USER MANUAL

Combining Immunohistochemistry or Immunofluorescence with the RNAscope® VS Universal HRP and AP Assays

For the Ventana DISCOVERY™ ULTRA System

Document Number MK 50-015

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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 48 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 immunohistochemistry (IHC) or immunofluorescence (IF) with the RNAscope® VS Universal Assay.

IMPORTANT! The procedures in this user manual differ depending on the type of detection you use. Steps required for HRP detection are marked **HRP DETECTION**. Steps required for AP detection are marked **AP DETECTION**.

Several versions of the RNAscope® VS Universal Assay are provided:

- **Chapter 4. Chromagenic VS Universal Assay/IHC dual stain** starting on page 22.
- **Chapter 5. Fluorescent VS Universal Assay/IF dual stain** starting on page 31.
- **Appendix A. Semi-automated RNAscope® VS Universal Assay** starting on page 41.

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.

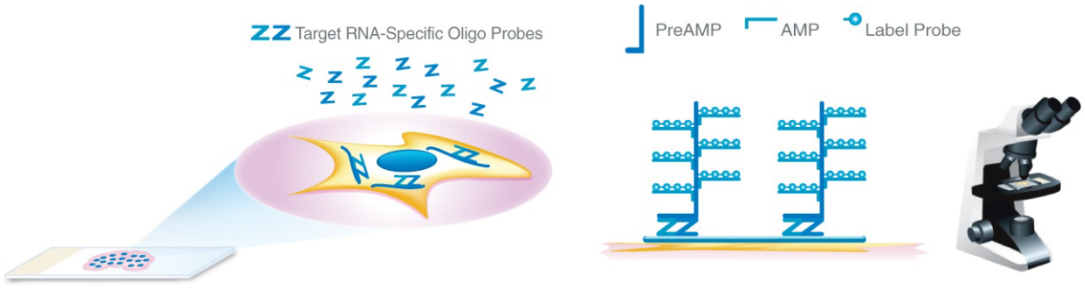
Background

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

Overview

Figure 1 on page 7 illustrates the RNAscope® VS Universal Assay procedure. 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 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 fluorescent 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-labeled or AP-labeled probes. The VS Universal 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.

Following the *in situ* hybridization (ISH) steps, the Ventana DISCOVERY™ ULTRA System will run immunofluorescence (IF) or immunohistochemistry (IHC) steps on your sample. The entire procedure can be completed on the instrument in ~15 hours.

Kit contents and storage

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

RNAscope® 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 tables:

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® VS FFPE Reagent Kit — Positive Control Probe – *[species]– PPIB*	Various	Probe targeting common housekeeping gene	7 mL x 1 bottle	2–8°C
	RNAscope® VS FFPE Reagent Kit — Positive Control Probe – *[species]– POLR2a*	Various	Probe targeting common housekeeping gene	7 mL x 1 bottle	2–8°C
	RNAscope® VS 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® 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 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 AP Detection Reagents (Cat. No. 323260)				
☑	Reagent	Cat. No.	Quantity	Storage
	RNAscope® VS Universal AP AMP 1	322211	14 mL x 1 bottle	2–8°C
	RNAscope® VS Universal AP AMP 2	322212	14 mL x 1 bottle	2–8°C
	RNAscope® VS Universal AP AMP 3	322213	14 mL x 1 bottle	2–8°C
	RNAscope® VS Universal AP AMP 4	322214	14 mL x 1 bottle	2–8°C
	RNAscope® VS Universal AP AMP 5	322261	14 mL x 1 bottle	2–8°C
	RNAscope® VS Universal AP AMP 6	322262	14 mL x 1 bottle	2–8°C
	RNAscope® VS Universal AP 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)
RNAscope® VS Accessory Kit (Cat. No. 320630)				
☑	Reagent	Cat. No.	Quantity	Storage
	RNAscope® VS Hematoxylin	320631	7 mL x 1 bottle	2–8°C
	RNAscope® VS Bluing Reagent	320632	7 mL x 1 bottle	2–8°C

RNAscope® VS Protease*				
☑	Reagent	Cat. No.	Quantity	Storage
	RNAscope® VS Protease	323218	14 mL x 1 bottle	2–8°C

*Use this protease for the antibody qualification procedure described in Chapter 3.

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, including RNAscope® VS Universal Reagent Kits or those having the same name.

Required materials from Roche Diagnostics

The RNAscope® VS Universal 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 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)

mRNA RED Probe Amplification Kit (Cat. No. 760-236; Ordering Code 7095341001)		
☑	Component	Storage
	mRNA AMP 1 dispenser — fill dispenser with Universal AP AMP 1	Room Temp (15–30°C)
	mRNA AMP 2 dispenser — fill dispenser with Universal AP AMP 2	Room Temp (15–30°C)
	mRNA AMP 3 dispenser — fill dispenser with Universal AP AMP 3	Room Temp (15–30°C)
	mRNA AMP 4 dispenser — fill dispenser with Universal AP AMP 4	Room Temp (15–30°C)
	mRNA AMP 5 dispenser — fill dispenser with Universal AP AMP 5	Room Temp (15–30°C)
	mRNA AMP 6 dispenser — fill dispenser with Universal AP AMP 6	Room Temp (15–30°C)
	mRNA AMP 7 dispenser — fill dispenser with Universal AP 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
mRNA RED Detection Kit (Cat. No. 760-234; Ordering Code 7099037001)		
☑	Component	Storage
	mRNA Inhibitor-prefilled	2–8°C
	mRNA Activator dispenser-prefilled	2–8°C
	mRNA Napthol dispenser-prefilled	2–8°C
	mRNA Fast Red dispenser-prefilled	2–8°C

Ventana HRP Detection Kits specific for IHC				
☑	Kit	Cat. No.	Ordering Code	Storage
	DISCOVERY ChromoMap DAB Kit	760-159	05266645001	2–8°C
	DISCOVERY Purple Kit	760-229	07053983001	2–8°C
	DISCOVERY Silver Kit	760-227	07053649001	2–8°C
	DISCOVERY Teal Kit	760-247	8254338001	2–8°C

Ventana AP Detection Kits specific for IHC				
☑	Kit	Cat. No.	Ordering Code	Storage
	DISCOVERY ChromoMap Blue Kit	760-161	5266661001	2–8°C
	DISCOVERY Red Kit	760-228	7425333001	2–8°C
	DISCOVERY Yellow Kit	760-239	7425333001	2–8°C

Ventana Fluorescent Detection Kits				
☑	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
Generic Dispensers				
☑	Component	Cat. No.	Ordering Code	Storage
	250 Test Counterstain 1 dispenser — fill dispenser with VS Hematoxylin	771-741	05271720001	Room Temp (15–30°C)
	250 Test Counterstain 2 dispenser — fill dispenser with Bluing Reagent	771-742	05271738001	Room Temp (15–30°C)
Ancillary Dispensers				
☑	Component	Cat. No.	Ordering Code	Storage
	250 Test Enzyme # 1— fill with VS Protease IHC Reagent	771-721	05271517001	2–8°C
	DISCOVERY Inhibitor – prefilled	760-4840	07017944001	2–8°C
	Ventana Antibody dispensers—prefilled	Various	Various	2–8°C
	Open Antibody dispensers—fill with non-Ventana primary and secondary antibodies*			Room Temp (15–30°C)
Counterstain				
☑	Component	Cat. No.	Ordering Code	Storage
	QD DAPI Counterstain	760-4196	05268826001	2–8°C

*You may use your own antibodies in place of Ventana primary and secondary antibodies.

