USER MANUAL

RNAscope™ HiPlex Pro for COMET™ Kit

For use with Lunaphore's COMET™ System

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Citing RNAscope in Publications

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

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1 Chapter 1. Product Information

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

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

About this guide

This user manual provides guidelines and protocols to use the RNAscope HiPlex Pro for COMET Kit on FFPE sections mounted on slides with Lunaphore's COMET System.

Product description

Background

The RNAscope HiPlex Pro Assay on COMET uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize up to 12 different RNA targets per cell in sectioned samples mounted on slides.

The assay is based on ACD's patented signal amplification and background suppression technology and incorporates multiplexed signal amplification systems, which enable users to investigate expression as well as positional relationship between multiple genes within a cellular context. The RNAscope HiPlex Pro Assay on Lunaphore's COMET system is fully automated, protease-free, and combines the RNAscope HiPlex Pro assay for RNA detection with sequential immunofluorescence (seqIF™) assay for protein detection on the same tissue section.

Overview

Figure 1 illustrates the fully automated multiomics procedure on COMET, which combines RNAscope HiPlex Pro and seqIF Assays for RNA and protein detection and can be completed on the instrument in ~22 hours when detecting 12 RNA and 24 protein targets on a 9x9 mm imaging area.

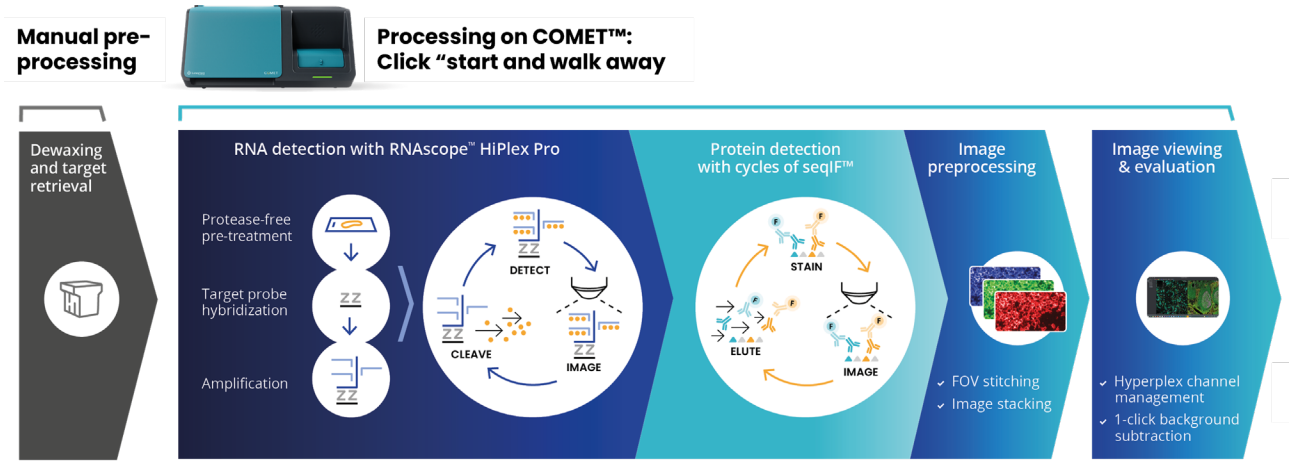
Starting with properly prepared samples, tissue sections are first dewaxed. Target retrieval is performed on samples before they are loaded on COMET followed by a protease-free pre-treatment on the instrument. Then, up to 12 RNA-specific target probes designed for different detection tails/channels are hybridized simultaneously. After a series of highly effective and specific signal amplifications, single RNA transcripts for up to four target genes at a time are imaged in distinct fluorescent channels (referred to as a round) using cleavable versions of the fluorophores AF488, Dylight 550, Dylight650 and AF750. The fluorophores from the first four targets are cleaved after imaging, and the next four targets are labeled and imaged. The kit for RNAscope HiPlex Pro on COMET is available in a 12-plex and 4-

plex version. The 12-plex version allows you to perform three rounds and the 4-plex version allows you to perform one round (T1-T4) of fluorescent target labeling and imaging.

Proteins are detected using the seqIF approach through staining, imaging, and elution cycles. You can adjust protocol conditions in each cycle to stain and image typically one or two protein markers per cycle (up to 4). COMET works with off-the-shelf, non-conjugated primary antibodies and fluorescent-labeled secondary antibodies (matching the excitation and emission filters of FITC, TRITC, Cy5 and Cy7 channels available in the instrument). Antibodies are removed after imaging with a gentle and efficient elution buffer, ensuring optimal tissue preservation.

To analyze the data, images are automatically stitched, aligned, and stacked in an OME-TIFF file.

Figure 1. Procedure overview



1. Manual pre-processing	2. RNA detection	3. Protein detection	4. Image pre-processing
Starting with properly prepared tissue sections, perform dewax and retrieval of target RNAs and proteins.	Protease-free pre-treatment with PretreatPro allows access to target RNA. Hybridization of gene-specific probe pairs to target RNAs is followed by a cascade of signal amplification steps, followed by binding of dye-labeled probes visible in different fluorescent channels. RNAs are detected in cycles of four. Dye-labeled probes for each cycle are cleaved after the fluorescent signal is imaged and before the next set of dye-labeled probes bind.	Proteins are detected in cycles. Up to four proteins per cycle are stained by incubating with primary and fluorescently labeled secondary antibodies. This is followed by imaging and elution to gently strip off primary and secondary antibodies before the next cycle.	Images are automatically stitched, aligned, and stacked on a final OME-TIFF file.

Compatible sample types

The RNAscope HiPlex Pro Assay on COMET is compatible with human FFPE tissue.

Kit contents and storage

The RNAscope HiPlex Pro Assay for COMET requires RNAscope HiPlex Probes and RNAscope HiPlex Pro for COMET Reagent Kit, available from Advanced Cell Diagnostics.

RNAscope HiPlex Probes

The RNAscope HiPlex Probes consist of user-specified Target Probes and Positive and Negative Control Probes. Each target probe contains a mixture of short oligonucleotides designed to bind to a specific target RNA and is detectable in one of four fluorescent channels specified in the following table:

Detection (3 rounds)	Probe Tail/Channel	Fluorophore	Emission	Color
Round 1	T1	Alexa Fluor 488	520 +/- 10nm	Green
	T2	Dylight 550	562 +/- 10 nm	Orange
	T3	Dylight 650	652 +/- 10 nm	Far Red
	T4	Alexa Fluor 750	775 +/- 10nm	Near IR
Round 2	T5	Alexa Fluor 488	520 +/- 10nm	Green
	T6	Dylight 550	562 +/- 10 nm	Orange
	T7	Dylight 650	652 +/- 10 nm	Far Red
	T8	Alexa Fluor 750	775 +/- 10nm	Near IR
Round 3	T9	Alexa Fluor 488	520 +/- 10nm	Green
	T10	Dylight 550	562 +/- 10 nm	Orange
	T11	Dylight 650	652 +/- 10 nm	Far Red
	T12	Alexa Fluor 750	775 +/- 10nm	Near IR

Note: The 4-plex kits only contain reagents to detect probes T1-T4.

