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USER MANUAL

Fluorescent Guide for the RNAscope® VS Universal HRP Assay

For Ventana DISCOVERY™ ULTRA System

Fluorescent ISH

Document Number MK 50-014

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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.

Disclaimers

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Contents

1 Chapter 1. Product Information

Before using this product, read and understand the information in **Appendix B. Safety** on page 31 in this document.

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

About this guide

This user manual describes how to combine the RNAscope® VS Universal HRP assay with fluorophores from Roche Tissue Diagnostics to create an RNAscope® VS Universal HRP fluorescent ISH assay.

Two versions of the RNAscope® VS Universal HRP Fluorescent Assay are provided:

- **Chapter 3. Automated RNAscope® VS Universal HRP Fluorescent** Assay starting on page 11.
- **Appendix A. Semi-automated RNAscope® VS Universal HRP Fluorescent** Assay starting on page 21.

RNAscope® assays are compatible with a variety of sample types. You must use both an RNAscope® Assay user manual and a sample preparation and pretreatment user guide or tech note to perform the entire assay. Go to **https:// acdbio.com/technical-support/user-manuals** for sample preparation user guides and tech notes.

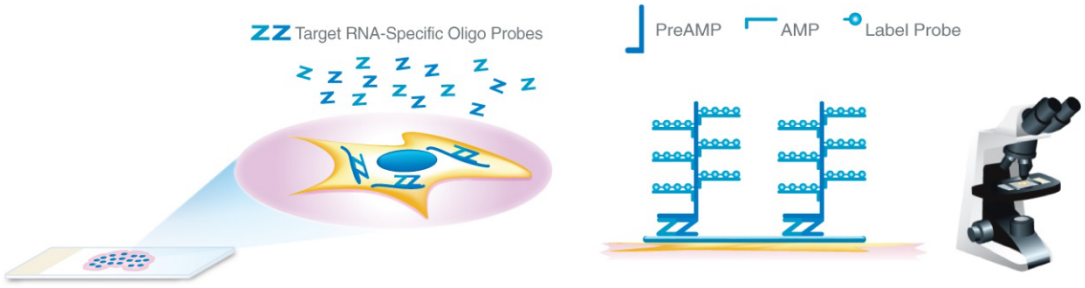
Background

The RNAscope® 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® VS Universal HRP Fluorescent Assay allows users to automate the highly sensitive RNAscope® Assay using the Ventana DISCOVERY™ ULTRA System.

Overview

Figure 1 on page 6 illustrates the RNAscope® VS Universal HRP Fluorescent Assay procedure, which 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)-labeled probes and detection using a fluorescent substrate. Use a common fluorescent microscope with the proper filters to visualize the RNA transcripts.

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-labeled probes. The VS Universal Fluorescent Assay enhances signal further with additional amplification steps. Add a fluorescent substrate to detect target RNA.	Visualize target RNA using a fluorescent microscope.

Kit contents and storage

The RNAscope® VS Universal HRP Fluorescent Assay requires the RNAscope® VS Probes and the RNAscope® VS Universal HRP Reagents, available from Advanced Cell Diagnostics.

RNAscope® 2.5 VS Probes

The RNAscope® VS Probes consist of the user-specified Target Probe and the Positive and Negative Control Probes. Visit **https://acdbio.com/products** to find a gene-specific Target Probe or appropriate Control Probes. Each probe is sufficient for staining ~30 standard slides. The probes have a shelf life of two years from the manufacturing date when stored as indicated in the following table:

Target Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® 2.5 VS Target Probe – *[species]– [gene]*	various	Probe targeting specific RNA	7 mL x 1 bottle	2–8°C
Control Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® 2.5 VS Positive Control Probe – *[species]– PPIB*	various	Probe targeting common housekeeping gene	7 mL x 1 bottle	2–8°C
	RNAscope® 2.5 VS Positive Control Probe – *[species]– POLR2a*	various	Probe targeting common housekeeping gene	7 mL x 1 bottle	2–8°C
	RNAscope® 2.5 VS 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® 2.5 VS Positive Control Probe and RNAscope® 2.5 VS Negative Control Probe. The slides have a shelf life of nine months from the manufacturing date when stored at 2–8°C with desiccants.

RNAscope® VS Universal Reagents

RNAscope® VS kits provide enough reagents to stain ~60 standard slides. The reagents are Ready-To-Use (RTU) and have a shelf life of nine months from the manufacturing date when stored as indicated in the following tables:

RNAscope® VS Universal HRP Detection Reagents (Cat. No. 323210)				
☑	Reagent	Cat. No.	Quantity	Storage
	RNAscope® VS Universal HRP AMP 1	322211	14 mL x 1 bottle	2–8°C
	RNAscope® Universal HRP AMP 2	322212	14 mL x 1 bottle	2–8°C
	RNAscope® Universal HRP AMP 3	322213	14 mL x 1 bottle	2–8°C
	RNAscope® Universal HRP AMP 4	322214	14 mL x 1 bottle	2–8°C
	RNAscope® Universal HRP AMP 5	322215	14 mL x 1 bottle	2–8°C
	RNAscope® Universal HRP AMP 6	322216	14 mL x 1 bottle	2–8°C
	RNAscope® Universal HRP AMP 7	322217	14 mL x 1 bottle	2–8°C
	RNAscope® VS Protease	322218	14 mL x 1 bottle	2–8°C
RNAscope® VS Universal Sample Prep Reagents (Cat. No. 323220)				
☑	Reagent	Cat. No.	Quantity	Storage
	RNAscope® VS Target Retrieval	322221	14 mL x 2 bottles	Room Temp (15–30°C)
	RNAscope® VS Dewax	322222	14 mL x 1 bottle	Room Temp (15–30°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! Use only RNAscope® 2.5 VS Probes. Do not substitute the reagent components of the RNAscope® VS Universal Reagent Kit with those of other RNAscope® Reagent Kits.