Equipment and buffers

☑	Component	Cat. No.	Ordering Code	Storage
	10X DISCOVERY Wash (RUO)	950-510	7311079001	Room Temp (15–30°C)
	ULTRA LCS (Predilute)	650-210	5424534001	Room Temp (15–30°C)
	SSC Buffer (10X)	950-110	5353947001	Room Temp (15–30°C)
	Reaction Buffer (10X)	760-107	5266262001	Room Temp (15–30°C)
	DISCOVERY CC1	950-500	6414575001	Room Temp (15–30°C)

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
	Cytoseal XYL or other xylene-based mounting medium (use with HRP detection)	Richard-Allen Scientific/MLS	8312-4
	ProLong® Gold Antifade Reagent	Life Technologies	P36930
	EcoMount (use with AP detection)	Biocare	EM897L
	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	—
	Drying oven, capable of holding temperature at 60 +/– 1°C	MLS	—
	Fume hood	MLS	—
	100% ethanol (EtOH)	MLS	—
	Xylene	MLS	—
	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 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 48 in this document for more information.

3 Chapter 3. IHC Qualification for the RNAscope® VS Universal Assay

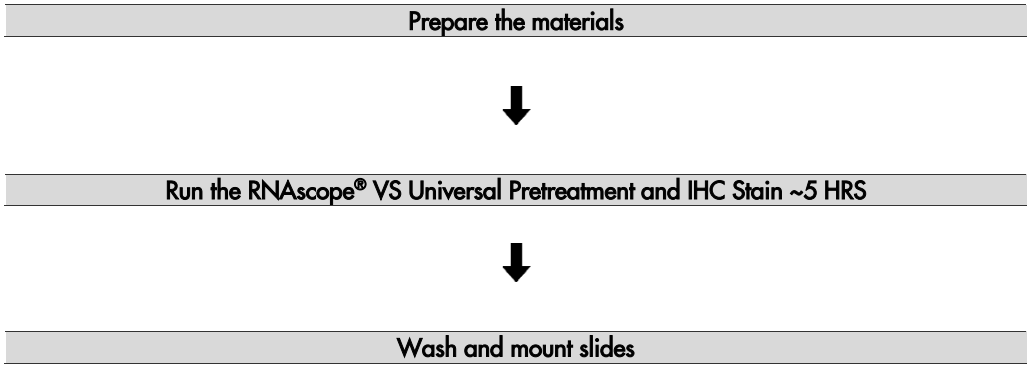
To determine antibody sensitivity to the RNAscope® VS protease digestion step, we recommend performing a qualification run.

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

Note: **Appendix A. Semi-automated RNAscope® VS Universal Assay** describes an offline boiling procedure for use with Cat. No. 322000 (RNAscope® Target Retrieval Reagents).

IMPORTANT! The procedures in this user manual differ depending on the type of detection you use. Steps required for HRP detection are marked **HRP DETECTION**. Steps required for AP detection are marked **AP DETECTION**.

Workflow



Prepare the materials

Materials required

Materials Provided by Advanced Cell Diagnostics	Materials Provided by Ventana™ Medical Systems	Other Materials and Equipment
• RNAscope® VS Universal Sample Prep Reagents • RNAscope® VS Accessory Kit • RNAscope® VS Protease	• DISCOVERY™ ULTRA — automated slide stainer • DISCOVERY Wash Buffer 10X • ULTRA LCS (Predilute) • SSC Buffer 10X • DISCOVERY CC1 • Reaction Buffer 10X • mRNA Sample Prep Kit • User fillable dispensers • mRNA Probe Amplification kits • mRNA Detection kits • Ventana Antibody or Open Antibody dispensers (for non-Ventana primary antibodies) • Species matched secondary enzyme conjugate compatible with detection • QD DAPI Counterstain • DISCOVERY Inhibitor • Enzyme 1 dispenser	• Distilled water • Dawn detergent or similar detergent • Fume hood • Xylene • Tissue-Tek® Staining Dish • Tissue-Tek® Clearing Agent Dish, xylene-resistant • Tissue-Tek® Vertical 24 Slide Rack • Cytoseal XYL xylene-based or EcoMount • Cover Glass, 24 mm x 50 mm

Prepare the instrument

Most sample types can be fully automated. Manual pretreatment may give a better result in some cases (see **Appendix A. Semi-automated RNAscope® VS Universal Assay** on page 41). 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

Prepare the bulk fluids according to the 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:

- 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 9 to determine the proper dispenser for each reagent.

1. Transfer the RNAscope® VS Pretreat 2–Dewax, both bottles of VS Target Retrieval, VS Protease (Enzyme 1), 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 use.

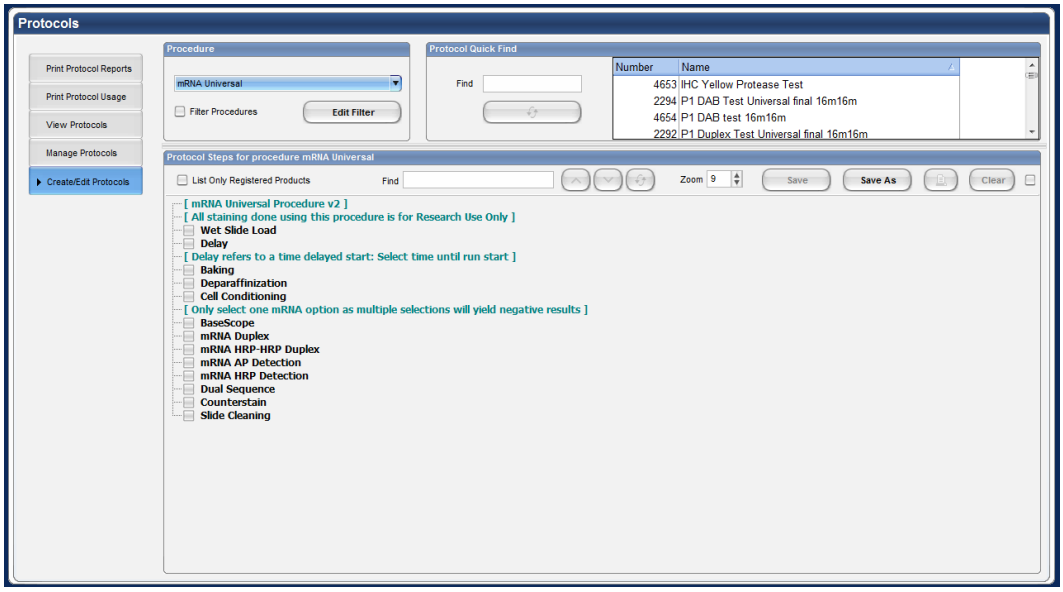
2. If using a non-Ventana antibody, dilute with an appropriate buffer and transfer the volume into a clean dispenser.
3. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
4. Store tightly-capped dispensers (except the Dewax dispenser) at **4°C** when not in use.
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! Use reagents that have not expired.

