USER MANUAL

ADVANCED CELL DIAGNOSTICS

RNAscope® VS Assay

For DISCOVERY™ XT

RED

Document Number 320523

For Research Use Only (RUO)

Rev. Date 20150318

For Reseach Use Only (RUO).

Trademarks

RNAscope® is a registered trademarks of Advanced Cell Diagnostics, Inc. VENTANA and DISCOVERY are trademarks of Roche. All other trademarks belong to their respective owners.

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.

Disclaimers

Advanced Cell Diagnostics, Inc. reserves the right to change its products and services at any time to incorporate technological developments. This manual is subject to change without notice.

Although this manual has been prepared with every precaution to ensure accuracy, Advanced Cell Diagnostics, Inc. assumes no liability for any errors, omissions, or for any damages resulting from the use of this information.

Copyright

ADVANCED CELL DIAGNOSTICS

Contents

ADVANCED CELL DIAGNOSTICS

1 Chapter 1. Product Information

Before using this product, read and understand the information in **Appendix C. Safety** on page 37 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. Semi-automated RNAscope® VS Assay** starting on page 14.
- **Chapter 5. Fully Automated RNAscope® VS Assay** starting on page 23.

Product description

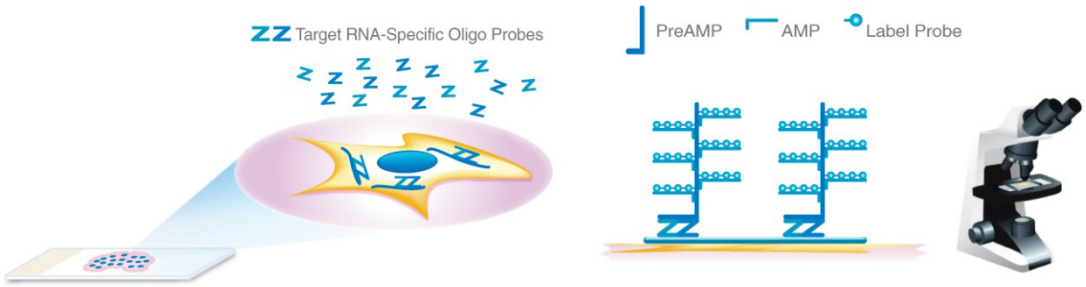
Background

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™ XT or 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



1: Tissue section	2: Hybridize to target RNA	3: Amplify signal	4: Image
Start with properly prepared sections and pretreat to allow access to target RNA.	Hybridize gene-specific probe pairs to the target mRNA.	Probes are hybridized to a cascade of signal amplification molecules, culminating in binding of HRP- or AP-labeled probes. The 2.0 Assay enhances signal further with additional amplification steps. Add DAB or Fast Red substrate to detect target RNA.	Visualize target RNA using a standard bright field microscope.

Kit contents and storage

The RNAscope® 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					
☑	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					
☑	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 dessicants.

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)			
☑	**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)			
☑	**Reagent**	**Quantity**	**Storage**
	VS Hematoxylin	7 mL x 1 bottle	4°C
	VS Bluing Reagent	7 mL x 1 bottle	4°C
RNAscope® VS Pretreat 2 Pack (Cat. No. 320043)			
☑	**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)		
☑	**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)		
☑	**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)		
☑	**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)		
☑	**Component**	**Storage**
	mRNA Inhibitor-prefilled	4°C
	mRNA Activator dispenser-prefilled	4°C
	mRNA Napthol dispenser-prefilled	4°C
	mRNA Fast Red dispenser-prefilled	4°C
Generic dispensers (Cat. No. 771-741 and 771-742)		
☑	**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)		
☑	**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

☑	Component	Cat. No.
	DISCOVERY™ XT — automated slide stainer	F-DISXT-750000
	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.

☑	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 C	MLS	—
	Water bath or incubator, capable of holding temperature at 40 +/- 1°C 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.

2 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™ XT 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 **Appendix A. Recommended Guidelines** on page 33 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 37 for more information.

ADVANCED CELL DIAGNOSTICS

3 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 **Appendix A. Recommended Guidelines** on page 33 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.

Dehydrate sample using a standard ethanol series, followed by xylene.

Embed sample in paraffin using standard procedures.

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

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

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.

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

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

ADVANCED CELL DIAGNOSTICS

4 Chapter 4. Semi-automated RNAscope® VS Assay

Most sample types require manual pretreatment prior to running the automated RNAscope® VS Assay. For brain and spinal cord sections, you may fully automate manual pretreatment steps. See **Chapter 5. Fully Automated RNAscope® VS Assay** on page 23.

Workflow

Prepare the materials

↓

Bake the slides 30 MIN

↓

Deparaffinize FFPE tissue ~20 MIN

↓

Pretreat the slides ~20–45 MIN

↓

Run the RNASCOPE® VS Assay ~8 HRS

Prepare the materials

Materials can be prepared ahead of time or while baking the slides, unless otherwise stated. See Bake the slides on page 20.

