Xenium In Situ - Fresh Frozen Tissue Preparation Handbook

Introduction

The Xenium In Situ Gene Expression workflow is designed to measure mRNA in tissue sections derived from fresh frozen (FF) and embedded tissue samples and requires a Xenium slide with intact tissue sections as input. Proper tissue handling, storage, and preparation techniques preserve the morphological quality of tissue sections and integrity of mRNA transcripts.

This FF Tissue Handbook provides guidance on:

- Freezing and embedding tissue samples prior to cryosectioning
- Best practices for handling tissue samples and Xenium slides before and after cryosectioning
- Hematoxylin and Eosin (H&E) staining to check tissue quality
- Cryosectioning of tissue samples and placement of sections on Xenium slides
- Fixation and permeabilization of tissue sections

Additional Guidance

Refer to the 10x Genomics Support website for additional resources. This protocol is compatible with Xenium In Situ (referred to as Xenium v1) and Xenium Prime reagents and downstream assay workflows as specified in the table.

Compatible Reagent Kits & Downstream Workflows		
	Xenium v1	**Xenium Prime**
Reagent Kits	Xenium Slides & Sample Prep Reagents PN-1000460	Xenium Prime Sample Preparation Reagents PN-1000720
	Xenium Decoding Consumables* PN-1000487	Xenium Decoding Consumables* v2 PN-1000726
	Xenium Instrument Accessory Kit Module A PN-1000530	Xenium Thermocycler Adaptor v2 PN-1000739
Assay Workflows	Xenium In Situ Gene Expression (CG000582)	Xenium Prime In Situ Gene Expression with optional Cell Segmentation Staining (CG000760)
	Xenium In Situ Gene Expression with Cell Segmentation Staining (CG000749)	

*Contains Xenium Cassettes

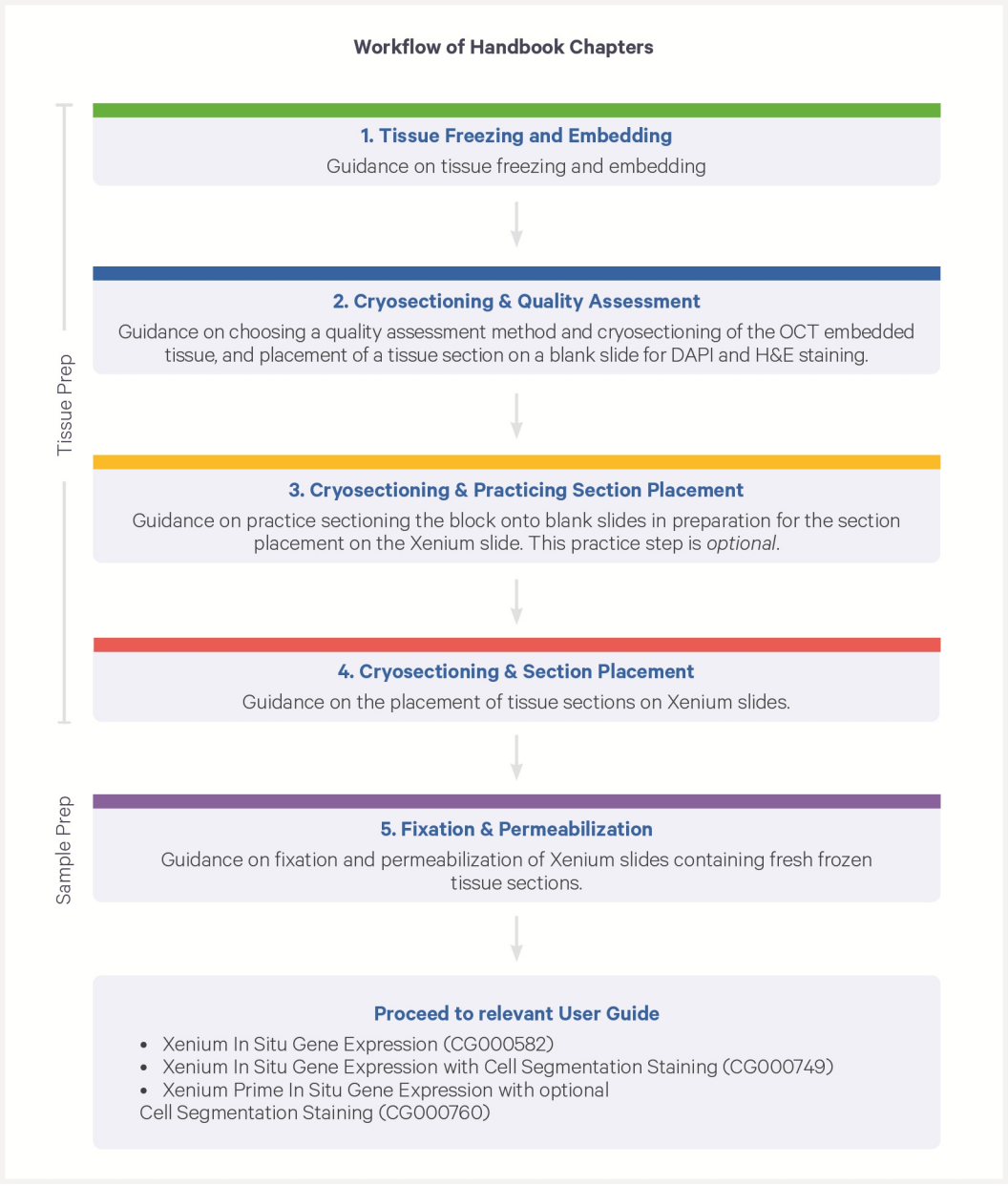
Contents

Document Revision Summary

Handbook Overview and Navigation

Overview

This handbook describes Fresh Frozen tissue and sample preparation for the Xenium In Situ Gene Expression workflow. Tabs on the right-hand side of the page denote different sections of this handbook.



Reagent Kits

Xenium In Situ Gene Expression Reagent Kits

Compatible only with the following Xenium v1 workflows:

- Xenium In Situ Gene Expression (CG000582)
- Xenium In Situ Gene Expression with Cell Segmentation Staining (CG000749)

Refer to SDS for handling and disposal information.

Xenium Slides & Sample Prep Reagents (2 slides, 2 rxns) PN-1000460

Xenium Slides & Sample Prep Reagents
(2 slides, 2 rxns), PN-1000460
Shipped in dry ice
Store at −20°C

	#	PN
Probe Hybridization Buffer*	1	2000390
Post Hybridization Wash Buffer*	1	2000395
Ligation Buffer*	1	2000391
Ligation Enzyme A*	1	2000397
Ligation Enzyme B*	1	2000398
Amplification Mix*	1	2000392
Amplification Enzyme*	1	2000399
Reducing Agent B	1	2000087
Autofluorescence Mix*	1	2000753
FFPE Tissue Enhancer*	1	2000798
Nuclei Staining Buffer*	1	2000762
Perm Enzyme B	1	3000553
Xenium Slides (2 pack)	1	3000941

10x GENOMICS

Only the Xenium Slides (2 pack) are needed for this workflow.

