

RNAscope[®] VS Assay

For DISCOVERY[™] ULTRA

RED

Document Number 320521

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Citing RNAscope® VS in Publications

When describing a procedure for publication using this product, please refer to it as the RNAscope® VS Assay and cite: Wang F, Flanagan J, Su N, Wang L-C, Bui S, Nielson A, Wu X, Vo H-T, Ma X-J and Luo Y. RNAscope®: A Novel *In Situ* RNA Analysis Platform for Formalin-Fixed Paraffin-Embedded Tissues. J. Mol. Diagnostics, 2012, 14:22–29.

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Chapter 1. Product Information



Before using this product, read and understand the information in **Appendix C. Safety** on page 39 in this document.

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

About this guide

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

- **Chapter 4. Fully Automated RNAscope® VS Assay** starting on page 14.
- **Chapter 5. Semi-automated RNAscope® VS Assay** starting on page 20.

Product description

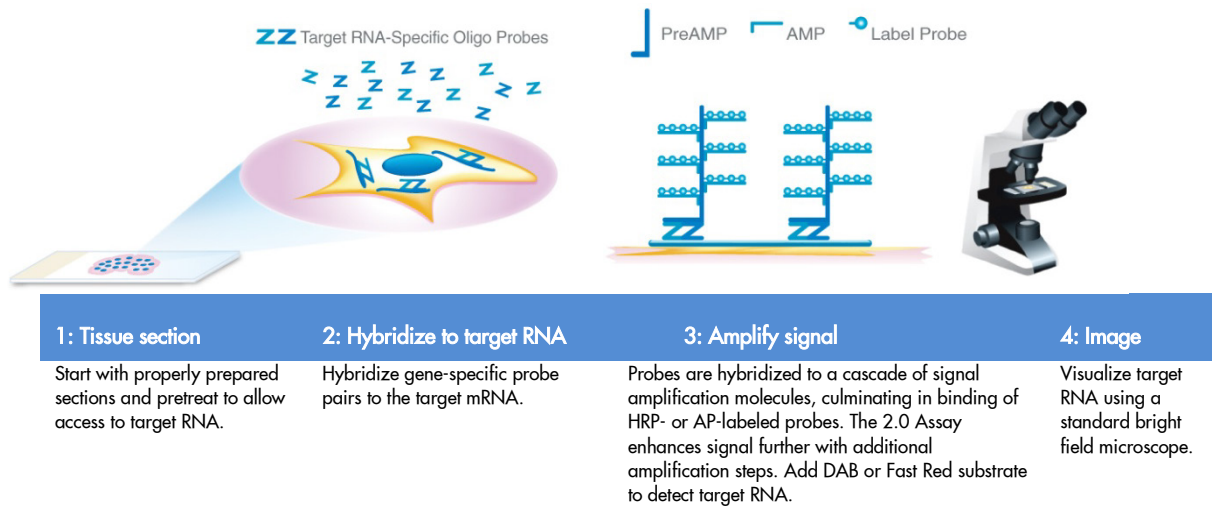
Background

The RNAscope® Assays use a novel and proprietary method of *in situ* hybridization (ISH) to visualize single RNA molecules per cell in 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 Assay allows users to automate the highly sensitive RNAscope® Assay using the Ventana™ DISCOVERY™ ULTRA systems.

Overview

The RNAscope® VS Assay procedure is illustrated in Figure 1 on page 6 and can be completed on the instrument in ~8–10 hours. Starting with properly prepared samples, sections are first pretreated, and then RNA-specific probes are hybridized to target RNA. The signal is amplified using multiple steps, followed by hybridization to horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-labeled probes and detection using a chromogenic substrate. Each single RNA transcript appears as a distinct dot of chromogen precipitate visible using a common bright-field microscope.

Figure 1 Procedure overview



Kit contents and storage

The RNAscope® VS Assay requires the RNAscope® VS Probes and the RNAscope® VS 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 www.acdbio.com/products/target-probes/search-product to find a gene-specific Target Probe. Visit www.acdbio.com/products/targetprobes/controls-housekeeping to order appropriate Control Probes.

Each probe is sufficient for staining ~30 standard slides. The probes have a shelf life of six months from the shipment date when stored as indicated in the following table:

Target Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® VS Target Probe – [species] – [gene]	Various	Probe targeting specific RNA	7 mL x 1 bottle	4°C
Control Probes					
<input checked="" type="checkbox"/>	Reagent	Cat. No.	Content	Quantity	Storage
	RNAscope® VS Positive Control Probe – [species] – PPIB	Various	Probe targeting common housekeeping gene	7 mL x 1 bottle	4°C
	RNAscope® VS Negative Control Probe – DapB	310043	Probe targeting bacterial gene dapB	7 mL x 1 bottle	4°C

RNAscope® Control Slides

The RNAscope® Control Slides (Cat. No. 310045 for Human control slide, Hela; Catalog 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 Positive Control Probe and RNAscope Negative Control Probes. The slides have a shelf life of 6 months from the shipment date when stored at 2–8°C with desiccants.

RNAscope® VS Reagents

The RNAscope® VS Reagents contain all the reagents needed to run the RNAscope® assay on the Ventana Discovery ULTRA, except for the RNA-specific probes. The contents of the RNAscope® VS Reagents consist of the RNAscope® VS Reagent Kit–RED (Cat. No. 320610), the RNAscope® VS Accessory Kit (Cat. No. 320630), and the RNAscope® Pretreat 2 Pack (Cat. No. 320043). The kits provide enough reagents to stain ~60 standard slides.

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

RNAscope® VS Reagent Kit–RED (Cat. No. 320610)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	VS Pretreat A–Red — Protease A	14 mL x 1 bottle	4°C
	VS Pretreat B–Red — Protease B	14 mL x 1 bottle	4°C
	VS Amp 1–Red	14 mL x 1 bottle	4°C
	VS Amp 2–Red	14 mL x 1 bottle	4°C
	VS Amp 3–Red	14 mL x 1 bottle	4°C
	VS Amp 4–Red	14 mL x 1 bottle	4°C
	VS Amp 5–Red	14 mL x 1 bottle	4°C
	VS Amp 6–Red	14 mL x 1 bottle	4°C
	VS Amp 7–Red	14 mL x 1 bottle	4°C
RNAscope® VS Accessory Kit (Cat. No. 320630)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	VS Hematoxylin — RTU	7 mL x 1 bottle	4°C
	VS Bluing Reagent — RTU	7 mL x 1 bottle	4°C
RNAscope® VS Pretreat 2 Pack (Cat. No. 320043)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	10X Pretreat 2 (antigen retrieval solution)	70 mL x 4 bottles	Room temperature (20–25°C)

IMPORTANT! Do not substitute the reagent components of the RNAscope® VS Reagent Kit with those of other RNAscope® Reagent Kits, even those having the same name.

Required materials from Ventana™ Medical Systems

The RNAscope® VS Assay requires specific materials and equipment available *only* from Ventana™ Medical Systems.