Required materials from Roche Diagnostics

The RNAscope® VS Universal HRP Fluorescent 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 Sample Prep Kit (Cat. No. 760-248; Ordering Code 08127166001)		
☑	Component	Storage
	mRNA Target Retrieval dispenser — fill dispenser with RNAscope® VS Universal Target Retrieval	Room Temp (15–30°C)
	mRNA Dewax dispenser — fill dispenser with RNAscope® VS Dewax	Room Temp (15–30°C)
	mRNA Protease dispenser — fill dispenser with RNAscope® VS 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 Universal HRP AMP 1	Room Temp (15–30°C)
	mRNA AMP 2 dispenser — fill dispenser with Universal HRP AMP 2	Room Temp (15–30°C)
	mRNA AMP 3 dispenser — fill dispenser with Universal HRP AMP 3	Room Temp (15–30°C)
	mRNA AMP 4 dispenser — fill dispenser with Universal HRP AMP 4	Room Temp (15–30°C)
	mRNA AMP 5 dispenser — fill dispenser with Universal HRP AMP 5	Room Temp (15–30°C)
	mRNA AMP 6 dispenser — fill dispenser with Universal HRP AMP 6	Room Temp (15–30°C)
	mRNA AMP 7 dispenser — fill dispenser with Universal HRP AMP 7	Room Temp (15–30°C)

Ventana Fluorescent Detection Kits (select one)				
☑	Kit	Cat. No.	Ordering Code	Storage
	DISCOVERY DCC kit	760-240	07988192001	2–8°C
	DISCOVERY FAM kit	760-243	07988150001	2–8°C
	DISCOVERY FITC kit	760-232	07259212001	2–8°C
	DISCOVERY Rhodamine kit	760-233	07259883001	2–8°C
	DISCOVERY Rhodamine 6G kit	760-244	07988168001	2–8°C
	DISCOVERY Red 610 kit	760-245	07988176001	2–8°C
	DISCOVERY Cy5 kit	760-238	07551215001	2–8°C

Counterstain (Cat. No. 760-4196; Ordering Code 05268826001)		
☑	Component	Storage
	DISCOVERY QD DAPI –prefilled Counterstain	2–8°C

Ancillary Dispenser (Cat. No.760-4840, Ordering Code 07017944001)		
☑	Component	Storage
	DISCOVERY Inhibitor – prefilled	2–8°C

Equipment and buffers

☑	Component	Cat. No./ Ordering Code
	10X DISCOVERY Wash (RUO)	950-510 / 7311079001
	ULTRA LCS (Predilute)	650-210 / 5424534001
	SSC Buffer (10X)	950-110 / 5353947001
	Reaction Buffer (10X)	760-107 / 5266262001
	DISCOVERY CC1	950-500 / 6414575001

IMPORTANT! To run the VS Universal Assay successfully, use DISCOVERY Wash (950-510) and not DISCOVERY EZ Prep. In the SSC bulk container, use 2X SSC (950-110) and not Ribowash. To properly operate and prime the instrument, you must fill the option bulk container with fluid (for example, 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
	ProLong Gold mounting medium	Life Technologies	P36930
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24
	Tissue-Tek® Staining Dishes	American Master Tech Scientific/MLS	LWT4457EA
	Cover Glass 24 x 50 mm	Fisher Scientific/MLS	12--545-F
	Distilled water	MLS	—
	Dawn detergent or similar detergent	MLS	—
	Optional: Drying oven, capable of holding temperature at 60 +/− 1°C	MLS	—
	Optional: fume hood	MLS	—
	Optional: 100% ethanol (EtOH)	MLS	—
	Optional: xylene	MLS	—
	Optional: Tissue-Tek® Clearing Agent Dishes, xylene-resistant	American Master Tech Scientific/MLS	LWT4456EA
	Optional: Glass beaker (1 or 2 L)	MLS	—
	Optional: Hot plate	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® VS Universal HRP Fluorescent Assay on your samples for the first time, we recommend that you:

- Be familiar with the Ventana™ DISCOVERY™ ULTRA 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; Catalog No. 310023 for Mouse control slide, 3T3) using the Positive and Negative Control Probes.