**The reagent name may or may not include the prefix "Xenium"; Irrespective of the prefix, the indicated part number is associated with the reagent name.*

Xenium Slides Kit (4 slides) PN-1000659

Xenium Slides Kit
(2 slides), PN-1000659
Store at –20°C

	#	PN
Xenium Slides (2 pack)	2	3000941

10x GENOMICS

Xenium Slides Kit (16 slides) PN-1000660

Xenium Slides Kit
(2 slides), PN-1000660
Store at –20°C

	#	PN
Xenium Slides (2 pack)	8	3000941

10x GENOMICS

Purchase the Xenium Slides Kit (4 or 16 slides) for additional slides as needed.

Xenium Prime In Situ Gene Expression Reagent Kits

Compatible only with the following Xenium Prime workflows:

- Xenium Prime In Situ Gene Expression with optional Cell Segmentation Staining (CG000760)

Refer to SDS for handling and disposal information.

Xenium Prime Sample Preparation Reagents with Slides - (2 rxns) PN-1000741

Contains Xenium Slides (2 pack) PN-1000465 and Xenium Prime Sample Preparation Reagents (2 rxns) PN-1000720. Only the slide kits are shown below, as the sample preparation reagents are not used in this protocol.

Xenium Slides Kit
(2 slides), PN-1000465
Store at –20°C

	#	PN
Xenium Slides (2 pack)	1	3000941

10x GENOMICS

Xenium Prime Sample Preparation Reagents with Cell Segmentation with Slides - (2 rxns) PN-1000742

Contains Xenium Slides (2 pack) PN-1000465, Xenium Cell Segmentation Staining Reagents (2 rxns) PN-1000661, and Xenium Prime Sample Preparation Reagents (2 rxns) PN-1000720. Only the slide kits are shown below, as the sample preparation reagents are not used in this protocol.

Xenium Slides Kit
(2 slides), PN-1000465
Store at –20°C

	#	PN
Xenium Slides (2 pack)	1	3000941

10x GENOMICS

Third Party Items

Successful execution of the Xenium In Situ Gene Expression 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 Xenium In Situ Gene Expression Protocol Planner (CG000601) 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 reagents at indicated temperatures. Thoroughly mix reagents before use.
- When pipette mixing reagents, unless otherwise specified, set pipette to 75% of total volume.
- Promptly move reagents back to the recommended storage.

Pipette Calibration

- Follow manufacturer's calibration and maintenance schedules.

Tissue Scoring

- OCT block with embedded tissue can be trimmed with a razor blade to fit the Sample Area on the Xenium slide.
- Large tissue samples can be scored during sectioning to generate smaller samples to fit the Sample Area.
- Scoring can be done by making a shallow incision (~1 mm deep) on the cutting surface of the tissue with a razor blade.
- The incision should be shallow. A deep incision may lead to tissue damage and disintegration.
- Once a tissue has been scored, use extra care during sectioning and section handling.

Cryosectioning Temperature

- Cryosectioning temperatures impact tissue section integrity. A temperature setting of −20°C for blade and −10°C for the specimen head is recommended.
- The temperature settings depend upon the local conditions, tissue types, and the cryostat used and 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 is dependent and impacted by 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 or damage to the tissue block or cryostat.

Section Thickness

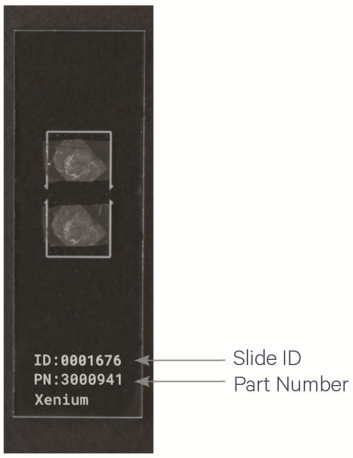
- Recommended section thickness is 10 μm.

The instructions apply to both Xenium v1 and Xenium Prime assays. The consumables are referred to generically without always citing the version suffix. Based on the assay choice, always ensure that either Xenium v1 or Xenium Prime specific reagents and consumables are used.

Xenium Slide

- Xenium slides include an imageable area outlined by a white line measuring 12 mm x 24 mm, with an available sample positioning area measuring 235 mm^2 (10.45 mm x 22.45 mm). The available sample positioning area will be referred to as the Sample Area for the remainder of this document.
- The Sample Area is surrounded by fiducials. Tissue sections are placed within the Sample Area without obstructing the fiducials. The imageable area includes the area within the fiducial frame + Sample Area.
- The Sample Area can accommodate as many tissue sections as can fit within the space. Ensure tissue sections (including OCT) DO NOT overlap.
- An etched label denoting the Slide ID, Part, and Version numbers is located at the bottom of the slide. Tissue sections should be placed on labeled-side of slide.

Xenium Slide with Tissue Sections



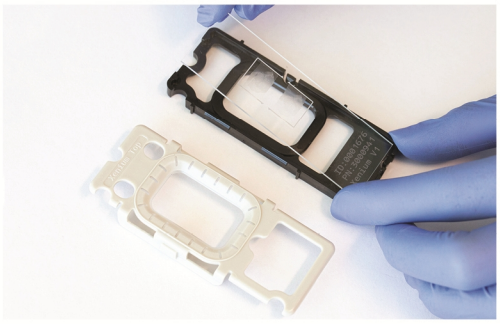
General Xenium Slide Handling

- Always wear gloves when handling slides.
- The bottom of the slide is indicated by the etched label, which should be readable when in the proper position.
- The tissue sections should always be placed within the Sample Area on etched label side of the slide.
- Hold the slide on the label. DO NOT touch the tissue sections or near fiducials.
- Minimize exposure of the slides to sources of particles and fibers.
- Keep the slide cassette flat on the bench when adding reagents to the Sample Area.
- Ensure that no absorbent surface is in contact with the reagents on the slide during incubation.
- When pipetting reagent onto a slide, avoid generating bubbles. Avoid pipetting directly onto the tissue.
- After aspirating reagent from a slide, pipette new reagent onto same slide before moving onto aspiration of second slide.

The Xenium Slide (PN-3000941) may not always include the suffix v1 in the etched label.

The instructions apply to both Xenium Cassette and Xenium Cassette v2. The image shows a Xenium Cassette.

Xenium Slide Handling



Note the Slide ID and Part numbers on the slide label will be required for downstream analysis.

Handling Xenium Slides Prior to and After Sectioning

Handling Xenium Slides Without Tissue Sections

- Store packaged slides at **−20ºC**.
- DO NOT touch the surface of the slide.
- Prior to sectioning, slides and slide mailers should be equilibrated to cryostat temperature for:
  - **10 min** if taken immediately from **−20ºC**.
  - At least **30 min** if stored at room temperature.
- Once opened, the slide can remain at room temperature in a desiccator for up to one week.

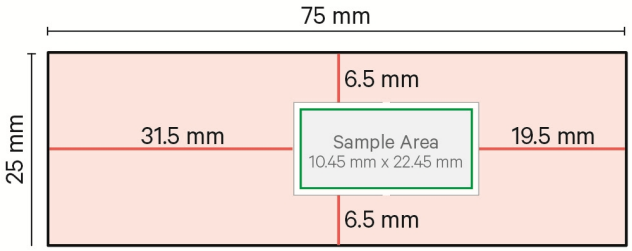
Handling Xenium Slides Containing Tissue Sections

- Slides containing tissue sections are stored in a slide mailer at **−80ºC** for up to **4 weeks** after sectioning.

Xenium Slide Template

- Use the following diagram to verify that freshly placed tissue sections are compatible with the Xenium Slide. Reference the image below to draw the sample area on the back of blank slides.

