USER MANUAL

RNAscope™ VS Duplex Assay

For VENTANA® DISCOVERY® ULTRA System

AP/HRP CHROMOGEN COMBINATIONS

Document Number UM 323300

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Citing RNAscope Assay in Publications

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

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Revision Updates

Revision	Effective Date	Change(s)	Location of change
D	7/10/2026	Inclusion of revision updates tables	Pg 2
		Addition of new "gentle" pretreatment condition	Pg 21, 63
		Note for adjustment of mRNA teal or green detection	Pg 31
		Change to target probe preparation volume/slide	Pg 24

Contents

1 Chapter 1. Product Information

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

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

About this guide

This user manual provides several workflows using the RNAscope VS Duplex Assay depending on the targets of interest:

Workflow	Reference
RNAscope Assay - mRNA detection	Chapter 5
Enabling smRNA detection	Chapter 6
Sequential RNA-Protein Co-Detection	Chapter 7
Semi-automated Assay (for completing Pretreatment steps manually)	Appendix A

Product description

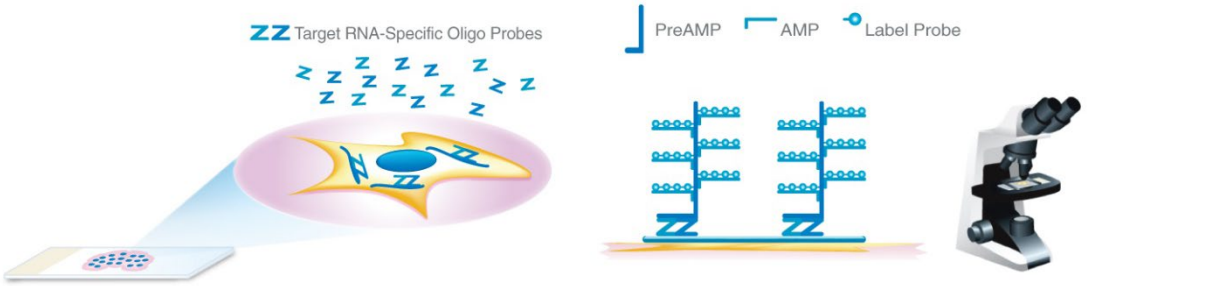
Background

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

Overview

Figure 1 illustrates the RNAscope VS Duplex Assay procedure, which can be completed on the instrument in ~14-16 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)- and alkaline phosphatase (AP)-labeled probes and detection using a chromogenic substrate. Each single RNA transcript appears as a distinct dot of chromogen precipitate visible using a common bright-field microscope.

Figure 1. Procedure overview



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

Kit contents and storage

This chapter includes a complete list of reagents needed to perform any of the workflows outlined at the beginning of the chapter. Users should select their appropriate combination(s).

The RNAscope VS Duplex Assay requires the RNAscope 2.5 VS Probes, VS C2 probes, and the RNAscope VS Duplex Reagents, available from Advanced Cell Diagnostics, a Bio-Techne brand.

RNAscope VS Probes

The RNAscope 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 date of manufacturing 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	Ready-To-Use (RTU) probe for color channel 1	7 mL x 1 bottle	2–8°C
	RNAscope 2.5 VS Target Probe – *[species] – [gene]* – C2	Various	16x probes for color channel 2	440 µL x 1 tube	2–8°C
mRNA Control Probes					
	Reagent	**Cat. No.**	**Content**	**Quantity**	**Storage**
	RNAscope 2.5 VS Duplex Control Probes – (PPIB-C1, Polr2A-C2)	Various	RTU mixture of two probes targeting PPIB in channel C1 and POLR2A in channel C2.	7 mL x 1 bottle	2–8°C
	RNAscope 2.5 VS Duplex Negative Control Probe – (DapB-C1, DapB-C2)	320759	RTU mixture negative control probes targeting a bacterial gene (not present in mammalian tissue) in channel C1 and in channel C2.	7 mL x 1 bottle	2–8°C

For small RNA probes for detection using miRNAscope Expansion Pack, we recommend species and channel specific control probes as follows:

Channel	**Positive Control Probe**	**Species**	**Part Number**
S1	RNU6	Human, Mouse, Rat, Rabbit, Monkey, Equine	727879-S1
S2	SR-Hs-SNORD54	Human	728939-S2
	SR-Mm-SNORD85	Mouse	728959-S2
	SR-Rn-SNORD95	Rat	729059-S2
Channel	**Negative Control Probe**	**Species**	**Part Number**
S1	Scramble	Any	727889
S2	Scramble		727889-S2

Note: Different colors are assigned to the C1 and C2 color channels (for mRNA) or S1 and S2 channels for small RNA targets depending on the particular RNAscope Assay. The color channels for the RNAscope VS Duplex Assay are shown in the following table:

Probe Channel ID	**Chromogenic Labels**	
	Enzyme	**Color**
C1* OR S1	HRP	Dark Brown/Teal/Green
C2 OR S2	AP	Red

* Default channel for RNAscope probes

Note: Channel C1/ S1 target probes are Ready-To-Use (RTU), while channel C2/S2 probes are shipped as a 16X concentrated stock. To prepare working stock, dilute the C2/S2 16x probe into the

C1 RTU probe. To independently detect two target RNAs in a Duplex assay, each target probe must be in a different color channel, C1 and C2.

If you wish to only use the C2 or S2 probes, please use "RNAscope 2.5 VS Blank Probe Diluent" (Cat. No. 300049) to dilute the C2 target probe 16X concentrate.

When ordering target probes for duplex detection, we suggest assigning the higher-expressing target to the first channel (C1/S1) and the lower-expressing target to the second channel (C2/S2) to achieve the clearest dual-signal visualization.

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 Duplex Positive Control Probe (PPIB-C1, Polr2A-C2) and RNAscope 2.5 VS Duplex Negative Control Probe (DapB-C1, DapB-C2). The slides have a shelf life of 9 months from the date of manufacturing when stored at 2–8°C with desiccants.

RNAscope VS Duplex Reagents

RNAscope VS Duplex kits provide enough reagents to stain ~60 standard slides. You will receive four boxes when you order the RNAscope VS Duplex Reagent Kit (Cat. No. 323300). RNAscope VS Duplex Reagents include:

- RNAscope VS Duplex Detection Reagents (Cat. No. 323310)
  - RNAscope VS Duplex 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 of nine months from the date of manufacturing when stored as indicated in the following table:

RNAscope VS Duplex Detection Reagents (Cat. No. 323310)				
RNAscope VS Duplex Detection Kit with PretreatPro (Cat. No. 323920)				
☑	Cat. No.	Reagent	Quantity	Storage
	323311	RNAscope VS Duplex AMP 1	14 mL x 1 bottle	2–8°C
	323312	RNAscope VS Duplex AMP 2	14 mL x 1 bottle	2–8°C
	323313	RNAscope VS Duplex AMP 3	14 mL x 1 bottle	2–8°C
	323314	RNAscope VS Duplex AMP 4	14 mL x 1 bottle	2–8°C
	323315	RNAscope VS Duplex AMP 5	14 mL x 1 bottle	2–8°C
	323316	RNAscope VS Duplex AMP 6	14 mL x 1 bottle	2–8°C
	323317	RNAscope VS Duplex AMP 7	14 mL x 1 bottle	2–8°C
	323318	RNAscope VS Duplex AMP 8	14 mL x 1 bottle	2–8°C
	323319	RNAscope VS Duplex AMP 9	14 mL x 1 bottle	2–8°C

RNAscope VS Duplex Detection Reagents (Cat. No. 323310)				
RNAscope VS Duplex Detection Kit with PretreatPro (Cat. No. 323920)				
☑	**Cat. No.**	**Reagent**	**Quantity**	**Storage**
	323320	RNAscope VS Duplex AMP Wash	14 mL x 2 bottles	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 Kit (Cat. No. 323740)				
☑	**Cat. No.**	**Reagent**	**Quantity**	**Storage**
	323741	RNAscope VS Universal Target Retrieval v2	10 mL x 2 bottles	Room Temp (15–30°C)
	323742	RNAscope VS Universal 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
RNAscope VS Expansion Pack: miRNAscope (Cat. No. 322120)				
☑	**Cat. No.**	**Reagent**	**Quantity**	**Storage**
	322118	smRNA Detection Reagent	14 mL x 1 bottle	2–8°C

IMPORTANT! Dewax must be in solution and at room temperature before use on the instrument. Warm in the hand, or place at 37°C for 15 MIN before each use regardless of the prior storage condition, since it may precipitate during shipment.

IMPORTANT! Use only RNAscope 2.5 VS Probes. Do not substitute the reagent components of the RNAscope VS Duplex Reagent Kit with those of other RNAscope Reagent Kits, including RNAscope VS Duplex Reagent Kits or those having the same name.

Required materials from Roche Diagnostics

The RNAscope VS Duplex Assay requires specific materials and equipment available *only* from Roche 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 960-785; for Ordering Code, please contact local Roche representative)		
☑	**Component**	**Storage**
	250 Test Probe #1–20 dispensers — fill dispensers with RNAscope 2.5 VS Probes. Use up to 20 probes at a time.	Room Temp (15–30°C)
mRNA Sample Prep Kit (Cat. No. 760-248; Ordering Code 08127166001)		
☑	**Component**	**Storage**
	mRNA Target Retrieval dispenser — fill dispenser with RNAscope VS Universal Target Retrieval v2†	Room Temp (15–30°C)
	mRNA Dewax — fill dispenser with RNAscope VS Universal Dewax	Room Temp (15–30°C)
	mRNA Protease dispenser — fill dispenser with RNAscope VS Protease†	Room Temp (15–30°C)
mRNA Duplex Amp Kit (Cat. No. 760-249; Ordering Code 08127174001)		
☑	**Component**	**Storage**
	mRNA AMP 1 dispenser — fill dispenser with VS Duplex AMP 1	Room Temp (15–30°C)
	mRNA AMP 2 dispenser — fill dispenser with VS Duplex AMP 2	Room Temp (15–30°C)
	mRNA AMP 3 dispenser — fill dispenser with VS Duplex AMP 3	Room Temp (15–30°C)
	mRNA AMP 4 dispenser — fill dispenser with VS Duplex AMP 4	Room Temp (15–30°C)
	mRNA AMP 5 dispenser — fill dispenser with VS Duplex AMP 5	Room Temp (15–30°C)
	mRNA AMP 6 dispenser — fill dispenser with VS Duplex AMP 6	Room Temp (15–30°C)
	mRNA AMP 7 dispenser — fill dispenser with VS Duplex AMP 7	Room Temp (15–30°C)
	mRNA AMP 8 dispenser — fill dispenser with VS Duplex AMP 8	Room Temp (15–30°C)
	mRNA AMP 9 dispenser — fill dispenser with VS Duplex AMP 9	Room Temp (15–30°C)
	mRNA AMP Wash dispenser — fill dispenser with VS Duplex AMP Wash	Room Temp (15–30°C)
mRNA Link (Cat. No. 760-6014; Ordering Code 08127115001)		
☑	**Component**	**Storage**
	mRNA Link	2–8°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

† Optional: This dispenser is only needed for Protease-Free Workflow

* Optional: This dispenser is only needed for smRNA detection (Chapter 6)

Roche Chromogenic Detection Kits

To run duplex chromogenic *in situ* hybridization on the Ventana DISCOVERY ULTRA System, you will need to order a combination of the following Detection reagents from Roche Tissue Diagnostics depending on desired color combination.

mRNA RED Detection Kit (Cat. No. 760-234; Ordering Code 7099037001)		
☑	**Component**	**Storage**
	mRNA Inhibitor-prefilled	2–8°C
	mRNA Activator dispenser-prefilled	2–8°C
	mRNA Napthol dispenser-prefilled	2–8°C
	mRNA Fast Red dispenser-prefilled	2–8°C

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

DISCOVERY Inhibitor (Cat. No. 760-4840; Ordering Code 07017944001)*		
☑	**Component**	**Storage**
	DISCOVERY Inhibitor dispenser-prefilled	2–8°C

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

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

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 Yellow Kit*	760-239	07698445001	2–8°C
	DISCOVERY Green HRP Kit †	760-278	07053983001	2–8°C
	DISCOVERY Teal HRP Kit †	760-247	08254338001	2–8°C
	DISCOVERY ChromoMap DAB Kit †	760-159	05266645001	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 an 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
	DISCOVERY Antibody Block‡	760-4204	05268869001	Pre-filled

*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.