You can select different combinations of targets in the RNAscope HiPlex Pro Assay on COMET. Each target probe is assigned to a different probe channel/tail (T1–T12). All RNAscope HiPlex target probes are shipped as 50X concentrated stocks, which are diluted in RNAscope HiPlex Pro Probe Diluent (included in the reagent kit).

IMPORTANT! Do not use RNAscope HiPlex probes for any other RNAscope assay.

Each probe is sufficient for staining ~10 sections, each with a staining area of approximately 21 mm x 21 mm ensuring this area will remain hydrated throughout protocol. The maximum imaging area on COMET is 12.5 mm x 12.5 mm. The probes have a shelf life of two years from the manufacturing date when stored as indicated in the following tables:

Target Probes					
☑	**Reagent**	**Cat. No.**	**Content**	**Quantity**	**Storage**
	RNAscope HiPlex CS Probe – *[species]* – *[gene]* – T1…T12	Various	50X probe	120 µL x 1 tube	2–8 °C
Control Probes					
☑	**Reagent**	**Cat. No.**	**Content**	**Quantity**	**Storage**
	RNAscope HiPlex12 CS Positive Control Probe-Hs	324317	RTU mixture of 12 probes targeting housekeeping transcripts *Polr2a*, *PPIB*, *UBC*, *HPRT1*, *TUBB*, *RPL28*, *RPL5*, *B2M*, *ACTB*, *LDHA-O1*, *RPLP0-X-RPLP0P2*, and *GAPDH* with T1- T12 tails respectively in each of the 12 channels.	5.5 mL x 1 bottle	2–8 °C
	RNAscope HiPlex12 CS Negative Control Probe	324347	RTU probe targeting a bacterial gene (*dapB*), with T1- T12 tails respectively in each of the 12 channels.	5.5 mL x 1 bottle	2–8 °C

Note: RNAscope HiPlex Probes (used for the manual assay) are compatible with RNAscope HiPlex Pro for COMET. However, approximately three slides can be processed due to the volume per slide differences between workflows. Using COMET target probes allows up to 10 slides to be processed.

RNAscope HiPlex Pro for COMET Reagent Kits

The RNAscope HiPlex Pro for COMET Assay is available in four formats of varying RNA plex-levels and kit sizes. The kits provide enough reagents to stain 20 tissue sections or eight tissue sections, respectively. Each section has a maximum imaging area of approximately 12.5 mm x 12.5 mm (0.75″ x 0.75″) which represents the maximum imaging area of COMET.

- RNAscope HiPlex Pro for COMET 12-plex, 20-slide Kit (Cat. No. 322075)
- RNAscope HiPlex Pro for COMET 12-plex, 8-slide Kit (Cat. No. Coming Soon!)
- RNAscope HiPlex Pro for COMET 4-plex, 20-slide Kit (Cat. No. 322085)
- RNAscope HiPlex Pro for COMET 4-plex, 8-slide Kit (Cat. No. Coming Soon!)

Note: 4-plex kits can only be used for detection of T1, T2, T3, and/or T4 probes.

The reagents have a shelf life of nine months from the manufacturing date when stored as indicated in the following tables:

RNAscope HiPlex Pro for COMET Detection Reagents (provided in box)		
RNAscope HiPlex Pro AMP 1	11.8 mL x 1 bottle (20-slide kits) 4.8 mL x 1 bottle (8-slide kits)	2–8 °C

RNAscope HiPlex Pro for COMET Detection Reagents (provided in box)		
RNAscope HiPlex Pro AMP 2	11.8 mL x 1 bottle (20-slide kits) 4.8 mL x 1 bottle (8-slide kits)	2–8 °C
RNAscope HiPlex Pro AMP 3	11.8 mL x 1 bottle (20-slide kits) 4.8 mL x 1 bottle (8-slide kits))	2–8 °C
RNAscope HiPlex Pro Fluoro T1–T4	11.8 mL x 1 bottle (20-slide kits) 4.8 mL x 1 bottle (8-slide kits)	2–8 °C
RNAscope HiPlex Pro Fluoro T5–T8 (not included in 4-plex kits)	11.8 mL x 1 bottle (12-plex, 20-slide kits) 4.8 mL x 1 bottle (12-plex, 8-slide kits)	2–8 °C
RNAscope HiPlex Pro Fluoro T9–T10 (not included in 4-plex kits)	11.8 mL x 1 bottle (12-plex, 20-slide kits) 4.8 mL x 1 bottle (12-plex, 8-slide kits)	2–8 °C
RNAscope HiPlex Pro DAPI (1 mg/mL)	1 mL x vial	2–8 °C
RNAscope HiPlex PretreatPro	11.8 mL x 1 bottle (20-slide kits) 4.8 mL x 1 bottle (8-slide kits)	2–8 °C
RNAscope HiPlex Pro Probe Diluent	11.8 mL x 1 bottle (20-slide kits) 4.8 mL x 1 bottle (8-slide kits)	2–8 °C

RNAscope HiPlex Pro for COMET (P/N 324399)		
RNAscope HiPlex Cleaving Stock Solution v2 P/N 324399	1.5 mL x 20 ampoules (20-slide kits); 4 Boxes 1.5 mL x 10 ampoules (8-slide kits); 2 Boxes	Room temperature (15–30 °C)

RNAscope HiPlex Pro Imaging Buffer		
RNAscope Imaging Buffer Part A	400 mg x 1 bottle	15-30 °C
RNAscope Imaging Buffer Part B	100 mL x 1 bottle	15-30 °C

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

Required materials and equipment

The following materials and equipment are necessary to perform the RNAscope HiPlex Pro Assay on COMET.