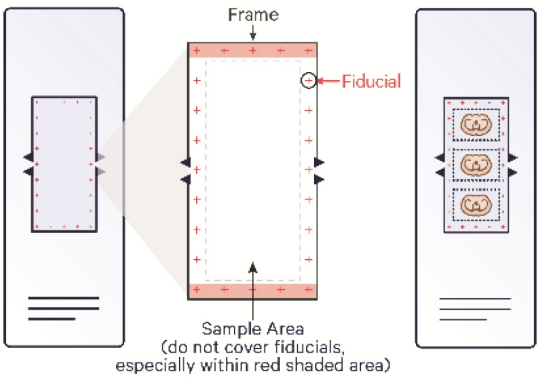
Images are to scale if scaling settings are not modified (select "actual size" or "100%" to print to scale).



- Slide thickness is 1.1 mm ± 0.05 mm.
- Practice correct section placement within the representative frames using non-experimental blocks.

Section Placement on Xenium Slides

- Discard the first couple of sections after facing the block before placing sections on Xenium slides.
- Place the tissue section within the Sample Area on Xenium slides. Avoid covering the fiducials with tissue. It is fine if paraffin covers the fiducials.



- To assist in section placement, trace the Sample Area on the back of the slide using the provided template in Xenium Slide Template on the previous page.
- The section on the slides should be uniform without any cracks, tears, or folds.
- The OCT surrounding each tissue section should not overlap other sections.

- Once sections are placed on Xenium slides, they cannot be repositioned as this would compromise slide integrity and assay performance.

Tissue Detachment on Xenium Slides

- Monitor section adhesion on Xenium slides throughout the workflow.
- Tissue detachment during the workflow can negatively impact performance. If observed, contact support@10xgenomics.com.
- For more information, refer to Troubleshooting.

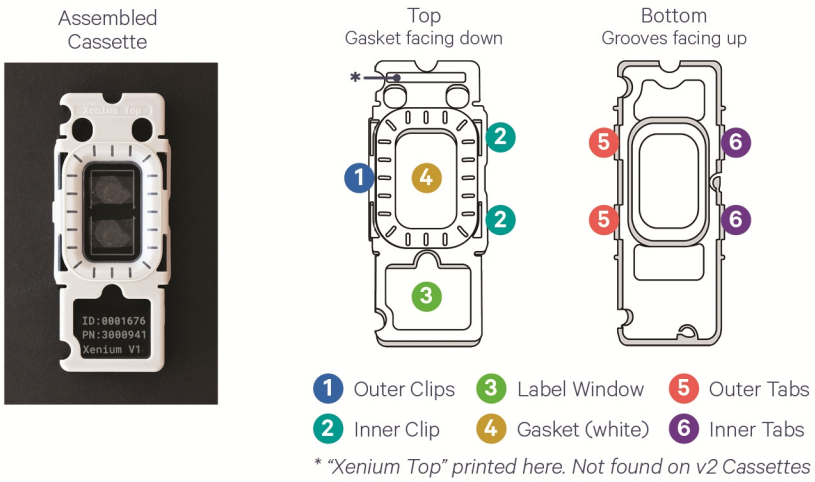
Slide Storage

- Always store unused slides at -20°C in their original packaging and keep sealed. Once opened, slides should remain at room temperature in a desiccator and be used within one week.
- After tissue placement, store slides in a sealed container. If using an unsealed slide mailer, store in a secondary sealed container, such as a resealable bag.
- Store the sealed container containing slides with fresh frozen tissue at -80°C for up to four weeks.

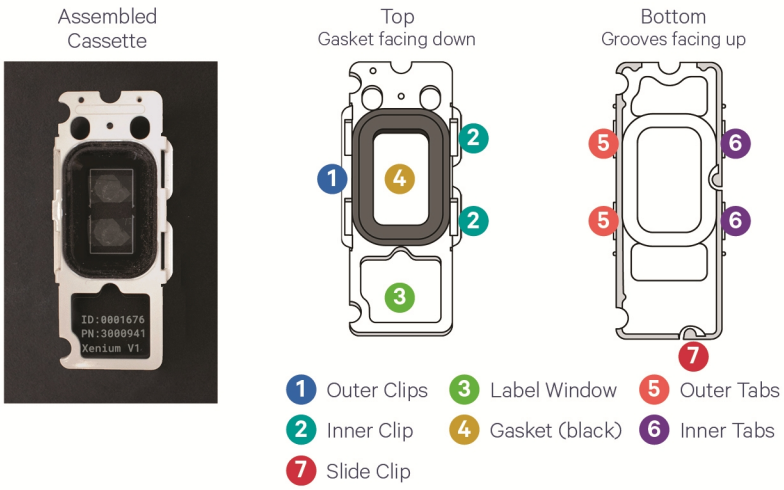
Store Slides in a Sealed Container

Xenium Cassette

Xenium Cassette
(Use with Xenium v1 Assays)



Xenium Cassette v2
(Use with Xenium Prime Assays)

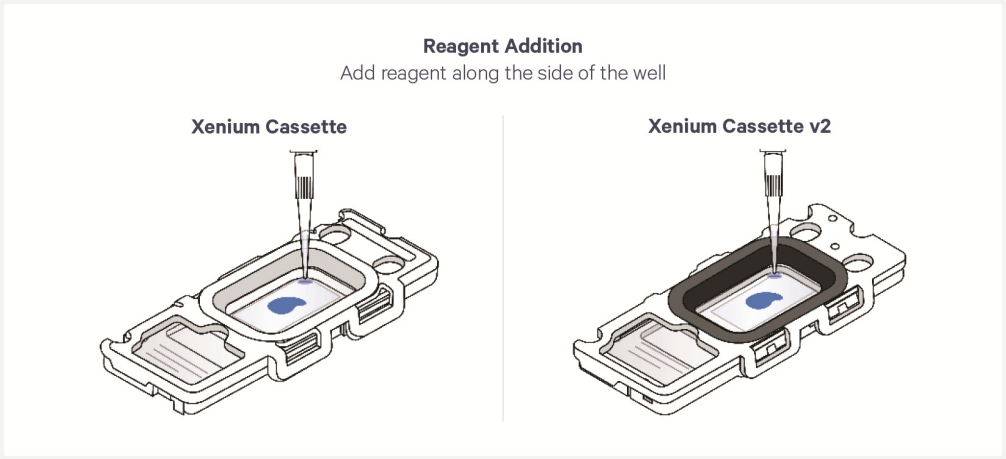


The following guidance applies to both Xenium Cassette and Xenium Cassette v2

- The Xenium Cassette is assembled and disassembled manually. Consult the Xenium Quick Reference Card for Slide Cassette Assembly (CG000623) for cassette assembly and removal instructions.
- The Xenium Cassette is a single use item.
- The Xenium Cassette encases the slide and creates a leakproof well for adding reagents.
- Place the slide in the Xenium Cassette only when specified.
- Inner and outer tabs on the bottom half of the Xenium Cassette are used for holding the slide in the cassette. Applying excessive force to the cassette may cause the slide to break.
- The Xenium Cassette includes an attached Xenium Gasket. The Xenium Gasket corresponds to the Sample Area on the slides.
- The etched slide label is visible in the label window when properly assembled.
- Ensure that the Xenium Cassette and gasket are free of debris before assembly. If placing the top half of the cassette on a surface, ensure the gasket faces up so it does not collect debris.
- Visually inspect the gasket to ensure it is seated properly. If the gasket appears warped, the Xenium Cassette is safe to use if the cassette can fully close and no reagent leakage is observed.

