Visium HD Fixed Frozen Tissue Preparation Handbook

Introduction

The Visium HD Spatial Gene Expression workflow is designed to analyze mRNA in tissue sections derived from fixed frozen (FxF) tissue samples. This workflow is facilitated via the CytAssist instrument, which enables the capture of ligated probe products onto the Visium HD Slide. A single CytAssist run accommodates up to two stained tissue slides (tissue placed on a blank slide) as sample input. Proper tissue handling and preparation techniques preserve the morphological quality of the tissue sections and the integrity of mRNA transcripts. Maintaining tissue adhesion and high-quality RNA is critical to assay performance.

This FxF Tissue Handbook provides guidance on:

- Best practices for handling tissue samples and blank slides before and after cryosectioning
- Fixing, cryopreserving, and embedding tissue samples prior to cryosectioning
- Cryosectioning of tissue samples and placement of sections on blank slides
- RNA quality and optional tissue morphology assessment
- Hematoxylin & eosin (H&E) Staining and imaging
- Immunofluorescence (IF) Staining and imaging

Additional Guidance

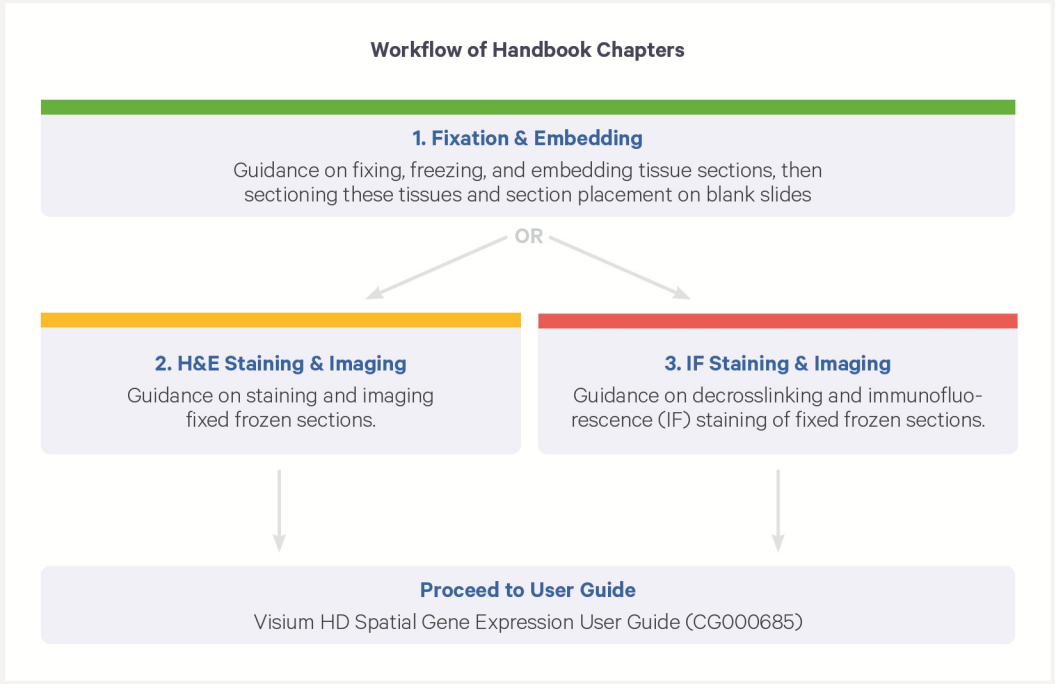
This protocol is compatible with most human and mouse tissue types. See the 10x Genomics Support website for additional resources, including a list of tissues tested.

Contents

Handbook Overview and Navigation

Overview

This handbook describes sample preparation for the Visium HD Spatial Gene Expression workflow. Tabs on the right-hand side of the page denote different sections of this handbook.



Visium HD Spatial Gene Expression Reagent Kits

Consult SDS for handling and disposal information

Visium HD Spatial Gene Expression Reagent Kits

Visium HD Reagent Kit – Small, PN-1000668

Visium HD Reagent Kit – Small
PN-1000668
(store at –20°C)

		#	PN
○	Amp Mix B	1	2000567
●	Extension Enzyme	1	2000389
●	Extension Buffer	1	2000409
●	RNase Enzyme	1	3000605
●	2X RNase Buffer	1	2000411
●	Perm Enzyme B	1	3000553
●	TS Primer Mix B	1	2000537
●	Decrosslinking Buffer B	1	2001094

10x GENOMICS

RNase Inhibitor 40X – PN-1000887

RNase Inhibitor 40X
PN-1000887
(store at –20°C)

		#	PN
●	RNase Inhibitor 40X	1	2001488

10x GENOMICS

Visium HD Cassettes, 6.5 mm, 4 rxns PN-1000669

Visium HD Cassettes, 6.5 mm
4 rxns PN-1000669
(store at ambient temperature)

	#	PN
Visium 2-port Cassette S3, 6.5 mm		
Visium Cassette 2-port gasket, 6.5 mm	2	3001831
Visium Cassette Bottom	2	3001830
Visium Tissue Slide Cassette S3, 6.5 mm		
Tissue Slide Cassette Top	4	3001826
Movable Tissue Gasket 6.5 mm (preassembled with translator)	4	3001828
Movable Tissue Gasket Translator (preassembled with gasket)	4	3001927
Tissue Slide Cassette Bottom	4	3001825
Visium Slide Seals, 12 pack	2	2000283

10x GENOMICS

*The Visium HD Workflow is run with Visium Tissue Slide Cassettes S3 and Visium Cassettes S3. These are referred to as Tissue Slide Cassettes and Visium Cassettes in this document, respectively. Consult the Visium S3 Tissue Slide Cassette Assembly Quick Reference Card (CG000730) for assembly and disassembly information.

Visium 8-port Cassette S3, 4 pk PN-1000685

Visium 8-port Cassette S3
4 pk PN-1000685
(store at ambient temperature)

	#	PN
8-port Gasket Top	4	3001827
Tissue Slide Cassette Bottom	4	3001825

10x GENOMICS

A Visium 8-port Cassette S3, 4 pk (PN-1000685) must be purchased to perform antibody optimization prior to IF staining.

Visium Tissue Slide Cassette S3, 6.5 mm, 4 pk PN-1000684

Visium Tissue Slide Cassette S3, 6.5 mm 4 pk PN-1000684
(store at ambient temperature)

	#	PN
Tissue Slide Cassette Top	4	3001826
Movable Tissue Gasket 6.5 mm	4	3001828
Movable Tissue Gasket Translator	4	3001927
Tissue Slide Cassette Bottom	4	3001825

10x GENOMICS

One additional Tissue Slide Cassette (Visium Tissue Slide Cassette S3 6.5 mm, 4 pk PN-1000684) is required for each processed Tissue Slide during IF staining

10x Genomics Accessories

Visium CytAssist Reagent Accessory Kit PN-1000499

Visium CytAssist Reagent Accessory Kit PN-1000499
(store at ambient temperature)

	#	PN
10x Magnetic Separator	1	2001212
Low Profile Thermocycler Adaptor	2	3000823

10x GENOMICS

Third-Party Items

Successful execution of the Visium HD workflow requires third-party reagents, kits, and equipment in addition to those provided by 10x Genomics. All third-party reagents and consumables should be obtained prior to starting this workflow.

Consult the Visium HD Spatial Gene Expression Protocol Planner (CG000698, Rev B or later) for a list of the following third-party items:

- Additional reagents, kits, and equipment
- Tested pipette tips
- Tested thermal cyclers
- Tested blank slides

10x Genomics has tested all items listed in the Protocol Planner. Unless otherwise noted, 10x Genomics recommends using these items to ensure optimal assay performance. Substituting materials may adversely affect assay performance.

Tips & Best Practices

Icons

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

General Reagent Handling

- Fully thaw and thoroughly mix reagents before use.
- When pipette mixing, unless otherwise specified, set pipette to 75% of total volume.

General Best Practices

- When handling tissues, use sterile technique and nuclease-free reagents/consumables.

Pipette Calibration

- Follow manufacturer's calibration and maintenance schedules.

RNA Quality Assessment

- Assess RNA quality of the tissue block by calculating the percentage of total RNA fragments › 200 nucleotides (DV200) of RNA extracted from tissue sections.
- A column-based method of RNA isolation should be used. Alternate methods of RNA isolation may affect score.
- Various factors could lead to variations in DV200 scores, such as:
  - Specific tissue types
  - Tissue heterogeneity

- Diseased or necrotic tissues
- Sample preparation and handling
- Loading concentration or ladder errors on the RNA QC platform

Cryosectioning Temperature

- Cryosectioning temperature impacts tissue section integrity. Use a temperature setting of –15°C to –20°C for the blade and –10°C to –15°C for the specimen head.
- Temperature settings depend upon local conditions, tissue types, and the cryostat used. Settings should be optimized based on the quality of resulting tissue sections.
- During prolonged sectioning periods, allow the cryostat temperature to equilibrate by briefly closing the chamber.

Sectioning Speed

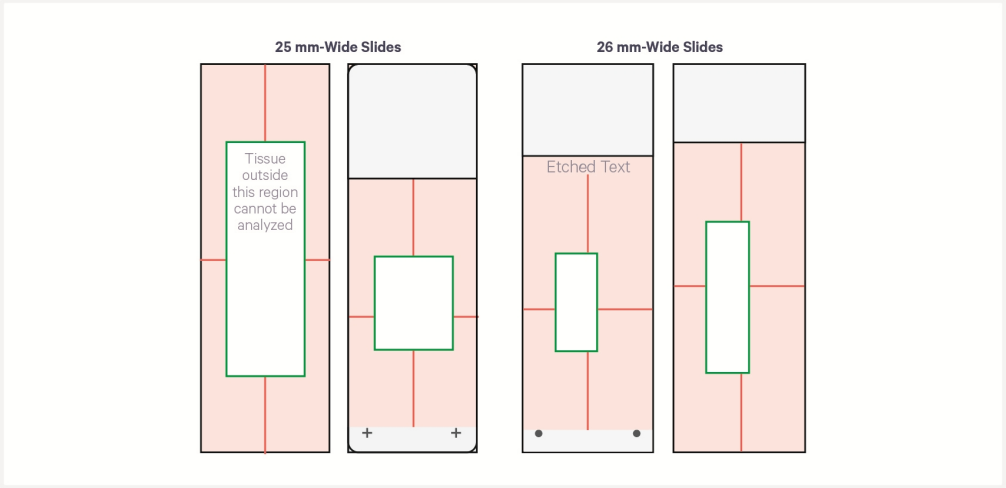
- Sectioning speed depends upon the desired thickness of the sections and the condition of the tissue. Harder and thicker sections require slow sectioning speed.
- Faster sectioning speed may lead to cracks or tears in the sections. Faster section speed may also damage the tissue block or cryostat.

Section Thickness

- Recommended section thickness for most tissue types is 10 µm, but tissues from 10–20 µm are compatible with the assay. Sections outside of that range may result in reduced performance.

Section Placement on Blank Slides

- After section placement, blank slides are referred to as tissue slides.
- Before section placement, draw an outline of the allowable area on the back of the blank slide to ensure compatibility with the Visium CytAssist instrument and Tissue Slide Cassette, as shown below. Example allowable area images below are not to scale.



Consult the Visium CytAssist Accessory Kit Quick Reference Card (CG000548) on the 10x Genomics support website for complete instructions on determining allowable area. Consult the Visium HD Spatial Gene Expression Protocol Planner (CG000698) for a list of tested slides.

- If working with sections on multiple tissue slides, ensure that sections are placed in the same location on the tissue slides for improved imaging efficiency.
- Each tissue slide can only be processed with the Visium CytAssist instrument once.
- 10x Genomics recommends placing tissues with large amounts of connective tissue (like breast, skin, or colon) on Schott Nexterion Slide H - 3D Hydrogel Coated Slides (Schott North America, PN-1800434) to minimize risk of tissue detachment. Schott H slides may also be used if prior detachment has been observed with tissues of interest.

Practice Section Placement

- Practice correct section placement using nonexperimental blocks.
- Practicing section placement also allows for determining an ideal tissue thickness for the tissue type or block.
- Sections should be placed on compatible blank slides listed in the Visium HD Spatial Gene Expression Protocol Planner (CG000698).

Optional - Area of Interest Annotation

If a tissue section does not fit completely within the Visium HD Slide Capture Area, a smaller area of interest (AOI) should be defined.

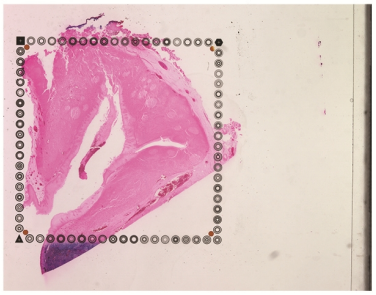
The AOI should be:

- Small enough to fit inside a well in a Tissue Slide Cassette gasket. Tissue outside of the gasket will not be processed during the assay workflow.
- Centered within the appropriate Capture Area size on the Tissue Slide Stage on the Visium CytAssist instrument.

Defining an AOI can occur after section placement by examining the tissue under a microscope or after H&E staining and imaging.

H&E Staining:

- Annotate the AOI using a marker on the back side of the tissue slide based on the assessment of tissue morphology from the H&E image. This annotation can serve as a reference for gasket placement when assembling the tissue slide in the Tissue Slide Cassette.
- Remove annotations with isopropanol or ethanol before loading the tissue slide onto the CytAssist instrument. Failure to remove annotations can lead to improper tissue detection, causing automatic tissue registration to fail. Should this occur, manual tissue registration on Loupe Browser will be required.
- When loading the tissue slide on the instrument, the pale eosin staining of the tissue area included within the gasket and the darker H&E staining of tissue areas outside the cassette will provide sufficient contrast to identify and align the correct tissue region on the CytAssist.



IF Staining:

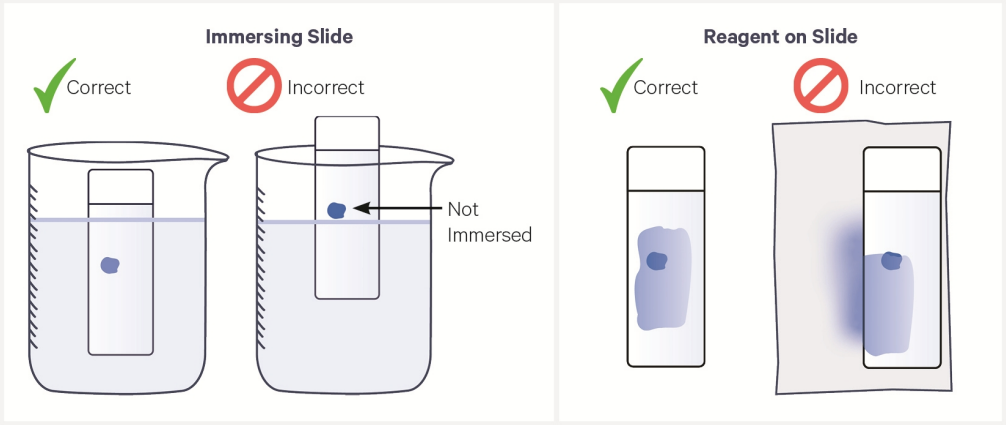
- Before immunostaining, the tissue slide must be assembled in the Tissue Slide Cassette for decrosslinking. Since decrosslinking requires placing the gasket over the AOI prior to staining, 10x Genomics recommends using tissue morphology information from an adjacent H&E or IF stained section to annotate the back of the tissue slide. This annotation can serve as a reference for the gasket placement when assembling the tissue slide in the Tissue Slide Cassette for the decrosslinking and immunostaining steps as well as help with reapplying the gasket after coverslip removal.
- Remove annotations with isopropanol or ethanol before loading the tissue slide onto the CytAssist instrument. Failure to remove annotations can lead to improper tissue detection, causing automatic tissue registration to fail. Should this occur, manual tissue registration on Loupe Browser will be required.
- When loading the slide on the instrument, the gasket imprint marks the boundary of the tissue area and can be used to align the AOI on the CytAssist.

Handling Tissue Slides

- If a laboratory wipe is required, use 100% polyester lint-free laboratory wipes. Lens paper or non-lint free laboratory wipes are not suitable alternatives.

  See Visium HD Spatial Gene Expression Protocol Planner (CG000698) for tested part numbers.

- When immersing slides in reagent, ensure all tissue sections are immersed.
- Maintain tissue slides in a mailer in dry ice after removal from the freezer. Do not remove from dry ice until ready to thaw slides on a thermal cycler during Tissue Slide Preparation.
- When placing a tissue slide in a slide mailer or 50-ml centrifuge tube after sectioning, mailer or tube should be pre-cooled to cryostat temperature for 10–15 min.
  - Immediately place storage container in dry ice for transport to a -80°C freezer for long-term storage.
  - Ensure freezer maintains a stable temperature.
  - Avoid tissue slides touching one another while in storage.
- Always wear gloves when handling slides.
- DO NOT touch the tissue sections on the slide.
- Minimize exposure of the slides to sources of particles and fibers.
- Inspect slides prior to tissue placement for particles and fibers. If found, remove with a lint-free laboratory wipe or compressed air. If using compressed air, do not introduce moisture to the slides.
- Keep the slide flat on the bench when adding reagents.
- Ensure that no absorbent surface is in contact with the reagents on the slide during incubation.



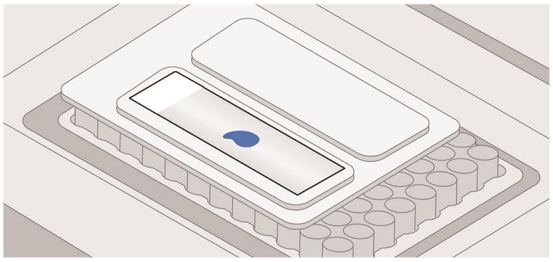
- DO NOT use a hydrophobic barrier (such as one applied by a PAP pen) on slides. These barriers may affect assay performance.
- Tissue slides may be annotated on the back of the slide to highlight an AOI. See Optional - Area of Interest Annotation on page 12 for more information.
- When imaging, avoid pressing down on the coverslip to ensure easy removal after imaging.

Tissue Slide Incubation

Incubation using a Thermal Cycler:

- Position a Low Profile Thermocycler Adapter on a thermal cycler that is set at the incubation temperature. Low Profile Thermocycler Adapter must come to temperature before placing tissue slide.
- Ensure that the Low Profile Thermocycler Adapter is in contact with the thermal cycler surface uniformly. Consult the Visium HD Spatial Gene Expression Protocol Planner (CG000698) for information on thermal cycler compatibility.
- Position tissue slides on the Low Profile Thermocycler Adapter with the side with tissue facing up.
- Ensure that the entire bottom surface of tissue slides is in contact with the Low Profile Thermocycler Adapter.
- DO NOT close the thermal cycler lid when incubating tissue slides.
- Allow Low Profile Thermocycler Adapter to cool before removing from thermal cycler.

Incubation on a Thermal Cycler

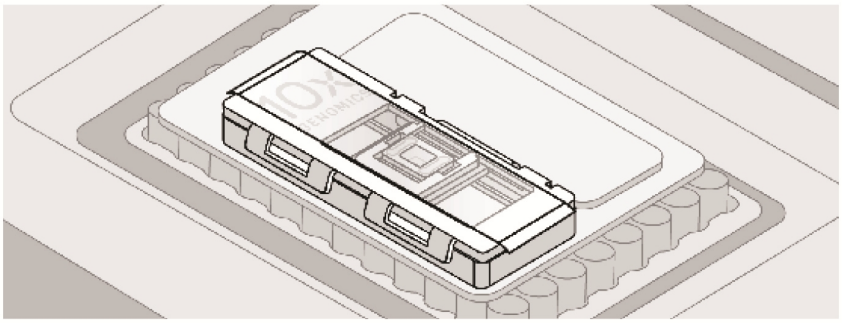


Cassette Incubation

Incubation using a Thermal Cycler:

- Position a Low Profile Thermocycler Adapter on a thermal cycler that is set at the incubation temperature. Low Profile Thermocycler Adapter must come to temperature before cassette placement.
- Ensure that the Low Profile Thermocycler Adapter is in contact with the thermal cycler surface uniformly. Consult the Visium HD Spatial Gene Expression Protocol Planner (CG000698) for information on thermal cycler compatibility.
- Ensure that the cassette sits flush with the raised portion of the Low Profile Thermocycler Adapter.
- Allow Low Profile Thermocycler Adapter to cool before removing from thermal cycler.

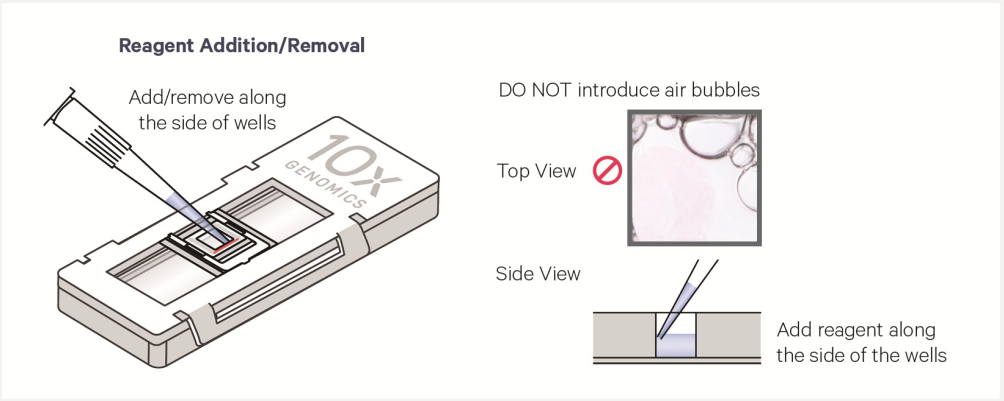
Ensure Cassette Sits Flush Against Thermocycler Adapter



Reagent Addition to & Removal from Wells

Reagent Addition

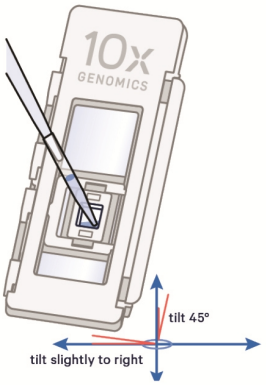
- Assemble slide into the cassette flat on a clean work surface. See Cassette Assembly Quick Reference Card (CG000577).
- Dispense and remove reagents along the side of the wells without touching the slide surface, tissue sections (when applicable), and without introducing bubbles.
- DO NOT forcefully insert pipette tip into well corners to avoid damaging the Visium HD Slide.
- When processing two or more Visium Tissue Slide Cassettes, remove and add reagent from the first cassette before proceeding to the next cassette. Ensure tissue sections are always covered with reagent in between removal and addition steps to avoid drying out of tissue samples.
- Always cover the Capture Area or tissue completely when adding reagents to the well. A gentle tap may help spread the reagent more evenly.