Probe Dispensers (Cat. No. 960-761 to 960-780)		
<input checked="" type="checkbox"/>	Component	Storage
	Probes 1–20 dispensers — fill dispensers with RNAscope® VS Probes. Use up to 20 probes at a time.	Room temperature (20–25°C)
mRNA Pretreatment Kit (Cat. No. 760-223)		
<input checked="" type="checkbox"/>	Component	Storage
	mRNA Pretreat A dispenser — fill dispenser with Pretreat A	Room temperature (20–25°C)
	mRNA Pretreat B dispenser — fill dispenser with Pretreat B	Room temperature (20–25°C)
mRNA Probe Amplification Kit (Cat. No. 760-222)		
<input checked="" type="checkbox"/>	Component	Storage
	mRNA Amp 1 dispenser — fill dispenser with Amp 1	Room temperature (20–25°C)
	mRNA Amp 2 dispenser — fill dispenser with Amp 2	Room temperature (20–25°C)
	mRNA Amp 3 dispenser — fill dispenser with Amp 3	Room temperature (20–25°C)
	mRNA Amp 4 dispenser — fill dispenser with Amp 4	Room temperature (20–25°C)
	mRNA Amp 5 dispenser — fill dispenser with Amp 5	Room temperature (20–25°C)
	mRNA Amp 6 dispenser — fill dispenser with Amp 6	Room temperature (20–25°C)
	mRNA Amp 7 dispenser — fill dispenser with Amp 7	Room temperature (20–25°C)
mRNA Red Detection Kit (Cat. No. 760-234)		
<input checked="" type="checkbox"/>	Component	Storage
	mRNA Inhibitor-prefilled	4°C
	mRNA Activator dispenser-prefilled	4°C
	mRNA Naphthol dispenser-prefilled	4°C
	mRNA Fast Red dispenser-prefilled	4°C
Generic dispensers (Cat. No. 771-741 and 771-742)		
<input checked="" type="checkbox"/>	Component	Storage
	Counterstain 1 dispenser — fill dispenser with Hematoxylin	Room temperature (20–25°C)
	Counterstain 2 dispenser — fill dispenser with bluing reagent	Room temperature (20–25°C)
mRNA Red, Amplification & Pretreatment PTO Kit (Cat. No. 760-235)		
<input checked="" type="checkbox"/>	Component	Storage
	mRNA Red Detection Kit (Cat. No. 760-234)	4°C
	mRNA Red Amplification Kit (Cat. No. 760-236)	Room temperature (20–25°C)
	mRNA Red Pretreatment Kit (Cat. No. 760-237)	Room temperature (20–25°C)

Equipment and buffers

<input checked="" type="checkbox"/>	Component	Cat. No.
	DISCOVERY™ ULTRA — automated slide stainer	N750-DISU-FS
	EZPrep Buffer	950-100
	LCS Buffer	650-010
	RiboWash Buffer	760-105
	RiboCC Buffer — used for automated cell conditioning (CC2)	760-107
	Reaction Buffer	950-300
	DISCOVERY™ 1X SSC Wash (in Option Bottle)	950-210

User-supplied materials

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

<input checked="" type="checkbox"/>	Description	Supplier	Cat. No.
	SuperFrost® Plus Slides (required)	Fisher Scientific	12-550-15
	100% ethanol (EtOH)	American Master Tech Scientific/MLS*	ALREAGAL
	Xylene	Fisher Scientific/MLS	X3P-1GAL
	10% neutral-buffered formalin (NBF)	MLS	—
	Paraffin wax	MLS	—
	1X PBS	MLS	—
	Microtome	MLS	—
	Drying oven, capable of holding temperature at 60 +/- 1°C	MLS	—
	Water bath or incubator, capable of holding temperature at 40 +/- 1°C	MLS	—
	EcoMount	Biocare	EM897L
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24
	Tissue-Tek® Staining Dish (3 required)	American Master Tech Scientific/MLS	LWT4457EA
	Tissue-Tek® Clearing Agent Dish, xylene resistant (3 required)	American Master Tech Scientific/MLS	LWT4456EA
	Cover Glass 24 x 50 mm	Fisher Scientific/MLS	12--545-F
	Distilled water	MLS	—
	Dawn detergent	MLS	—
	Fume hood	MLS	—
	Glass beaker (1 or 2 L)	MLS	—
	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.

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Chapter 2. Before You Begin

Prior to running the RNAscope® VS 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.
- View the video demonstrations available at www.acdbio.com/technical-support/online-training-videos.
- Run the assay on FFPE RNAscope® Control Slides (Cat. No. 310045 for Human control slide, Hela; Catalog No. 310023 for Mouse control slide, 3T3) using the Positive and Negative Control Probes.

Important procedural guidelines

- Start with properly fixed and prepared sections. Refer to **Chapter 3. Prepare and Pretreat Samples** on page 12 for preparation of FFPE slides. For preparation of other sample types, contact support@acdbio.com.
- Follow the recommended pretreatment conditions for your sample. Refer to **Recommended Guidelines** on page 28 for pretreatment conditions and to the technical notes available at www.acdbio.com/technical-support/downloads/technical-doc/category/tech-notes.
- 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.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix C. Safety** on page 39 for more information.

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Chapter 3. Prepare and Pretreat Samples

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

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

For samples treated differently from the following protocol, please see the sample pretreatment optimization procedure described in **Recommended Guidelines** on page 28 and technical notes available at www.acdbio.com/technical-support/downloads/technical-doc/category/tech-notes.

Prepare FFPE sections

Materials required

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

Fix the sample

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



CAUTION! Handle biological specimens appropriately.

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

Dehydrate, embed, and cut the sample

IMPORTANT! Use fresh reagents.

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

Note: Embedded samples may be stored at **RT** for years.

4. Trim paraffin blocks as needed, and cut embedded tissue into 5 +/- 1 µm sections using a microtome.

5. Place paraffin ribbon in a **40–45°C** water bath, and mount sections on **SUPERFROST® PLUS SLIDES**.

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

6. Air dry slides **OVERNIGHT** at **RT**.

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

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Chapter 4. Fully Automated RNAscope® VS Assay

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

Workflow

Prepare the materials



Run the RNASCOPE® VS Assay ~10 hours

Prepare the materials

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

Materials required

Materials provided by Advanced Cell Diagnostics	Materials provided by Ventana™ Medical Systems	Other materials and equipment
<ul style="list-style-type: none"> • VS Target Probe • VS Positive Control Probe • VS Negative Control Probe • VS Pretreat A-Red • VS Pretreat B-Red • VS Amp 1-Red • VS Amp 2-Red • VS Amp 3-Red • VS Amp 4-Red • VS Amp 5-Red • VS Amp 6-Red • VS Amp 7-Red • VS Hematoxylin • VS Bluing reagent 	<ul style="list-style-type: none"> • DISCOVERY™ ULTRA — automated slide stainer • EZPrep Buffer • LCS Buffer • RiboWash Buffer • CC1 and RiboCC Buffers (used for automated cell conditioning) • Reaction Buffer • DISCOVERY™ 1X SSC (Option Bottle) • Probe dispensers • mRNA Pretreatment Kit • mRNA Probe Amplification Kit • mRNA Red Detection Kit • Generic dispensers 	<ul style="list-style-type: none"> • Distilled water • Dawn detergent • Fume hood • Xylene • Tissue-Tek® Staining Dish (1) • Tissue-Tek® Clearing Agent Dish, xylene-resistant (1) • Tissue-Tek® Vertical 24 Slide Rack • EcoMount • 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 **Appendix A. Maintain the Instrument** on page 32.

Dilute bulk reagents

Prepare EZPrep, RiboWash, Reaction Buffer, and SSC (optional) by diluting them 1:10 with distilled water.

Register new reagents

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

Prepare instrument reagents

1. For AMP 1 to AMP 7, transfer the entire volume of each RNAscope® VS FFPE Reagent Kit component into the correspondingly labeled dispenser.
2. Transfer the rest of the RNAscope® VS Reagents to the correspondingly labeled dispensers.

IMPORTANT! Avoid cross contamination between reagents.

