USER MANUAL

RNAscope® VS Reagent Kit (BROWN) User Manual

For Use with Ventana DISCOVERY™ XT System

Catalog Number 320493

Complete guide: From Sample Pretreatment to RNAscope® Detection.

For Molecular Biology Applications, not intended for diagnosis.

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

Contents

1 Chapter 1. Product Information

Before using this product, read and understand the information in **Appendix C. Safety** on page 41 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 15, for use with most sample types.
- **Chapter 5. Fully Automated RNAscope® VS Assay** starting on page 25, for use with brain and spinal cord sections.

Product description

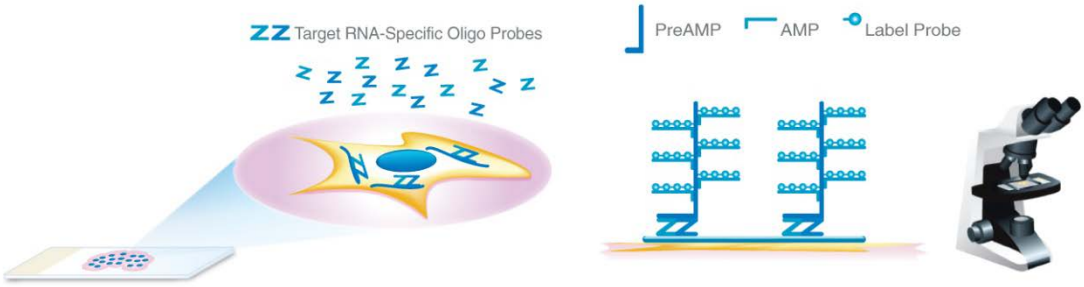
Background

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/target-probes/controls-housekeeping** to order appropriate Control Probes.

Each probe is sufficient for staining ~30 standard slides. The probes have a shelf life of 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® VS Reagents

The RNAscope® VS Reagents consist of the RNAscope® VS Reagent Kit, the RNAscope® VS Accessory Kit, and the RNAscope® VS Offline CC Kit. 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 (Cat. no. 320600)			
☑	**Reagent**	**Quantity**	**Storage**
	VS Pretreat A — Ready-To-Use (RTU) Protease A	14 mL x 1 bottle	4°C
	VS Pretreat B — RTU Protease B	14 mL x 1 bottle	4°C
	VS Amp 1 — RTU	14 mL x 1 bottle	4°C
	VS Amp 2 — RTU	14 mL x 1 bottle	4°C
	VS Amp 3 — RTU	14 mL x 1 bottle	4°C
	VS Amp 4 — RTU	14 mL x 1 bottle	4°C
	VS Amp 5–Brown — RTU	14 mL x 1 bottle	4°C
	VS Amp 6 –Brown — RTU	14 mL x 1 bottle	4°C
	VS Amp 7 — RTU	14 mL x 1 bottle	4°C
RNAscope® VS Accessory Kit (Cat. no. 320630)			
☑	**Reagent**	**Quantity**	**Storage**
	VS Hematoxylin — RTU	7 mL x 1 bottle	4°C
	VS Blueing Reagent — RTU	7 mL x 1 bottle	4°C
RNAscope® VS Offline CC Kit (Cat. no. 320043)			
☑	**Reagent**	**Quantity**	**Storage**
	10X Pretreat 2	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 DAB Detection Kit (Cat. no. 760-224)		
☑	**Component**	**Storage**
	mRNA Inhibitor-prefilled	4°C
	mRNA DAB dispenser-prefilled	4°C
	mRNA H_2O_2 dispenser-prefilled	4°C
	mRNA Copper 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 blueing reagent	Room temperature (20–25°C)
mRNA DAB, Amplification & Pretreatment Kit (Cat. No. 760-225)		
☑	**Component**	**Storage**
	mRNA DAB Detection Kit (Cat. No. 760-224)	4°C
	mRNA Pretreatment Kit (Cat. no. 760-223)	Room temperature (20–25°C)
	mRNA Probe Amplification Kit (Cat. no. 760-222)	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
	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	American Master Tech Scientific/MLS*	ALREAGAL
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24
	Tissue-Tek® Staining Dish (6 required)	American Master Tech Scientific/MLS	LWT4457EA
	Tissue-Tek® Clearing Agent Dish, xylene resistant (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/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 13 for preparation of FFPE slides. For preparation of other sample types, contact **support@acdbio.com**.
- Optimize pretreatment conditions for your sample. Refer to **Appendix A. Recommended Guidelines** on page 35 for pretreatment conditions.
- 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 41 for more information.

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 suboptimally treated samples, follow the recommended guidelines in **Appendix A. Recommended Guidelines** on page 35.

Prepare FFPE sections

Materials required

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

Fix the sample

1. Remove sample and **CUT 3–4 mm** pieces prior to fixing.

   CAUTION! Handle biological specimens appropriately.

2. Fix sample in **FRESH 10% NBF** for **16–32 HOURS** at **ROOM TEMPERATURE**. Fixation time will vary depending on tissue type.

IMPORTANT! Under-fixation will result in significant signal loss when performing 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 room temperature 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.