‡ Optional depending on defined working IHC protocol

Instrument buffers

☑	Component	Cat. No./ Ordering Code
	10x DISCOVERY Wash (RUO)	950-510 / 07311079001
	ULTRA LCS (Predilute)	650-210 / 05424534001
	SSC Buffer (10X)	950-110 / 05353947001
	Reaction Buffer (10X)	950-300 / 05353955001
	DISCOVERY CC1	950-500 / 06414575001

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

User-supplied materials

☑	Description	Supplier	Cat. No.
	SuperFrost Plus Slides (required)	Fisher Scientific	12-550-15
	100% ethanol (EtOH)	American Master Tech Scientific/MLS*	ALREAGAL
	Xylene	Fisher Scientific/MLS	X3P-1GAL
	10% neutral-buffered formalin (NBF)	MLS	—
	Paraffin wax	MLS	—
	1X PBS	MLS	—
	Microtome	MLS	—
	Drying oven, capable of holding temperature at 60 +/– 1°C	MLS	—
	EcoMount	Biocare or ACD	EM897L or 320409
	Tissue-Tek Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24
	Tissue-Tek Staining Dish (6 required)	American Master Tech Scientific/MLS	LWT4457EA
	Tissue-Tek Clearing Agent Dish, xylene resistant (4 required)	American Master Tech Scientific/MLS	LWT4456EA
	Cover Glass 24 x 50 mm	Fisher Scientific/MLS	12-545-F
	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
	Primary antibody – RTU or concentrate	User	Various

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

* 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 Duplex Assay on your samples for the first time, we recommend that you:

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

Important procedural guidelines

- Start with properly fixed and prepared sections. Refer to **Chapter 3. Prepare and Pretreat Samples** and to our VS Duplex Assay user manual available at **https://www.bio-techne.com/p/in-situ-hybridization-assays/rnascope-vs-duplex-reagent-kit_323300#product-documents**.
- Regularly maintain and clean your automated staining instrument.
- Always run positive and negative control probes on your sample to assess sample RNA quality and optimal permeabilization.
- Do *not* substitute required materials. Assay has been validated with these materials only.
- Follow the protocol exactly for best results. Do not substitute buffers or diluents.
- Use good laboratory practice and follow all necessary safety procedures. Refer to **Appendix C. Safety** in this document for more information.

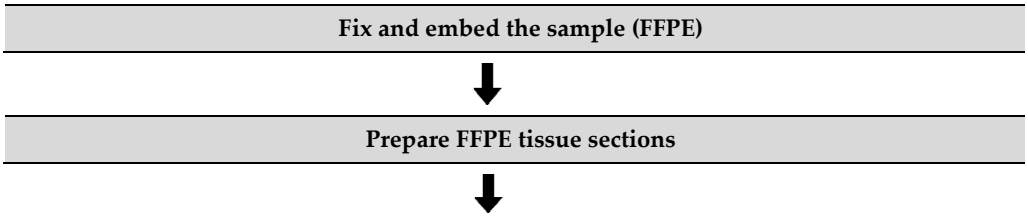
3 Chapter 3. Prepare and Pretreat Samples

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.

For samples treated differently from the following protocol, you may need to optimize pretreatment conditions. Contact **support.acd@bio-techne.com** if you need additional guidance.

Workflow



Materials required

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

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.

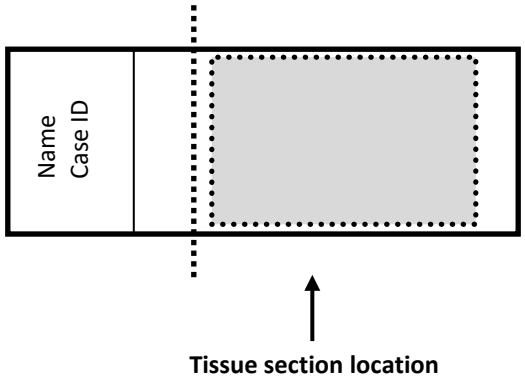
IMPORTANT! Use fresh reagents. Embed samples as quickly as possible to preserve RNA quality.

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

OPTIONAL STOPPING POINT. 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 Duplex Assay is compatible with both protease-based and protease-free pretreatment approaches. We recommend using protease-free pretreatment if you plan to combine RNA with protein detection in future downstream applications.

Target retrieval and permeabilization

Two options are available:

- Protease-free permeabilization is recommended when there is interest in combining RNA with Protein detection downstream. This approach uses an IHC-style cell conditioning for Target Retrieval, using Roche CC1 bulk reagent, combined with permeabilization using ACD's VS PretreatPro reagent. This allows co-detection of RNA and proteins that were previously incompatible with protease on the same tissue section using the same cell conditioning as immunohistochemistry (IHC). We recommend the protease-free pretreatment to be considered as the default pretreatment.
- Protease-based pretreatment is recommended for experiments that stain only RNA for established protocols when maintenance of performance compared to historical ISH experiments is critical. This option 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 table below 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 for the protease 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 Target Retrieval v2 (Cell Conditioning) to maximize the positive RNA control signal while minimizing or eliminating the negative RNA control signal.

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 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 Duplex Assay

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.

The RNAscope VS Duplex assay can be used to stain mRNA targets. smRNA targets can be stained with the use of materials and adjustments outlined in **Chapter 6**.

Appendix A. Semi-automated RNAscope VS Duplex Assay describes an offline boiling procedure for use with Cat. No.322000.

Workflow

Prepare the materials

⬇

Run the RNAscope Duplex Assay ~14 HRS. Wash and mount slides

Prepare the 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 Duplex Positive Control Probe
• RNAscope 2.5 VS Duplex Negative Control Probe
• RNAscope VS Dewax
• VS PretreatPro*
• RNAscope VS Protease†
• RNAscope VS Universal Target Retrieval v2†
• RNAscope VS Duplex AMP 1
• RNAscope VS Duplex AMP 2
• RNAscope VS Duplex AMP 3
• RNAscope VS Duplex AMP 4
• RNAscope VS Duplex AMP 5
• RNAscope VS Duplex AMP 6
• RNAscope VS Duplex AMP 7
• RNAscope VS Duplex AMP 8
• RNAscope VS Duplex AMP 9
• RNAscope VS Duplex AMP Wash
• 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 Universal Sample Prep v2 Kit
	• mRNA Duplex Amp Kit
	• mRNA Red Detection Kit
	• One of the following:
	  • mRNA DAB Detection Kit
	  • mRNA Teal Detection Kit
	  • mRNA Purple Detection Kit
	  • mRNA Green Detection Kit
	• User fillable dispensers
	• mRNA Link
		• Distilled water
		• Dawn detergent or similar detergent
		• Fume hood
		• xylene
		• Tissue-Tek Staining Dish
		• Tissue-Tek Clearing Agent Dish, xylene resistant-resistant
		• Tissue-Tek Vertical 24 Slide Rack
		• EcoMount
		• Cover Glass, 24 mm x 50 mm

* 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.

Prepare the instrument

Most tissue types can be fully automated using the DISCOVERY ULTRA Kits. Manual pretreatment may give better results in some cases. Use the semi-automated procedure in **Appendix A** for tissues that do not have a satisfactory result when using the fully automated procedure.

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

Dilute bulk reagents

Please prepare the bulk fluids as per manufacturer's instructions.

Register new reagents

Reagent dispensers come with appropriate barcode labels and registration buttons for dispensing RNAscope VS Duplex 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".

Note: For the duplex target probe, user's should log them into the software as 1 reagent. Then probes are pooled into one dispenser prior to registration.

2. Use the wand or scanner that comes with the instrument to register *new* reagent kits.

Prepare instrument reagents

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

1. For RNAscope VS Duplex **AMP 1** to **AMP 9 and Amp Wash**, transfer the entire volume of each component into the correspondingly labeled dispenser.
   a. Transfer the remaining RNAscope VS Duplex Reagents (VS Positive Control Probe, VS Negative Control Probe, VS Protease, VS Hematoxylin, and VS Bluing Reagent) to the correspondingly labeled dispensers.
   b. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
2. Continue by preparing the VS Target Probe(s), for the duplex target probe(s)
   a. Determine the volume of duplex probe pool needed. Calculate 200µl per slide, then add an additional 500µl to account for any potential loss during dispenser priming or testing.
   b. In a clean container, dilute the 16X C2 probe concentrate stock into the appropriate volume of Ready-to-Use C1 probe to prepare required volume for the staining run.
   c. Invert the solution minimally 5 times to mix the probes.
   d. Transfer the solution to a clean Ventana Open Probe dispenser.
   e. Properly prime the dispenser and place it on the instrument.

Note: 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.

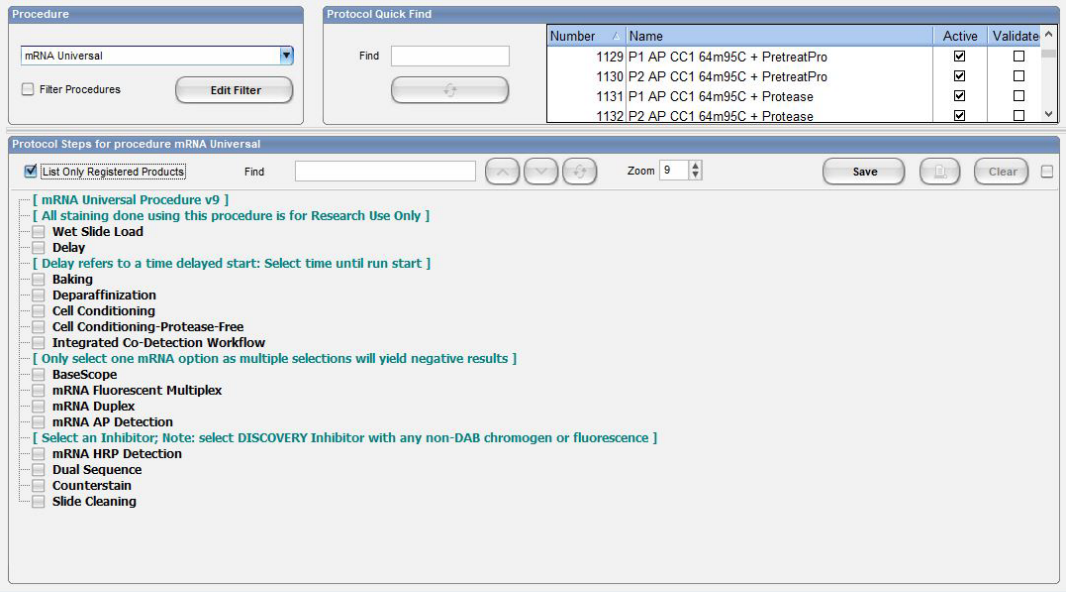
3. Check solution levels: DISCOVERY Wash, 2X SSC, Reaction Buffer, ULTRA LCS (Predilute), and CC1. Refill if they are less than half full. To run a full tray of 30 slides, fully fill the 2X SSC and Reaction Buffer containers.

IMPORTANT! Do not use expired reagents.