Reagent Removal from Wells

- Slightly tilt the cassette while removing the reagent. DO NOT completely tilt the cassette at a 90 degree angle to avoid liquid from entering other grooves within the gasket. See image below for proper technique.
- Place the pipette tip on the bottom edge of the wells.
- Remove reagents along the side of the wells without touching the tissue sections (when applicable) and without introducing bubbles.
- Remove all liquid from the wells in each step. To ensure complete removal, check the bottom of the well by tilting the cassette slightly. A meniscus at

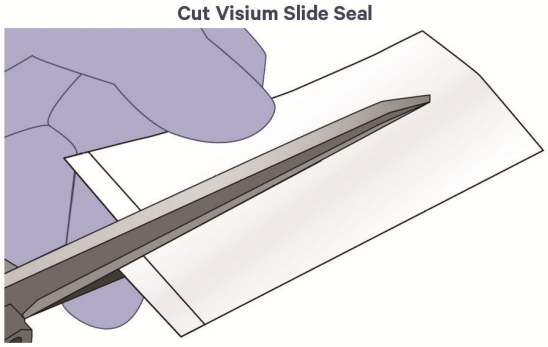
the bottom of the well will indicate the presence of liquid in the well. Repeat removal steps until no reagent remains.



Visium Slide Seal Application & Removal

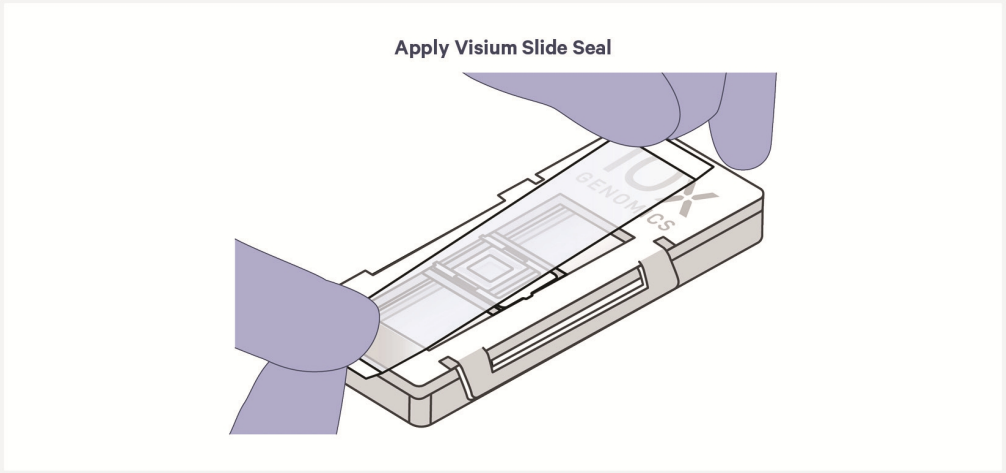
Application

- If applying a Visium Slide Seal to a Tissue Slide Cassette, the seal must be cut in half lengthwise. Cut the seal as shown in the image below. Six pre-cut seals per tissue slide are necessary for this assay.



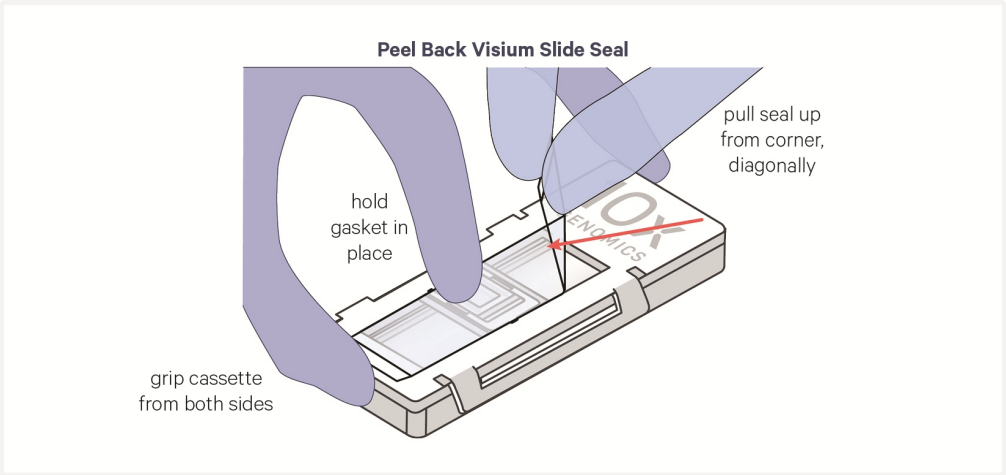
- Place the cassette flat on a clean work surface. Ensure surface of cassette is dry.
- Remove the back of the adhesive Visium Slide Seal.
- Align the Visium Slide Seal with the surface of the cassette and apply while firmly holding the cassette with one hand.
- Press on the Visium Slide Seal to ensure uniform adhesion.

- Use a fresh Visium Slide Seal when prompted during the protocol. Steps that do not require a new slide seal will specify that the slide seal should be pulled back and reapplied instead.



Removal

- Place the cassette flat on a clean work surface.
- Grasp a Visium Slide Seal corner. Carefully pull on the Visium Slide Seal up and across the cassette while firmly holding the cassette. Ensure that no liquid splashes out of the wells. Movement of the gasket during slide seal removal could damage the tissue section.



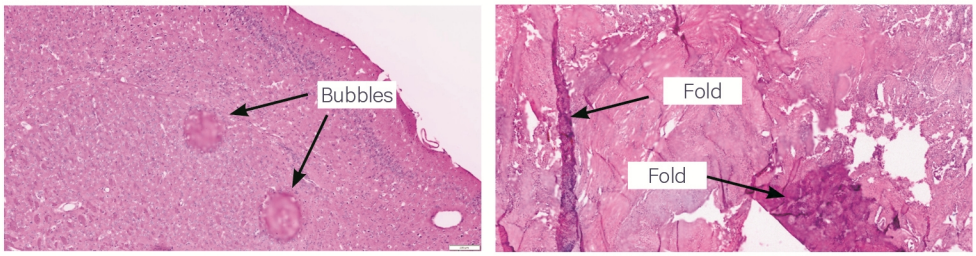
1. Tissue Handling, Fixation, Embedding, Sectioning, Section Placement, and Quality Assessment

Overview

This chapter provides guidance on tissue handling, fixation, cryopreservation, embedding, sectioning, section placement, and quality assessment. Fixed frozen tissues can be challenging to work with for the following reasons:

- Sectioning of fixed frozen tissue blocks can be more challenging and is typically more prone to folds and tears due to the composition of this sample type.
- Localized folds across the tissue section can result in nonuniform adhesion of the tissue section to the slide. These areas are prone to localized micro-detachments that appear as bubbles or folds in the H&E image.

Bubbles and Folds in Tissue



The above factors typically lead to compromised tissue adhesion of varying degrees depending on the tissue type (tissues with large amounts of connective tissue such as breast, colon or cellularly less dense such as lung, are more prone to detachment) and slide type used. Refer to the Visium HD Spatial Gene Expression Protocol Planner (Document CG000698) for a full list of tested slides. Proper tissue handling, storage, and preparation techniques preserve the morphological quality of tissue sections and the integrity of mRNA transcripts.

Freshly obtained tissue samples are fixed in a formaldehyde or paraformaldehyde (PFA) solution to preserve tissues in their current state. Fixed tissues are then transferred into a cryopreservant sucrose solution to prevent ice crystal formation in tissues. Once fixed and cryopreserved, tissue samples are embedded in a freezing and embedding compound, Optimal Cutting Temperature (OCT), to preserve the morphology of the tissue and to

provide structural support during cryosectioning. Other methods of freezing and embedding for fixed frozen samples have not been validated.

For large tissues, segment fresh tissue so that the thickness of the tissue does not exceed 5 mm. This ensures optimal penetration of the fixative and uniform fixation. To help determine if the tissue is of an appropriate thickness for fixation, a standard biopsy/tissue embedding cassette may be used. A tissue of appropriate thickness should fit easily in a cassette of this type.

To assess RNA quality, DV200 scores may be calculated from the chosen fresh frozen sample. DV200 is a measure of RNA fragments with sizes greater than >200 bases. Tissue sections with DV200 ≥ 50% are optimal for the Visium HD assay. Low DV200 scores do not necessarily result in poor data, but high scores are more likely to correlate with higher sensitivity.

While DV200 values provide insights into RNA integrity, it is also important to consider the following key factors that help maintain RNA quality and tissue integrity:

- Gentle handling of fresh tissue
- Minimizing post-mortem intervals
- Washes with RNAse-free, cold isotonic storage conditions (such as cold 1X PBS) prior to fixation
- Immediate fixation of fresh samples
- Assessment of your H&E image for signs of necrosis or other impacts to tissue integrity

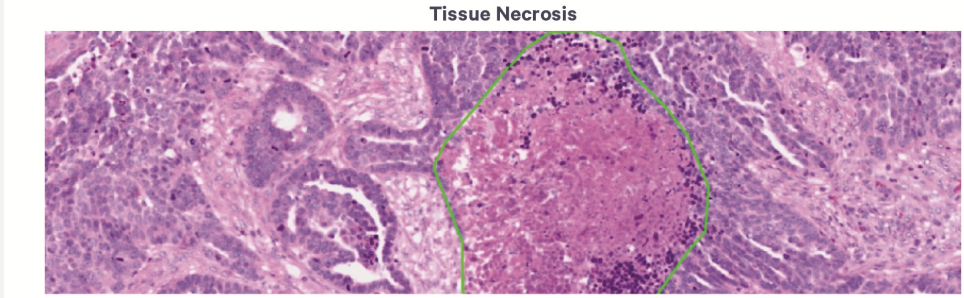
If additional sections are available for testing, optional tissue morphology assessments via DAPI and H&E staining are recommended.

Tissue Handling

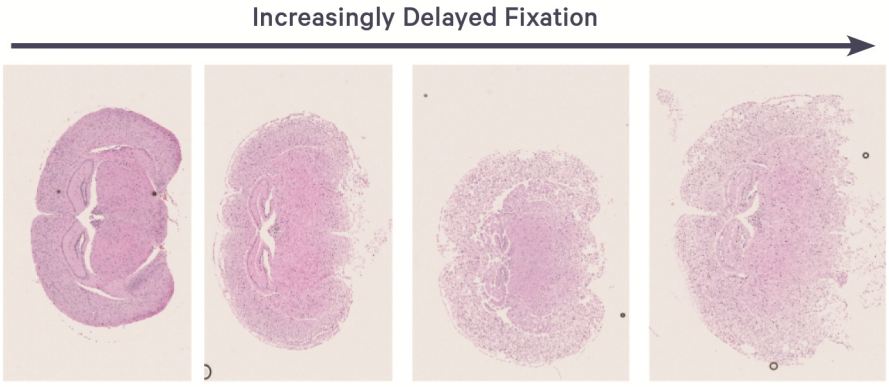
Prior to fixation and embedding, tissues should be handled according to the following guidelines to maximize RNA quality and prevent degradation.

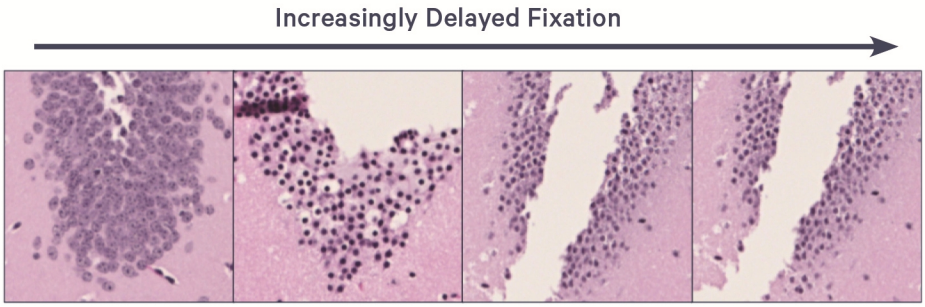
- Gentle Handling - Tissues should be handled gently to avoid mechanical stress. Mechanical stress may damage tissue structure. Examples of processes that introduce mechanical stress include ischemia, coagulative necrosis from electrocautery, and hemorrhages from surgical trauma. In the example image below, necrotic regions marked by green lines have signs of nuclear condensation (pyknosis) and nuclear fragmentation (karyorrhexis). These regions are typically characterized by poor RNA quality. The

surrounding regions have normal-appearing nuclei.



- Minimizing Ischemia/Post Mortem Interval (PMI) and Fixation Timing - Prolonged ischemia and PMI can negatively affect tissue quality. If processing delays occur, keep tissues in a cold isotonic solution such as cold 1X PBS and avoid exceeding four hours between tissue resection and freezing. However, this allowable time may vary across tissues. For instance, some tissues have higher levels of RNases (lung, pancreas, etc.) and can be more prone to degradation. Long-term storage of fresh tissue in isotonic solutions is not recommended and tissue samples should be fixed immediately after resection. Delayed tissue fixation may lead to autolysis, degrading tissue, and negatively impacting results.
- In the following example H&E images, considerable degradation of tissue quality or autolysis occurs as the time from fresh mouse brain tissue collection to fixation increases. In the zoomed in image, the dentate gyrus region shows considerable deterioration of nuclear architecture (signs of nuclear condensation and nuclear fragmentation) with increasing delays to fix the tissue





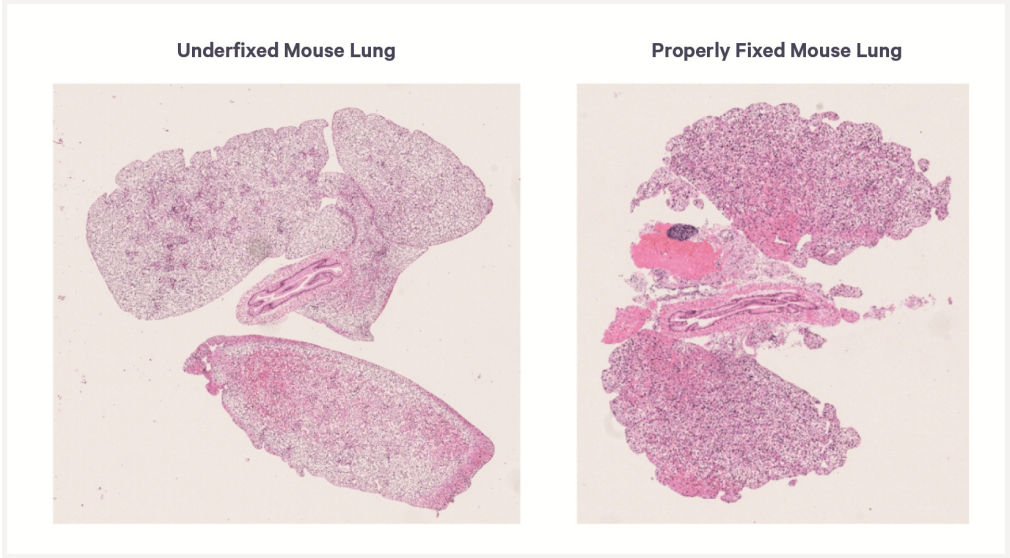
Tissue Fixation

Tissue blocks are fixed in cold 4% formaldehyde or PFA solution (pH 7.4) until the tissue sinks to the bottom of the solution, approximately 12-16 hours at 4°C. Tissue block size can impact fixation time. If necessary, tissue should be trimmed so that tissue thickness does not exceed 5-10 mm. The fixed tissue blocks are then cryopreserved upon placement in 30% sucrose solution until the tissue sinks to the bottom of the solution, approximately 6-12 hours. Ensuring that tissues are fully submerged in both fixative and cryopreservant solutions is critical for success.

Improper sample fixation (over or underfixation) can lead to poor transcript density, either across the entire tissue sample or in the interior of the sample. It can also lead to nuclei degradation, which negatively affects segmentation and transcript assignment to cells.

- Underfixation can lead to the continued activity of certain enzymes postmortem or post-operation, which can contribute to the degradation of proteins, nucleic acids, and lipids. Tissues may not adequately preserve the RNA if underfixed, leading to fragmented strands of nucleotides. Underfixation can lead to the presence of artifacts in the tissue such as irregular chromatin patterns, overstained cytoplasm (eosin), and common autolysis artifacts (such as separation of epithelium from connective tissue).
- Overfixation can lead to the loss of structural integrity of certain molecular features, the oxidation of lipids, excessive cross-linking, and decreased antigenicity. Tissue hardening is a sign of overfixation, which may make sectioning difficult. Overfixation can lead to artifacts in the tissue such as

irregularly shaped or smaller cells or hyperchromatic nuclei staining.

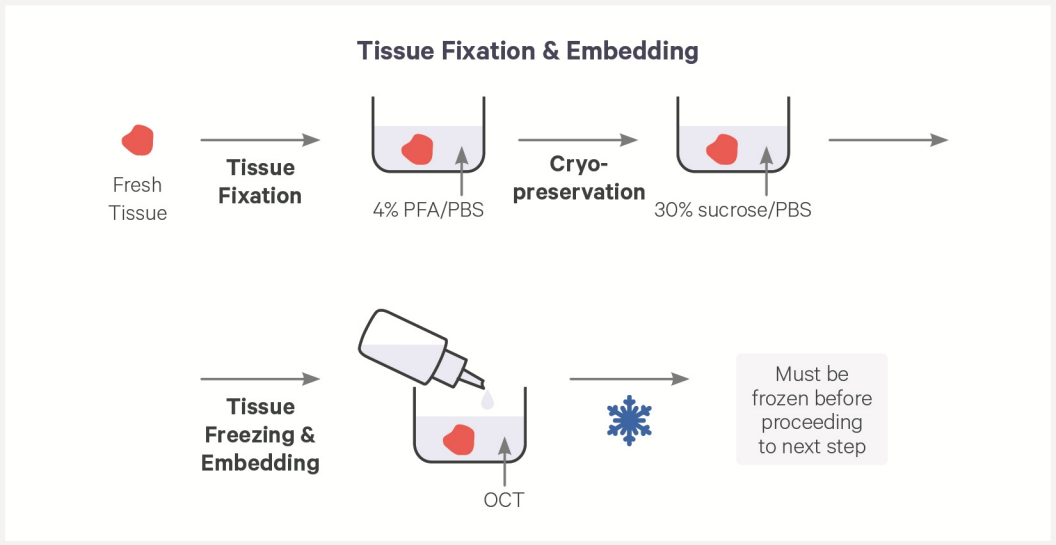


Simultaneous Tissue Freezing & Embedding

After fixation and prior to cryosectioning, tissue blocks are simultaneously frozen and embedded in OCT utilizing an isopentane bath in dry ice. If dry ice is unavailable, use an isopentane bath in liquid nitrogen.

OCT embedding of the tissue offers the following advantages:

- Preserves the morphology of the tissue and provides structural support during cryosectioning.
- Maintains an optimal temperature during sectioning, thus leading to smooth sections.



Cryosectioning

OCT embedded tissue blocks are removed from –80°C storage and cryosectioned in a cryostat to generate sections for blank slides while keeping samples in a cold environment.

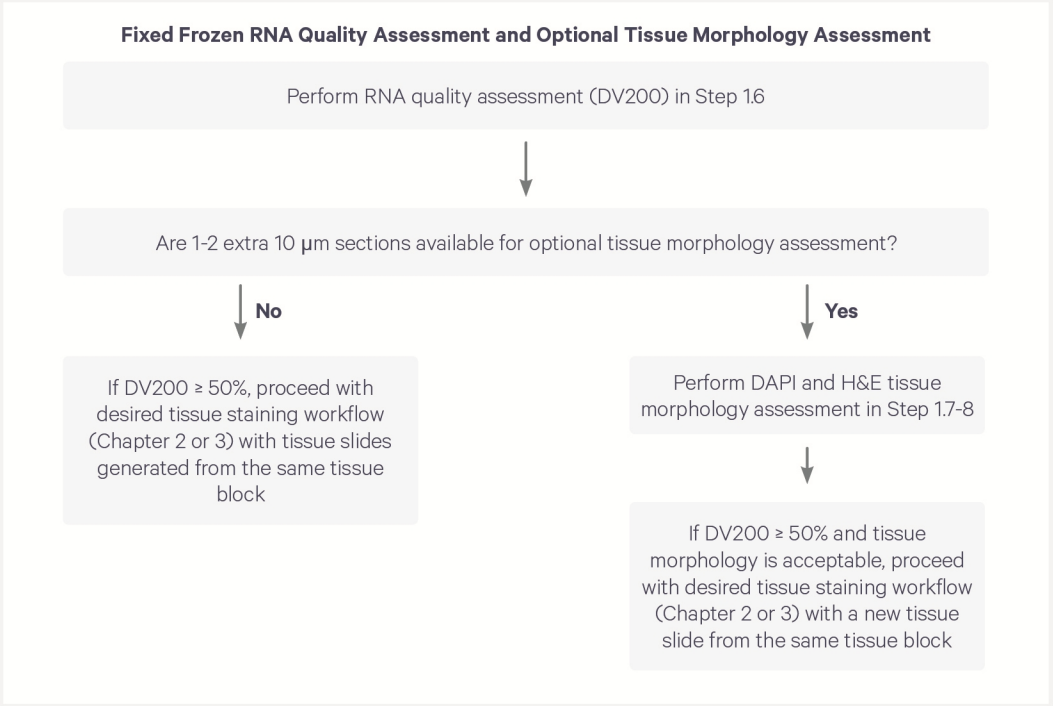
Section Placement

Tissue sections are placed within the allowable area on compatible blank slides. Placing only one section per slide is recommended.