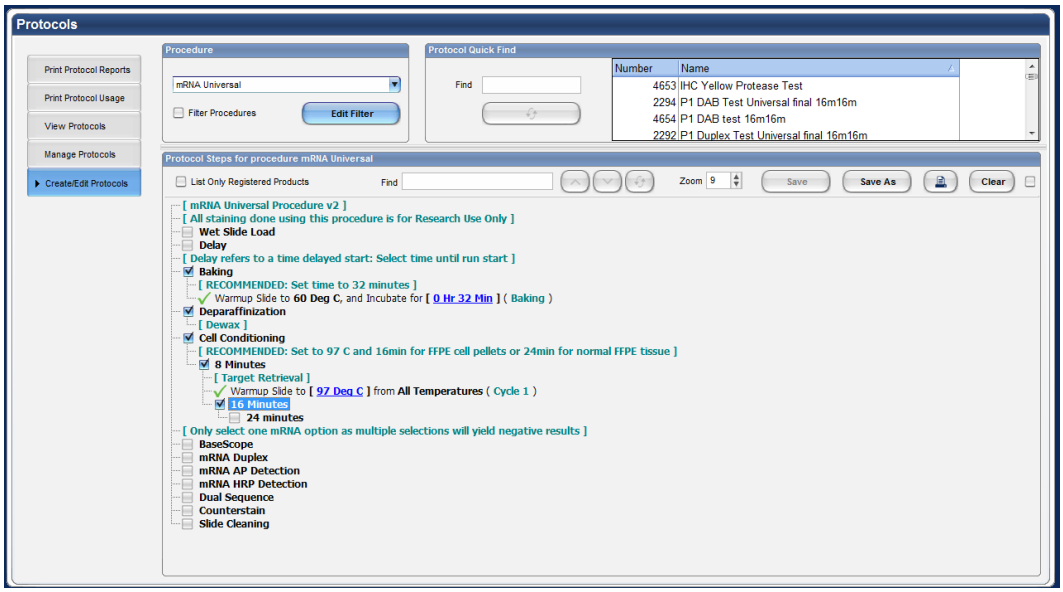
6. Empty the waste bottle if needed.

Create an instrument protocol

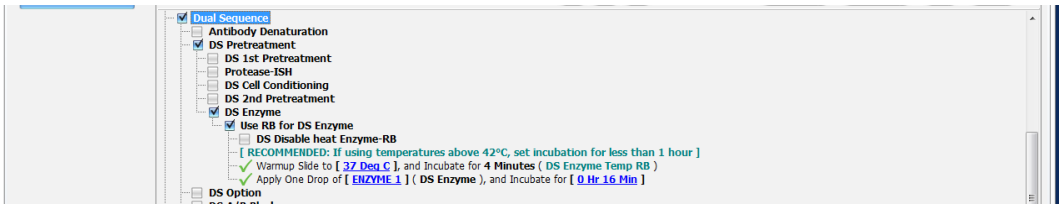
1. Open the VSS software and click on the **Protocol** button.
2. Click on **Create/Edit Protocols**, go to the Procedure drop down menu and select **mRNA Universal**.
3. Main protocol steps appear as shown:



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



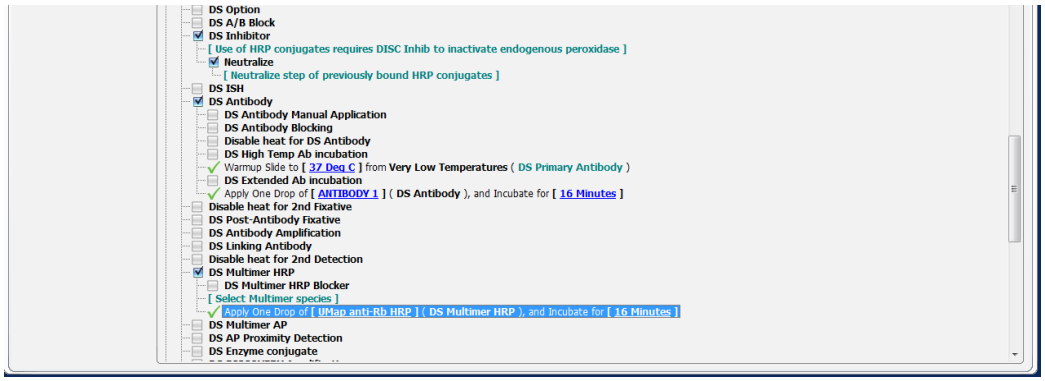
5. Select **Dual Sequence** to see the IHC options.
6. Next select **DS Pretreatment** followed by **DS Enzyme**:



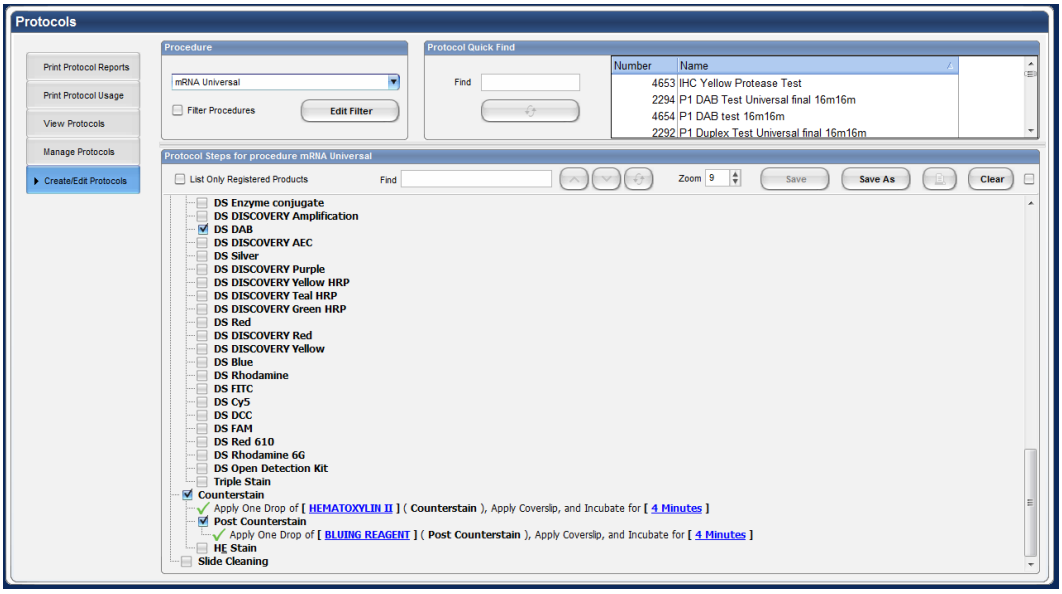
7. Select 37°C and **16 MIN** as your enzyme pretreatment conditions. Select the barcode of the dispenser that contains the VS Protease (Enzyme 1).

   Note: We strongly recommend running two positive control slides; one without protease, and one using the preceding conditions.

8. **HRP DETECTION:** If using HRP detection, select **DS Inhibitor** followed by **Neutralize**. Do not select these options if using AP detection.
9. Select the antibody to be used for IHC, then select a multimer that is compatible with the chosen antibody and the final detection kit used (for example, use an anti-rabbit horseradish peroxidase (HRP) multimer with a rabbit monoclonal antibody and the DAB detection kit):



10. Select the appropriate detection kit, and preferred counterstain/post-counterstain to be used in the assay. See page 10 for a list of Ventana kits compatible with AP or HRP detection:



11. Select the appropriate target retrieval 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
Prostate	97°C	24 MIN
Spleen	97°C	24 MIN
Tonsil	97°C	24 MIN

12. Select **Save as**, then select a protocol number from the drop down menu and choose a protocol name for each probe.
13. Select **Save**.
14. Select **Close** to go back to the main screen.
15. 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 IHC Qualification 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. Proceed to the next page.