Materials required

Materials provided by Advanced Cell Diagnostics	Materials provided by Ventana™ Medical Systems	Other materials and equipment
• 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	• DISCOVERY™ XT — 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	• 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 B** on page 34.
- If your instrument has been used recently, run the Prime-XT protocol two times to clear the fluid lines before setting up the experiment. Refer to the *Ventana™ System User Manual* and **Appendix B** on page 34 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).

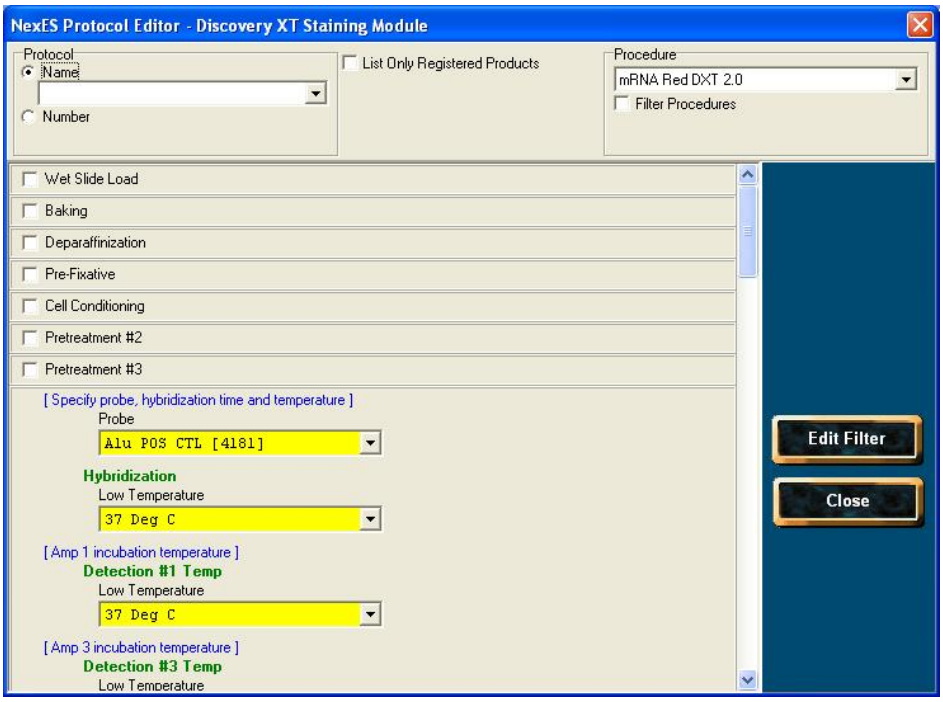
1. Prepare 700 mL of fresh 1X Pretreat 2 by adding 630 mL distilled water to 1 bottle (70 mL) 10X Pretreat 2 solution in the beaker.
2. Mix well and cover the beaker with foil.

Create an instrument protocol

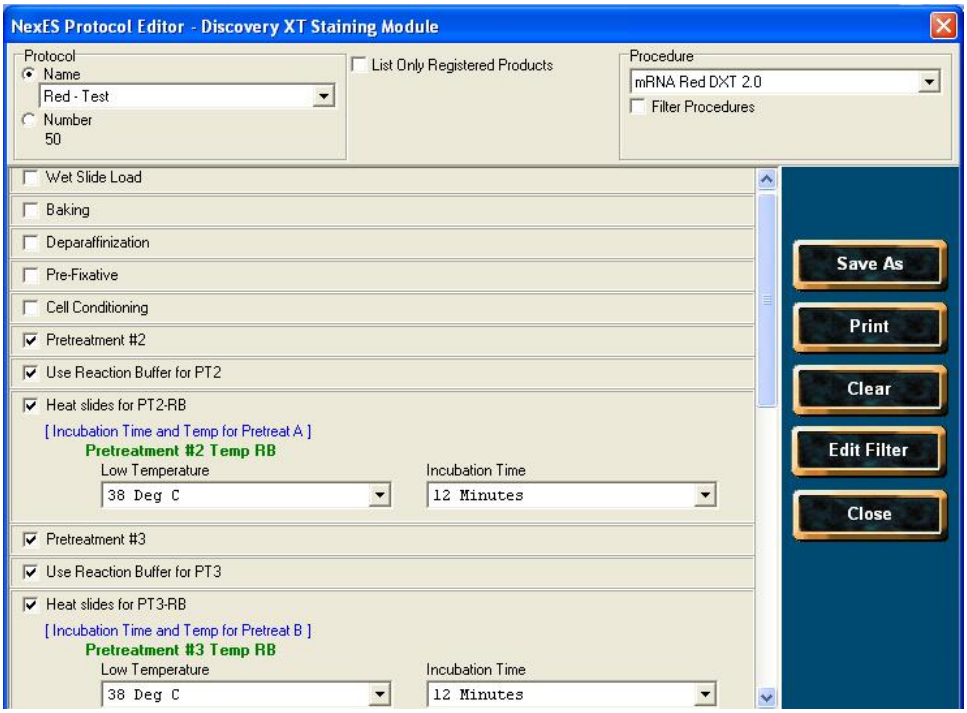
1. Open the NexES software and click on the **Protocol** button.