10x Genomics recommends placing tissues with large amounts of connective tissue (like breast, skin, or colon) on Schott Nexterion Slide H - 3D Hydrogel Coated Slides (Schott North America, PN-1800434) to minimize risk of tissue detachment. Schott H slides may also be used if prior detachment has been observed with tissues of interest.

RNA Quality Assessment

Assess tissue block RNA quality by calculating DV200 of freshly collected tissue sections. RNA quality assessment should be done before placing the tissue sections on blank slides. Use sections from the same tissue block that will produce sections for the main assay. If extra sections are available, placing them on slides for Optional Tissue Morphology Assessment is recommended. Use the decision tree below to determine what quality assessment protocols should be followed.



See RNA Quality Assessment for details. DV200 should be ≥ 50%. Low DV200 scores do not necessarily result in poor data, but high scores are more likely to correlate with good data. Various factors could lead to low DV200 scores, such as specific tissue types, diseased or necrotic tissues, ischemic tissue, and suboptimal sample preparation and handling.

Optional Tissue Morphology Assessment

Assessment of tissue morphology prior to performing the Visium HD assay is recommended, but not mandatory. This assessment is composed of DAPI and H&E staining. After staining, tissues are assessed to determine suitability for the Visium HD assay.

DAPI and H&E may be performed on the same tissue section or on serial sections. If extra tissue sections are not available to perform this assessment, H&E or IF images generated later in this handbook may be evaluated to gain additional insights on sample quality.

Estimation of DV200 scores as well as the morphological assessments based on H&E and/or DAPI help identify potential tissue morphology and RNA quality issues. However, these methods are not all-encompassing and may not always correlate exactly to final assay performance.

1.0 Preparation

Consult the Visium HD Spatial Applications Protocol Planner (CG000698) for a list of third-party items.

For Fixation

Items		Preparation & Handling
Maintain on ice		
☐	1X PBS	If using 10X PBS stock, PBS must be diluted to 1X using nuclease-free water. Pre-chill large volume of 1X PBS on ice or at 4°C prior to use. Following preparation, fill a 50-ml centrifuge tube with 40 ml cold 1X PBS. Prepare one centrifuge tube per tissue. Maintain on ice. Return 1X PBS to ice in between use.
☐	Fixation Solution	Weigh 4 g paraformaldehyde in chemical hood. Add 1X PBS to volume of 99 ml. Slowly heat while stirring (DO NOT heat >60°C). Cool to room temperature. Adjust to pH 7.4 with HCl. Add 1X PBS to final volume of 100 ml. If prepared ahead of time, store at 4°C. Alternatively, use commercial 16% paraformaldehyde or 37% formaldehyde and dilute to 4% concentration using 1X PBS. Following preparation, fill a 50-ml centrifuge tube with 40 ml 4% PFA solution. Prepare one centrifuge tube per tissue. Maintain on ice.
Maintain at room temperature		
☐	30% Sucrose/PBS Solution	Weigh 30 g sucrose. Add 1X PBS for final volume of 100 ml. Vortex briefly. Filter using a sterile 0.22 µm filter. If prepared ahead of time, store at room temperature. Following preparation, fill a 50-ml centrifuge tube with 40 ml 30% sucrose solution. Prepare one centrifuge tube per tissue block. Maintain at room temperature.

For Freezing & Embedding

Items		Preparation & Handling
Prepare		
☐	Isopentane bath in dry ice (preferred) OR liquid nitrogen	Fill two-thirds of a polypropylene beaker with isopentane (sufficient to fully submerge the cryomold) and place in dry ice (same level as isopentane) to allow sufficient contact. Incubate for 15 min. **Tissue Freezing & Embedding** Isopentane bath setup  If dry ice is unavailable and the isopentane bath must be prepared with liquid nitrogen, fill two-thirds of a metal beaker with isopentane sufficient to fully

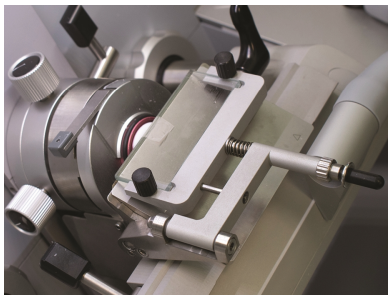
Items		Preparation & Handling
		submerge the tissue (separate freezing & embedding) or cryomold (simultaneous freezing & embedding). Place in a liquid nitrogen dewar (same level as isopentane). Incubate 15 min. Isopentane will slowly freeze if left too long in the liquid nitrogen bath. When this occurs, briefly remove the metal beaker containing isopentane from the liquid nitrogen bath. Once thawed, place the beaker back in the liquid nitrogen bath. DO NOT use the same isopentane bath for different tissue types.
☐	Pre-cooled OCT	Place OCT on ice for ≥30 min.
☐	Pre-cooled forceps	Place forceps on dry ice for ≥30 min.
☐	Pre-cooled cryovial or resealable bag	Place cryovial or resealable bag on dry ice for ≥30 min.
Confirm		
☐	Cryomold	The cryomold used for embedding should be of appropriate size to fit the tissue sample.

For Cryosectioning

Items		Preparation & Handling
Adjust		
☐	Cryostat temperature settings	Turn cryostat on to pre-cool chamber. Recommended sectioning temperature is –10°C to –20°C for cryostat blade and specimen head. Follow manufacturer's manual for detailed operations. Impact of improper cryostat specimen head can be found in the Troubleshooting section.
Equilibrate		
☐	Blank slides to the cryostat chamber temperature	Slides should be cooled down to cryostat temperature for ≥30 min. Warm slides will lead to quick melting of the sections and degradation of RNA. If using a Nexterion Schott H slide, ensure the slide has been equilibrated to room temperature for 30 min after removing from storage before cooling down to cryostat temperature for ≥30 min.
☐	OCT-embedded tissue block to cryostat chamber temperature	Freshly prepared or OCT-embedded tissue block stored at –80°C must be equilibrated to cryostat chamber temperature for at least 30 min before sectioning. If the tissue block is too cold, it will lead to section cracking. If the tissue block is too warm, it will lead to section compression or crumpling.

For Section Placement

Items		Preparation & Handling
Confirm		
☐	Section thickness setting	Recommended section thickness is 10 µm, but tissue section thicknesses of 10–20 µm have been validated with the assay.

Items		Preparation & Handling
☐	Anti-roll plate is in place *Optional*	Anti-roll plate prevents rolling of tissue sections. Optimize the position of anti-roll plate based on the tissue block size. If possible, adjust the position of anti-roll plate before reaching area of interest. 
☐	Specimen head temperature	Confirm the temperature of the specimen head. If the sections appear cracked, the specimen head is too cold. If the sections appear crumpled, the specimen head is too warm. Adjust temperature accordingly.
☐	Slide storage	Pre-cool slide mailer or 50-ml centrifuge tube (one container per tissue slide) to cryostat temperature for 10–15 min.
Practice		
☐	Section placement on a blank slide.	Create a representative allowable area on a blank slide. Optimize section quality and practice section placement within the allowable area before working with experimental blocks.

For RNA Quality Assessment

Items		Preparation & Handling
Equilibrate		
☐	Microcentrifuge tube to cryostat chamber temperature	Microcentrifuge tube should be cooled down to cryostat temperature by placing the microcentrifuge tube in the cryostat chamber for ≥30 min or at -20°C for ≥30 min
☐	Forceps to the cryostat chamber temperature	Forceps should be cooled down to cryostat temperature by placing the forceps in the cryostat chamber for ≥30 min or at -20°C for ≥30 min

For Optional DAPI Staining

Prepare		
☐	DAPI Solution	Prepare DAPI Solution on ice, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain on ice. 500 µl of DAPI solution is sufficient for one slide.

DAPI Solution	Stock	Final	Total Amount (µl)
1X PBS	-	-	497.5
DAPI	100X	0.5X	2.5
Total	**-**	**-**	**500.0**

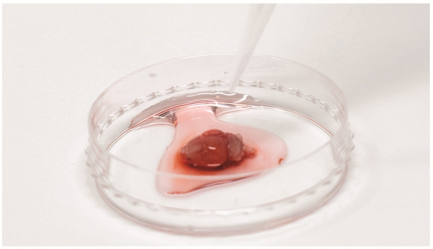
1.1 Tissue Fixation

a. Fill bottom of petri dish with cold 1X PBS. Transfer freshly obtained tissue to petri dish using forceps or spatula.

TIPS *Work quickly between tissue harvest and fixation steps.*

b. Rinse tissue using a pipette with cold 1X PBS to remove residual blood.

Tissue Rinse



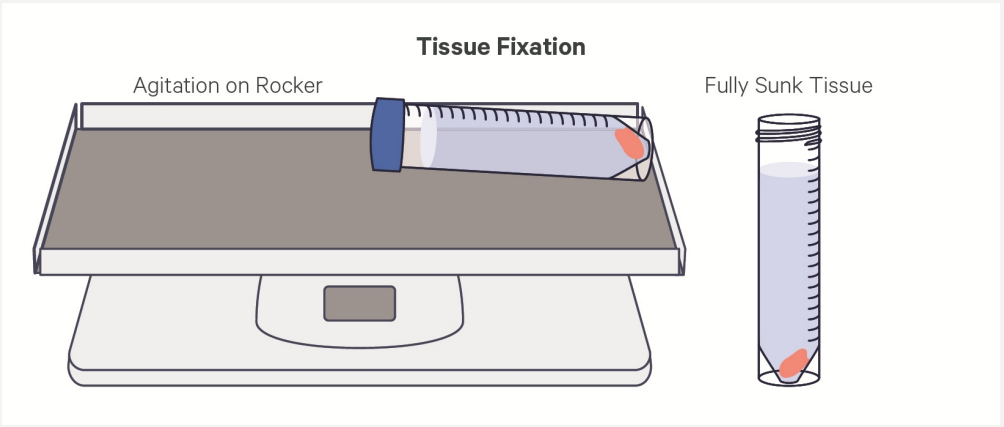
c. If necessary, segment fresh tissue with a scalpel so that the thickness of the tissue does not exceed 5 mm. Fixing tissue that is too large may lead to incomplete fixation.

d. Using either forceps or a spatula, transfer tissue into a 50-ml centrifuge tube containing **40 ml** freshly prepared, cold Fixation Solution.

e. Gently agitate centrifuge tube containing tissue on a rocker at lowest setting until tissue sinks to bottom of tube, around **12–16 hrs** at **4°C**.

After 12–16 hrs, take tube off rocker and check that tissue has sunk to tube bottom. If not, check every 2–3 hrs for up to 24 hrs.

It is essential that the tissue sinks to the bottom of the solution, indicating proper fixation. Underfixation can lead to a reduction in RNA quality.



f. Using either forceps or a spatula, transfer tissue into a 50-ml centrifuge tube containing **40 ml** cold 1X PBS.

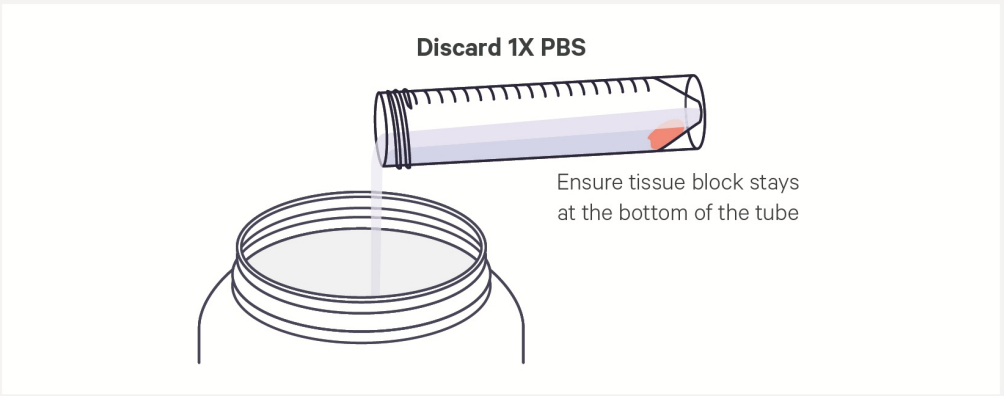
g. Incubate for **1 min** at **room temperature**.

h. Carefully discard cold 1X PBS, ensuring tissue block stays at tube bottom.

TIPS

The tube cap may be held near the mouth of the tube to catch the tissue in case the tissue block dislodges.

i. **Repeat** cold 1X PBS wash two more times.



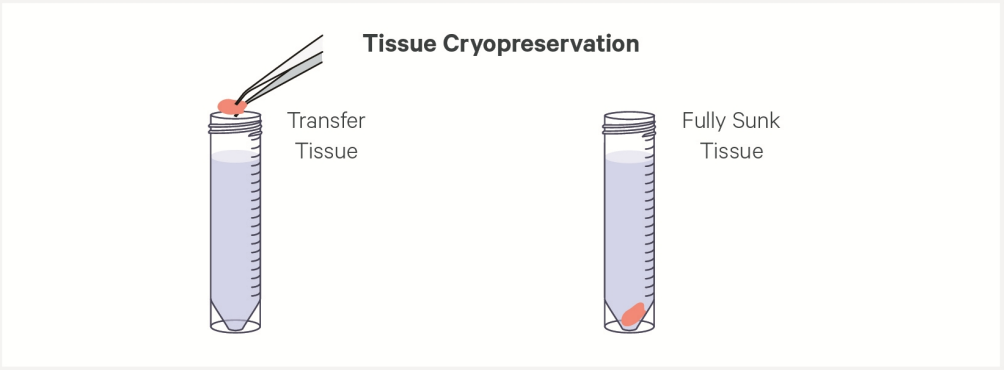
j. Discard excess 1X PBS.

k. Using either forceps or a spatula, transfer tissue onto a folded lint-free laboratory wipe. Gently blot the tissue with the lint-free laboratory wipe to remove any residual 1X PBS.

Alternatively, cover the top of the tube with a lint-free laboratory wipe and tilt the tube so that the tissue falls onto the lint-free laboratory wipe.

l. Using either forceps or a spatula, transfer tissue into a 50-ml centrifuge tube containing 30% sucrose/PBS solution and incubate until tissue sinks to bottom of tube, approximately **6–12 h** at **4°C**.

After 6 hrs, check that tissue has sunk to tube bottom. If not, check every 2–3 hrs for up to 12 hrs.



m. Proceed **immediately** to 1.2 Simultaneous Freezing & Embedding below.

1.2 Simultaneous Freezing & Embedding

a. Label an appropriately sized cryomold to mark tissue orientation and place at room temperature.

TIPS

Label the cryomold before adding OCT and tissue. OCT will quickly turn white once frozen, making it hard to determine tissue orientation later.

b. Fill cryomold with pre-cooled OCT without introducing bubbles.

c. Using pre-cooled forceps, place tissue into OCT, covering any exposed surfaces with additional OCT. Confirm there are no bubbles, especially near tissue.

d. Immediately fully submerge cryomold containing tissue and OCT in isopentane bath.

e. Wait until OCT is completely frozen.

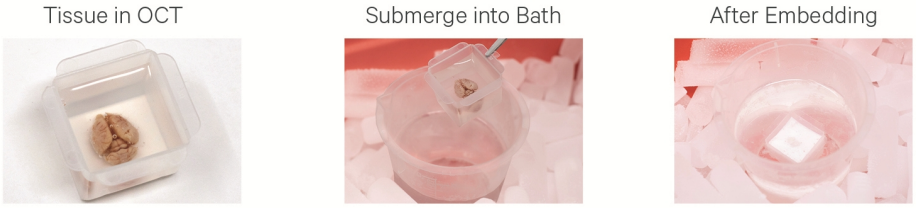
f. Store OCT-embedded tissue block in a sealed container at **–80°C** for **long-term** storage or **immediately** proceed to 1.3 Cryosectioning on the next page.

A WHEATON CryoELITE cryovial or a resealable bag should be used for storing the tissue block. Failure to use a sealed container for storage may dehydrate and damage the tissue.

If using a cryovial, apply a thin layer of OCT to the tissue to form a protective layer. Allow OCT to freeze. Score the OCT to mark tissue

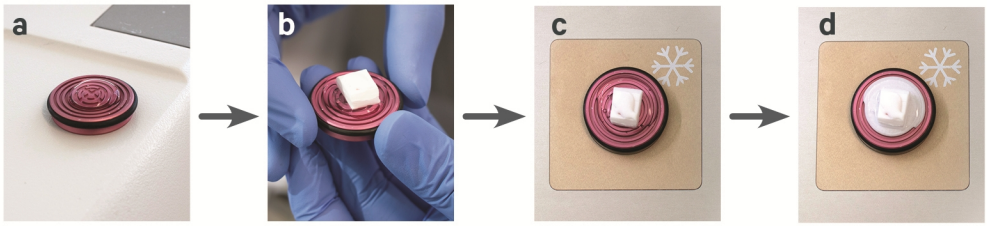
orientation, as this will be lost when tissue block is removed from cryomold. Remove tissue block from cryomold. If needed, trim using a razor blade to fit into the cryovial.

Tissue Freezing & Embedding



1.3 Cryosectioning

OCT Embedded Tissue Block Mounting



a. Fill specimen stage (chuck) with OCT.

Ensure OCT-embedded tissue block has been equilibrated to cryostat temperature as described in 1.0 Preparation on page 27

b. Place OCT-embedded tissue block on stage with cutting surface facing away from stage.

c. Place stage and tissue block on cryobar inside cryostat chamber.

d. Allow OCT and tissue block to freeze and adhere to specimen stage.

e. Once frozen, install stage with tissue block on specimen head of cryostat and start sectioning to remove excess OCT.

Install Block onto Specimen Head

Sectioning conditions vary across different tissues and cryostats. Follow manufacturer's recommendation for cryosectioning.

f. Continue sectioning until tissue is visible. Once tissue is visible, subsequent sections may be used for section placement. See Section Thickness on page 10 for information on recommended thickness.

g. Prior to section placement, acquiring sections from the tissue block for RNA quality assessment is recommended. See 1.6 RNA Quality Assessment on page 38 for more information.

1.4 Section Placement

a. Once desired tissue section is obtained, carefully flatten it out by gently touching the surrounding OCT with cryostat brushes.

b. Gently touch section to front of pre-chilled blank slide within allowable area.

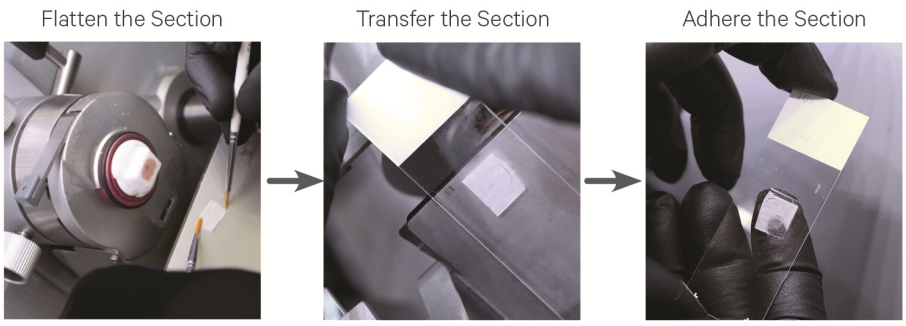
DO NOT place sections on a room temperature slide. Slide should be equilibrated to cryostat chamber.

c. Immediately place a finger on backside of the slide for a few seconds to allow section to adhere.

Ensure that entire tissue section is fully adhered.

DO NOT remove slide from cryostat chamber at any point during sectioning and tissue placement.

d. Immediately place tissue slide with the tissue facing up on cryobar to freeze the section. Continue transferring sections on remaining slides.



Immediately place the slide on the cryobar to allow section to freeze

e. Transfer slides containing tissue sections to a pre-cooled slide mailer. Alternatively, use a pre-cooled 50-ml centrifuge tube.

Store slides individually (one slide per 50-ml centrifuge tube, if using) in a sealed container. If using an unsealed slide mailer, store in a secondary sealed container, such as a resealable bag.

f. Transfer slides within slide mailer or centrifuge tube to dry ice.

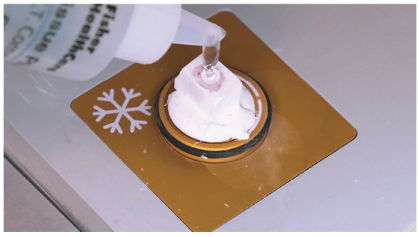
STOP

g. Store slides at **–80°C** for up to **2 months** or immediately proceed to either optional morphology assessment or tissue staining.

1.5 Tissue Block Storage and Tissue Slide Shipping

Leftover Tissue Block Storage

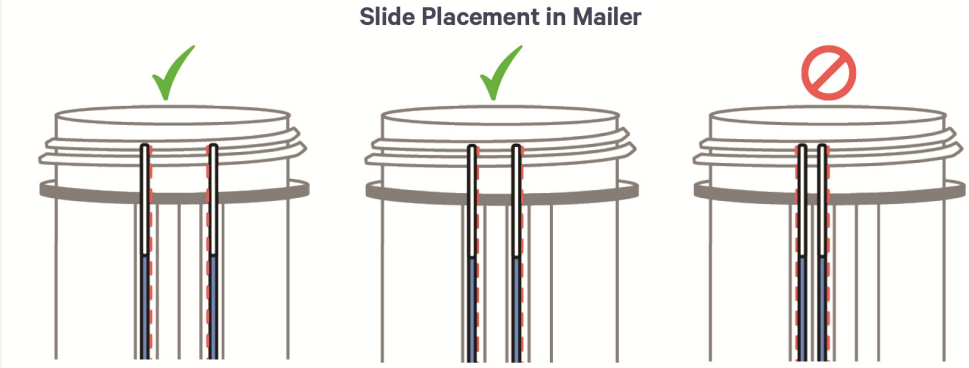
- Remove leftover tissue block attached to the specimen stage from the cryostat's specimen head and place onto cryobar.
- Cover exposed tissue with a thin layer of pre-cooled OCT and allow to freeze.
- Frozen tissue block can be stored attached to specimen stage in a sealed container at **–80°C**. To separate frozen tissue block from specimen stage, lift tissue block and stage from cryobar and lightly warm stage with hands or an aluminum block at room temperature.
- DO NOT let block and tissue fully melt, as this will severely damage the tissue. Separation of the tissue block from the specimen stage is optional.
- Immediately place tissue block in dry ice. Ensure that melted areas have refrozen.
- Store in a sealed container at **–80°C** for **long-term** storage.



