USER MANUAL

RNAscope™ VS Universal HRP Assay

For VENTANA® DISCOVERY® ULTRA System

Document Number UM 323200

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

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Revision Updates

Revision	Effective Date	Change(s)	Location of change
D	7/10/2026	Inclusion of revision updates table	Pg 2
		Additional post-processing and mounting option	Pg 30-31, 55-56
		Addition of new “gentle” pretreatment condition	Pg 20, 57
		Modification of recommended mRNA purple detection incubation time	Pg 27, 38

Contents

1 Chapter 1. Product Information

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

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

About this guide

This user manual provides several versions of the RNAscope VS Universal HRP Assay:

- Chapter 5. Automated RNAscope VS Universal HRP Assay
- Chapter 6. Automated smRNA (miRNA) RNAscope VS Universal HRP Assay
- Chapter 7. Protease-Free, Sequential RNA-Protein Co-Detection
- Appendix A. Semi-automated RNAscope VS Universal HRP Assay

Product description

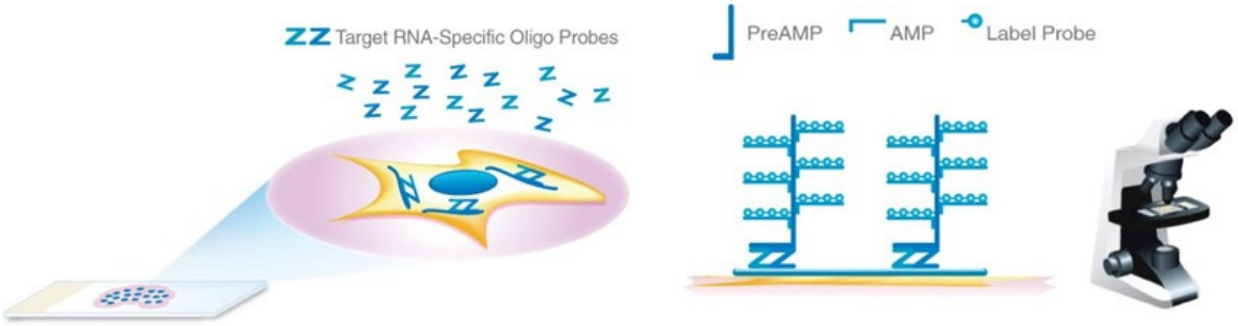
Background

The RNAscope VS Universal Assays uses a novel and proprietary method of in situ hybridization (ISH) to visualize single RNA molecules per cell in formalin-fixed, paraffin-embedded (FFPE) tissue mounted on slides. The assays are based on Advanced Cell Diagnostic's patented signal amplification and background suppression technology and can detect RNA molecules in archival samples and partially degraded specimens. The RNAscope VS Universal Assay allows users to automate the highly sensitive RNAscope Assay using the Ventana DISCOVERY ULTRA System.

Overview

Figure 1 illustrates RNAscope VS Universal HRP Assay procedure, which can be completed on the instrument in ~11 hours. Starting with properly prepared samples, sections are first pretreated, and then RNA-specific probes are hybridized to target RNA. The signal is amplified using multiple steps, followed by hybridization to horseradish peroxidase (HRP)- labeled probes and detection using a 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	4. Image
Start with properly prepared sections and pretreat to allow access to target RNA.	Hybridize gene-specific probe pairs to the target mRNA.	Probes are hybridized to a cascade of signal amplification molecules, culminating in binding of HRP-labeled probes. Add desired chromogen substrate to detect target RNA.	Visualize target RNA using a standard bright field microscope.

Kit contents and storage

The RNAscope VS Universal HRP Assay requires the RNAscope 2.5 VS Probes and the RNAscope VS Universal Reagents, available from Advanced Cell Diagnostics.

RNAscope VS Probes

The RNAscope 2.5 VS Probes consist of the user-specified Target Probe and the Positive and Negative Control Probes. Visit **https://www.bio-techne.com/reagents/rnascope-ish-technology/probes** to find a gene-specific Target Probe or appropriate Control Probes. Each probe is sufficient for staining ~30 standard slides. The probes have a shelf life of two years from the manufacturing date when stored as indicated in the following table:

Target Probes					
☑	**Reagent**	**Cat. No.**	**Content**	**Quantity**	**Storage**
	RNAscope 2.5 VS Target Probe *([species] – [gene]*	Various	Probe targeting specific RNA	7 mL x 1 bottle	2–8°C
Control Probes					
☑	**Reagent**	**Cat. No.**	**Content**	**Quantity**	**Storage**
	RNAscope 2.5 VS Positive Control Probe – *[species]* – PPIB)	Various	Probe targeting common housekeeping gene	7 mL x 1 bottle	2–8°C
	RNAscope 2.5 VS— Negative Control Probe – DapB	312039	Probe targeting bacterial gene dapB	7 mL x 1 bottle	2–8°C

RNAscope VS Control Slides

The RNAscope VS Control Slides (Cat. No. 310045 for Human control slide, HeLa; Cat. No. 310023 for Mouse control slide, 3T3) contain FFPE cell pellets sectioned and mounted on slides. The control slides can be used for assay control with the RNAscope 2.5 VS Positive Control Probe and RNAscope 2.5 VS Negative Control Probe. The slides have the shelf life listed on the labels when stored at 2–8°C with desiccants.

RNAscope VS Universal Reagents

RNAscope VS Universal kits provide enough reagents to stain ~60 standard slides. You will receive four boxes when you order the RNAscope VS Universal HRP Reagent Kit (Cat. No. 323200). RNAscope VS Universal HRP Reagents include:

- RNAscope VS Universal HRP Detection Reagents (Cat. No. 323210)
  - RNAscope VS Universal HRP Standard Reagents
  - RNAscope VS Pro Reagents
- RNAscope VS Universal Sample Prep Reagents v2 (Cat. No. 323740)
- RNAscope VS Accessory Kit (Cat. No. 320630)

The reagents are Ready-To-Use (RTU) and have a shelf life as listed on the label when stored as indicated in the following tables:

RNAscope VS Universal HRP Detection Reagents (Cat. No. 323210)				
RNAscope VS Universal HRP Standard Reagents (Cat. No. 322045)				
☑	Cat. No.	Reagent	Quantity	Storage
	323211	RNAscope VS Universal HRP AMP 1	14 mL x 1 bottle	2–8°C
	323212	RNAscope VS Universal HRP AMP 2	14 mL x 1 bottle	2–8°C
	323213	RNAscope VS Universal HRP AMP 3	14 mL x 1 bottle	2–8°C
	323214	RNAscope VS Universal HRP AMP 4	14 mL x 1 bottle	2–8°C
	323215	RNAscope VS Universal HRP AMP 5	14 mL x 1 bottle	2–8°C
	323216	RNAscope VS Universal HRP AMP 6	14 mL x 1 bottle	2–8°C
	323217	RNAscope VS Universal HRP AMP 7	14 mL x 1 bottle	2–8°C
	323218	RNAscope VS Protease	14 mL x 1 bottle	2–8°C
RNAscope VS Pro Reagents (Cat. No. 322035)				
☑	Cat. No.	Reagent	Quantity	Storage
	322031	RNAscope VS PretreatPro	18 mL x 1 bottle	2–8°C
	322032	RNAscope VS CoDetectPro	18 mL x 1 bottle	2–8°C

RNAscope VS Universal Sample Prep Reagents v2 (323740)				
☑	Cat. No.	Reagent	Quantity	Storage
	323741	RNAscope VS Target Retrieval v2	14 mL x 2 bottles	Room Temp (15–30°C)
	323742	RNAscope VS Dewax	14 mL x 1 bottle	Room Temp (15–30°C)

RNAscope VS Accessory Kit (Cat. No. 320630)				
☑	Cat. No.	Reagent	Quantity	Storage
	320631	RNAscope VS Hematoxylin — RTU	7 mL x 1 bottle	2–8°C
	320632	RNAscope VS Bluing Reagent — RTU	7 mL x 1 bottle	2–8°C

IMPORTANT! Ensure Dewax is in solution and at room temperature before use on the instrument. Warm the solution in your hand or place it at **37°C** for **15 MIN** prior to each use, regardless of previous storage conditions, as it may precipitate during shipment.

IMPORTANT! Do not interchange the reagent components of the reagent kits, even those having the same name.

Required materials from Roche Diagnostics

The RNAscope VS Universal Assay requires specific materials and equipment available only from Roche Tissue Diagnostics. Catalog numbers are valid in the U.S. only. For other regions, please check catalog or ordering numbers with your local lab supplier.

Probe Dispensers (Cat. No. 960-761 to 960-780; for Ordering Code, please contact local Roche rep)		
☑	Component	Storage
	250 Test Probe #1–20 dispensers — fill dispensers with RNAscope 2.5 VS Probes. Use up to 20 probes at one time.	Room Temp (15–30°C)
mRNA Sample Prep Kit (Cat. No. 760-248; Ordering Code 08127166001)		
☑	Component	Storage
	mRNA Target Retrieval dispenser — fill dispenser with RNAscope VS Universal Target Retrieval v2 (Optional: This dispenser is not needed for Protease-Free Workflow)	Room Temp (15–30°C)
	mRNA Dewax — fill dispenser with RNAscope VS Dewax	Room Temp (15–30°C)
	mRNA Protease dispenser — fill dispenser with RNAscope VS Protease (Optional: This dispenser is not needed for Protease-Free Workflow)	Room Temp (15–30°C)
mRNA Probe Amplification Kit (Cat. No. 760-222; Ordering Code 06614337001)		
☑	Component	Storage
	mRNA AMP 1 dispenser — fill dispenser with VS Universal HRP AMP 1	Room Temp (15–30°C)
	mRNA AMP 2 dispenser — fill dispenser with VS Universal HRP AMP 2	Room Temp (15–30°C)
	mRNA AMP 3 dispenser — fill dispenser with VS Universal HRP AMP 3	Room Temp (15–30°C)
	mRNA AMP 4 dispenser — fill dispenser with VS Universal HRP AMP 4	Room Temp (15–30°C)
	mRNA AMP 5 dispenser — fill dispenser with VS Universal HRP AMP 5	Room Temp (15–30°C)
	mRNA AMP 6 dispenser — fill dispenser with VS Universal HRP AMP 6	Room Temp (15–30°C)
	mRNA AMP 7 dispenser — fill dispenser with VS Universal HRP AMP 7	Room Temp (15–30°C)

Additional Dispensers for RNAscope				
☑	Component	Cat. No.	Ordering Code	Fill with:
	Pretreatment dispenser†	960-901 to 960-909	Contact local Roche representative	VS PretreatPro
	Detection dispenser	960-911 to 960-930	Contact local Roche representative	VS smRNA Detection Reagent
	Counterstain 1 dispenser	771-741	05271720001	VS Hematoxylin
	Counterstain 2 dispenser	771-742	05271738001	VS Bluing Reagent

†Use VS PretreatPro for Protease-Free RNAscope. If performing Protease-Free ISH-IHC, choose distinct barcode numbers for VS PretreatPro and VS CoDetectPro. Ensure that staining protocols reflect the barcode numbers assigned to each reagent.

Roche Chromogenic Detection Kits

To run single plex chromogenic *in situ* hybridization on the Ventana DISCOVERY ULTRA System, you will need to order one of the following chromogenic detection kits from Roche Tissue Diagnostics. The RNAscope VS Universal Reagent Kit works with any of the following:

DISCOVERY Inhibitor (Cat. No. 760-4840; Ordering Code 07017944001)*		
☑	Component	Storage
	DISCOVERY Inhibitor dispenser-prefilled	2–8°C
mRNA DAB Detection Kit (Cat. No. 760-224; Ordering Code 06614353001)		
☑	Component	Storage
	mRNA Inhibitor-prefilled	2–8°C
	mRNA DAB dispenser-prefilled	2–8°C
	mRNA H_2O_2 dispenser-prefilled	2–8°C
	mRNA Copper dispenser-prefilled	2–8°C
mRNA Teal Detection Kit (Cat. No.760-256; Ordering Code 08352941001)		
☑	Component	Storage
	mRNA Teal Substrate dispenser-prefilled	2–8°C
	mRNA Teal H_2O_2 dispenser-prefilled	2–8°C
	mRNA Teal Activator dispenser-prefilled	2–8°C
mRNA Green Detection Kit (Cat. No.760-278; Ordering Code 08952612001)		
☑	Component	Storage
	mRNA Green Substrate dispenser-prefilled	2–8°C
	mRNA Green H_2O_2 dispenser-prefilled	2–8°C
	mRNA Green Activator dispenser-prefilled	2–8°C
mRNA Purple Detection Kit (Cat. No. 760-225; Ordering Code 08352909001)		
☑	Component	Storage
	mRNA Purple Substrate dispenser-prefilled	2–8°C
	mRNA Purple H_2O_2 dispenser-prefilled	2–8°C

Generic Dispensers (Cat. No. 771-741; Ordering Code 05271720001, Cat. No. 771-742; Ordering Code 05271738001)		
☑	**Component**	**Storage**
	250 Test Pretreatment dispenser – fill dispenser with VS PretreatPro	Room Temp (15–30°C)
	250 Test Counterstain 1 dispenser — fill dispenser with VS Hematoxylin	Room Temp (15–30°C)
	250 Test Counterstain 2 dispenser — fill dispenser with VS Bluing Reagent	Room Temp (15–30°C)

*If using the mRNA DAB Detection Kit for brown chromogenic detection, the DISCOVERY Inhibitor is not needed

Roche Fluorophore Detection Kits

To run single plex fluorescent *in situ* hybridization on the Ventana DISCOVERY ULTRA System, you will need to order at least one of the following fluorophore kits from Roche Tissue Diagnostics. The RNAscope VS Universal Reagent Kit works with any of the following:

☑	**Fluorophore Kit**	**Cat. No./Ordering Code**
	Cy5 Kit, DISCOVERY	760-238/07551215001
	Rhodamine Kit (RUO), DISCOVERY	760-233/07259883001
	FITC Kit (RUO), DISCOVERY	760-232/07259212001
	FAM Kit, DISCOVERY	760-243/07988150001
	DCC Kit, DISCOVERY	760-240/07988192001
	Rhodamine 6G Kit, DISCOVERY	760-244/07988168001
	Red 610 Kit. DISCOVERY	760-245/07988176001
☑	**Additional Materials for Fluorescent Detection**	Cat. No./Ordering Code
	DISCOVERY Inhibitor dispenser-prefilled	760-4840/07017944001
	DISCOVERY QD DAPI RUO	760-4196/05268826001

Roche materials for Sequential RNA-Protein Co-Detection

Sequential RNA-Protein Co-Detection can be performed with either Roche RTU primary antibody or with your choice of primary antibody concentrate diluted for use on the DISCOVERY ULTRA. For a list of available Roche antibodies and ordering information, please contact your local Roche representative.

