may give a better result in some cases. Use the semi-automated procedure for tissues that do not have a satisfactory result when using the fully automated procedure.

Workflow



Kit contents and storage

RNAscope 2.5 VS Reagents

For Offline Boiling: RNAscope® Target Retrieval Kit (Cat. No. 322000)				
☑	Cat. No.	Reagent	Quantity	Storage
	322000	RNAscope® Target Retrieval Reagent	70 mL x 4 bottles	Room Temp (15–30°C)

IMPORTANT! Do not substitute the reagent components of the RNAscope® 2.5 VS Reagent Kit with those of other RNAscope® Reagent Kits, even those having the same name. The Target Retrieval solution in the RNAscope® 2.5 VS Kit CANNOT be used for offline boiling. Please use the RNAscope® Target Retrieval Reagents (Cat. No. 322220) to boil samples offline.

Prepare the materials

Materials can be prepared ahead of time or while baking the slides, unless otherwise stated. See **Bake the slides** on page 28.

Materials required

Materials Provided by Advanced Cell Diagnostics	Materials Provided by Ventana™ Medical Systems	Other Materials and Equipment
• RNAscope® VS 2.5 Target Probe • RNAscope® VS 2.5 Positive Control Probe • RNAscope® VS2.5 Negative Control Probe • RNAscope® Target Retrieval Reagents (Offline) • RNAscope® 2.5 VS Pretreat 3-Protease • RNAscope® 2.5 VS Amp 1 • RNAscope® 2.5 VS Amp 2 • RNAscope® 2.5 VS Amp 3 • RNAscope® 2.5 VS Amp 4 • RNAscope® 2.5 VS Amp 5 • RNAscope® 2.5 VS Amp 6 • RNAscope® 2.5 VS Amp 7 • RNAscope® VS Hematoxylin • RNAscope® VS Bluing Reagent	• DISCOVERY™ XT — automated slide stainer • DISCOVERY Wash • LCS • RiboWash Buffer • Reaction Buffer • Cell Conditioning 1 (CC1) Buffer • Probe dispensers • mRNA Pretreatment Kit • mRNA Probe Amplification Kit • mRNA DAB Detection Kit • User fillable dispensers • Option 10 Dispenser	• Distilled water • Glass beaker (1 or 2 L) • Hot plate • Dawn detergent or similar detergent • Fume hood • Xylene • 100% ethanol (EtOH) • Tissue-Tek® Staining Dish (3) • Tissue-Tek® Clearing Agent Dish, xylene-resistant (3) • Tissue-Tek® Vertical 24 Slide Rack • Cytoseal XYL xylene-based • Cover Glass, 24 mm x 50 mm

Prepare the instrument

If your instrument has been used recently, run the Prime-XT protocol two times to clear the fluid lines before setting up the experiment. Refer to the *Ventana DISCOVERY XT System User Manual*.

Dilute bulk reagents

Please prepare the bulk fluids as per manufacturer's instructions.

Prepare instrument reagents

Log all ACD reagents and probes into the software as "log user-fillable reagents" or "'log user-fillable probes". Then, register *new* reagent kits using the wand that comes with the instrument. Refer to the table on page 9 to determine the proper dispenser for each reagent.

1. From RNAscope® 2.5 VS AMP 1 to AMP 7, transfer the entire volume of each RNAscope® 2.5 VS Reagent Kit component into the correspondingly labeled dispenser.
2. Transfer the rest of the RNAscope® VS Reagents (RNAscope® VS 2.5 Pretreat 3-Protease, RNAscope® VS Hematoxylin, and RNAscope® VS Bluing Reagent) to the correspondingly labeled dispensers.

IMPORTANT! Avoid cross contamination between reagents.

3. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
4. Press the dispenser caps down tightly.

Note: Store tightly capped dispensers at **4°C** when not in use.