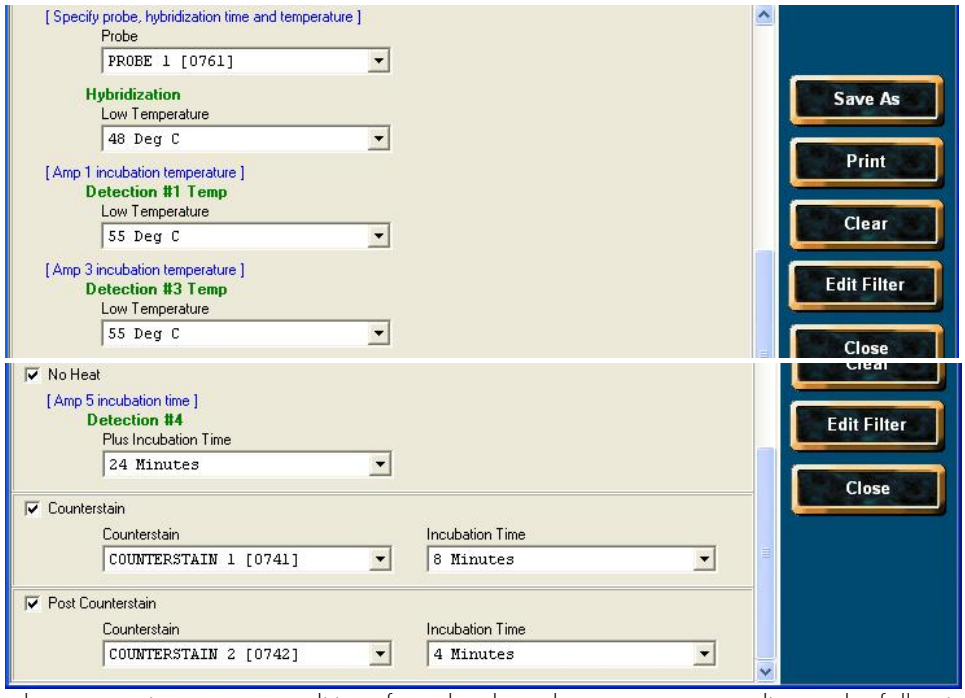
Click on **Create/Edit Protocols**, go to the **Procedure** drop down menu and select **mRNA Red DXT 2.0**.

2. Main protocol steps appear as shown:



3. 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:





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
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

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 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.

Select your preferred template or create a new template. To create a new template, refer to the *Ventana™ System User Manual* for details.

Select the protocol you created for the RNAscope® VS Assay.

Click on **Protocol** to add and print the label.

ADVANCED CELL DIAGNOSTICS

Manually pretreat the samples

Materials required

Materials provided by the RNAscope® VS Reagents	Other materials and equipment
• 10X Pretreat 2	• Drying oven • FFPE slides • Tissue-Tek® Vertical 24 Slide Rack • Distilled water • Fume hood • Xylene • 100% ethanol (EtOH) • Tissue-Tek® Clearing Agent Dish (2) • Tissue-Tek® Staining Dish (2) • Glass beaker (1 or 2 L) • Hot plate

Bake the slides

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

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

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 21, and **Run the RNAscope® VS Assay** on page 23. See **Prepare the materials** on page 15.

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 16–19.

1. Place slides in a Tissue-Tek® Slide Rack and submerge in the first xylene-containing clearing agent dish in the fume hood.

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.

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.

Repeat Step 2.

Remove the slide rack from the second xylene-containing dish and *immediately* place in the staining dish containing 100% EtOH.

Incubate the slides in 100% EtOH for **1 MIN** at **RT** with agitation.

Repeat Step 6 with fresh 100% EtOH.

Remove the slides from the rack, and place on absorbent paper with the section face-up. Air dry for **5 MIN** at **RT**.

While slides are drying, place printed labels on the slides.

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

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.

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

* This procedure can be automated for these tissue types. See page 23.

Use the forceps to *immediately* transfer the hot slide rack from the 1X Pretreat 2 to the staining dish containing distilled water. Do not let the slides cool in Pretreat 2.

Wash slides 3–5 times by moving the Tissue-Tek® Slide Rack up and down in the distilled water.

Repeat Step 4 with fresh distilled water.

Directly proceed to **Loading the slides** into slide holders on the instrument.

Run the RNAscope® VS Assay

Materials required

- Prepared slides
- Prepared instrument reagents
- 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

Loading the slides

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

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

Note: Avoid pouring distilled water directly on the sections.