The following additional materials from Roche can be used for Sequential RNA-Protein Co-Detection on Ventana DISCOVERY ULTRA.

Reagent Options for Ventana IHC AP Detection				
☑	Component	Cat. No.	Ordering Code	Storage
	DISCOVERY UltraMap anti-Ms Alk Phos*	760-4312	05269687001	2–8°C
	DISCOVERY UltraMap anti-Rb Alk Phos*	760-4314	05269709001	2–8°C
	DISCOVERY Yellow Kit*	760-239	07698445001	2–8°C
	DISCOVERY Red Kit	760-228	07425333001	2–8°C
	DISCOVERY ChromoMap Red Kit*	760-160	05266653001	2–8°C
Reagent Options for Ventana IHC HRP Detection				
☑	Product	Cat. No.	Ordering Code	Storage
	DISCOVERY UltraMap anti-Ms HRP*	760-4313	05269695001	2–8°C
	DISCOVERY UltraMap anti-Rb HRP*	760-4315	05269717001	2–8°C
	DISCOVERY UltraMap anti-Rat HRP*	760-4456	05891884001	2–8°C
	DISCOVERY UltraMap anti-Gt HRP*	760-4648	06607241001	2–8°C
	DISCOVERY Purple Kit*	760-229	07053983001	2–8°C
	DISCOVERY Green HRP Kit †	760-271	08478295001	2–8°C
	DISCOVERY Teal HRP Kit †	760-247	08254338001	2–8°C
	DISCOVERY ChromoMap DAB Kit †	760-159	05266645001	2–8°C
	DISCOVERY DCC Kit †	760-240	07988192001	2–8°C
	DISCOVERY FAM Kit †	760-243	07988150001	2–8°C
	DISCOVERY FITC Kit†	760-232	07259212001	2–8°C
	DISCOVERY Rhodamine Kit †	760-233	07259883001	2–8°C
	DISCOVERY Rhodamine 6G Kit †	760-244	07988168001	2–8°C
	DISCOVERY Red 610 Kit†	760-245	07988176001	2–8°C
	DISCOVERY Cy5 Kit †	760-238	07551215001	2–8°C

* Choose one secondary detection antibody depending on the primary antibody species and desired IHC chromogen
† Choose one IHC chromogen per protein target

Dispensers for Sequential RNA-Protein Co-Detection				
☑	Component	Cat. No.	Ordering Code	Fill with:
	Antibody dispensers (*Optional**)	770-001 to 770-099	Contact local Roche representative	User-sourced Primary Antibody Concentrate diluted in Co-Detection Antibody Diluent
	Pretreatment dispenser†	960-901 to 960-909	Contact local Roche representative	VS PretreatPro
	Pretreatment dispenser†	960-901 to 960-909	Contact local Roche representative	VS CoDetectPro
	Counterstain 1 dispenser	771-741	05271720001	VS Hematoxylin
	Counterstain 2 dispenser	771-742	05271738001	VS Bluing Reagent
	Roche RTU Counterstain Reagents	Various	Contact local Roche representative	Pre-filled
	DISCOVERY Antibody	760-4204	05268869001	Pre-filled

Dispensers for Sequential RNA-Protein Co-Detection				
☑	Component	Cat. No.	Ordering Code	Fill with:
	Block			

*Sequential RNA-Protein Co-Detection can be performed with either Roche RTU primary antibody or your choice of primary antibody.

†Choose distinct barcode numbers for VS PretreatPro and VS CoDetectPro. Ensure that staining protocols reflect the barcode numbers assigned to each reagent.

Equipment and buffers

☑	Component	Cat. No./Ordering Code
	10X DISCOVERY Wash (RUO)	950-510 / 07311079001
	ULTRA LCS (Predilute)	650-210 / 05424534001
	SSC (10X)	950-110 / 5353947001
	Reaction Buffer (10X)	760-107 / 5266262001
	DISCOVERY CC1	950-500 / 6414575001

IMPORTANT! To run the VS Universal assay successfully, use DISCOVERY Wash (950-510). Do not use DISCOVERY EZ Prep. Place 2X SSC (950-110) in the SSC bulk container. You may fill the option bulk container with reaction buffer.

User-supplied materials

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

☑	Description	Supplier	Cat. No.
	SuperFrost Plus Slides (required)	Fisher Scientific	12-550-15
	100% ethanol (EtOH)	American Master Tech Scientific/MLS*	ALREAGAL
	Xylene (or Histo-Clear)	Fisher Scientific/MLS	X3P-1GAL
	10% neutral-buffered formalin (NBF)	MLS	—
	Paraffin wax	MLS	—
	1X PBS	MLS	—
	Microtome	MLS	—
	Drying oven, capable of holding temperature at 60 +/– 1°C	MLS	—
	Cytoseal XYL xylene-based mounting medium	Richard-Allen Scientific/MLS	8312-4
	Prolong Gold Antifade	Thermo Fisher	P36930; P10144; P36934
	Tissue-Tek Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24

☑	Description	Supplier	Cat. No.
	Tissue-Tek Staining Dishes	American Master Tech Scientific/MLS	LWT4457EA
	Tissue-Tek Clearing Agent Dishes, xylene resistant	American Master Tech Scientific/MLS	LWT4456EA
	Cover Glass, 24 x 50 mm	Fisher Scientific/MLS	12-545F
	Distilled water	MLS	—
	Dawn detergent or similar detergent	MLS	—
	Fume hood	MLS	—
	Optional: Glass beaker (1 or 2 L)	MLS	—
	Optional: Hot plate	Fisher Scientific/MLS	11-300-49SHP

* Major Laboratory Supplier in North America. For other regions, please check Catalog Numbers with your local lab supplier.

2 Chapter 2. Before You Begin

Prior to running the RNAscope VS Universal Assay on your samples for the first time, we recommend that you:

- Be familiar with the Ventana DISCOVERY ULTRA system. Refer to the *Ventana DISCOVERY ULTRA System User Manual*.
- Run the assay on FFPE RNAscope VS Control Slides (Cat. No. 310045 for Human control slide, HeLa; Catalog No. 310023 for Mouse control slide, 3T3) using the Positive and Negative Control Probes.

Important procedural guidelines

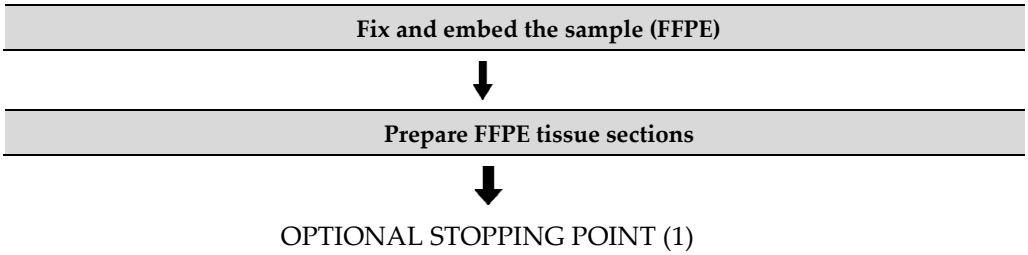
- Start with properly fixed and prepared sections. Refer to **Chapter 3. Prepare and Pretreat Samples**.
- Regularly maintain and clean your automated staining instrument.
- Always run positive and negative control probes on your sample to assess sample RNA quality and background, respectively.
- Do not substitute required materials. Assay has been qualified with these materials only.
- Follow the protocol exactly for the best results. Do not substitute buffers or diluents.
- Store reagents accordingly right after use. To avoid any contamination or evaporation, do not leave reagents open for an extended period.
- Do not let your sections dry out during the procedure unless specified in the protocol.
- Use good laboratory practice and follow all necessary safety procedures. Refer to **Appendix C. Safety** for more information.

3 Chapter 3. Prepare Samples

This chapter describes the formalin-fixed, paraffin-embedded (FFPE) sample preparation method and slide pre-processing procedure. If you have already embedded your FFPE samples, you can proceed to "Prepare FFPE Tissue Sections."
For other sample types and preparation methods, contact **support.acd@bio-techne.com** for the latest protocols and guidelines.

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

Workflow



Materials required

- 10% Neutral Buffered Formalin (NBF)
- 1X PBS
- Paraffin wax
- Tissue-Tek Clearing Agent Dishes
- Tissue-Tek Staining Dishes
- Tissue-Tek Vertical 24 Slide Rack
- 100% alcohol (EtOH)
- Histo-Clear (or xylene)
- Microtome
- Water bath
- SuperFrost Plus slides
- Drying oven
- Fume hood

Fix and embed samples

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 assay.

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

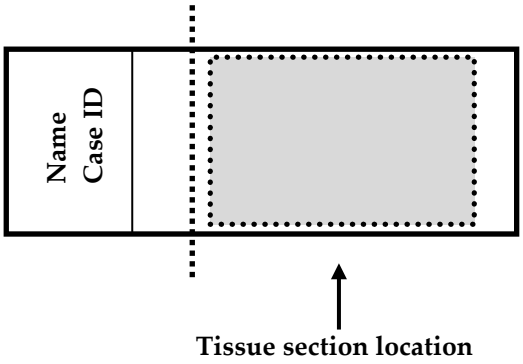
IMPORTANT! Use fresh reagents. Embed samples as quickly as possible to preserve RNA quality.

4. Embed sample in paraffin using standard procedures.

Note: Embedded samples can be stored at room temperature with desiccants. For best preservation of RNA quality over an extended period (>1 yr), storage at **2–8°C** with desiccants is recommended.

Prepare FFPE tissue sections

1. Trim paraffin blocks as needed and cut embedded tissue into 5 +/– 1 µm sections using a microtome.
2. Place paraffin ribbon in a **40–45 °C** water bath and mount the sections on **SUPERFROST PLUS SLIDES.**



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

3. Air dry the slides **OVERNIGHT** at **RT**. Do not bake slides unless they are used within a week.

OPTIONAL STOPPING POINT (1). You can store sections with desiccants at room temperature. For best preservation of RNA quality, storage at **2–8°C** with desiccants is recommended. Use sectioned tissue within 3 months.

4 Chapter 4. Determine Pretreatment Conditions

The following protocols describe sample pretreatment for formalin-fixed, paraffin-embedded (FFPE) samples. For other sample types and preparation methods, contact **support.acd@bio-techne.com** for the latest protocols and guidelines.

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

Pretreat FFPE sections

Pretreatment workflow selection

FFPE samples must be de-crosslinked with a target retrieval step, and then permeabilized for best RNA access. The RNAscope VS Universal RNAscope HRP Assay is compatible with both protease-based and protease-free pretreatment approaches. We recommend using protease-free pretreatment if you may be combining RNA with protein detection downstream.

Target retrieval and permeabilization

Two options are available:

- Protease-free permeabilization is recommended when there is a possibility of combining RNA with protein detection downstream. This method uses an IHC-style cell conditioning for target retrieval with Roche CC1 bulk reagent, paired with permeabilization using ACD's VS PretreatPro reagent. It enables co-detection of RNA and proteins on the same tissue section—previously incompatible with protease treatment—while maintaining the same cell conditioning used in immunohistochemistry (IHC). We recommend considering protease-free pretreatment as the default option.
- Protease-based pretreatment is recommended for experiments staining only RNA, especially when maintaining performance consistent with established in situ hybridization (ISH) protocols is critical. This method uses ACD Target Retrieval v2 and VS Protease. Tissue pretreatment recommendations for the protease-free workflow

Start with the following conditions when tissues are prepared as described in **Chapter 3**. Depending on the tissue type, you may need to adjust the time and temperature of CC1 to maximize the positive RNA control signal while minimizing or eliminating the negative RNA control signal.

Recommended pretreatment selections:

Reagent	Mild	Standard	Extended
CC1 Cell Conditioning – Protease-Free*	32 MIN @95C	64 MIN @95C	92 MIN @95C
VS PretreatPro	32 MIN @40C	32 MIN @40C	32 MIN @40C

*Sample types, such as certain xenografts and cell pellets, might require shorter incubation time. For these tissue types, the CC1 time and or temperature can be reduced. VS PretreatPro incubation time is

adjustable but is rarely needed. Refer to the following table for recommendations for optimization and to **Appendix B** for recommended pretreatment strength by tissue type. If you have a tissue type not listed, contact ACD Support at **support.acd@bio-techne.com.**

Optimization guidance (if needed):

Optimization	
Troubleshooting:	**Recommended Optimization**
Tissue detachment	Reduce CC1 target retrieval time
Low ISH signal	Extend CC1 target retrieval time
High dot background	Reduce CC1 target retrieval time
Low staining patches	Reduce CC1 target retrieval temperature

Tissue pretreatment recommendations using Protease Workflow

Use these conditions as a starting point when tissues are prepared as described in **Chapter 3**. Depending on your tissue type, vary the time and temperature of Target Retrieval v2 (Cell Conditioning) until positive RNA control signal is maximized with minimal/no negative RNA control signal (see **Appendix B** for a list of tissues).

Recommended Pretreatment Selections:

Reagent	Gentle	Mild	Standard	Extended
ACD VS Target Retrieval v2	24 MIN @ 85°C	16 MIN @ 97°C	24 MIN @ 97°C	48 MIN @ 97°C
RNAscope VS Protease	16 MIN @ 37°C	16 MIN @ 37°C	16 MIN @ 37°C	16 MIN @ 37°C

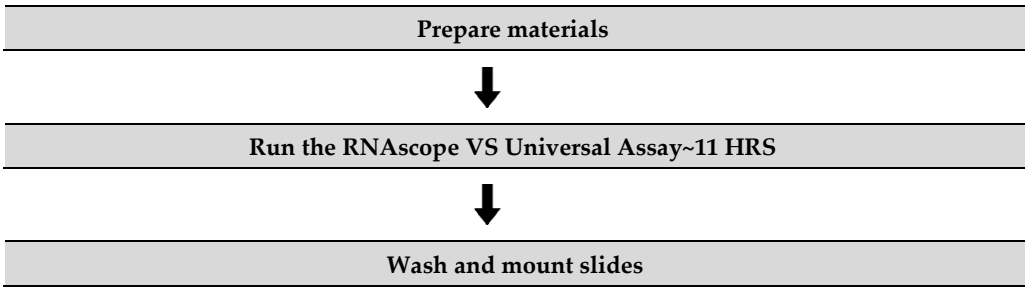
Sample types, such as certain xenografts and cell pellets, may require further reduction to the Target Retrieval incubation time. RNAscope VS Protease incubation time can also be adjusted but is rarely needed. Refer to **Appendix B** for recommended pretreatment strength by tissue type. If you have a tissue type not listed, contact ACD Support at **support.acd@bio-techne.com.**

5 Chapter 5. Automated RNAscope VS Universal HRP Assay – Chromogenic or Fluorescent

IMPORTANT! We strongly recommend you run the RNAscope VS Control Slides (Cat. No. 310045 or 310023) using the RNAscope 2.5 VS Positive and Negative Control Probes along with your samples in every run.