Run the RNAscope® VS Universal IHC Qualification Assay

Materials required

- Prepared slides
- Prepared instrument reagents
- Prepared detergent
- Distilled water
- Prepared dehydrating materials
- Tissue-Tek® Vertical 24 Slide Rack
- Tissue-Tek® Staining Dish
- Cytoseal XYL xylene-based mounting medium
- Cover Glass, 24 mm x 50 mm
- Fume hood
- Xylene

Load the reagents

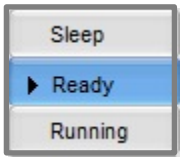
1. Remove the nozzle caps from the filled dispensers and place each cap on the post located on the back of the dispenser.
2. If needed, remove any air bubbles at the nozzle tips by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle

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 all of the prefilled dispensers. Refer to the instructions provided by Ventana™ Medical Systems.
5. Load the reagent racks onto the reagent carousel.

Start the run

1. Select the **Ready** button.

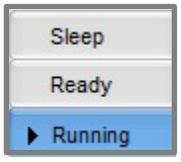


2. Eject slide drawers.

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. Select the **Running** button. Automated assay will finish in **~5 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**.

Prepare dehydrating reagents

- In a fume hood, fill two clearing agent dishes with ~200 mL fresh xylene.
- **HRP DETECTION:** If using HRP detection, fill three staining dishes with ~200 mL fresh 100% EtOH.

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

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.

Dehydrate the slides

1. **AP DETECTION:** Place slides using AP detection in a drying oven at 60°C for at least **30 MIN**, then proceed to step 5.
2. **HRP DETECTION:** If using HRP detection, 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.
3. **HRP DETECTION:** Move the Tissue-Tek® Slide rack into the second Staining Dish containing 100% EtOH for **2 MIN** with occasional agitation.
4. **HRP DETECTION:** Move the Tissue-Tek® Slide rack into the third Staining Dish containing 100% EtOH for **2 MIN** with occasional agitation.
5. Move the Tissue-Tek® Slide rack into the Staining Dish containing xylene for **1 MIN** with occasional agitation.
6. 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 each slide by adding 1–2 drops of mounting medium compatible with your chosen detection, and then 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 6. Evaluate the Results** on page 39.

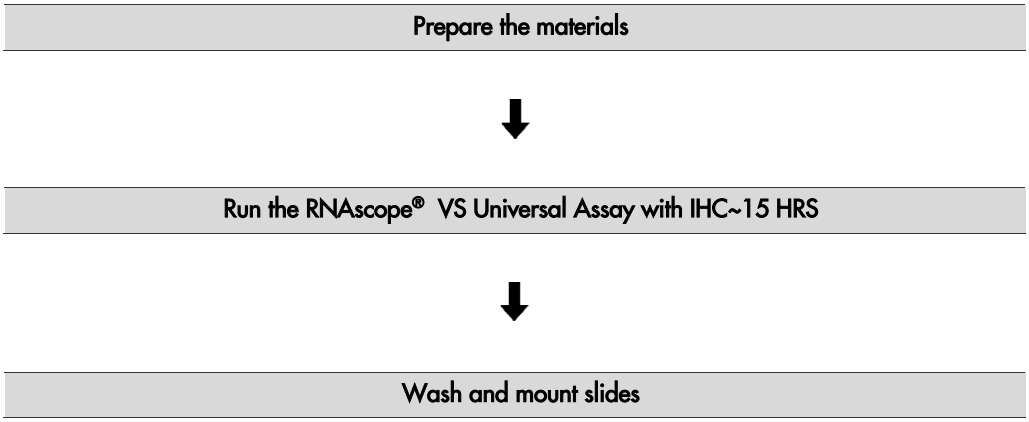
4 Chapter 4. Chromagenic VS Universal Assay/IHC Dual Stain

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 Fluorescent Assay describes an offline boiling procedure for use with Cat. No. 322000 (RNAscope® Target Retrieval Reagents).

IMPORTANT! The procedures in this user manual differ depending on the type of detection you use. Steps required for HRP detection are marked **HRP DETECTION**. Steps required for AP detection are marked **AP DETECTION**.

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 Control Probes • RNAscope® VS Universal Sample Prep Reagents • RNAscope® VS Accessory Kit • RNAscope® VS Universal HRP Detection Reagents *or* RNAscope® VS Universal AP Detection Reagents	• DISCOVERY™ ULTRA — automated slide stainer • DISCOVERY Wash Buffer 10X • ULTRA LCS (Predilute) • SSC Buffer 10X • DISCOVERY CC1 • Reaction Buffer 10X • mRNA Sample Prep Kit • User fillable dispensers • mRNA Probe Amplification kits • mRNA Detection kits • Ventana Antibody or Open Antibody dispensers (for non-Ventana primary antibodies) • Species matched secondary enzyme conjugate compatible with detection • DISCOVERY Inhibitor	• Distilled water • Dawn detergent or similar detergent • Fume hood • Xylene • Tissue-Tek® Staining Dish • Tissue-Tek® Clearing Agent Dish, xylene-resistant • Tissue-Tek® Vertical 24 Slide Rack • Cytoseal XYL xylene-based or EcoMount • Cover Glass, 24 mm x 50 mm

Prepare the instrument

Most sample types can be fully automated. Manual pretreatment may give a better result in some cases (see **Appendix A. Semi-automated RNAscope® VS Universal Fluorescent Assay** on page 41). 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

Prepare the bulk fluids according to the 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:

- 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 9 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.

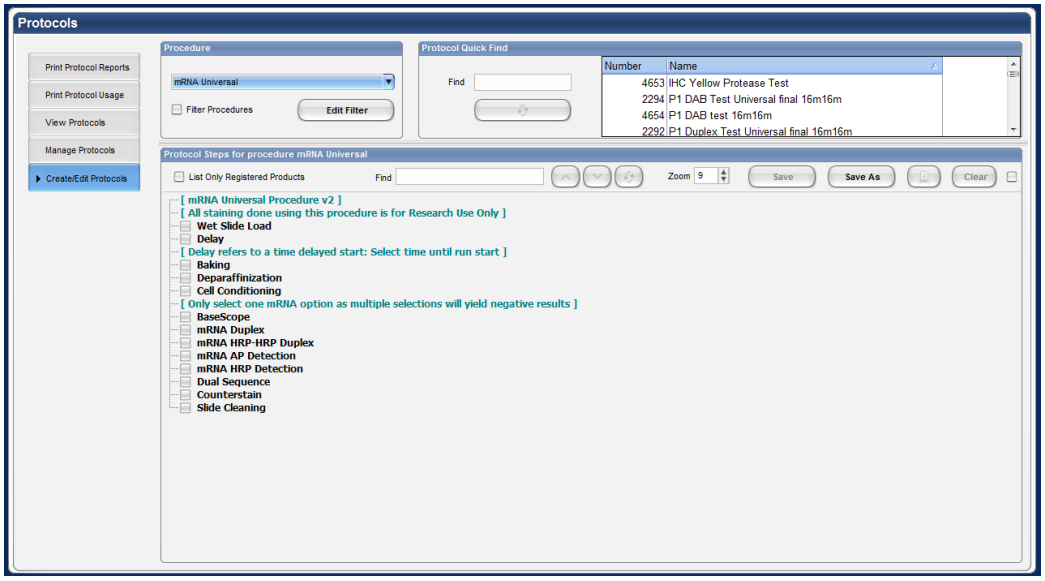
3. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
4. Store tightly-capped dispensers (except the Dewax dispenser) at **4°C** when not in use.
5. Check solution levels: DISCOVERY Wash, 2X SSC, Reaction Buffer, ULTRA LCS (Predilute), and CC1. Refill if they 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.

