[USER MANUAL]

RNAscope™ HiPlex Pro for COMET™

bio-techne spatial™

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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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Document Revisions

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Contents

Chapter 1. Product Information 5

About this guide 5

Product description 5

- Background 5
- Overview 5
- Compatible sample types 7

Kit contents and storage 7

- RNAscope HiPlex Probes 7
- RNAscope HiPlex Pro for COMET Materials 8

Required materials and equipment 10

Chapter 2. Before You Begin 12

Important procedural guidelines 12

Chapter 3. Prepare Kit Reagents 13

Prepare and store reagents 13

- Prepare probes 13
- Prepare RNA Imaging Buffer Part A stock 13
- Prepare 4X SSC 14

Prepare fresh reagents 14

- Prepare RNAscope HiPlex Pro Imaging Buffer 14
- Prepare RNAscope HiPlex Pro DAPI 14
- Prepare RNAscope HiPlex Cleaving Reagent 14
- Prepare MSB with RNase inhibitor 15

Chapter 4. Prepare and Pre-process Samples 16

Workflow 16

Materials required 17

Fix and embed samples 17

Prepare FFPE tissue sections 17

Bake slides 18

Deparaffinize FFPE sections 18

Perform target retrieval with the PT Module 19

- Storing and preparing HIER buffer 19
- The day before staining 19
- The day of staining 19
- Cleaning the tanks 20
- Troubleshooting 20

Chapter 5. Set Up and Run a Protocol 21

Workflow 21

Materials required....21
Create and edit the reagents....22
Create and edit a protocol....22
Execute the protocol....23

Chapter 6. Evaluate the Results....25

Evaluate the samples....25
Troubleshooting....25

Appendix A. Frozen tissues....26

Recommended tissue block preparation and sectioning guidance....26
Fixed-frozen tissue block preparation and tissue sectioning....26
Fresh frozen tissue block preparation and tissue sectioning....26
Prepare tissue sections for staining (Day of Staining)....27
Post-Fixation....27
Dehydrate the tissue....28
Perform target retrieval using the PT Module....28
Tissue specific protocol Recommendations....29

Appendix B. Safety....31

Chemical safety....31
Biological hazard safety....31
In the U.S.:....31
In the EU:....32

Documentation and Support....33

Obtaining SDSs....33
Obtaining support....33
Contact information....33
Limited product warranty....33

1 Chapter 1. Product Information

Before using this product, read and understand the information in **Appendix B. 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 for utilizing the RNAscope HiPlex Pro for COMET Kit to stain slides with mounted FFPE and frozen sections on the 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 RNAscope'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 the 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

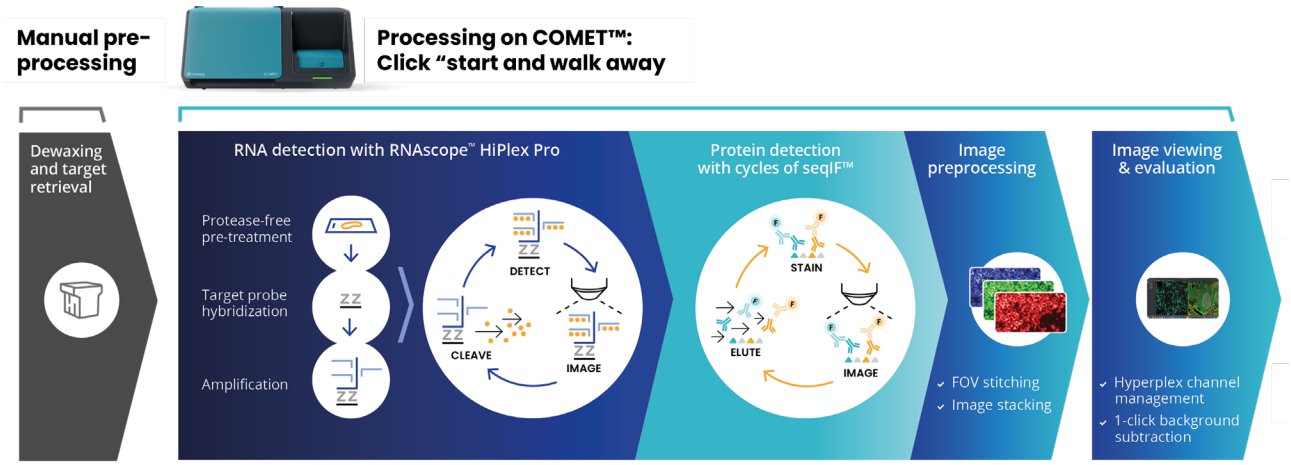
Figure 1 illustrates the fully automated multiomics procedure on COMET, which combines RNAscope HiPlex Pro and seqIF Assays for RNA and protein detection and can be completed on the instrument in ~22 hours when detecting 12 RNA and 24 protein targets on a 9x9 mm imaging area.