Important procedural guidelines

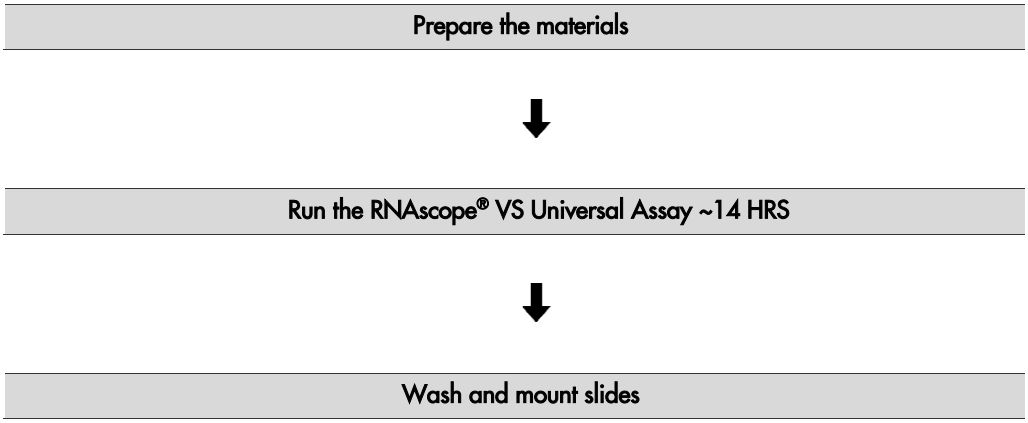
- Start with properly fixed and prepared sections. Refer to our sample preparation and pretreatment user guides and tech notes available at **https:// acdbio.com/technical-support/user-manuals**.
- Regularly maintain and clean your automated staining instrument.
- Always run positive and negative control 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.
- Do *not* let your sections dry out during the procedure unless specified in the protocol.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix B. Safety** on page 31 in this document for more information.

3 Chapter 3. Automated RNAscope® VS Universal HRP Fluorescent Assay

IMPORTANT! We strongly recommend you run the RNAscope® VS Control Slides (Cat. No. 310045 or 310023) using the RNAscope® 2.5 VS positive and negative control probes along with your samples in every run.

Appendix A. Semi-automated RNAscope® VS Universal HRP Fluorescent Assay describes an offline boiling procedure for use with Cat. No.322000.

Workflow



Prepare the materials

Materials required

Materials Provided by Advanced Cell Diagnostics	Materials Provided by Ventana™ Medical Systems	Other Materials and Equipment
• RNAscope® 2.5 VS Target Probe • RNAscope® 2.5 VS Positive Control Probe • RNAscope® 2.5 VS Negative Control Probe • RNAscope® VS Pretreat 2– Dewax • RNAscope® VS Protease • RNAscope® VS Target Retrieval • RNAscope® VS Universal HRP AMP 1 • RNAscope® VS Universal HRP AMP 2 • RNAscope® VS Universal HRP AMP 3 • RNAscope® VS Universal HRP AMP 4 • RNAscope® VS Universal HRP AMP 5 • RNAscope® VS Universal HRP AMP 6 • RNAscope® VS Universal HRP AMP 7	• DISCOVERY™ ULTRA — automated slide stainer • DISCOVERY Wash Buffer 10X • ULTRA LCS (Predilute) • SSC Buffer 10X • DISCOVERY CC1 • Reaction Buffer 10X • Probe dispensers • mRNA Sample Prep Kit • mRNA Probe Amplification Kit • Flourescent Detection Kit • User fillable dispensers • DISCOVERY Inhibitor • DISCOVERY QD DAPI Counterstain	• Distilled water • Dawn detergent or similar detergent • Tissue-Tek® Staining Dish • Tissue-Tek® Vertical 24 Slide Rack • ProLong Gold mounting medium • Cover Glass, 24 mm x 50 mm

Prepare the instrument

Most sample types can be fully automated using the DISCOVERY Universal HRP Kits. Manual pretreatment may give a better result in some cases (see **Appendix A. Semi-automated RNAscope® VS Universal HRP Fluorescent** Assay on page 21). Use the semi-automated procedure for tissues that do not have a satisfactory result when using the fully automated procedure.

If the instrument has not been used for ≥1 week, follow the guidelines for instrument maintenance in the *Ventana™ System User Manual*.

Dilute bulk reagents

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

Register new reagents

Reagent dispensers come with appropriate barcode labels and registration buttons for dispensing RNAscope® VS Universal Reagents. Refer to the *Ventana™ DISCOVERY ULTRA System User Manual* for details. To register reagents, use the wand that comes with the instrument to register *new* reagent kits.

Prepare instrument reagents

Refer to the table on page 7 to determine the proper dispenser for each reagent.

1. For RNAscope® VS Universal HRP AMP 1 to AMP 7, transfer the entire volume of each component into the correspondingly labeled dispenser.
2. Transfer the RNAscope® 2.5 VS Target Probe, 2.5 VS Positive Control Probe, 2.5 VS Negative Control Probe, VS Dewax, VS Protease, and both bottles of VS Target Retrieval to the correspondingly labeled dispensers.

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

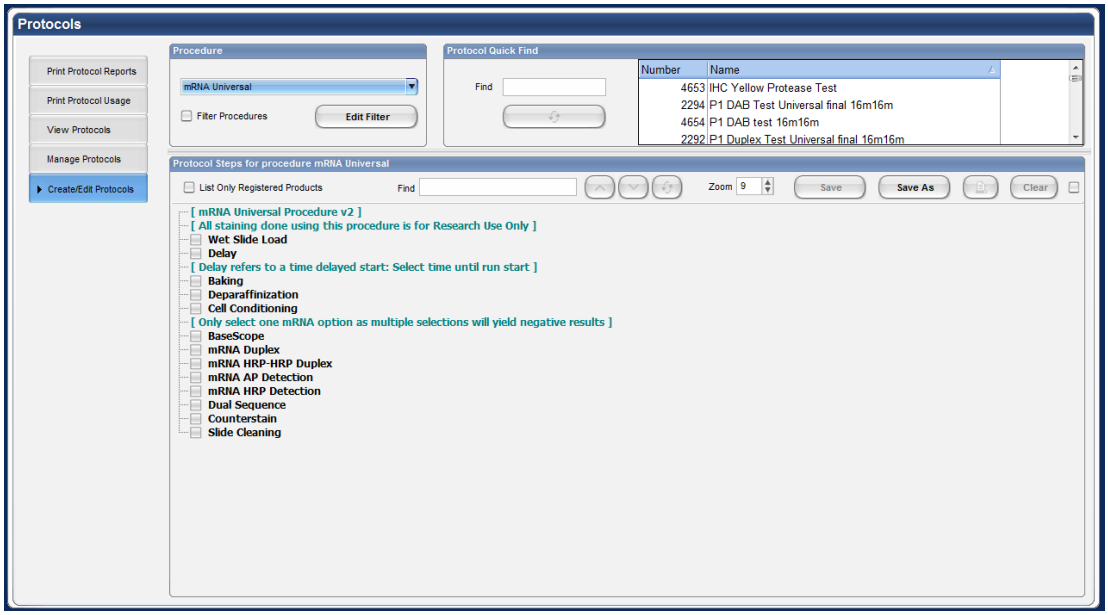
3. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
4. Store tightly-capped dispensers (except the Dewax dispenser) at **4°C** when not in use.