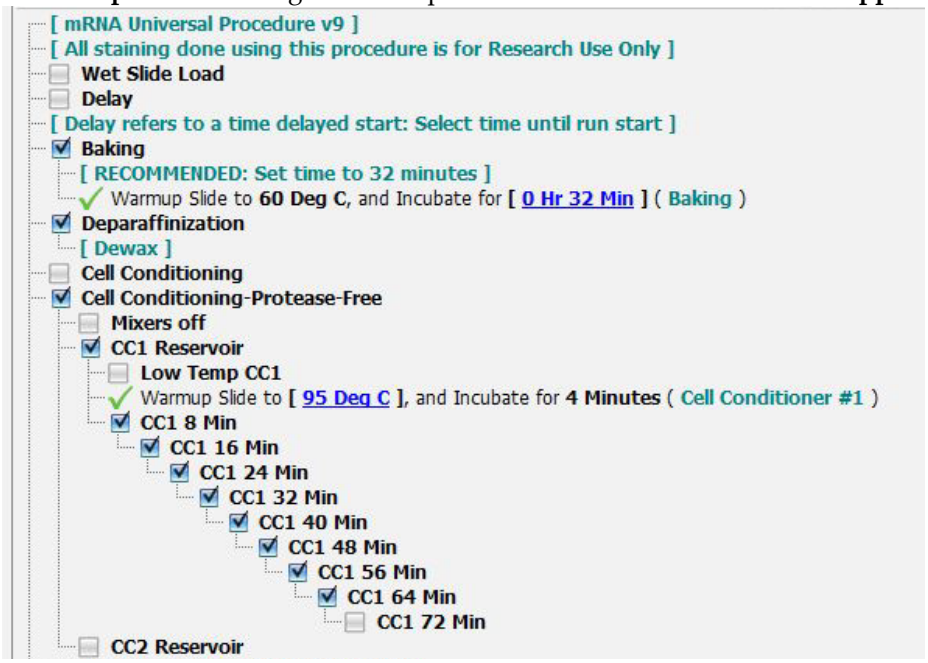
1. Empty the waste carboy if needed.

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**.
3. Main protocol steps appear as shown:



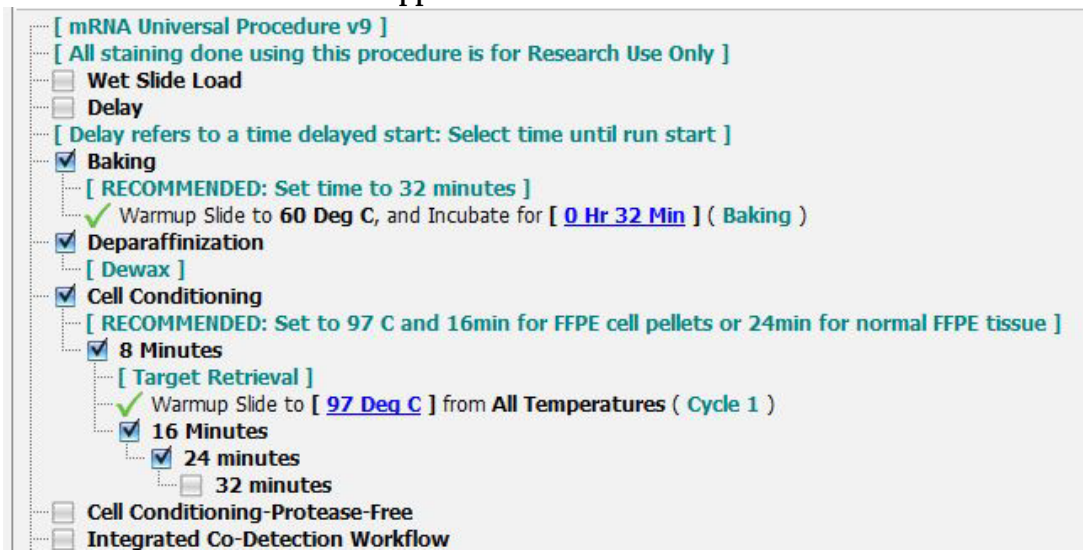
4. Do one of the following, depending on your selected workflow:
   - For target retrieval using the **Protease-Free Workflow**, select the appropriate pretreatment conditions according to the following screenshot. After selecting the main steps, drop-down menus become available. We recommend selecting **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.**



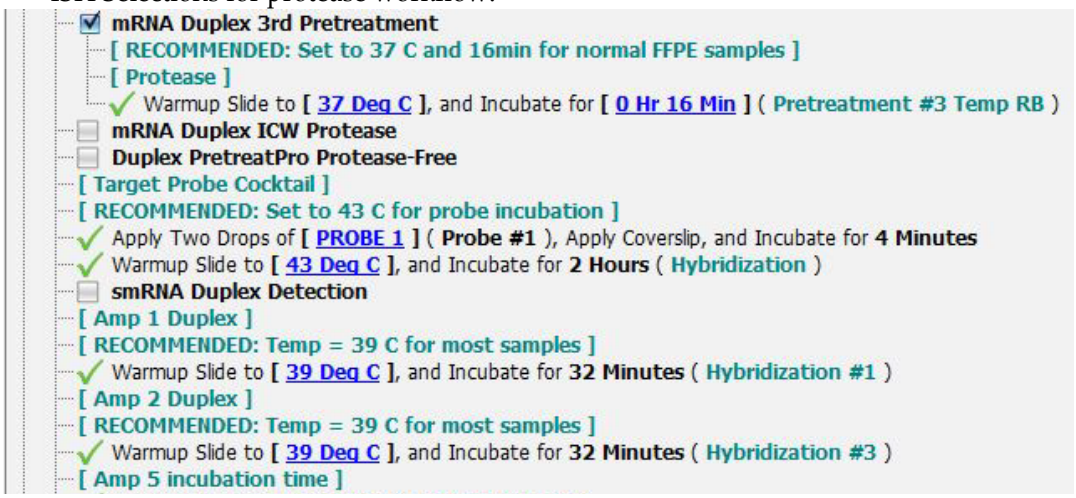
Note: The duration of tissue cell conditioning corresponds to the longest selected time. It is not the sum of all selected times.

5. For ISH staining with RNAscope VS Duplex, select **mRNA Duplex**.

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

6. Do one of the following, depending on your selected workflow:
   - If using the **Protease-Free Workflow**, select **Duplex PretreatPro Protease-Free** 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 **mRNA Duplex 3rd Pretreatment** to apply and incubate the VS Protease reagent. We recommend incubating this reagent for **16 MIN** at **37°C**.
   - ISH Selections for protease-free workflow:

- ISH Selections for protease workflow:



7. Select the remaining ISH staining conditions according to the following screenshots, depending on your selected workflow. There are three color combination available: Red/Brown, Red/Teal, and Red/Green.
   - For Red/Brown:
     a. After selecting mRNA Duplex, select Inhibitor, then choose Red-DAB Inhibitor.

     Note: The inhibitor selection must match the chromogen pairing used in the assay.

     b. After setting the Amp 8 incubation time, select the **Red-DAB AP-HRP Duplex** option.

c. Select the appropriate protocol steps by clicking on the associated check boxes as shown:

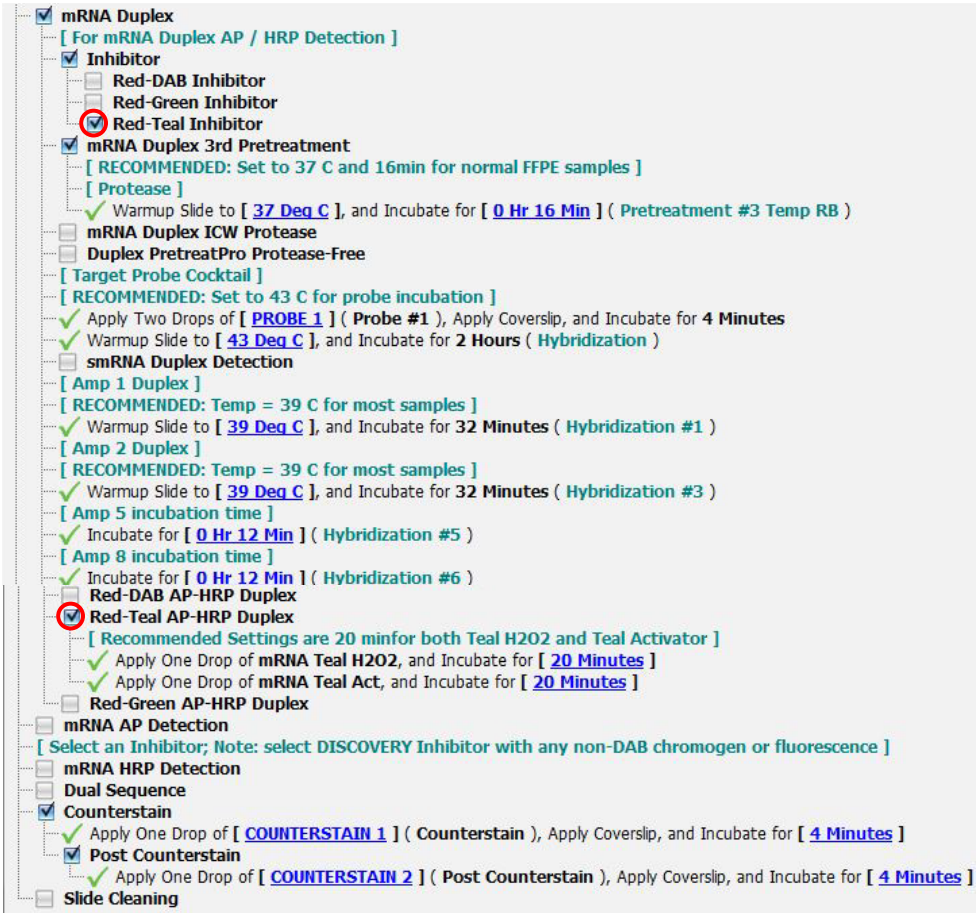
- For Red/Teal:

  a. After selecting **mRNA Duplex**, select **Inhibitor**, then choose **Red-Teal Inhibitor**.

  Note: The inhibitor selection must match the chromogen pairing used in the assay.

  b. After setting the Amp 8 incubation time, select the **Red-Teal AP-HRP Duplex** option. Recommended times for Teal incubation are **20 min mRNA Teal H_2O_2** and **20 min mRNA Teal Act**.

c. Select the appropriate protocol steps by clicking on the associated check boxes as shown:



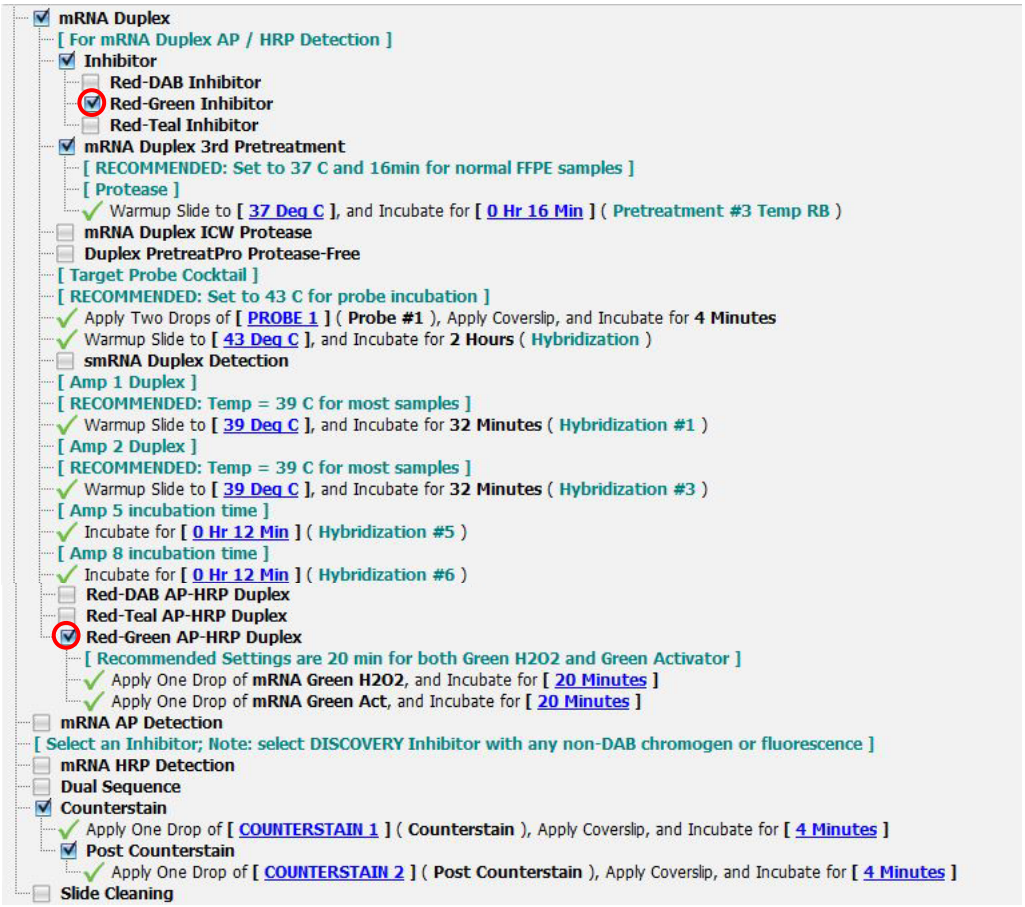
- **For** Red/Green:

  a. After selecting **mRNA Duplex**, select **Inhibitor**, then choose **Red-Green Inhibitor**.