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, Dylight 650 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 kit enables 12-plex detection using three rounds 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 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 FFPE and frozen tissue sections.

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

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 Positive Control Probe-Mm	324437	RTU mixture of 12 probes targeting housekeeping transcripts *Polr2a*, *PPIB*, *UBC*, *HPRT1*, *ACTB*, *SDHA*, *TFRC*, *LDHA*, *GAPDH*, *RPL5*, *YWHAZ*, *and RPL28* with T1-T12 tails respectively in each of the 12 channels.	5.5 mL x 1 bottle	2–8 °C
	RNAscope HiPlex12 CS Positive Control Probe-Rn	324337	RTU mixture of 12 probes targeting housekeeping transcripts *Polr2a*, *PPIB*, *UBC*, *HPRT1*, *ACTB*, *SDHA*, *TFRC*, *LDHA*, *GAPDH*, *RPL5*, *YWHAZ*, *and RPL28* 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 Materials

The RNAscope HiPlex Pro for COMET Assay is available as a 12-plex kit. The kit provides enough reagents to stain 10 tissue sections Each section has a maximum imaging area of approximately 12.5 mm x 12.5 mm which represents the maximum imaging area of COMET. Users can purchase the complete Reagent kit or individual components:

RNAscope HiPlex Pro for COMET Reagent Kit (Cat. No. 323680)		
RNAscope HiPlex Pro for COMET 12-plex, 10-slide Detection Reagents	Cat. No. 323675	Quantity 1, sufficient for 10-slides
RNAscope HiPlex Cleaving Stock Solution v2	Cat. No. 324399	Quantity 2 (5/box), sufficient for 10 runs
RNAscope HiPlex Pro Imaging Buffer	Cat. No. 322080	Quantity 1, sufficient for 20 slides

The sub-component quantities and storage conditions for each part are listed below. 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 (Cat. No. 323675, provided in box)		
RNAscope HiPlex Pro AMP 1	5.2 mL x 1 bottle	2–8 °C
RNAscope HiPlex Pro AMP 2	5.2 mL x 1 bottle	2–8 °C
RNAscope HiPlex Pro AMP 3	5.2 mL x 1 bottle	2–8 °C
RNAscope HiPlex Pro Fluoro T1–T4	5.2 mL x 1 bottle	2–8 °C
RNAscope HiPlex Pro Fluoro T5–T8	5.2 mL x 1 bottle	2–8 °C
RNAscope HiPlex Pro Fluoro T9–T12	5.2 mL x 1 bottle	2–8 °C
RNAscope HiPlex Pro DAPI (1 mg/mL)	1 mL x vial	2–8 °C
RNAscope HiPlex PretreatPro	5.2 mL x 1 bottle	2–8 °C
RNAscope HiPlex Pro Probe Diluent	5.2 mL x 1 bottle	2–8 °C
RNAscope post hybridization wash	8 mL x 1 bottle	2–8 °C

RNAscope HiPlex Cleaving Stock Solution v2 (Cat. No. 324399)		
RNAscope HiPlex Cleaving Stock Solution v2	1.5 mL x 5 ampoules	Room temperature (15–30 °C)

RNAscope HiPlex Pro Imaging Buffer (Cat. No. 322080)		
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 additional 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/.**

☑	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
	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 (MSB)	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	

☑	Component	Supplier	Cat. No.	Details
	10% neutral-buffered formalin (NBF)	MLS	—	
	Paraffin wax	MLS	—	
	1X PBS	MLS	—	
	RNase Inhibitor, murine	New England Biolabs	M0314S M0314L	Required for using slide loading protocol "RNA integrity preservation"
	Microtome	MLS	—	
	Drying oven, capable of holding temperature at 60 +/– 1 °C (optional)	MLS	—	
	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 the 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 activate it using the instructions in the "Add-Ons" chapter 5.9.5 of the COMET user manual.