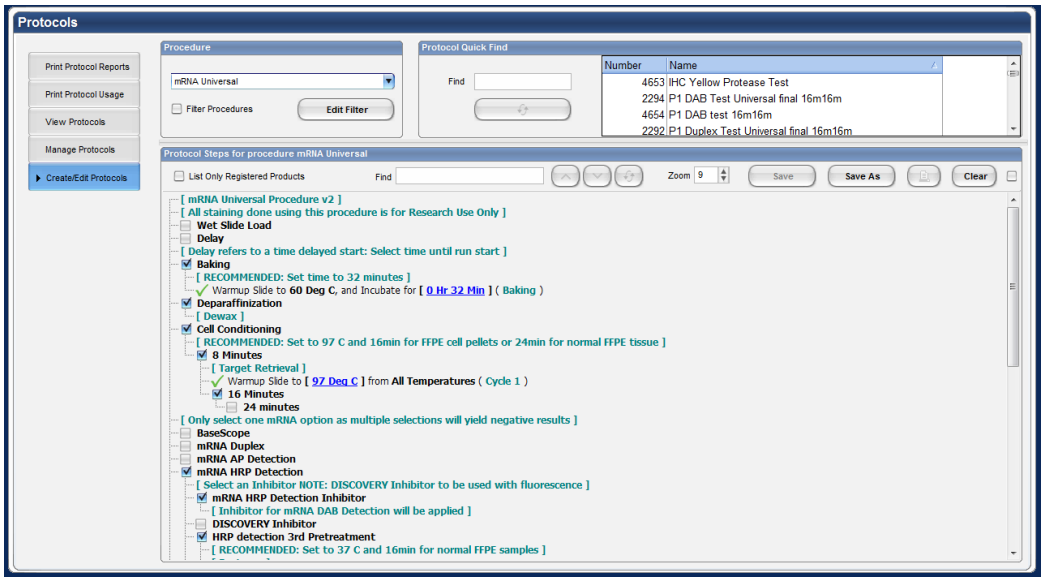
6. Empty the waste bottle, if needed.

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 by clicking on the associated check boxes as shown in the following figures:

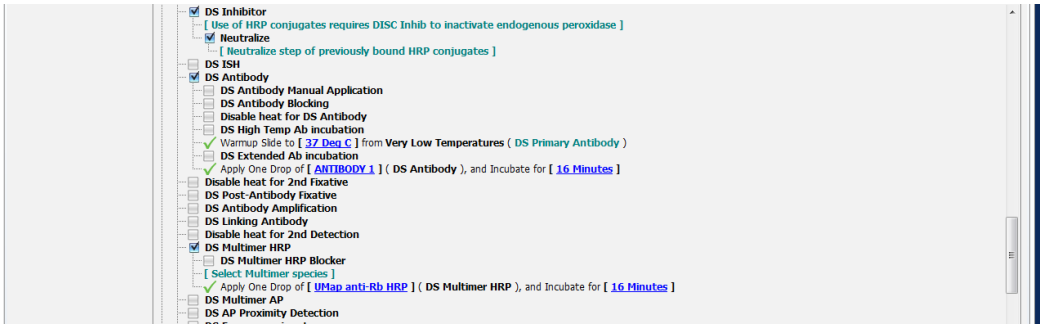


Add IHC Conditions

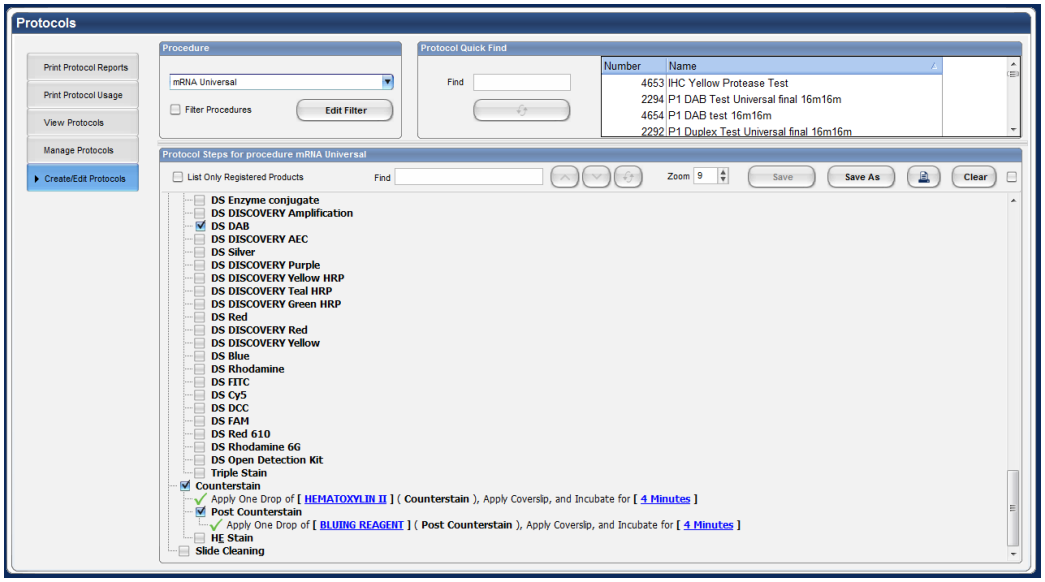
1. Select **Dual Sequence** to open up the IHC options.
2. Select the detection:
   - **AP DETECTION:** If using AP detection with the RNAscope Universal AP kit, select **DS Pretreatment**, followed by **DS Cell Conditioning** and **DS CC1**. Then select ≥90°C for at least **16 MIN**:

   - **HRP DETECTION:** If using HRP detection, select **DS Inhibitor** followed by **Neutralize**, and use a dispenser of DISCOVERY Inhibitor.

3. Select the antibody to be used for IHC, then select a multimer that is compatible with the chosen antibody and the final detection kit used (for example, use an anti-rabbit horseradish peroxidase (HRP) multimer with a rabbit monoclonal antibody and the DAB detection kit):



4. Select the appropriate detection kit, and preferred counterstain/post-counterstain to be used in the assay:



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

Tissue Type	Cell Conditioning Temperature	Time
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.

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 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 procedure.

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
- Tissue-Tek® Staining Dish
- Cytoseal XYL xylene-based mounting medium
- Cover Glass, 24 mm x 50 mm
- Fume hood
- Xylene

Load the reagents

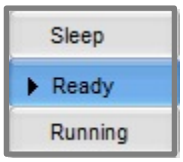
1. Remove the nozzle caps from the filled dispensers and place each cap on the post located on the back of the dispenser.
2. If needed, remove any air bubbles at the nozzle tips by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle

   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 all of the prefilled dispensers. Refer to the instructions provided by Ventana™ Medical Systems.
5. Load the reagent racks onto the reagent carousel.

Start the run

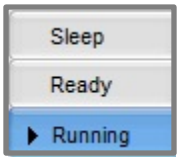
1. Select the **Ready** button.



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. Select the **Running** button. Automated assay will finish in **~15 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.

Prepare dehydrating reagents

- In a fume hood, fill two clearing agent dishes with ~200 mL fresh xylene.
- **HRP DETECTION:** If using HRP detection, 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 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.