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

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

5. Check solution levels: EZprep, RiboWash, Reaction Buffer, and 0.1X SSC (optional wash). Refill if they are less than half full. To run a full tray of 30 slides, fully fill the RiboWash Buffer container.
6. For RiboCC or CC1, the optimal volume is approximately 25% of the container capacity. Refill to this level if the volume in the container falls below 10% capacity.

IMPORTANT! Use reagents that have not expired.

7. Empty the waste bottle if needed.

Prepare detergent

1. Prepare 200 mL of diluted Dawn detergent by adding 4–5 mL Dawn 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.

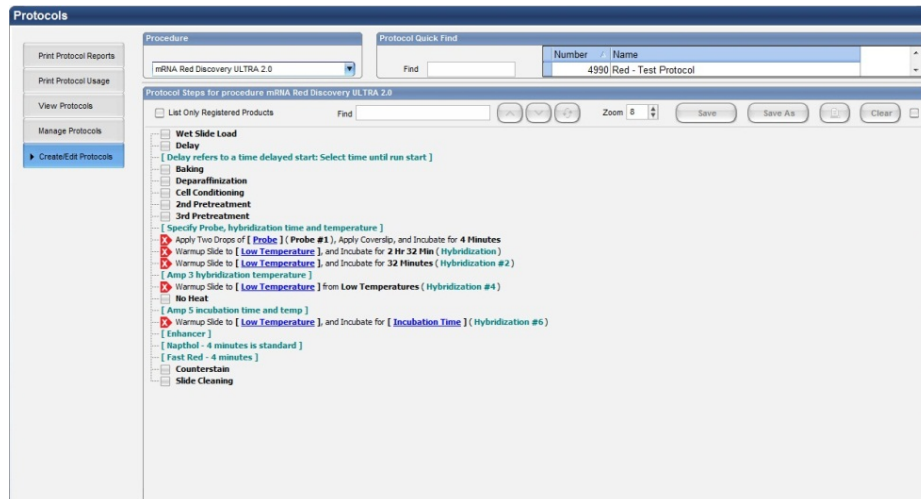
Prepare mounting reagents

- In a fume hood, fill a clearing agent dish with ~200 mL fresh xylene.

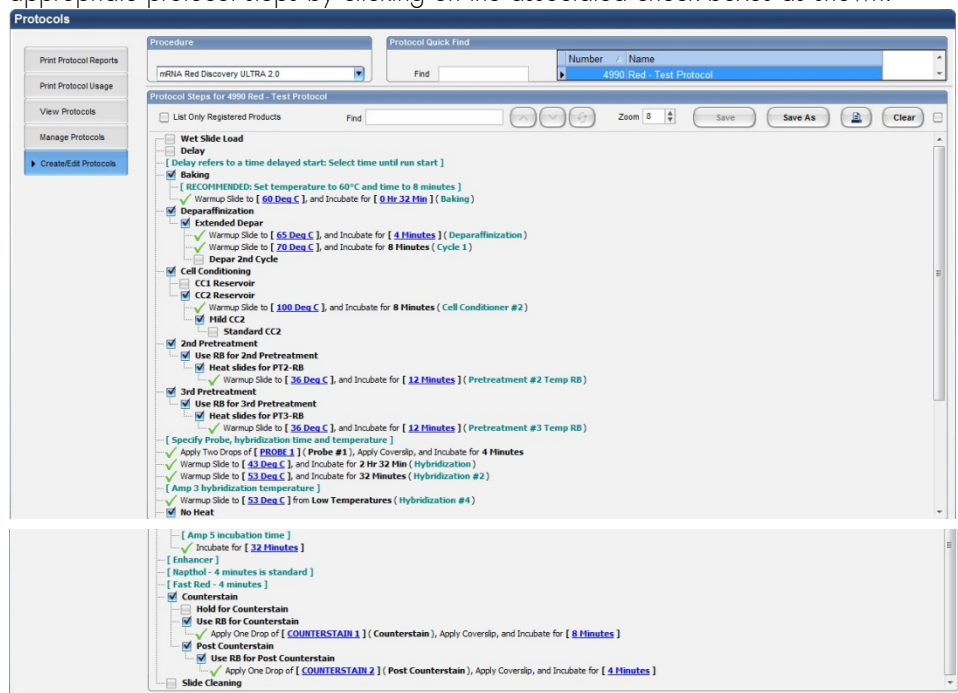
Note: Ensure all containers remain covered.

Create an instrument protocol

1. Open the NexES software and click on the **Protocol** button.
2. Click on **Create/Edit Protocols**, go to the Procedure drop down menu and select **mRNA Red Discovery ULTRA 2.0**.
3. Main protocol steps appear as shown:



- 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:



IMPORTANT! Optimal temperatures for the Amp 1 and Amp 3 hybridization steps (Hybridization #2 and Hybridization #4) may vary depending on your instrument. To optimize temperatures, follow the procedure in **Appendix B. Calibrate the Instrument** on page 36.

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

Tissue Type	Cell Conditioning (CC) Solution	CC Temp.	Time per Cycle	Cycle No.
Brain	CC1	100°C	12 MIN	1 cycle
Cell pellet	CC1	100°C	12 MIN	1 cycle
Colon	CC1	100°C	12 MIN	2–3 cycles
Heart	CC1	100°C	12 MIN	1 cycle
Intestine	CC1	100°C	12 MIN	2–3 cycles
Kidney	CC1	100°C	12 MIN	1 cycle
Liver	CC1	100°C	12 MIN	3 cycles
Lung	RiboCC	100°C	12 MIN	2–3 cycles
Prostate	CC1	100°C	12 MIN	1 cycle
Spleen	RiboCC	100°C	12 MIN	1 cycle
Tonsil	CC1	87°C	12 MIN	1 cycle

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

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

Print the labels

- Select the **Print Label** icon from the upper right corner of the home page screen.
- Select your preferred template or create new template. To create a new template, refer to the *Ventana™ System User Manual* for details.
- Click on **Protocol**.
- Select the protocol you created for the RNAscope® VS Assay and click on the **Add** button. When the protocols for all of the slides have been assigned, click on **Close/Print**.
- Fill in the template for each slide. Select **Print** when completed.
- Proceed to the following procedure **Loading the reagents**.

Run the RNAscope® VS Assay

Materials required

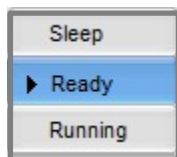
-
- Prepared slides
 - Prepared instrument reagents
 - Prepared detergent
 - Prepared mounting materials
 - Tissue-Tek® Vertical 24 Slide Rack
 - EcoMount
 - Cover Glass, 24 mm x 50 mm
-

Loading the reagents

- Remove the nozzle caps from the filled dispensers and place each cap on the post located on the back of the dispenser.
- If needed, remove any air bubbles at the nozzle tips by squeezing out one drop of reagent.
- Load dispensers onto the reagent racks.
- Remove the yellow locking ring from the dispensers in the prefilled mRNA RED Detection Kit. Refer to the instructions provided by Ventana™ Medical Systems.
- Load the reagent racks onto the reagent carousel.

Start the run

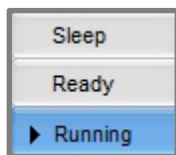
- Click the **Ready** button.



- Load each slide onto a heater pad with the label facing away from you. 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.

- Click the **Running** button. 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.

Complete the run

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

Wash the slides

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

Mount the samples

- Remove the slide rack from the staining dish and dry slides in a **60°C** dry oven for **15 MIN**.

IMPORTANT! The Red substrate is alcohol sensitive. Do not dehydrate the slides in alcohol.