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 and processing of frozen tissues, refer to Appendix A.
- 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 per recommendations. 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 B. 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.

Notes:

- Do not reuse or refreeze unused stock solution.
- Once prepared, the RNAscope HiPlex Pro Imaging Buffer Working Solution can be used for up to 120 hours if stored at room temperature.

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 (MSB, 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).
- Once prepared, the RNAscope HiPlex Pro DAPI Working Solution can be used for up to 120 hours if stored at room temperature

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.

Notes:

- Once prepared, the RNAscope HiPlex Cleaving Reagent Working Solution can be used for up to 120 hours if stored at room temperature

Prepare MSB with RNase inhibitor

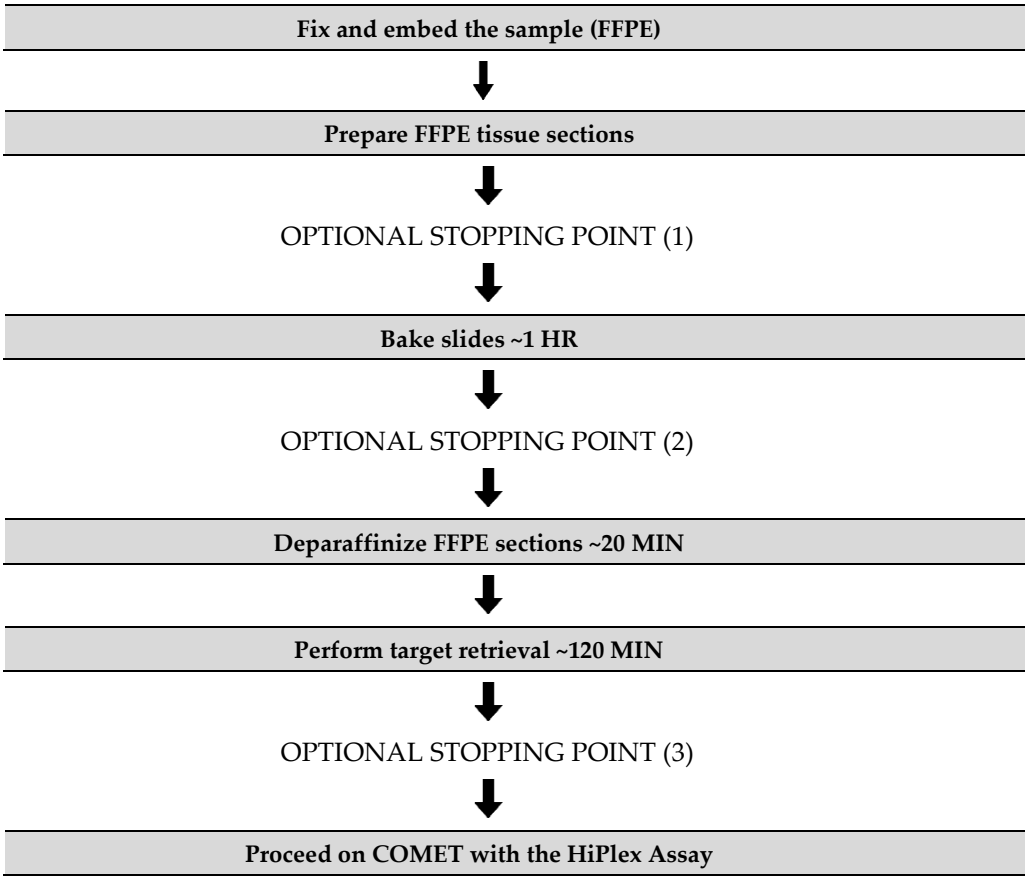
1. To prepare MSB buffer with RNase inhibitor murine (blocking reagent used in the RNA Integrity Preservation protocol), dilute the stock solution of RNase inhibitor murine (40,000 U/mL) with 1X MSB by pipetting one volume of stock solution with 39 volumes of 1X Multistaining buffer (final working concentration: 1,000 U/mL).
2. Mix by pipetting the solution several times

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 instructions to process and stain frozen tissues, refer to Appendix A.