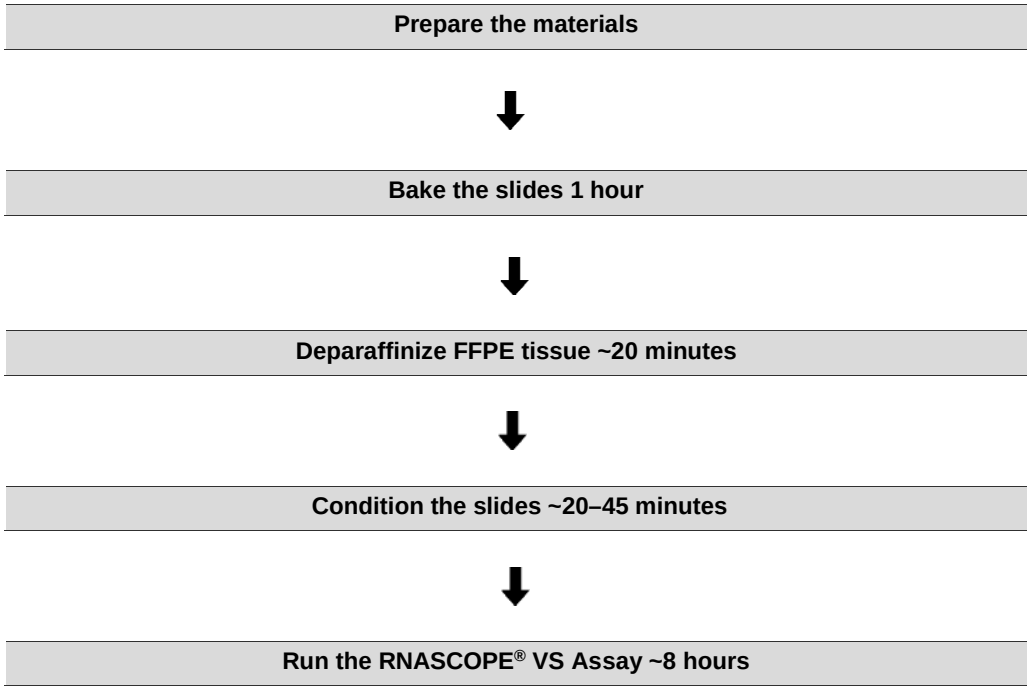
6. **AIR DRY** slides **OVERNIGHT** at **ROOM TEMPERATURE**. Do **NOT** bake slides unless they will be used for RNAscope within 1 week.

OPTIONAL STOPPING POINT. Use sectioned tissue within 3 months. Store sections with dessicants at room temperature.

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® Assay** on page 25.

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 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 • Pretreat 2 • VS Pretreat A • VS Pretreat B • VS Amp 1 • VS Amp 2 • VS Amp 3 • VS Amp 4 • VS Amp 5–Brown • VS Amp 6–Brown • VS Amp 7 • VS Hematoxylin • VS Blueing reagent	• DISCOVERY™ XT — automated slide stainer • EZPrep Buffer • LCS Buffer • RiboWash Buffer • Reaction Buffer • 1X SSC (Option Bottle) • Probe dispensers • mRNA Pretreatment Kit • mRNA Probe Amplification Kit • mRNA DAB Detection Kit • Generic dispensers	• Distilled water • Glass beaker (1 or 2 L) • Paper towels or absorbent paper • Aluminum foil • Thermometer • Forceps, large • Hot plate • Dawn detergent • Fume hood • Xylene • 100% ethanol • Tissue-Tek® Staining Dish (6) • Tissue-Tek® Clearing Agent Dish, xylene-resistant (4) • Tissue-Tek® Vertical 24 Slide Rack • Cytoseal XYL xylene-based • Cover Glass, 24 mm x 50 mm • Laboratory tissue paper

Prepare the instrument

- If the instrument has not been used for ≥1 week, follow the guidelines for instrument maintenance in **Appendix B** on page 37.
- 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 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, 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 room temperature.

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% ETHANOL**.

Note: Ensure all containers remain covered.

Prepare dehydrating reagents

IMPORTANT! Do not reuse deparaffinization reagents.

- In a fume hood, fill **TWO** Clearing Agent **DISHES** with **~200 mL FRESH XYLENE**.
- In a fume hood, fill **THREE** Staining **DISHES** with **~200 mL FRESH 100% ETHANOL**.

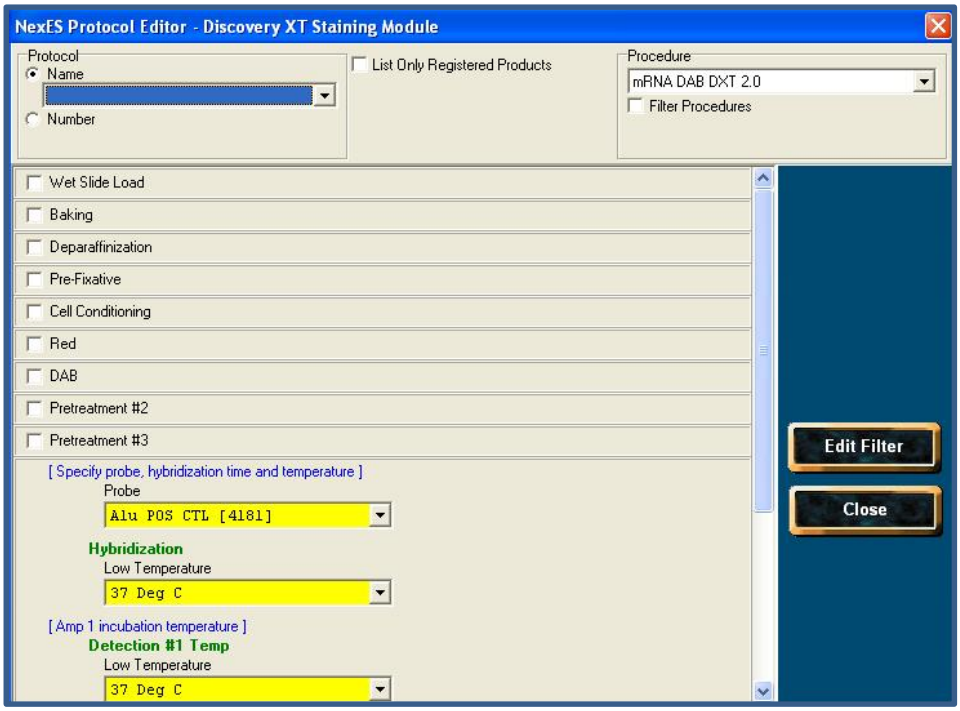
Note: Ensure all containers remain covered.