Slide Shipping

- Place tissue slides in a slide mailer and keep cold. If shipping multiple slides, ensure that there is sufficient space in between slides to avoid contact. If placing a slide in the last slot, ensure slide does not face outward. This guidance also applies to storing slides in a 50-ml centrifuge tube.
- Place mailer in a tightly sealed secondary container to limit exposure and keep cold.
- Samples can be shipped overnight in dry ice, provided there is enough dry ice to account for transit and delivery times. Ensure that samples are in an environment with a stable temperature.

- See local institution or delivery service for detailed instructions on shipping samples in dry ice.



1.6 RNA Quality Assessment

RNA quality assessment is recommended. If RNA quality will not be assessed, proceed directly to optional tissue morphology assessment or appropriate staining section within this workbook.

a. Cryosection **20-30 mg** of tissue sections from OCT-embedded tissue block (~4 sections at 24 µm thickness). If tissues contain extensive connective or adipose tissue, cryosection up to **50 mg** of tissue.

b. If OCT is excessive (**≥1** mm surrounding the tissue), remove excess OCT with a razor blade or with cooled forceps.

Excess OCT may reduce RNA quality score.

c. Using cooled forceps, transfer sections to a pre-cooled microcentrifuge tube.

d. Place pre-cooled microcentrifuge tube containing sections on dry ice. Store at -80°C or proceed to RNA extraction.

⚠ *DO NOT allow samples to melt, as this will lead to degradation of RNA and a poor DV200 score.*

e. Proceed with RNA extraction following manufacturer's instructions (See Visium HD Protocol Planner (CG000698), for tested part numbers). After RNA isolation, place sample on ice.

⚠ *Fixed frozen tissue is not paraffin embedded and therefore should not undergo the deparaffinization steps during RNA extraction.*

f. Store purified RNA at **–80°C** for **long-term** storage or immediately proceed to DV200 calculation using either Bioanalyzer or TapeStation. Follow manufacturer's instructions (Agilent) for DV200 calculation.

⚠ *Ensure RNA is quantified using a Qubit or Nanodrop and loaded within specifications of the relevant assay kit before running RNA on TapeStation or Bioanalyzer.*

1.7 Optional DAPI Staining for Tissue Morphology Assessment

If extra tissue slides are available, optional tissue morphology assessment via DAPI and H&E staining is recommended.

Prior to DAPI and H&E staining, rehydration is required. Rehydrate the tissue as described in 2.1 Rehydration on page 50.

DAPI Staining

a. Place tissue slide on a flat, clean, nonabsorbent work surface.

b. Add **500 µl** DAPI solution per slide to uniformly cover all tissue sections.

c. Incubate **1 min** in the dark at **room temperature**.

d. Discard reagent by holding slides at an angle with the bottom edge in contact with a laboratory wipe

e. Add **1 ml** 1X PBS per slide to uniformly cover all tissue sections.

f. Incubate **1 min** in the dark at **room temperature**.

g. Discard reagent by holding slides at an angle with the bottom edge in contact with a laboratory wipe

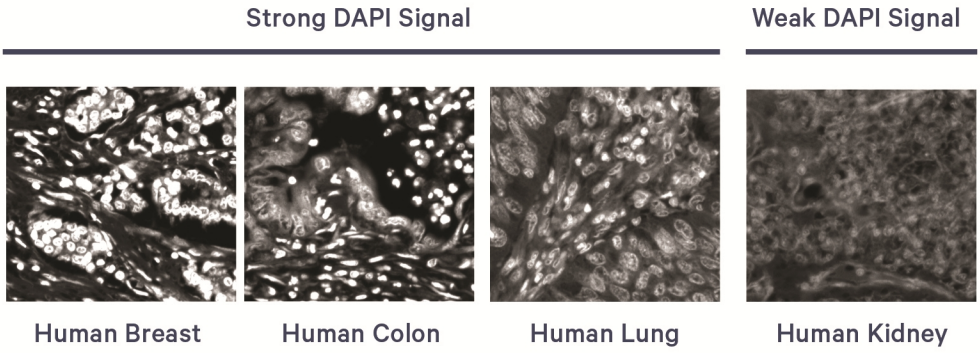
h. Repeat steps e-g twice more for a total of three washes.

i. If the same tissue section will be used for H&E morphology assessment, mount coverslip as described in 3.6 Coverslip Mounting on page 84 and image as described in 3.7 Imaging on page 85. If a serial section will be used for the optional H&E morphology assessment, mounting a coverslip is not necessary.

Quality Assessment

Review the DAPI image under the microscope at 20x magnification. Examine several sections within a single DAPI-stained section to ensure assessment is comprehensive. Tissue sections optimal for the assay should have:

- Punctate nuclei that are clearly defined
- DAPI staining that does not appear washed out



Sections with poor DAPI staining are unlikely to generate good data. All samples in the images below were imaged under the same conditions (405 nm channel). After evaluating DAPI staining, remove coverslip as described in 2.5 Coverslip Removal on page 55 and proceed to 1.8 Optional H&E Staining for Tissue Morphology Assessment below.

1.8 Optional H&E Staining for Tissue Morphology Assessment

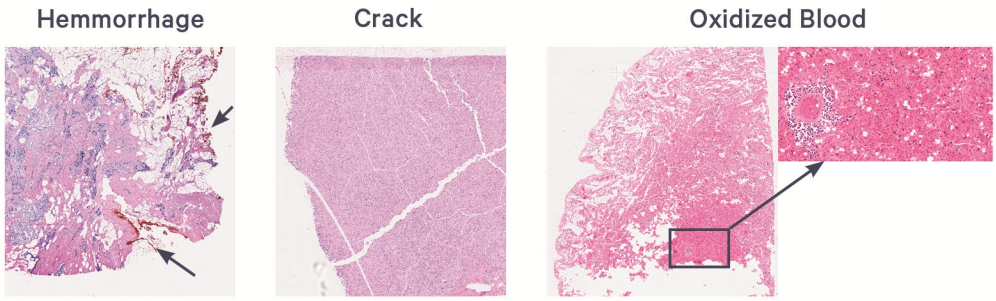
Prior to H&E staining, rehydration is required. Rehydrate the tissue as described in 2.1 Rehydration on page 50.

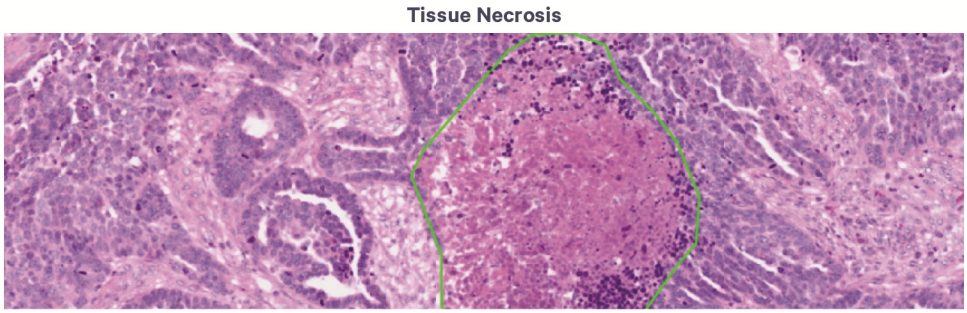
H&E Staining

Perform H&E staining as described in 2.2 H&E Staining on page 51, coverslip as described in Coverslip Mounting and image as described in 2.4 Imaging on page 54.

Quality Assessment

Ensure that the tissue section is free from tissue processing and sectioning artifacts as shown in the images below and in Troubleshooting on page 88. Tissue processing artifacts may include improper snap freezing, squeeze/crush artifacts, ice crystal artifacts, and hemorrhaging. If imaging reveals satisfactory tissue morphology, proceed with the appropriate staining protocol using a different tissue slide.





Staining protocol:

- 2. H&E Staining & Imaging on page 42
- 3. IF Staining & Imaging on page 61

2. H&E Staining & Imaging

Overview

This chapter provides guidance on the preparation, rehydration, H&E staining, imaging, destaining, and decrosslinking of fixed frozen tissue slides. Ensure that microscope settings have been verified and imaging programs have been created before starting this protocol. Consult the Visium HD Spatial Applications Imaging Guidelines Technical Note (CG000688) for more information. After completing Decrosslinking, proceed immediately to the Visium HD Spatial Gene Expression User Guide (CG000685).

2.0 Preparation

Consult the Visium HD Spatial Gene Expression Protocol Planner (CG000698) for a list of third-party items.

Washing tissue sections with RVC and adding RNase Inhibitor to mounting media is optional if tissue sections will be imaged and hybridized with probes on the same day. These RNA-protecting reagents are required for all supported stopping points in this document.

For All Steps

The amounts of buffer in this table are sufficient for all remaining steps in this chapter.

Items		Preparation & Handling
Prepare fresh.		
☐	1X PBS	Prepare 525 ml of 1X PBS. If using 10X stock, prepare 1X PBS using nuclease-free water.

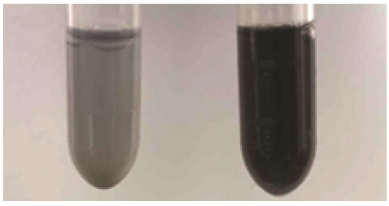
For Rehydration

Items		Preparation & Handling
Prepare fresh. If using 50-ml centrifuge tubes, two slides can be faced back to back (tissue facing out) per tube.		
☐	100% Ethanol	Label one 50-ml centrifuge tube as 100% Ethanol Tube. Dispense 30 ml 100% ethanol. Alternatively, use a coplin jar.
☐	70% Ethanol	Label one 50-ml centrifuge tube as 70% Ethanol Tube. Dispense 30 ml 70% ethanol. Alternatively, use a coplin jar.
☐	Milli-Q or UltraPure Water	Label two 50-ml centrifuge tubes as Water Tube 1 and 2. Dispense 30 ml water in each. Alternatively, use a coplin jar.
☐	1X PBS	Label one 50-ml centrifuge tube as 1X PBS Tube. Dispense 30 ml 1X PBS. Alternatively, use a coplin jar.

For H&E Staining

Items		Preparation & Handling
☐	Milli-Q or UltraPure Water	Label six 1000-ml beakers as Water Beakers 1–6. Dispense 800 ml of water into each beaker. Dispensed volumes in each beaker can be used for two slides. Alternatively, use 50-ml centrifuge tubes instead of beakers (one tissue slide per 50-ml centrifuge tube).
☐	Alcoholic Eosin	Prepare 30 ml in a 50-ml conical tube for each tissue slide.
☐	Gill II Hematoxylin	
☐	Bluing Buffer	

Optional Staining Reagents - Only Required if Using Stopping Point

Items		Preparation & Handling
☐	Ribonucleoside Vanadyl Complex (RVC)	Preparing aliquots: Incubate sealed vial at 65°C in a heating block, bead bath, or water bath for 20 min or until solution is reconstituted to a dark green solution with no visible particulates. Aliquot into single-use tubes (30 µl per slide). Store tubes at -20°C. DO NOT exceed 2 freeze-thaw cycles per aliquot. If using an aliquot immediately, no freezing is necessary. Store at room temperature until use. Gently invert every 10 min. Using a frozen aliquot: remove aliquot from -20°C and incubate at 65°C for 10 min to remove precipitate. Before Incubation / After Incubation 
☐	RVC Working Solution	Prepare Ribonucleoside Vanadyl Complex stock as described above prior to generating working solution. Pipette mix. Maintain at room temperature.

RVC Working Solution *1 slide = 1 Tissue Slide*	**Stock**	**Final**	**1 Slide (µl)**	**2 Slides +15% (µl)**
Nuclease-free Water	-	-	497.5	1,144.2
Ribonucleoside Vanadyl Complex	200 mM	1 mM	2.5	5.8
Total	**-**	**-**	**500.0**	**1,150.0**

For Coverslip Mounting

Items		Preparation & Handling
Prepare - Select One Mounting Medium		
☐	Mounting Medium	Prepare this Mounting Medium if stopping point will **not** be used. Invert to mix. Briefly centrifuge to remove bubbles. Inspect for bubbles. Continue centrifuging until no bubbles remain. Maintain at room temperature.

Mounting Medium *1 slide = 1 Tissue Slide*	**Stock**	**Final**	**1 Slide (µl)**	**2 Slides +15% (µl)**
Glycerol	100%	85%	127.5	293.3
Nuclease-free Water	-	-	22.5	51.7
Total	**-**	**-**	**150.0**	**345.0**

☐	Stopping Point Mounting Medium	Prepare this Mounting Medium if stopping point **will** be used. Invert to mix. Briefly centrifuge to remove bubbles. Inspect for bubbles. Continue centrifuging until no bubbles remain. Maintain at room temperature.

Stopping Point Mounting Medium *1 slide = 1 Tissue Slide*	**Stock**	**Final**	**1 Slide (µl)**	**2 Slides +15% (µl)**
Glycerol	100%	85%	127.5	293.3
Nuclease-free Water	-	-	15.0	34.5
RNase Inhibitor	40X	2X	7.5	17.3
Total	**-**	**-**	**150.0**	**345.0**

Obtain		
☐	Coverslip	-

For Destaining

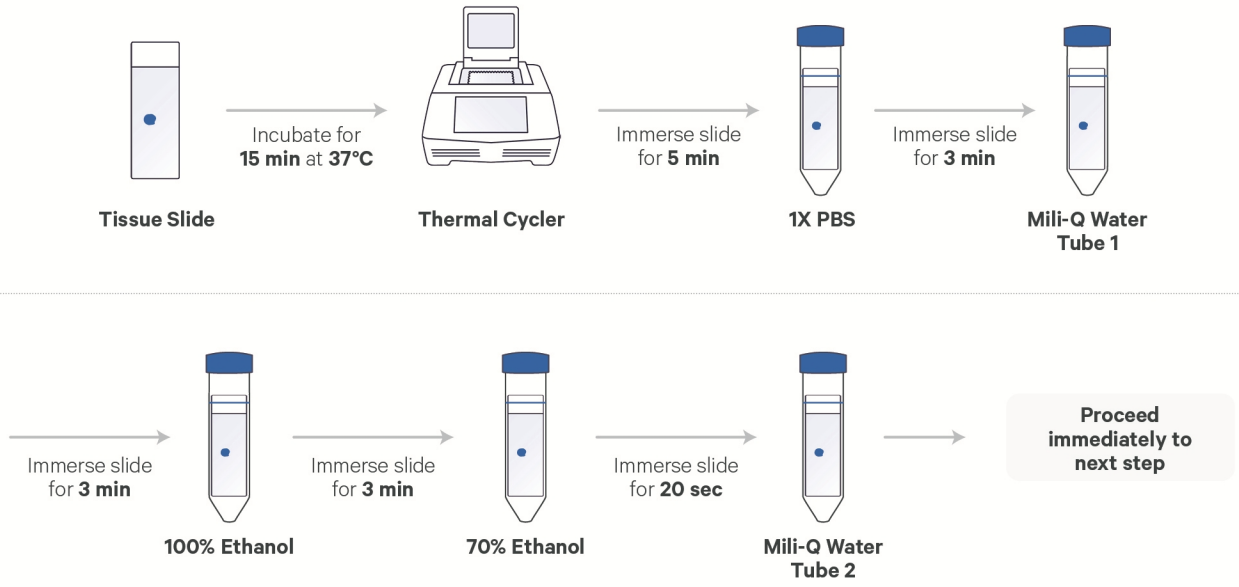
Items		10x PN	Preparation & Handling	Storage
Obtain				
☐	0.1 N HCl	-	If necessary, prepare 0.1 N HCl using nuclease-free water.	Ambient
☐	TE Buffer, pH 8.0	-	-	Ambient

For Decrosslinking

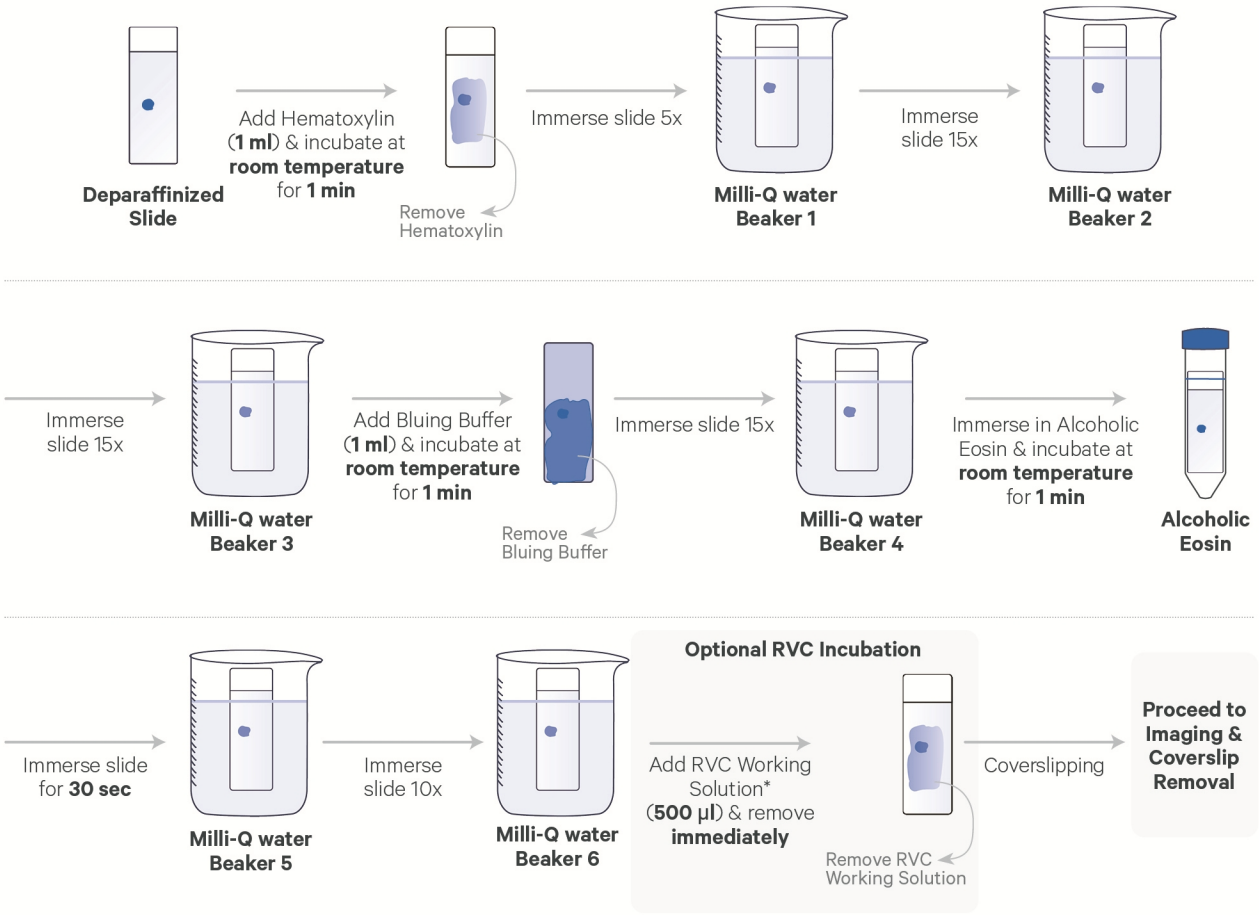
Items		10x PN	Preparation & Handling	Storage
Heat to 37°C				
☐	Decrosslinking Buffer B	2001094	Pre-heat thermomixer or water bath to 37°C. Thaw in a thermomixer (300 rpm with shaking) or water bath for 30 min at 37°C during Destaining.	-20°C
Equilibrate to room temperature				
☐	Perm Enzyme B	3000553	Remove from -20°C shortly before use. Maintain at room temperature. Pipette mix, centrifuge briefly. DO NOT vortex.	-20°C
☐	Decrosslinking Buffer B	2001094	After heating to 37°C as indicated above, cool to room temperature for 5 min. Vortex for 30 sec and centrifuge briefly.	-20°C
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	8M Urea	-	-	Ambient

Protocol Overview

Rehydration ~20 min

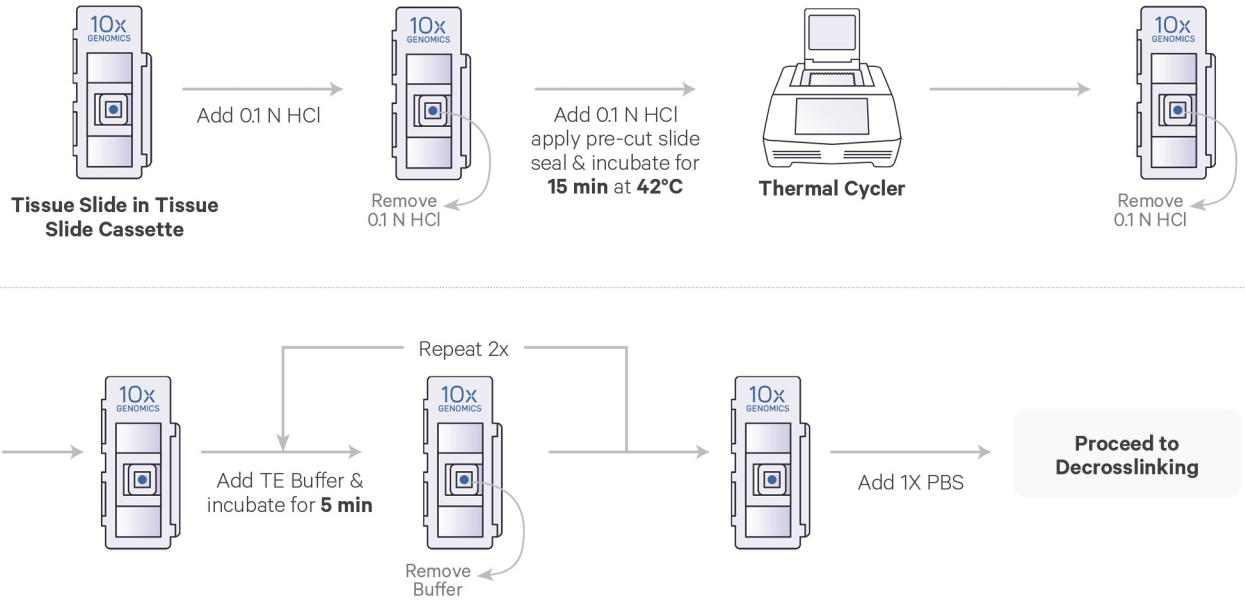


H&E Staining & Coverslipping ~15 min

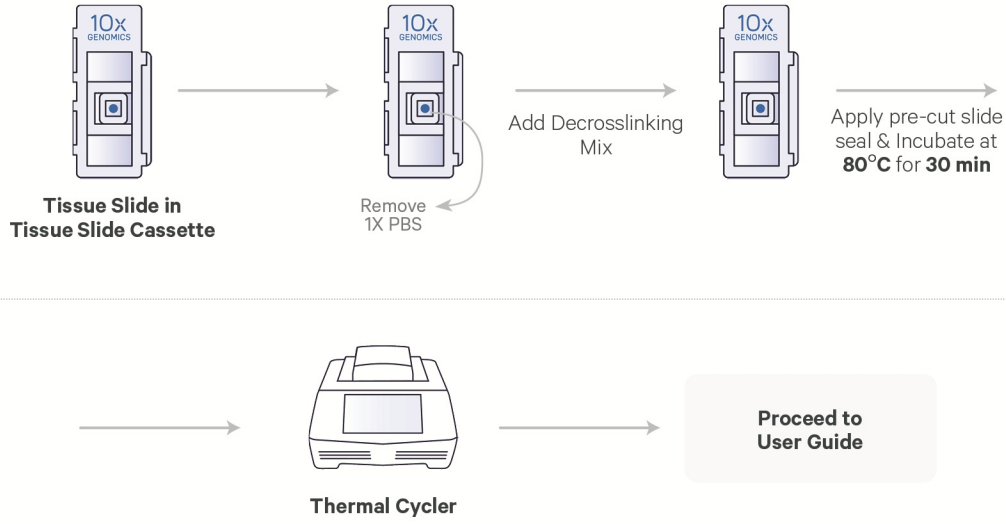


Wipe excess liquid from the back of the slide after each immersion series
**Only required if using stopping points*

Destaining ~30 min



Decrosslinking ~30 min



2.1 Rehydration

a. Place a Low Profile Thermocycler Adapter on a thermal cycler and preheat thermal cycler to **37°C**.

b. Retrieve slide containing tissue from **-80°C** and place on dry ice.

c. Place slide on Low Profile Thermocycler Adapter with tissue side facing up and incubate **15 min** at **37°C**. DO NOT close thermal cycler lid.