For instructions on how to use the COMET System, refer to the *COMET Instrument User Manual* available within Lunaphore's customer portal **https://my.lunaphore.com/instrument-materials.**

☑	Component	Supplier	Cat. No.	Details
	COMET System	Lunaphore	CM10-S	COMET Instrument and Control Station
	COMET Chip	Lunaphore	MK03	
	Glass slides	MLS*	—	Epredia™ SuperFrost Plus™ adhesion slides

☑	Component	Supplier	Cat. No.	Details
	2 mL microtubes	MLS	—	See COMET Instrument User Manual for a list of compatible products
	50 mL conical tubes	MLS	—	See COMET Instrument User Manual for a list of compatible products
	Multistaining Buffer	Lunaphore	BU06	See COMET Instrument User Manual for preparation instructions
	Elution Buffer Kit	Lunaphore	BU07-L	See COMET Instrument User Manual for preparation instructions
	Quenching Buffer Kit	Lunaphore	BU08-L	Optional, not recommended for RNAscope HiPlex Pro on COMET as it could also decrease positive signal. See COMET Instrument User Manual for preparation instructions
	Blocking Buffer Kit	Lunaphore	BU10	Optional, see COMET Instrument User Manual for preparation instructions
	Imaging Buffer	Lunaphore	BU09	See COMET Instrument User Manual for preparation instructions
	PT Module	Epredia	AP07	Provided by Lunaphore. Used for dewaxing and antigen-retrieval during slide pre-processing.
	Dewax and HIER Buffer H (pH9)	Epredia	AR03	Provided by Lunaphore. Dilute 1:15 in deionized (DI) water.
	Primary antibodies	MLS	—	User's choice
	Secondary antibodies	MLS	—	Matching primary antibody and detection channel characteristics
	20X SSC	MLS	—	
	Histo-Clear	MLS	—	
	Xylene	Fisher Scientific/MLS	X3P-1GAL	
	100% alcohol (EtOH)	American Master Tech Scientific/MLS*	ALREACS	
	10% neutral-buffered formalin (NBF)	MLS	—	
	Paraffin wax	MLS	—	
	1X PBS	MLS	—	
	Microtome	MLS	—	
	Drying oven, capable of holding temperature at 60 +/– 1 °C (optional)	MLS	—	

☑	Component	Supplier	Cat. No.	Details
	Water bath or incubator, capable of holding temperature at 40 +/– 1 °C	MLS	—	
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24	
	Tissue-Tek Staining Dish (4 required)	American Master Tech Scientific/MLS	LWT4457EA	
	Tissue-Tek Clearing Agent Dish, xylene resistant (2 required)	American Master Tech Scientific/MLS	LWT4456EA	
	Distilled water	MLS	—	
	Fume Hood	MLS	—	

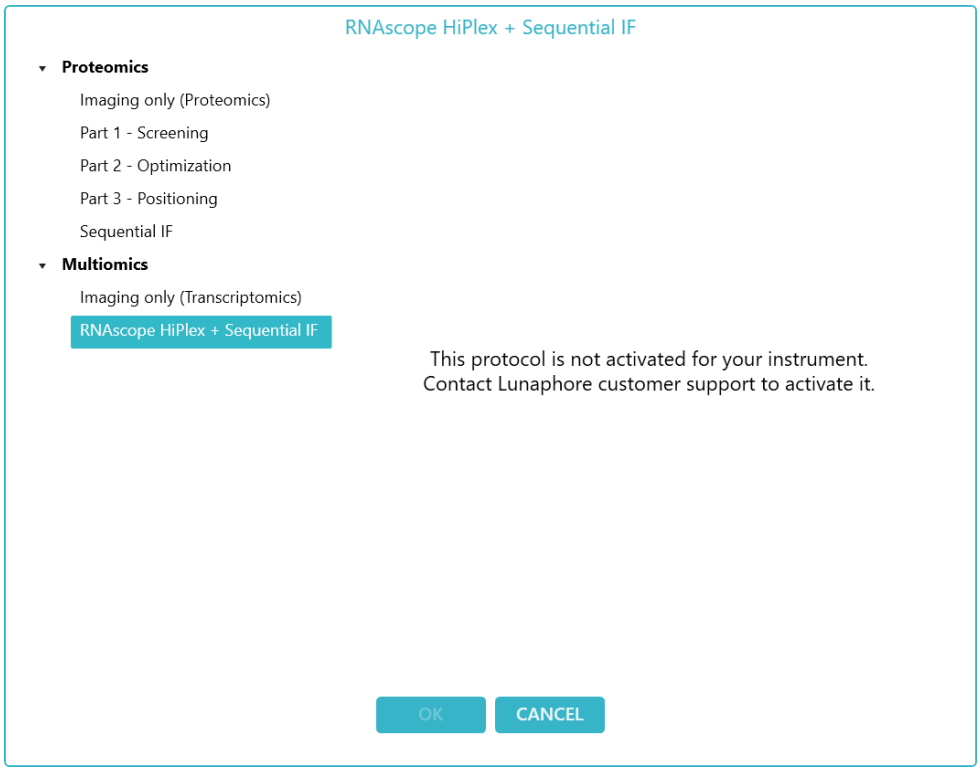
* Major Laboratory Supplier in North America. For other regions, please check Catalog Numbers with your local lab supplier.

2 Chapter 2. Before You Begin

Prior to running the RNAscope HiPlex Pro for COMET on your samples for the first time, we recommend that you:

- Become familiar with Lunaphore's COMET System. Refer to the *COMET User Manual*.
- Prepare to run the assay on Control Slides (Cat. No. 310045 for Human HeLa Cell Pellet, using the RNAscope HiPlex Positive (Hs) and Negative Control Probes.
- Make sure that the "RNAscope HiPlex + seqIF" protocol template is activated in the COMET control software prior to use. Go to **Plan > Protocols > Add new** and select the "RNAscope HiPlex + Sequential IF" template. If the template is inactive (see the following figure) activate it using the instructions in the "Add-Ons" chapter 5.8.5 of the COMET user manual.

Figure 2. Inactive "RNAscope HiPlex + Sequential IF" template.



Important procedural guidelines

- Start with properly fixed and prepared sections. Refer to **Chapter 4. Prepare and Pre-process Samples** for preparation of FFPE slides. For preparation of other sample types, contact **support.acd@bio-techne.com**.
- Regularly maintain and clean your automated staining instrument.
- Always run positive and negative control probes on your sample to assess sample RNA quality and background, respectively.
- Do not substitute required materials. Assay has been qualified with these materials only.
- Follow the protocol exactly for the best results. Do not substitute buffers or diluents.
- Store reagents accordingly right after use. To avoid any contamination or evaporation, do not leave reagents open for an extended period.
- Reagents are more viscous than water. Bring reagents to room temperature before use and take care when pipetting to avoid pipetting errors.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix A. Safety** for more information.

3 Chapter 3. Prepare Kit Reagents

This chapter describes the preparation of reagents required for the RNAscope HiPlex Pro Assay on COMET. Ensure that you use appropriate containers for reagent preparation and storage.