Prepare 1X Pretreat 2

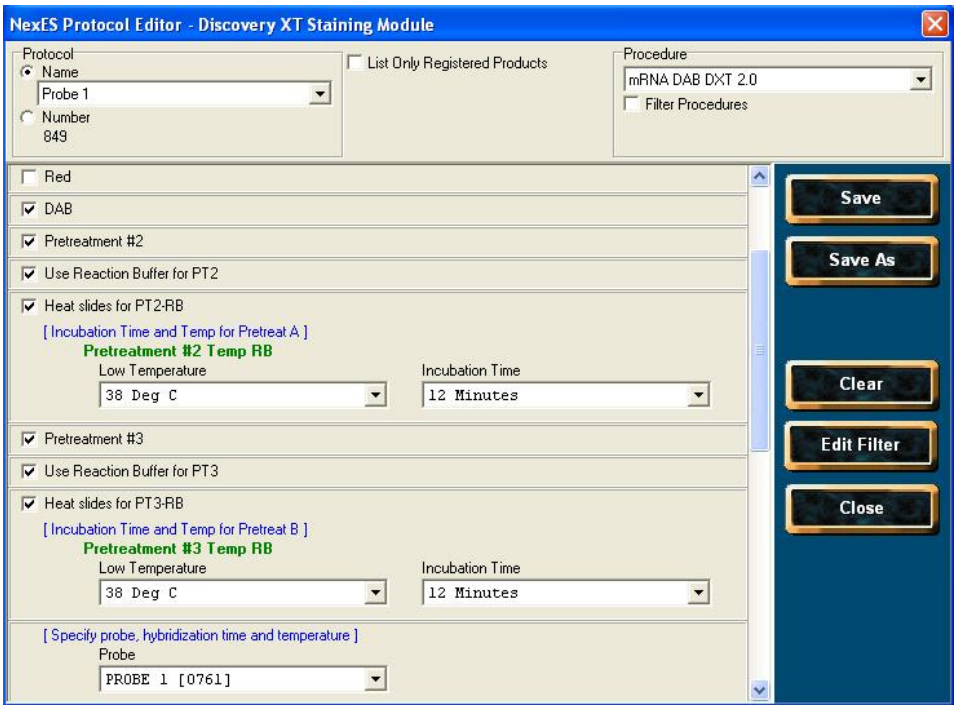
1. Prepare **1X PRETREAT 2** while FFPE slides are baking at 60°C, or the following day if you choose the optional stopping point on page 14. 1X Pretreat 2 is used in manual cell conditioning (CC).
2. Prepare **700 mL** of **FRESH 1X PRETREAT 2** by adding 630 mL distilled water to 1 bottle (70 mL) 10X Pretreat 2 solution in the beaker.
3. **MIX WELL** and cover the beaker with foil.

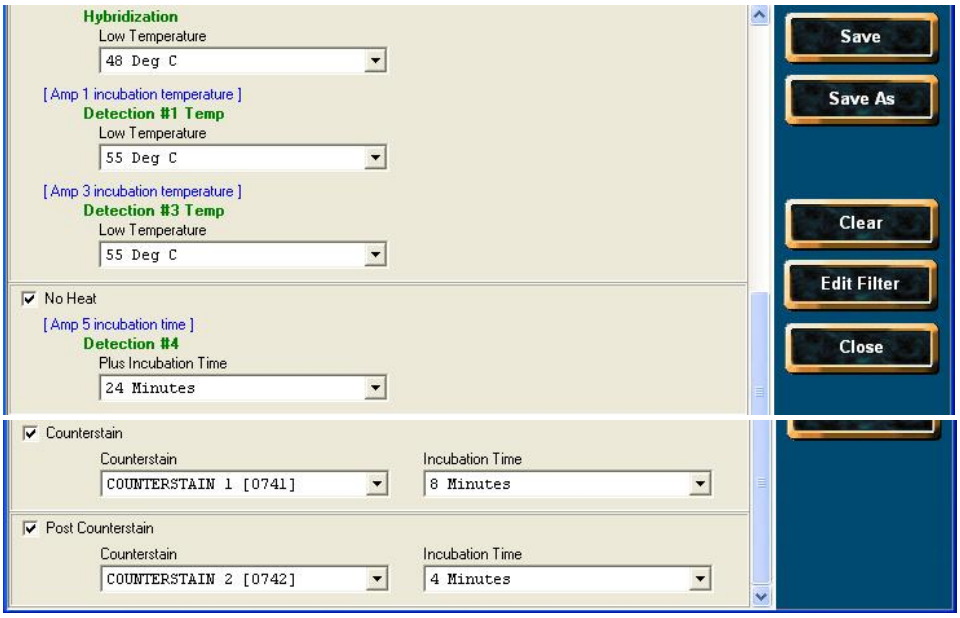
Create an instrument protocol

1. **OPEN** the NexES **SOFTWARE** and **CLICK** on the **"PROTOCOL" BUTTON**.
2. **CLICK** on "**CREATE/EDIT PROTOCOL**", go to the "Procedure" drop down menu and **SELECT** "**mRNA DAB DXT 2.0**". 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:





4. **SELECT** the appropriate assay **CONDITIONS** from the drop down menus according to the following table:

Tissue type	Pretreatment #2	Pretreatment #3
Brain and spinal cord	12 minutes	12 minutes
Breast cancer	12 minutes	12 minutes
Cell lines	12 minutes	12 minutes
Colon	12 minutes	12 minutes
GI tract	12 minutes	12 minutes
Head and neck cancer	12 minutes	12 minutes
Heart	12 minutes	12 minutes
Kidney	12 minutes	12 minutes
Liver	12 minutes	12 minutes
Lung	12 minutes	12 minutes
Lymphoma	8 minutes	8 minutes
Placenta	12 minutes	12 minutes
Prostate	12 minutes	12 minutes
Skin	12 minutes	12 minutes
Stomach	12 minutes	12 minutes
Thymus	8 minutes	8 minutes
Tonsil	8 minutes	8 minutes
Xenografts derived from cell lines	12 minutes	12 minutes
Xenografts derived from primary tumor	12 minutes	12 minutes

5. **CLICK** "**SAVE AS**", then **SELECT** a protocol **NUMBER** from the drop down menu and **CHOOSE** a protocol **NAME** for each probe. **CLICK** "**SAVE**".

6. **CLICK** "**CLOSE**" to go back to the main screen.
7. 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** 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
• Pretreat 2	• Drying oven • FFPE slides • Tissue-Tek® Vertical 24 Slide Rack • Distilled water • Fume hood • Xylene • 100% ethanol • Tissue-Tek® Clearing Agent Dish (2) • Tissue-Tek® Staining Dish (2) • Glass beaker (1 or 2 L) • Paper towel or absorbent paper • Hot plate • Aluminum foil • Forceps, large • Thermometer

Bake the slides

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

STOPPING POINT Use immediately or store at room temperature 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, **Condition the slides** on page 21, and **Run the RNAscope® VS Assay** on page 25. See **Prepare the materials** on page 16.