Dehydrate the slides

1. **AP DETECTION:** Place slides using AP detection in a drying oven at 60°C for at least **30 MIN**, then proceed to step 5.
2. **HRP DETECTION:** If using HRP detection, 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.
3. **HRP DETECTION:** Move the Tissue-Tek® Slide rack into the second Staining Dish containing 100% EtOH for **2 MIN** with occasional agitation.
4. **HRP DETECTION:** Move the Tissue-Tek® Slide rack into the third Staining Dish containing 100% EtOH for **2 MIN** with occasional agitation.
5. Move the Tissue-Tek® Slide rack into the Staining Dish containing xylene for **1 MIN** with occasional agitation.
6. 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 each slide by adding 1–2 drops of mounting medium compatible with your chosen detection, and then 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 6. Evaluate the Results** on page 39.

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 6. 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**.

5 Chapter 5. Fluorescent VS Universal Assay/Immunofluorescence Dual Stain

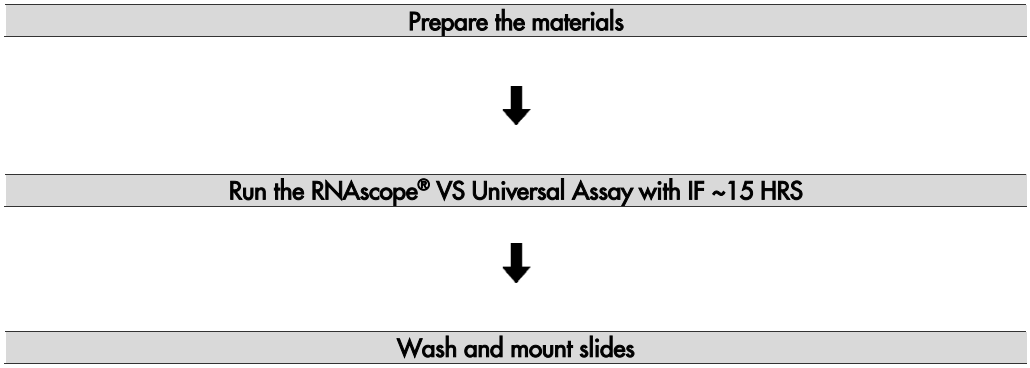
This chapter describes how to run the VS HRP Universal Fluorescent Assay for ISH detection, followed by immunofluorescence for protein detection.

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

Note: **Appendix A. Semi-automated RNAscope® VS Universal Assay** describes an offline boiling procedure for use with Cat. No. 322000 (RNAscope Target Retrieval Reagents).

IMPORTANT! The procedures in this user manual differ depending on the type of detection you use. Steps required for HRP detection are marked **HRP DETECTION**.

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 Control Probes • RNAscope® VS Universal Sample Prep Reagents • RNAscope® VS Accessory Kit • RNAscope® VS Universal HRP Detection Reagents	• DISCOVERY™ ULTRA — automated slide stainer • DISCOVERY Wash Buffer 10X • ULTRA LCS (Predilute) • SSC Buffer 10X • DISCOVERY CC1 • Reaction Buffer 10X • mRNA Sample Prep Kit • User fillable dispensers • mRNA Probe Amplification kits • DISCOVERY Fluorescent Detection kit • Second Fluorescent Detection kit (for use with an enzymatic secondary) • Ventana Antibody or Open Antibody dispensers (for non-Ventana primary antibodies) • Species matched secondary enzyme conjugate compatible with detection or fluorescently labeled secondary antibody • DISCOVERY QD DAPI Counterstain • DISCOVERY Inhibitor	• Distilled water • Dawn detergent or similar detergent • Fume hood • Xylene • Tissue-Tek® Staining Dish • Tissue-Tek® Clearing Agent Dish, xylene-resistant • Tissue-Tek® Vertical 24 Slide Rack • ProLong® Gold • Cover Glass, 24 mm x 50 mm

Prepare the instrument

Most sample types can be fully automated. Manual pretreatment may give a better result in some cases (see **Appendix A. Semi-automated RNAscope® VS Universal Assay** on page 41). 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:

- 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 9 to determine the proper dispenser for each reagent.

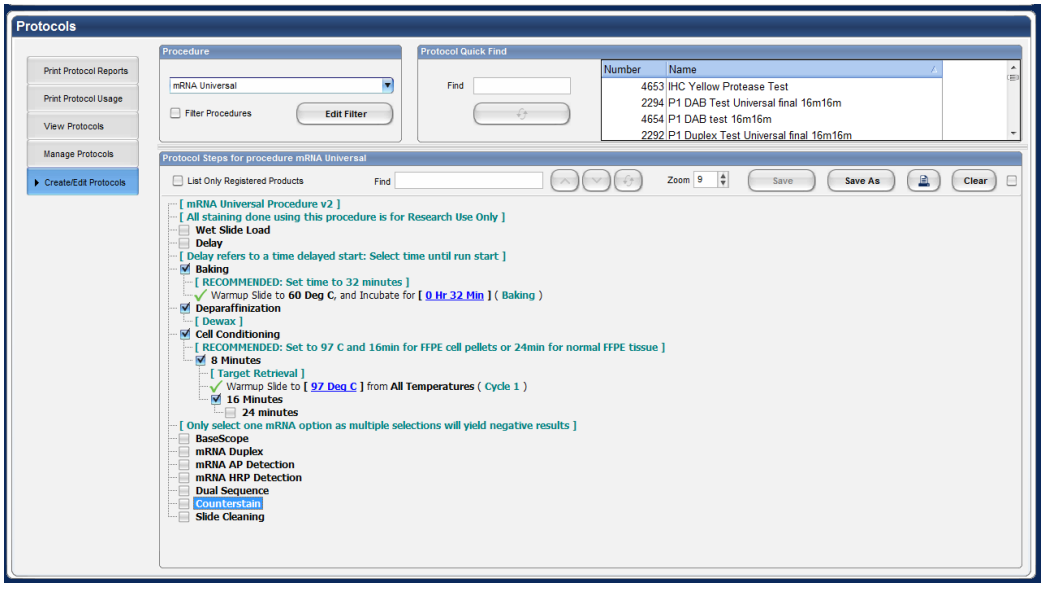
1. For RNAscope® VS Universal AMP 1 to AMP 7, transfer the entire volume of each RNAscope® VS Universal Reagent Kit component into the correspondingly labeled dispenser.
2. Transfer the remaining RNAscope® 2.5 VS Target Probe, 2.5 VS Positive Control Probe, 2.5 VS Negative Control Probe, VS Dewax, VS Protease, and both bottles of VS Target Retrieval to the correspondingly labeled dispensers.
3. Avoid cross contamination between reagents. Dewax must be warmed to room temperature and be completely in solution before use.
4. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
5. Store tightly-capped dispensers (except the Dewax dispenser) at **4°C** when not in use.
6. 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! Use reagents that have not expired.

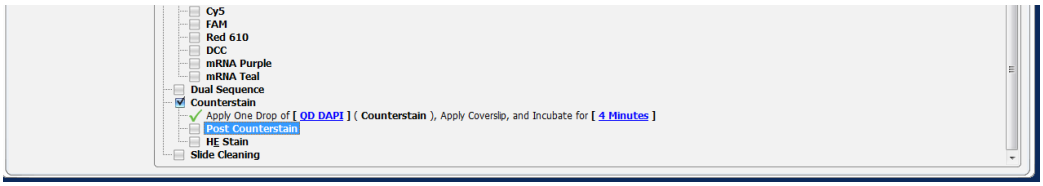
7. Empty the waste bottle if needed.