5. Check solution levels: DISCOVERY Wash, RiboWash, Reaction Buffer, and LCS Buffer. Refill if they are less than half full. Refill if they are less than half full. To run a full tray of 30 slides, fully fill the RiboWash Buffer container.
6. For CC1, the optimal volume is approximately 25% of the container capacity. Refill to this level if the volume in the container falls below 10% capacity.

IMPORTANT! Use reagents that have not expired.

7. Empty the waste carboy if needed.

Prepare deparaffinization reagents

IMPORTANT! Do not use deparaffinization solutions for dehydration.

1. In a fume hood, fill two clearing agent dishes with ~200 mL fresh xylene.
2. In a fume hood, fill two staining dishes with ~200 mL fresh 100% EtOH.

Note: Ensure all containers remain covered when not in use.

Prepare 1X Target Retrieval

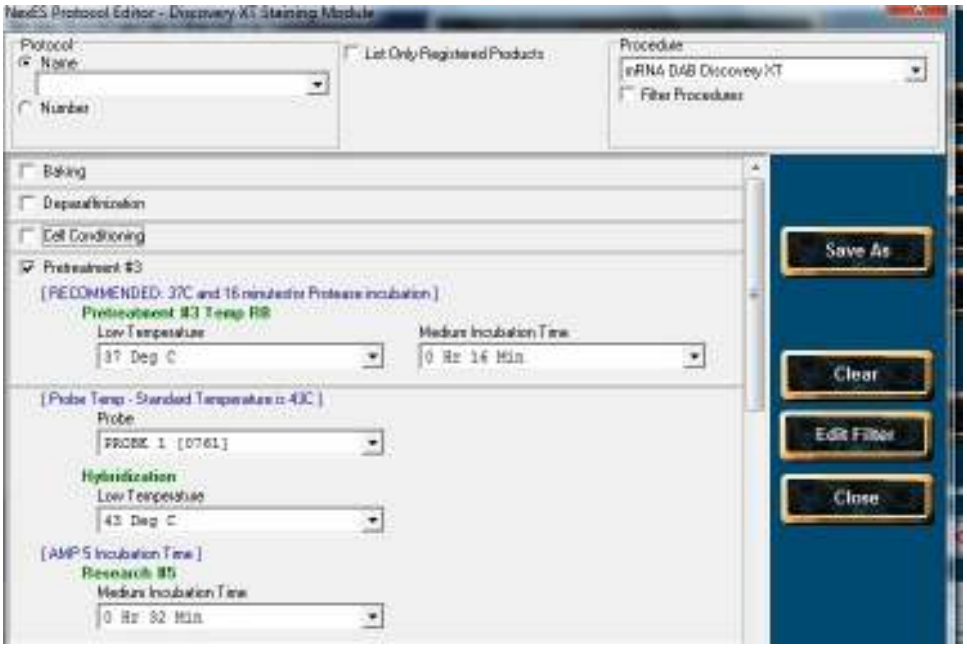
Prepare 1X Target Retrieval while FFPE slides are baking at 60°C, or the following day if you choose the optional stopping point on page 12. 1X Target Retrieval Buffer is used in manual Cell Conditioning

1. Prepare 700 mL of fresh 1X Target Retrieval by adding 630 mL distilled water to 1 bottle (70 mL) 10X Target Retrieval in the beaker.
2. Mix well and cover the beaker with foil.

IMPORTANT! Do not use RNAscope® 2.5 VS Target Retrieval for offline boiling.