- Cool the slides for **5 MIN** at **RT**.
- Briefly dip one slide into fresh pure xylene and *immediately* place 1–2 drops of EcoMount on the slide before the xylene dries.

IMPORTANT! Use the EcoMount mounting medium only.

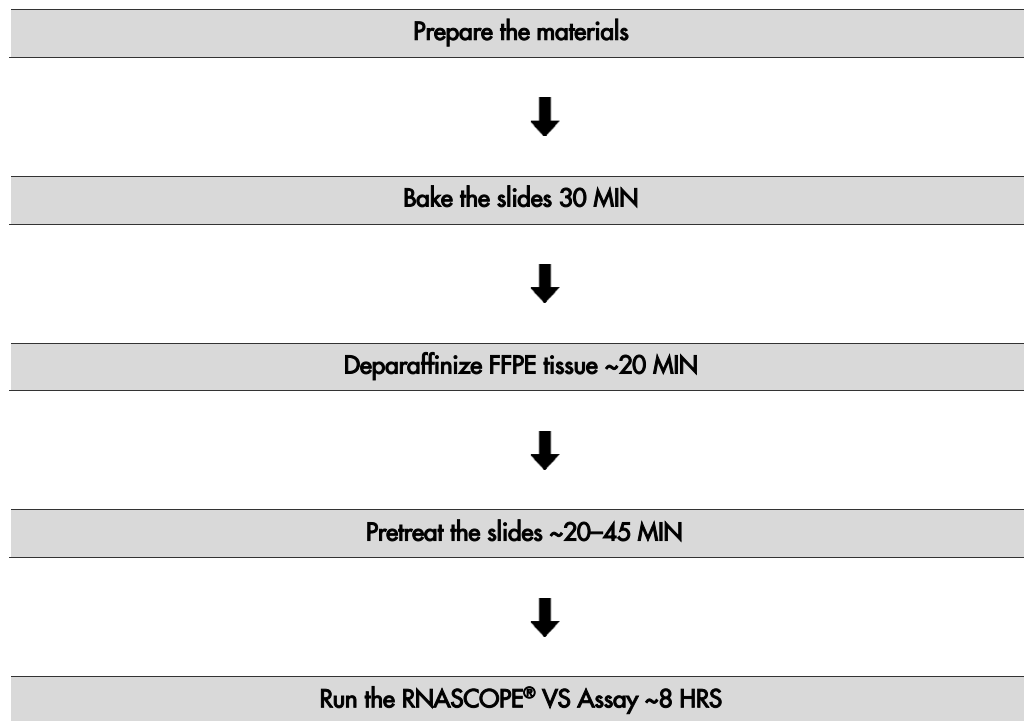
- Carefully place 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
- Repeat steps 2 and 3 for each slide.
- Air dry slides for **5 MIN**.
- Proceed to **Chapter 6. Evaluate the results** on page 30.

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Chapter 5. Semi-automated RNAscope® VS 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. Fully Automated RNAscope® VS Assay** on page 14.

Workflow



Prepare the materials

Materials can be prepared ahead of time or while baking the slides, unless otherwise stated. See **Bake the slides** on page 25.

Materials required

Materials provided by Advanced Cell Diagnostics	Materials provided by Ventana™ Medical Systems	Other materials and equipment
<ul style="list-style-type: none"> • VS Target Probe • VS Positive Control Probe • VS Negative Control Probe • 10X Pretreat 2 • VS Pretreat A-Red • VS Pretreat B-Red • VS Amp 1-Red • VS Amp 2-Red • VS Amp 3-Red • VS Amp 4-Red • VS Amp 5-Red • VS Amp 6-Red • VS Amp 7-Red • VS Hematoxylin • VS Bluing reagent 	<ul style="list-style-type: none"> • DISCOVERY™ ULTRA — automated slide stainer • EZPrep Buffer • LCS Buffer • RiboWash Buffer • Reaction Buffer • DISCOVERY™ 1X SSC (Option Bottle) • Probe dispensers • mRNA Pretreatment Kit • mRNA Probe Amplification Kit • mRNA Red Detection Kit • Generic dispensers 	<ul style="list-style-type: none"> • Distilled water • Glass beaker (1 or 2 L) • Hot plate • Dawn detergent • Fume hood • Xylene • 100% ethanol (EtOH) • Tissue-Tek® Staining Dish (3) • Tissue-Tek® Clearing Agent Dish, xylene-resistant (3) • Tissue-Tek® Vertical 24 Slide Rack • EcoMount • 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 **Appendix A. Maintain the Instrument** on page 32.

Dilute bulk reagents

Prepare EZPrep, RiboWash, Reaction Buffer, and SSC (optional) by diluting them 1:10 with distilled water.

Register new reagents

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

Prepare instrument reagents

Register *new* reagent kits using the wand that comes with the instrument.

1. In reverse order from AMP 7 to AMP 1, transfer the entire volume of each RNAscope® VS FFPE Reagent Kit component into the correspondingly labeled dispenser.
2. Transfer the rest of the RNAscope® VS Reagents to the correspondingly labeled dispensers.

IMPORTANT! Avoid cross contamination between reagents.

3. Follow the dispenser product insert instructions to properly prime and handle the dispensers.

4. Press the dispenser caps down tightly.

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

5. Check solution levels: EZPrep, RiboWash, Reaction, LCS Buffer, and 1X SSC. Refill if they are less than half full.

IMPORTANT! Use reagents that have not expired.

6. Empty the waste bottle if needed.

Prepare detergent

1. Prepare 200 mL of diluted Dawn detergent by adding 4–5 mL Dawn 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 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.

Prepare mounting reagents

- In a fume hood, fill a clearing agent dish with ~200 mL fresh xylene.

Note: Ensure all containers remain covered.

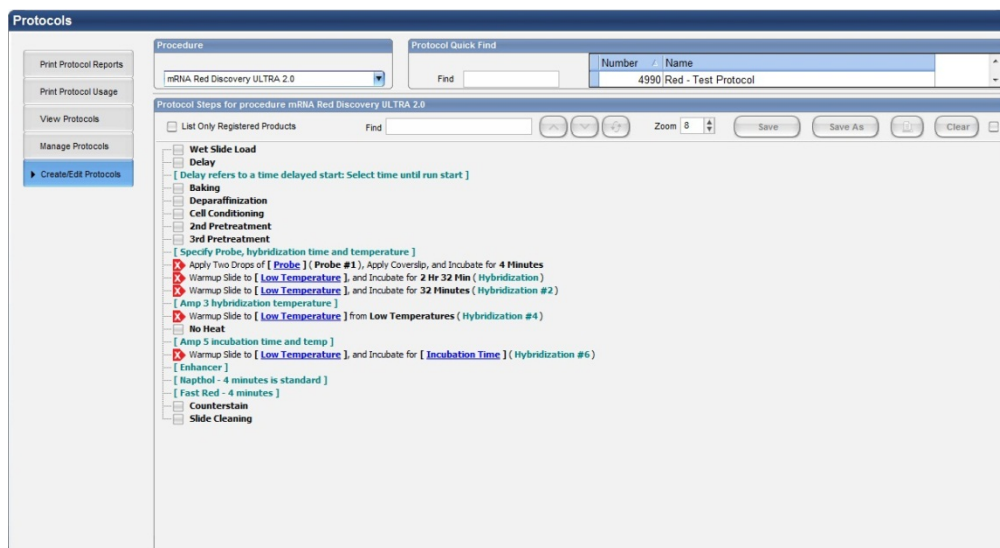
Prepare 1X Pretreat 2

Prepare 1X PRETREAT 2 while FFPE slides are baking at 60°C, or the following day if you choose the optional stopping point on page 13. 1X Pretreat 2 is used in manual cell conditioning (CC).