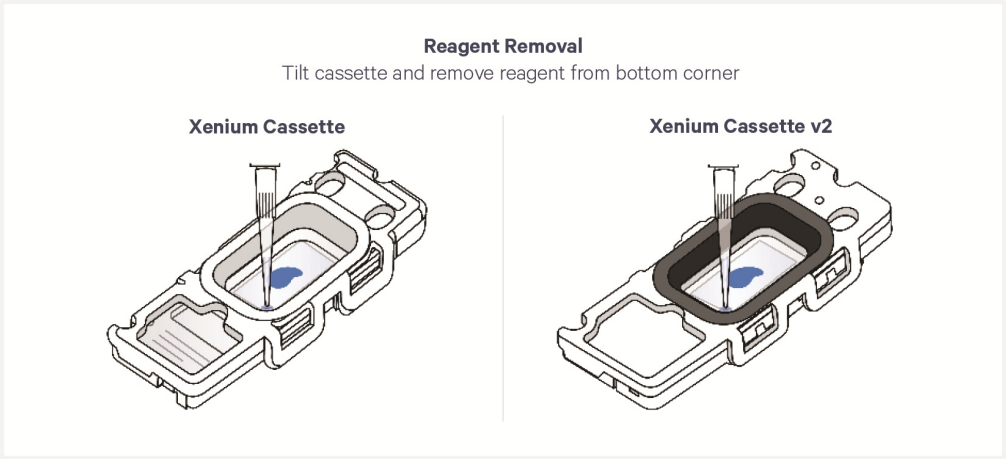
Reagent Addition to Wells

- Place assembled cassette flat on a clean work surface.
- Dispense and remove reagents along the side of the well without touching the tissue sections and without introducing bubbles.
- Always cover the Sample Area completely when adding reagents to the well. A gentle tap may help spread the reagent more evenly.



Reagent Removal from Wells

- Place assembled cassette flat on a clean work surface.
- Slightly tilt the cassette while removing the reagent.
- Place the pipette tip on the bottom edge of the well.
- Remove reagents along the side of the well without touching the tissue sections.
- Remove all liquid from the well in each step.



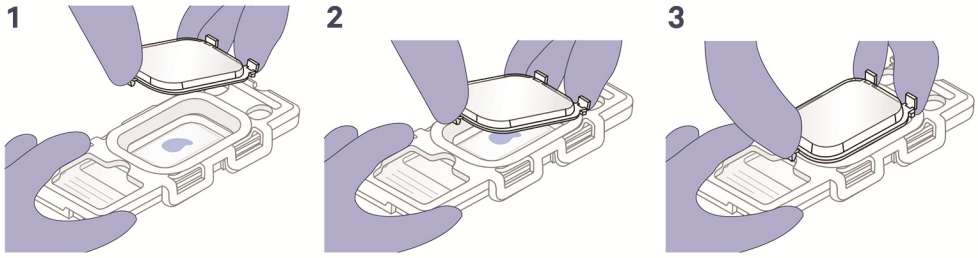
Xenium Cassette Lid Application & Removal

- Work on a clean surface.
- Use a new lid or reapply a used lid based on the instructions provided for a specific protocol step.

When handling an assembled cassette with the lid applied, always hold from the bottom of the cassette and not the lid.

Application

Applying the Xenium Cassette Lid

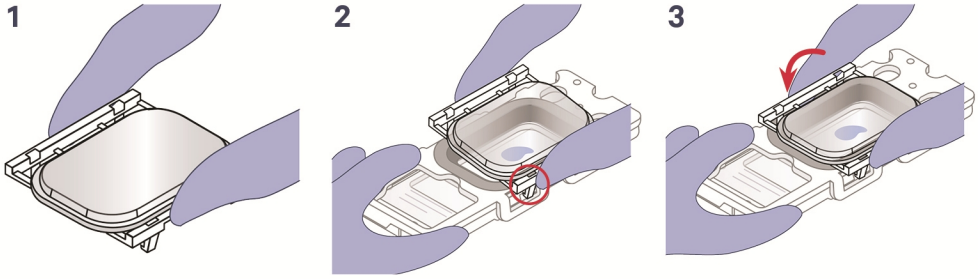


Hold the Xenium Cassette Lid with index and middle finger on two upper tabs and thumb on the lower clip.

Align lid with the surface of the Xenium Cassette. Hook the two upper clips into the two holes on the top of the cassette.

Push the lid down until the lower clip clicks into place. Inspect the lid to confirm placement.

Applying the Xenium Cassette Lid v2



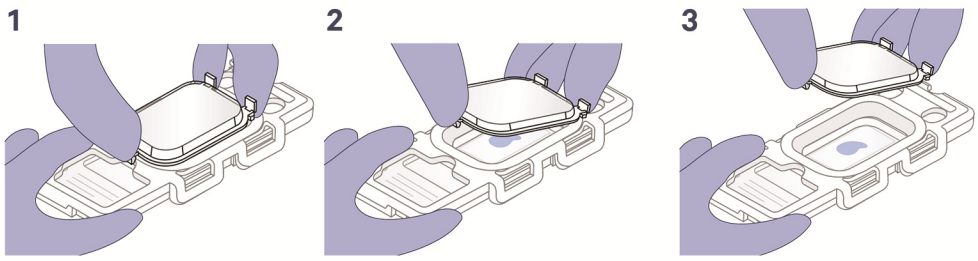
Hold the Xenium Cassette Lid v2 with thumb and index finger on the long sides.

On one side, align lid the lid feet to the notches on the cassette clip.. Hook the feet into the two side holes of the cassette.

Slowly push down on the other side of the lid until that side clicks into place. Inspect the lid to confirm placement.

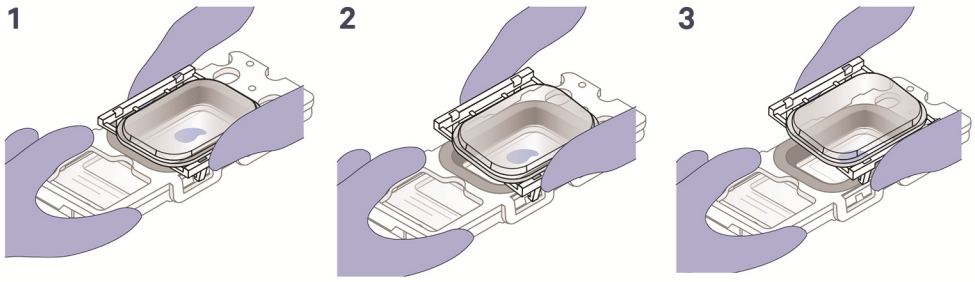
Removal

Removing the Xenium Cassette Lid



1. Push on the top of the two upper tabs with index and middle fingers.
2. Use thumb to push in on the lower clip. While maintaining inward pressure, pull upward with thumb to disengage lower clip.
3. Lift cassette up slowly to ensure no liquid splashes out of the well.