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

Workflow



IMPORTANT! Samples should be pre-processed on the same day they are going to be stained 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
- Dewax and HIER Buffer H (pH 9); Lunaphore product code AR03
- Multistaining Buffer (MSB); 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.

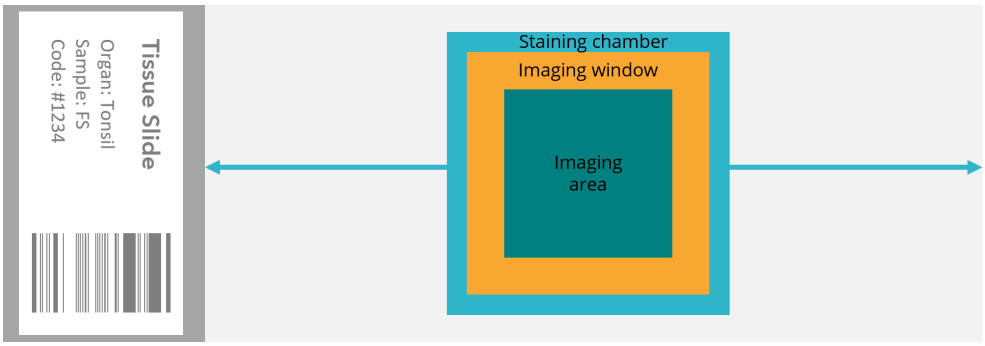
4. Embed sample in paraffin using standard procedures.

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 into hot (65°C) buffer that is remaining in the PT Module tank.
Extensive paraffin dots/crystals	The solution is saturated with paraffin. Use fresh Dewax and HIER Buffer.

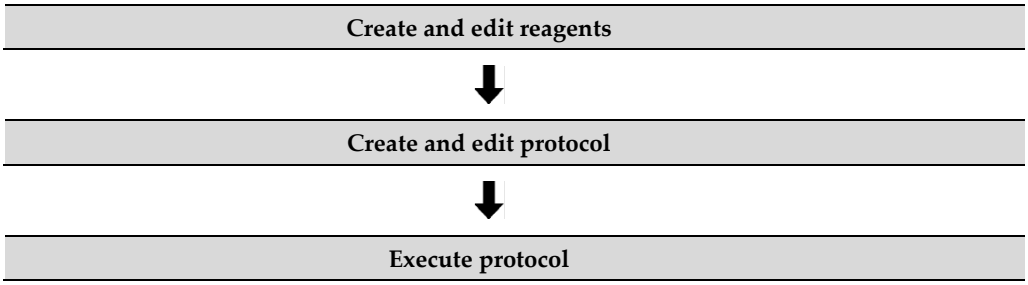
5 Chapter 5. Set Up and Run a Protocol

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 1.6.0.0 or later. 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 12-plex, 10-slide 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 • RNAscope HiPlex Pro Fluoro T9–T12 • 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 (MSB) • Imaging Buffer (for protein) • Prepared sections • Tubes (various sizes) • Antibodies (primary and secondary) • COMET chip • RNase inhibitor, Murine

Materials provided in the RNAscope HiPlex 12-plex, 10-slide Detection Reagents	Materials provided by RNAscope HiPlex Probes	Other materials and equipment
• RNAscope HiPlex Pro Probe diluent • RNAscope post hybridization wash		

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 Chapter 1 of this user manual. Use the following procedure to create the reagents on the instrument. For more detailed instructions, consult chapter 4.4.1 of the COMET user manual.

Other required reagents such as Amplifications probes, Detection probes, Cleaving reagent, Pretreatment reagent, post hybridization wash 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).
Blocking	Create RNase inhibitor, Murine and enter desired dilution (1:40) and dilution buffer (1X MSB)

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 open the protocol editor window, and edit parameters as needed.