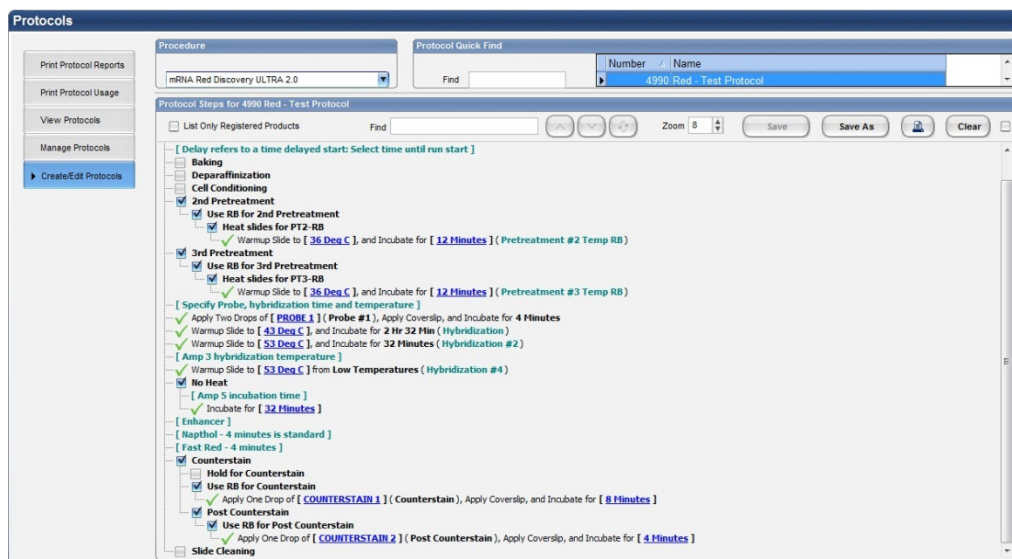
1. Prepare 700 mL of fresh 1X Pretreat 2 by adding 630 mL distilled water to 1 bottle (70 mL) 10X Pretreat 2 solution in the beaker.
2. Mix well and cover the beaker with foil.

Create an instrument protocol

1. Open the NexES software and click on the **Protocol** button.
2. Click on **Create/Edit Protocols**, go to the Procedure drop down menu and select **mRNA Red Discovery ULTRA 2.0**.
3. Main protocol steps appear as shown:



- 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:



IMPORTANT! Optimal temperatures for the Amp 1 and Amp 3 hybridization steps (Hybridization #2 and Hybridization #4) may vary depending on your instrument. To optimize temperatures, follow the procedure in **Appendix B. Calibrate the Instrument** on page 36.

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

Tissue type	Pretreatment #2 (Pretreat A)	Pretreatment #3 (Pretreat B)
Brain and spinal cord	12 MIN	12 MIN
Breast cancer	12 MIN	12 MIN
Cell lines	12 MIN	12 MIN
Colon	12 MIN	12 MIN
GI tract	12 MIN	12 MIN

Tissue type	Pretreatment #2 (Pretreat A)	Pretreatment #3 (Pretreat B)
Head and neck cancer	12 MIN	12 MIN
Heart	12 MIN	12 MIN
Kidney	12 MIN	12 MIN
Liver	12 MIN	12 MIN
Lung	12 MIN	12 MIN
Lymphoma	8 MIN	8 MIN
Placenta	12 MIN	12 MIN
Prostate	12 MIN	12 MIN
Skin	12 MIN	12 MIN
Stomach	12 MIN	12 MIN
Thymus	8 MIN	8 MIN
Tonsil	8 MIN	8 MIN
Xenograft derived from cell lines	12 MIN	12 MIN
Xenograft derived from primary tumor	12 MIN	12 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 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 Assay.
4. Click on **Protocol** to add and print the label.

Manually pretreat the samples

Materials required

Materials provided by the RNAscope® VS Reagents	Other materials and equipment
<ul style="list-style-type: none"> • 10X Pretreat 2 	<ul style="list-style-type: none"> • Drying oven • FFPE slides • Tissue-Tek® Vertical 24 Slide Rack • Tissue-Tek® Staining Dish • Distilled water • Prepared deparaffinization materials • Glass beaker (1 or 2 L) • Hot plate

Bake the slides

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

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

2. If you continue, prepare the materials for the following protocols while the slides are baking: **Deparaffinize FFPE sections** in the next section, **Pretreat the slides** on page 26, and **Run the RNAscope® VS Assay** on page 27. See **Prepare the materials** on page 21.

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 pages 22–24.

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 the next section.

Pretreat the slides

Begin heating 1X Pretreat 2 while FFPE slides are baking at 60°C or during the previous section.

IMPORTANT! Do not boil 1X Pretreat 2 more than **30 MIN** before use.

1. Heat 1X Pretreat 2 to **98–104°C**:
 - a. Place the beaker containing 1X Pretreat 2 on the hot plate. Cover the beaker with foil and turn the hot plate on high for **10–15 MIN**.
 - b. Once 1X Pretreat 2 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 Pretreat 2 solution. Cover the beaker with foil and boil the slides for the amount of time specified in the following table:

Tissue type	Treatment time
Brain and spinal cord	15 MIN
Breast cancer	15 MIN
Cell lines	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 Pretreat 2 to a staining dish containing distilled water. Do not let the slides cool in Pretreat 2.
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. Directly proceed to **Loading the reagents** on page 27.

Run the RNAscope® VS Assay

Materials required

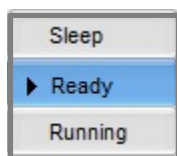
- Prepared slides
 - Prepared instrument reagents
 - Prepared detergent
 - Distilled water
 - Prepared mounting materials
 - Tissue-Tek® Vertical 24 Slide Rack
 - EcoMount
 - Cover Glass, 24 mm x 50 mm
-

Loading the reagents

1. Remove the nozzle caps of the filled dispensers and place them on their holders.
2. If needed, remove any air bubbles at the nozzle tips by squeezing out one drop of reagent.
3. Load dispensers onto the reagent racks.
4. Remove the yellow locking ring from the dispensers in the prefilled mRNA RED Detection Kit. Refer to the instructions provided by Ventana™ Medical Systems.
5. Load the reagent racks onto the reagent carousel.

Start the run

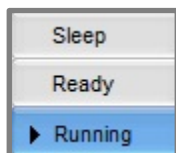
1. Click the **Ready** button.



2. Load each slide onto a heater pad with the label facing away from you. 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.

3. Click the **Running** button. 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.

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.

Wash the slides

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

Mount the samples

1. Remove the slide rack from the staining dish and dry slides in a **60°C** dry oven for **15 MIN**.

IMPORTANT! The Red substrate is alcohol sensitive. Do not dehydrate the slides in alcohol.

2. Cool the slides for **5 MIN** at **RT**.
3. Briefly dip one slide into fresh pure xylene and *immediately* place 1–2 drops of EcoMount on the slide before the xylene dries.

IMPORTANT! Use the EcoMount mounting medium only.

4. Carefully place 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat steps 2 and 3 for each slide.
6. Air dry slides for **5 MIN**.
7. Proceed to **Chapter 6. Evaluate the results** on page 30.