Loading the reagents

1. Remove the nozzle caps of the filled dispensers and place them on their holders.

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

1. Click the **Run** button.
2. Follow the instructions on the instrument screen. Select the **Reagent/Reagent Tray Loaded**, and **Reagent Caps Removed** check boxes.
3. Enter the number of slides.

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.

Store reagent racks at **4°C** until next use.

Wash the slides

1. Add 200 mL diluted detergent to a Tissue-Tek® Staining Dish.

Submerge a Tissue-Tek® Slide Rack into the staining dish.

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 6, 3–5 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 into fresh pure xylene and *immediately* place 1–2 drops of EcoMount on the slide before the xylene dries.

WARNING! Use the EcoMount mounting medium only.

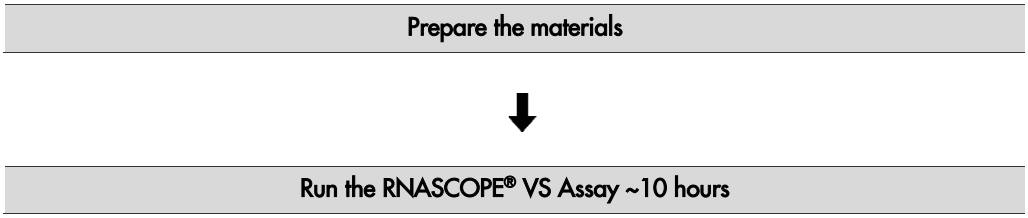
4. Carefully place 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat steps 2 and 3 for each slide.
6. Air dry slides for **5 MIN**.
7. Proceed to **Chapter 6. Evaluate the results** on page 31.

5 Chapter 5. Fully Automated RNAscope® VS Assay

Use this protocol for brain and spinal cord sections. The Fully Automated RNAscope® VS Assay shares many of the same steps as the Semi-automated RNAscope® VS Assay. Do not perform the manual pretreatment steps.

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

Workflow



Prepare the materials

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

Materials required

Materials provided by Advanced Cell Diagnostics	Materials provided by Ventana™ Medical Systems	Other materials and equipment
• 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	• DISCOVERY™ XT —slide stainer • EZPrep Buffer • LCS Buffer • RiboWash Buffer • RiboCC Buffer— used for automated cell conditioning (CC2) • Reaction Buffer • DISCOVERY™ 1X SSC (Option Bottle) • Probe dispensers • mRNA Pretreatment Kit • mRNA Probe Amplification Kit • mRNA Red Detection Kit • Generic dispensers	• 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 B** on page 34.
- If your instrument has been used recently, run the Prime-XT protocol two times to clear the fluid lines before setting up the experiment. Refer to the *Ventana™ System User Manual* and **Appendix B** 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.

Transfer the rest of the RNAscope® VS Reagents to the correspondingly labeled dispensers.

IMPORTANT! Avoid cross contamination between reagents.

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

Press the dispenser caps down tightly.