This workflow utilizes two protocols: one for RNA preservation and one to execute multiomics staining. The RNA preservation protocol is recommended for slide loading prior to RNA detection protocols to reduce RNA degradation prior to probe hybridization. Create protocols using:

PLAN>Protocols>Add new>Multiomics>RNA integrity preservation and
PLAN>Protocols>Addnew>Multiomics>RNAscope HiPlex + Sequential IF.

Note: Consult chapter 4.4.2 and 6.1 in COMET User manual for more detailed instructions on protocol creation and protocol parameters, respectively.

1. For **RNA integrity preservation** protocol click OK, and for parameters, enter:

    a. Washing buffer: Multistaining Buffer (Lunaphore)

    b. Blocking reagent: RNase inhibitor, Murine (1:40) or name used when blocking reagent created.

2. For **RNAscope HiPlex + Sequential IF**, Enter the desired values for the protocol creation parameters displayed on the right.:
    a. In **Common section**, choose tissue type.
    b. In **RNA detection** section, enter 1, 2 or 3 under **Detection cycles**.
    c. In **Protein detection** section, enter between 0 and a maximum of 12, 13, or 14 **Staining cycles** depending on the number of selected RNA detection cycles in step b.
3. Click **OK** to open the protocol editing window.
4. 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).
    d. Save the protocol or create an edited copy using **Save as**.

Execute the protocol

When running multiomics on COMET, it is necessary to initially execute the **RNA integrity preservation** protocol for all slides. This is especially important for multi-slide runs and for frozen tissue to reduce RNA degradation prior to probe hybridization.

1. Proceed with regular COMET slide loading for all slides
2. Load the selected *Blocking Reagent* (RNase inhibitor) and start the protocol on all slides

It is important to launch the RNA integrity preservation protocol shortly after slide loading on COMET to maximize RNA quality preservation. After the RNA integrity preservation protocol is complete, do not remove the slides. You can leave the blocking reagent in place and proceed or you can wash the reservoir holding the blocking reagent (see note below).
Note: The reservoir holding the Blocking Reagent can be washed before proceeding with the RNAscope HiPlex + Sequential IF run to make an additional reservoir available in the following RNAscope HiPlex + Sequential IF protocol.

The RNAscope HiPlex + Sequential IF is executed as a separate event following completion of the RNA integrity preservation protocol. Load the reagents needed for multiomics staining per Preparation PDF and start the multiomics (RNAscope HiPlex + Sequential IF) run.

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.

Notes:

- For detailed instructions, including slide loading and unloading, refer to section 4.5 of the COMET user manual.
- 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.
- Assess positive control signal strength. Positive control signal should be visible as punctate dots within the cell. Highly expressed targets might appear as clusters.
- Ensure exposure times for image acquisition result in acceptable background levels.
- Exposure times for negative control and positive control or target probes 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 or contact us; **support-tech@lunaphore.com**

Appendix A. Frozen tissues

This appendix describes steps to prepare and stain fixed- and fresh-frozen tissues using RNAscope HiPlex Pro for COMET with seqIF. If you are starting with sectioned tissue, proceed to second section within this Appendix, Prepare tissue sections for staining. Please note sections should not be >10 µm in thickness for use on COMET™.

Recommended tissue block preparation and sectioning guidance

Fixed-frozen tissue block preparation and tissue sectioning

Fix samples

1. If needed, perfuse tissue with freshly prepared 4% paraformaldehyde (PFA) in 1X PBS or go directly to Step 2.

Note: We recommend perfusing tissues with 1X PBS followed by freshly prepared 4% paraformaldehyde (PFA) in 1X PBS. For sub-optimally prepared samples, you may need to adjust pretreatment conditions.

2. Dissect tissue and fix in freshly prepared 10% NBF or 4% PFA for **24 HRS** at **4°C**.

Freeze tissues and section

1. Immerse the tissue in 10% sucrose in 1X PBS at 4°C until the tissue sinks to the bottom of the container (approximately **18 HRS** for brain tissue).

2. Repeat this step with 20% sucrose in 1X PBS, followed by 30% sucrose in 1X PBS, each time allowing the tissue to sink to the bottom of the container.