Recommended Guidelines

We highly recommend following the guidelines for Cell Conditioning (Pretreat 2), Pretreatment #2 (Pretreat A), and Pretreatment #3 (Pretreat B) conditions for:

- Any new or previously untested FFPE tissue types.
- Samples prepared differently than the protocol in **Chapter 3. Prepare and Pretreat Samples** on page 12.
 1. Stain six representative slides using the positive and negative control probes according to the following table:

Slide No.	Probe	Manual Cell Conditioning	Pretreatment #2	Pretreatment #3
1	Positive control	10 MIN	12 MIN	12 MIN
2	Negative control	10 MIN	12 MIN	12 MIN
3	Positive control	15 MIN	12 MIN	12 MIN
4	Negative control	15 MIN	12 MIN	12 MIN
5	Positive control	30 MIN	12 MIN	12 MIN
6	Negative control	30 MIN	12 MIN	12 MIN

2. Evaluate staining and tissue morphology as in **Chapter 6. Evaluate the results** on page 30, and determine which pretreatment condition yielded the highest positive control signal and lowest negative control signal. Positive control signal should have a staining score of 3 or higher, and the negative control signal should be 0.
3. Use the optimized pretreatment conditions to run the assay with the target probe.
4. If none of the conditions are satisfactory, contact technical support at support@acdbio.com.

6

Chapter 6. Evaluate the results

Examine tissue sections under a standard bright field microscope at 20–40X magnification:

- Assess tissue and cell morphology.
- Assess positive control signal strength. Positive control signal should be visible as punctate dots within cell nuclei at 20–40X magnification.
- Assess negative control background. One dot to every 10 cells displaying background staining per 20X microscope field is acceptable.
- Evaluate target probe signal using the scoring guidelines in the next section.

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/cell (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.

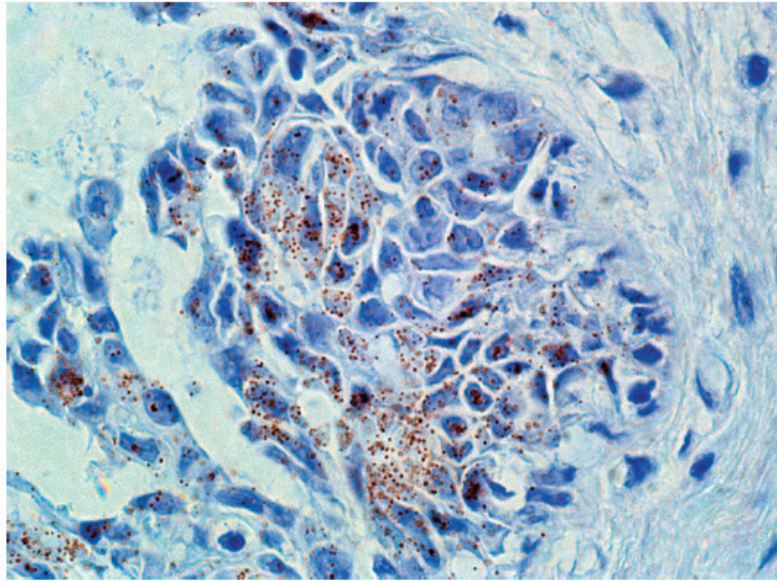
Troubleshooting

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

Control example

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

Figure 2 RNAscope® VS Assay detection of HPV E6/E7 mRNA in cervical cancer FFPE tissue.



A

Appendix A. Maintain the Instrument

If you have not used your DISCOVERY™ instrument for one week or longer, bacterial growth and/or salt crystallization may partially clog the valves that dispense bulk reagents possibly affecting assay performance.

Check all buffer containers for any visible signs of bacterial growth. Disinfect the containers with Lysol IC if necessary, rinse well with distilled water, and refill with fresh, unexpired buffers.

Note: We recommend decontaminating the instrument and buffer containers every three months.

Flush the instrument

If you have used the instrument to run assays other than the RNAscope® Assay, we recommend performing this procedure prior to running the RNAscope® Assay.

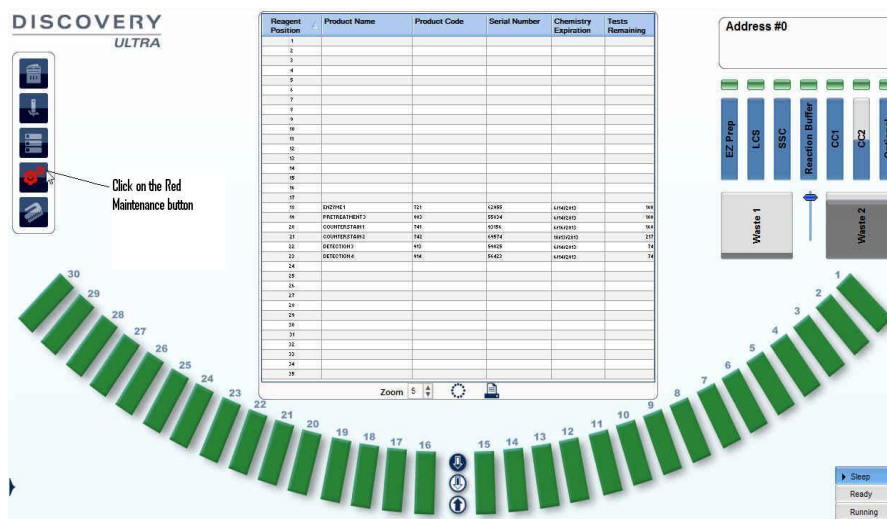
Purge and prime the system with clean distilled water

Purge and prime the system with clean distilled water several times to remove debris and bacterial growth:

1. Remove all the bulk reagent containers from the instrument.
2. Connect the yellow tubing manifold usually used for quarterly decontamination to the instrument.
3. Place the open end of the tubing manifold into a bottle containing 4 L of distilled water. Ensure that the tubing end touches the bottom of the bottle.

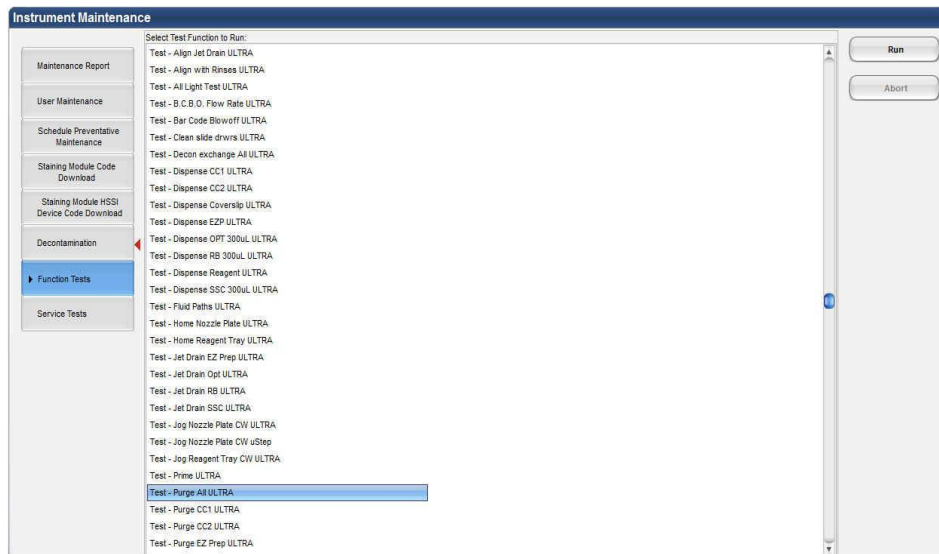
Note: Refill the bottle with distilled water as needed.