  Note: The inhibitor selection must match the chromogen pairing used in the assay.

  b. After setting the Amp 8 incubation time, select the **Red-Green AP-HRP Duplex** option. Recommended times for Green incubation are **20 min mRNA Green H_2O_2** and **20 min Green Act**.

c. Select the appropriate protocol steps by clicking on the associated check boxes as shown:



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

Standard Times/Temperatures for mRNA Duplex Detection	
Cell Conditioning*	24 MIN @97°C
Cell-Conditioning–Protease-Free (CC1)*	64 MIN @95°C
VS Protease temperature and time*	37°C, 16 MIN
Duplex PretreatPro (protease-free) temperature and time*	40°C, 32 MIN
Suggested probe temperature	Duplex Probe combinations - 43°C HPV Pooled Probes - 50°C
Suggested RNAscope Duplex AMP 1 and AMP 2 temperature	39°C†
RNAscope Duplex AMP 5 & AMP 8 incubation time	Instrument titrated ‡
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 Duplex Amp 5 and Amp 8 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 H2O2 incubation time.

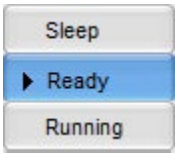
7. Select your preferred counterstain and post-counterstain settings. For optimal visualization of multiplex chromogenic staining, a light counterstain is recommended.
8. At the top of the Protocol Steps window, click **Save As**, then select a unique protocol number from the drop-down menu and choose a protocol name.
9. Click **Active**, specify any relevant comments in the available field, and then click **Save**.
10. Click **Close** to go back to the main screen.
11. 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.
12. 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.
13. Click on **Protocol**.
14. Select the protocol you created in the section above and click the **Add** button.
15. When the protocols for all the slides have been assigned, click on **Close/Print**.
16. Fill in the template for each slide. Click **Print** when completed.

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. Prime the user-fillable dispensers. For guidance, refer to the instructions provided by Roche Tissue Diagnostics.
3. 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
17. Remove the yellow locking ring from the dispensers for all prefilled reagents. Refer to the instructions provided by Ventana Medical Systems.
18. Load the reagent racks onto the reagent carousel.
6. Select the **Ready** button.

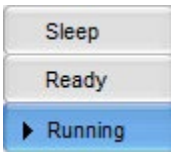


19. Eject slide drawers.

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

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

21. Close slide drawers.
22. Select the **Running** button.



23. The assay duration varies based on assay selections, approximately **14 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 mRNA Dewax reagent and Target Retrieval v2, place nozzle caps on the dispensers, and store at room temperature.
2. For the remaining reagents, place nozzle caps back on the dispensers and place racks onto magnet locking tray. Store at **4°C**.

Wash and dry the slides

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

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

10. Submerge a Tissue-Tek Slide Rack into the Tissue-Tek Staining Dish containing 200 mL diluted detergent.
11. Open the instrument slide drawers and unload slides.
12. Decant solution on the slides into the slide drawer, then *immediately* load slides into the Tissue-Tek Slide Rack submerged in detergent.
13. Rinse oil off the slides by moving the slide rack up and down in the dish 10 times.
14. Replace the detergent with distilled water and rinse slides by moving the slide rack up and down a minimum of **10** times.
15. Repeat Step 8 three to five times.
8. Place slides in a drying oven at **60°C** for at least **30 MIN**.

Mount the samples

1. Remove the slide rack from the staining dish and dry slides in a **60°C** dry oven for **30 MIN**.

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

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

IMPORTANT! Use the EcoMount mounting medium only.

4. Carefully place 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat steps 2 and 3 for each slide.
6. Air dry slides for at least **5 MIN.**
7. Proceed to **Chapter 8. Evaluate the Results**.

6 Chapter 6. Guidance for staining smRNA (miRNA) targets with the chromogenic VS Duplex assay

The RNAscope VS Duplex Assay can be used for chromogenic detection of up to two smRNA targets or for combined chromogenic detection of 1 smRNA and 1 RNAscope target. For duplex detection, you can choose either C1 (mRNA) or S1 (smRNA) for the first-channel probe, and either C2 (mRNA) or S2 (smRNA) for the second-channel probe . Do not combine C- and S-probes with the same number designation. For duplex detection, we recommend assigning the target with the higher expected or known expression level to the first channel (C1 or S1), while the target with the lower expected or known expression level should be assigned to the second channel (C2 or S2). If you are combining RNA and smRNA detection where both are expected to have low expression levels, place the smRNA target in S2.

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 the 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. These control probes are single-channel (not pre-mixed) and should be combined in the same way as RNAscope VS Duplex target probes.

Control probes: smRNA (miRNA)

Both RNU6 and SNORD54 (human), SNORD85 (mouse), and SNORD95 (rat) are non-coding RNAs expressed in the nucleus. RNU6 is highly expressed and is recommended as a positive control in S1. The SNORD targets have lower relative expression levels and are recommended as positive controls in S2.

Probe name	Species	Cat. No.
miRNAscope VS Positive Control Probe - SR-RNU6-S1	Human	727879-S1
	Mouse	727879-S1
	Rat	727879-S1
miRNAscope VS Positive Control Probe - SR-Hs-SNORD54	Human	728939-S2
miRNAscope VS Positive Control Probe - SR-Mm-SNORD85	Mouse	728959-S2
miRNAscope VS Positive Control Probe - SR-Rn-SNORD95	Rat	729059-S2
miRNAscope VS Negative Control Probe - SR-Scramble-	Any (Negative)	727889S1 727889-S2

Control probes: RNAscope

For assays combining smRNA and RNAscope detection, single-channel RNAscope control probes can be combined with the smRNA control probes listed in the previous table. Both targets are messenger RNAs, with most expression localized in the cytoplasm. Generally, PPIB shows medium expression levels, while POLR2A exhibits lower expression.

When combining mRNA and smRNA detection, we recommend matching your control probe combination to your target probe combination. For example, in human tissue, if your target mRNA is in C1 and your target smRNA is in S2, your positive control would be Hs-PPIB-C1 / SNORD54-S2. Conversely, if your target smRNA is in S1 and your target mRNA is in C2, your positive control would be RNU6-S1 / Hs-POLR2A-C2.

Species	Probe name	Cat. No.
Human	RNAscope 2.5 VS Positive Control Probe_Hs-PPIB	313909 313909-C2
	RNAscope 2.5 VS Positive Control Probe - Hs-POLR2A	310459 310459-C2
Mouse	RNAscope™ 2.5 VS Positive Control Probe_Mm-PPIB	313919 313919-C2
	RNAscope 2.5 VS Positive Control Probe - Mm-Polr2a	312479 312479-C2
Rat	RNAscope 2.5 VS Positive Control Probe - Rn-Ppib	313929 313929-C2
	RNAscope 2.5 VS Positive Control Probe - Rn-Polr2a	312489 312489-C2
Any	RNAscope 2.5 VS Negative Control Probe - DapB	312039 312039-C2

Register new reagents

Using the instructions in **Chapter 5**, register the reagents along with the following additions:

- 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

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

1. Fill the Probe dispenser with your cocktailed duplex smRNA or mRNA/smRNA probe.
2. Transfer the smRNA reagent into the corresponding test detection dispenser.
3. 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**:**

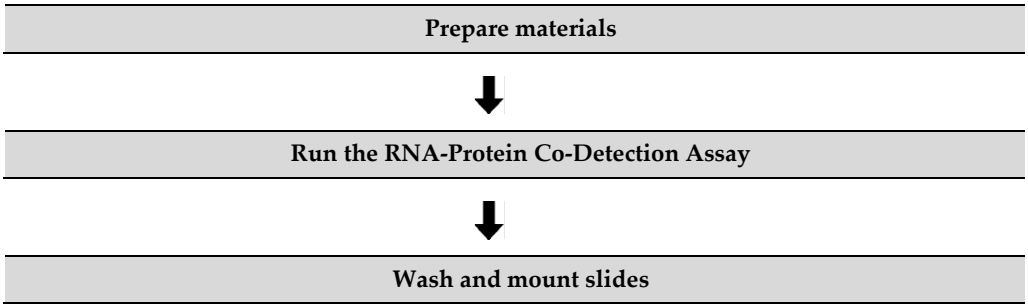
1. After selecting the target probes, choose **smRNA Duplex Detection.**
2. Select the corresponding test dispenser.
3. Set the recommended temperature to **39°C**.

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
• RNAscope VS Protease†
• VS PretreatPro*
• RNAscope VS Duplex AMP 1
• RNAscope VS Duplex AMP 2
• RNAscope VS Duplex AMP 3
• RNAscope VS Duplex AMP 4
• RNAscope VS Duplex AMP 5
• RNAscope VS Duplex AMP 6
• RNAscope VS Duplex AMP 7
• RNAscope VS Duplex AMP 8
• RNAscope VS Duplex AMP 9
• RNAscope VS Duplex AMP Wash
• 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 Universal Sample Prep v2 Kit
	• mRNA Duplex Amp Kit
	• mRNA Red Detection Kit
	• One of the following
	  • mRNA DAB Detection Kit
	  • mRNA Teal Detection Kit
	  • mRNA Green Detection Kit
	User fillable dispensers
	IHC detection options (see Chapter 1)
		• Distilled water
		• Dawn detergent or similar detergent
		• Fume hood
		• Xylene
		• Tissue-Tek Staining Dish
		• Tissue-Tek Clearing Agent Dish, xylene-resistant
		• Tissue-Tek Vertical 24 Slide Rack
		• EcoMount
		• Cover Glass, 24 mm x 50 mm
		• Primary antibody
		ProLong Gold Antifade Reagent

* 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.

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 or scanner 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.