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

Workflow



Prepare materials

Materials required

Materials Provided by Advanced Cell Diagnostics	Materials Provided by Roche Tissue Diagnostics	Other Materials and Equipment

• RNAscope 2.5 VS Target Probe
• RNAscope 2.5 VS Positive Control Probe
• RNAscope 2.5 VS Negative Control Probe
• RNAscope VS Dewax
• VS PretreatPro*
• RNAscope VS Protease†
• RNAscope VS Target Retrieval†
• RNAscope VS Universal HRP AMP 1
• RNAscope VS Universal HRP AMP 2
• RNAscope VS Universal HRP AMP 3
• RNAscope VS Universal HRP AMP 4
• RNAscope VS Universal HRP AMP 5
• RNAscope VS Universal HRP AMP 6
• RNAscope VS Universal HRP AMP 7
• RNAscope VS Hematoxylin‡
• RNAscope VS Bluing Reagent‡
	• DISCOVERY ULTRA — automated slide stainer
	• DISCOVERY Wash Buffer 10X
	• ULTRA LCS (Predilute)
	• SSC Buffer 10X
	• DISCOVERY CC1
	• Reaction Buffer 10X
	• Probe dispensers
	• mRNA Probe Amplification Kit
	• mRNA Universal Sample Prep v2 Kit
	• One of the following:
	  • mRNA DAB Detection Kit
	  • mRNA Purple Detection Kit
	  • mRNA Teal Detection Kit
	  • mRNA Green Detection Kit
	  • Fluorescent Detection Kit
	• DISCOVERY Inhibitor (unless using mRNA DAB Detection Kit)
	• DISCOVERY QD DAPI‡
	• User fillable dispensers
		• Distilled water
		• Dawn detergent or similar detergent
		• Fume hood
		• Ethanol (optional)
		• Xylene
		• Tissue-Tek Staining Dish
		• Tissue-Tek Clearing Agent Dish, xylene resistant
		• Tissue-Tek Vertical 24 Slide Rack
		• Cover Glass, 24 mm x 50 mm
		• Cytoseal XYL xylene-based mounting medium (as needed)
		• EcoMount mounting medium (as needed)
		• ProlongGold Antifade Mountant (as needed)

* This dispenser is only used for the protease-free workflow
† These dispensers are used for the protease workflow
‡VS Hematoxylin and VS Bluing Reagent can be used for chromogenic counterstaining. For fluorescent counterstaining, DISCOVERY QD DAPI can be used.

Prepare the instrument

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

Dilute bulk reagents

Please prepare the bulk fluids per manufacturer instructions.

Register new reagents

Reagent dispensers come with appropriate barcode labels and registration buttons for dispensing RNAscope VS Universal Reagents. Refer to the *Ventana DISCOVERY ULTRA System User Manual* for details. To register reagents:

1. Log all ACD reagents and probes into the software as “log user-fillable reagents” or “‘log user- fillable probes”.
2. Use the wand or barcode scanner that comes with the instrument to register new reagent kits.

Prepare instrument reagents

After selecting the pretreatment workflow, refer to the dispenser table in **Chapter 1** to determine the proper dispenser for each reagent.

1. Transfer VS Protease and both bottles of VS Target Retrieval v2 **OR** VS PretreatPro to the corresponding labeled dispenser.
2. For RNAscope VS Universal HRP reagents AMP 1 to AMP 7, transfer the entire volume of each component into the correspondingly labeled dispenser.
3. Transfer the VS Dewax, VS Hematoxylin, and VS Bluing Reagent to the correspondingly labeled dispenser.
4. Use probe dispensers for 2.5 VS Target Probe, 2.5 VS Positive Control Probe, 2.5 VS Negative Control Probe

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

5. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
6. Store the tightly-capped mRNA Dewax and Target Retrieval v2 dispensers at **15–30°C**. Store all other capped dispensers at **4°C** when not in use.
7. Check solution levels: DISCOVERY Wash, 2X SSC, Reaction Buffer, ULTRA LCS (Predilute), and CC1. Refill if they are less than half full. To run a full tray of 30 slides, completely fill the 2X SSC and Reaction Buffer containers.

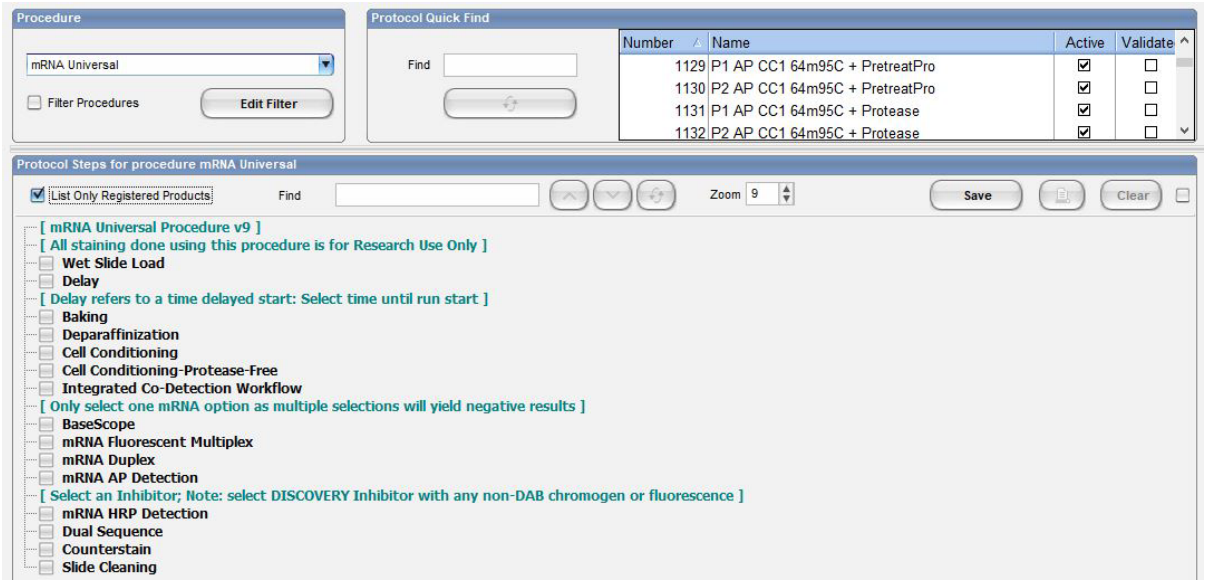
IMPORTANT! Do not use expired reagents.

8. Empty the waste bottle if needed.

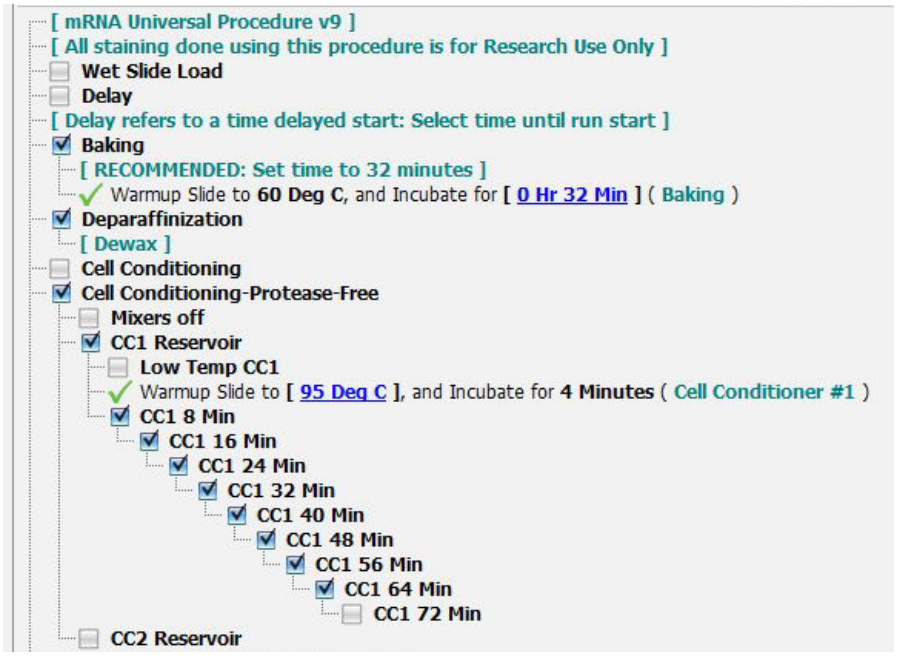
Create an instrument protocol

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 Universal**.

Main protocol steps appear as shown:



3. Do one of the following, depending on your selected workflow:
   - For target retrieval using the **Protease-Free Workflow**, select the appropriate pretreatment conditions according to the following screenshot. After selecting the main steps, drop-down menus become available. We recommend selecting **Cell-Conditioning-Protease-Free** with CC1 for the time and temperature specified in **Chapter 4** according to tissue-specific recommendations found in **Appendix B**.



Note: The duration of tissue cell conditioning corresponds to the longest selected time. It is not the sum of all selected times.

- For target retrieval using the **Protease Workflow**, select the appropriate pretreatment conditions according to the following screenshot. After selecting the main steps, drop-down menus become available. We recommend selecting **Cell Conditioning** for the time and temperature specified in **Chapter 4** according to tissue-specific recommendations found in **Appendix B.**

Note: The duration of tissue cell conditioning corresponds to the longest selected time. It is not the sum of all selected times.

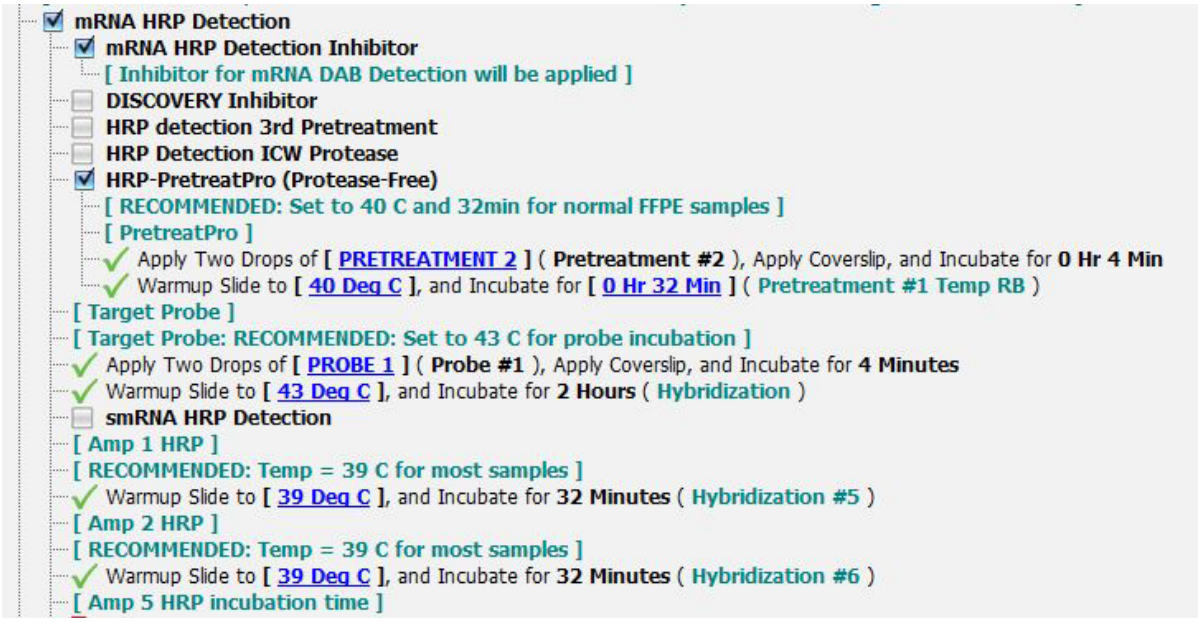
4. For ISH staining with RNAscope VS Universal HRP, select **mRNA HRP Detection**

Note: Select only one mRNA option per protocol. Choosing multiple mRNA options gives negative results.

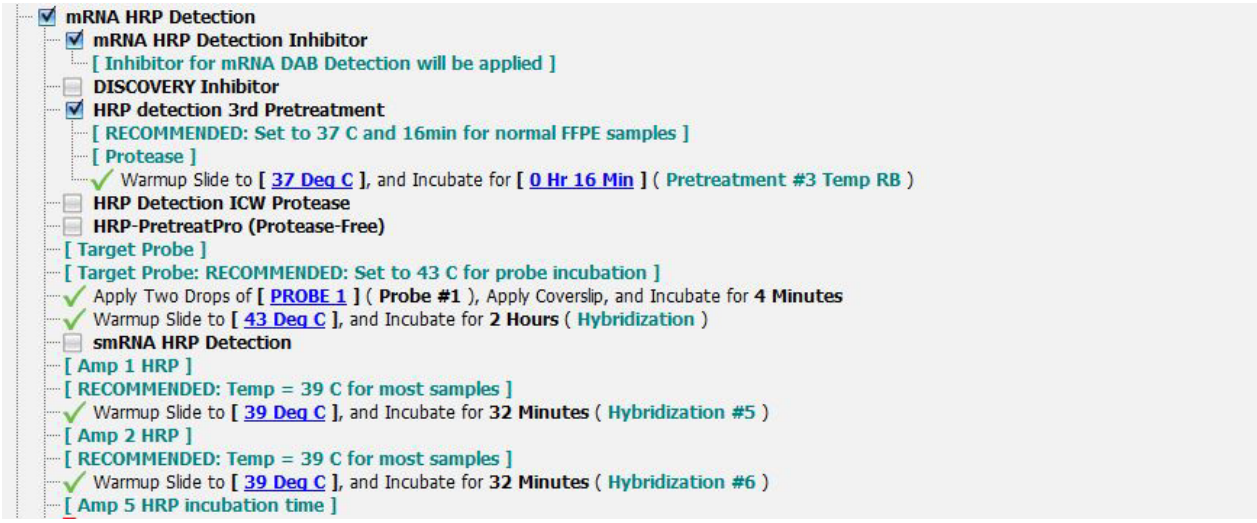
5. If you are using the mRNA DAB Detection Kit, select **mRNA HRP Detection Inhibitor**. mRNA DAB is applied by default, so no detection kit selection is needed.
6. If you are using any other chromogenic or fluorescent detection kit, select **DISCOVERY Inhibitor**, along with your detection choice.
7. Do one of the following, depending on your selected workflow:
   - If using the **Protease-Free Workflow**, select **HRP–PretreatPro** to apply and incubate the VS PretreatPro reagent. We recommend incubating this reagent for **32 MIN** at **40°C**.
   - If using the **Protease Workflow**, select **HRP detection 3rd Pretreatment** to apply and incubate the VS Protease reagent. We recommend incubating this reagent for **16 MIN** at **37°C**.
8. Select the remaining ISH staining conditions according to the following screenshots and table depending on your selected workflow.