4. Click on the **Red Maintenance** button.

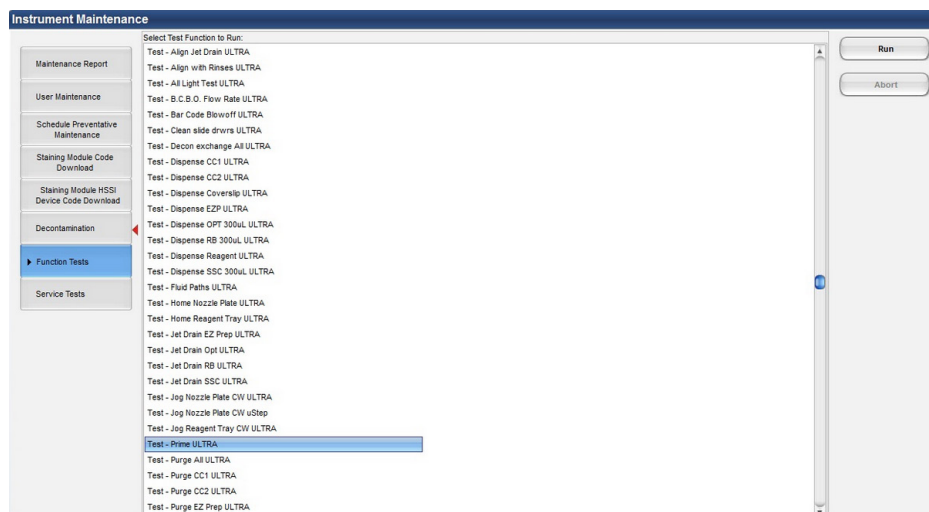


5. Click on **Function Tests** on the main screen.
6. Double click on **Test - Purge All ULTRA**:

Note: You will hear a warning sound and see an error message stating that the pressure is too low. This is normal. Click on **Sign off** to turn off the warning sound.



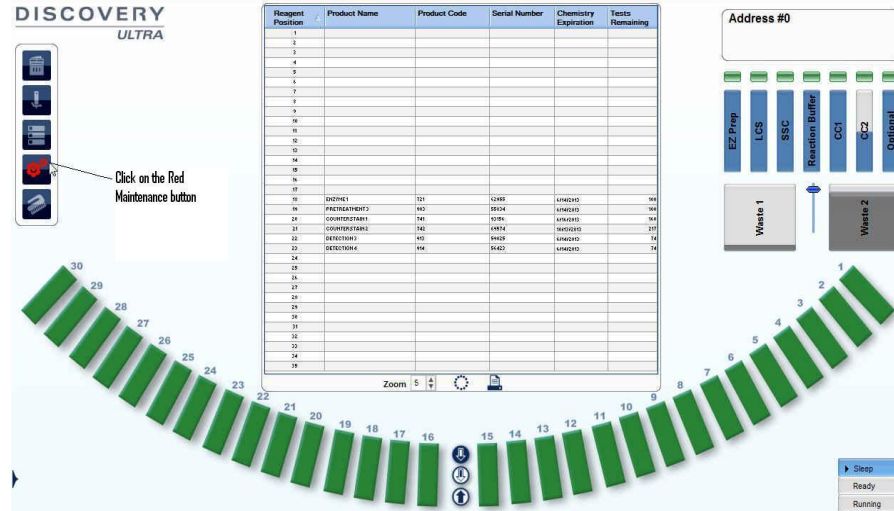
7. Repeat Steps 4—6 four more times.
8. Click on the **Red Maintenance** button.
9. Click **Function Tests** on the main screen.
10. Double click on **Test - Prime ULTRA**:



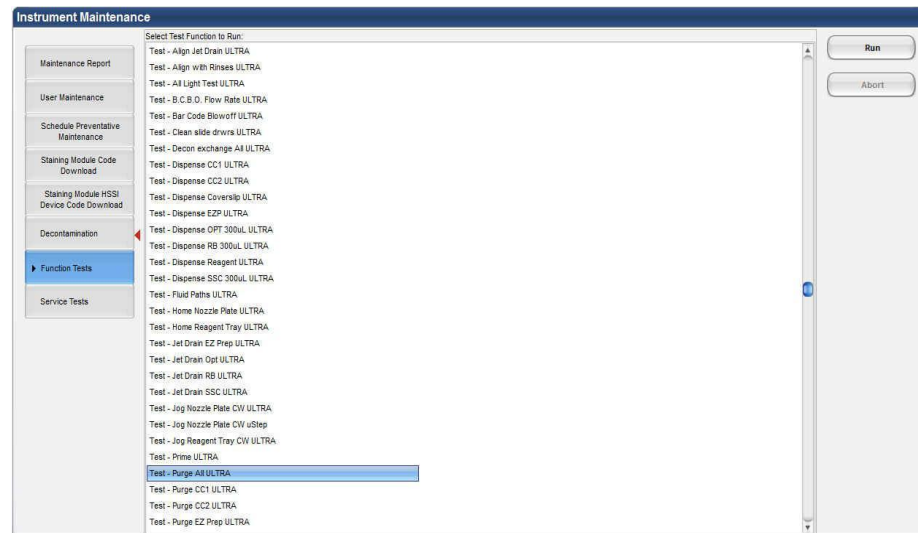
11. Repeat Steps 8—10 four more times.

Purge and prime the system with fresh reagents

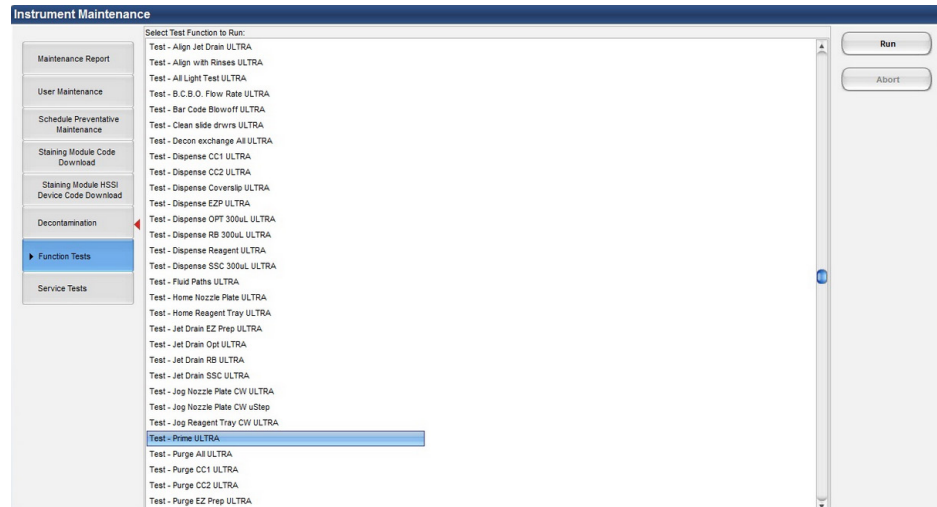
1. Unplug the yellow tubing manifold, and then load the bulk reagent containers containing fresh buffers back on the instrument.
2. Click on the **Red Maintenance** button.



3. Click **Function Tests** on the main screen.
4. Double click on **Test - Purge All ULTRA**:



5. Repeat Steps 2—4 once.
6. Click on the **Red Maintenance** button.
7. Click **Function Tests** on the main screen.
8. Double click on **Test - Prime ULTRA**.



9. Repeat Steps 6—8 once.

B

Appendix B. Calibrate the Instrument

Heating capabilities may vary between instruments and could impact assay results. To maximize assay performance, we recommend performing the following protocol prior to running the RNAscope® Assay for the first time on an instrument.