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

5. Check solution levels: EZprep, RiboWash, RiboCC, 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**.

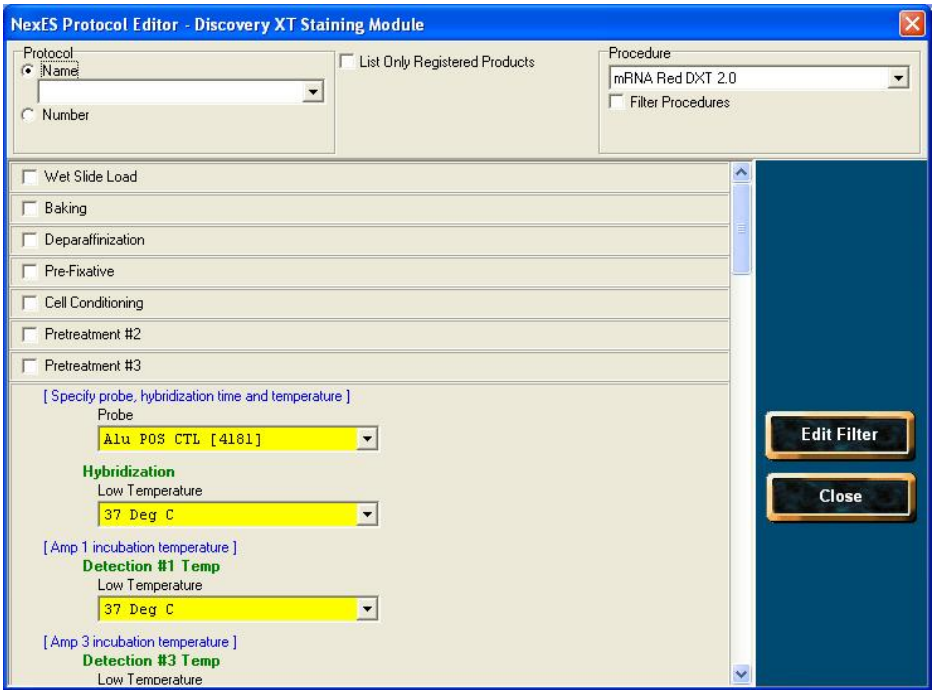
Prepare mounting reagents

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

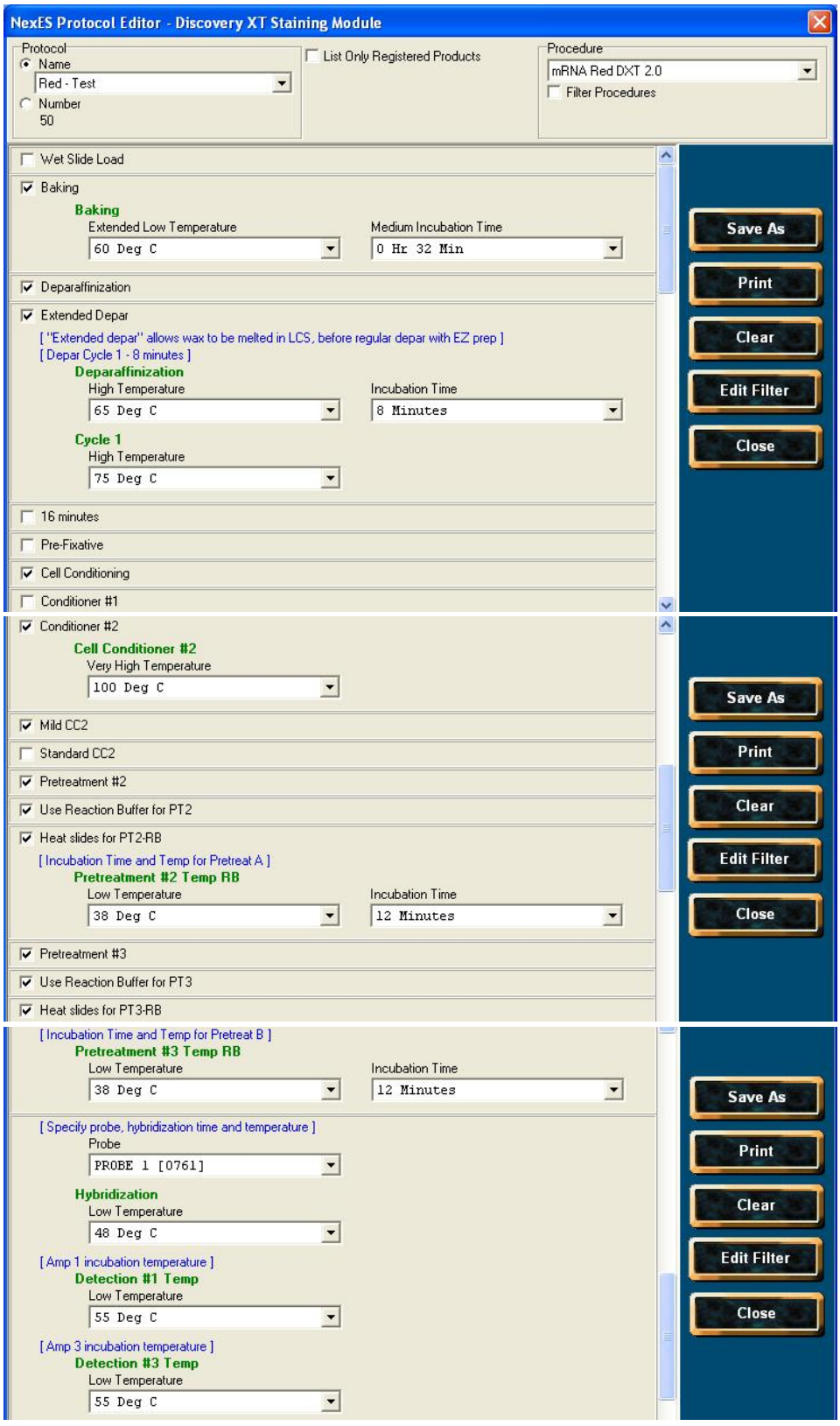
Note: Ensure all containers remain covered.

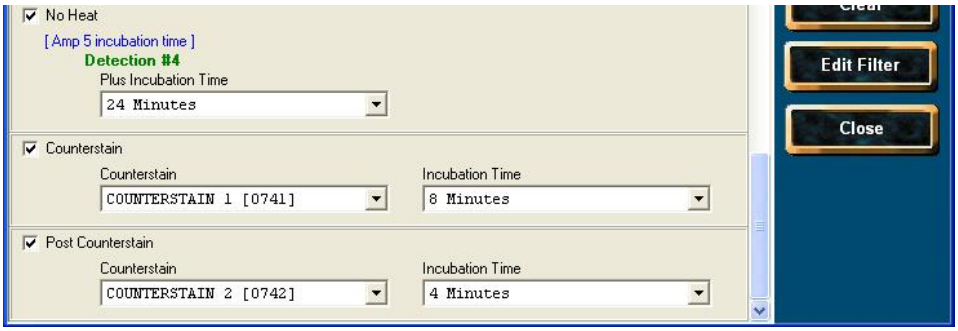
Create an instrument protocol

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 DXT 2.0**.
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:


NexES Protocol Editor - Discovery XT Staining Module
Protocol
Name
Red - Test
Number
50
List Only Registered Products
Procedure
mRNA Red DXT 2.0
Filter Procedures
Wet Slide Load
Baking
Baking
Extended Low Temperature
60 Deg C
Medium Incubation Time
0 Hr 32 Min
Deparaffinization
Extended Depar
["Extended depar" allows wax to be melted in LCS, before regular depar with EZ prep]
[Depar Cycle 1 - 8 minutes]
Deparaffinization
High Temperature
65 Deg C
Incubation Time
8 Minutes
Cycle 1
High Temperature
75 Deg C
16 minutes
Pre-Fixative
Cell Conditioning
Conditioner #1
Conditioner #2
Cell Conditioner #2
Very High Temperature
100 Deg C
Mild CC2
Standard CC2
Pretreatment #2
Use Reaction Buffer for PT2
Heat slides for PT2-RB
[Incubation Time and Temp for Pretreat A]
Pretreatment #2 Temp RB
Low Temperature
38 Deg C
Incubation Time
12 Minutes
Pretreatment #3
Use Reaction Buffer for PT3
Heat slides for PT3-RB
[Incubation Time and Temp for Pretreat B]
Pretreatment #3 Temp RB
Low Temperature
38 Deg C
Incubation Time
12 Minutes
[Specify probe, hybridization time and temperature]
Probe
PROBE 1 [0761]
Hybridization
Low Temperature
48 Deg C
[Amp 1 incubation temperature]
Detection #1 Temp
Low Temperature
55 Deg C
[Amp 3 incubation temperature]
Detection #3 Temp
Low Temperature
55 Deg C
Save As
Print
Clear
Edit Filter
Close



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

Tissue type	Automated cell conditioning	Pretreatment #2	Pretreatment #3
Brain and spinal cord	100°C, mild CC2	12 MIN	12 MIN

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 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.

Select your preferred template or create a new template. To create a new template, refer to the *Ventana™ System User Manual* for details.

Select the protocol you created for the RNAscope® VS Assay.

Click on **Protocol** to add and print the label.

Directly proceed to **Loading the slides** on page 29.

Run the RNAscope® VS Assay

Materials required

- Prepared instrument reagents
- 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

Loading the slides

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

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

Note: Avoid pouring distilled water directly on the sections.

Loading the reagents

1. Remove the nozzle caps of the filled dispensers and place them on their holders.

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

1. Click the **Run** button.

Follow the instructions on the instrument screen. Select the **Reagent/Reagent Tray Loaded**, and **Reagent Caps Removed** check boxes.

Enter the number of slides.

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.

Store reagent racks at **4°C** until next use.

Wash the slides

1. Add 200 mL diluted detergent to a Tissue-Tek® Staining Dish.

Submerge a Tissue-Tek® Slide Rack into the staining dish.

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 6, 3–5 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 into fresh pure xylene and *immediately* place 1–2 drops of EcoMount on the slide before the xylene dries.

WARNING! 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**.

Proceed to **Chapter 6. Evaluate the results** on page 31.

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**.

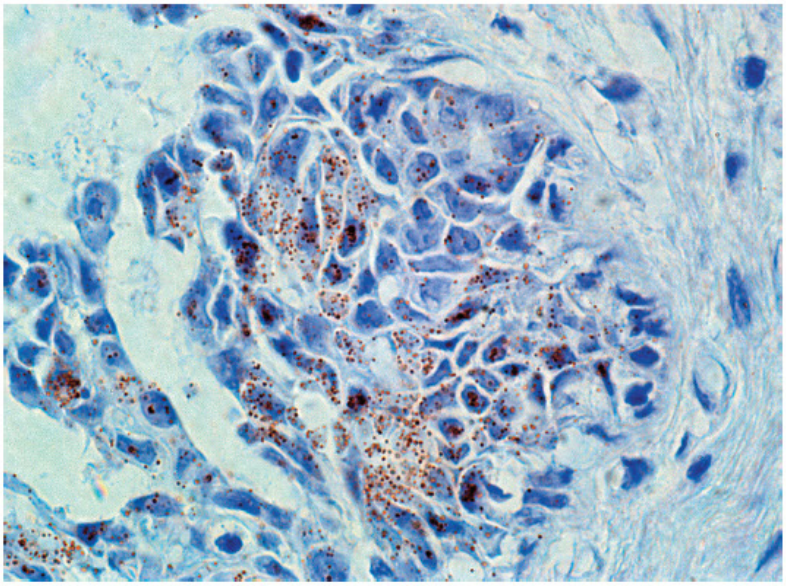
Troubleshooting

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. 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

Evaluate staining and tissue morphology as in **Chapter 6. Evaluate the results** on page 31, 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.