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 17–19.

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 MINUTES** at **ROOM TEMPERATURE**. 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% ethanol.
6. Incubate the slides in **100% ETHANOL** for **1 MINUTE** at **ROOM TEMPERATURE** with agitation.
7. **REPEAT STEP 6** with **FRESH 100% ETHANOL**.
8. Remove the slides from the rack, and place on absorbent paper with the section face-up. **AIR DRY** for **5 MINUTES** at **ROOM TEMPERATURE**.
9. While slides are drying, **PLACE** printed **LABELS** on the slides.

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

10. **INSERT** the **SLIDES** into a Tissue-Tek® Slide **RACK** and proceed to **CONDITION THE SLIDES.**

Condition the slides

1. 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 minutes before use.

2. **HEAT 1X PRETREAT 2** to 95–104°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 minutes.
   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.
3. 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 minutes
Breast cancer	15 minutes
Cell lines	10 minutes
Colon	15 minutes
GI tract	15 minutes
Head and neck cancer	15 minutes

Tissue type	Treatment time
Heart	15 minutes
Kidney	15 minutes
Liver	30 minutes
Lung	15 minutes
Lymphoma	10 minutes
Placenta	15 minutes
Prostate	15 minutes
Skin	15 minutes
Stomach	15 minutes
Thymus	10 minutes
Tonsil	10 minutes
Xenografts derived from cell lines	7 minutes
Xenografts derived from primary tumor	15 minutes

* This procedure can be automated for these tissue types. See page 25.

4. 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.
5. **WASH** slides **3–5 TIMES** by moving the Tissue-Tek® Slide Rack up and down in the **DISTILLED WATER**.
6. **REPEAT STEP 4** with **FRESH DISTILLED WATER**.
7. Proceed to **LOADING THE SLIDES**.

Run the RNAscope® VS Assay

Materials required

- Prepared instrument reagents
- Distilled water
- Paper towels or absorbent paper
- Dawn detergent
- Fume hood
- Xylene
- 100% ethanol
- Tissue-Tek® Staining Dish (3)
- Tissue-Tek® Clearing Agent Dish, xylene-resistant (2)
- Tissue-Tek® Vertical 24 Slide Rack
- Cytoseal XYL xylene-based
- 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. Following the "**CONDITION THE SLIDES**" step on page 21, **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.
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 DAB DETECTION KIT**. Refer to the instructions provided by Ventana™ Medical Systems.
5. **LOAD** the reagent **RACKS** onto the reagent **CAROUSEL**.

Start the run

1. **CLICK** the "**RUN**" button
2. 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**.
4. **CLICK** the "**RUNNING**" button. Automated assay will finish in ~8 hours.

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. Add **200 mL** diluted **DETERGENT** to a Tissue-Tek® Staining **DISH**.
2. **SUBMERGE** a Tissue-Tek® Slide **RACK** into the Staining **DISH**.
3. **OPEN** the instrument **SLIDE TRAY** and **UNLOAD SLIDES**.
4. **DECANT SOLUTION** on the slides into the slide tray, then *immediately* **LOAD SLIDES** into the Tissue-Tek® Slide **RACK** submerged in detergent.
5. **RINSE OIL** off the slides by moving the slide rack up and down in the dish **10 TIMES**.
6. **REPLACE** the detergent with **DISTILLED WATER** and **RINSE SLIDES** by moving the slide rack up and down **10 TIMES**.
7. **REPEAT STEP 6 3–5 TIMES**.

Dehydrate the slides

1. Move the Tissue-Tek® Slide rack into the first Staining Dish containing **100% ETHANOL** in the fume hood for **2 MINUTES**. Agitate the slides by occasionally lifting the slide rack up and down.
2. Move the Tissue-Tek® Slide rack into the second Staining Dish containing **100% ETHANOL** for **2 MINUTES** with occasional agitation.
3. Move the Tissue-Tek® Slide rack into the third Staining Dish containing **100% ETHANOL** for **2 MINUTES** with occasional agitation.
4. Move the Tissue-Tek® Slide rack into the Staining Dish containing **XYLENE** for **1 MINUTE** with occasional agitation.
5. Move the Tissue-Tek® Slide rack **RACK** into the Staining Dish containing **XYLENE** for **1 MINUTE** with occasional agitation.

Mount the samples

1. Remove the slides from the Tissue-Tek® Slide Rack and lay flat with the sections facing up in the fume hood.
2. Mount one slide at a time by adding **1–2 DROPS** of **CYTOSEAL** or other xylene-based mounting medium to each slide and carefully placing a **24 mm x 50 mm COVERSLIP** over the section. Avoid trapping air bubbles.
3. **AIR DRY** slides for **5 MINUTES**.
4. Proceed to **Chapter 6. Evaluate the results** on page 33.

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! 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.

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
• VS Target Probe • VS Positive Control Probe • VS Negative Control Probe • Pretreat 2 • VS Pretreat A • VS Pretreat B • VS Amp 1 • VS Amp 2 • VS Amp 3 • VS Amp 4 • VS Amp 5–Brown • VS Amp 6–Brown • VS Amp 7 • VS Hematoxylin • VS Blueing reagent	• DISCOVERY™ XT — automated slide stainer • EZPrep Buffer • LCS Buffer • RiboWash Buffer • RiboCC Buffer — used in the fully automated assay (CC2) • Reaction Buffer • 1X SSC (Option Bottle) • Probe dispensers • mRNA Pretreatment Kit • mRNA Probe Amplification Kit • mRNA DAB Detection Kit • Generic dispensers	• Distilled water • Paper towels or absorbent paper • Dawn detergent • Fume hood • Xylene • 100% ethanol • Tissue-Tek® Staining Dish (3) • Tissue-Tek® Clearing Agent Dish, xylene-resistant (2) • Tissue-Tek® Vertical 24 Slide Rack • Cytoseal XYL xylene-based • Cover Glass, 24 mm x 50 mm • Laboratory tissue paper

Prepare the instrument

- If the instrument has not been used for ≥1 week, follow the guidelines for instrument maintenance in **Appendix B** on page 37.
- 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**.
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, 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**:
   a. Add 4–5 mL Dawn detergent to 200 mL distilled water in a container with a cap.
   b. Mix well by inverting the container 4–5 times.
   c. Add diluted detergent to a Tissue-Tek® Staining Dish.