You can aliquot reagents to preserve reagent quality. We recommend discarding any reagents that remain at room temperature for an extended period.

During protocol execution, the user can generate a Preparation PDF (referenced in this chapter), which will provide the final volumes of reagents required for the run.

Prepare and store reagents

The following reagents can be prepared and stored in advance.

Prepare probes

RNAscope HiPlex Control Probes for COMET are supplied in a ready-to-use format and do not need to be diluted.

Use the following guideline to prepare the 50X RNAscope HiPlex Pro Target Probes for COMET (T1…T12).

1. Mix each unique target probe set by diluting 50X probe stocks with RNAscope HiPlex Pro Probe diluent. Dilute probes to 1X by pipetting 1 volume of each stock to 50 volumes of probe diluent.
   - For example, to make 1 mL of solution containing all 12 probes, add 20 µL of each probe stock and 760 µL of RNAscope HiPlex Pro Probe diluent to an Eppendorf tube.
2. Mix gently.

Notes:

- The mixed probes can be stored at **2–8°C** for up to two years.
- The "Preparation PDF" protocol only determines the final pooled probe volume, not volumes of the individual probes, required for the run.
- For details on the COMET control software, consult the COMET user manual.

Prepare RNA Imaging Buffer Part A stock

1. To prepare 40X imaging buffer stock solution, add 25 mL DI water directly to the RNAscope HiPlex Pro Imaging Buffer Part A (bottle containing 400 mg powder). Mix well.
2. Pipette 1 mL aliquots into 1.5- or 2-mL tubes. Freeze aliquots at **–20°C**.

Prepare 4X SSC

1. To prepare 4X SSC, dilute 20X SSC with distilled water by pipetting one volume of 20X SSC with four volumes of distilled water.
2. Mix thoroughly by inverting the container at least ten times.

Note: Prepare 20X SSC by dissolving 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of distilled water and adjusting the pH to 7.0 with a few drops of 1M HCl. Use water to adjust the volume to 1 liter. Sterilize by autoclaving or filtering under vacuum.

Prepare fresh reagents

The following reagents need to be prepared and used the day of the run.

Prepare RNAscope HiPlex Pro Imaging Buffer

1. Follow the Preparation PDF instructions to thaw the correct number of aliquots of stock required to prepare the final volume of Imaging Buffer Working Solution.
   - For example, to prepare 40 mL of working solution mix 1 mL of thawed RNA Imaging Buffer Part A stock, and 4 mL of 10X RNAscope HiPlex Pro Imaging Buffer Part B to 35 mL of DI water.

Note: Do not reuse or refreeze unused stock solution.

Prepare RNAscope HiPlex Pro DAPI

We recommend the following preparation when using the procedures described in **Chapter 4.** Prepare and
Pre-process Samples.

1. Refer to the Preparation PDF for the recommended final volume.
2. Dilute the RNAscope HiPlex Pro DAPI (1 mg/mL) 1:200 in 1X Multistaining Buffer (BU06).

Notes:

- You can adjust DAPI concentration if DAPI detection is saturated or sub-optimal.
- If Multiomics protocols are selected, staining cycles for protein detection following RNA detection cycles should also include RNAscope HiPlex Pro DAPI at the same selected concentration (either as a stand-alone counterstaining reagent or mixed with secondary antibody).

Prepare RNAscope HiPlex Cleaving Reagent

1. Immediately prior to starting the run, break open an ampoule of RNAscope HiPlex Pro Cleaving Reagent.
2. Refer to the Preparation PDF for the required final volume.
3. Dilute 1:10 in 4X SSC. Do not reuse.
   - For example, to prepare 10 mL of working solution mix 1 mL of Cleaving Solution stock, and 9 mL of 4X SSC.

4 Chapter 4. Prepare and Pre-process Samples

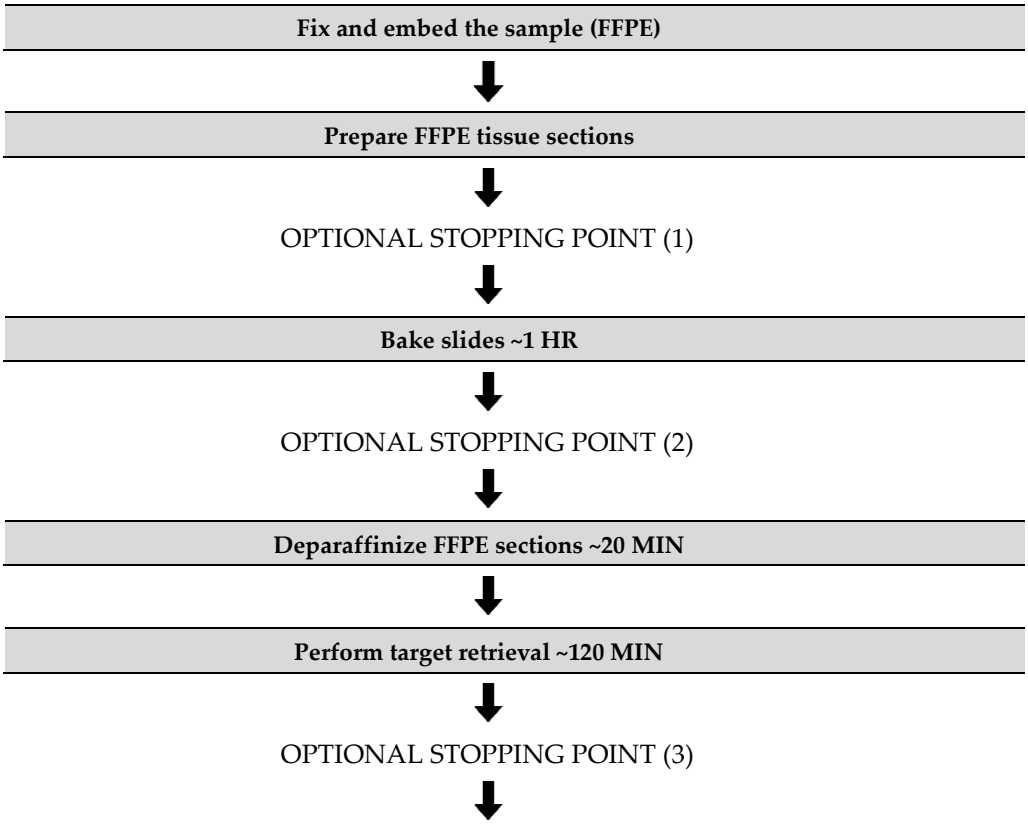
This chapter describes the formalin-fixed, paraffin-embedded (FFPE) sample preparation method and slide pre-processing procedure. If you have already embedded your FFPE samples, you can proceed directly to the **Prepare FFPE tissue sections** procedure.