Use the optimized pretreatment conditions to run the assay with the target probe.

If none of the conditions are satisfactory, contact technical support at **support@acdbio.com**.

ADVANCED CELL DIAGNOSTICS

B

Appendix B. Maintain the Instrument

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

Check all buffer containers for any visible signs of bacterial growth. Disinfect the containers with Lysol IC (NEED Cat. No?) if necessary, rinse well with distilled water, and refill with fresh, unexpired buffers.

Note: We recommend decontaminating the instrument and buffer containers every 3 months.

If you have never run an assay using these buffers, perform the following procedure.

Flush the instrument

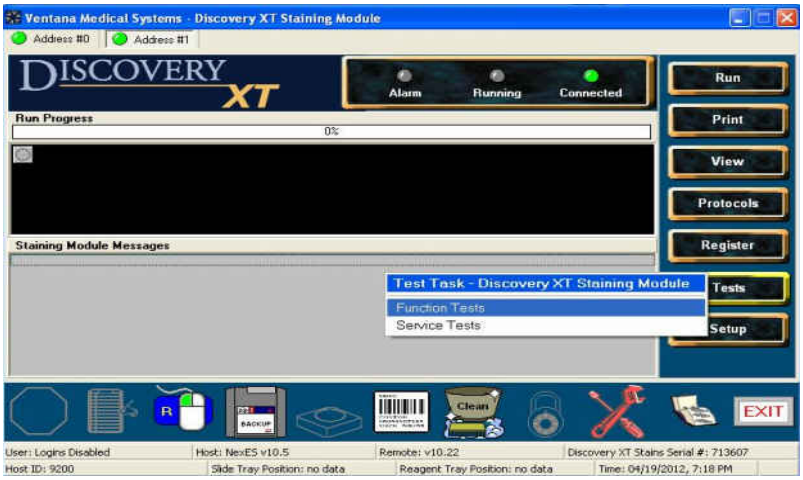
Purge and prime the system with clean distilled water

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

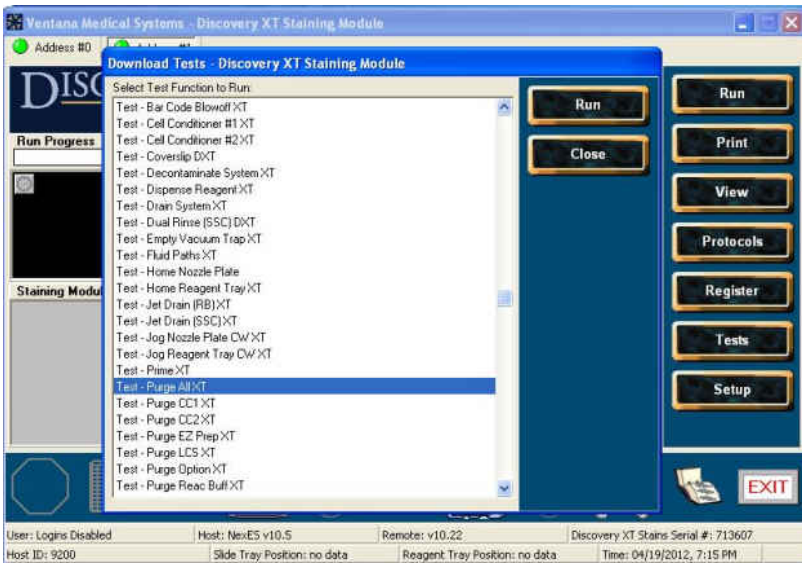
1. Remove all the bulk reagent containers from the instrument.
2. Connect the yellow tubing manifold usually used for quarterly decontamination to the instrument.
3. Place the open end of the tubing manifold into a bottle containing 4 L of distilled water. Ensure that the tubing end touches the bottom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

Note: Refill the bottle with distilled water as needed.

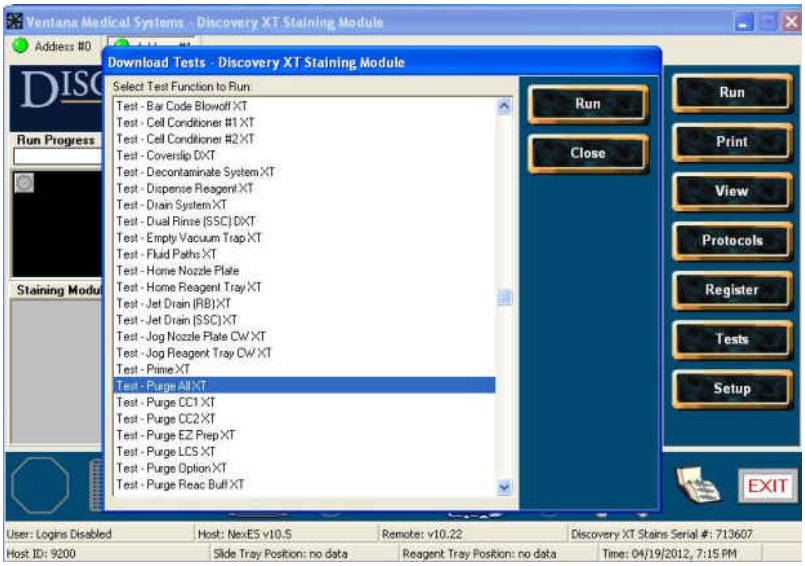
4. Click **Test** on the main screen, and then select **Function Test**:



5. Double click on **Test - Purge All XT**:



6. You will hear a warning sound and see an error message stating that the pressue is too low. This is normal. Click on **Sign off** to turn off the warning sound.
7. Repeat Step 5 four more times.
8. Click **Test** on the main screen, and then select **Function Test**:

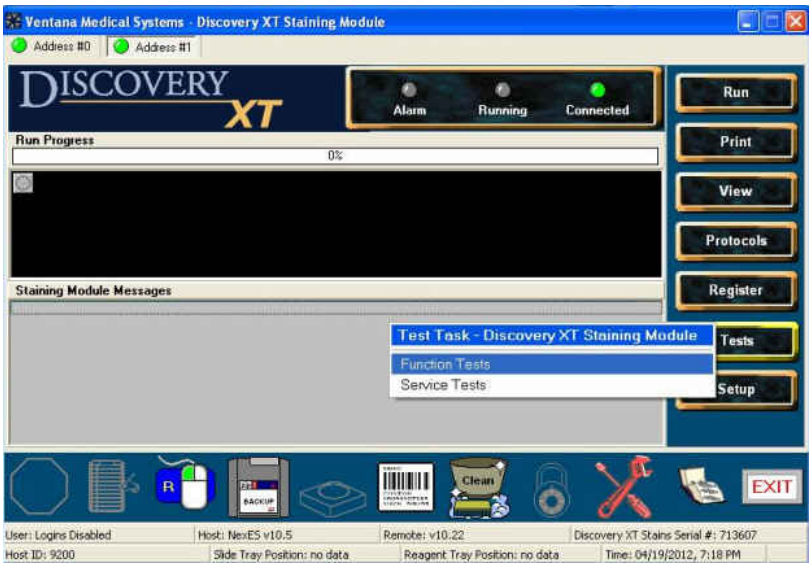


9. Double click on **Test - Prime XT**.
10. Repeat Step 9 four more times.

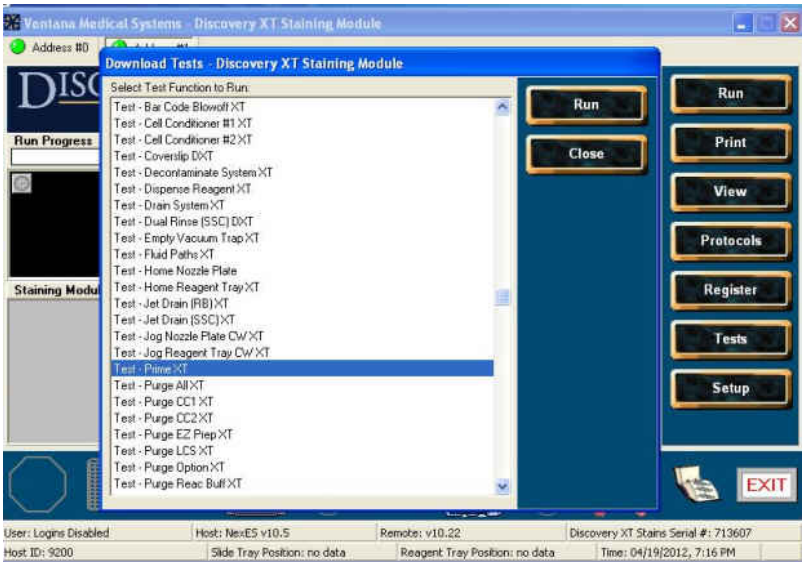
Purge and prime the system with fresh reagents

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 **Test** on the main screen, and then select **Function Test**:



3. Double click on **Test - Purge All XT**:



4. Repeat Step 3.
5. Click **Test** on the main screen, and then select **Function Test**.
6. Double click on **Test - Prime XT**.
7. Repeat Step 6.

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_support.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 ADC General Terms and Conditions of Sale found on the ADC website at **www.acdbio.com/product_support.html**. If you have any questions, please contact Advanced Cell Diagnostics at **www.acdbio.com/product_support.html**.

Headquarters
3960 Point Eden Way | Hayward, CA 94545 | Phone 1-510-576-8800 | Toll Free 1-877-576-3636

For support, email support@acdbio.com.
www.acdbio.com

ADVANCED CELL DIAGNOSTICS