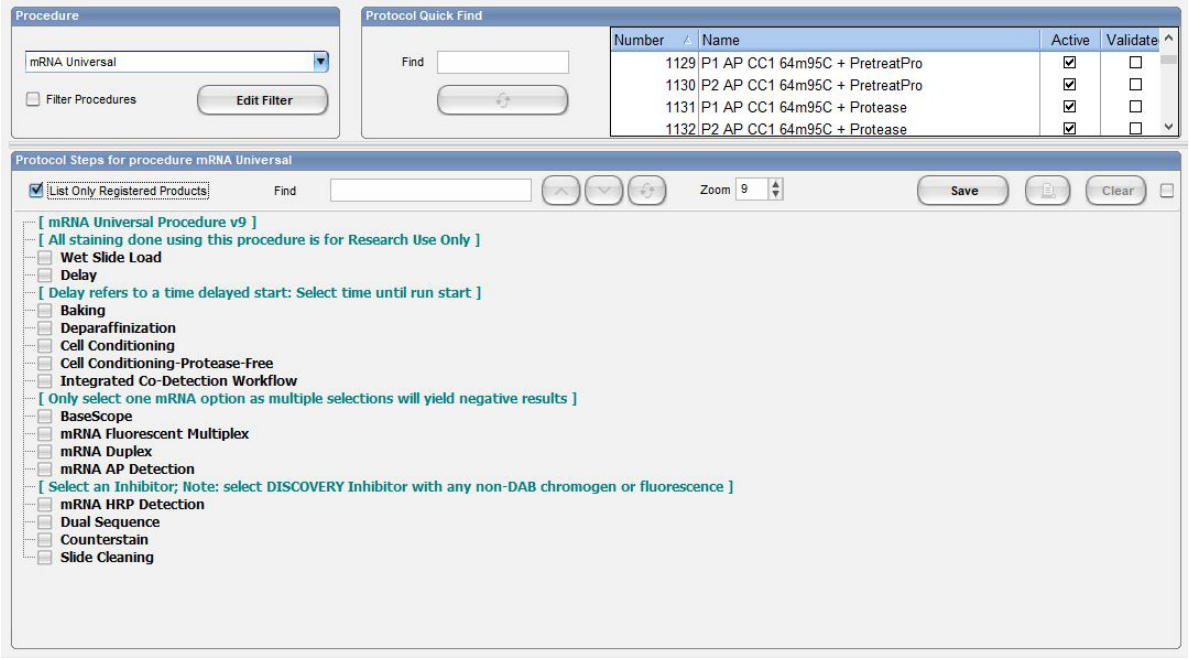
1. Transfer the entire volume of each AMP component of the Detection Kit to the corresponding labeled dispenser from the appropriate mRNA Amplification kit.
2. Fill the mRNA Sample Prep Kit by transferring the VS Dewax reagent from the RNAscope VS Sample Prep Reagent Kit v2 to the mRNA Dewax Dispenser.

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

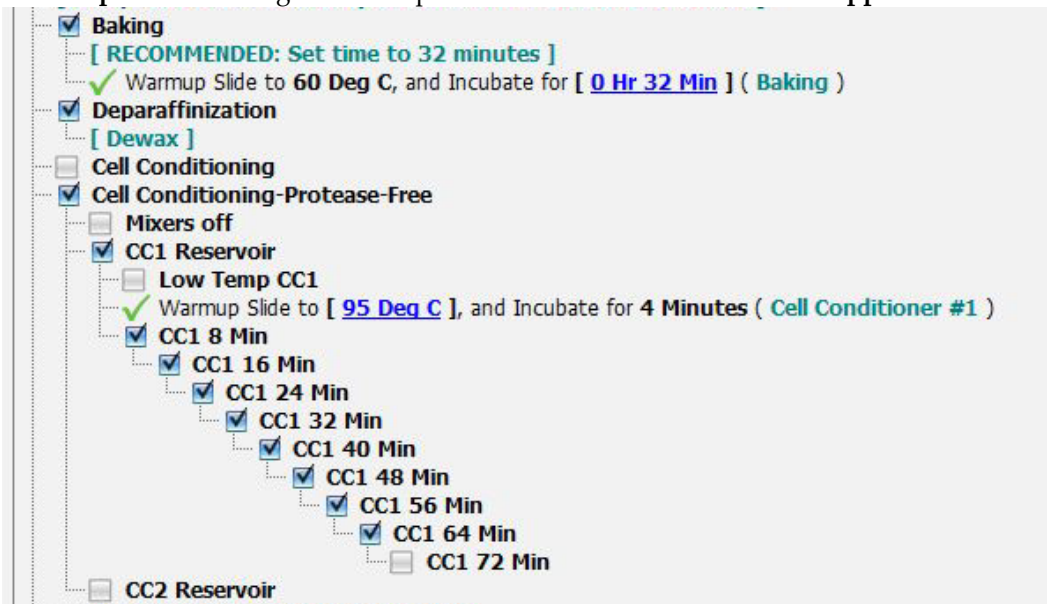
3. Fill the following dispensers:
   a. Transfer the RNAscope 2.5 VS Target Probe and control probes to the corresponding probe dispensers.
   b. Transfer the VS PretreatPro and VS CoDetectPro from the RNAscope VS Pro Reagents to the open pretreatment dispensers. Ensure the barcode number aligns with 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.

3. To enable ISH staining with RNAscope VS Duplex, select **mRNA Duplex**.

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

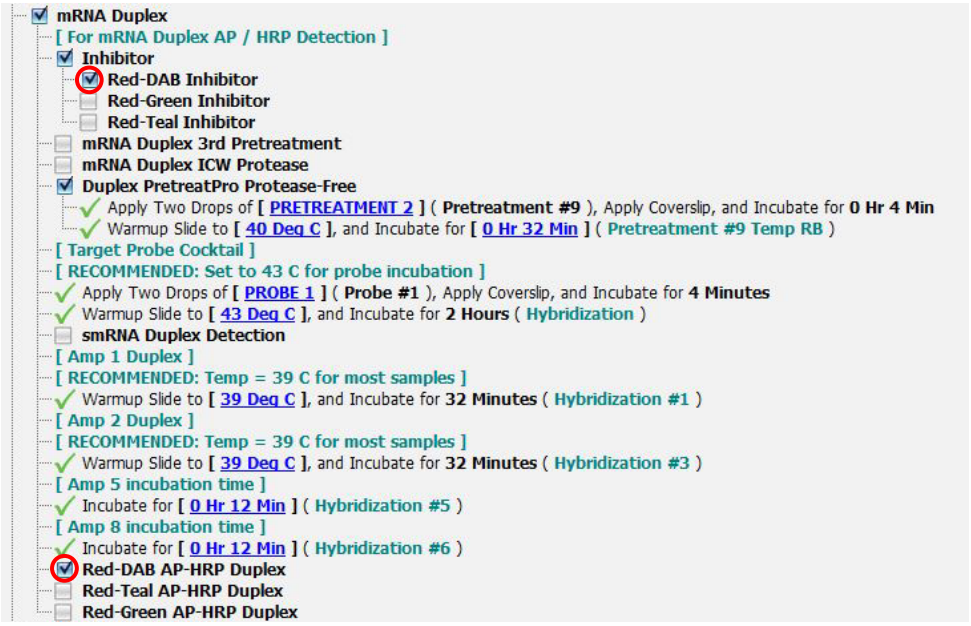
6. Select **Duplex PretreatPro Protease-Free** to apply and incubate the VS PretreatPro reagent. We recommend incubating this reagent for **32 MIN** at **40°C**.

7. Select the remaining ISH staining conditions according to the following screenshots, depending on your selected workflow. There are three color combinations available: Red/Brown, Red/Teal, and Red/Green.

- For Red/Brown:
  a. After selecting mRNA Duplex, select Inhibitor, then choose Red-DAB Inhibitor.

  Note: The inhibitor selection must match the chromogen pairing used in the assay.

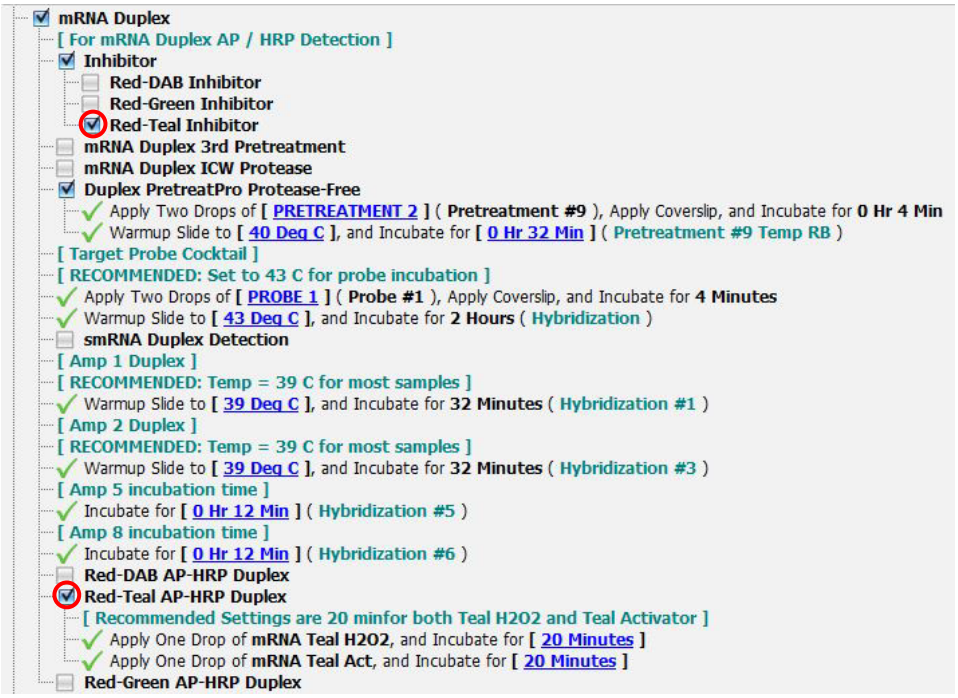
  b. After setting the Amp 8 incubation time, select the **Red-DAB AP-HRP Duplex** option.
  c. Select the appropriate protocol steps by clicking on the associated check boxes as shown:



- For Red/Teal:
  a. After selecting **mRNA Duplex**, select **Inhibitor**, then choose **Red-Teal Inhibitor**.

  Note: The inhibitor selection must match the chromogen pairing used in the assay.

  b. After setting the Amp 8 incubation time, select the **Red-Teal AP-HRP Duplex** option. Recommended times for Teal incubation are **20 min mRNA Teal H_2O_2** and **20 min mRNA Teal Act**.
  c. Select the appropriate protocol steps by clicking on the associated check boxes as shown:



- **For** Red/Green:
  a. After selecting **mRNA Duplex**, select **Inhibitor**, then choose **Red-Green Inhibitor**.

Note: The inhibitor selection must match the chromogen pairing used in the assay.

b. After setting the Amp 8 incubation time, select the **Red-Green AP-HRP Duplex** option. Recommended times for Green incubation are **20 min mRNA Green H_2O_2** and **20 min Green Act**.

c. Select the appropriate protocol steps by clicking on the associated check boxes as shown:

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

Standard Times/Temperatures for Protease-Free mRNA Duplex Detection	
Cell-Conditioning–Protease-Free (CC1)	64 MIN @95°C
Duplex PretreatPro (protease-free) temperature and time	40°C, 32 MIN
Suggested probe temperature	Duplex Probe combinations - 43°C HPV Pooled Probes - 50°C
Suggested RNAscope Duplex AMP 1 and AMP 2 temperature	39°C†
RNAscope Duplex AMP 5 & AMP 8 incubation time	Instrument titrated ‡
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

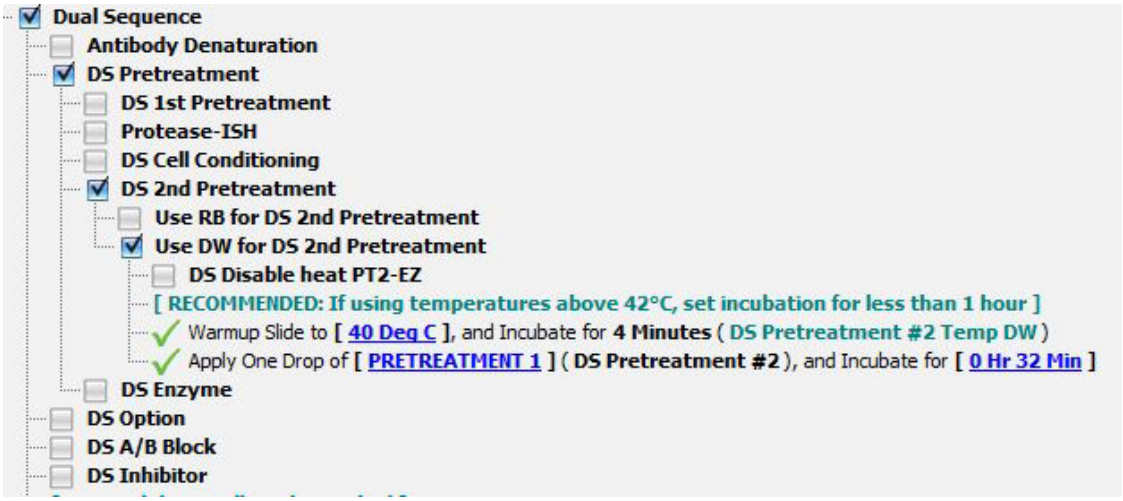
† If very high nuclear background is observed, you can raise temperatures to 40 or 41°C. However, some signal sensitivity may be lost. Please contact support or your FAS before making these changes.