IMPORTANT! Do not use expired reagents.

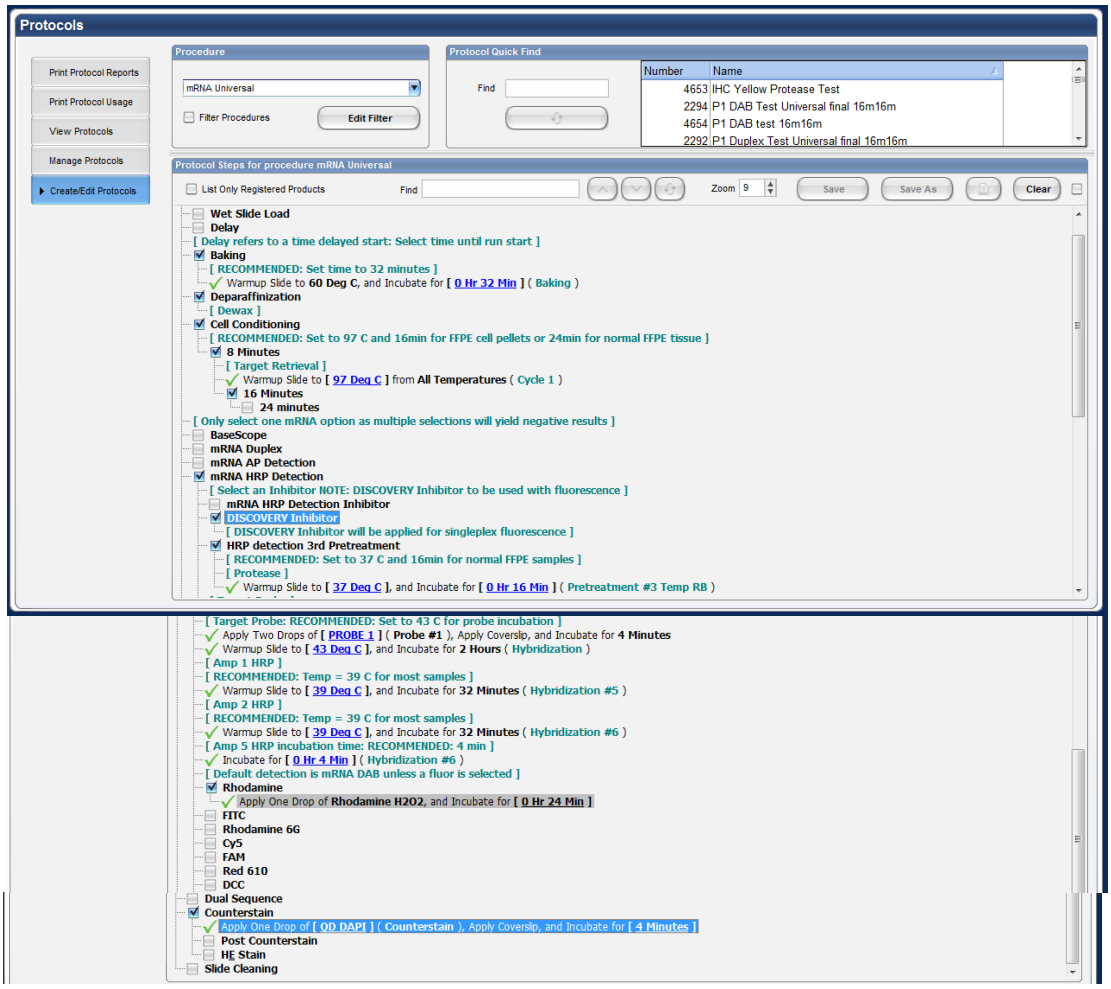
5. Empty the waste bottle, if needed.

Create an instrument protocol

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



4. After selecting the main protocol steps, drop down menus become available. Select the appropriate protocol steps under **mRNA HRP Detection** by clicking on the associated check boxes as shown in the following figures:



Note: When selecting the detection, select DISCOVERY Inhibitor. The VS Fluorescent Detection kits do not contain an inhibitor dispenser.

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

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
Prostate	97°C	24 MIN
Spleen	97°C	24 MIN
Tonsil	97°C	24 MIN

Suggested Temperatures/Times	
VS Protease	37°C
Suggested probe temperatures	Single Probes 43°C
	Pooled Probes 50°C
Suggested Amp 1 and Amp 2 temperatures	39°C
AMP 5 incubation time*	4 MIN

*Staining intensity can be modified by adjusting AMP 5 incubation time.