Note: Store diluted detergent at room temperature.

Prepare dehydrating reagents

IMPORTANT! Do not reuse deparaffinization reagents.

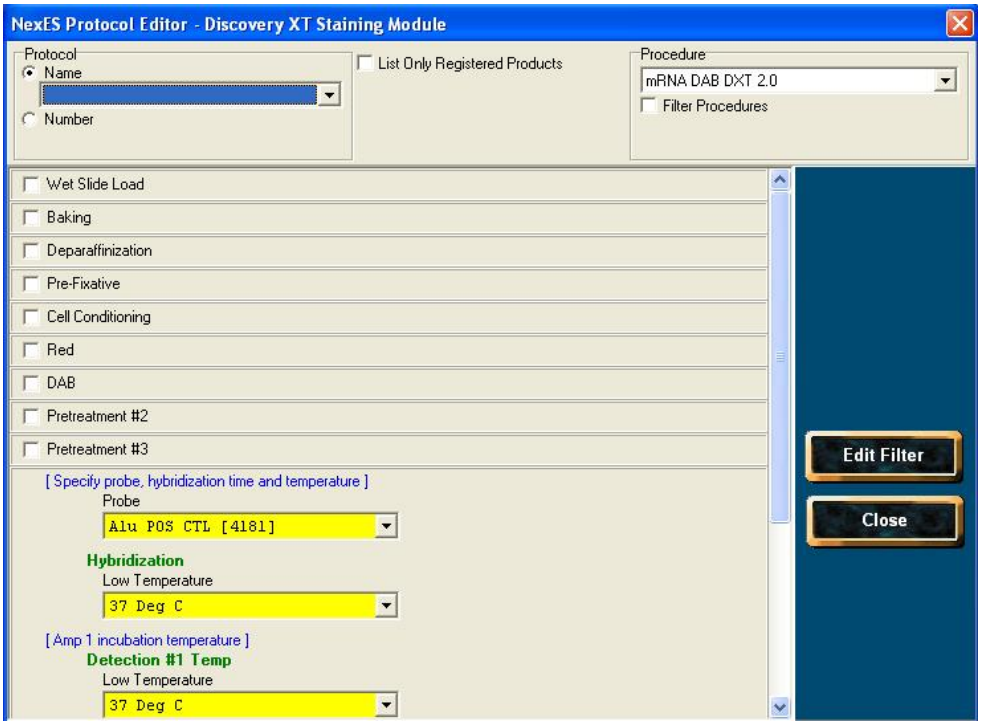
- In a fume hood, fill **TWO** Clearing Agent **DISHES** with **~200 mL FRESH XYLENE**.
- In a fume hood, fill **THREE** Staining **DISHES** with **~200 mL FRESH 100% ETHANOL**.

Note: Ensure all containers remain covered.