Note: If you are interested in smRNA staining refer to Chapter 6 at this point to understand correct protocol settings

- ISH selections for the protease-free workflow:

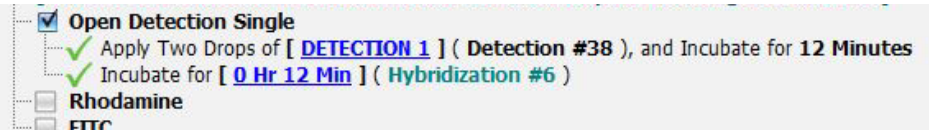


- ISH selections for the protease workflow:

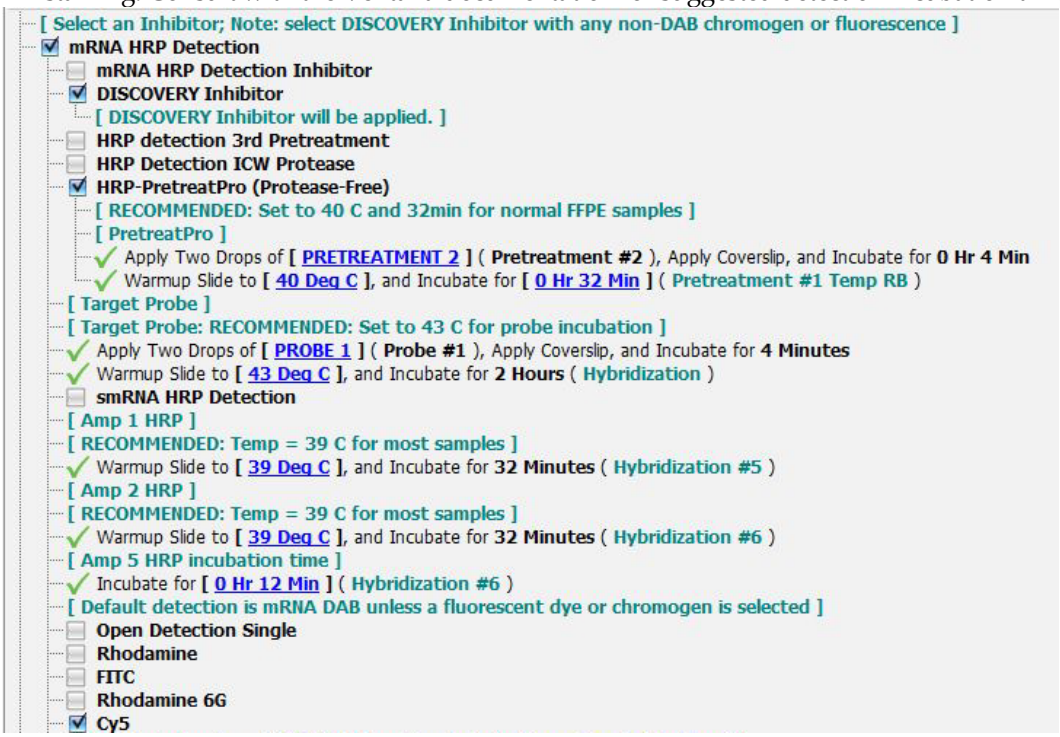


9. For fluorescent detection with user-diluted TSA dye, select Open **Detection Single** and the appropriate detection reagent. We recommend incubation for **12 MIN**. Roche Fluorophores can also be selected.

Note: Roche fluorophores can also be selected but are provided at a fixed concentration. To adjust the detection intensity, vary the incubation time between 12 and 92 minutes.



10. If you are using fluorescent detection or the purple, teal or green chromogens that do not ship with inhibitor, ensure that **DISCOVERY Inhibitor** is selected. The following example is for Cy5 staining. Consult with the Ventana documentation for suggested detection incubation times.



Standard Times/Temperatures for mRNA HRP Detection	
Cell Conditioning*	24 MIN @97C
Cell-Conditioning–Protease-Free (CC1)*	64 MIN @95C
Select appropriate inhibitor depending on chemistry	Check required box
VS Protease temperature and time*	37°C, 16 MIN
HRP-PretreatPro (Protease-Free) temperature and time*	40°C, 32 MIN
Suggested probe temperature	Single Probes - 43°C HPV Pooled Probes - 50°C
Suggested RNAscope HRP AMP 1 and AMP 2 temperature	39°C†
RNAscope HRP AMP 5 incubation time	Instrument titrated‡
mRNA Purple detection incubation time	12-40 MIN
mRNA Teal H_2O_2 incubation time	20 MIN^
mRNA Teal Activator incubation time	20 MIN
mRNA Green H_2O_2 incubation time	20 MIN^
mRNA Green Activator incubation time	20 MIN

*Choose either Cell Conditioning/VS Protease or Cell-Conditioning–Protease-Free/VS PretreatPro depending on the workflow. Do not select both within the same protocol. Refer to **Chapter 4** for pretreatment recommendations.

† If very high nuclear background is observed, you can raise temperatures to 40 or 41°C. However, some signal sensitivity may be lost. Please contact support or your FAS before making these changes.

‡ VS Universal HRP Amp 5 incubation time is determined by instrument calibration. Use the instrument setting previously optimized for the mRNA Universal software. For assistance, consult your local ACD FAS for more information.

^ The strength of mRNA Teal and mRNA Green detection can be modulated through adjustment of the mRNA Teal/Green H_2O_2 incubation time.

11. Select your preferred counterstain and post-counterstain settings. For optimal visualization of multiplex chromogenic staining, a light counterstain is recommended. Example below is for use of ACD Hematoxylin and Bluing Reagent counterstains using open Counterstain dispensers. If fluorescent counterstain is needed, QD DAPI may be selected.

12. At the top of the Protocol Steps window, click **Save As**, then select a unique protocol number from the drop-down menu and choose a protocol name.
13. Click **Active**, specify any relevant comments in the available field, and then click **Save**.
14. Click **Close** to go back to the main screen.
15. Assign a probe number from the list to each probe of interest. For each probe selected, assign a protocol.

Print the labels

1. Select the **Print Label** icon from the upper right corner of the home page screen.
2. Select your preferred template or create a new template. To create a new template, refer to the *Ventana DISCOVERY ULTRA System User Manual* for details.
3. Click on **Protocol**.
4. Select the protocol you created for the RNAscope VS Universal Assay and click on the **Add** button.
5. When the protocols for all the slides have been assigned, click on **Close/Print**.
6. Fill in the template for each slide. Select **Print** when completed.
7. Proceed to **Load the reagents** in the next section.

Run the RNAscope VS Universal HRP Assay

Load the reagents

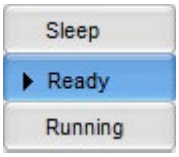
1. Remove the nozzle caps from the filled dispensers and place each cap on the post located on the back of the dispenser.
2. If needed, remove any air bubbles at the nozzle tips by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle.

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

3. Load dispensers onto the reagent racks.
4. Remove the yellow locking ring from the dispensers in the prefilled **mRNA BROWN Detection Kit.** Refer to the instructions provided by Roche Tissue Diagnostics.
5. Load the reagent racks onto the reagent carousel.

Start the run

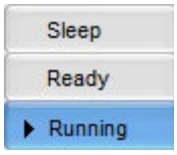
1. Select the **Ready** button.



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

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

4. Close slide drawers.
5. Select the **Running** button. Automated assay will finish in **~11 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, remove the Dewax reagent and Target Retrieval v2, place nozzle caps on the dispensers, and store them at room temperature.
2. For the remaining reagents, place nozzle caps back on the dispensers and place racks onto magnet locking tray and store at **4°C**.

Wash and dry the slides

1. Prepare 200 mL of diluted detergent by adding 1 to 2 drops detergent to 200 mL distilled water in a container with a cap.
2. Mix well by inverting the container 4–5 times.
3. Add diluted detergent to a Tissue-Tek Staining Dish.

Note: Store diluted detergent at **RT**.

4. Submerge a Tissue-Tek Slide Rack into the Tissue-Tek Staining Dish containing **200 mL** diluted detergent.
5. Open the instrument slide drawers and unload slides.
6. Decant solution on the slides into the slide drawer, then *immediately* load slides into the Tissue-Tek Slide Rack submerged in detergent.
7. Rinse oil off the slides by moving the slide rack up and down in the dish 10 times.
8. Replace the detergent with distilled water and rinse the slides by moving the slide rack up and down a minimum of **10** times.
9. Repeat Step 5 three to five times.
10. Transfer the slides to a Tissue-Tek staining Dish containing 200ml distilled water
    1. To mount with ProLong Gold, proceed directly to Option 2 under "Mount the Samples"
11. For chromogenic detection, slides may be dried by air or by ethanol dehydration.
    NOTE: Pan-tissue chromogen blush can occur when using mRNA Green or mRNA Teal chromogens, and can be mitigated through ethanol dehydration. However, ethanol dehydration is not recommended if performing co-detection with AP-based chromogen.
    1. To **Air Dry** the slides:
       a. Place slides in a drying oven at **60°C** for at least **30 MIN.**
    2. To perform **Ethanol Dehydration**:
       a. In a fume hood, prepare three Tissue Teck staining dishes, containing 70% EtOH, 95% EtOH, 95% EtOH, and 100% EtOH.
       b. Place slides in a Tissue-Tek staining dish containing 70% EtOH and incubate for **2 MIN** at RT with occasional agitation.
       c. Move the slides to the first Tissue-Tek staining dish containing 95% EtOH and incubate for **2 MIN** at RT with occasional agitation.
       d. Move the slides to the second Tissue-Tek staining dish containing 95% EtOH and incubate for **2 MIN** at RT with occasional agitation.
       e. Move the slides to the second Tissue-Tek staining dish containing 100% EtOH and incubate for **2 MIN** at RT with occasional agitation.

Mount the samples

IMPORTANT! mRNA Teal, mRNA Green, DISCOVERY Teal HRP and DISCOVERY Green HRP chromogens are light sensitive and can fade over time. If slides are mounted with EcoMount or Cytoseal (Option 1), we recommend to protect stored slides from the light and image within one week of staining. Alternatively, slides may be mounted with ProLong Gold mounting media to preserve signal (Option 2).

Option 1: Mounting chromogenic slides using EcoMount or Cytoseal mounting media:

1. In a fume hood, fill two Tissue-Tek staining dishes with ~200 mL fresh xylene.
2. Once slides have been dehydrated, move the Tissue-Tek Slide rack into the staining dish containing xylene for **1 MIN** with occasional agitation.
3. Move the Tissue-Tek Slide rack into the staining dish containing xylene for **1 MIN** with occasional agitation.
4. Lay each slide flat with the sections facing up in the fume hood then add 1 to 2 drops of EcoMount or Cytoseal depending on assay run. Carefully place a 24 mm x 50 mm coverslip over the section and avoid trapping air bubbles.
4. Air dry slides for at least **15 MIN** before evaluation.
6. Proceed to **Chapter 8. Evaluate the Results**.

Option 2: Mounting chromogenic or fluorescent slides using ProLong Gold mounting media:

1. Keep the slide rack in the staining dish containing distilled water. One at a time, remove a slide from the slide rack and lay each slide flat with the sections facing up.
2. Add 1 to 2 drops of mounting media to the slide. Carefully place a 24 mm x 50 mm coverslip over the section and avoid trapping air bubbles.
3. Dry slides overnight in the dark before evaluation.
4. Proceed to **Chapter 8. Evaluate the Results**.

6 Chapter 6. Automated smRNA (miRNA) RNAscope VS Universal HRP Assay

The RNAscope VS Universal HRP Assay can be used for detection of one smRNA target using a S1 (smRNA) probe.

Refer to **Chapter 5** for instructions. Only sections requiring changes are listed in this chapter.

Prepare materials

Materials required

Follow **Chapter 5** for materials listed with the following changes:

- In addition to materials listed in Chapter 5, you will also need RNAscope VS Expansion Pack: miRNAscope (Cat. No. 322120), which provides RNAscope VS smRNA Detection Reagent sufficient for 60 slides of smRNA detection
- Additional, unique barcode 250 test Detection dispenser (fill with smRNA Detection Reagent)
- Omit the control and target probes listed in **Chapter 5** and see the following recommendations.

Control probes: smRNA (miRNA)

RNU6 (U6 small nuclear RNA) is an endogenous, highly expressed small non-coding RNA found across many species and is recommended as a positive control in S1.

Probe name	Species	Cat. No.
RNU6	Human, Mouse, Rat, Rabbit, Monkey, Equine	727879-S1
Scramble	Any	727889-S1

Register new reagents

Follow **Chapter 5** to register materials with the following changes:

- Log smRNA reagent (ACD) and new probes into the software as “log user-fillable reagents” or “‘log user-fillable probes.”
- To ensure accurate volume tracking, input the reagent fill volume during registration.

Prepare **instrument reagents**

Follow **Chapter 5** to prepare instrument reagents with the following changes:

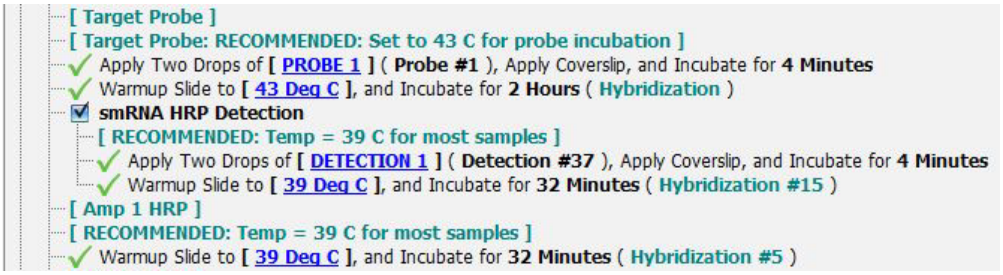
- Fill your smRNA probe into the Probe dispenser.
- Transfer the smRNA reagent into its corresponding test detection dispenser.
- Ensure both probe and smRNA reagent dispensers are properly primed and load them onto the reagent carousel.

Create an instrument protocol

Refer to **Chapter 5** for protocol selections with the following modification**:**

1. After selecting the target probes, choose **smRNA AP Detection**.
2. Select the corresponding test dispenser.
3. Set the recommended temperature to **39°C**.