For optimal results, we recommend using the PreTreatment Module (PTM) and Heat-Induced Epitope Retrieval (HIER) Solution from Epredia in the antigen and target retrieval protocol performed on slides before the staining on COMET. Please refer to the PreTreatment Module user manual and related documents for detailed instructions.

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



Proceed on COMET with the HiPlex Assay

IMPORTANT! Samples should be pre-processed on the same day they are going to be analyzed on COMET.

Materials required

- 10% Neutral Buffered Formalin (NBF)
- 1X PBS
- Paraffin wax
- Tissue-Tek Clearing Agent Dishes
- Tissue-Tek Staining Dishes
- Tissue-Tek Vertical 24 Slide Rack
- 100% alcohol (EtOH)
- Histo-Clear (or xylene)
- Microtome
- Water bath
- SuperFrost Plus slides
- Drying oven
- Fume hood
- PreTreatment (PT) Module; Lunaphore product code AP07
- HIER Buffer H (pH 9); Lunaphore product code AR03
- Multistaining Buffer; Lunaphore product code BU06

Fix and embed samples

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

⚠ **CAUTION!** Handle biological specimens appropriately.

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

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

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

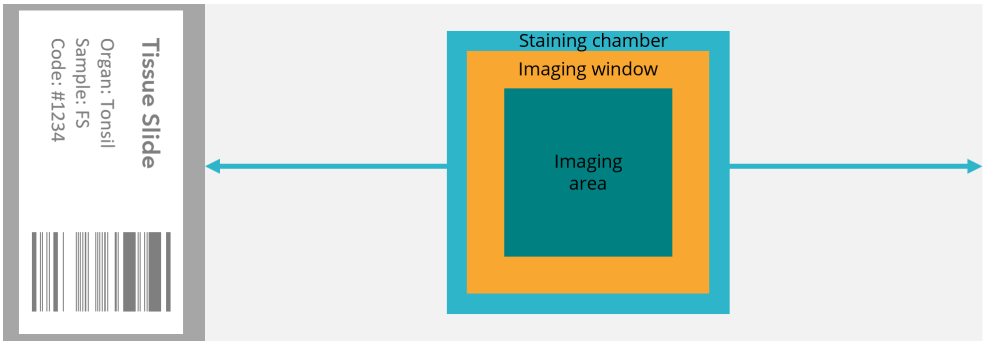
4. Embed sample in paraffin using standard procedures.

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

Prepare FFPE tissue sections

1. Trim paraffin blocks as needed and cut embedded tissue into 5 +/– 1 µm sections using a microtome.
2. Place paraffin ribbon in a **40–45 °C** water bath and mount the sections on **SUPERFROST PLUS SLIDES.** Use the following figure as a guideline for placing the tissue within the COMET imaging area The imaging area can be moved along the blue arrow when loading the slide onto COMET.

Figure 2: Imaging area of a slide on COMET.



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

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

Bake slides

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

OPTIONAL STOPPING POINT (2). Use immediately, or store at **RT** with desiccants for ≤ 1 week. Prolonged storage could degrade sample RNA.

Note: If you continue with the procedure, you can prepare materials for the next steps while the slides are baking.

Deparaffinize FFPE sections

Reagents can be prepared ahead of time. Ensure that all containers remain covered.

1. Prepare reagents:
   - Fill two Tissue-Tek Clearing Agent dishes with ~200 mL fresh Histo-Clear (or xylene).
   - Fill two Tissue-Tek Staining dishes with ~200 mL fresh 100% ethanol.
2. Place slides in a Tissue-Tek Slide Rack and submerge in the first Histo-Clear-containing dish.
3. Incubate the slides in Histo-Clear for **5 MIN** at **RT**. Agitate the slides by occasionally lifting the slide rack up and down in the dish.
4. Remove the slide rack from the first Histo-Clear-containing dish and immediately place it in the second Histo-Clear-containing dish.
5. Incubate the slides in Histo-Clear for **5 MIN** at **RT** with agitation.

6. Remove the slide rack from the second Histo-Clear-containing dish and immediately place it in a dish containing 100% ethanol.
7. Incubate the slides in 100% ethanol for **2 MIN** at **RT** with agitation.
8. Remove the slide rack from the first ethanol-containing dish and immediately place it in the second ethanol-containing dish.
9. Incubate the slides in 100% ethanol for **2 MIN** at **RT** with agitation.
10. Remove the slides from the rack and place them on absorbent paper with the section face-up. Dry slides in a drying oven for **5 MIN** at **60 °C** or until completely dry.

Perform target retrieval with the PT Module

Storing and preparing HIER buffer

- Store the HIER Buffer H (pH 9) stock solution at room temperature (RT) or 2-8°C if storing for longer than three months.
- Discard the stock solution if it becomes cloudy or shows a large amount of precipitate.
- Dilute a 100 mL buffer bottle in 1400 mL of DI water to fill one PT Module tank with 1.5 L of fresh 1X target retrieval solution. The working dilution is 1:15 in DI water.
- HIER solution can be reused for a maximum of five runs within five days. For maximum reproducibility, replace the HIER solution in the PT Module after every target retrieval run.
- Dispose of the HIER solution according to local disposal regulations and based on the pH (see MSDS).
- Follow the instructions in the PT Module documentation to dispose of waste generated by the PT Module.

The day before staining

1. Fill a PT Module tank with 1.5 L of HIER Buffer H (pH 9).
2. (Optional) If you are programming a preheating, set the current day and time in the **1 – Set Clock and Delay Start** menu.
3. Set the run time to **01:00 h** and the temperature to **99°C** in the **2 – Setup Cycle (Time and Temp)** menu.

Note: For FFPE tissues**, 1 HR** is the standard retrieval time. For ACD FFPE Hela cells pellets, **15 MIN** is the recommended time.

4. Set the preheat temperature to **85°C** and the cooling end temperature to **65°C** in the **3 - Preheat Settings** tab.
5. (Optional) If you are programming a preheating, set the desired **Preheat Start Time** and select either the **One Time** mode for a single preheating or the **Continuous** mode for a daily preheating.
6. Place the PT Module lid on the tank and latch it.

The day of staining

1. If the PT Module was not preheated previously, press **Run** on the home tab.
2. Once **85°C** is reached, mount the slides into the slide rack. Open the PT Module, remove the tank lid and insert the slide rack. Replace and latch the lid.

3. Press **Run** a second time to start the heating cycle. The lid locks and the retrieval protocol will begin.

   Note: If needed, press **Reset** to stop the run and unlock the lid. Check the temperature and wear proper protective equipment in case it is necessary to handle the slides already in the bath.