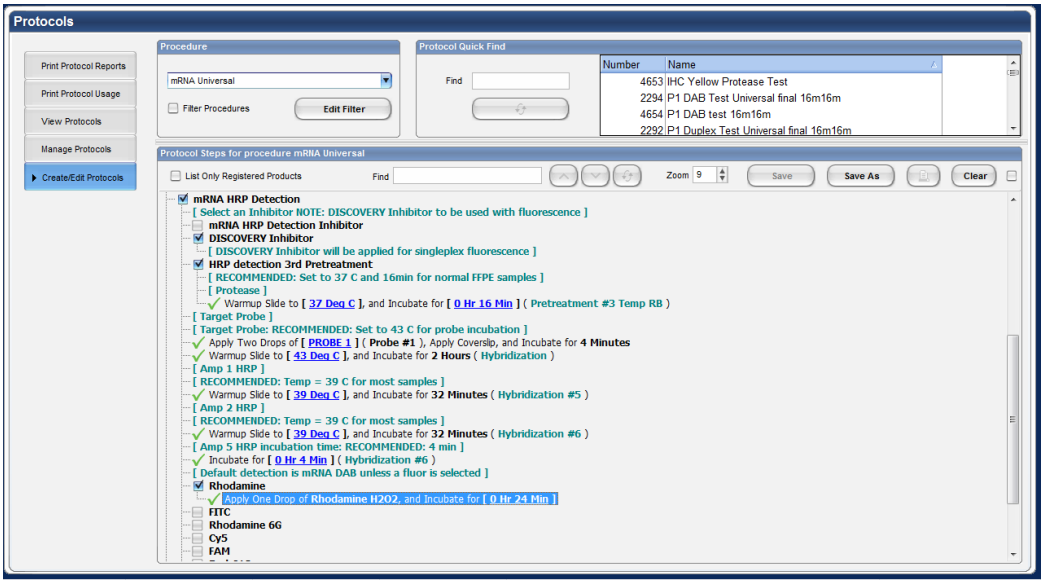
Create an instrument protocol

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 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:



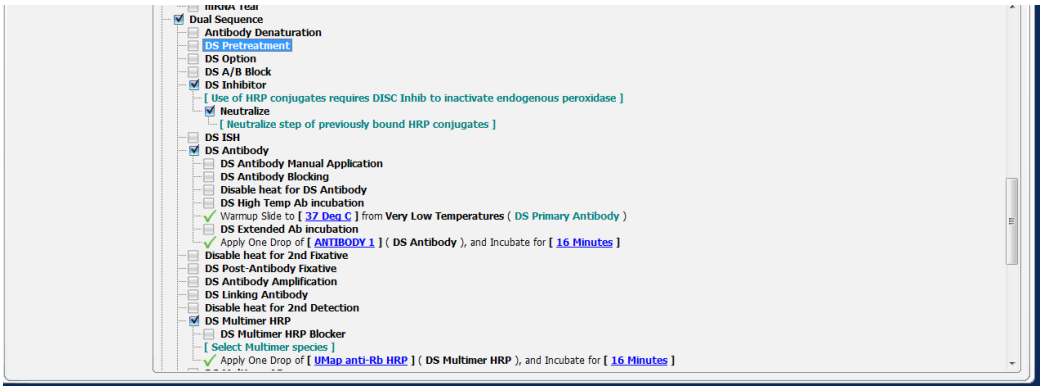


Add IF Conditions

1. Select **Dual Sequence** to open up the IF options.
2. Select **DS Inhibitor** then **Neutralize**, and use a dispenser of DISCOVERY Inhibitor.

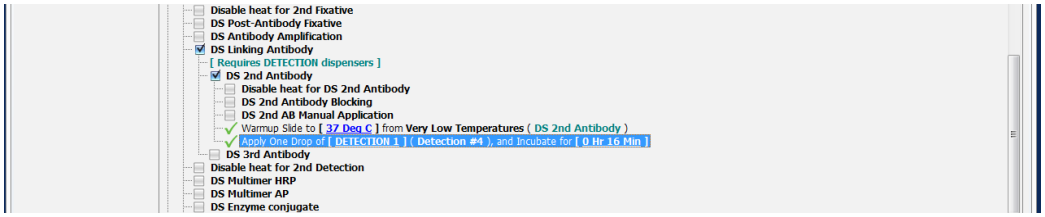
   Note: DISCOVERY Inhibitor is not required if you are using directly labeled primary or secondary antibodies.
3. Select between the following two options depending on your detection of choice:

   - Select the antibody to be used for IF, and then select a multimer that is compatible with the chosen antibody and the final detection kit used (for example, use an anti-rabbit horseradish peroxidase (HRP) multimer with a rabbit monoclonal antibody).

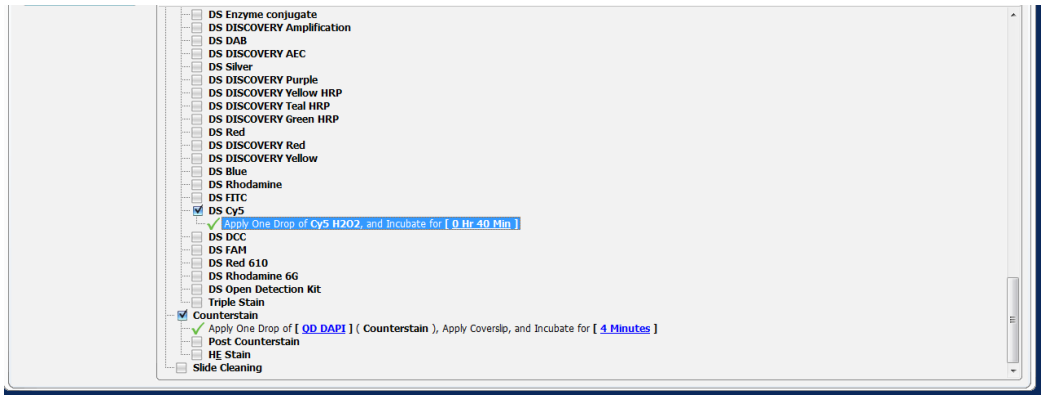


   - If you are using a fluorescently labeled secondary, select **DS Linking Antibody**, then choose the temperature and incubation time. Make sure that there is wavelength separation ofatleast50 nm between the antibody and the fluorescent label used for mRNA detection.

Note: You will need a special Detection Open Barcode, and the antibody must be transferred into an unused Ventana dispenser labeled with the barcode.



4. Select the fluorescent detection kit making sure that there is no overlapping emission with the mRNA signal. Select the counterstain (for example, QD DAPI).



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	
Pretreatment 3	Protease: 37°C, / 16 MIN
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

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 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 to the next procedure.

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
- Tissue-Tek® Staining Dish
- ProLong® Gold
- 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

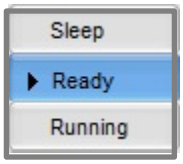
3. or forms a small meniscus at the tip of the nozzle

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

4. Load dispensers onto the reagent racks.
5. Remove the yellow locking ring from the dispensers in all of the prefilled dispensers. Refer to the instructions provided by Ventana™ Medical Systems.
6. Load the reagent racks onto the reagent carousel.

Start the run

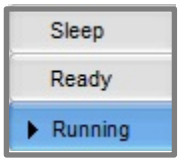
1. Select the **Ready** button.