Suggested Fluorescent Detection Times*	
DISCOVERY DCC Kit	32 MIN
DISCOVERY FAM kit	20 MIN
DISCOVERY FITC kit	20 MIN
DISCOVERY Rhodamine kit	32 MIN
DISCOVERY Rhodamine 6G kit	32 MIN
DISCOVERY Red 610 kit	32 MIN
DISCOVERY Cy5 kit	40 MIN

* Staining intensity/dot size can be modified by adjusting detection times.

6. Select **Save as**, then select a protocol number from the drop down menu and choose a protocol name for each probe.
7. Select **Save**.
8. Select **Close** to go back to the main screen.
9. 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 upper right corner 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 ULTRA System User Manual* for details.
3. Click on **Protocol**.
4. Select the protocol you created for the RNAscope® VS Universal Assay and click on the **Add** button. When the protocols for all of the slides have been assigned, click on **Close/Print**.
5. Fill in the template for each slide. Select **Print** when completed.
6. Continue with the next section.

Run the RNAscope® VS Universal Assay

Materials required

- Prepared slides
- Prepared instrument reagents
- Prepared detergent
- Distilled water
- Tissue-Tek® Vertical 24 Slide Rack
- Tissue-Tek® Staining Dishes
- ProLong Gold mounting medium
- Cover Glass, 24 mm x 50 mm

Load the reagents

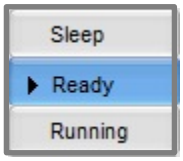
1. Remove the nozzle caps from the filled dispensers and place each cap on the post located on the back of the dispenser.
2. If needed, remove any air bubbles at the nozzle tips by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle

   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, DISCOVERY Fluorescent Detection Kit of your choice. Refer to the instructions provided by Ventana™ Medical Systems.
5. Load the reagent racks onto the reagent carousel.

Start the run

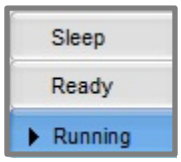
1. Click the **Ready** button.



2. Eject slide drawers.
3. Load each slide onto a heater pad with the label facing upward and inward. 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 drawers.
5. Click the **Running** button. Automated assay will finish in **~14 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 1-2 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.

Complete the run

1. After the run is complete, remove the Dewax 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 drawers and unload slides.
3. Decant solution on the slides into the slide 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 three to five times.
7. Transfer the slides into a Tissue-Tek® Staining Dish containing 200 mL distilled water.

Mount the samples

1. Remove the slides from the Tissue-Tek® Slide Rack and lay flat with the sections facing up.
2. Add 1–2 drops of ProLong Gold mounting medium or other qualified fluorescent medium to a slide.
3. Carefully place 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 at least **5 MIN**.
8. Proceed to **Chapter 4. Evaluate the Results** on page 19.

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 protocols in our sample preparation and pretreatment user guides and tech notes available at **https:// acdbio.com/technical-support/user-manuals**.

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 in **Chapter 4. Evaluate the Results** and determine which pretreatment condition yielded the highest positive control signal and lowest negative control signal. Using PPIB, 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**.

4 Chapter 4. Evaluate the Results

Examine tissue sections under a fluorescent 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 cell 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.
- For kit information regarding excitation and emission wavelengths to ensure proper filter compatibility, see the following table:

DISCOVERY Detection Kit	Excitation Wavelength (nm)	Emission Wavelength (nm)
DCC	436	480
FAM	490	520
FITC	490	525
Rhodamine	542	568
Rhodamine 6G	546	572
Red 610	580	625

Scoring guidelines

The RNAscope® 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**.

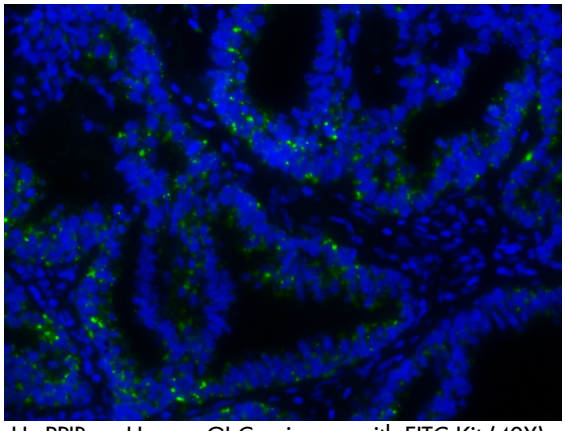
Troubleshooting

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

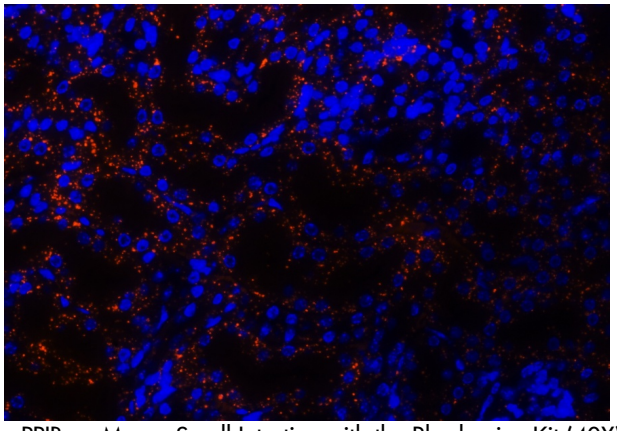
Tissue example

The following figures display examples of positive RNA staining with three different fluorescent detection kits:

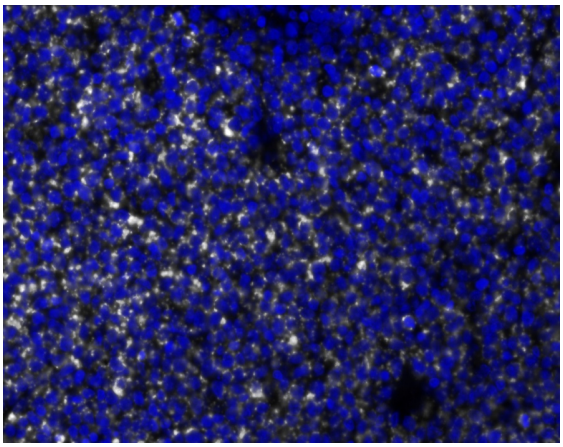
Figure 2. RNAscope® VS Universal Fluorescent Assay results



Hs-PPIB on Human GI Carcinoma with FITC Kit (40X)



Mm-PPIB on Mouse Small Intestine with the Rhodamine Kit (40X)

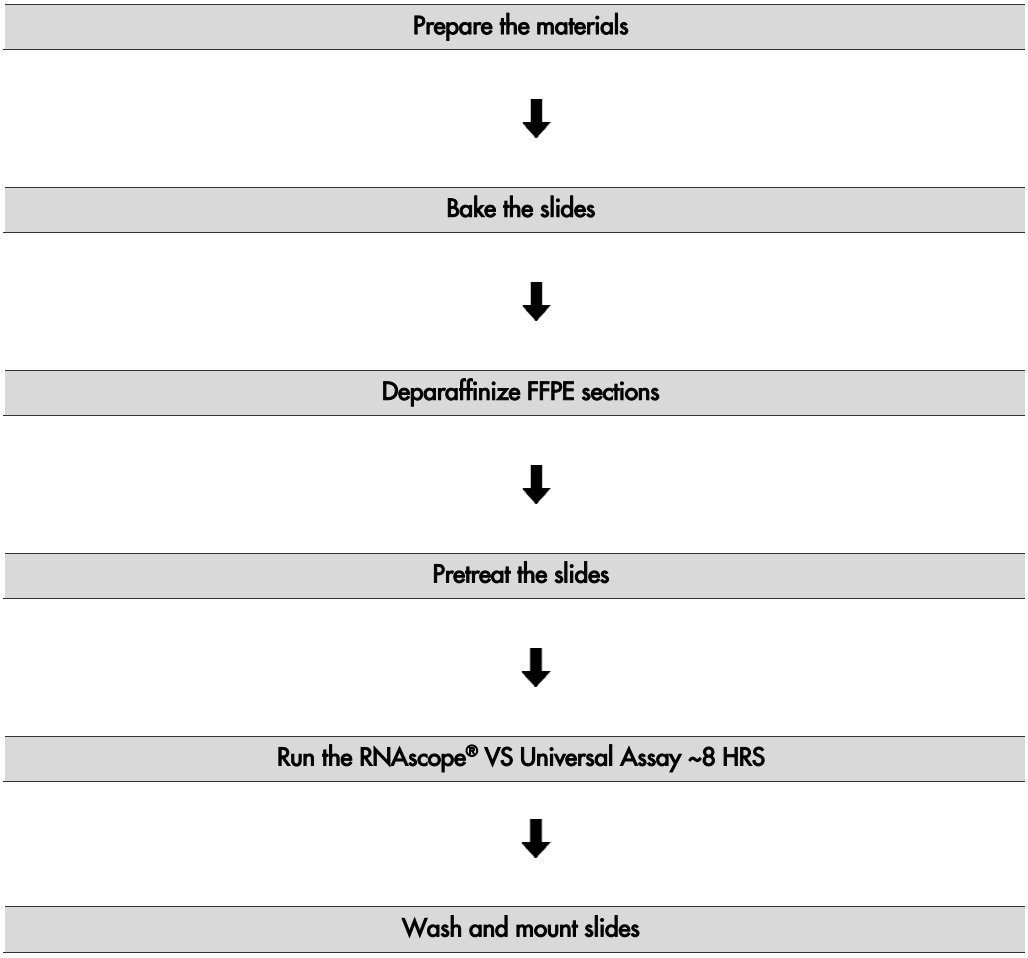


Hs-PPIB on Human Tonsil with Cy5 Kit (40X)

Appendix A. Semi-automated RNAscope® VS Universal HRP Fluorescent Assay

Most sample types can be fully automated on the Discovery ULTRA. 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. See **Chapter 3. Automated RNAscope® VS Universal HRP Fluorescent Assay** on page 11.

Workflow



Kit contents and storage

RNAscope Reagents

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

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

Prepare the materials

Materials can be prepared ahead of time or while baking the slides, unless otherwise stated. See **Bake the slides** on page 26.