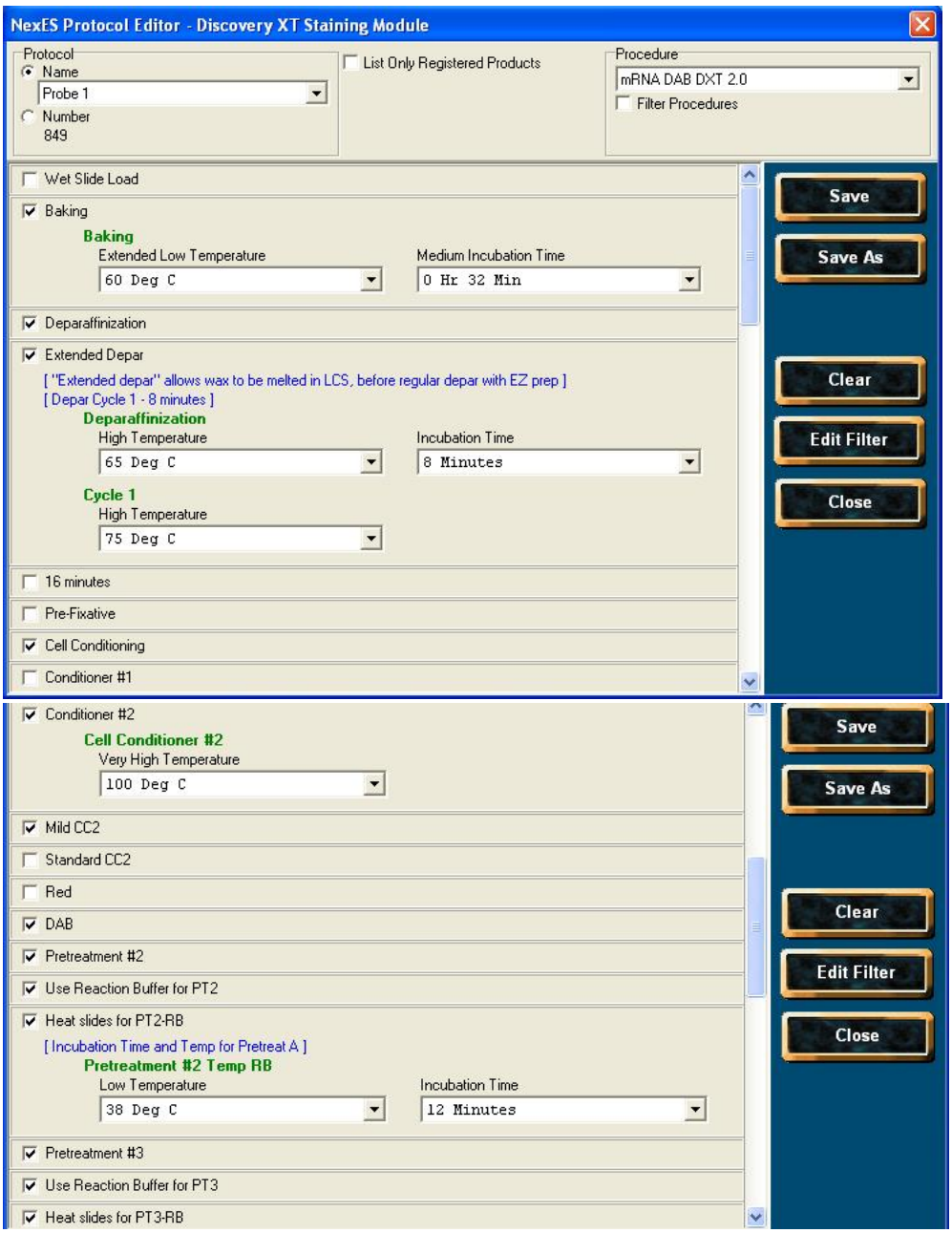
Create an instrument protocol

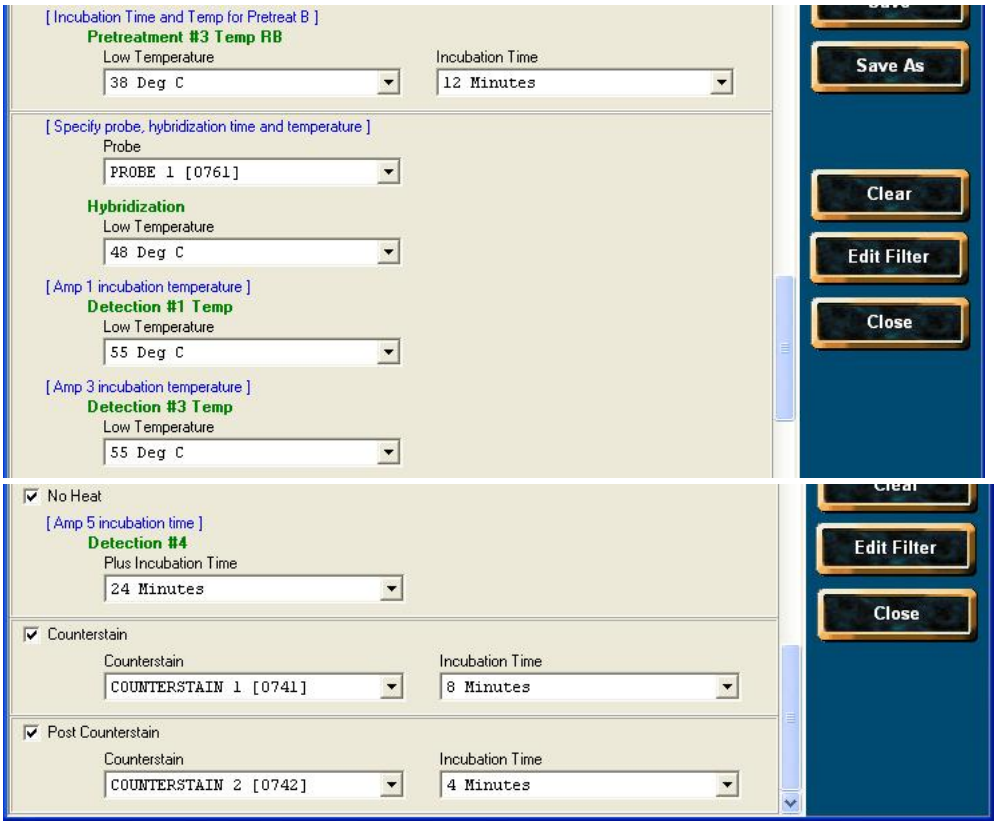
1. **OPEN** the NexES **SOFTWARE** and **CLICK** on the "**PROTOCOL" BUTTON**.
2. **CLICK** on "**CREATE/EDIT PROTOCOL**", go to the "Procedure" drop down menu and **SELECT** "**mRNA DAB DXT 2.0**".

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:





4. **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 minutes	12 minutes

5. **CLICK** "**SAVE AS**", then **SELECT** a protocol **NUMBER** from the drop down menu and **CHOOSE** a protocol **NAME** for each probe. **CLICK** "**SAVE**".
6. **CLICK** "**CLOSE**" to go back to the main screen.
7. 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** 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**.
5. **PROCEED** to **LOADING THE SLIDES** on page 30.

Run the RNAscope® VS Assay

Materials required

- Prepared instrument reagents
- Distilled water
- Paper towels or absorbent paper
- Dawn detergent
- Fume hood
- Xylene
- 100% ethanol
- Tissue-Tek® Staining Dish (3)
- Tissue-Tek® Clearing Agent Dish, xylene-resistant (2)
- Tissue-Tek® Vertical 24 Slide Rack
- Cytoseal XYL xylene-based
- 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.
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 DAB DETECTION KIT**. Refer to the instructions provided by Ventana™ Medical Systems.
5. **LOAD** the reagent **RACKS** onto the reagent **CAROUSEL**.

Start the run

1. **CLICK** the "**RUN**" button.
2. 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**.
4. **CLICK** the "**RUNNING**" button. Automated assay will finish in ~8 hours.

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. Add **200 mL** diluted **DETERGENT** to a Tissue-Tek® Staining **DISH**.
2. **SUBMERGE** a Tissue-Tek® Slide **RACK** into the Staining **DISH**.
3. **OPEN** the instrument **SLIDE TRAY** and **UNLOAD SLIDES**.
4. **DECANT SOLUTION** on the slides into the slide tray, then *immediately* **LOAD SLIDES** into the Tissue-Tek® Slide **RACK** submerged in detergent.
5. **RINSE OIL** off the slides by moving the slide rack up and down in the dish **10 TIMES**.
6. **REPLACE** the detergent with **DISTILLED WATER** and **RINSE SLIDES** by moving the slide rack up and down **10 TIMES**.
7. **REPEAT STEP 6 3–5 TIMES**.

Dehydrate the slides

1. Move the Tissue-Tek® Slide rack into the first Staining Dish containing **100% ETHANOL** in the fume hood for **2 MINUTES**. Agitate the slides by occasionally lifting the slide rack up and down.
2. Move the Tissue-Tek® Slide rack into the second Staining Dish containing **100% ETHANOL** for **2 MINUTES** with occasional agitation.
3. Move the Tissue-Tek® Slide rack into the third Staining Dish containing **100% ETHANOL** for **2 MINUTES** with occasional agitation.
4. Move the Tissue-Tek® Slide rack into the Staining Dish containing **XYLENE** for **1 MINUTE** with occasional agitation.
5. Move the Tissue-Tek® Slide rack into the Staining Dish containing **XYLENE** for **1 MINUTE** with occasional agitation.