Ensure tissue is dry before proceeding to next step. Damp tissue may lead to detachment. DO NOT exceed 20 min incubation time.

d. Remove slide from thermal cycler. Gently immerse slide in 1X PBS Tube and incubate for **5 min**.

TIPS

When immersing slides in solutions, ensure that tissue sections are completely submerged.

e. Gently immerse slide in Milli-Q Water Tube 1 and incubate for **3 min**.

f. Gently immerse slide in 100% Ethanol Tube for **3 min**.

g. Gently immerse slide in 70% Ethanol Tube for **3 min**.

h. Gently immerse slide in Milli-Q Water Tube 2 for **20 sec**.

i. Proceed immediately to next step.

To ensure even staining, DO NOT let slides dry.

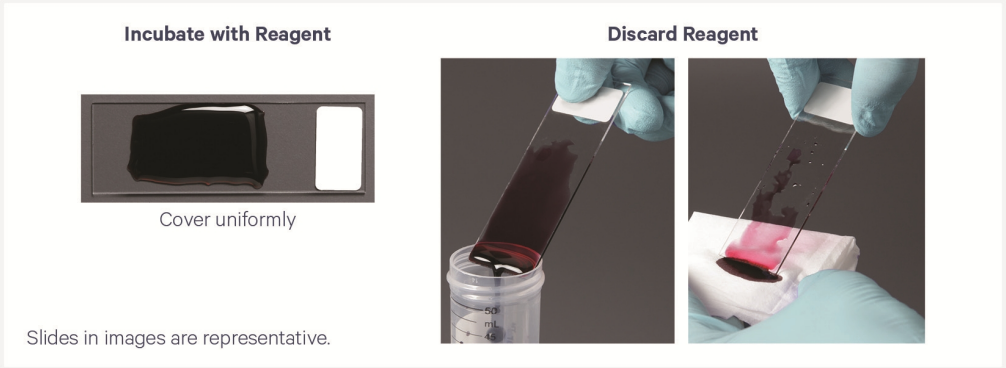
2.2 H&E Staining

a. Place slide on a flat, clean, nonabsorbent work surface. Some residual droplets may remain.

b. Add **1 ml** Hematoxylin per slide to uniformly cover all tissue sections. DO NOT pipette directly onto tissue sections.

If staining two tissue slides at a time, ensure that hematoyxlin is discarded quickly after incubation to prevent overstaining.

c. Incubate **1 min** at **room temperature**.



d. Discard reagent by holding slides at an angle with the bottom edge in contact with a lint-free laboratory wipe.

e. Immerse slides 5x in Water Beaker 1.

f. Immerse slides 15x in Water Beaker 2.

g. Immerse slides 15x in Water Beaker 3.

h. Wipe excess liquid from back of slides with a lint-free laboratory wipe without touching the tissue section.

i. Place slide on a flat, clean, nonabsorbent work surface. Some droplets may remain.

j. Add **1 ml** Bluing Buffer per slide to uniformly cover all tissue sections.

k. Incubate **1 min** at **room temperature**.

l. Discard reagent by holding slides at an angle with the bottom edge in contact with a lint-free laboratory wipe.

m. Immerse slides 15x in Water Beaker 4.

n. Wipe excess liquid from back of slides with a lint-free laboratory wipe without touching the tissue section. Place on a flat, clean, nonabsorbent work surface. Some droplets may remain.

o. Gently immerse slides in alcoholic Eosin solution in separate 50-ml centrifuge tubes. DO NOT use diluted Eosin.

p. Incubate **1 min** at **room temperature**.

q. Discard reagent by holding slides at an angle with the bottom edge in contact with a lint-free laboratory wipe.

r. Immerse slides for **30 sec** in Water Beaker 5.

s. Immerse slides 10x in Water Beaker 6.

t. Wipe excess liquid from back of slides with a lint-free laboratory wipe without touching the tissue section. Place on a flat, clean, nonabsorbent work surface.

u. Inspect the tissue for signs of bubbles or folds, which may result in detachment or UMI loss. Tissue sections with bubbles or any large folds typically have localized regions that appear uneven or wavy upon visual examination during the various staining and washing steps. If bubbles or folds are observed, use a different section for the Visium HD assay.

v. *Optional RNA protection - only if using pre or post-imaging stopping point*

- Add **500 µl** RVC Working Solution per slide to uniformly cover all tissue sections.
- Immediately discard reagent by holding slides at an angle with the bottom edge in contact with a lint-free laboratory wipe and gently tapping the slide against the wipe.

  Ensure RVC Working Solution is completely removed before proceeding. Failure to remove RVC Working Solution may lead to weaker eosin staining. DO NOT allow slides to air dry.

w. Proceed to Coverslip Mounting.

2.3 Coverslip Mounting

a. Place slides on a flat, clean, nonabsorbent work surface. Some residual droplets may remain.

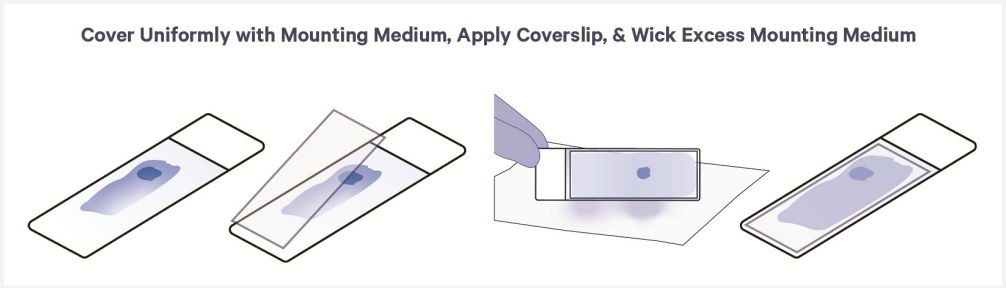
b. Using a **wide-bore** pipette tip:

- If **not** using stopping point, add **100–150 µl** Mounting Medium to cover all tissue sections uniformly.
- If **using** stopping point, add **100–150 µl** Stopping Point Mounting Medium to cover all tissue sections uniformly.

⚠ *Ensure that the appropriate Mounting Medium is used. Mounting Medium is prepared differently if using stopping points. Tissue slides with large sections or multiple sections may require more Mounting Medium.*

c. Slowly apply coverslip at an angle on one end of the slides, without introducing bubbles. Allow Mounting Medium to spread and settle.

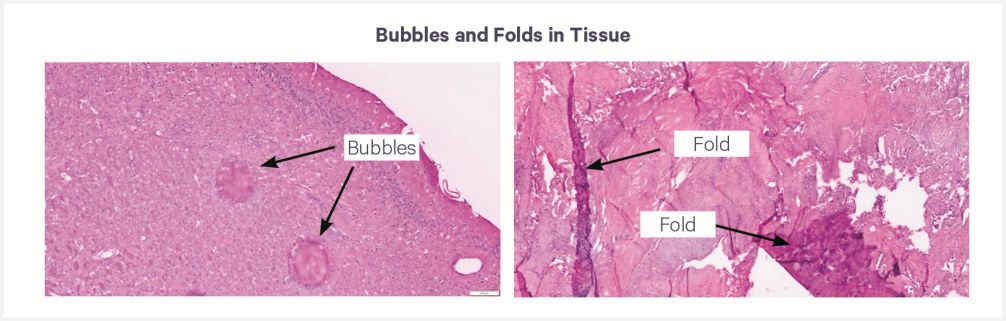
⚠ *DO NOT use Cytoseal or nail polish for securing the coverslip.*



d. Remove any large excess of Mounting Medium by carefully wicking away from the edge of the coverslip using a lint-free laboratory wipe. Do not move coverslip and disturb tissue.

e. Inspect the tissue for signs of bubbles or folds, which may result in detachment or UMI loss. Bubbles and folds may appear as regions of tissue that appear uneven or wavy. If bubbles or folds are observed, use a different section for the Visium HD assay.

Images below are representative.



STOP

f. **Immediately** proceed with imaging or if Stopping Point Mounting Medium was used, store slides laying flat in a slide mailer or a slide holder. Store slides in the dark at **4°C** for up to **72 h**. Ensure that slides are laid flat to prevent loss of Mounting Medium.

Stopping points may only be used if staining guidance for stopping points was followed, which includes an RVC incubation and appropriate mounting media with RNase inhibitor.

If storing slides for longer is desired, **immediately** proceed with imaging and follow stop instructions in 2.4 Imaging below. The stopping point after coverslip mounting and the stopping point in 2.4 Imaging below cannot be combined.

2.4 Imaging

a. Image tissue section of interest at desired magnification using brightfield imaging settings. Consult the Visium HD Spatial Applications Imaging Guidelines Technical Note (CG000688) for additional information.

STOP

b. If slides were immediately imaged after coverslip mounting and Stopping Point Mounting Medium was used, slides may be stored in the dark at **4°C** for up to **1 week** prior to Coverslip Removal. Ensure that slides are laid flat to prevent loss of Mounting Medium.

Stopping points may only be used if staining guidance for stopping points was followed, which includes an RVC incubation.

DO NOT let the attached coverslip dry out.

c. Proceed **immediately** to Coverslip Removal.

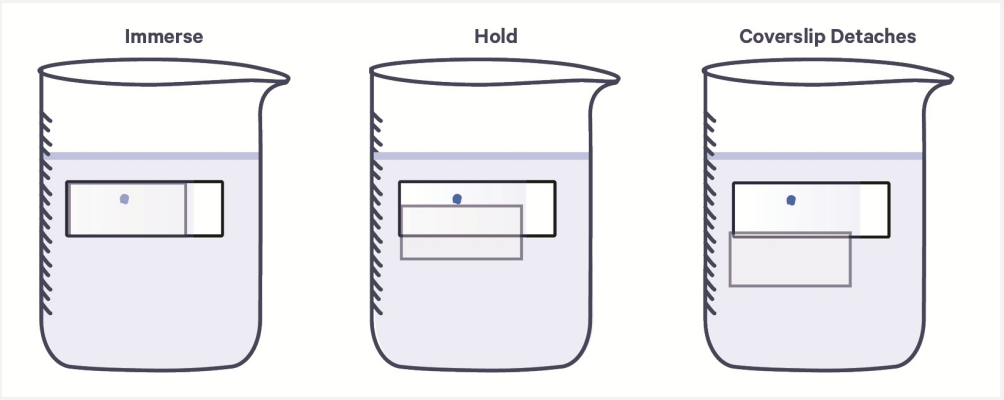
2.5 Coverslip Removal

a. Dispense **800 ml** Milli-Q water in a beaker. Up to 10 slides may be processed using this beaker.

b. Immerse slides sideways in the beaker containing **800 ml** water with coverslipped surface fully sideways to prevent coverslip from dragging across tissue.

c. Hold slides in water until the coverslip slowly separates away from the slide.

To avoid tissue section damage or detachment, DO NOT move slide up and down, shake forcibly, or manually move coverslip.



d. Gently immerse slides 30x in water to ensure all Mounting Medium is removed.

e. Wipe back of slide with a lint-free laboratory wipe.

f. Place slide on a flat, clean, nonabsorbent work surface and air dry for **5 min**.

g. Incubate slide on Low Profile Thermocycler Adapter with thermal cycler lid open for **3 min** at **37°C**. Ensure tissue is completely dry. If necessary, wipe droplets around tissue with a lint-free laboratory wipe.

h. Proceed **immediately** to Destaining.

2.6 Destaining

a. Place a Low Profile Thermocycler Adapter in the thermal cycler. Prepare thermal cycler with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
42°C (lid may be set to lowest temperature if instrument does not enable 42°C)	100 µl	15 min

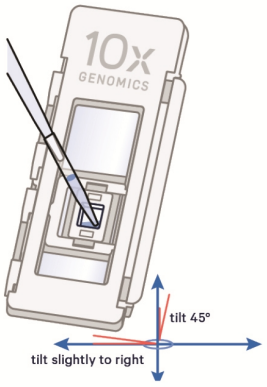
Step	Temperature	Time *hh:mm:ss*
Pre-equilibrate	42°C	Hold
Destaining	42°C	00:15:00
Hold	22°C	Hold

b. Place slide in a Tissue Slide Cassette, centering the tissue or area of interest, and label the top of the cassette with tissue slide information.

See Visium Cassette S3 Quick Reference Card (CG000730) for assembly instructions. Practice assembly with a blank slide.

c. Add **150 µl** 0.1 N HCl along the side of the wells to uniformly cover tissue sections, without introducing bubbles. Tap cassette gently to ensure uniform coverage.

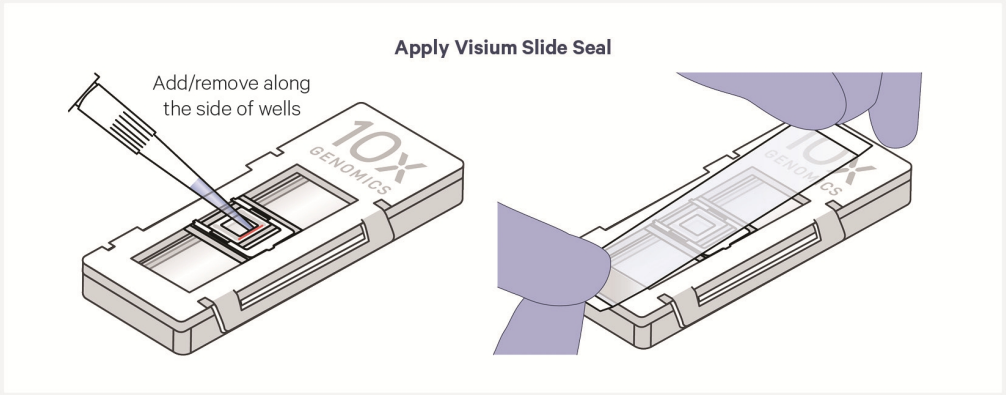
d. Remove HCl from wells.



e. Add **100 µl** 0.1 N HCl along the side of the wells to uniformly cover tissue sections, without introducing bubbles. Tap cassette gently to ensure uniform coverage.

TIPS

f. Apply pre-cut slide seal on cassette and place cassette on Low Profile Thermocycler Adapter at **42°C**. See Tips & Best Practices for more information on cassette incubation in a thermal cycler.

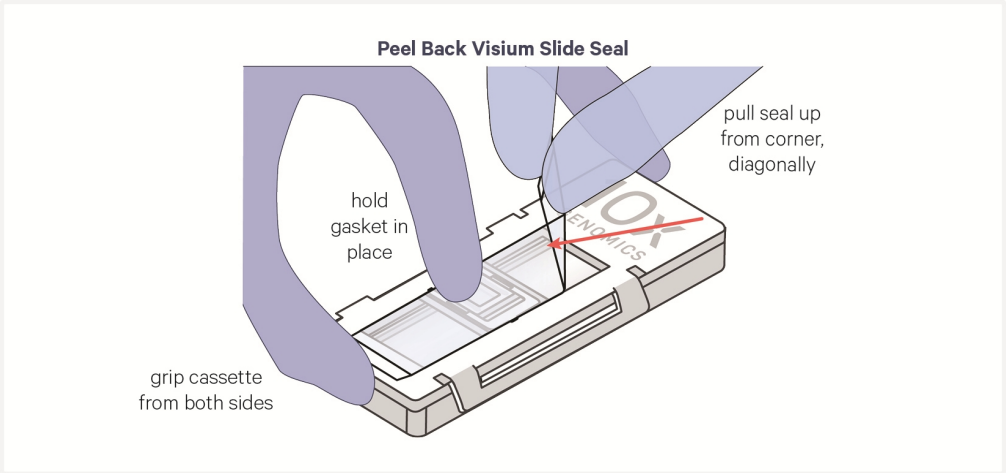


g. Close thermal cycler lid. Skip Pre-equilibrate step to initiate Destaining.

h. Remove cassette from Low Profile Thermocycler Adapter and place on a flat, clean work surface.

Some color remaining in the tissue after thermal cycler incubation is normal.

i. Peel back slide seal and using a pipette, remove all the HCl from well corners.



Three TE Buffer washes:

j. **Wash 1:** Add **150 µl** TE Buffer (pH 8.0) along the side of the wells. Incubate for **5 min** at **room temperature**. Remove TE Buffer from the wells.

k. **Wash 2:** Add **150 µl** TE Buffer (pH 8.0) along the side of the wells. Incubate for **5 min** at **room temperature**. Remove TE Buffer from the wells.

l. **Wash 3:** Add **150 µl** TE Buffer (pH 8.0) along the side of the wells. Incubate for **5 min** at **room temperature**. Remove TE Buffer from the wells.

m. Add **100 µl** 1X PBS along the side of the wells.

n. Re-apply slide seal.

2.7 Decrosslinking

Ensure Decrosslinking Buffer B has been prepared according to Preparation - Buffers.

a. Prepare thermal cycler with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
80°C	100 µl	40 min
Step	**Temperature**	**Time** *hh:mm:ss*
Hold	22°C	Hold
Decrosslinking	80°C	00:30:00
Re-equilibrate	22°C	00:10:00
Hold	22°C	Hold

b. Prepare Diluted Perm Enzyme B according to the table below. Add reagents in the order listed. Mix thoroughly with a 1-ml pipette set to 600 µl. Maintain at room temperature.

Diluted Perm Enzyme B	Stock	Final	Total Amount (µl)
1X PBS	-	-	998.0
Perm Enzyme B *(Maintain at room temperature. Pipette mix, centrifuge briefly. DO NOT vortex).*	-	-	2.0
Total	**-**	**-**	**1,000.0**

c. Prepare Decrosslinking Mix according to the table below. Add reagents in the order listed. Pipette mix thoroughly. Centrifuge briefly. Maintain at room temperature.

Decrosslinking Mix *1 slide = 1 Tissue Slide*	Stock	Final	1 slide (µl)	2 slides + 10% (µl)	4 slides + 10% (µl)
Decrosslinking Buffer B	-	-	92.50	203.50	407.00
Urea	8 M	0.5 M	6.25	13.75	27.50
Diluted Perm Enzyme B	-	-	1.25	2.75	5.50
Total	**-**	**-**	**100.00**	**220.00**	**440.0**

d. Remove slide seal and remove 1X PBS from the wells.

e. Add **100 µl** Decrosslinking Mix along the side of the wells

TIPS

f. Apply a new pre-cut slide seal on the cassette and place cassette on the Low Profile Thermocycler Adapter. See Tips & Best Practices for more information on cassette incubation in a thermal cycler.

g. Close and tighten thermal cycler lid. Skip Pre-equilibrate step and initiate Decrosslinking.

h. Proceed **immediately** to Visium HD Spatial Gene Expression Reagent Kits User Guide (CG000685).