‡ VS Universal Duplex Amp 5 and Amp 8 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.

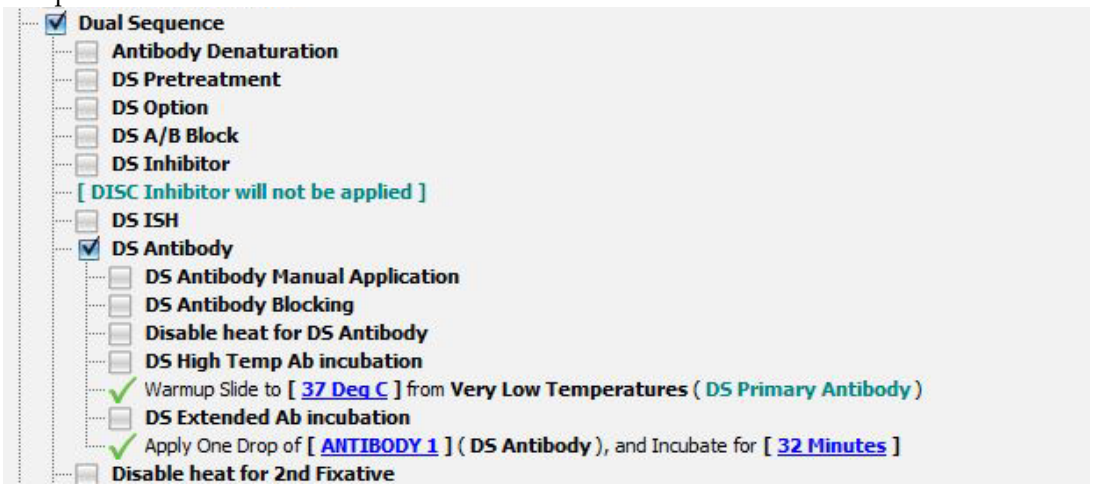
^ The strength of mRNA Teal and mRNA Green detection can be modulated through adjustment of the mRNA Teal/Green H2O2 incubation time.

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.

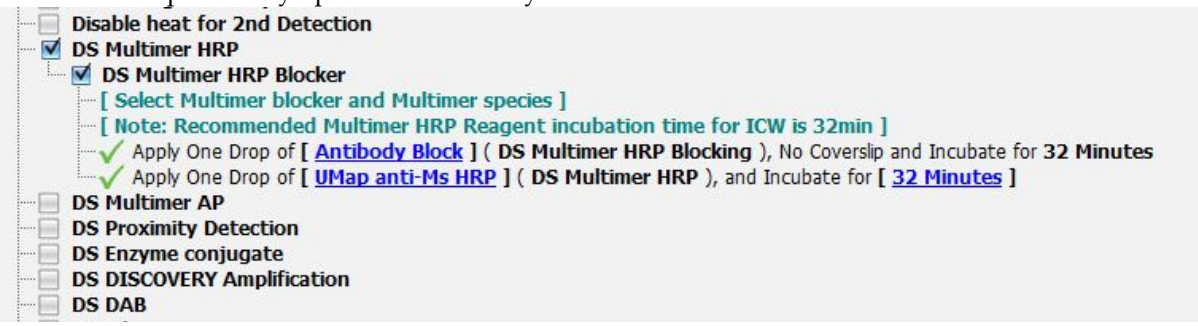


12. Select the settings for secondary detection:

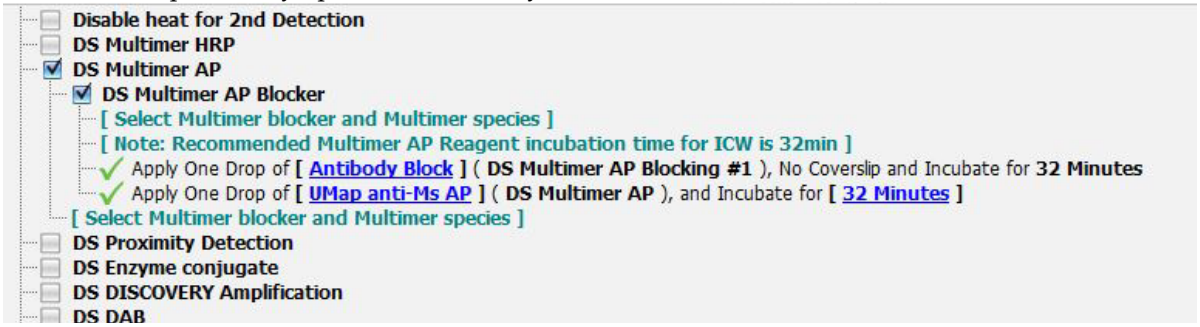
Note: To prevent non-specific cross-detection of RNAscope, we recommend using Roche's Antibody Block reagent immediately before applying the secondary antibody. Skipping the Antibody Block step could cause a hue shift in the RNA dots, making it difficult to accurately interpret RNA-protein colocalization.

- Do the following for HRP-based secondary detection:

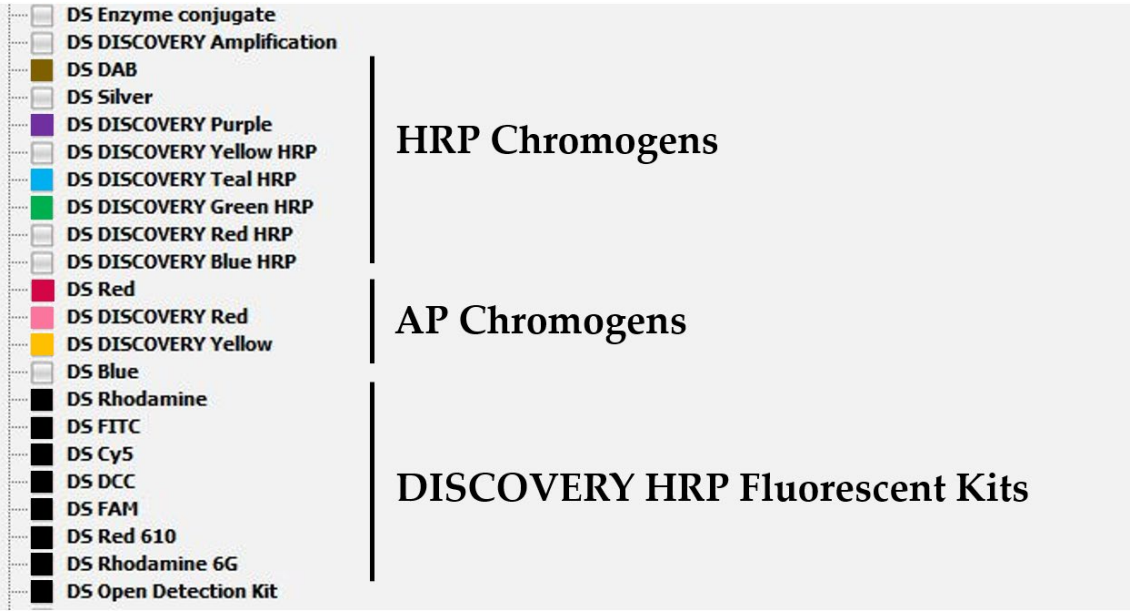
a. Choose a Roche Multimer HRP reagent corresponding to the primary host species.
b. To enable secondary incubation, select **DS Multimer HRP**.
c. To apply Antibody Block, select **DS Multimer HRP Blocker**, then select Antibody Block reagent.
d. Select your Multimer HRP reagent of choice and desired 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:

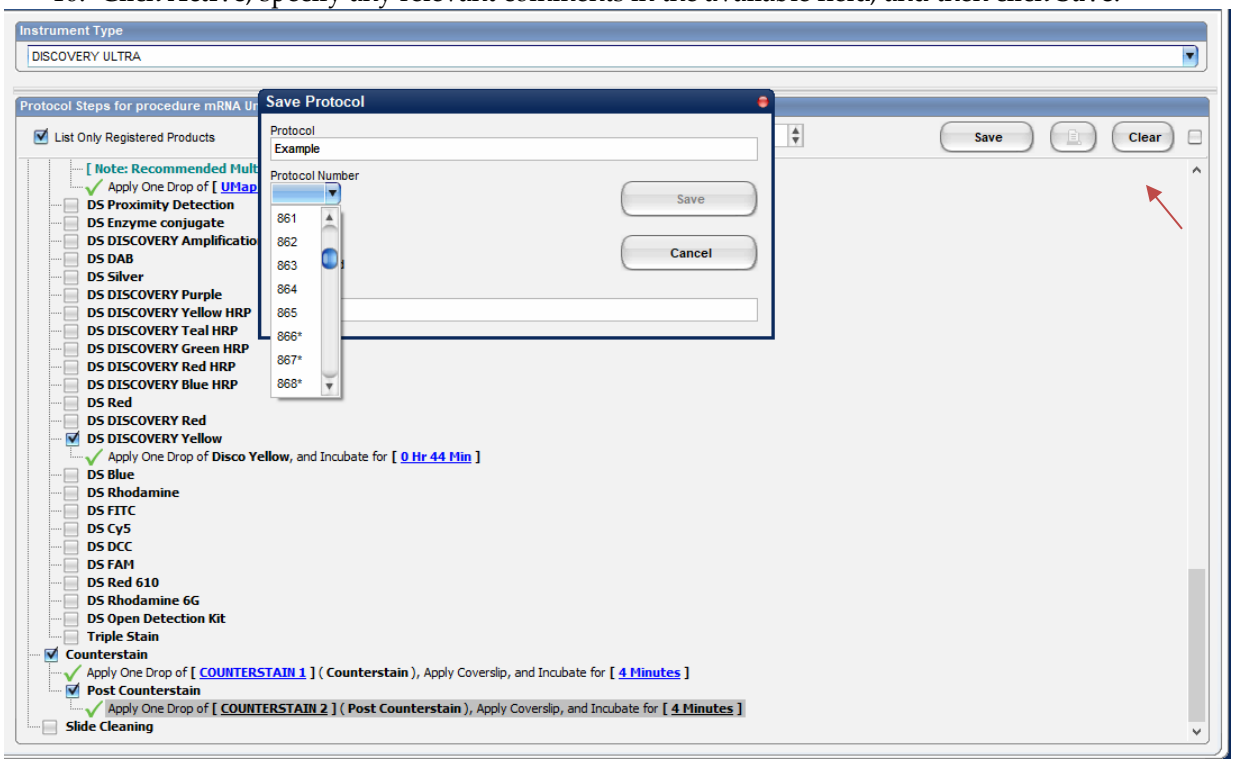


IHC Chromogen Selection and Settings		
IHC Enzyme	**IHC Chromogen**	**Recommended Chromogen Settings***
AP	DISCOVERY Yellow	44 MIN – 2 HRS
	DISCOVERY Red	12 MIN†
	DS Red / ChromoMap Red‡	Default
HRP (chromogenic)	DS DAB / ChromoMap DAB	Default
	DISCOVERY Purple	40 MIN
	DISCOVERY Teal HRP	DISCOVERY Teal 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**).

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.
    - 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.

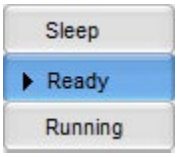
Run the RNA-Protein Co-Detection Assay

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.
1. 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.

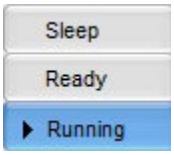
2. Remove the yellow locking ring from the dispensers in all the prefilled dispensers. Refer to the instructions provided by Roche Tissue Diagnostics.
3. Load the dispensers onto the reagent racks.
4. Load the reagent racks onto the reagent carousel.
5. Select the **Ready** button.