Materials required

Materials provided by Advanced Cell Diagnostics	Materials Provided by Ventana™ Medical Systems	Other Materials and Equipment
• RNAscope® 2.5 VS Target Probe • RNAscope® 2.5 VS Positive Control Probe • RNAscope® 2.5 VS Negative Control Probe • RNAscope® VS Pretreat 2– Dewax • RNAscope® VS Protease • RNAscope® VS Target Retrieval • RNAscope® VS Universal HRP AMP 1 • RNAscope® VS Universal HRP AMP 2 • RNAscope® VS Universal HRP AMP 3 • RNAscope® VS Universal HRP AMP 4 • RNAscope® VS Universal HRP AMP 5 • RNAscope® VS Universal HRP AMP 6 RNAscope® VS Universal HRP AMP 7	• DISCOVERY™ ULTRA — automated slide stainer • DISCOVERY Wash 10X • ULTRA LCS (Predilute) • SSC Buffer 10X • Reaction Buffer 10X • Probe dispensers • mRNA Sample Prep Kit • mRNA Probe Amplification Kit • Fluorescent Detection Kit • User fillable dispensers • CC1 Buffer • DISCOVERY Inhibitor • DISCOVERY QDMap Counterstain	• Distilled water • Glass beaker (1 or 2 L) • Hot plate • Dawn detergent or similar detergent • Fume hood • Xylene • 100% ethanol (EtOH) • Tissue-Tek® Staining Dishes • Tissue-Tek® Clearing Agent Dishes, xylene-resistant • Tissue-Tek® Vertical 24 Slide Rack • ProLong Gold mounting medium • Cover Glass, 24 mm x 50 mm

Prepare the instrument

If the instrument has not been used for ≥1 week, follow the guidelines for instrument maintenance in the *Ventana™ System User Manual*.

Dilute bulk reagents

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

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:

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

Prepare instrument reagents

Refer to the table on page 7 to determine the proper dispenser for each reagent.

1. For RNAscope® VS Universal HRP AMP 1 to AMP 7, transfer the entire volume of each component into the correspondingly labeled dispenser.
2. Transfer the RNAscope® VS 2.5 VS Target Probe, 2.5 VS Positive Control Probe, 2.5 VS Negative Control Probe, and VS Protease to the correspondingly labeled dispensers.
3. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
4. Store tightly-capped dispensers at **4°C** when not in use.
5. Check solution levels: DISCOVERY Wash, 2X SSC, Reaction Buffer, ULTRA LCS (Predilute), and CC1. Refill if they are less than half full. To run a full tray of 30 slides, fully fill the 2X SSC and Reaction Buffer containers.

   IMPORTANT! Do not use expired reagents.

6. Empty the waste carboy, if needed.

Prepare deparaffinization reagents

- In a fume hood, fill two clearing agent dishes with ~200 mL fresh xylene.
- 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 26. 1X Target Retrieval is used in manual cell conditioning (CC).

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

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

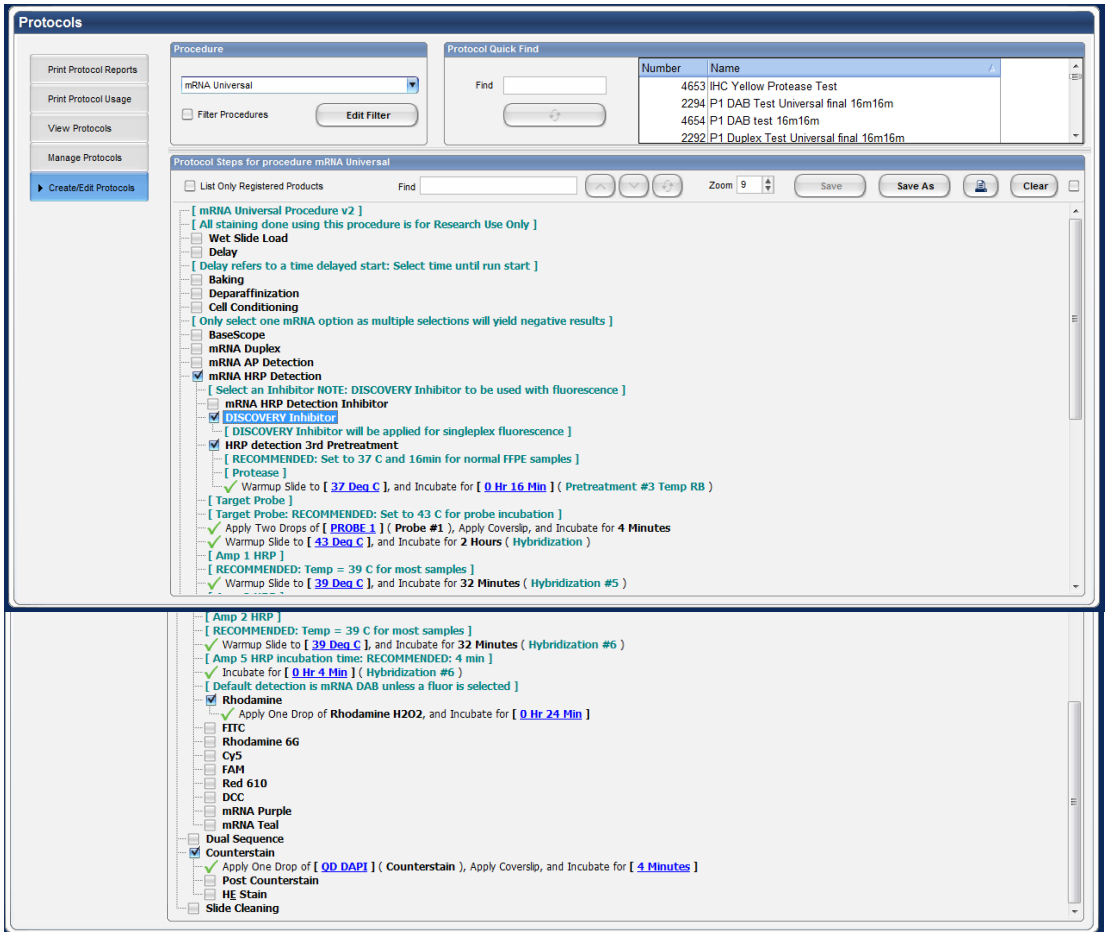
Create an instrument protocol

1. Open the VSS software and click on the **Protocol** button.
2. Click on **Create/Edit Protocols**, go to the Procedure drop down menu and select **mRNA Universal**.