Removing the Xenium Cassette Lid v2



1. Apply even pressure on both sides of the lid using thumb and index finger.
2. While maintaining inward pressure, pull upward with index finger to unhook the side feet from the cassette.
3. Slowly lift cassette lid up to unhook the remaining lid feet. Ensure no liquid splashes out of the well

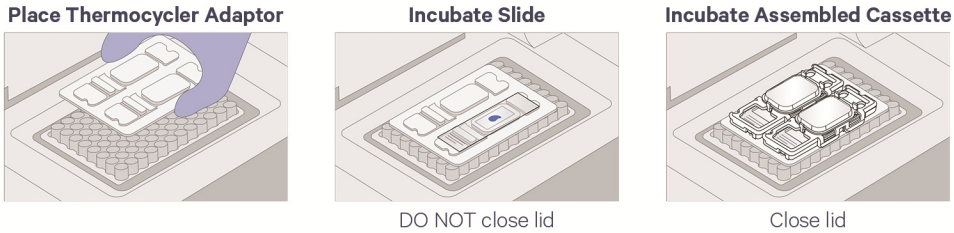
Slide Incubation Guidance

Incubation at a Specified Temperature

The instructions apply to both Thermocycler Adaptor and Thermocycler Adaptor v2. The illustrations show a Thermocycler Adaptor.

Incubation using a Thermal Cycler:

- Position a Xenium Thermocycler Adaptor on a thermal cycler that is set at the incubation temperature. Ensure thermal cycler has reached appropriate temperature before starting incubation.
- Ensure that the Thermocycler Adaptor is fully inserted into the thermal cycler and is in contact with the thermal cycler block surface uniformly.
- When incubating a slide, position the slide on the Thermocycler Adaptor with the tissue side facing up.
- Ensure the Sample Area is aligned with the corresponding area on the Thermocycler Adaptor. DO NOT close lid.
- When incubating a slide encased in a Xenium Cassette, place the assembled unit on the Thermocycler Adaptor with the well facing up. The cassette should always be sealed with a Xenium Cassette Lid when on the Thermocycler Adaptor unless indicated otherwise.



Incubation on Ice

- Place Xenium slides with label toward the top of the slide mailer for incubations on ice.
- Separate multiple slides by at least one slotted channel inside the mailer.
- Avoid placing slides in the last slotted channel of the mailer. Slides with tissues in this position may get scratched if facing the mailer wall.
- Ensure slide mailer is submerged in the ice up to the lower part of the pink cap and is in standing position during incubation.

Incubation at room temperature

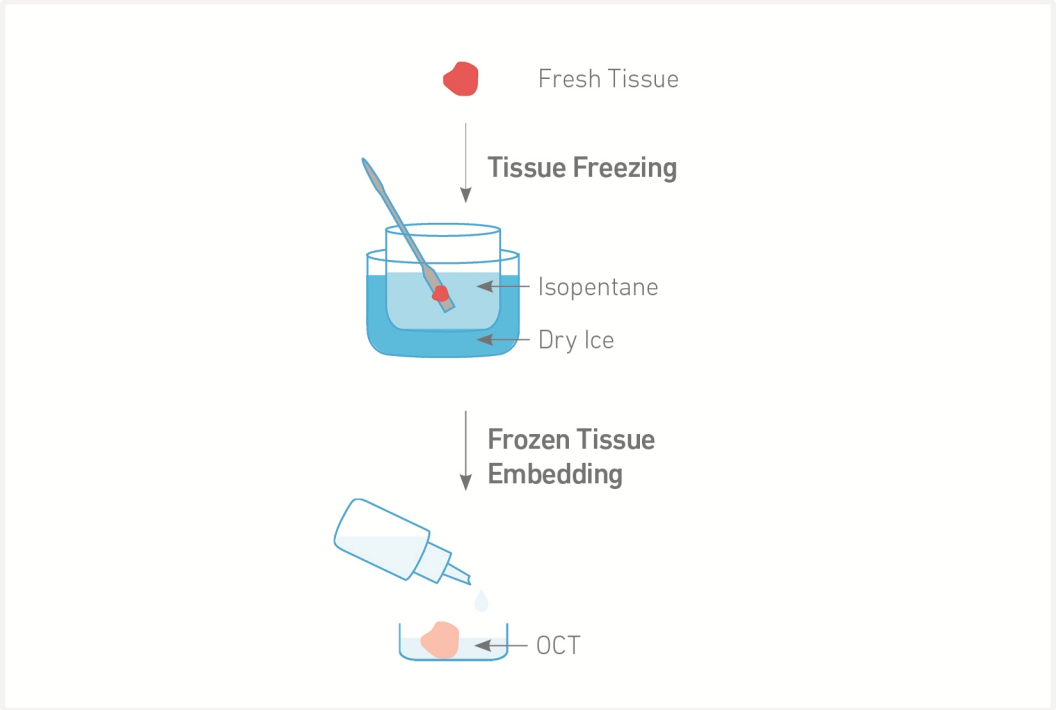
- Place Xenium slides with label toward the top of the slide mailer for incubations at room temperature.
- Separate multiple slides by at least one slotted channel inside the mailer.
- Avoid placing slides in the first or last slotted channel of the mailer. Slides with tissues in these positions may get scratched if facing the mailer wall.
- Ensure the slide mailer is in standing position during incubation.

1. Tissue Freezing and Embedding

Overview

This section provides guidance on tissue freezing and embedding. Freshly obtained tissue samples must be snap frozen to prevent RNA degradation and avoid crystal formation, which can lead to morphological damage to the tissue. Once frozen, tissue samples are embedded in a freezing and embedding compound, Optimal Cutting Temperature (OCT), to preserve the structure of the tissue and to provide structural support during cryosectioning.

Alternatively, perform simultaneous freezing and embedding in OCT for tissues with crevices/gaps or tissues that have a tendency to curl.



Tissue Freezing

A bath of isopentane on dry ice is used to freeze the freshly obtained tissue.

Tissue should not be placed directly in liquid nitrogen as the temperature difference may cause boiling on the surface of the tissues, leading to air pockets and uneven freezing. This may crack and morphologically damage the tissue.

Frozen Tissue Embedding

Prior to cryosectioning, frozen tissue samples are embedded in OCT. Unlike paraffin or resin-based embedding techniques, OCT does not interact with proteins and other molecules that impact antigenicity.

OCT embedding of the tissue offers the following advantages:

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

1.0 Simultaneous Tissue Freezing & Embedding

Items		Preparation & Handling
Prepare		
	Isopentane and dry ice	Fill a polypropylene beaker with ~**200 ml** of isopentane and place in a styrofoam box with dry ice pellets. Let sit for ≥ **30 min.**
	OCT	OCT should be at room temperature.
	Tissue	Using a rolled up laboratory wipe, absorb excess moisture from the surface of the tissue to limit ice crystal formation.

a. Label the cryomold to mark the orientation of the tissue.

b. Add a few drops of room temperature OCT into the crymold.

c. Using forceps, transfer tissue into the cryomold with OCT. If necessary, adjust tissue orientation and add additional OCT to ensure tissue is fully covered. Confirm there are no bubbles, especially near the tissue. Let sit for **20 sec**.

d. Using forceps, lower the cryomold containing embedded tissue into the isopentane without fully submerging. Keep cryomold in contact with isopentane until the OCT has solidified and turned white.

e. Once frozen, place the cryomold on a piece of aluminum foil on dry ice for **30 min**.

f. Store frozen embedded tissue in an airtight sealed container at **–80°C** for **long- term** storage or immediately proceed to Cryosectioning and Section Placement.

Failure to use a sealed container for storage may dehydrate and damage the tissue.