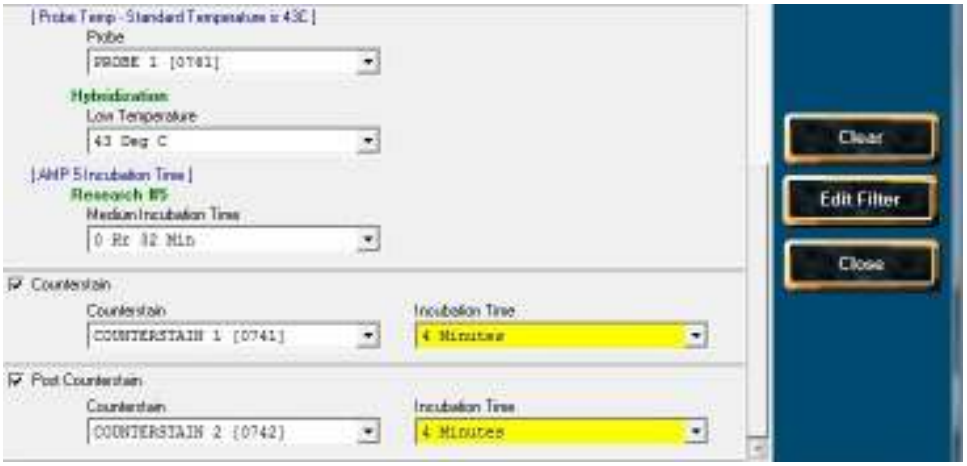
Create an instrument protocol

1. Open the NexES software and click on the **Protocol** button.
2. Click on Create/Edit Protocols, go to the Procedure drop down menu, and select **mRNA DAB Discovery XT**
3. Main protocol steps appear as shown:



IMPORTANT! Do not select Baking, Deparaffinization, or Cell Conditioning.

4. After selecting the main protocol steps, drop down menus become available. Select the appropriate protocol steps by clicking on the associated check boxes as shown:



5. Select the appropriate assay conditions from the drop down menus according to the following table:

Suggested Temperatures/Times	
Pretreatment 3	37°C at 16 MIN
Suggested probe temperatures	Single Probes 43°C
	Pooled Probes 50°C
AMP 5 incubation time*	32 MIN

* Staining intensity can be modified by adjusting Amp 5 incubation times.

6. Click **Save As**, then select a protocol number from the drop down menu and choose a protocol name for each probe. Click **Save**.
7. Click **Close** to go back to the main screen

8. Assign a probe number from the list to each probe of interest. For each probe selected, assign a protocol.

Print the labels

1. Select the **Print Label** icon from the bottom of the home page screen.
2. Select your preferred template or create a new template. To create a new template, refer to the *Ventana™ DISCOVERY XT System User Manual* for details.
3. Select the protocol you created for the RNAscope® 2.5 VS Assay.
4. Click on **Protocol** to add and print the label.

Manually pretreat the samples

Materials required

Materials Provided by the Target Retrieval Reagents (Cat. No. 322000)	Other Materials and Equipment
• RNAscope® Target Retrieval Reagents	• Drying oven • FFPE slides • Tissue-Tek® Vertical 24 Slide Rack • Distilled water • Fume hood • Xylene • 100% ethanol (EtOH) • Tissue-Tek® Clearing Agent Dish (2) • Tissue-Tek® Staining Dish (2) • Glass beaker (1 or 2 L) • Hot plate

Bake the slides

1. Bake slides in a dry oven for up to **30-60 MIN** at **60°C**.

OPTIONAL STOPPING POINT Use immediately or store at RT with desiccants for ≤1 week. Prolonged storage may degrade sample RNA.

2. If you continue, prepare the materials for the following protocols while the slides are baking: **Deparaffinize FFPE sections** in the next section, **Pretreat the slides** on page 29, and **Run the RNAscope® 2.5 VS Assay** on page 30. See **Prepare the materials** on page 30.

Deparaffinize FFPE sections

IMPORTANT! If you have not already done so, create a protocol for your instrument and print slide labels during this procedure. See **Create an instrument protocol** on page 26.

1. Place slides in a Tissue-Tek® Slide Rack and submerge in the first xylene-containing clearing agent dish in the fume hood.
2. Incubate the slides in xylene for **5 MIN** at **RT**. Agitate the slides by occasionally lifting the slide rack up and down in the clearing agent dish.
3. Repeat Step 2.
4. Remove the slide rack from the second xylene-containing dish and *immediately* place in the staining dish containing 100% EtOH.
5. Incubate the slides in 100% EtOH for **1 MIN** at **RT** with agitation.