3. IF Staining & Imaging

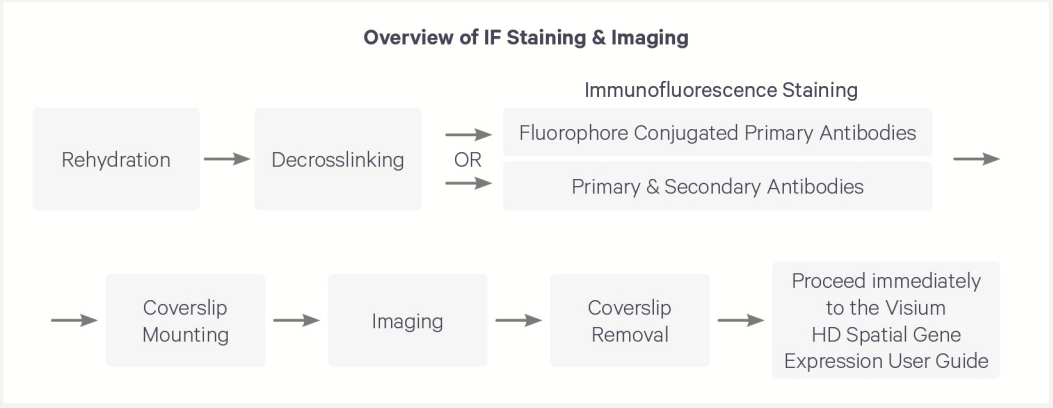
Overview

This chapter provides guidance on decrosslinking, and immunofluorescence (IF) staining, coverslip mounting, imaging, and coverslip removal for tissue slides. These tissue slides should be prepared according to 1. Tissue Handling, Fixation, Embedding, Sectioning, Section Placement, and Quality Assessment on page 20 prior to starting this chapter. This chapter also provides guidance on optimizing antibodies. After coverslip removal, proceed immediately to the Visium HD Spatial Gene Expression User Guide (CG000685).

This chapter provides guidance on the rehydration, decrosslinking, IF staining, and imaging of fixed frozen tissue slides. This chapter also provides guidance on optimizing antibodies. After coverslip removal, proceed immediately to the Visium HD Spatial Gene Expression User Guide (CG000685).

One additional Tissue Slide Cassette (Visium Tissue Slide Cassette S3 6.5 mm, 4 pk PN-1000684) is required for each processed Tissue Slide.

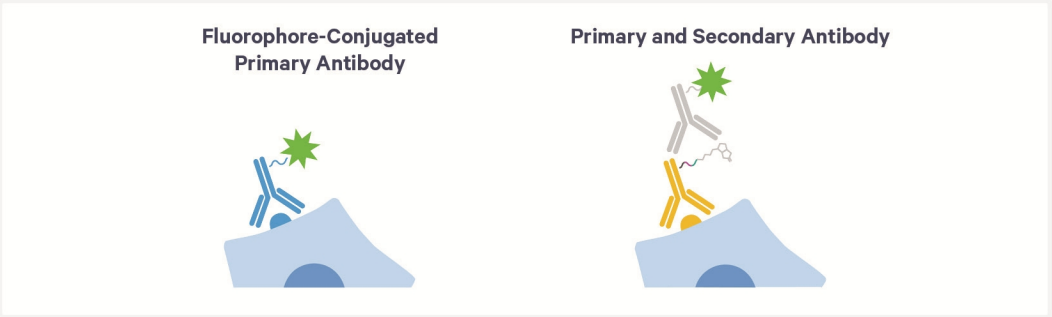
A Visium 8-port Cassette S3, 4 pk (PN-1000685) must be purchased to perform antibody optimization.



Verify microscope settings and create imaging programs prior to starting this step. Consult the Visium HD Spatial Applications Imaging Guidelines Technical Note (CG000688) for more information.

Separate protocols are provided in this chapter if fluorophore-conjugated primary antibodies are used vs. if primary and secondary antibodies are used.

- 3.4 Immunofluorescence Staining - Fluorophore Conjugated Primary Antibodies on page 77
- 3.5 Immunofluorescence Staining - Primary & Secondary Antibodies on page 80.



Antibody Optimization

Prior testing of the antibodies is recommended on the same tissue block before performing immunofluorescence staining in combination with the Visium HD Spatial Gene Expression workflow. Determination of the optimal antibody concentration is crucial for successfully executing this protocol.

The amount of Decrosslinking Buffer provided in the kit is enough to perform antibody optimization, while still leaving enough reagent for the main assay.

6.5 mm Slides Reagent	PN	Reactions* Available for Optimization
Decrosslinking Buffer B	2001094	8

*Reactions refer to the number of antibody dilutions that can be prepared, given the volume of each well in the Visium Cassette. For example, the example dilution series below tests seven antibody optimization reactions.

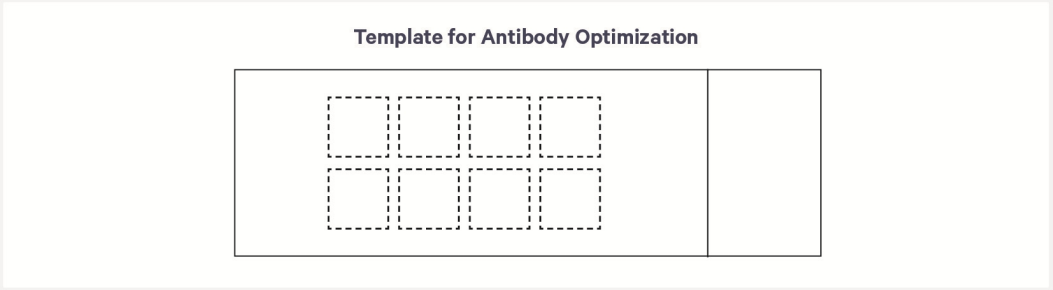
Optimal antibody concentrations for this Demonstrated Protocol may differ from other applications. Composition of reagents and buffers may also differ from other immunofluorescence applications.

Ensure that enough reactions are available for the main assay after running optimization experiments.

When optimizing the antibody for a single tissue type, ensure that stained slides can be imaged according to the imaging guidelines listed in Visium HD Spatial Applications Imaging Guidelines Technical Note (CG000688).

Below is a suggested optimization workflow. Antibody optimization may be performed according to preference, as long as tissues are tested using the immunofluorescence staining protocol described in this document.

- Draw representative frames on the back of a blank slide using the example slide layout for a 25 mm wide slide.



- Trim tissue sections to fit within each 6.5 mm area on the template.
- Place tissue sections in the frames on the front of the slide for compatibility with the Visium Cassette.
- Execute the Decrosslinking & Immunofluorescence Staining protocol using a range of antibody concentrations, testing multiple concentrations on the same tissue slide. A starting concentration of 0.01 µg/µl (0.7 µg/sample) is recommended.
- To reduce autofluorescence, TrueBlack reagent may be added.
- Select the antibody concentration that results in the specific staining of desired cells, while minimizing nonspecific background staining.

Autofluorescence quenchers are added following immunofluorescence staining and may result in the reduction of fluorescence signal. Additional optimization and increase in antibody concentration may be required to properly visualize immunostaining.

An example dilution layout is provided below. DAPI and merged images are provided to show the presence of breast cancer tissue for each antibody dilution. Dilutions are of recombinant Anti-Vimentin antibody conjugated to Alexa Fluor 594 (BioLegend, PN677804, 0.5 mg/ml). A 1:100 dilution (0.35 µg/sample) was considered optimal in this example.

Example calculations:

Stock antibody concentration is 0.5 µg/µl and desired concentration is 0.01 µg/µl (50X dilution).

Reagents	Stock	Final	Volume (µl)
PBS	10X	1X	7.0
BSA	10%	2%	14.0
RNase Inhibitor	40X	2X	3.5
Tween-20	10%	0.1%	0.7
Antibody	0.5 µg/µl	0.01 µg/µl	1.4
DAPI Solution	100X	0.5X	0.4
Nuclease-free Water			43.0
Total			**70.0**

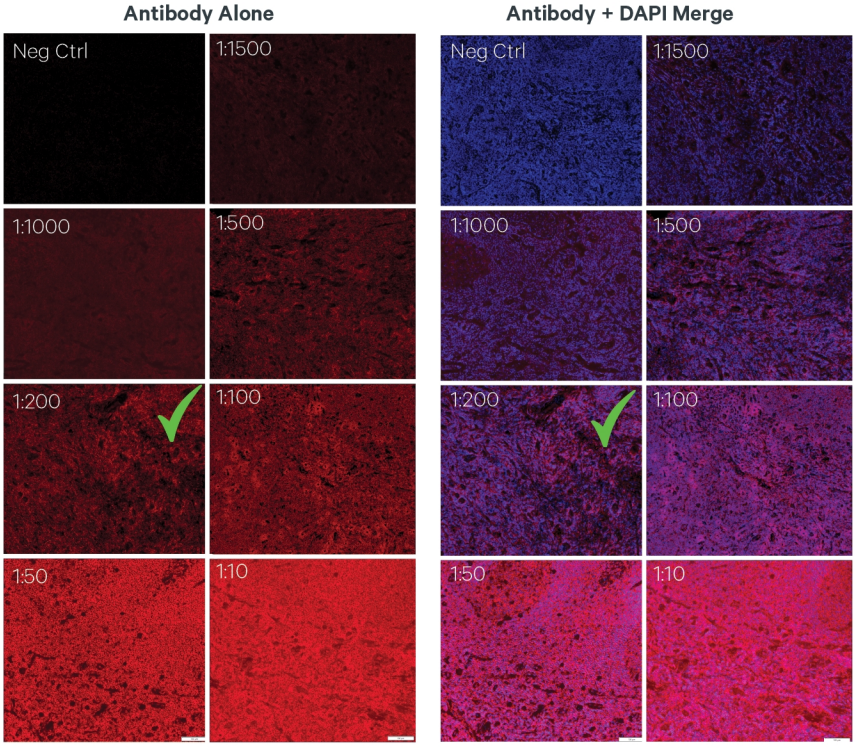
Stock antibody concentration is 0.5 µg/µl and desired concentration is 0.0025 µg/µl (200X dilution).

- This calculation requires pipetting a very small volume of antibody. Dilute the stock concentration first. In the example below, the stock antibody is diluted 10X to 0.05 µg/µl. Proceed with the following reagent table:

Reagents	Stock	Final	Volume (µl)
PBS	10X	1X	7.0
BSA	10%	2%	14.0
RNase Inhibitor	40X	2X	3.5
Tween-20	10%	0.1%	0.7
Antibody	0.05 µg/µl*	0.0025 µg/µl	3.5
DAPI Solution	100X	0.5X	0.4
Nuclease-free Water			40.9
Total			**70.0**

*Diluted antibody stock

Example Fluorophore Conjugated Primary Antibody Dilution Series



3.0 Preparation

Consult the Visium HD Spatial Gene Expression Protocol Planner (CG000698) for a list of third-party items. If using slide containers other than slide mailers (where applicable), reagent volumes will need to be scaled appropriately.

Washing tissue sections with RVC and adding RNase Inhibitor to mounting media is optional if tissue sections will be imaged and hybridized with probes on the same day. These RNA-protecting reagents are required for all supported stopping points in this document.

For All Steps

The amounts of buffer in this table are sufficient for all remaining steps in this chapter.

Items		Preparation & Handling
Prepare fresh.		
☐	1X PBS	Prepare 900 ml of 1X PBS. If using 10X stock, prepare 1X PBS using nuclease-free water.

For Rehydration

Items		Preparation & Handling
Prepare fresh. If using 50-ml centrifuge tubes, two slides can be faced back to back (tissue facing out) per tube.		
☐	100% Ethanol	Label one 50-ml centrifuge tube as 100% Ethanol Tube. Dispense 30 ml 100% ethanol. Alternatively, use a coplin jar.
☐	70% Ethanol	Label one 50-ml centrifuge tube as 70% Ethanol Tube. Dispense 30 ml 70% ethanol. Alternatively, use a coplin jar.
☐	Milli-Q or UltraPure Water	Label two 50-ml centrifuge tubes as Water Tube 1 and 2. Dispense 30 ml water in each. Alternatively, use a coplin jar.
☐	1X PBS	Label one 50-ml centrifuge tube as 1X PBS Tube. Dispense 30 ml 1X PBS. Alternatively, use a coplin jar.

For Decrosslinking

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	Perm Enzyme B	3000553	Equilibrate to room temperature. Pipette mix, centrifuge briefly. DO NOT vortex.	-20°C
☐	Decrosslinking Buffer B	2001094	Thaw in a thermomixer for 30 min at 37°C, 300 rpm with shaking. Cool to room temperature for 5 min. Vortex for 30 sec and centrifuge briefly. Alternatively, thaw in a water bath for 30 min at 37°C. Cool to room temperature for 5 min. Vortex for 30 sec and centrifuge briefly.	-20°C
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	10% Tween-20	-	-	Ambient
☐	8M Urea	-	-	Ambient

For IF Staining

Items		Preparation & Handling
☐	1X PBS	Dispense 50 ml of 1X PBS into a 50-ml centrifuge tube. Up to four tissue slides may be processed with this 50 ml of 1X PBS.
☐	70% Ethanol	Optional, if performing TrueBlack Quenching. Prepare 1 ml of 70% Ethanol by diluting stock ethanol with nuclease-free water.

Items		Preparation & Handling
☐	Wash Buffer	Prepare Wash Buffer according to the appropriate table below, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain on ice.

If using a fluorophore conjugated primary antibody:

Wash Buffer *1X = 1 Tissue Slide Cassette Gasket*	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
PBS	10X	1X	60.0	132.0	264.0
Tween-20	10%	0.4%	24.0	52.8	105.6
Nuclease-free Water	-	-	516.0	1,135.2	2,270.4
Total	**-**	**-**	**600.0**	**1,320.0**	**2,640.0**

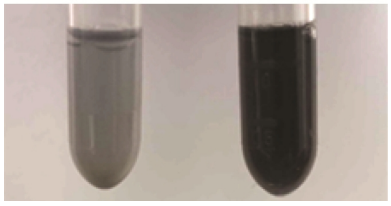
If using a primary and secondary antibody:

Wash Buffer *1X = 1 Tissue Slide Cassette Gasket*	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
PBS	10X	1X	120.0	264.0	528.0
Tween-20	10%	0.4%	48.0	105.6	211.2
Nuclease-free Water	-	-	1,032.0	2,270.4	4,540.8
Total	**-**	**-**	**1,200.0**	**2,640.0**	**5,280.0**

☐	1X Blocking Buffer	Prepare Blocking Buffer on ice according to the appropriate table below, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain on ice.

1X Blocking Buffer	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
PBS	10X	1X	10.0	22.0	44.0
BSA	10%	2%	20.0	44.0	88.0
RNase Inhibitor	40X	1X	2.5	5.5	11.0
Tween-20	10%	0.1%	1.0	2.2	4.4
Nuclease-free Water	-	-	66.5	146.3	292.6
Total	**-**	**-**	**100.0**	**220.0**	**440.0**

Optional Staining Reagents - Only Required if Using Stopping Point

Items		Preparation & Handling
☐	Ribonucleoside Vanadyl Complex (RVC)	Preparing aliquots: Incubate sealed vial at 65°C for 20 min or until solution is reconstituted to a dark green solution with no visible particulates. Aliquot into single-use tubes (30 µl per slide). Store tubes at -20°C. DO NOT exceed 2 freeze-thaw cycles per aliquot. If using an aliquot immediately, no freezing is necessary. Store at room temperature until use. Using a frozen aliquot: remove aliquot from -20°C and incubate at 65°C for 10 min to remove precipitate. Before Incubation / After Incubation 
☐	RVC Working Solution	Prepare Ribonucleoside Vanadyl Complex stock as described above prior to generating working solution. Pipette mix. Maintain at room temperature.

RVC Working Solution *1 slide = 1 Tissue Slide*	**Stock**	**Final**	**1 Slide (µl)**	**2 Slides +15% (µl)**	**4 Slides +15% (µl)**
1X PBS	-	-	497.5	1,144.2	2,288.5
Ribonucleoside Vanadyl Complex	200 mM	1 mM	2.5	5.8	11.5
Total	**-**	**-**	**500.0**	**1,150.0**	**2,300.0**

For Coverslip Mounting

Items		Preparation & Handling
Prepare - Select One Mounting Medium		
☐	Mounting Medium	Prepare this Mounting Medium if stopping point will **not** be used. Invert to mix. Briefly centrifuge to remove bubbles. Inspect for bubbles. Continue centrifuging until no bubbles remain. Maintain at room temperature.

Mounting Medium *1 slide = 1 Tissue Slide*	**Stock**	**Final**	**1 Slide (µl)**	**2 Slides +15% (µl)**
Glycerol	100%	80%	120	276
Slowfade Diamond	100%	20%	30	69
Total	**-**	**-**	**150**	**345**

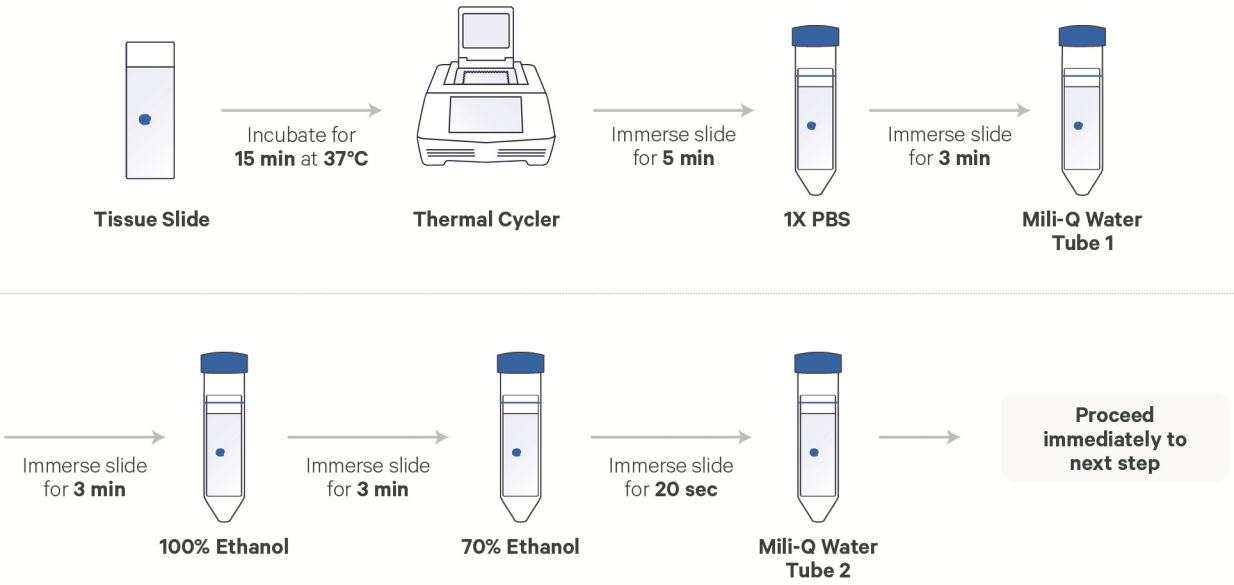
☐	Stopping Point Mounting Medium	Prepare this Mounting Medium if stopping point **will** be used. Invert to mix. Briefly centrifuge to remove bubbles. Inspect for bubbles. Continue centrifuging until no bubbles remain. Maintain at room temperature.

Stopping Point Mounting Medium *1 slide = 1 Tissue Slide*	**Stock**	**Final**	**1 Slide (µl)**	**2 Slides +15% (µl)**
Glycerol	100%	75%	112.5	258.7
Slowfade Diamond	100%	20%	30.0	69.0
RNase Inhibitor	40X	2X	7.5	17.3
Total	**-**	**-**	**150.0**	**345.0**