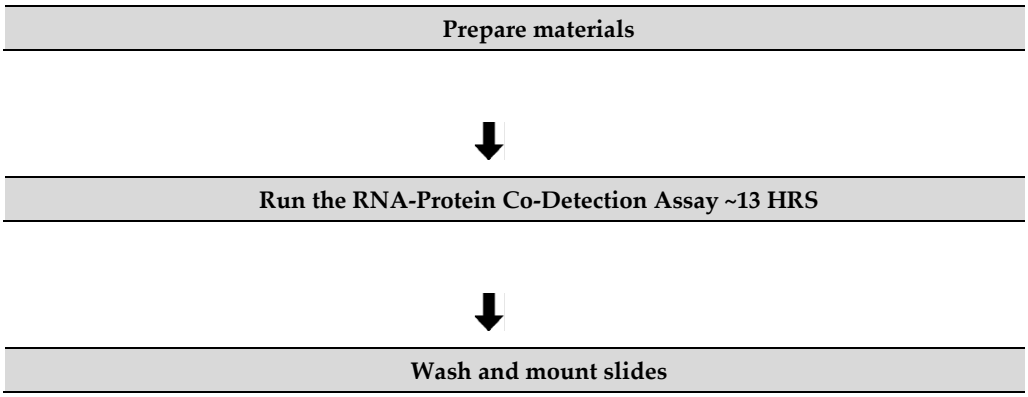
See the following screenshot for reference:



7 Chapter 7: Protease-Free, Sequential RNA-Protein Co-Detection

In the protease-free workflow for sequential RNA-Protein Co-Detection, tissue samples undergo pretreatment using Roche IHC-style cell conditioning with CC1, followed by further digestion using ACD's VS PretreatPro. This is followed by ISH detection, and then IHC detection and counterstaining. We recommend first establishing successful detection of RNA and IHC separately in your tissue of interest before proceeding to combined detection.

Workflow



Prepare materials

Materials required

Materials Provided by Advanced Cell Diagnostics	Materials Provided by Roche Tissue Diagnostics	Other Materials and Equipment
• RNAscope 2.5 VS Target Probe • RNAscope 2.5 VS Positive Control Probe • RNAscope 2.5 VS Negative Control Probe • RNAscope VS Dewax • VS PretreatPro* • RNAscope VS Target Retrieval† • RNAscope VS Universal HRP AMP 1 • RNAscope VS Universal HRP AMP 2 • RNAscope VS Universal HRP AMP 3 • RNAscope VS Universal HRP AMP 4 • RNAscope VS Universal HRP AMP 5 • RNAscope VS Universal HRP AMP 6 • RNAscope VS Universal HRP AMP 7 • RNAscope VS Hematoxylin (optional) ‡ • RNAscope VS Bluing Reagent (optional) ‡	• DISCOVERY ULTRA — automated slide stainer • DISCOVERY Wash Buffer 10X • ULTRA LCS (Predilute) • SSC Buffer 10X • DISCOVERY CC1 • Reaction Buffer 10X • Probe dispensers • mRNA Probe Amplification Kit • mRNA Universal Sample Prep v2 Kit • One of the following: • mRNA DAB Detection Kit • mRNA Purple Detection Kit • mRNA Teal Detection Kit • mRNA Green Detection Kit • Fluorescent Detection Kit • DISCOVERY Inhibitor (unless using mRNA DAB Detection Kit) • DISCOVERY QD DAPI (optional)‡ • User fillable dispensers • IHC detection options (see Chapter 1)	• Distilled water • Dawn detergent or similar detergent • Fume hood • Ethanol (optional) • Xylene • Tissue-Tek Staining Dish • Tissue-Tek Clearing Agent Dish, xylene-resistant • Tissue-Tek Vertical 24 Slide Rack • Cover Glass, 24 mm x 50 mm • Primary antibody • Cytoseal XYL xylene-based mounting medium (as needed) • EcoMount mounting medium (as needed) • ProlongGold Antifade Mountant (as needed)

* This dispenser is only used for the protease-free workflow
†These dispensers are used for the protease workflow
‡VS Hematoxylin and VS Bluing Reagent can be used for chromogenic counterstaining. For fluorescent counterstaining, DISCOVERY QD DAPI can be used.

Prepare the instrument

If the instrument has not been used for > 1 week, follow guidelines for instrument maintenance from Roche Tissue Diagnostics. Before use, empty the waste carboys if needed.

Dilute instrument bulk reagents

1. Prepare the instrument bulk fluids according to the manufacturer's instructions.
2. Fill bulk solution containers for 1X DISCOVERY Wash, ULTRA LCS (predilute), and CC1 (predilute) to be at least half full. Completely fill bulk solution containers for 2X SSC and 1X Reaction Buffer.

IMPORTANT! Do not use expired reagents.

Register new reagents

Reagent dispensers come with appropriate barcode labels and registration buttons for dispensing RNAscope VS Universal Reagents. Refer to the *Ventana DISCOVERY ULTRA System User Manual* for details. To register reagents:

- Log all ACD reagents and probes into the software as **log user-fillable reagents** and **log user-fillable probes**, respectively**.**
- Use the reagent registration wand that comes with the instrument to register new reagent kits from Roche Tissue Diagnostics

Prepare user-fillable reagents for RNA-Protein Co-Detection

Refer to **Chapter 1** to determine the proper dispenser for each reagent.

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

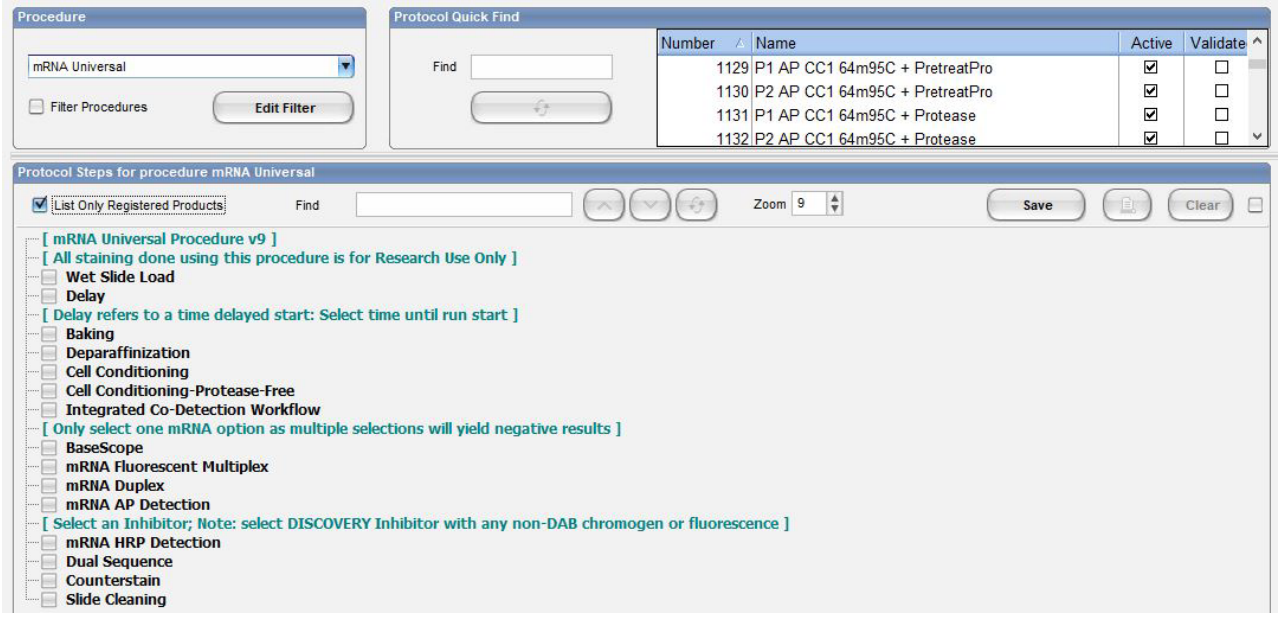
1. Transfer the entire volume of each AMP component of the Detection Kit to the corresponding labeled dispenser from the appropriate mRNA Amplification kit.
2. Fill the mRNA Sample Prep Kit by transferring the VS Dewax reagent from the RNAscope VS Sample Prep Reagent Kit v2 to the mRNA Dewax Dispenser.

Note: mRNA Target Retrieval and mRNA Protease dispensers are not needed for the protease-free workflow.

3. Fill the following dispensers:
   a. Transfer the RNAscope 2.5 VS Target Probe and control probes to the corresponding probe dispensers.
   b. Transfer the VS PretreatPro and VS CoDetectPro from the RNAscope VS Pro Reagents to the open pretreatment dispensers. Ensure the barcode number aligns with the protocol used.
   c. Transfer the VS Hematoxylin and VS Bluing to the Counterstain 1 and Counterstain 2 dispensers.
4. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
5. Store tightly capped dispensers at **4°C** when not in use.
6. Store mRNA Dewax and Target Retrieval v2 dispenser at room temperature when not in use.

Create an instrument protocol

1. Open the NexES software and click the **Protocol** button.
2. Click **Create/Edit Protocols**, then select **mRNA Universal** from the Procedure drop-down menu**.**
3. Confirm that "mRNA Universal v9" is displayed. The main protocol selections appear as shown:



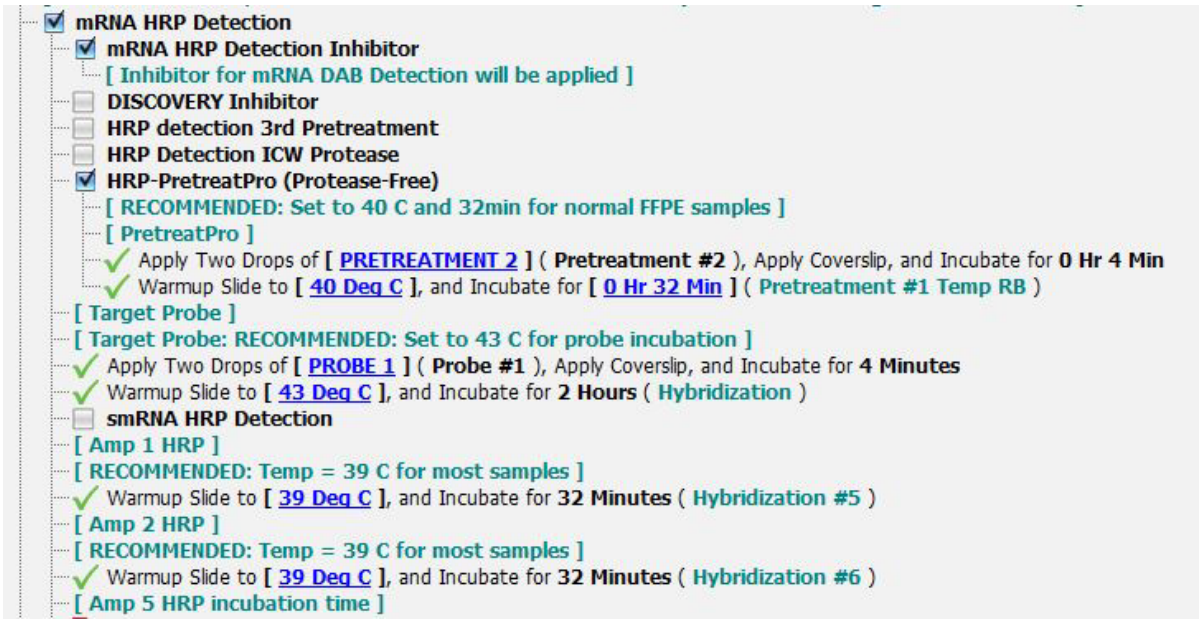
4. Select the appropriate pretreatment conditions according to the following screenshot. After selecting the main steps, drop-down menus become available. We recommend selecting **Cell-Conditioning-Protease-Free** with CC1 for the time and temperature specified in **Chapter 4** according to tissue-specific recommendations found in **Appendix B**.

Note: The duration of tissue cell conditioning corresponds to the longest selected time. It is not the sum of all selected times.

5. To enable ISH staining with RNAscope VS Universal HRP, select **mRNA HRP Detection**.

Note: Select only one mRNA option per protocol. Choosing multiple mRNA options gives negative results.

6. Select **mRNA HRP Detection Inhibitor** if staining with DAB or select **DISCOVERY Inhibitor** if staining with fluorescence or other chromogens.
7. Select **HRP–PretreatPro** to apply and incubate the VS PretreatPro reagent. We recommend incubating this reagent for **32 MIN** at **40°C**.
8. Select the remaining ISH staining conditions according to the following screenshot and table:



Standard Times/Temperatures for mRNA HRP Detection	
Cell Conditioning*	24 MIN @97C
Cell-Conditioning–Protease-Free (CC1)*	64 MIN @95C
Select appropriate inhibitor depending on chemistry	Check required box
VS Protease temperature and time*	37°C, 16 MIN
HRP-PretreatPro (Protease-Free) temperature and time*	40°C, 32 MIN
Suggested probe temperature	Single Probes - 43°C HPV Pooled Probes - 50°C
Suggested RNAscope HRP AMP 1 & AMP 2 temperature	39°C†
RNAscope HRP AMP 5 incubation time	Instrument titrated ‡
mRNA Purple detection incubation time	12-40 MIN
mRNA Teal H_2O_2 incubation time	20 MIN^
mRNA Teal Activator incubation time	20 MIN
mRNA Green H_2O_2 Incubation time	20 MIN^
mRNA Green Activator incubation time	20 MIN

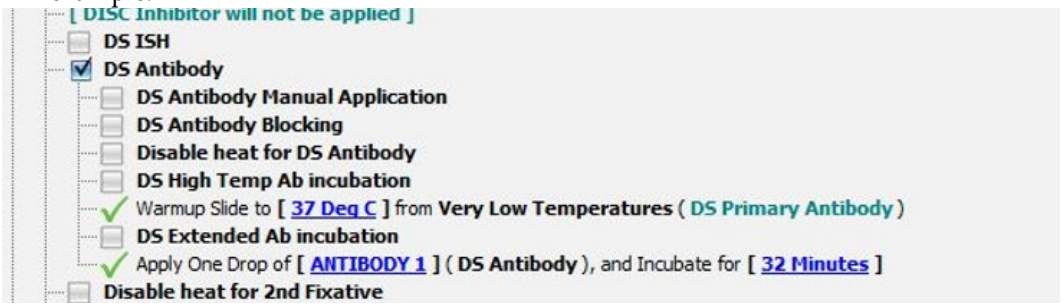
*Refer to **Chapter 4** for the CC1 time and temperature recommended for your tissue type.
† If very high nuclear background is observed, you can raise temperatures to 40 or 41°C. However, some signal sensitivity may be lost. Please contact support or your FAS before making these changes.
‡ VS Universal HRP Amp 5 incubation time is determined by instrument calibration. Use the instrument setting previously optimized for the mRNA Universal software. For assistance, consult your local ACD FAS for more information.
^ The strength of mRNA Teal and mRNA Green detection can be modulated through adjustment of the mRNA Teal/Green H_2O_2 incubation time.