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 under **mRNA HRP Detection** by clicking on the associated check boxes as shown.



Note: When selecting the detection, select DISCOVERY Inhibitor. The VS Fluorescent Detection kits do not contain an inhibitor dispenser.

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

Suggested Temperatures/Times	
VS Protease	Protease: 37°C
Suggested probe temperatures	Single Probes 43°C
	Pooled Probes 50°C
Suggested AMP 1 and Amp 2 temperatures	39°C
AMP 5 incubation time*	4 MIN

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

Suggested Fluorescent Detection Times*	
DISCOVERY DCC Kit	32 MIN
DISCOVERY FAM kit	20 MIN
DISCOVERY FITC kit	20 MIN
DISCOVERY Rhodamine kit	32 MIN
DISCOVERY Rhodamine 6G kit	32 MIN
DISCOVERY Red 610 kit	32 MIN
DISCOVERY Cy5 kit	40 MIN

* Staining intensity/dot size can be modified by adjusting detection 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™ System User Manual* for details.
3. Select the protocol you created for the RNAscope® VS Universal 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 Dishes • Tissue-Tek® Staining Dishes • Glass beaker (1 or 2 L) • Hot plate

Bake the slides

1. Bake slides in a dry oven for **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.

IMPORTANT! If you continue, prepare the materials for the following protocols while the slides are baking: **Deparaffinize FFPE sections**, **Pretreat the slides**, and **Run the RNAscope® VS Universal Assay**.

Deparaffinize FFPE sections

IMPORTANT! If you have not done so already, create a protocol for your instrument and print slide labels during this procedure. See page 23.

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. Remove the slide rack from the first xylene-containing dish and *immediately* place in the second xylene-containing clearing agent dish in the fume hood.
4. Repeat Step 2.
5. Remove the slide rack from the second xylene-containing dish and *immediately* place in the staining dish containing 100% EtOH.
6. Incubate the slides in 100% EtOH for **1 MIN** at **RT** with agitation.
7. Repeat Step 6 with fresh 100% EtOH.
8. Remove the slides from the rack, and place on absorbent paper with the section face-up. Air dry for **5 MIN** at **RT**.
9. While slides are drying, place printed labels on the slides.

   IMPORTANT! Labels must be in place prior to the next section.
10. Insert the slides into a Tissue-Tek® Slide Rack and proceed to condition the slides.

Pretreat the slides

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

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	Target Retrieval Time
Brain and spinal cord	15 MIN
Breast cancer	15 MIN
Cell lines	10 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 a 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 **Load the reagents** on page 28.

Run the RNAscope® VS Universal Assay

Materials required

- Prepared slides
- Prepared instrument reagents
- Prepared detergent
- Distilled water
- Prepared dehydrating materials
- Tissue-Tek® Vertical 24 Slide Rack
- ProLong Gold mounting medium
- Cover Glass, 24 mm x 50 mm

Load the reagents

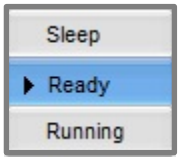
1. Remove the nozzle caps from the filled dispensers and place each cap on the post located on the back of the dispenser.
2. If needed, remove any air bubbles at the nozzle tips by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle

   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 DISCOVERY Fluorescent Detection Kit of your choice. Refer to the instructions provided by Ventana™ Medical Systems.
5. Load the reagent racks onto the reagent carousel.

Start the run

1. Click the **Ready** button.

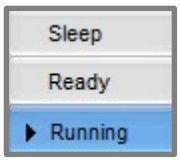


2. Eject slide drawers.
3. Load each slide onto a heater pad with the label facing upward and inward. Ensure that the slides sit securely on the pads.

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

4. Close slide drawers.

5. Click the **Running** 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.

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 and unload slides.
3. Decant solution on the slides into the slide 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 three to five times.
7. Transfer the slides into a Tissue-Tek® Staining Dish containing 200 mL distilled water.

Mount the samples

1. Remove the slides from the Tissue-Tek® Slide Rack and lay flat with the sections facing up.
2. Add 1–2 drops of ProLong Gold mounting medium or other qualified fluorescent medium to a slide.
3. Carefully place 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 at least **5 MIN**.
8. Proceed to **Chapter 4. Evaluate the Results** on page 19.

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 protocols in our sample preparation and pretreatment user guides and tech notes available at **https:// acdbio.com/technical-support/user-manuals**.

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 4. Evaluate the Results**, and determine which pretreatment condition yielded the highest positive control signal and lowest negative control signal. Using PPIB, 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**.

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

Biological hazard safety

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

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **https://www.cdc.gov/biosafety/**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **https://www.osha.gov/pls/oshaweb/owadisp.show_document?p_id=10051&p_table=STANDARDS**

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

In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at:
  http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/
- Information about the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) can be found at: **https://echa.europa.eu/regulations/reach**

Documentation and Support

Obtaining support

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

At the website, you can:

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

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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. If you have any questions, please contact Advanced Cell Diagnostics at **https://acdbio.com/about/contact**.

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