Calibrate the temperature of the instrument

Run an RNAscope® VS Assay to test three sets of temperatures for the Amp 1 (Hybridization #2) and Amp 3 (Hybridization #4) steps. Follow the instructions in **Chapter 4. Fully Automated RNAscope® VS Assay** on page 14.

Temperatures tested:

- 49°C (Amp 1)/49°C (Amp 3)
- 51°C (Amp 1)/51°C (Amp 3)
- 53°C (Amp 1)/53°C (Amp 3)

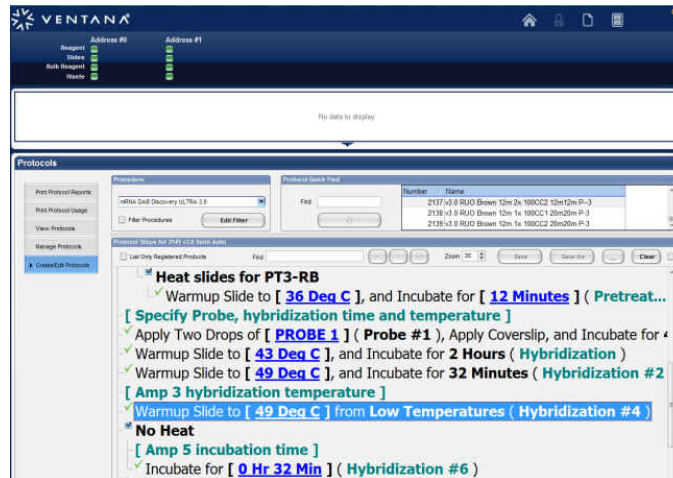
Materials required

-
- RNAscope® Control Slides – Cat. No. 310045 for Human control slide, HeLa or Catalog No. 310023 for Mouse control slide, 3T3 (6 slides total)
 - VS Positive Control Probe – *[species]*– PPIB
 - VS Negative Control Probe – DapB
 - All other reagents required to run the RNAscope® VS Assay
-

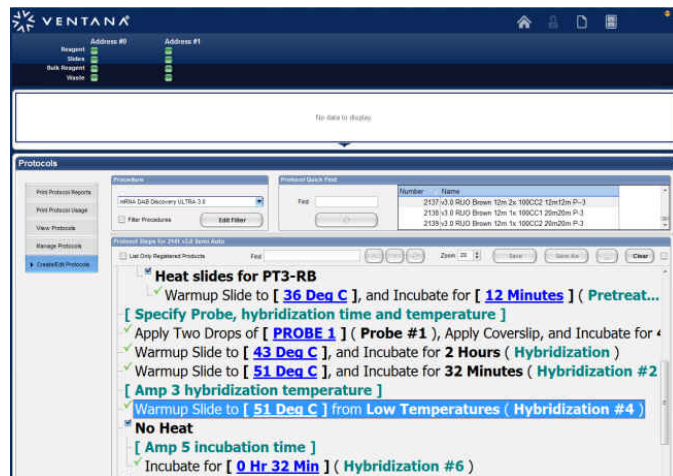
Procedure

1. Prepare six control slides to test the three temperature sets. Run each temperature set using the Positive and Negative Control Probes.
2. Prepare the instrument and reagents as described in **Chapter 4. Fully Automated RNAscope® VS Assay**.
3. See **Create an instrument protocol** on page 16 to create three sets of protocols. Each set should contain two protocols to run the Positive and Negative Control Probes. Use the provided screenshots as a guide:

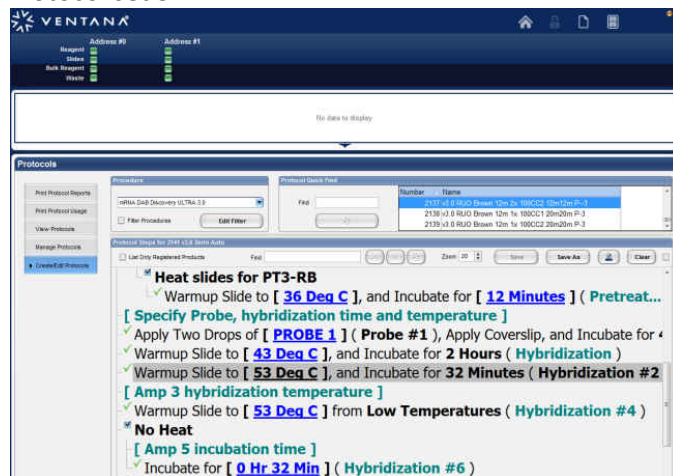
Protocol set 1



Protocol set 2

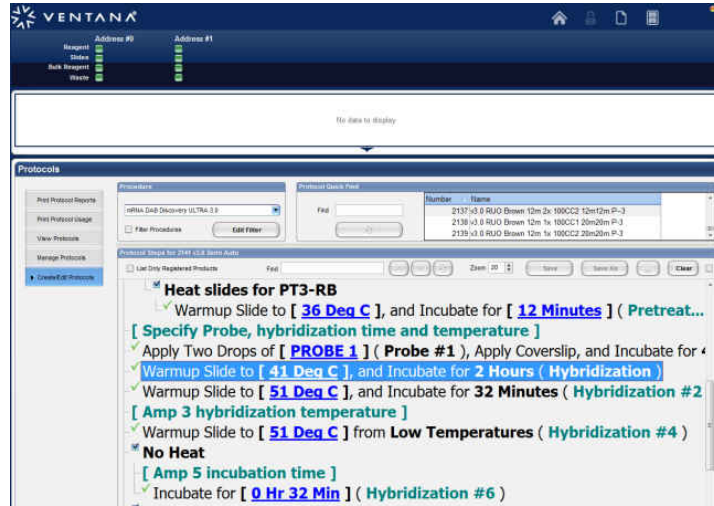


Protocol set 3



4. Run the assay as described in **Run the RNAscope® VS Assay** on page 18.
5. Using the images on page Error! Bookmark not defined. as references, determine the optimal temperatures for the Amp 1 and 3 hybridization steps.

- Adjust the Target Probe hybridization temperature to match the same scale as the hybridization temperatures for the Amp1 and Amp 3 steps. For example, if the optimal temperature is 51°C, lower the Target Probe hybridization temperature to 41°C instead of 43°C. See the following example:



C

Appendix C. Safety

Chemical safety



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

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

Biological hazard safety



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

In the U.S.:

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

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

In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at:
www.who.int/csr/resources/publications/biosafety/who_cds_csr_lyo_2004_11/en/
- Information about the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) can be found at:
eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:133:0001:0043:EN:PDF

Documentation and support

Obtaining MSDSs

Material Safety Data Sheets (MSDSs) are available at: www.acdbio.com/product_literature.html. For the MSDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

Obtaining support

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

At the website, you can:

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

Contact information

Advanced Cell Diagnostics, Inc.

3960 Point Eden Way

Hayward, CA 94545

Toll Free: 1-877-576-3636

Direct: 1-510-576-8800

Fax: 1-510-576-8801

Information: info@acdbio.com

Orders: orders@acdbio.com

Support Email: support@acdbio.com

Limited product warranty

Advanced Cell Diagnostics, Inc. and/or its affiliate(s) warrant their products as set forth in the ACD General Terms and Conditions of Sale found on the ACD website at www.acdbio.com/product_literature.html. If you have any questions, please contact Advanced Cell Diagnostics at www.acdbio.com/about/contact.

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For support, email support@acdbio.com.
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