1.1 Separate Tissue Freezing

Items		Preparation & Handling
Prepare		
	Isopentane and dry ice	Fill a polypropylene beaker with ~**200 ml** of isopentane and place in a styrofoam box with dry ice pellets. Let sit for ≥ **30 min.**
	Tissue	Using a rolled up laboratory wipe, absorb excess moisture from the surface of the tissue to limit ice crystal formation.

a. Using forceps, transfer the tissue onto a piece of aluminum foil.

b. Wrap the tissue in aluminum foil, ensuring the tissue is completely wrapped.

c. Using forceps, lower the wrapped tissue into the isopentane.

- Freezing time is dependent on tissue size and composition. For a 10 x 10 mm tissue, freezing time is ~**1 min**.
- Avoid long freezing times as they can lead to morphological changes.

d. Once frozen, use chilled forceps to lift the wrapped tissue out of the isopentane and placed on dry ice

e. Use chilled forceps to unwrap the foil and transfer tissue to a chilled and airtight sealed container or resealable bag.

f. Store frozen tissue at **–80°C** for **long-term** storage or immediately proceed to the next step (Frozen Tissue Embedding).

To prevent evaporation and dehydration of the tissue sample, snap-frozen tissue sample must be stored in a sealed container.

1.2 Frozen Tissue Embedding

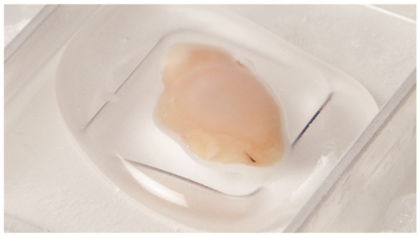
Prepare	
Item	**Preparation & Handling**
Chilled OCT	Place OCT on ice for ≥ **30 min**
Chilled forceps	Chill forceps on dry ice for ≥ **30 min**
Chilled beaker	In a fume hood, pour **200 ml** isopentane into a polypropylene beaker and place the beaker on dry ice for ≥ **30 min**

Confirm	
Item	**Preparation & Handling**
Cryomold	The crymold used for embedding should be of appropriate size to fit the tissue sample.

a. Label an appropriately sized cryomold to mark the orientation of the tissue.

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

OCT Before Freezing



Frozen OCT

b. Fill the cryomold with chilled OCT without introducing bubbles.

c. Place the cryomold on a piece of aluminum foil on dry ice.

d. Using chilled forceps, place the frozen tissue into the OCT, covering any exposed surfaces with additional OCT and noting the tissue orientation. Confirm there are no bubbles, especially near the tissue.

e. Using chilled forceps, immediately transfer the cryomold containing tissue and OCT to the isopentane for **1 min**.

f. Transfer cryomold onto a piece of aluminum foil on dry ice for **30 min**.

STOP

g. Store the OCT embedded tissue block in an airtight sealed container at –

80°C for **long-term** storage or immediately proceed to Cryosectioning & Section Placement.

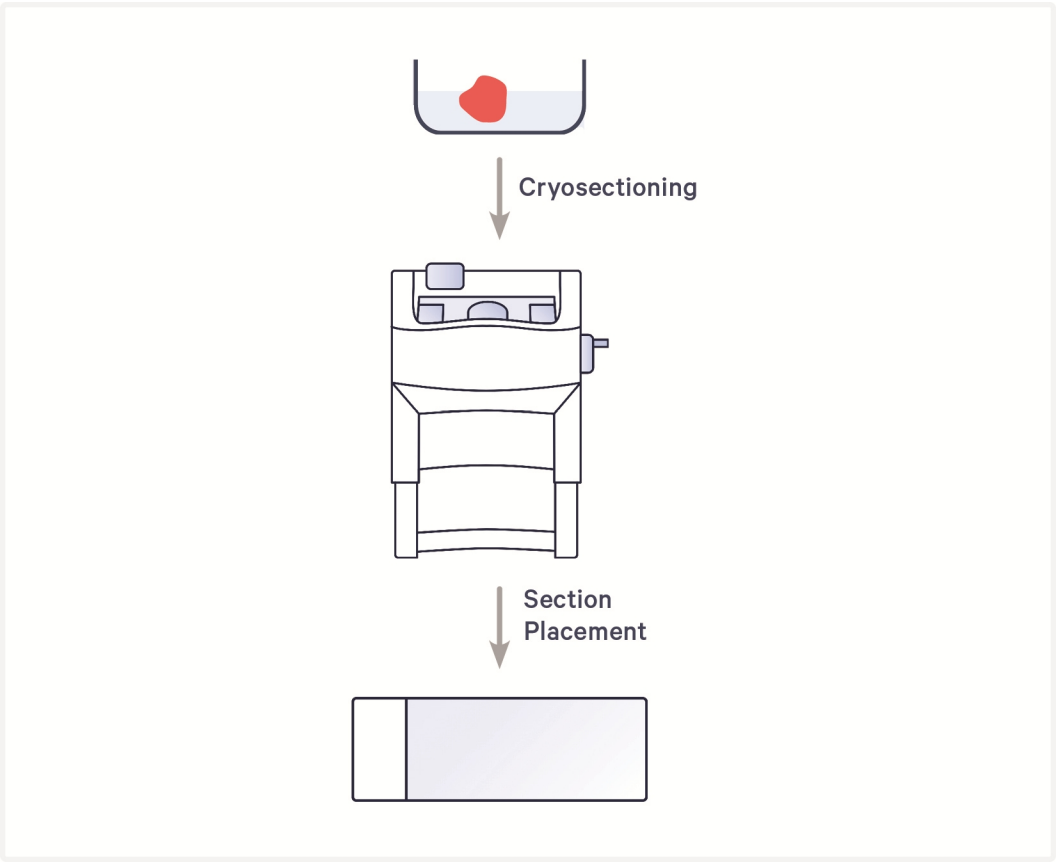
Failure to use a sealed container for storage may dehydrate and damage the tissue.

2. Cryosectioning & Quality Assessment

Overview

This section provides guidance on choosing a quality assessment method and cryosectioning of the OCT embedded tissue and placement of a tissue section onto a blank slide for DAPI and H&E Staining.

Xenium slides are not used for the quality assessment. Sectioning and placement on Xenium slides for the full workflow is described in step 4.



Choose a Quality Assessment Method

Choose one or more quality assessment methods from the following options. Though quality assessment is optional, 10x Genomics strongly recommends performing DAPI and H&E quality assessment on one or more serial sections prior to starting the Xenium assay. Quality assessment is performed after sections are placed onto blank slides.

DAPI Staining

DAPI is a nuclear stain that enables assessment of nuclei quality, a predictor of assay success. However, good nuclei quality is not an absolute determinant of assay success.

H&E Staining

H&E staining allows for observation of tissue damage and artifacts that may result from poor tissue handling, sectioning, or fixation. These artifacts are correlated with poor assay outcomes. However, identification of artifacts may require histology expertise. Additionally, blood in the H&E image may result in high autofluorescence. HIgh autofluorescence does not necessarily correlate with poor assay outcomes.

Cryosectioning

OCT embedded tissue blocks are removed from the **–80°C** storage and cryosectioned in a cryostat to generate appropriately sized sections for blank slides while keeping the samples frozen.

Cryostat Chamber Specifications

This protocol describes the use of a Cryostar NX70 Cryostat with specific capabilities. Alternatively, use a different cryostat with the following features.