4. When the heating cycle is finished and the tank has cooled down to **65°C**, the module beeps and the lid unlocks. Take the rack(s) out of the PT Module and place the slide(s) in 1X Lunaphore Multistaining Buffer (1X).

OPTIONAL STOPPING POINT (3). Place and keep the slide(s) in fresh Multistaining Buffer (1X) at room temperature until proceeding with the COMET run as described in **Chapter 5**.

IMPORTANT! We recommend keeping slides in Multistaining buffer for less than three hours prior to loading the slides and beginning the COMET protocol.

Cleaning the tanks

1. After emptying the tanks, wipe them with a paper towel to remove any wax deposits.
2. Wash the PT Module tanks in the sink with soap and hot water, then rinse them with DI water.

Troubleshooting

Issue	Solution
Sparse dots or paraffin crystals on the slide	Carefully dip the slide in hot (65°C) buffer remaining PT Module tank.
Extensive paraffin dots/crystals	The solution is saturated with paraffin. Use fresh Dewax and HIER Buffer.

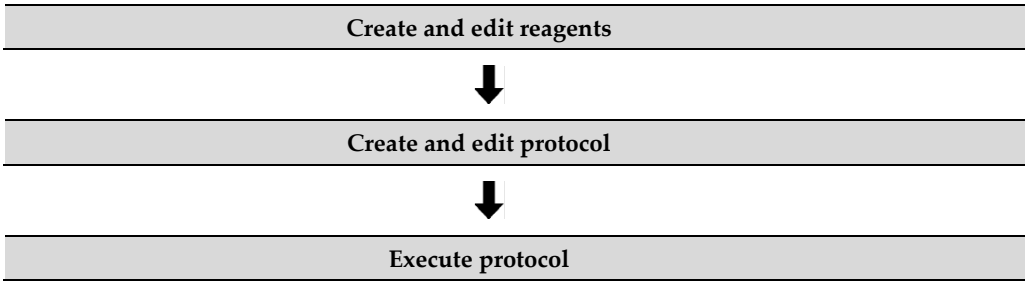
5 Chapter 5. Set Up and Run a Protocol

Use the instructions in this chapter to set up the RNAscope HiPlex Pro on COMET Assay. Please refer to the COMET user manual *Chapter 4 Instrument Operation* for detailed instructions.

IMPORTANT! COMET 1.0 or higher is required to run the following setup on software version 1.2.1.0. Please contact Lunaphore customer support to upgrade if needed before starting the assay.

IMPORTANT! We strongly recommend you first run the Control Slides (Cat. No. 310045 for human) using the RNAscope positive and negative control probes to assess RNA quality and background.

Workflow



Materials required

Materials provided in the RNAscope HiPlex Detection Reagents	Materials provided by RNAscope HiPlex Probes	Other materials and equipment
• RNAscope HiPlex Pro AMP 1 • RNAscope HiPlex Pro AMP 2 • RNAscope HiPlex Pro AMP 3 • RNAscope HiPlex Pro Fluoro T1–T4 • RNAscope HiPlex Pro Fluoro T5–8 (for 12-plex kits) • RNAscope HiPlex Pro Fluoro T9–T12 (for 12-plex kits) • RNAscope HiPlex Pro DAPI • RNAscope HiPlex Cleaving Reagent v2 • RNAscope HiPlex Pro Imaging Buffer (A and B)	• RNAscope HiPlex CS Target Probes (50X) • RNAscope HiPlex12 CS Negative Control Probe (RTU) • RNAscope HiPlex12 CS Positive Control Probe-Hs (RTU)	• 20X SSC • Water • Multistaining buffer • Imaging Buffer (for protein) • Prepared sections • Tubes (various sizes) • Antibodies (primary and secondary)

Materials provided in the RNAscope HiPlex Detection Reagents	Materials provided by RNAscope HiPlex Probes	Other materials and equipment
• RNAscope HiPlex Pro Probe diluent		

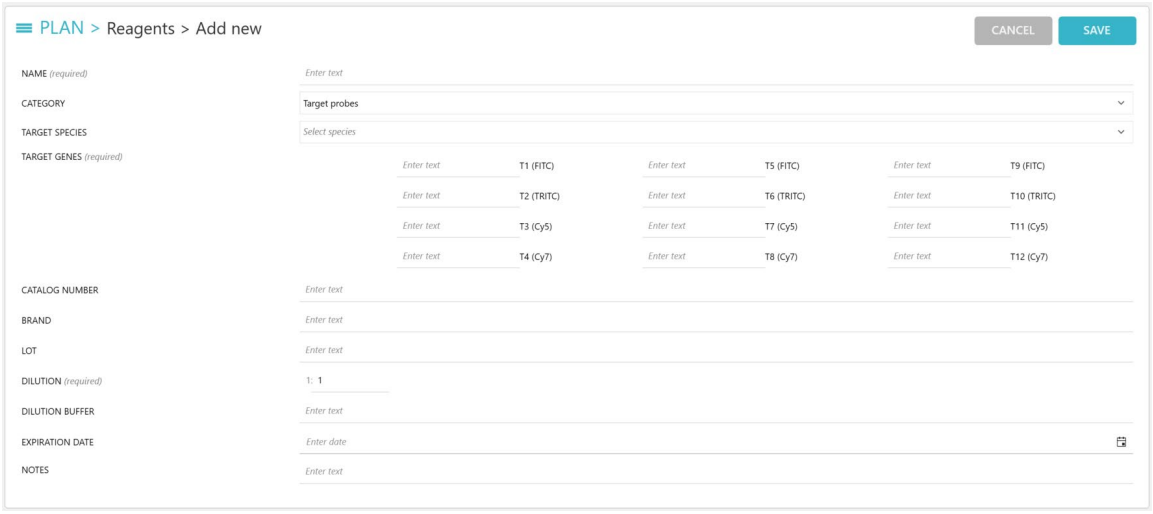
Create and edit the reagents

Perform the COMET start up procedure as described in chapter 4.3 of the COMET user manual.

The reagents necessary to create and execute a RNAscope HiPlex Pro on COMET Kit protocol are listed in **Table 1**. Use the following procedure to create the reagents on the instrument. For more detailed instructions, consult chapter 4.4.2 of the COMET user manual.

Other required reagents such as Amplifications probes, Detection probes, Cleavage reagent, Pretreatment reagent and RNA Imaging Buffer are already pre-loaded in the Reagent library of the COMET Control Software. You can modify some of the fields for these reagents, such as LOT number information.