Mount the samples

1. Remove the slides from the Tissue-Tek® Slide Rack and lay flat with the sections facing up in the fume hood.
2. Mount one slide at a time by adding **1–2 DROPS** of **CYTOSEAL** or other xylene-based mounting medium to each slide and carefully placing a 24 mm x 50 mm **COVERSLIP** over the section. Avoid trapping air bubbles.
3. **AIR DRY** slides for **5 MINUTES**.
4. Proceed to **Chapter 6. Evaluate the Results** on page 33.

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 punctuate dots within cell nuclei at 20–40X magnification.
- Assess negative control background. One dot to every 10 cells displaying background DAB 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 to every ten cells (40X magnification)
1	1–3 dots/cell (visible at 20–40X magnification)
2	4–10 dots/cell. 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**.

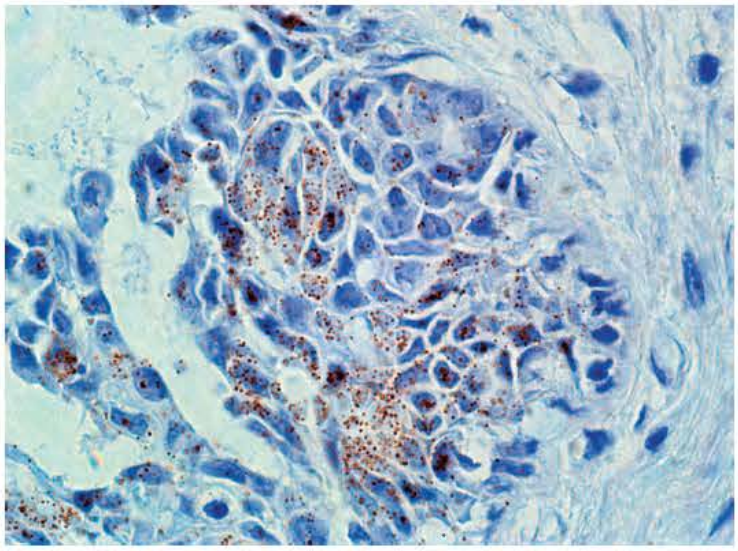
Troubleshooting

For troubleshooting information, refer to the *Troubleshooting User Manual* (Cat. no. 320519) available at: **www.acdbio.com/support/technical-doc**

Control example

Figure 2 is an example of staining in cervical cancer FFPE tissue.

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, Pretreatment #2, and Pretreatment #3 conditions for:

- Any new or previously untested FFPE tissue types.
- Suboptimally prepared samples.

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 minutes	12 minutes	12 minutes
2	Negative control	10 minutes	12 minutes	12 minutes
3	Positive control	15 minutes	12 minutes	12 minutes
4	Negative control	15 minutes	12 minutes	12 minutes
5	Positive control	30 minutes	12 minutes	12 minutes
6	Negative control	30 minutes	12 minutes	12 minutes

2. Evaluate staining and tissue morphology as in **Chapter 6. Evaluate the results** on page 33.
3. Use the optimized pretreatment conditions to run the assay with the target probe.
4. If none of the conditions are satisfactory, refer to the *Troubleshooting User Manual* (Cat. no. 320519) available at: **www.acdbio.com/support/technical-doc.**

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.

RiboCC Buffer is used specifically for automated cell conditioning (CC2), while the RiboWash Buffer is used only during the semi-automated RNAscope® VS Assay. Check the RiboCC and RiboWash bulk containers for any visible signs of bacterial growth. Disinfect the containers with Lysol if necessary, rinse well with distilled water, and refill with fresh, unexpired buffers.

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

Flush the instrument

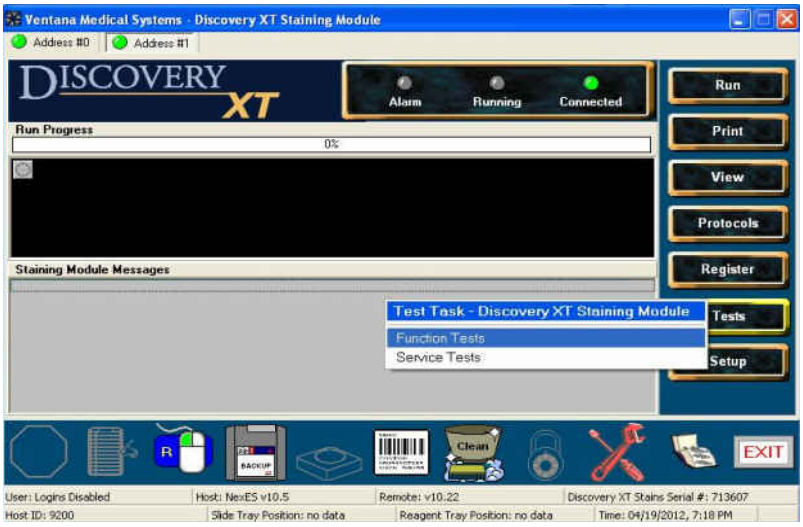
Purge and prime the system with clean distilled water