Function	Notes
Main Cryochamber	Maintains stable temperatures from –10°C to –20°C
Cryostat Blade	Separate and adjustable temperature control Maintains stable temperatures from –35°C to –5°C
Specimen Head	Separate and adjustable temperature control Maintains stable temperatures from –50°C to +10°C X-axis and Y-axis adjustment
Blade Holder Base	Adjustable cutting angle Adjustable blade position
Cryobar	Rapid cooling

Section Placement

The tissue section is placed on a blank slide.

Fixation & Quality Assessment

Tissue sections are fixed and stained to allow for inspection. First, tissue sections are stained with a DAPI solution. Perform DAPI staining according to

any preferred protocol. Stained tissue sections are then coverslipped and imaged. Tissue sections with bright, clear nuclei are considered good quality and will likely generate good data with the Xenium workflow. Next, the same tissue section has their coverslip removed and is stained with Hematoxylin and Eosin (H&E) solutions. Perform H&E staining according to any preferred protocol. A fixation and H&E protocol are provided in the Appendix. Stained tissue sections are then coverslipped and ready to image. Proceed immediately to imaging following the H&E Staining workflow. Review the H&E image thoroughly to assess tissue quality. Inspect tissue morphology for tissue processing and sectioning artifacts that may contribute to poor assay performance. Refer to 2.4 H&E Quality Assessment on page 42 and Troubleshooting on page 63 for more guidance. If quality is satisfactory, proceed with 3. Cryosectioning & Practicing Section Placement on page 44 if additional sectioning practice is needed or proceed to 4.2 Section Placement on Xenium slides on page 51.

2.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Obtain				
☐	Blank Slides	-	Slides should be chilled to cryostat temperature for **≥30 min**. Warm slides will lead to quick melting of the sections.	Ambient
☐	Slide Mailer	-	Slide mailer should be chilled to cryostat temperature for **≥30 min**.	Ambient
☐	OCT-embeded Tissue Block	-	OCT embedded tissue block stored at **–80°C** must be equilibrated to cryostat chamber temperature for **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.	–80°C
☐	Specimen Chuck	-	Specimen chuck should be chilled to cryostat temperature for **≥5 min**.	Ambient
☐	Cryostat	-	Turn cryostat on to chill chamber. Recommended sectioning temperature is **–20°C** for cryostat blade and **–10°C** for the specimen head. Follow manufacturer's manual for detailed operations.	Ambient
☐	Razor Blade *(optional, if scoring)*	-	Chill to cryostat temperature.	Ambient
☐	Anti-roll Plate *(optional)*	-	Anti-roll plate prevents rolling of tissue sections. Chill to cryostat temperature for **≥30 min**. Warm anti-roll plate will damage tissue during sectioning. 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.	Ambient

2.1 Cryosectioning

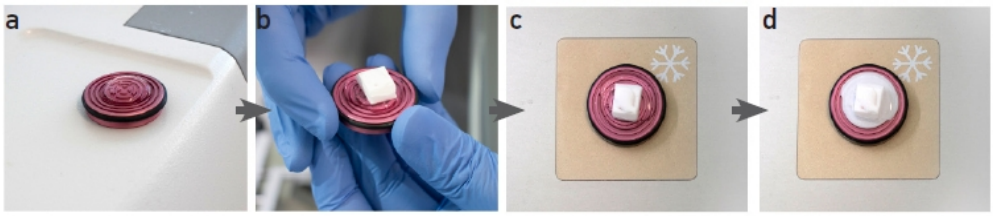
Mount OCT Embedded Tissue Block on the Specimen Stage

a. Fill the specimen stage (chuck) with OCT.

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

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

d. Allow the OCT and the tissue block to freeze and adhere to the specimen stage for **5 min**.



Remove Excess OCT by Cryosectioning

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

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

c. Continue sectioning until the tissue is visible.

Tissue Scoring

- Large tissue samples can be scored during sectioning to generate smaller samples to fit the Sample Area. To score, make a shallow incision (~1 mm deep) on the cutting surface of the tissue with a pre-cooled razor blade. The incision should be shallow. A deep incision may lead to tissue damage and disintegration. Once the tissue has been scored, extra care must be taken during sectioning and section handling.
- Example: To examine a specific region within one hemisphere of the mouse brain, scoring can be done by making a ~1 mm shallow incision at the midline of the brain.

Tissue Scoring

2.2 Tissue Sectioning

Confirm	
Item	**Preparation & Handling**
Section thickness setting	Recommended section thickness is **10 µm**.
Anti-roll plate is in place (*optional*)	Anti-roll plate prevents rolling of tissue sections. Chill to cryostat temperature for **≥30 min**. Warm anti-roll plate will damage tissue during sectioning. 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.

Position of Anti-roll Plate

a. Acquire a tissue section and carefully flatten it out by gently touching the surrounding OCT with cryostat brushes.

If re-sectioning a used tissue block:

- For tissues prone to freeze/thaw damage, such as mouse brain, cut and discard ~7 sections with thickness set to 10 µm prior to collecting first section.
- For other tissues, cut and discard ~5 sections with thickness set to 10 µm prior to collecting first section.

b. Adhere tissue section to slide by gently touching the tissue section to the blank slide.

c. Immediately place a finger on the backside of the slide where the tissue section was placed to allow section to adhere to the slide.

d. Apply OCT to the exposed surface of the block for storage.

e. Prior to proceeding to DAPI and H&E quality assessments, sections should be fixed. A fixation protocol is provided in the Appendix.

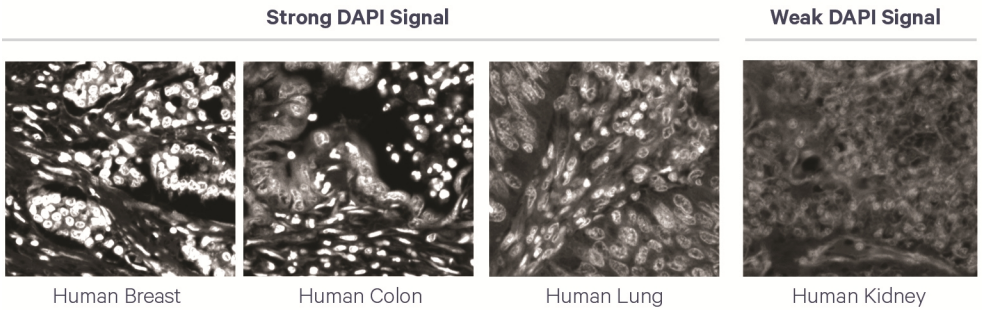
f. After fixation, proceed to DAPI Staining.

2.3 DAPI 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 suitable for the Xenium 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, proceed to for placement of tissue sections on Xenium slides or proceed with additional quality assessment methods.