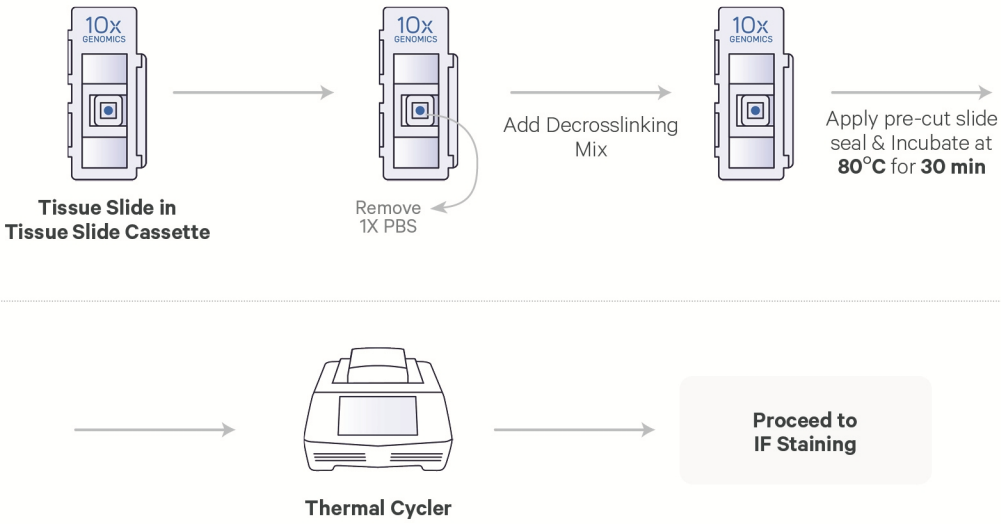
Obtain		
☐	Coverslip	-

Protocol Overview

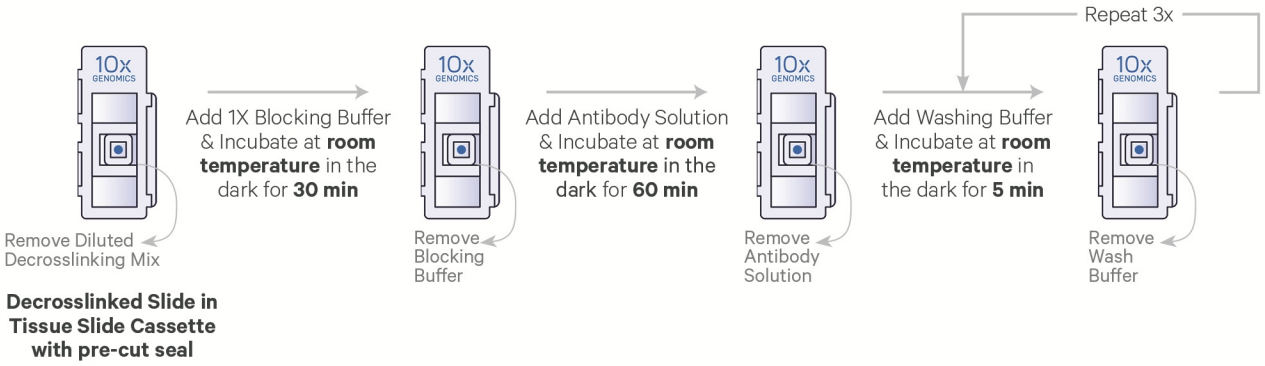
Rehydration ~20 min

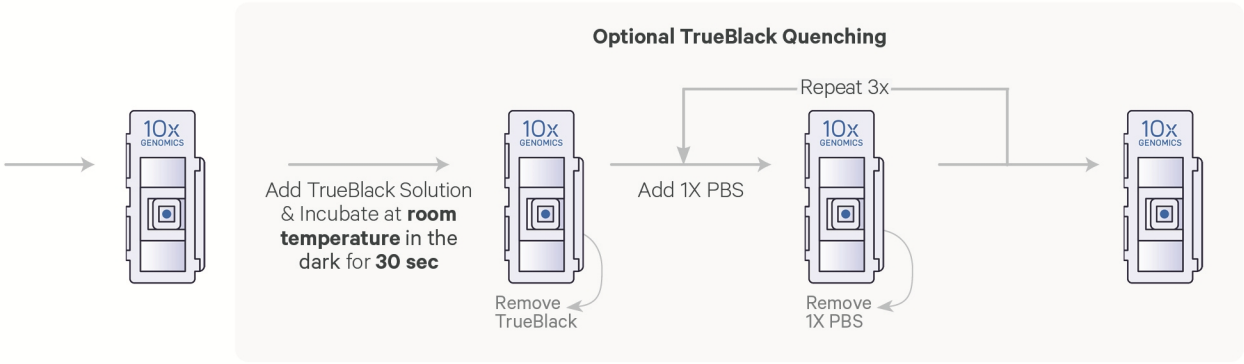


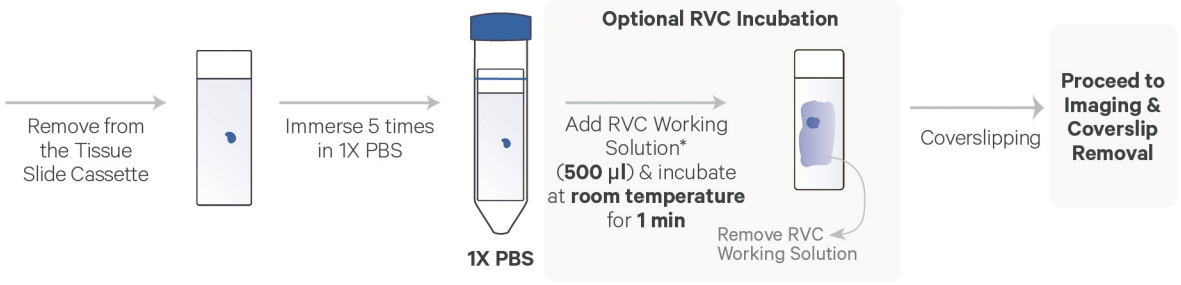
Decrosslinking ~30 min



Immunofluorescence Staining – Fluorophore Conjugated Primary Antibodies ~2 h

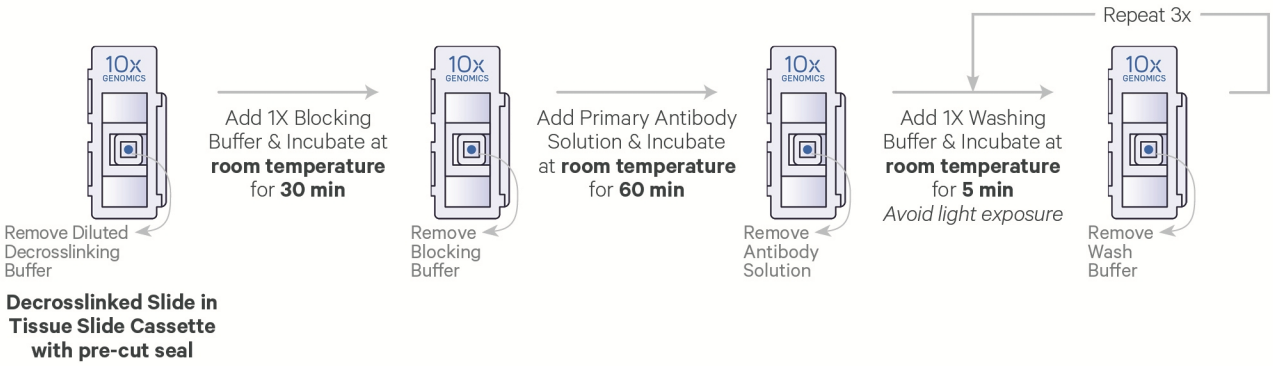


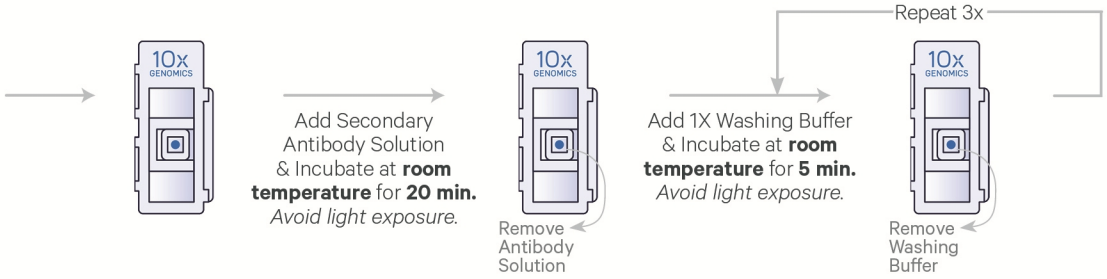


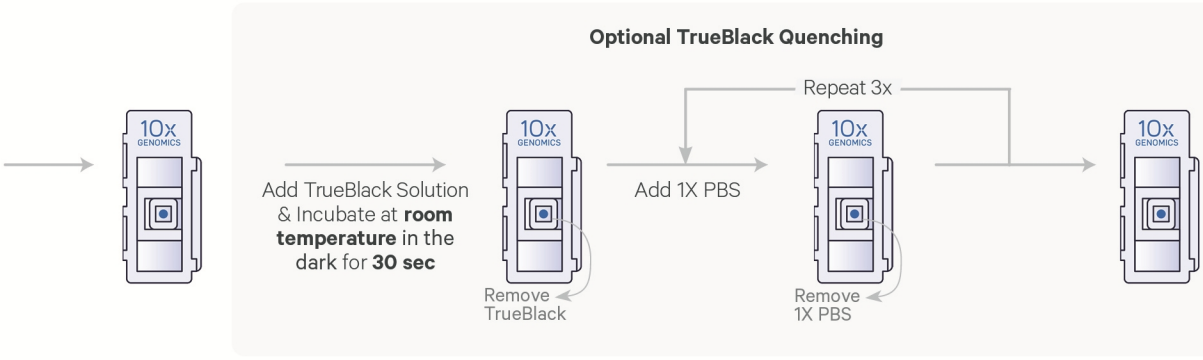


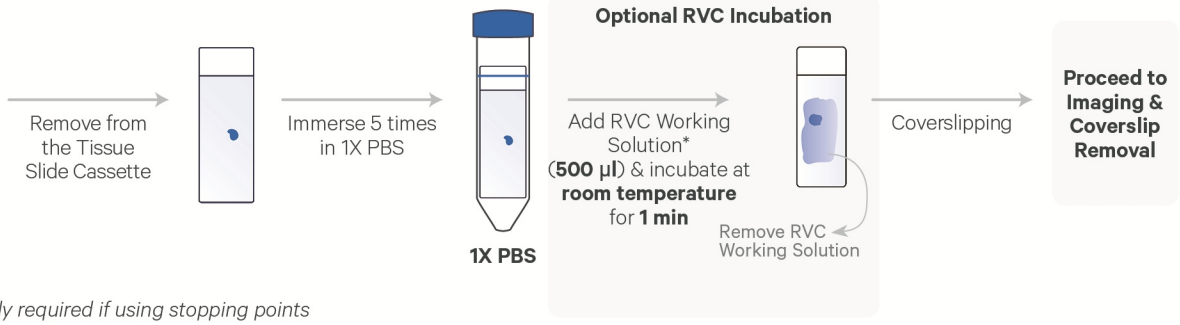
**Only required if using stopping points*

Immunofluorescence Staining – Primary & Secondary Antibodies ~2.5 h









**Only required if using stopping points*

3.1 Rehydration

a. Place a Low Profile Thermocycler Adapter on a thermal cycler and preheat thermal cycler to **37°C**.

b. Retrieve slide containing tissue from **-80°C** and place on dry ice.

c. Place slide on Low Profile Thermocycler Adapter with tissue side facing up and incubate **15 min** at **37°C**. DO NOT close thermal cycler lid.

Ensure tissue is dry before proceeding to next step. Damp tissue may lead to detachment. DO NOT exceed 20 min incubation time.

d. Remove slide from thermal cycler. Gently immerse slide in 1X PBS Tube and incubate for **5 min**.

TIPS

When immersing slides in solutions, ensure that tissue sections are completely submerged.

e. Gently immerse slide in Milli-Q Water Tube 1 and incubate for **3 min**.

f. Gently immerse slide in 100% Ethanol Tube for **3 min**.

g. Gently immerse slide in 70% Ethanol Tube for **3 min**.

h. Gently immerse slide in Milli-Q Water Tube 2 for **20 sec**.

i. Proceed immediately to next step.

To ensure even staining, DO NOT let slides dry.

3.2 Decrosslinking

Ensure Decrosslinking Buffer B has been prepared according to Preparation - Buffers.

a. Prepare thermal cycler with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
80°C	100 µl	40 min
Step	**Temperature**	**Time** *hh:mm:ss*
Hold	22°C	Hold
Decrosslinking	80°C	00:30:00
Re-equilibrate	22°C	00:10:00
Hold	22°C	Hold

b. Prepare Diluted Perm Enzyme B according to the table below. Add reagents in the order listed. Mix thoroughly with a 1-ml pipette set to 600 µl. Maintain at room temperature.

Diluted Perm Enzyme B	Stock	Final	Total Amount (µl)
1X PBS	-	-	998.0
Perm Enzyme B *(Maintain at room temperature. Pipette mix, centrifuge briefly. DO NOT vortex).*	-	-	2.0
Total	**-**	**-**	**1,000.0**

c. Prepare Decrosslinking Mix according to the table below. Add reagents in the order listed. Pipette mix thoroughly. Centrifuge briefly. Maintain at room temperature.

Decrosslinking Mix *1 slide = 1 Tissue Slide*	Stock	Final	1 slide (µl)	2 slides + 10% (µl)	4 slides + 10% (µl)
Decrosslinking Buffer B	-	-	92.4	203.4	407.0
Urea	8 M	0.5 M	6.3	13.8	27.5
Diluted Perm Enzyme B	-	-	1.3	2.8	5.5
Total	**-**	**-**	**100.0**	**220.0**	**440.0**

d. Place slide in a Tissue Slide Cassette.

See Visium Cassette S3 Quick Reference Card (CG000730) for assembly instructions. Practice assembly with a blank slide.

e. Add **100 µl** 1X PBS along the side of the wells.

f. Remove 1X PBS from wells.

g. Add **100 µl** Decrosslinking Mix along the side of the wells

TIPS

h. Apply a new pre-cut slide seal on the cassette and place cassette on Low Profile Thermocycler Adapter. See Tips & Best Practices for more information on cassette incubation in a thermal cycler.

i. Close and tighten thermal cycler lid. Skip Pre-equilibrate step and initiate Decrosslinking.

j. After decrosslinking is complete, remove cassette from Low Profile Thermocycler Adapter and place on a flat, clean work surface.

k. Peel back slide seal and using a pipette, remove all Decrosslinking Mix from well corners.

l. Add **150 µl** 1X PBS along the side of the wells to uniformly cover tissue sections, without introducing bubbles.

m. Proceed to one of the following:

- 3.4 Immunofluorescence Staining - Fluorophore Conjugated Primary Antibodies on the next page
- 3.5 Immunofluorescence Staining - Primary & Secondary Antibodies on page 80

3.3 Immunofluorescence Staining

Choose appropriate staining protocol depending upon the type of antibodies used. If using a primary and secondary antibody, proceed directly to 3.5 Immunofluorescence Staining - Primary & Secondary Antibodies on page 80.

TIPS

Antibody dilution may vary depending on the antibody, ranging from 1:50 down to 1:1000. Antibody volumes will depend on concentrations determined during antibody optimization.

3.4 Immunofluorescence Staining - Fluorophore Conjugated Primary Antibodies

a. Prepare Antibody Solution on ice, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain on ice.

Antibody Solution	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
1X = 1 Tissue Slide Cassette Gasket					
PBS	10X	1X	7.0	15.4	30.8
BSA	10%	2%	14.0	30.8	61.6
RNase Inhibitor	40X	2X	3.5	7.7	15.4
Tween-20	10%	0.1%	0.7	1.5	3.1
Antibody #1	Variable	Variable	Variable	Variable	Variable
Antibody #2 (Optional)	Variable	Variable	Variable	Variable	Variable
Antibody #3 (Optional)	Variable	Variable	Variable	Variable	Variable
DAPI Solution	100X	0.5X	0.4	0.8	1.5
Nuclease-free Water	Variable	Variable	Variable	Variable	Variable
Total	**-**	**-**	**70.0**	**154.0**	**308.0**

b. *Optional* - Prepare 1X TrueBlack Solution according to the appropriate table, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain at room temperature.

1X TrueBlack Solution *1X = 1 Tissue Slide Cassette Gasket*	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
TrueBlack Lipofuscin Quencher	20X	1X	3.5	7.7	15.4
70% Ethanol	-	_	66.5	146.3	292.6
Total	**-**	**-**	**70.0**	**154.0**	**308.0**

c. Add **100 µl** 1X Blocking Buffer along the side of the wells.

d. Re-apply Visium Slide Seal to Tissue Slide Cassette.

e. Incubate for **30 min** at **room temperature**.

f. Peel back slide seal and remove all Blocking Buffer from wells.

g. Add **70 µl** Antibody Solution along the side of the wells. Tap gently to ensure uniform coverage.

h. Re-apply slide seal to cassette.

i. Incubate for **1 h** at **room temperature** in the dark.

j. Peel back slide seal and remove Antibody Solution.

Four Wash Buffer washes:

k. **Wash 1:** Add **150 µl** Wash Buffer along the side of the wells. Incubate for **5 min** at **room temperature**. Remove all Wash Buffer from wells.

l. **Wash 2:** Add **150 µl** Wash Buffer along the side of the wells. Incubate for **5 min** at **room temperature**. Remove all Wash Buffer from wells.

m. **Wash 3:** Add **150 µl** Wash Buffer along the side of the wells. Incubate for **5 min** at **room temperature**. Remove all Wash Buffer from wells.

n. **Wash 4:** Add **150 µl** Wash Buffer along the side of the wells. Incubate for **5 min** at **room temperature**. Remove all Wash Buffer from wells.

o. *Optional* - TrueBlack Quenching

- Add **70 µl** 1X TrueBlack Solution along the side of the wells. Tap cassette gently to ensure uniform coverage.
- Incubate for **30 sec** at **room temperature**.
- Remove all 1X TrueBlack Solution from wells.

Four PBS washes:

- **Wash 1:** Add **150 µl** 1X PBS along the side of the wells. Remove all PBS from wells.

- **Wash 2:** Add **150 µl** 1X PBS along the side of the wells. Remove all PBS from wells.
- **Wash 3:** Add **150 µl** 1X PBS along the side of the wells. Remove all PBS from wells.
- **Wash 4:** Add **150 µl** 1X PBS along the side of the wells. Remove all PBS from wells.

p. Remove slide from cassette.

TIPS *See Tips & Best Practices for removal instructions.*

q. Gently immerse slide 5x in 1X PBS in 50-ml centrifuge tube. If processing multiple slides, use a separate 50-ml centrifuge tube filled with PBS for each slide.

r. Remove slide from PBS and place on a flat, clean, nonabsorbent work surface.

s. *Optional RNA protection - only if using pre or post-imaging stopping point*

- Add **500 µl** RVC Working Solution per slide to uniformly cover all tissue sections.
- Incubate **1 min** at **room temperature**.
- Discard reagent by holding slides at an angle with the bottom edge in contact with a lint-free laboratory wipe and gently tapping the slide against the wipe.

t. Proceed immediately to Coverslip Mounting.

3.5 Immunofluorescence Staining - Primary & Secondary Antibodies

a. Prepare Primary Antibody Solution on ice, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain on ice.

Primary Antibody Solution *1X = 1 Tissue Slide Cassette Gasket*	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
PBS	10X	1X	7.0	15.4	30.8
BSA	10%	2%	14.0	30.8	61.6
RNase Inhibitor	40X	2X	3.5	7.7	15.4
Tween-20	10%	0.1%	0.7	1.5	3.1
Antibody #1	Variable	Variable	Variable	Variable	Variable
Antibody #2 (Optional)	Variable	Variable	Variable	Variable	Variable
Antibody #3 (Optional)	Variable	Variable	Variable	Variable	Variable
Nuclease-free Water	Variable	Variable	Variable	Variable	Variable
Total	**-**	**-**	**70.0**	**154.0**	**308.0**

b. Prepare Secondary Antibody Solution on ice, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain on ice.

Secondary Antibody Solution *1X = 1 Tissue Slide Cassette Gasket*	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
PBS	10X	1X	7.0	15.4	30.8
BSA	10%	2%	14.0	30.8	61.6
RNase Inhibitor	40X	2X	3.5	7.7	15.4
Tween-20	10%	0.1%	0.7	1.5	3.1
Antibody #1	Variable	Variable	Variable	Variable	Variable
Antibody #2 (Optional)	Variable	Variable	Variable	Variable	Variable
Antibody #3 (Optional)	Variable	Variable	Variable	Variable	Variable
DAPI Solution	100X	1X	0.7	1.5	3.1
Nuclease-free Water	Variable	Variable	Variable	Variable	Variable
Total	**-**	**-**	**70.0**	**154.0**	**308.0**

TIPS Antibody dilution can change depending on the antibody, ranging from 1:10 up

to 1:1500. Antibody volumes will depend on concentrations determined during antibody optimization.

c. *Optional* - Prepare 1X TrueBlack Solution according to the appropriate table, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain at room temperature.

1X TrueBlack Solution *1X = 1 Tissue Slide Cassette Gasket*	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
TrueBlack Lipofuscin Quencher	20X	1X	3.5	7.7	15.4
70% Ethanol	-	-	66.5	146.3	292.6
Total	**-**	**-**	**70.0**	**154.0**	**308.0**

d. Peel back slide seal and remove all 1X PBS from wells.

e. Add **100 µl** 1X Blocking Buffer along the side of the wells.

f. Re-apply Visium Slide Seal to Tissue Slide Cassette.

g. Incubate for **30 min** at **room temperature**.

h. Peel back slide seal and remove all Blocking Buffer from wells.

i. Add **70 µl** Primary Antibody Solution along the side of the wells. Tap gently to ensure uniform coverage.

j. Re-apply slide seal to cassette.

k. Incubate for **1 h** at **room temperature** in the dark.

l. Peel back slide seal and remove Primary Antibody Solution.

Four Wash Buffer washes:

m. **Wash 1:** Add **150 µl** Wash Buffer along the side of the wells. Incubate for **5 min** at **room temperature**. Remove all Wash Buffer from wells.

n. **Wash 2:** Add **150 µl** Wash Buffer along the side of the wells. Incubate for **5 min** at **room temperature**. Remove all Wash Buffer from wells.

o. **Wash 3:** Add **150 µl** Wash Buffer along the side of the wells. Incubate for **5 min** at **room temperature**. Remove all Wash Buffer from wells.

p. **Wash 4:** Add **150 µl** Wash Buffer along the side of the wells. Incubate for **5 min** at **room temperature**. Remove all Wash Buffer from wells.

q. Add **70 µl** Secondary Antibody Solution along the side of the wells. Tap gently to ensure uniform coverage.

r. Re-apply slide seal to cassette.

s. Incubate for **20 min** at **room temperature** in the dark.

t. Peel back slide seal and remove Secondary Antibody Solution.

Four Wash Buffer washes:

u. **Wash 1:** Add **150 µl** Wash Buffer along the side of the wells. Incubate for **5 min** at **room temperature**. Remove all Wash Buffer from wells.

v. **Wash 2:** Add **150 µl** Wash Buffer along the side of the wells. Incubate for **5 min** at **room temperature**. Remove all Wash Buffer from wells.

w. **Wash 3:** Add **150 µl** Wash Buffer along the side of the wells. Incubate for **5 min** at **room temperature**. Remove all Wash Buffer from wells.

x. **Wash 4:** Add **150 µl** Wash Buffer along the side of the wells. Incubate for **5 min** at **room temperature**. Remove all Wash Buffer from wells.

y. *Optional* - TrueBlack Quenching

- Add **70 µl** 1X TrueBlack Solution along the side of the wells. Tap cassette gently to ensure uniform coverage.
- Incubate for **30 sec** at **room temperature**.
- Remove all 1X TrueBlack Solution from wells.

Four PBS washes:

- **Wash 1:** Add **150 µl** 1X PBS along the side of the wells. Remove all PBS from wells.
- **Wash 2:** Add **150 µl** 1X PBS along the side of the wells. Remove all PBS from wells.
- **Wash 3:** Add **150 µl** 1X PBS along the side of the wells. Remove all PBS from wells.
- **Wash 4:** Add **150 µl** 1X PBS along the side of the wells. Remove all PBS from wells.

z. Remove slide from cassette.

TIPS *See Tips & Best Practices for removal instructions.*

aa. Gently immerse slide 5x in 1X PBS in a 50-ml centrifuge tube. If processing multiple slides, use a separate 50-ml centrifuge tube filled with PBS for each slide.

ab. Remove slide from PBS and place on a flat, clean, nonabsorbent work surface

ac. *Optional RNA Protection - only if using pre or post-imaging stopping point*

- Add **500 µl** RVC Working Solution per slide to uniformly cover all tissue sections.
- Incubate **1 min** at **room temperature**.
- Discard reagent by holding slides at an angle with the bottom edge in contact with a lint-free laboratory wipe and proceed immediately to Coverslip Mounting.