9. Select **Dual Sequence** to enable IHC following ISH detection.
10. Apply VS CoDetectPro from the RNAscope VS Pro Reagents by selecting **DS Pretreatment**, **DS 2nd Pretreatment** and **Use DW for DS 2nd Pretreatment**. Select a pretreatment barcode to

correspond to the VS CoDetectPro reagent. We recommend incubating at **50°C** for **32 MIN** prior to protein detection to quench RNAscope enzymes prior to IHC detection.

IMPORTANT! Make sure that VS CoDetectPro uses a unique Pretreatment number, different from VS PretreatPro, to ensure the protocol runs correctly.

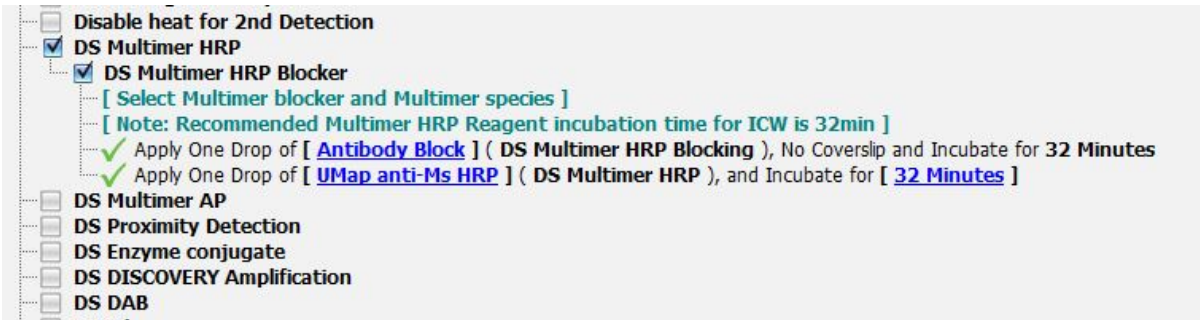
11. To apply primary antibody, select **DS Antibody** and your desired incubation time and temperature, using previously optimized IHC-only conditions. See the following for an example.



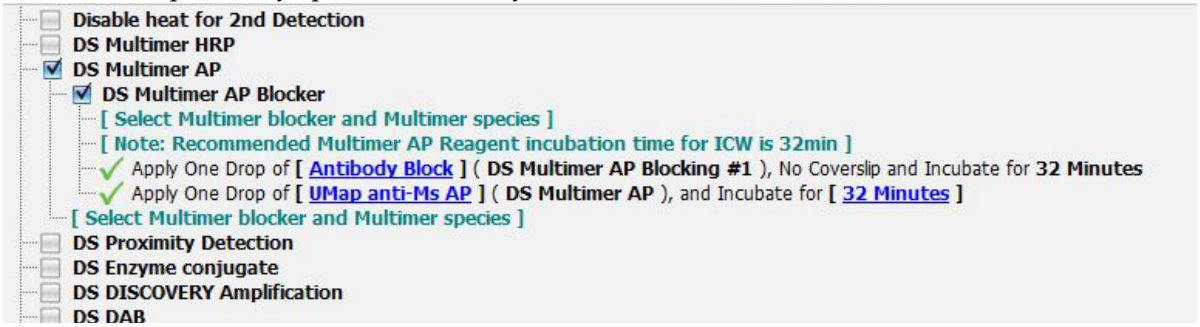
12. Select the settings for secondary detection:

Note: To prevent non-specific cross-detection of RNAscope, we recommend using Roche's Antibody Block reagent immediately before applying the secondary antibody. Skipping the Antibody Block step could cause a hue shift in the RNA dots, making it difficult to accurately interpret RNA-protein colocalization.

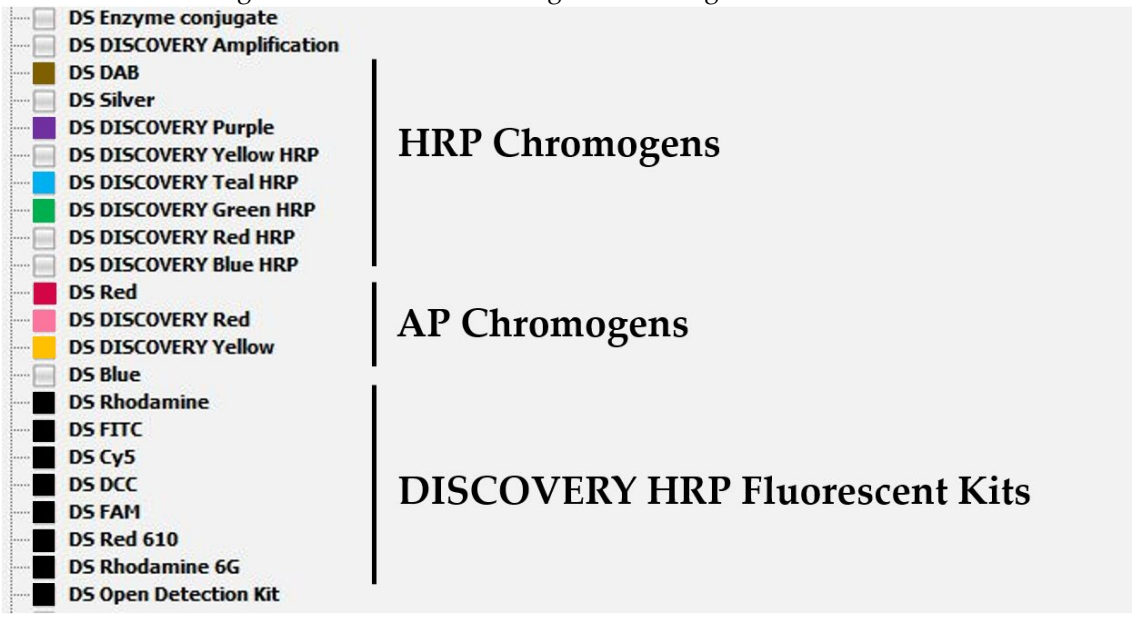
- Do the following for HRP-based secondary detection:
  a. Choose a Roche Multimer HRP reagent corresponding to the primary host species.
  b. To enable secondary incubation, select **DS Multimer HRP**.
  c. To apply Antibody Block, select **DS Multimer HRP Blocker**, then select **Antibody Block** reagent.
  d. Select your Multimer HRP reagent of choice and desired incubation time based on previously optimized IHC-only conditions.



- Do the following for AP-based secondary detection:
  a. Choose a Roche Multimer AP reagent corresponding to the primary host species.
  b. To enable secondary incubation, select **DS Multimer AP**.
  c. To apply Antibody Block, select **DS Multimer AP Blocker**, then select Antibody Block reagent.
  d. Select your Multimer AP reagent of choice and desired incubation time based on previously optimized IHC-only conditions.



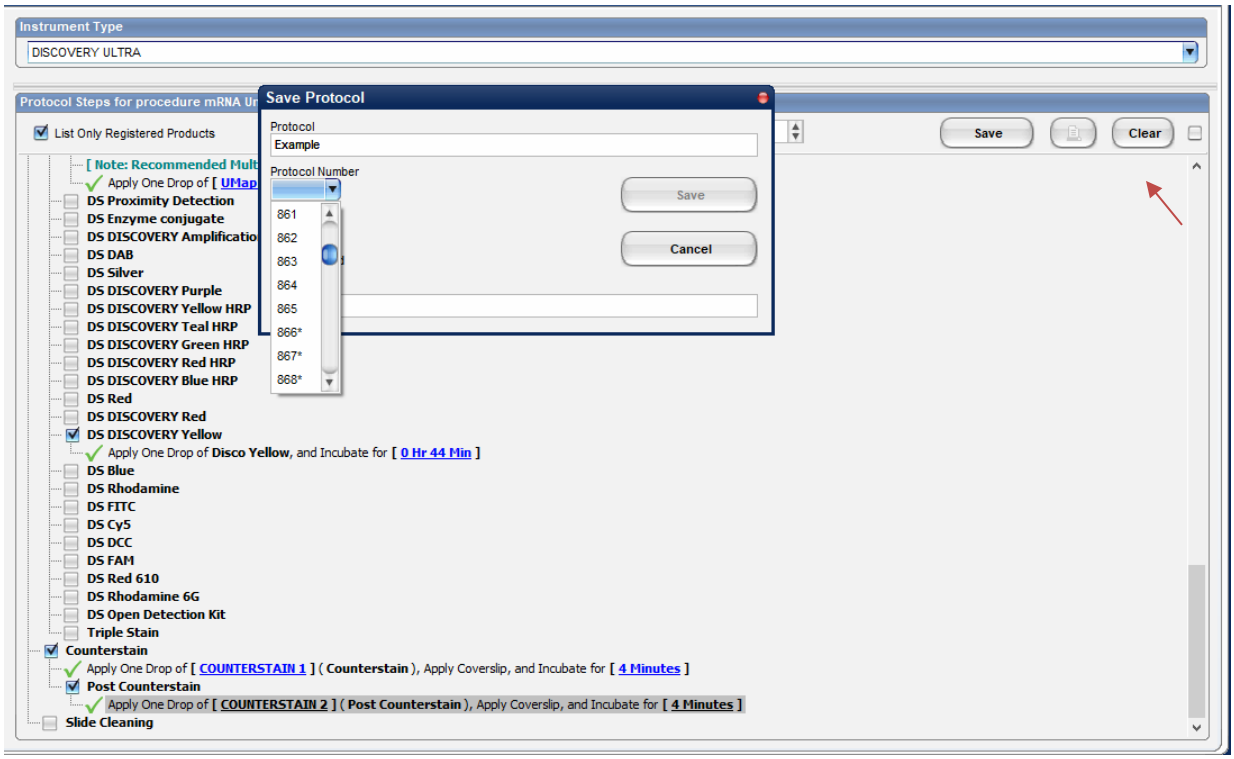
13. Select chromogens for IHC detection using the following recommendations:



IHC Chromogen Selection and Settings		
IHC Enzyme	**IHC Chromogen**	**Recommended Chromogen Settings***
AP	DISCOVERY Yellow	44 MIN – 2 HRS
	DISCOVERY Red	12 MIN†
	DS Red / ChromoMap Red‡	Default
HRP (chromogenic)	DS DAB / ChromoMap DAB	Default
	DISCOVERY Purple	40 MIN
	DISCOVERY Teal HRP^	DISCOVERY Teal H_2O_2 — 16–32 MIN DISCOVERY Teal Act – 16 MIN
	DISCOVERY Green HRP^	DISCOVERY Green H_2O_2 — 16–32 MIN DISCOVERY Green Act — 16 MIN
HRP (fluorescent)	DISCOVERY DCC Kit	32 MIN
	DISCOVERY FAM Kit	20 MIN
	DISCOVERY FITC Kit	20 MIN
	DISCOVERY Rhodamine Kit	32 MIN
	DISCOVERY Rhodamine 6G Kit	32 MIN
	DISCOVERY Red 610 Kit	32 MIN
	DISCOVERY Cy5 Kit	40 MIN

*We recommend using the same IHC chromogen settings for sequential ISH-IHC as optimized for IHC alone.
†Extending DISCOVERY Red incubation is not recommended, as it can result in a dot-like background.
‡For stronger AP-based Red IHC detection, ChromoMap Red is recommended (select **DS Red**).
^The strength of DISCOVERY Teal HRP and DISCOVERY Green HRP detection can be modulated through adjustment of the DISCOVERY Teal/Green H_2O_2 incubation time.

14. Select your preferred counterstain and post-counterstain settings. A light counterstain is recommended for best visualization of multiplex chromogenic staining.
15. At the top of the Protocol Steps window, click **Save As**, then select a unique protocol number from the drop-down menu and choose a protocol name.
16. Click **Active**, specify any relevant comments in the available field, and then click **Save**.



17. Create a new protocol for each probe/antibody/chromogen combination. Click **Save**.

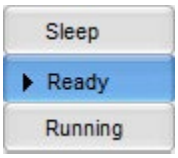
Print the labels

1. Select the **Print Label** icon from the upper right corner of the home screen.
2. Select your preferred template or create a new template. To create a new template, refer to the *Ventana DISCOVERY ULTRA System User Manual* for details.
3. Click Protocol.
4. Select the protocols you created in the section above. Click the **Add** button.
5. When the protocols for all slides have been assigned, click **Close/Print**.
6. Fill in the template for each slide. Click **Print** when complete.

Load the reagents

Remove the nozzle caps from the filled dispensers and place each cap on the post located on the back of the dispenser.

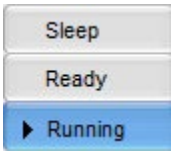
1. Prime the user-fillable dispensers. For guidance, refer to the instructions provided by Roche Tissue Diagnostics.
2. If needed, remove any air bubbles at the nozzle tip by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle.
3. Remove the yellow locking ring from the dispensers in all the prefilled dispensers. Refer to the instructions provided by Roche Tissue Diagnostics.
4. Load the dispensers onto the reagent racks.
5. Load the reagent racks onto the reagent carousel.
6. Select the **Ready** button.



7. Open the slide drawers.
8. Load each slide onto a heater pad with the label facing upward and inward. Ensure that the slides sit securely on the pads.

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

9. Close the slide drawers.
10. Select the **Running** button.



11. The assay duration varies based on assay selections, approximately **15 – 20 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, remove the Dewax reagent and Target Retrieval v2, place nozzle caps on the dispensers, and store them at room temperature.
2. For the remaining reagents, place nozzle caps back on the dispensers and place racks onto magnet locking tray and Store at **4°C.**

Wash and dry the slides

1. Prepare 200 mL of diluted detergent by adding 1 to 2 drops detergent to 200 mL distilled water in a container with a cap.
2. Mix well by inverting the container four to five times.
3. Add diluted detergent to a Tissue-Tek Staining Dish.

Note: Store diluted detergent at **RT**.

4. Submerge a Tissue-Tek Slide Rack into the Tissue-Tek Staining Dish containing 200 mL diluted detergent.
5. Open the instrument slide drawers and unload slides.
6. Decant solution on the slides into the slide drawer, then *immediately* load slides into the Tissue-Tek Slide Rack submerged in detergent.
7. Rinse oil off the slides by moving the slide rack up and down in the dish 10 times.
8. Replace the detergent with distilled water and rinse slides by moving the slide rack up and down a minimum of **10** times.
9. Repeat Step 8 three to five times.
12. Transfer the slides to a Tissue-Tek staining Dish containing 200ml distilled water
    1. To mount with ProLong Gold, proceed directly to Option 2 under "Mount the Samples"
13. For chromogenic detection, slides may be dried by air or by ethanol dehydration.

NOTE: Pan-tissue chromogen blush can occur when using mRNA Green or mRNA Teal chromogens, and can be mitigated through ethanol dehydration. However, ethanol dehydration is not recommended if performing co-detection with AP-based chromogen.