3. Freeze the tissue in Optimal Cutting Temperature (OCT) embedding media with dry ice, liquid nitrogen, or 2-methyl butane, and store it in an airtight container at **–80°C**.

4. Before tissue sectioning, equilibrate the tissue blocks at **–20°C** for at least **1 HR** in a cryostat.

5. Section the blocks by cutting 7–10 µm thick sections. Mount the sections on **SUPERFROST PLUS SLIDES**. Sections thicker than 10 µm are not compatible with the COMET instrument.

6. Air dry the slides for **60–120 MIN** at **–20°C**.

OPTIONAL STOPPING POINT . Use sectioned tissue within three months. Store sections with desiccants in a sealed bag at –80°C.

7. Proceed to **Prepare tissue sections for staining**.

Fresh frozen tissue block preparation and tissue sectioning

Prepare samples

1. Remove tissue and cut to fit into cryomolds.
2. Freeze the specimen within 5 MIN of tissue harvest.
3. Embed the frozen tissue in cryo-embedding medium (OCT):
    a. Add two drops of OCT into a cryomold.
    b. Place the frozen tissue on the OCT in the correct orientation for cutting.
    c. Add more OCT to fill the cryomold. Do not allow any air bubbles to form.
    d. Freeze the block by holding the cryomold with forceps on the surface of the liquid nitrogen or isopentane cooled by dry ice or liquid nitrogen or place the cryomold on dry ice.
4. Store the frozen block in an air-tight container at –80°C prior to sectioning.

OPTIONAL STOPPING POINT. Section tissue within three months.

5. Section the block:
    a. Equilibrate block to –20°C in a cryostat ~1 HR.
    b. Cut 10 µm thick sections and mount onto SUPERFROST PLUS SLIDES.
    c. Dry the sections at 60 –120 MIN at –20°C to retain tissue adherence.
6. Store the sections in slide boxes wrapped air-tight with aluminum foil or zip-lock bags at –80°C until use. Note: Sections may be stored for up to three months.

IMPORTANT! Do not fix the sections prior to this step.

OPTIONAL STOPPING POINT. Use sectioned tissue within three months.

7. Proceed to **Prepare tissue sections for staining**.

Prepare tissue sections for staining (Day of Staining)

Post-Fixation

IMPORTANT! Use FRESH fixatives. Do NOT reuse. Do NOT use 10% NBF that has been stored for more than six months, exposed to air for more than a week, or used repeatedly. If using 4% PFA, do not use if stored for more than one week or used repeatedly.

Using fixative that is not fresh can result in suboptimal tissue fixation.

Fixed-frozen tissues

1. Prepare 10% NBF (10% NBF or freshly made 4% PFA in 1X PBS) and chill at 4°C.
2. Bake the slides in a dry oven for 30 MIN at 60°C.
3. Place slides in a slide rack or holder and wash with freshly prepared 1X PBS (diluted in RNase-free water) in slide jar for 5 MIN at RT while moving the rack up and down or with gentle agitation (30 rpm) to remove the embedding media.

4. Post-fix the slides by immersing them in prechilled 10% NBF for 15 MIN at 4°C.
5. Proceed to Dehydrate the tissue.

Fresh frozen tissues

Prepare 10% NBF (10% NBF or freshly made 4% PFA in 1X PBS).

1. Remove the slides from –80°C and place them in a slide rack or holder.
2. Immediately immerse slides in fresh 10% NBF or 4% PFA in 1X PBS. Fix for 60 MIN at RT. The fixation time may need to be optimized for each tissue type.
3. Wash slides with 1X PBS (diluted in RNase-free water) by moving the rack up and down 3–5 times or with gentle agitation (30 rpm) to remove the embedding media.
4. Repeat with fresh 1X PBS
5. Proceed to Dehydrate the tissue.