1. In the navigation bar of the COMET Control Software, select **PLAN > Reagents** then click **Add new**. The Add new window opens.



2. Select the desired reagent category from the drop-down menu and fill in the required fields.
3. Filling in any of the other fields as desired. The fields available depend on the selected category.

Table 1 RNAscope HiPlex Pro on COMET Assay reagents that need to be created in the COMET Control Software

Reagent category	Info
Target probes	Create one **Target probes** reagent per RNA panel and enter all target genes in the respective tail fields. Enter 1:1 in the dilution field.
Counterstaining	Create the RNAscope DAPI Solution reagent and enter the desired dilution factor (1:200 is the recommended dilution) in the dilution field.

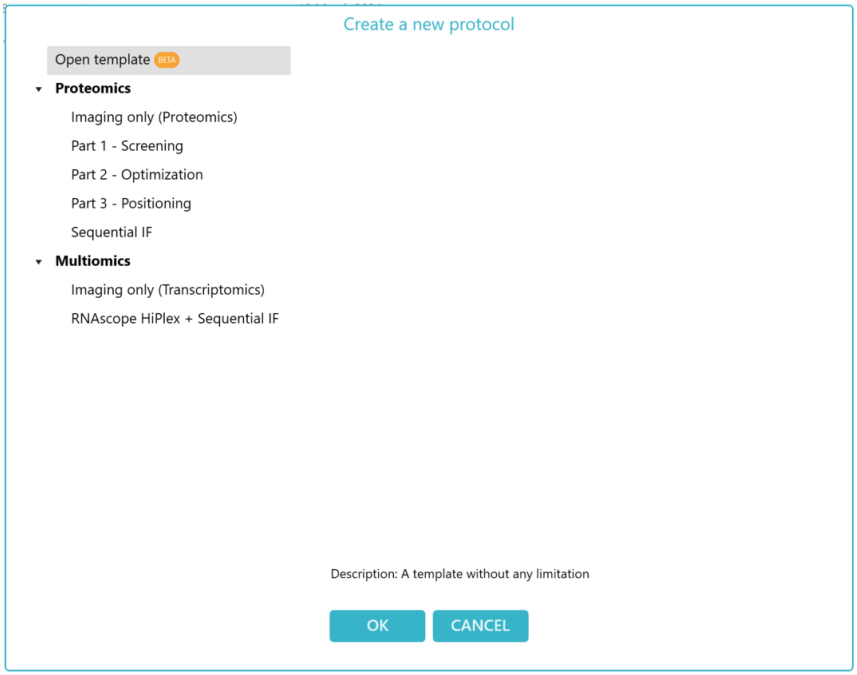
Primary antibody	Create a reagent for every primary antibody in your assay. You can also create a **Primary Antibody Mix** or **Primary Antibody (fluorophore-labeled)**.
Secondary antibody	Create a reagent for every secondary antibody in your assay. You can also create a **Secondary Antibody Mix** or **Secondary Antibody and Counterstaining Mix** (recommended).

Create and edit a protocol

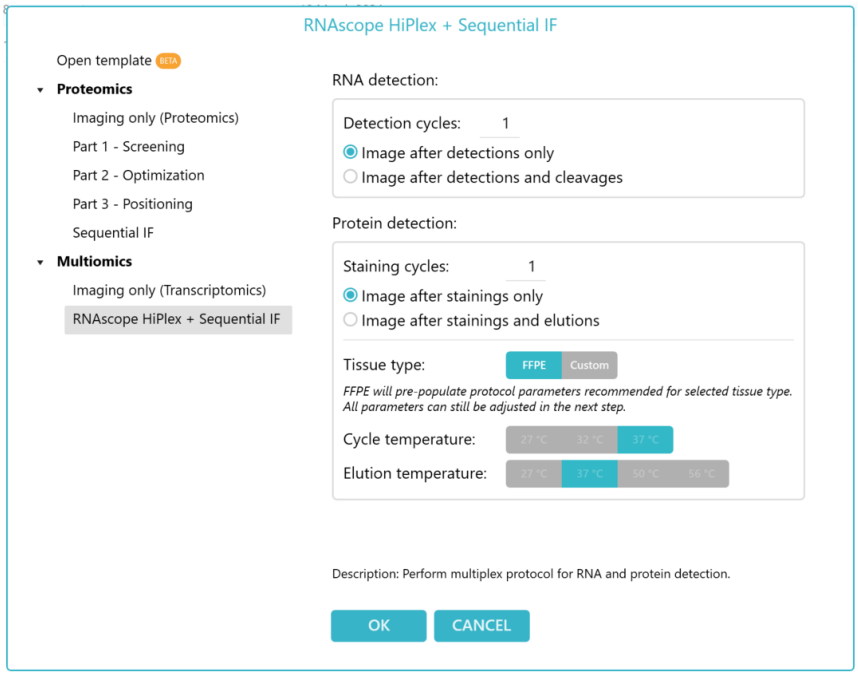
Use the following procedure to create a new protocol. To edit an existing protocol, select a protocol in the library (PLAN > Protocols), click **Edit** in the overview panel on the right side to pen the protocol editor window, and follow the procedure beginning at step 5.

Note: For more detailed instructions on protocol creation consult chapter 4.4.2 of the COMET user manual.

1. Click **PLAN > Protocols>Add new**. The **Create a new protocol** window opens.



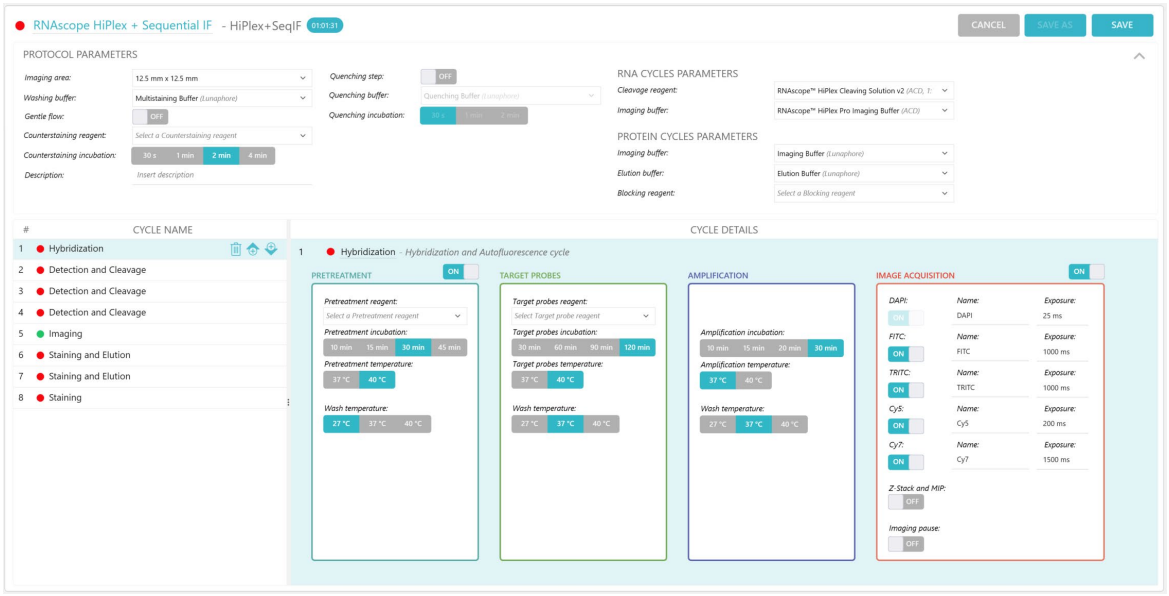
2. On the left, choose the **RNAscope HiPlex + Sequential IF** protocol template.