6. Repeat Step 6 with fresh 100% EtOH.
7. Remove the slides from the rack, and place on absorbent paper with the section face-up. Air dry for **5 MIN** at **RT**.
8. While slides are drying, place printed labels on the slides.

IMPORTANT! Labels must be in place prior to the next section.

9. Insert the slides into a Tissue-Tek® Slide Rack and proceed to the next section.

Pretreat the slides

Begin heating 1X Target Retrieval Buffer while FFPE slides are baking at 60°C or during deparaffinization.

IMPORTANT! Do not boil 1X Target Retrieval more than **30 MIN** before use.

1. Heat 1X Target Retrieval Buffer to **98–104°C**:
   a. Place the beaker containing 1X Target Retrieval Buffer on the hot plate. Cover the beaker with foil and turn the hot plate on high for **10–15 MIN**.
   b. Once 1X Target Retrieval Buffer reaches a slow boil (**98–104°C**), turn the hot plate to a lower setting to maintain the correct temperature. Check the temperature with a thermometer.
2. With a pair of forceps *very slowly* submerge the slide rack containing the slides into the boiling 1X Target Retrieval Buffer solution. Cover the beaker with foil and boil the slides for the amount of time specified in the following table:

Tissue Type	Treatment Time
Brain and spinal cord	15 MIN
Breast cancer	15 MIN
Cell lines	8 MIN
Colon	15 MIN
GI tract	15 MIN
Head and neck cancer	15 MIN
Heart	15 MIN
Kidney	15 MIN
Liver	30 MIN
Lung	15 MIN
Lymphoma	10 MIN
Placenta	15 MIN
Prostate	15 MIN
Skin	15 MIN
Stomach	15 MIN
Thymus	10 MIN
Tonsil	10 MIN
Xenograft derived from cell lines	7 MIN
Xenograft derived from primary tumor	15 MIN

3. Use the forceps to *immediately* transfer the hot slide rack from the 1X Target Retrieval Buffer to the staining dish containing distilled water. Do not let the slides cool in Target Retrieval.

4. Wash slides 3–5 times by moving the Tissue-Tek® Slide Rack up and down in the distilled water.
5. Repeat Step 4 with fresh distilled water.
6. Proceed directly to
7. **Load the** slides in the next section.

Run the RNAscope® 2.5 VS Assay

Materials required

- Prepared slides
- Prepared instrument reagents
- Prepared detergent
- Distilled water
- Prepared dehydrating materials
- Tissue-Tek® Vertical 24 Slide Rack
- Cytoseal XYL xylene-based
- Cover Glass, 24 mm x 50 mm

Load the slides

IMPORTANT! Prior to loading the slides, ensure heater pads are completely dry. Wipe off any solution using laboratory tissue paper.

1. Load each slide onto a heater pad with the label positioned away from you. Ensure that the slides sit securely on the pads.

Load the reagents

1. Remove the nozzle caps of the filled dispensers and place each cap in their holders (found on the post located on the back of the dispenser).
2. If needed, remove any air bubbles at the nozzle tips by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle.

IMPORTANT! Do not dispense any drops as this could compromise your drop inventory.

3. Load dispensers onto the reagent racks.
4. Remove the yellow locking ring from the dispensers in the prefilled mRNA BROWN Detection Kit. Refer to the instructions provided by Ventana™ Medical Systems.
5. Load the reagent racks onto the reagent carousel.

Start the run

1. Click the **Run** button.

2. Select both checkboxes as shown below from the Pre-Run Checklist window, and enter number of slides.

3. Ensure that the slides sit securely on the pads.

IMPORTANT! Prior to loading the slides, ensure heater pads are completely dry. Wipe off any solution using laboratory tissue paper.

4. Close slide drawer.
5. Click the **Run** button. Semi-automated assay will finish in **~8 HRS**.

IMPORTANT! Before leaving the instrument unattended, ensure all reagents and slides are successfully registered and the instrument is running.