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

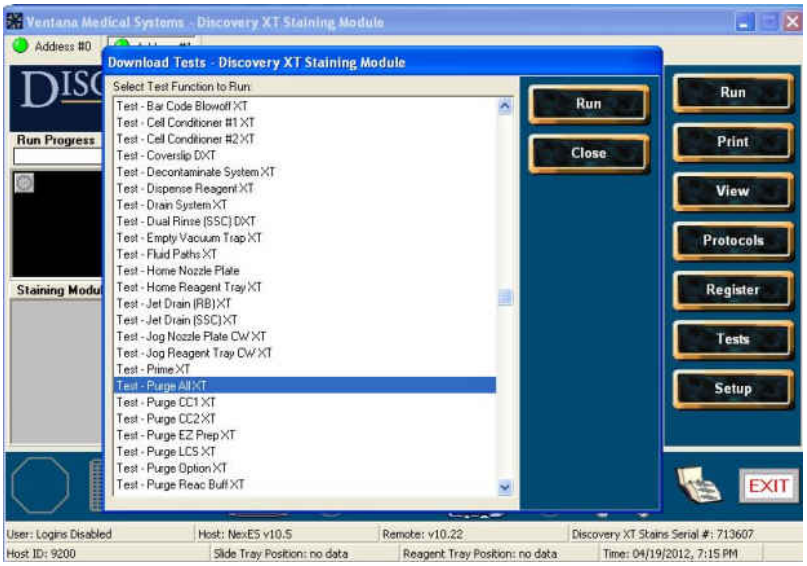
1. Remove all the bulk reagent containers from the instrument.
2. Connect the yellow tubing manifold usually used for quarterly decontamination to the instrument.
3. Place the open end of the tubing manifold into a bottle containing 4 L of distilled water. Ensure that the tubing end touches the bottom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

Note: Refill the bottle with distilled water as needed.

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

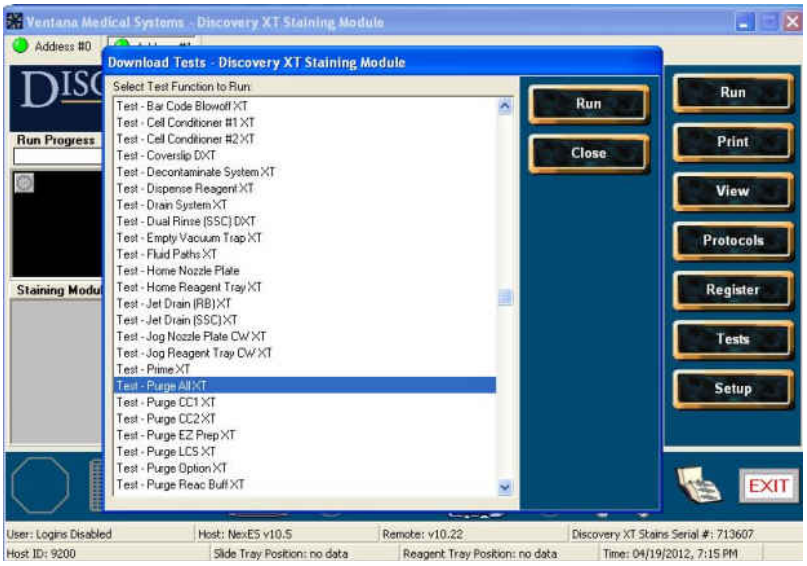


5. Double click on “Purge All **XT**”:



Note: 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.

6. Repeat **STEP 5** four more times.
7. Click “Test” on the main screen, and then select “Function Test”:

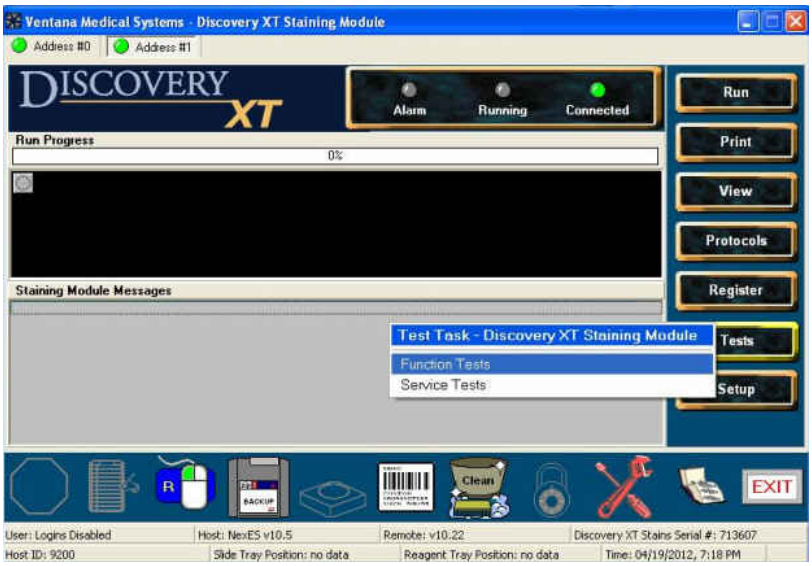


8. Double click on “Prime **XT**”.
9. Repeat **STEP 8** four more times.

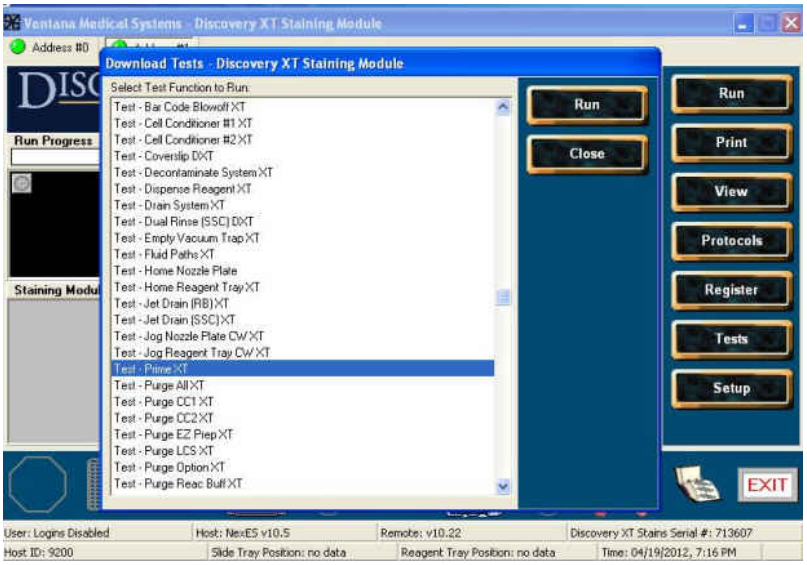
Purge and prime the system with fresh reagents

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 "Purge **All XT**":



4. Repeat **STEP 3**.
5. Click "Test" on the main screen, and then select "Function Test".
6. Double click on "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/support/technical-doc/category/msds**. 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/support**

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/tos/terms-and-conditions-of-sale**. If you have any questions, please contact Advanced Cell Diagnostics at **www.acdbio.com/support**.

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, INC.