6. Open the slide drawers.
7. 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.

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



Note: 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, 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. 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.
10. Place slides in a drying oven at 60°C for at least **30 MIN**.

Mount the samples

1. Remove the slide rack from the staining dish and dry slides in a **60°C** dry oven for **30 MIN**.

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

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

IMPORTANT! Use the EcoMount mounting medium only.

4. Carefully place 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat steps 2 and 3 for each slide.
6. Air dry slides for at least **5 MIN.**
7. Proceed to **Chapter 8. Evaluate the Results**.

8 Chapter 8. Evaluate the Results

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

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

Scoring guidelines

The RNAscope VS Duplex Assay enables a semi-quantitative scoring guideline utilizing the estimated number of punctate dots present within each cell boundary.

An example of how to develop such a guideline for semi-quantitative assessment of RNAscope staining intensity is given for a gene with expression level varying between 1 to > 10 copies per cell.

Note: If your gene expression level is higher or lower than this range, you may need to scale the criteria accordingly.

Categorize staining into five grades: 0, 1+, 2+, 3+, and 4+ according to the following table:

Staining Score	Scoring Definition
0	No staining, or less than 1 dot/10 cells
1	1–3 dots/cell
2	4–9 dots/cell or very few dot clusters
3	10-15 dots/cell. < 10% positive cells are in clusters
4	>15 dots/cell and >10% dots are in clusters (visible at 20X magnification)

* Discount cells with artificially high nuclear background staining.

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.

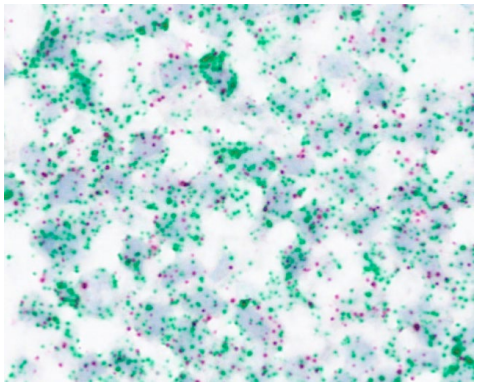
Troubleshooting

For troubleshooting information, please contact your local Field Application Scientist or technical support at **support.acd@bio-techne.com**.

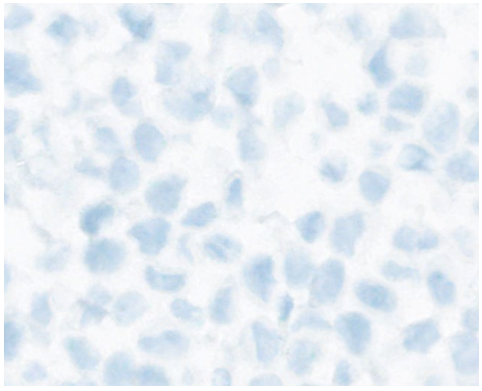
Tissue example

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

Figure 1. RNAscope VS Duplex Assay results in HeLa cells using control probes

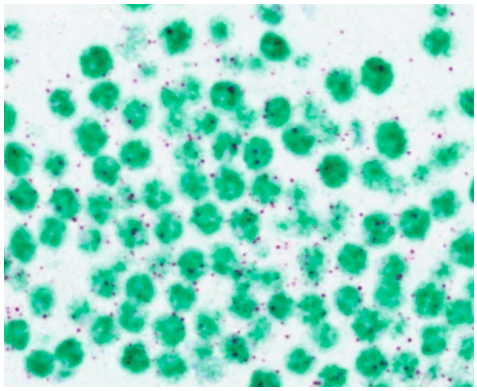


Hs-POLR2A-C1 (Green) / Hs-TBP-C2 (Red) (Positive Control)

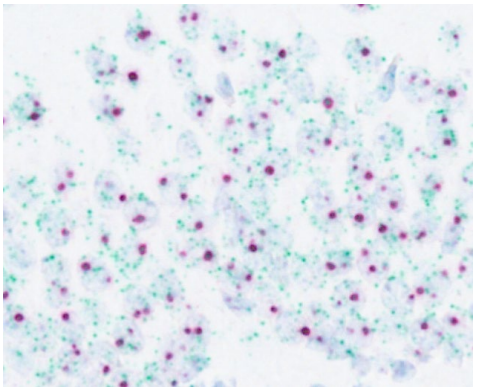


DapB / DapB (Negative Control)

Figure 2. smRNA/mRNA results with the RNAscope VS Duplex Assay in mouse brain tissue using control probes



RNU6-S1 (Green) / POLR2A-C2 (Red) (Positive Control)



PPIB-C1 (Green) / Snord85-S2 (Red) (Positive Control)

Scramble / Scramble (Negative Control)

A Appendix A. Semi-automated RNAscope VS Duplex Assay

Most sample types can be fully automated on the Discovery ULTRA. 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! We highly recommend following these guidelines. We cannot guarantee assay results with other preparation methods.

Workflow

Prepare the materials

↓

Bake the slides

↓

Deparaffinize FFPE sections

↓

Pretreat the slides

↓

Run the RNAscope Duplex Assay ~12 HRS

↓

Wash and mount slides

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)

* Not provided with the kit and needs to be purchased separately.

IMPORTANT! Do not substitute the reagent components of the RNAscope VS Duplex 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 separately purchase 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..

Materials required

Materials provided by Advanced Cell Diagnostics	Materials Provided by Ventana Medical Systems	Other Materials and Equipment

• RNAscope 2.5 VS Target Probe
• RNAscope 2.5 VS Duplex Positive Control Probe
• RNAscope 2.5 VS Duplex Negative Control Probe
• RNAscope Target Retrieval Reagents
• RNAscope VS Protease
• RNAscope VS Duplex AMP 1
• RNAscope VS Duplex AMP 2
• RNAscope VS Duplex AMP 3
• RNAscope VS Duplex AMP 4
• RNAscope VS Duplex AMP 5
• RNAscope VS Duplex AMP 6
• RNAscope VS Duplex AMP 7
• RNAscope VS Duplex AMP 8
• RNAscope VS Duplex AMP 9
• RNAscope VS Duplex AMP Wash
• RNAscope VS Hematoxylin
• RNAscope VS Bluing Reagent
	• DISCOVERY™ ULTRA — automated slide stainer
	• DISCOVERY Wash 10x
	• ULTRA LCS (Predilute)
	• SSC Buffer 10X
	• DISCOVERY CC1
	• Reaction Buffer 10X
	• Probe dispensers
	• mRNA Sample Prep Kit
	• mRNA Duplex Amp Kit
	• mRNA Red Detection Kit
	• One of the following
	  • mRNA DAB Detection Kit
	  • mRNA Teal Detection Kit
	  • mRNA Purple Detection Kit
	  • mRNA Green Detection Kit
	• User fillable dispensers
	• mRNA Link
		• Distilled water
		• Glass beaker (1 or 2 L)
		• Hot plate
		• Dawn detergent or similar detergent
		• Fume hood
		• xylene
		• 100% ethanol (EtOH)
		• Tissue-Tek Staining Dish (3)
		• Tissue-Tek Clearing Agent Dish, xylene-resistant (3)
		• Tissue-Tek Vertical 24 Slide Rack
		• EcoMount
		• Cover Glass, 24 mm x 50 mm

Prepare the instrument

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

Dilute bulk reagents

Please prepare the bulk fluids as per manufacturer's instructions.

Register new reagents

Reagent dispensers come with appropriate barcode labels and registration buttons for dispensing RNAscope VS 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 scanner that comes with the instrument to register *new* reagent kits.

Prepare instrument reagents

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

1. For RNAscope VS Duplex **AMP 1** to **AMP 9 and Amp Wash**, transfer the entire volume of each component into the correspondingly labeled dispenser.
2. Transfer the remaining RNAscope VS Duplex Reagents (VS Target Probe, VS Positive Control Probe, VS Negative Control Probe, VS Protease, VS Hematoxylin, and VS Bluing Reagent) to the correspondingly labeled dispensers.
3. Follow the dispenser product insert instructions to properly prime and handle the dispensers.
4. Continue by preparing the VS Target Probe(s), for the duplex target probe(s)
   a. Determine the volume of duplex probe pool needed. Calculate 200µl per slide, then add an additional 500µl to account for any potential loss during dispenser priming or testing
   b. In a clean container, Dilute the 16X C2 probe concentrate stock into the appropriate volume of Ready-to-Use C1 probe to prepare required volume for the staining run.
   c. Invert the solution minimally 5 times to mix the probes.
   d. Transfer the solution to a clean Ventana open Probe dispenser.
   e. Properly prime the dispenser and place it on the instrument.

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

5. Check solution levels: DISCOVERY Wash, 2X SSC, Reaction Buffer, ULTRA LCS (Predilute), and CC1. Refill if they are less than half full. To run a full tray of 30 slides, fully fill the 2X SSC and Reaction Buffer containers.

IMPORTANT! Do not use expired reagents.

6. Empty the waste carboy if needed.

Prepare deparaffinization reagents

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

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

Prepare 1X Target Retrieval

Prepare **1X** Target Retrieval while FFPE slides are baking at 60°C, or the following day if you choose the optional stopping point on page 59.

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

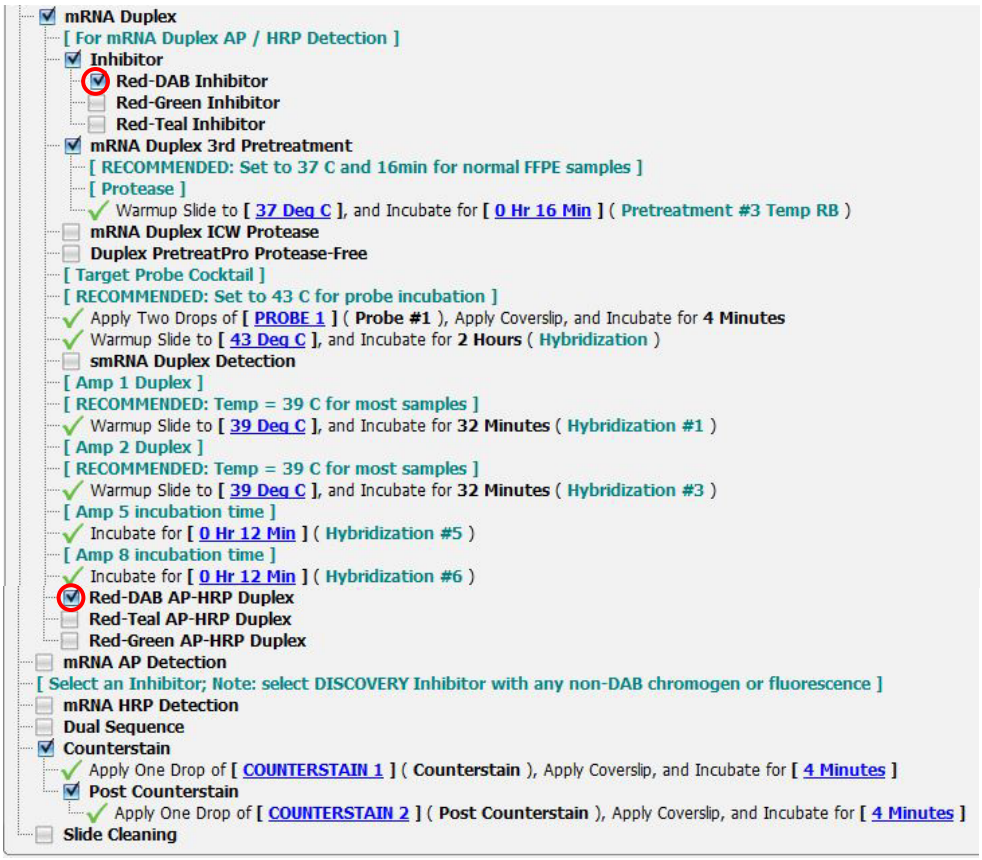
IMPORTANT! Do not use RNAscope VS Universal Target Retrieval v2 for offline boiling.