1. To **Air Dry** the slides:
   a. Place slides in a drying oven at **60°C** for at least **30 MIN.**
2. To perform **Ethanol Dehydration**:
   a. In a fume hood, prepare three Tissue Teck staining dishes, containing 70% EtOH, 95% EtOH, 95% EtOH, and 100% EtOH.
   b. Place slides in a Tissue-Tek staining dish containing 70% EtOH and incubate for **2 MIN** at RT with occasional agitation.
   c. Move the slides to the first Tissue-Tek staining dish containing 95% EtOH and incubate for **2 MIN** at RT with occasional agitation.
   d. Move the slides to the second Tissue-Tek staining dish containing 95% EtOH and incubate for **2 MIN** at RT with occasional agitation.
   e. Move the slides to the second Tissue-Tek staining dish containing 100% EtOH and incubate for **2 MIN** at RT with occasional agitation.

Mount the samples

IMPORTANT! mRNA Teal, mRNA Green, DISCOVERY Teal HRP and DISCOVERY Green HRP chromogens are light sensitive and can fade over time. If slides are mounted with EcoMount or Cytoseal (Option 1), we recommend to protect stored slides from the light and image within one week of staining. Alternatively, slides may be mounted with ProLong Gold mounting media to preserve signal (Option 2).

Option 1: Mounting chromogenic slides using EcoMount or Cytoseal mounting media:

5. In a fume hood, fill two Tissue-Tek staining dishes with ~200 mL fresh xylene.
6. Once slides have been dehydrated, move the Tissue-Tek Slide rack into the staining dish containing xylene for **1 MIN** with occasional agitation.
7. Move the Tissue-Tek Slide rack into the staining dish containing xylene for **1 MIN** with occasional agitation.
8. Lay each slide flat with the sections facing up in the fume hood then add 1 to 2 drops of EcoMount or Cytoseal depending on assay run. Carefully place a 24 mm x 50 mm coverslip over the section and avoid trapping air bubbles.
5. Air dry slides for at least **15 MIN** before evaluation.
12. Proceed to **Chapter 8. Evaluate the Results**.

Option 2: Mounting chromogenic or fluorescent slides using ProLong Gold mounting media:

5. Keep the slide rack in the staining dish containing distilled water. One at a time, remove a slide from the slide rack and lay each slide flat with the sections facing up.
6. Add 1 to 2 drops of mounting media to the slide. Carefully place a 24 mm x 50 mm coverslip over the section and avoid trapping air bubbles.
7. Dry slides overnight in the dark before evaluation. .
8. Proceed to **Chapter 8. Evaluate the Results**.

8 Chapter 8. Evaluate the Results

Evaluate the samples

Examine the experimental results.

- Assess tissue and cell morphology.

  Assess the negative control background first. For fluorescence imaging, set exposure times of image acquisition to acceptable background levels.
- Assess positive control signal strength. Positive control signal should be visible as punctate dots within the cell. Highly expressed targets might appear as clusters. For fluorescence imaging, exposure times for negative control and positive control should be the same for each unique sample.
- Determine which pretreatment condition yielded the highest positive control signal and lowest negative control signal.

If none of the conditions are satisfactory, contact technical support at **support.acd@bio-techne.com.**

Staining Score	Scoring Definition
0	No staining or <1 dot/10 cells
1	1 – 3 dots/cell
2	4 – 9 dots/ cell or very few dot clusters
3	10 – 15 dots/ cell and <10% dots are in clusters
4	>15 dots/ cell and >10% dots are in clusters

The miRNAscope VS Assay provides a semi-quantitative scoring guideline based on the estimated number of punctate dots within each cell boundary. Below is an example guideline for assessing miRNAscope staining intensity for a gene with expression levels ranging from 1 to over 10 copies per cell.

Note: If your miRNA expression is outside this range, adjust the scoring criteria accordingly.

Categorize staining into four grades: 0, 1, 2, and 3 according to the following table:

Staining Score	Scoring Definition
0	No staining, or less than 1 dot/ cell (40X magnification)
1	2–10 dots/cell (visible at 20–40X magnification)
2	11–20 dots/cell and /or <25% dots are in clusters (visible at 20-40X magnification)
3	>20 dots/cell and/or >25% dots are in clusters (visible at 20X magnification)

* Discount cells with artificially high nuclear background staining.

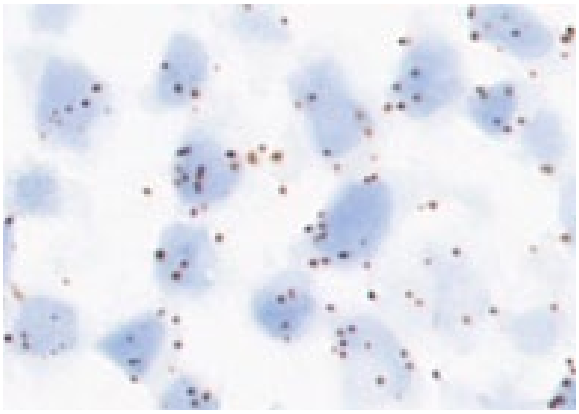
Troubleshooting

For troubleshooting information, please contact technical support at **support.acd@bio-techne.com.**

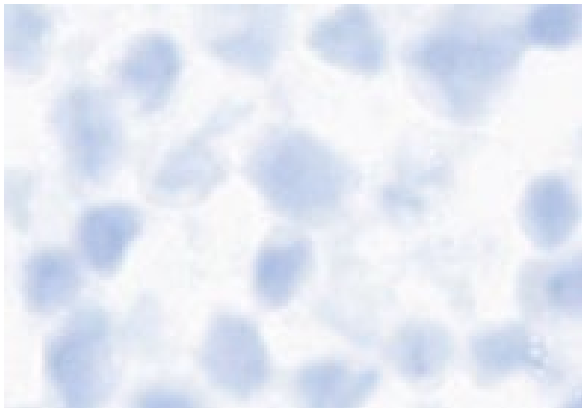
Tissue example

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

Figure 2. RNAscope VS Universal HRP Assay results in HeLa cells

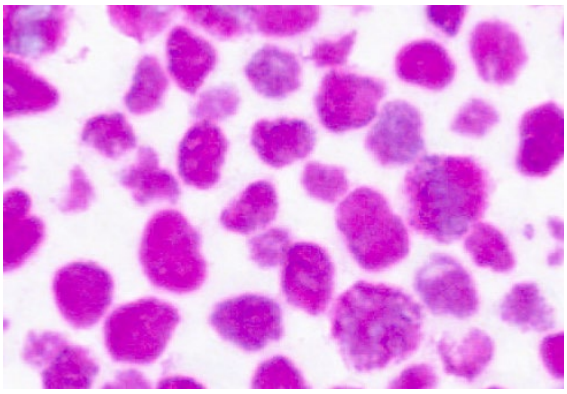


Hs-TBP (Positive Control)

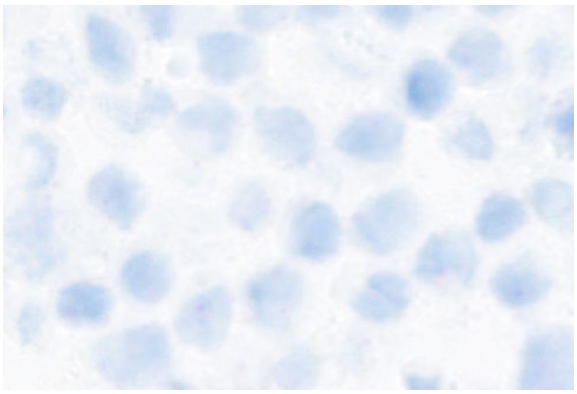


DapB (Negative Control)

Figure 3. smRNA (miRNA) RNAscope VS Universal HRP Assay (Purple) results in HeLa cells



RNU6 (smRNA Positive Control)



Scramble (smRNA Negative Control)

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

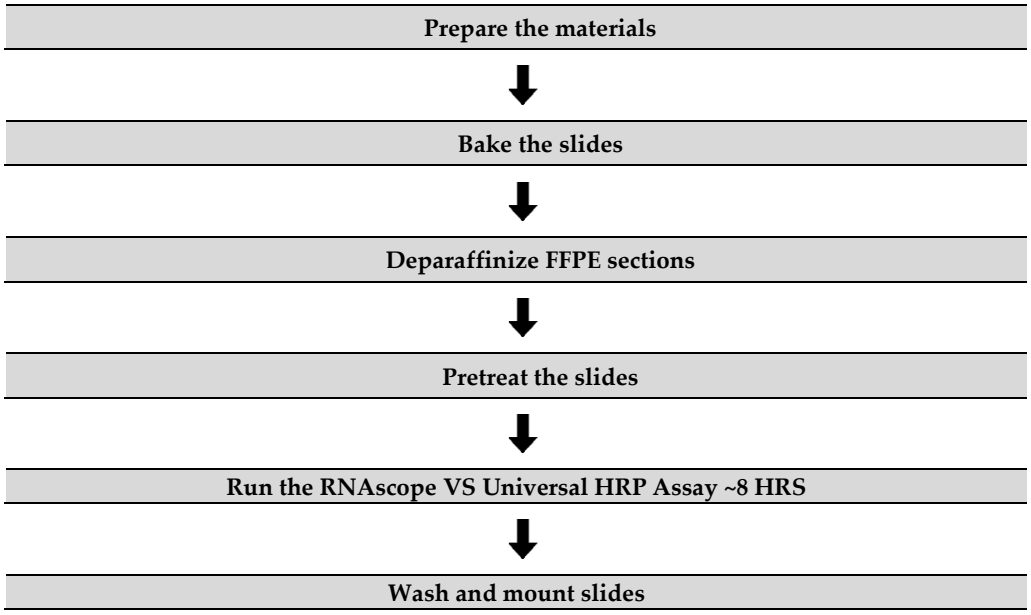
Most sample types can be fully automated on the Discovery ULTRA. However, manual pretreatment may yield better results in some cases. For tissues that do not achieve satisfactory results with the fully automated procedure and conditions in **Chapter 4**, use the semi-automated procedure.

The following protocols describe formalin-fixed, paraffin-embedded (FFPE) sample pretreatment. For other sample types and preparation methods, contact **support.acd@bio-techne.com** for the latest protocols and guidelines.

IMPORTANT! This workflow has only been tested with the VS Protease reagent. Using the VS PretreatPro results in sub-optimal retrieval.

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

Workflow



Kit contents and storage

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

IMPORTANT! Do not substitute the reagent components of the RNAscope VS Universal Reagent Kit with those of other RNAscope Reagent Kits, even those having the same name. The RNAscope VS Target Retrieval v2 solution in the RNAscope VS Universal Sample Prep Kit CANNOT be used for offline boiling. Please use the RNAscope Target Retrieval Reagents (Cat. No. 322000) to boil samples off the instrument.

Prepare the materials

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

Materials required

Materials provided by Advanced Cell Diagnostics	Materials Provided by Roche Tissue Diagnostics	Other Materials and Equipment

• RNAscope 2.5 VS Target Probe
• RNAscope 2.5 VS Positive Control Probe
• RNAscope 2.5 VS Negative Control Probe
• RNAscope Target Retrieval Reagents (Offline)
• RNAscope VS Protease*
• RNAscope VS Universal HRP AMP 1
• RNAscope VS Universal HRP AMP 2
• RNAscope VS Universal HRP AMP 3
• RNAscope VS Universal HRP AMP 4
• RNAscope VS Universal HRP AMP 5
• RNAscope VS Universal HRP AMP 6
• RNAscope VS Universal HRP AMP 7
• RNAscope VS Hematoxylin (optional)†
• RNAscope VS Bluing Reagent (optional)†
	• DISCOVERY ULTRA — automated slide stainer
	• DISCOVERY Wash 10x
	• ULTRA LCS (Predilute)
	• SSC Buffer 10X
	• Reaction Buffer 10X
	• Probe dispensers
	• mRNA Probe Amplification Kit
	• One of the following:
	  • mRNA DAB Detection Kit
	  • mRNA Purple Detection Kit
	  • mRNA Teal Detection Kit
	  • mRNA Green Detection Kit
	  • Fluorescent Detection Kit
	• DISCOVERY Inhibitor (unless using mRNA DAB Detection Kit)
	• DISCOVERY QD DAPI (optional)**
	• User fillable dispensers
	• mRNA Universal Sample Prep Kit
	• CCI Buffer
		• Distilled water
		• Glass beaker (1 or 2 L)
		• Hot plate
		• Dawn detergent or similar detergent
		• Fume hood
		• Ethanol (optional)
		• Xylene
		• 100% ethanol (EtOH)
		• Tissue- Staining Dishes
		• Tissue-Tek Clearing Agent Dishes, xylene-resistant
		• Tissue-Tek Vertical 24 Slide Rack
		• Cover Glass, 24 mm x 50 mm
		• Cytoseal XYL xylene-based mounting medium (as needed)
		• EcoMount mounting medium (as needed)
		• ProlongGold Antifade Mountant (as needed)

* This dispenser is only used for the protease-based workflow

**Can be used for fluorescent detection

†VS Hematoxylin and VS Bluing Reagent can be used for chromogenic counterstaining. For fluorescent counterstaining, DISCOVERY QD DAPI can be used.

Prepare the instrument

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

Dilute bulk reagents

Please prepare the bulk fluids per manufacturer instructions.

Register new reagents

Reagent dispensers come with appropriate barcode labels and registration buttons for dispensing RNAscope VS Reagents. Refer to the *Ventana DISCOVERY ULTRA System User Manual* for details. To register reagents:

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

Prepare instrument reagents

Refer to the table in Chapter 1 to determine the proper dispenser for each reagent.

1. For RNAscope VS Universal HRP **AMP 1** to **AMP 7**, transfer the entire volume of each component into the correspondingly labeled dispenser.
2. Transfer the VS Dewax, VS Protease, VS Hematoxylin, and VS Bluing Reagent to the correspondingly labeled dispenser.
3. Use Roche Tissue Diagnostics probe dispensers for 2.5 VS Target Probe, 2.5 VS Positive Control Probe, 2.5 VS Negative Control Probe
4. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
5. Store the tightly-capped mRNA Dewax dispensers at **15–30°C**. Store all other capped dispensers at **4°C** when not in use.
6. Check solution levels: DISCOVERY Wash, 2X SSC, Reaction Buffer, ULTRA LCS (Predilute), and CC1. Refill if they are less than half full. To run a full tray of 30 slides, fully fill the 2X SSC and Reaction Buffer containers.

IMPORTANT! Do not use expired reagents.

13. Empty the waste carboy if needed.

Prepare deparaffinization reagents

- In a fume hood, fill two clearing agent dishes with ~200 mL fresh xylene.
- In a fume hood, fill two staining dishes with ~200 mL fresh 100% EtOH.