Dehydrate the tissue

1. Prepare 100 mL of 50% EtOH (diluted in RNase-free water), 100 mL of 70% EtOH (diluted in RNase-free water) and 200 mL of 100% EtOH (or enough to fill staining dishes).
2. Remove the slides from the previous solution and immerse them in 50% EtOH for 5 MIN at RT, with gentle agitation (30 rpm).
3. Remove the slides from 50% EtOH and immerse them in 70% EtOH for 5 MIN at RT, with gentle agitation (30 rpm).
4. Remove the slides from 70% EtOH and immerse them in 100% EtOH for 5 MIN at RT, with gentle agitation (30 rpm).
5. Repeat step 4 with fresh 100% EtOH.
6. Remove the slides from 100% EtOH and let them air dry for 15 MIN at RT (or until completely dry).

Perform target retrieval using the PT Module

Note: refer to the COMET Instrument User Manual available within Lunaphore's customer portal **https://my.lunaphore.com/** for detailed instructions including buffer preparation and storage and instrument operating instructions.

1. The recommended starting retrieval parameters for tissue sections are listed in the tables below, and tissues tested are listed in the next section **"Tissue-specific protocol recommendations."**
2. Start with the recommended retrieval condition listed corresponding to each tissue preparation method.

Tissue Preparation	Retrieval Condition	Temperature/Time
Fixed-frozen	HIER Buffer H (pH 9), 1:15	99°C/10 min
Fresh-frozen	HIER Buffer H (pH 9), 1:15	90°C/10 min

3. Use the preheat setting in the table below.

Preheat Settings	Temperature
Preheat temperature	85°C
Cooling end temperature	65°C

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

Proceed immediately to the next step.

5. Transfer the slides to a separate container with DIW at RT and rinse the slides for 10 SEC.
6. Transfer the slides to 100% EtOH for 3 MIN at RT, incubating with gentle agitation (30 rpm).
7. Air-dry the slide until completely dry. Do not seal the container to allow residual ethanol to evaporate completely.

OPTIONAL STOPPING POINT. Store the slides dry until just before slide loading on COMET. The reagents/COMET protocol can be prepared after slide preparation. It is recommended to launch the COMET run within the same day. Just before the slide loading on COMET, rehydrate the slides by inserting them in a MSB 1X solution

IMPORTANT! Do not load slides dry on COMET. Right before performing the slide loading, insert them on a MSB 1X solution to rehydrate them.

8. Proceed to **Chapter 5 to Set-up and run the protocol**.

Tissue specific protocol Recommendations

This section includes recommended starting conditions for multiple tissue types when prepared as described in the "Fixed frozen block preparation and sectioning" or "Fresh frozen block preparation and sectioning" guidance provided in Appendix D. For tissues with unknown or modified preparation or for tissues not listed, you can contact support for additional recommendations.

For best understanding of performance, it is recommended to assess staining with the species-specific positive control probe and the negative control probe on the tissue of interest. In most tissues, when assay performance is optimized, the *PPIB* control probe, staining is expected to show 5-10 dots/cell with minimal clustering when the corresponding negative control signal is less than or equal to 5 dots per every 10 cells.

For some tissue types or for tissues prepared differently from our guidance, the tissue pretreatment may require optimization to achieve best results.

Preparation	Species	Tissue Type	Fixation	PTM retrieval	PretreatPro
Fixed-frozen	Mouse	Brain	15 min, 4°C	99°C, 10 min	yes
		Liver	15 min, 4°C	99°C, 10 min	yes
		Colon	15 min, 4°C	99°C, 10 min	yes
		Spleen	15 min, 4°C	99°C, 10 min	yes
		Small intestine	15 min, 4°C	99°C, 10 min	yes
Fresh-frozen	Human	Lung	1 hr, RT	90°C, 10 min	yes
		Breast	1 hr, RT	90°C, 10 min	yes
		Tonsil	1 hr, RT	90°C, 10 min	yes
	Mouse	Brain	1 hr, RT	90°C, 10 min	yes
		Liver	1 hr, RT	90°C, 10 min	yes
		Colon	1 hr, RT	90°C, 10 min	yes
		spleen	1 hr, RT	90°C, 10 min	yes

B Appendix B. 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, visit product pages on **https://www.bio-techne.com/**
- 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 on product pages at: **https://www.bio-techne.com/** . 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://www.bio-techne.com/support/contact-us

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.

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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://www.bio-techne.com/support/contact-us.

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