Create an instrument protocol

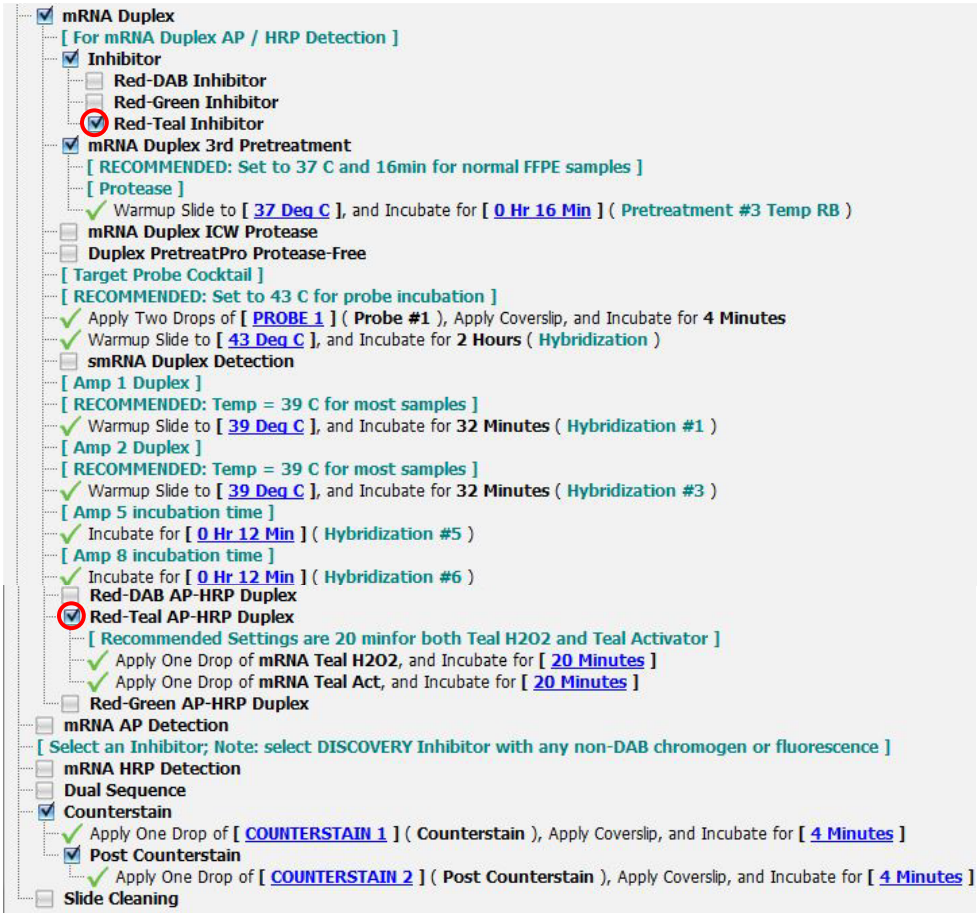
1. Open the VSS software and click on the Protocol button**.**
2. Click on Create/Edit Protocols, go to the Procedure drop down menu and select mRNA Universal.
3. Main protocol steps appear as shown:
   - For Red/Brown:
     a. After selecting mRNA Duplex, select Inhibitor, then choose Red-DAB Inhibitor.

     Note: The inhibitor selection must match the chromogen pairing used in the assay.

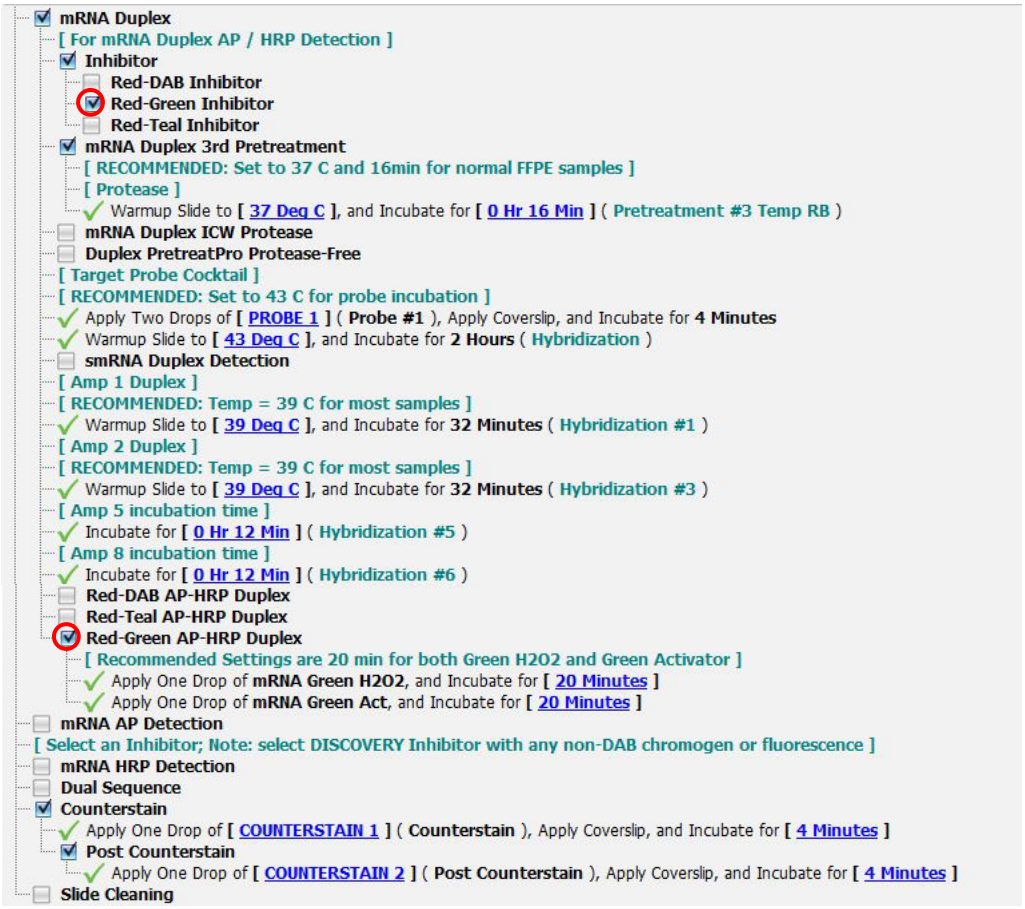
     b. After setting the Amp 8 incubation time, select the **Red-DAB AP-HRP Duplex** option.
     c. Select the appropriate protocol steps by clicking on the associated check boxes as shown:



- **For** Red/Teal:
  a. After selecting **mRNA Duplex**, select **Inhibitor**, then choose **Red-Teal Inhibitor**.
  Note: The inhibitor selection must match the chromogen pairing used in the assay.
  b. After setting the Amp 8 incubation time, select the **Red-Teal AP-HRP Duplex** option. The suggested times for Teal incubation are *20 min mRNA Teal* H_2O_2 and *20 min mRNA Teal Act*.
  c. Select the appropriate protocol steps by clicking on the associated check boxes as shown:



- **For** Red/Green:
  a. After selecting **mRNA Duplex**, select **Inhibitor**, then choose **Red-Green Inhibitor**.
  Note: The inhibitor selection must match the chromogen pairing used in the assay.
  b. After setting the Amp 8 incubation time, select the **Red-Green AP-HRP Duplex** option. The suggested times for Green incubation are *20 min mRNA Green* H_2O_2 and *20 min Green Act*.
  c. Select the appropriate protocol steps by clicking on the associated check boxes as shown:



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

4. After selecting the main protocol steps, drop down menus become available. Select the appropriate protocol steps by clicking on the associated check boxes as shown above.
5. Select the appropriate assay conditions from the drop-down menus according to the following table:

Standard Times/Temperatures for mRNA Duplex Detection	
Cell Conditioning*	24 MIN @97°C
Cell-Conditioning–Protease-Free (CC1)*	64 MIN @95°C
VS Protease temperature and time*	37°C, 16 MIN
Duplex PretreatPro (protease-free) temperature and time*	40°C, 32 MIN
Suggested probe temperature	Duplex Probe combinations - 43°C HPV Pooled Probes - 50°C
Suggested RNAscope Duplex AMP 1 and AMP 2 temperature	39°C†
RNAscope Duplex AMP 5 & AMP 8 incubation time	Instrument titrated ‡

*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 Duplex Amp 5 and Amp 8 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.

6. Click **Save As**, then select a protocol number from the drop-down menu and choose a protocol name for each probe. Click **Save.**
7. Click **Close** to go back to the main screen.
8. Assign a probe number from the list to each probe of interest. For each probe selected, assign a protocol.

Print the labels

1. Select the Print Label icon from the bottom of the home page screen.
2. Select your preferred template or create a new template. To create a new template, refer to the *Ventana™ System User Manual* for details.
3. Select the protocol you created for the RNAscope VS Duplex Assay.
4. Click on **Protocol** to add and print the label.

Manually pretreat the samples

Materials required

Materials Provided by the Target Retrieval Reagents (Cat. No. 322000)	Other Materials and Equipment
• RNAscope Target Retrieval Reagents	• Drying oven • FFPE slides • Tissue-Tek Vertical 24 Slide Rack • Distilled water • Fume hood • Xylene • 100% ethanol (EtOH) • Tissue-Tek Clearing Agent Dish (2) • Tissue-Tek Staining Dish (2) • Glass beaker (1 or 2 L) • Hot plate

Bake the slides

1. Bake slides in a dry oven for **30-60 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 Duplex 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. See page 56.

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

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.

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
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

Run the RNAscope VS Duplex Assay

Materials required

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

Load the reagents

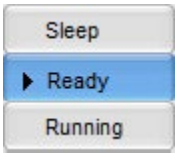
1. Remove the nozzle caps from the filled dispensers and place each cap on the post located on the back of the dispenser.
2. If needed, remove any air bubbles at the nozzle tips by pushing down on the nozzle until the liquid reaches the tip of the nozzle or forms a small meniscus at the tip of the nozzle

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 RED Detection Kit, mRNA DAB, Teal or Green Detection Kits, and mRNA Link**.** Refer to the instructions provided by Ventana™ Medical Systems.
5. Load the reagent racks onto the reagent carousel.

Start the run

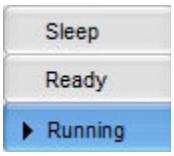
1. Click the **Ready** button.



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

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

4. Close slide drawers.
5. Click the **Running** button. Semi- automated assay will finish in **~12 HRS**.



IMPORTANT! Before leaving the instrument unattended, ensure all reagents and slides are successfully registered and the instrument is running.

Prepare detergent

1. Prepare 200 mL of diluted detergent by adding 1 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**.

Prepare dehydrating reagents

IMPORTANT! Do not use deparaffinization solutions for dehydration.

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

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

Complete the run

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

IMPORTANT! Store the Dewax dispenser at room temperature.

Wash the slides

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

Mount the samples

1. Remove the slide rack from the staining dish and dry slides in a **60°C** dry oven for **30 MIN**.

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

2. Cool the slides for **5 MIN** at **RT**.

3. Briefly **dip** one slide into fresh pure xylene and *immediately* place 1 to 2 drops of EcoMount on the slide before the xylene dries.

IMPORTANT! Use the EcoMount mounting medium only.

4. Carefully place 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat steps 2 and 3 for each slide.
6. Air dry slides for at least **5 MIN.**
7. 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 **https://acdbio.com/technical-support/user-manuals**.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

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

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **https://www.cdc.gov/biosafety/**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **https://www.osha.gov/lawsregs/regulations/standardnumber/1910/1910.1030**

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

In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/
- Information about the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) can be found at: https://echa.europa.eu/regulations/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: info.acd@bio-techne.com **.**

7707 Gateway Blvd. Newark, CA 94560
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