Prepare detergent

1. Prepare 200 mL of diluted detergent by adding 1 to 2 drops detergent to 200 mL distilled water in a container with a cap.
2. Mix well by inverting the container 4–5 times.
3. Add diluted detergent to a Tissue-Tek® Staining Dish.

Note: Store diluted detergent at **RT**.

Prepare dehydrating reagents

IMPORTANT! Do not reuse deparaffinization reagents.

1. In a fume hood, fill two clearing agent dishes with ~200 mL fresh xylene.
2. In a fume hood, fill three staining dishes with ~200 mL fresh 100% EtOH.

Note: Ensure all containers remain closed when not in use.

Complete the run

1. After the run is complete, place nozzle caps back on the dispensers.
2. Store reagent racks at **4°C** until next use.

IMPORTANT! Store the Dewax dispenser at room temperature.

Wash the slides

1. Submerge a Tissue-Tek® Slide Rack into the Tissue-Tek® Staining Dish containing 200 mL diluted detergent.
2. Open the instrument slide drawer tray and unload slides.
3. Decant solution on the slides into the slide drawer tray, then *immediately* load slides into the Tissue-Tek® Slide Rack submerged in detergent.
4. Rinse oil off the slides by moving the slide rack up and down in the dish 10 times.
5. Replace the detergent with distilled water and rinse slides by moving the slide rack up and down a minimum of 10 times.
6. Repeat Step 5, 3–5 times.

Dehydrate the slides

1. Move the Tissue-Tek® Slide Rack into the first staining dish containing 100% EtOH in the fume hood for **2 MIN**. Agitate the slides by occasionally lifting the slide rack up and down.
2. Move the Tissue-Tek® Slide rack into the second Staining Dish containing 100% EtOH for **2 MIN** with occasional agitation.
3. Move the Tissue-Tek® Slide rack into the third Staining Dish containing 100% EtOH for **2 MIN** with occasional agitation.
4. Move the Tissue-Tek® Slide rack into the Staining Dish containing xylene for **1 MIN** with occasional agitation.
5. Move the Tissue-Tek® Slide rack into the Staining Dish containing xylene for **1 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 1–2 drops 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 at least **5 MIN**.
4. Proceed to **Chapter 5. Evaluate the Results** on page 22.

Recommended guidelines

We highly recommend following the guidelines for Cell Conditioning (Target Retrieval) and Protease conditions for:

- Any new or previously untested FFPE tissue types.
- Samples prepared differently than the protocol in Chapter 3. Prepare Samples.

1. Stain six representative slides using the positive and negative control probes according to the following table:

Slide No.	Probe	Target Retrieval	Protease
1	Positive control	10 MIN	24 MIN
2	Negative control	10 MIN	24 MIN
3	Positive control	10 MIN	16 MIN
4	Negative control	10 MIN	16 MIN
5	Positive control	15 MIN	16 MIN
6	Negative control	15 MIN	16 MIN

2. Evaluate staining and tissue morphology as in **Chapter 5. Evaluate the Results** on page 22, and determine which pretreatment condition yielded the highest positive control signal and lowest negative control signal. Positive control signal should have a staining score of 3 or higher if using PPIB, and the negative control signal should be 0.
3. Use the optimized pretreatment conditions to run the assay with the target probe.
4. If none of the conditions are satisfactory, contact technical support at **support@acdbio.com**.

ADVANCED CELL DIAGNOSTICS

B Appendix B. Safety

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) 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: **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 SDSs

Safety Data Sheets (SDSs) are available at: **http://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 **http://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.
3960 Point Eden Way
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** . If you have any questions, please contact Advanced Cell Diagnostics at **http://www.acdbio.com/technical-support/support-overview**.

Headquarters
3960 Point Eden Way Hayward, CA 94545 Phone 1-510-576-8800 Toll Free 1-877-576-3636
For support, email support@acdbio.com.
www.acdbio.com

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