3.6 Coverslip Mounting

a. Place slides on a flat, clean, nonabsorbent work surface. Some residual droplets may remain.

b. Using a **wide-bore** pipette tip:

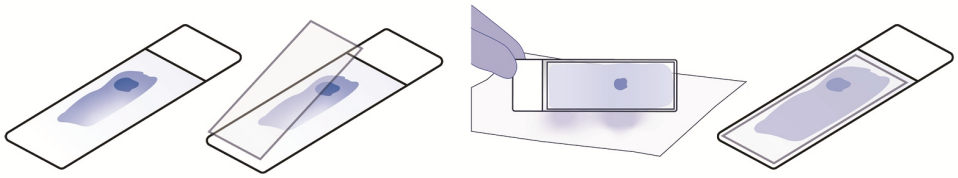
- If **not** using stopping point, add **100–150 µl** Mounting Medium to cover all tissue sections uniformly.
- If **using** stopping point, add **100–150 µl** Stopping Point Mounting Medium to cover all tissue sections uniformly.

Ensure that the appropriate Mounting Medium is used. Mounting Medium is prepared differently if using stopping points. Tissue slides with large sections or multiple sections may require more Mounting Medium.

c. Slowly apply coverslip at an angle on one end of the slides, without introducing bubbles. Allow Mounting Medium to spread and settle.

DO NOT use Cytoseal or nail polish for securing the coverslip.

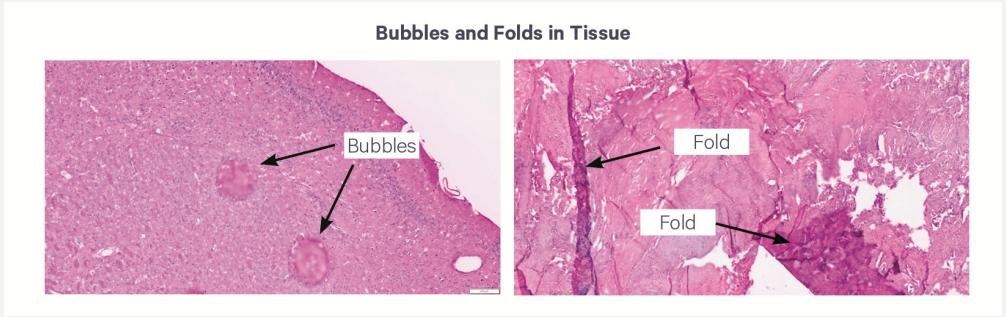
Cover Uniformly with Mounting Medium, Apply Coverslip, & Wick Excess Mounting Medium



d. Remove any large excess of Mounting Medium by carefully wicking away from the edge of the coverslip using a lint-free laboratory wipe. Do not move coverslip and disturb tissue.

e. Inspect the tissue for signs of bubbles or folds, which may result in detachment or UMI loss. Bubbles and folds may appear as regions of tissue that appear uneven or wavy. If bubbles or folds are observed, use a different section for the Visium HD assay.

Images below are representative.



STOP

f. **Immediately** proceed with imaging or if Stopping Point Mounting Medium was used, store slides laying flat in a slide mailer or a slide holder. Store slides in the dark at **4°C** for up to **72 h**. Ensure that slides are laid flat to prevent loss of Mounting Medium.

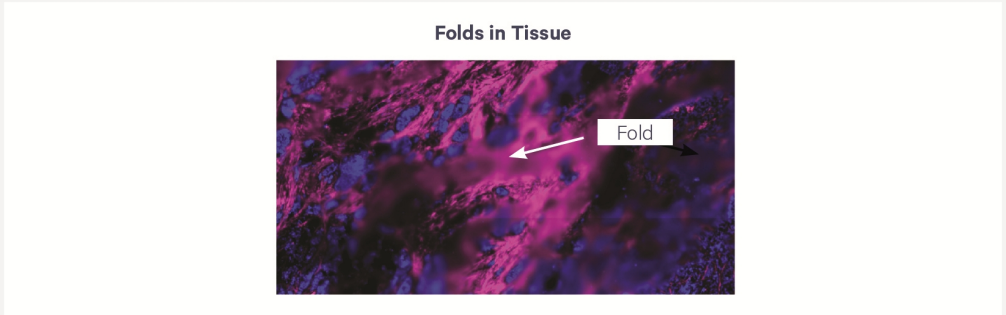
Stopping points may only be used if staining guidance for stopping points was followed, which includes an RVC incubation and appropriate mounting media with RNase inhibitor.

If storing slides for longer is desired, **immediately** proceed with imaging and follow stop instructions in 3.7 Imaging below. The stopping point after coverslip mounting and the stopping point in 3.7 Imaging below cannot be combined.

3.7 Imaging

a. Image tissue section of interest at desired magnification using fluorescence imaging settings. Consult the Visium HD Spatial Applications Imaging Guidelines Technical Note (CG000688) for additional information.

b. Inspect the tissue for signs of bubbles or folds, which may result in detachment or UMI loss. Folds may appear as blurry regions in the IF image. If bubbles or folds are observed, use a different section for the Visium HD assay.



c. If slides were immediately imaged after coverslip mounting and Stopping Point Mounting Medium was used, slides may be stored in the dark at **4°C** for up to **1 week** prior to Coverslip Removal. Ensure that slides are laid flat to prevent loss of Mounting Medium.

Stopping points may only be used if staining guidance for stopping points was followed, which includes an RVC incubation.

DO NOT let the attached coverslip dry out.

d. Proceed **immediately** to Coverslip Removal.

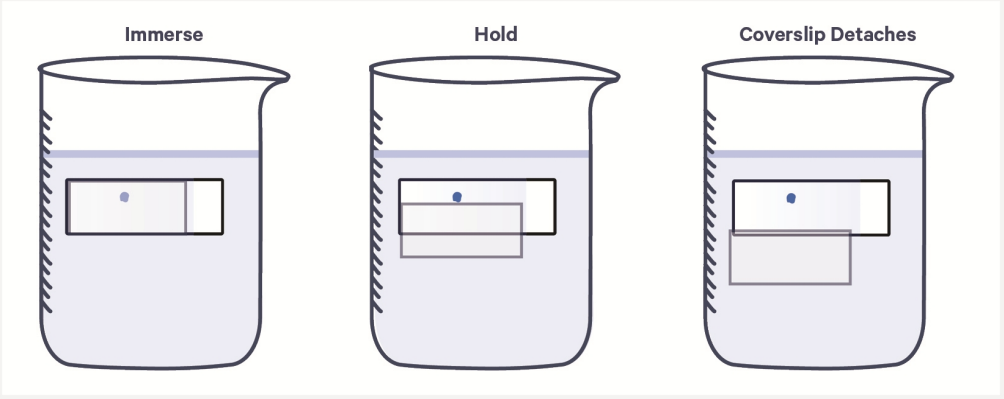
3.8 Coverslip Removal

a. Dispense **800 ml** 1X PBS in a beaker. Up to 10 slides may be processed using this beaker.

b. Immerse slides sideways in the beaker containing **800 ml** 1X PBS with coverslipped surface fully sideways to prevent coverslip from dragging across tissue.

c. Hold slides in PBS until the coverslip slowly separates away from slide.

To avoid tissue section damage or detachment, DO NOT move slide up and down, shake forcibly, or manually move coverslip.



d. Gently immerse slides 30x in 1X PBS to ensure all Mounting Medium is removed.

e. Wipe back of slide with a lint-free laboratory wipe.

f. Place slide on a flat, clean, nonabsorbent work surface and air dry for **5 min**.

g. Place slide in a new Tissue Slide Cassette.

h. Add **100 µl** 1X PBS along the side of the wells.

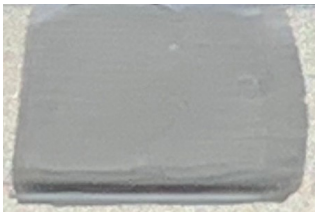
i. Apply a new pre-cut slide seal to cassette.

j. Proceed immediately to Visium HD Spatial Gene Expression Reagent Kits User Guide (CG000685).

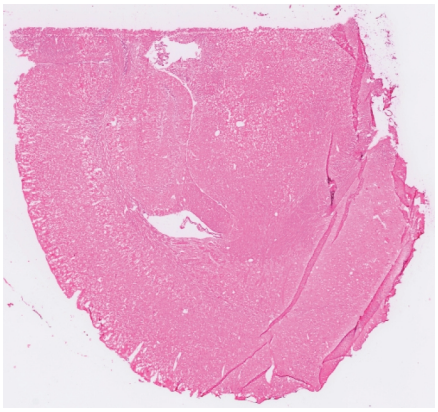
Troubleshooting

Tissue Preparation Troubleshooting

Cryosectioning - Impact of Cryostat Specimen Head Temperatures on Tissue Tearing

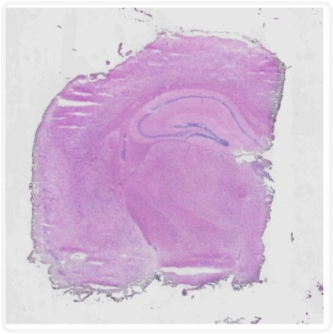
-10°C	-14°C	-20°C	-30°C
			
Normal Section			Tissue section has significant tearing.

Cryosectioning - Impact of Warm Tissue Block on Cryosectioning

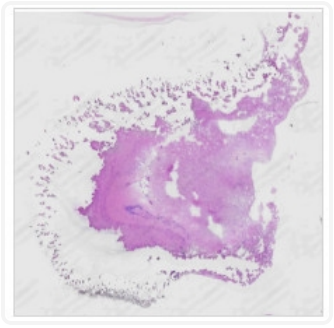


Folds in tissue are a result of warmer than optimal sectioning temperature.

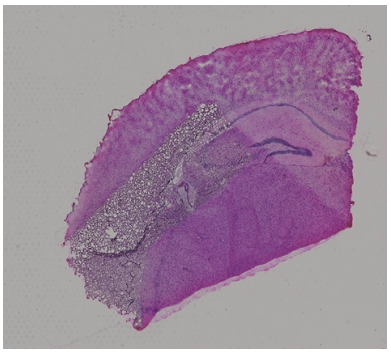
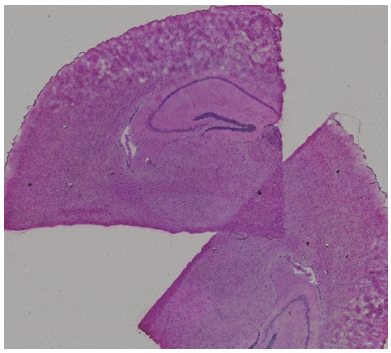
Section Placement - Impact of Condensation on Tissue Sections



No Condensation. Intact tissue section.

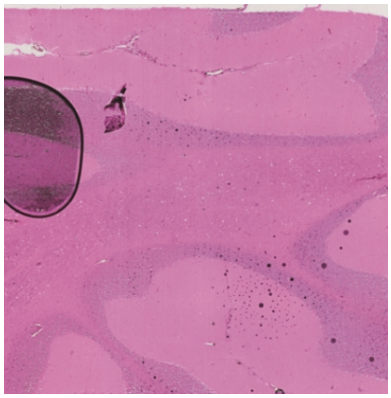


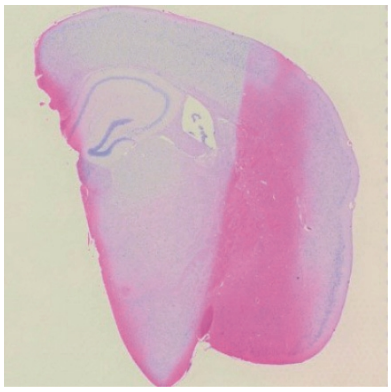
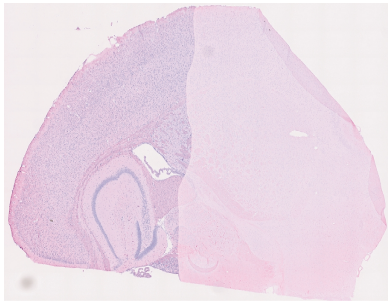
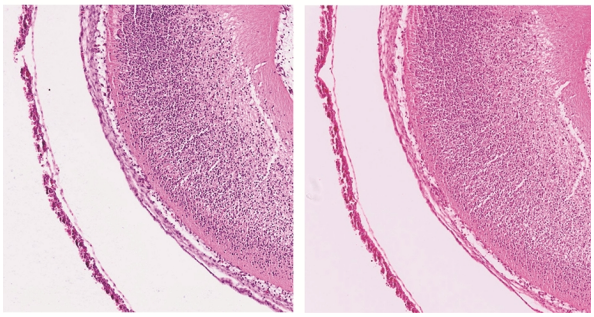
Tissue degraded due to condensation. DO NOT leave slides at room temperature, especially with fresh sections as the resulting condensation will cause tissue disintegration.

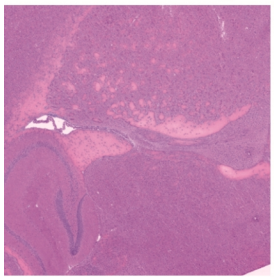
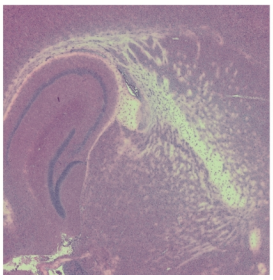
Section Placement - Incorrect Placement of Tissue Sections	
	
Folded tissue section	Overlapping sections

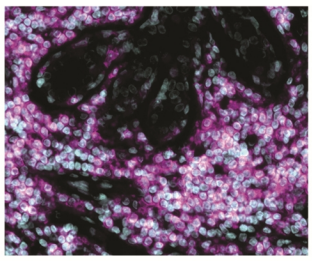
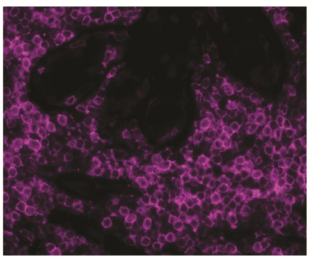
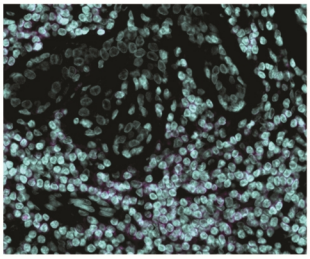
Practice correct section placement on blank slides.

Fixation Troubleshooting	
Tissue does not Sink after Fixation	If tissue still does not sink to the bottom of the tube after 24 h of fixation, proceed with protocol to avoid risk of overfixation. Ensure that tissue is trimmed so that tissue thickness does not exceed 5 mm.

H&E Staining Troubleshooting	
Tissue Detachment	Ensure compatible blank slides are used to minimize tissue detachment. Consult the Visium HD Protocol Planner (CG000698). Tissues with large amounts of connective tissue (like breast or colon) should be placed on Schott Nexterion Slide H 3D Slides (Schott North America, PN-1800434) to minimize risk of tissue detachment Ensure that all steps and timings during tissue fixation, freezing, and embedding are followed.
Bubbles	Avoid bubble formation during coverslip mounting. Bubbles can be mitigated by applying the coverslip at an angle and slowly lowering it onto the slide, allowing air to escape. Centrifuge mounting medium to remove bubbles before use. Avoid introducing bubbles when pipetting mounting medium onto slide. Bubbles may cause blackening of tissue. 

H&E Staining Troubleshooting	
Uneven Staining	**Causes** Ensure fresh, unexpired staining reagents are applied to the tissue uniformly and adequate washes are performed. A gentle tap may help reagents spread uniformly. Consider filtering hematoxylin.  Uneven staining may also be caused by allowing slides to dry during staining. Ensure that slides do not air dry prior to coverslip mounting. 
Incorrect Staining Protocol	The staining protocol in this handbook was optimized for tissues that will be processed with the Visium HD assay. Using an alternative H&E staining protocol may result in reduced staining performance. In the mouse embryo examples below, the Visium HD H&E protocol results in better contrast between the hematoxylin (blue) stained nuclei and the eosin (pink) stained cytoplasm compared to the incorrect protocol. **Visium HD H&E Protocol** **Incorrect Protocol** 
Inadequate RVC Working Solution Removal	Failure to remove RVC Working Solution from the slide may lead to Eosin leaching. In the images below, incubating tissue with RVC for longer than the recommended time has led to a loss in pink Eosin staining.

H&E Staining Troubleshooting	
	No Eosin Leaching  **RVC-Induced Eosin Leaching** 
Tissue Section not washed with RVC Working Solution	RVC Working Solution is not required if stopping points in this Handbook will not be used. However, if stopping points will be used and tissue sections are not washed with RVC, a drop in assay sensitivity is likely.

IF Staining Troubleshooting	
Weak Antibody Signal	Weak antibody signal in areas of the tissue where strong signal is expected may indicate that a nonideal concentration of antibody was used. Perform antibody optimization to determine the optimal concentration. Good Signal - DAPI and Antibody Stain  Good Signal - Antibody Stain Only  Weak Signal - DAPI and Antibody Stain  Weak Signal - Antibody Stain Only 

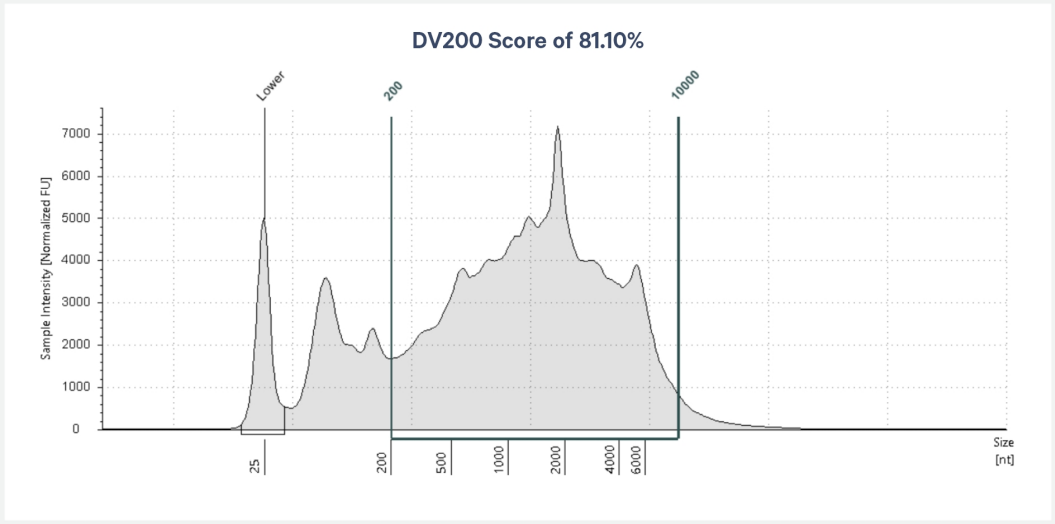
Appendix

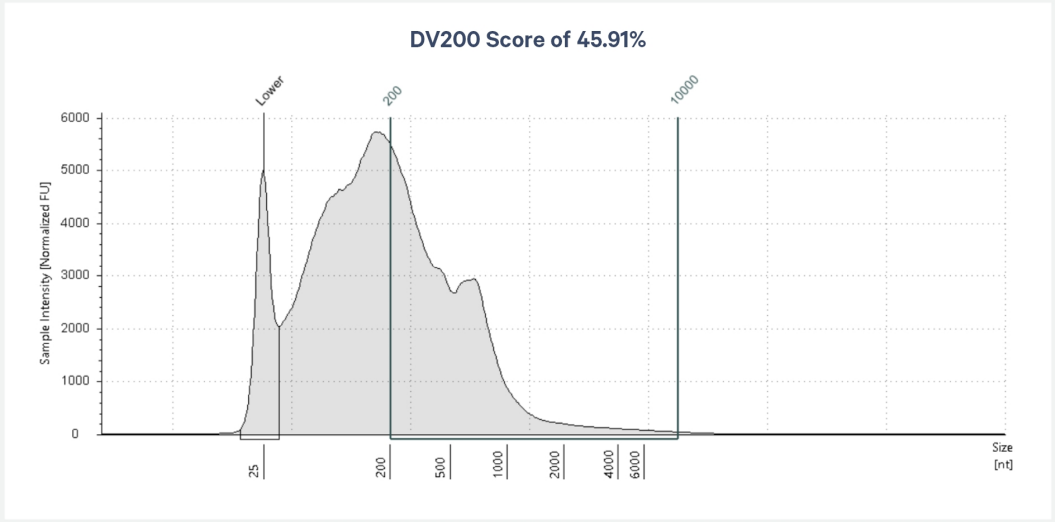
DV200 Performance and Recommendations

DV200 is a broad measurement of RNA quality and is influenced by factors including:

- Tissue block age, type and composition
- Region selected for RNA extraction
- Presence of diseased or necrotic regions
- Depth of section
- Fixation method
- Miscellaneous upstream tissue handling and processing

10x Genomics recommends that fixed frozen tissues used with the Visium HD Spatial Gene Expression should have a DV200 of > 50%. Below are examples of DV200 traces.




DV200 Score of 45.91%
Lower
200
10000
6000
5000
4000
3000
2000
1000
0
Sample Intensity [Normalized FU]
25
200
500
1000
2000
4000
6000
Size [nt]

Notices

Document Number

CG000764 | Rev C

Legal Notices

Instrument & Licensed Software Updates Warranties

Updates to existing Instruments and Licensed Software may be required to enable customers to use new or existing products.

Support

Email: support@10xgenomics.com
10x Genomics, Inc.
6230 Stoneridge Mall Road
Pleasanton, CA

Document Revision Summary

Document Number

CG000764

Title

Visium HD FxF Tissue Preparation Handbook

Revision

Rev C

Revision Date

April 16, 2025

Description of Changes

- Added 10x Genomics RNase Inhibitor 40X (PN-2001488) to Visium HD Spatial Gene Expression Reagent Kits on page 5.
- Added additional notes on requiring an additional 8-port cassette for antibody optimization and an additional Tissue Slide Cassette for each IF-stained tissue slide in Visium HD Spatial Gene Expression Reagent Kits on page 5.

Take 1 minute to evaluate this protocol. Scan this code or click here.