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. Select the **Running** button. Automated assay will finish in **~15 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 (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 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 in the fume hood.
2. Mount one slide at a time by adding 1–2 drops of ProLong® Gold Antifade Reagent or other qualified pro-fluorescent 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**.

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 6. 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**.

6 Chapter 6. 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**.

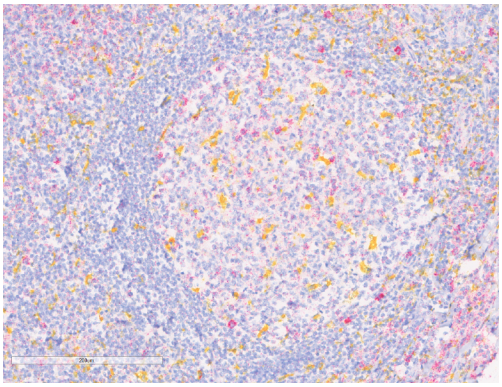
Troubleshooting

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

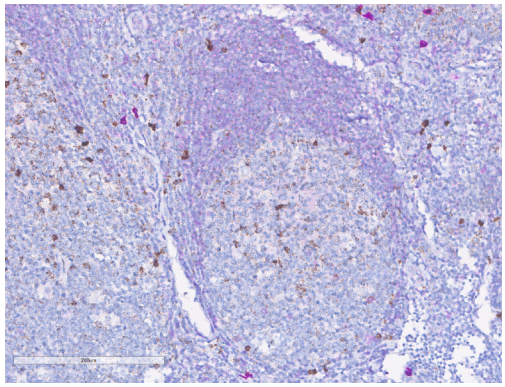
Tissue examples

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

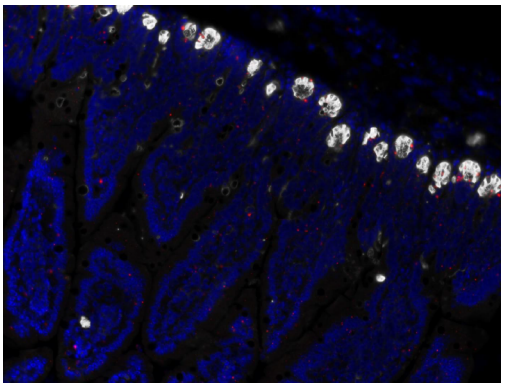
Figure 2. RNAscope® VS Universal Assay with IHC



Hs-PPIB (Red) with CD68 (Yellow) on Human Tonsil (20X)



Hs-PPIB (Brown) with IgD (Purple) on Human Tonsil (20X)

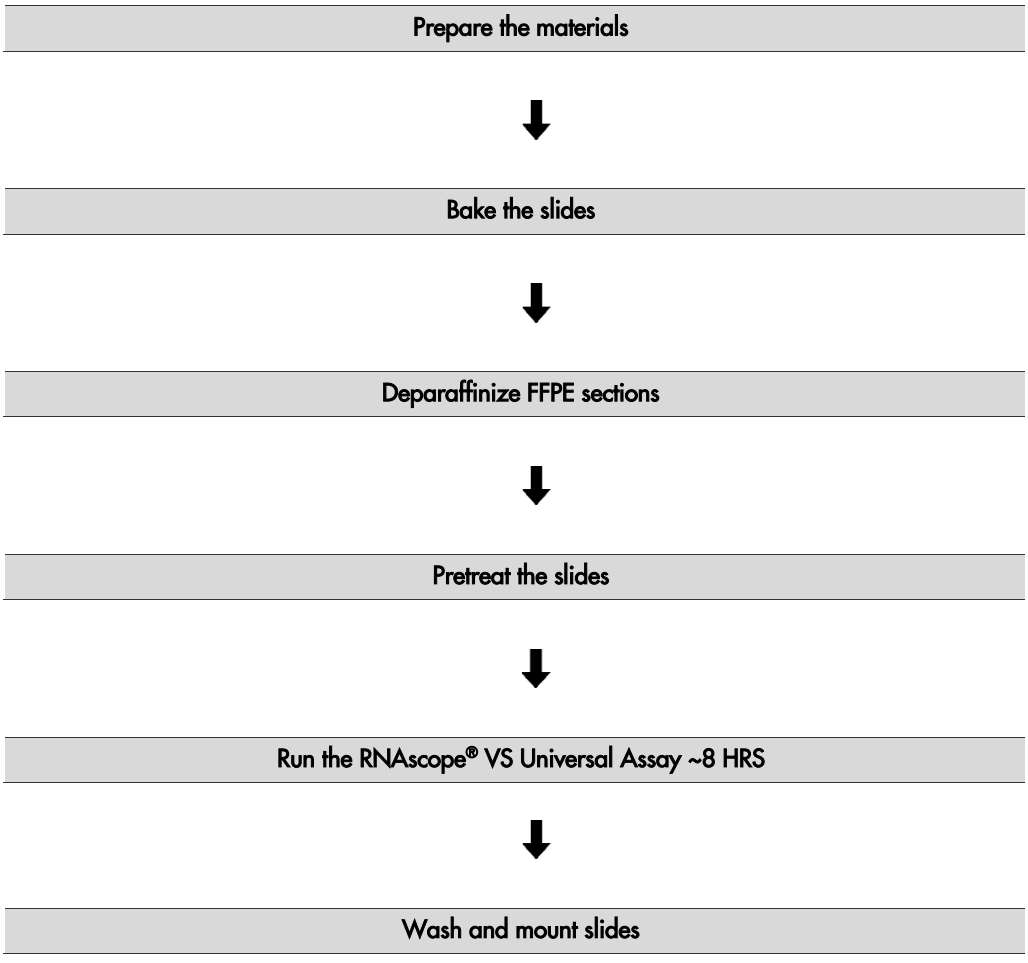


Mm-LGR5 (Red) with Lysozyme (White) on Mouse Intestine (20X)

Appendix A. Semi-automated RNAscope® VS Univeral 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 4. Chromagenic VS Universal Assay/IHC dual stain** starting on page 22, and **Chapter 5. Fluorescent VS Universal Assay/IF dual stain** starting on page 31.

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 45.

Materials required

Materials provided by Advanced Cell Diagnostics	Materials Provided by Ventana™ Medical Systems	Other Materials and Equipment
• RNAscope® VS Universal Sample Prep Reagents • RNAscope® VS Accessory Kit See Chapters 3–5 for materials required for specific applications	• DISCOVERY™ ULTRA — automated slide stainer • DISCOVERY Wash Buffer 10X • ULTRA LCS (Predilute) • SSC Buffer 10X • DISCOVERY CC1 • Reaction Buffer 10X • User fillable dispensers • mRNA Probe Amplification kits • See Chapters 3–5 for materials required for specific applications	• 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 • Cytoseal XYL xylene-based or ProLong® Gold • 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

Prepare the bulk fluids according to the 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 9 to determine the proper dispenser for each reagent. See Chapter 3–5 to prepare the reagents required for each specific procedure.

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

4. 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 45. 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 **VS Ultra**.

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. See Chapters 3–5 to set up protocol steps for specific applications.
5. See Chapters 3–5 to print labels.

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 • Prepared deparaffinization materials • 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.

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.

Run the RNAscope® VS Universal Assay

Refer to this section in Chapters 3–5 to run the assay, and mount your slides.

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 6. 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, Authorization 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.

Contact information

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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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