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

Prepare 1X Target Retrieval

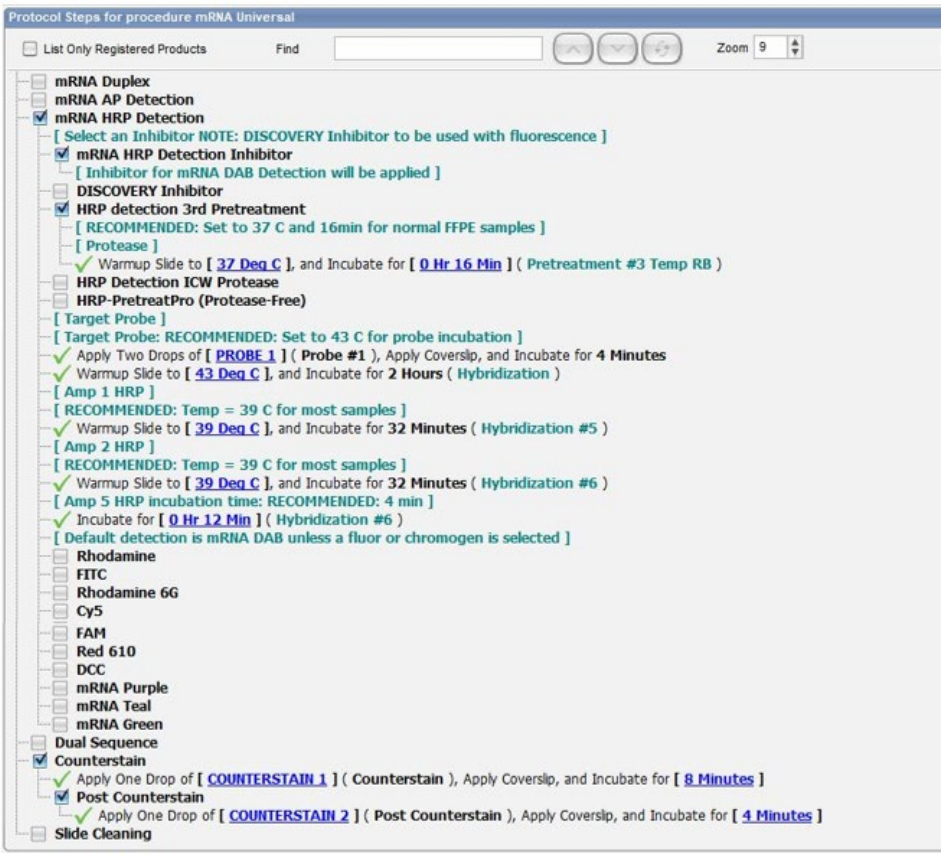
1X Target Retrieval is used in manual cell conditioning (CC).

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

IMPORTANT! Do not use RNAscope VS Target Retrieval v2 for offline boiling.

Create an instrument protocol

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



IMPORTANT! Do not select Baking, Deparaffinization, or Cell Conditioning.

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. If you are using the DAB Detection Kit, select **mRNA HRP Detection Inhibitor** along with your detection choice. If you are using fluorescent detection, or alternate chromogen kit choose the **DISCOVERY Inhibitor** (see the Ventana documentation for suggested detection incubation times to use in **Chapter 6**).
5. Select the appropriate assay conditions from the drop-down menus according to the following table:

Standard Times/Temperatures for mRNA HRP Detection	
Cell Conditioning*	24 MIN @97C
Cell-Conditioning–Protease-Free (CC1)*	64 MIN @95C
Select appropriate inhibitor depending on chemistry	Check required box
VS Protease temperature and time*	37°C, 16 MIN
HRP-PretreatPro (Protease-Free) temperature and time*	40°C, 32 MIN
Suggested probe temperature	Single Probes - 43°C HPV Pooled Probes - 50°C
Suggested RNAscope HRP AMP 1 and AMP 2 temperature	39°C†
RNAscope HRP AMP 5 incubation time	Instrument titrated‡

Standard Times/Temperatures for mRNA HRP Detection	
mRNA Purple detection incubation time	12-40 MIN
mRNA Teal H_2O_2 incubation time	20 MIN^
mRNA Teal Activator Incubation time	20 MIN
mRNA Green H_2O_2 incubation time	20 MIN^
mRNA Green Activator incubation time	20 MIN

* Refer to **Chapter 4** for the CC1 time and temperature recommended for your tissue type
† If very high nuclear background is observed, you can raise temperatures to 40 or 41°C. However, some signal sensitivity may be lost. Please contact support or your FAS before making these changes.
‡ VS Universal HRP Amp 5 incubation time is determined by instrument calibration. Use the instrument setting previously optimized for the mRNA Universal software. For assistance, consult your local ACD FAS for more information.
^ The strength of mRNA Teal and mRNA Green detection can be modulated through adjustment of the mRNA Teal/Green H_2O_2 incubation time.

6. At the top of the Protocol Steps window, click **Save As**, then select a unique protocol number from the drop-down menu and choose a protocol name.
7. Click **Active**, specify any relevant comments in the available field, and then click **Save**.
8. Click **Close** to go back to the main screen.
9. Assign a probe number from the list to each probe of interest. For each probe selected, assign a protocol.

Print the labels

1. Select the Print Label icon from the bottom of the home page screen.
2. Select your preferred template or create a new template. To create a new template, refer to the *Ventana DISCOVERY ULTRA System User Manual* for details.
3. Select the protocol you created for the RNAscope VS Universal Assay.

Manually pretreat the samples

Bake the slides

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

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

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

Deparaffinize FFPE sections

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

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

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

10. Insert the slides into a Tissue-Tek Slide Rack and proceed to condition the slides.

Pretreat the slides

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

IMPORTANT! Do not boil 1X Target Retrieval for more than 30 MIN before use.

1. Heat 1X Target Retrieval Buffer to **98–104°C**:
   a. Place the beaker containing 1X Target Retrieval Buffer on the hot plate. Cover the beaker with foil and turn the hot plate on high for **10–15 MIN**.
   b. Once 1X Target Retrieval Buffer reaches a slow boil (**98–104°C**), turn the hot plate to a lower setting to maintain the correct temperature. Check the temperature with a thermometer.
2. With a pair of forceps *very slowly* submerge the slide rack containing the slides into the boiling 1X Target Retrieval Buffer solution. Cover the beaker with foil and boil the slides for the time indicated below, according to target retrieval strength recommended in the following tables:

Extra Mild	Mild	Standard	Extended
7 MIN	10 MIN	15 MIN	30 MIN

Tissue-specific pretreatment recommendations:

Tissue Type	Offline Target Retrieval Time
Brain and spinal cord	Standard
Breast cancer	Standard
Cell lines	Mild
Colon	Standard
GI tract	Standard
Head and neck cancer	Standard
Heart	Standard

Tissue Type	Offline Target Retrieval Time
Kidney	Standard
Liver	Extended
Lung	Standard
Lymphoma	Mild
Placenta	Standard
Prostate	Standard
Skin	Standard
Stomach	Standard
Thymus	Mild
Tonsil	Mild
Xenograft derived from cell lines	Extra Mild
Xenograft derived from primary tumor	Standard
ACD control cell pellet	Mild

3. Immediately transfer the hot slide rack from the 1X Target Retrieval Buffer to a staining dish containing distilled water. Do not let the slides cool in Target Retrieval.
4. Wash slides 3–5 times by moving the Tissue-Tek Slide Rack up and down in the distilled water.
5. Repeat Step 4 with fresh distilled water.
6. Proceed directly to **Load the reagents** in the next section.

Run the RNAscope VS Universal HRP Assay

Load the reagents

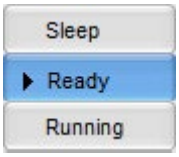
1. Remove the nozzle caps from the filled dispensers and place each cap on the post located on the back of the dispenser.
2. If needed, remove any air bubbles at the nozzle tips by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle.

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

3. Load dispensers onto the reagent racks.
4. Remove the yellow locking ring from the dispensers in the prefilled **mRNA BROWN Detection Kit.** Refer to the instructions provided by Roche Tissue Diagnostics.
5. Load the reagent racks onto the reagent carousel.

Start the run

1. Select the **Ready** button.

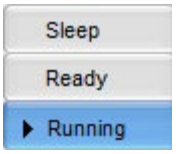


2. Eject slide drawers.

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

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

4. Close slide drawers.
5. Select the **Running** button. Automated assay will finish in **~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, remove the Dewax reagent and Target Retrieval v2, place nozzle caps on the dispensers, and store them at room temperature.
2. For the remaining reagents, place nozzle caps back on the dispensers and place racks onto magnet locking tray and Store at **4°C.**

Wash and dry the slides

1. Prepare 200 mL of diluted detergent by adding 1 to 2 drops detergent to 200 mL distilled water in a container with a cap.
2. Mix well by inverting the container four to five times.
3. Add diluted detergent to a Tissue-Tek Staining Dish.

Note: Store diluted detergent at **RT**.

4. Submerge a Tissue-Tek Slide Rack into the Tissue-Tek Staining Dish containing 200 mL diluted detergent.
5. Open the instrument slide drawers and unload slides.
6. Decant solution on the slides into the slide drawer, then *immediately* load slides into the Tissue-Tek Slide Rack submerged in detergent.
7. Rinse oil off the slides by moving the slide rack up and down in the dish 10 times.
8. Replace the detergent with distilled water and rinse slides by moving the slide rack up and down a minimum of **10** times.
9. Repeat Step 8 three to five times.
14. Transfer the slides to a Tissue-Tek staining Dish containing **200mL** distilled water
    1. To mount with ProLong Gold, proceed directly to Option 2 under "Mount the Samples"
15. For chromogenic detection, slides may be dried by air or by ethanol dehydration.
    NOTE: Pan-tissue chromogen blush can occur when using mRNA Green or mRNA Teal chromogens, and can be mitigated through ethanol dehydration. However, ethanol dehydration is <u>not recommended</u> if performing co-detection with AP-based chromogen.
    1. To **Air Dry** the slides:
        a. Place slides in a drying oven at **60°C** for at least **30 MIN.**

2. To perform **Ethanol Dehydration**:
   a. In a fume hood, prepare four Tissue Teck staining dishes, containing 70% EtOH, 95% EtOH, 95% EtOH, and 100% EtOH.
   b. Place slides in a Tissue-Tek staining dish containing 70% EtOH and incubate for **2 MIN** at RT with occasional agitation.
   c. Move the slides to the first Tissue-Tek staining dish containing 95% EtOH and incubate for **2 MIN** at RT with occasional agitation.
   d. Move the slides to the second Tissue-Tek staining dish containing 95% EtOH and incubate for **2 MIN** at RT with occasional agitation.
   e. Move the slides to the Tissue-Tek staining dish containing 100% EtOH and incubate for **2 MIN** at RT with occasional agitation.

Mount the samples

IMPORTANT! mRNA Teal, mRNA Green, DISCOVERY Teal HRP and DISCOVERY Green HRP chromogens are light sensitive and can fade over time. If slides are mounted with EcoMount or Cytoseal (Option 1), we recommend to protect stored slides from the light and image within one week of staining. Alternatively, slides may be mounted with ProLong Gold mounting media to preserve signal (Option 2).

Option 1 (Standard Recommendation): Mounting chromogenic slides using EcoMount or Cytoseal mounting media:

9. In a fume hood, fill two Tissue-Tek staining dishes with ~200 mL fresh xylene.
10. Once slides have been dehydrated, move the Tissue-Tek Slide rack into the staining dish containing xylene for **1 MIN** with occasional agitation.
11. Move the Tissue-Tek Slide rack into the staining dish containing xylene for **1 MIN** with occasional agitation.
12. Lay each slide flat with the sections facing up in the fume hood then add 1 to 2 drops of EcoMount or Cytoseal depending on assay run. Carefully place a 24 mm x 50 mm coverslip over the section and avoid trapping air bubbles.
6. Air dry slides for at least **15 MIN** before evaluation.
7. Proceed to **Chapter 8. Evaluate the Results**.

Option 2 (Enhanced mRNA teal or green chromogen stability): Mounting chromogenic or fluorescent slides using ProLong Gold mounting media:

9. Keep the slide rack in the staining dish containing distilled water. One at a time, remove a slide from the slide rack and lay each slide flat with the sections facing up.
10. Add 1 to 2 drops of mounting media to the slide. Carefully place a 24 mm x 50 mm coverslip over the section and avoid trapping air bubbles.
11. Dry slides overnight in the dark before evaluation.
12. Proceed to **Chapter 8. Evaluate the Results**.

B Appendix B. Pretreatment Guidance for FFPE Samples

Follow the recommended pretreatment conditions based on your tissue type for:

- Any new or previously untested FFPE tissue types
- Samples prepared differently than the sample preparation protocol found in **Chapter 3**.
- For specific guidance on other sample preparations contact ACD Support at **support.acd@bio-techne.com**

Tissue-specific pretreatment conditions

Refer to the following table for tissue specific FFPE pretreatment conditions. For information about species or tissue type not listed here, contact support at **support.acd@bio-techne.com.**

Tissue Type	Protease-Free Workflow Recommended CC1 Target Retrieval (online)		Protease Workflow Recommended ACD Target Retrieval (online)	
	mRNA	smRNA	mRNA	smRNA
Cell Pellet	Mild	Mild	Mild	Extra Mild
Brain	Standard	Standard	Gentle	Gentle
Spleen	Standard	Standard	Standard	Mild
Tonsil	Standard	Standard	Standard	Standard
Breast	Standard*	Standard	Standard	Standard
Prostate	Standard*	Standard	Standard	Standard
Intestine	Standard	Standard	Standard	Standard
Colon	Standard	Standard	Standard	Standard
Stomach	Standard	Standard	Standard	Standard
Kidney	Standard	Standard	Standard	Standard
Bladder	Standard	Standard	Standard	Standard
Heart	Standard	Standard	Standard	Standard
Lung	Standard	Standard	Standard	Standard
Skin	Standard	Standard	Standard	Standard
Head and Neck	Standard	Standard	Standard	Standard
Ovary	Standard	Standard	Standard	Standard
Cervical	Standard*	Standard*	Standard	Standard
Pancreas	Standard	Standard	Standard	Standard
Liver	Standard	Standard†	Standard†	Standard

*Mild pretreatment can help reduce tissue detachment
† Extended pretreatment may be needed

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

Biological hazard safety

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

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030)

- 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.
- Registration, Evaluation, Authorization and Restriction of Chemicals (REACH)

Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available at: **https://acdbio.com/documents/product-documents**. For the SDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

Obtaining support

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

At the website, you can:

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

Contact information

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Newark, CA 94560
Toll Free: 1-877-576-3636
Direct: 1-510-576-8800
Fax: 1-510-576-8801
Information: **info.acd@bio-techne.com**
Orders: **orders.acd@bio-techne.com**
Support Email : **support.acd@bio-techne.com**

Limited product warranty

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

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