3. Enter the desired values for the protocol creation parameters displayed on the right.:
   a. Enter 1, 2 or 3 under **Detection cycles**.
   b. Enter between 0 and a maximum of 12, 13, or 14 depending on the number of selected RNA cycles in step a.

Note: For more detailed instructions refer to section **Error! Reference source not found.**.7 of the COMET User Manual.

4. Click **OK** to open the protocol editing window.

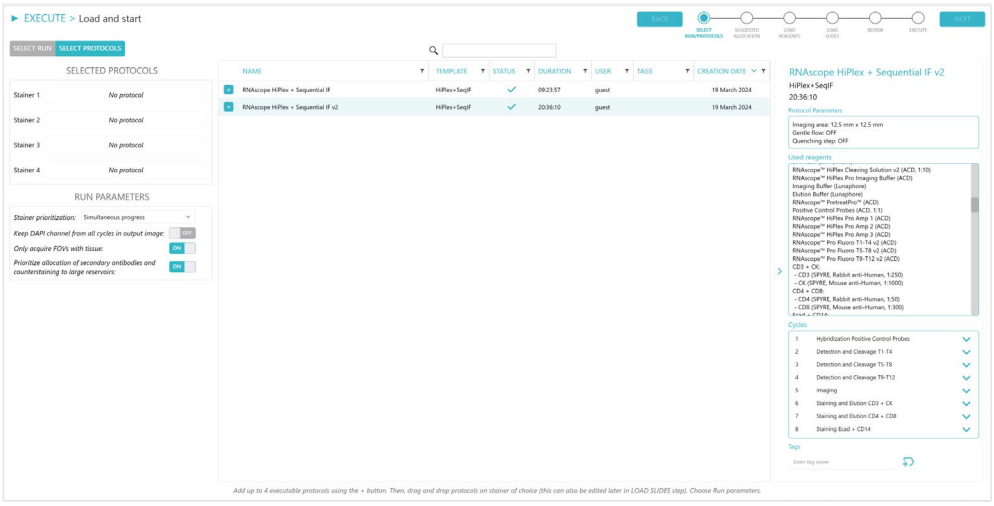


5. Edit the protocol details if desired. In each image acquisition step, you can activate **Z stack and MIP** to improve the detection of punctate RNA signals. If this setting is turned on, "additional images above and below the autofocus plane along the Z-axis are taken and combined to build one image using maximum intensity projection (MIP) to select the maximum intensity for each pixel among the Z-stack images:
   a. Toggle the switch **On** or **Off**. Default is **Off**.
   b. Under **Number of steps** define the number of extra images (±1, ±2 or ±3) above and below the autofocus plane.
   c. Under **Z-Step size**: define the focus distance between the Z-stack images (1.0, 1.5, 2.0, 2.5 or 3.0µm).
6. Save the protocol or create an edited copy using **Save as**.

Execute the protocol

1. In EXECUTE, select the **Select protocols** mode to start the wizard.
2. Follow the wizard by navigating with the **Next** and **Back** buttons and complete each step. During "Load Reagent" the user can click the "Preparation PDF" button to generate a PDF with the complete details of the reagent preparation for the run. Refer to Chapter 3.

Note: For detailed instructions, including slide loading and unloading, refer to section 4.5 of the COMET user manual.



Note: When a protocol in a run is completed (or aborted), the report and image are automatically generated and saved in a folder named after the protocol's execution date, the stainer number, a unique execution code and the name of the protocol (e.g., 20210928_175826_1_8pjInb_Imaging only). The report and image are similarly named. The folder is saved at the path defined in the **Output folder** field in **SETTINGS > Configuration**; the image will be opened by the viewer software defined in the **Viewer path** field in **SETTINGS > Configuration**.

6 Chapter 6. Evaluate the Results

Evaluate the samples

Examine the experimental results.

- Assess tissue and cell morphology.

  Assess the negative control background first. For each target, five dots per every 10 cells in a 20X microscope field is acceptable. Set exposure times of image acquisition to acceptable background levels.
- Assess positive control signal strength. Positive control signal should be visible as punctate dots within the cell. Highly expressed targets might appear as clusters. Exposure times for negative control and positive control should be the same for each unique sample.

Troubleshooting

If you obtain less than satisfactory results, troubleshoot your assay by following the dedicated Troubleshooting section of the COMET User Manual.

- For troubleshooting information, please contact our technical support teams: **support-tech@lunaphore.com** for COMET related questions or **support.acd@bio-techne.com** RNAscope questions.

A Appendix A. Safety

Chemical safety

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

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

Biological hazard safety

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

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at **www.cdc.gov/biosafety**

- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030)
- Your company's/institution's Biosafety Program protocols for working with/handling
  potentially infectious materials
- Additional information about biohazard guidelines is available at **www.cdc.gov/**

In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition
- Registration, Evaluation, Authorization and Restriction of Chemicals (REACH)

Documentation and Support

Obtaining SDSs

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

Obtaining support

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

At the website, you can:

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

Contact information

Advanced Cell Diagnostics, Inc.
7707 Gateway Blvd Suite 200
Newark, CA 94560
Toll Free: 1-877-576-3636
Direct: 1-510-576-8800
Fax: 1-510-576-8801
Information: **info.acd@bio-techne.com**
Orders: **orders.acd@bio-techne.com**
Support Email : **support.acd@bio-techne.com**

Limited product warranty

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

Headquarters
7707 Gateway Blvd Suite 200, Newark, CA 94560 **Phone 1-510-576-8800** **Toll Free 1-877-576-3636**
For support, email support.acd@bio-techne.com
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