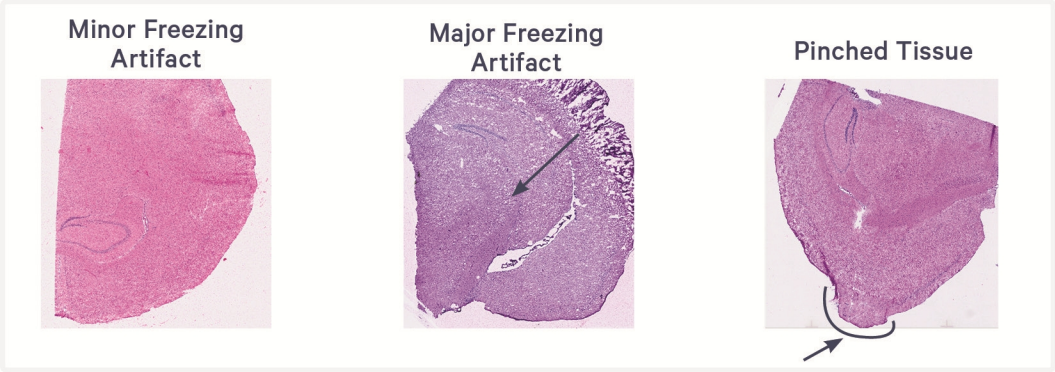


2.4 H&E Quality Assessment

Perform H&E staining according to any preferred protocol. An H&E protocol is provided in the Appendix. Review the H&E image thoroughly to assess tissue quality and select area(s) of interest. If scoring the block is necessary, refer to 2.1 Cryosectioning on page 39.

Ensure that the tissue section is free from tissue processing and sectioning artifacts as shown in the images below and in Troubleshooting on page 63. Tissue processing artifacts may include improper snap freezing, squeeze/crush artifacts, ice crystal artifacts, and hemorrhaging. If imaging reveals satisfactory tissue morphology, proceed with 3. Cryosectioning & Practicing Section Placement on page 44 if additional sectioning practice is needed or 4.2 Section

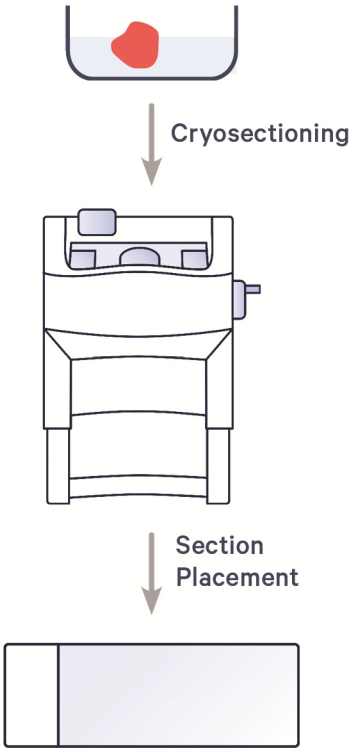
Placement on Xenium slides on page 51 for placement of tissue sections on Xenium slides.



3. Cryosectioning & Practicing Section Placement

Overview

After examining the H&E tissue section, practice sectioning the block onto blank slides in preparation for the section placement on the Xenium slide. This practice step is optional.



Cryosectioning

OCT embedded tissue blocks are removed from the **–80°C** storage and cryosectioned in a cryostat to generate appropriately sized sections for slides while keeping the samples frozen. Cryosection as described in step 2.

Section Placement

Draw the template shown in Xenium Slide Template on page 16 onto a blank slide to simulate the Sample Area on the Xenium slide. The Sample Area can

accommodate two tissue sections of 1 cm^2 each, or more if the sections are smaller. Perform section placement as described in step 2.

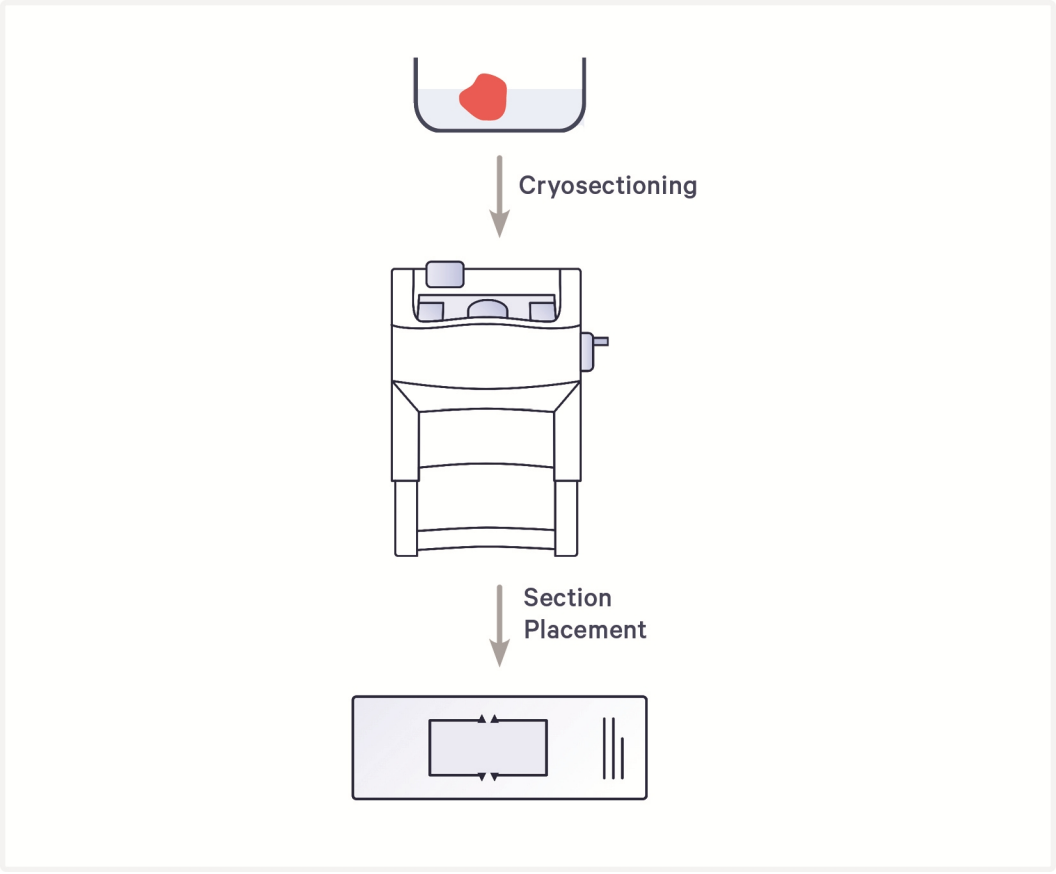
3.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Obtain				
☐	Blank Slides	-	Slides should be chilled to cryostat temperature for **≥30 min**. Warm slides will lead to quick melting of the sections.	Ambient
☐	Slide Mailer	-	Slide mailer should be chilled to cryostat temperature for **≥30 min**.	Ambient
☐	OCT-embeded Tissue Block	-	OCT embedded tissue block stored at **–80°C** must be equilibrated to cryostat chamber temperature for **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.	–80°C
☐	Specimen Chuck	-	Specimen chuck should be chilled to cryostat temperature for **≥5 min**.	Ambient
☐	Cryostat	-	Turn cryostat on to chill chamber. Recommended sectioning temperature is **–20°C** for cryostat blade and **–10°C** for the specimen head. Follow manufacturer's manual for detailed operations.	Ambient
☐	Razor Blade *(optional, if scoring)*	-	Chill to cryostat temperature.	Ambient
☐	Anti-roll Plate *(optional)*	-	Anti-roll plate prevents rolling of tissue sections. Chill to cryostat temperature for **≥30 min**. Warm anti-roll plate will damage tissue during sectioning. 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.	Ambient

4. Cryosectioning & Section Placement

Overview

This section provides guidance the placement of tissue sections on Xenium slides.



Cryosectioning

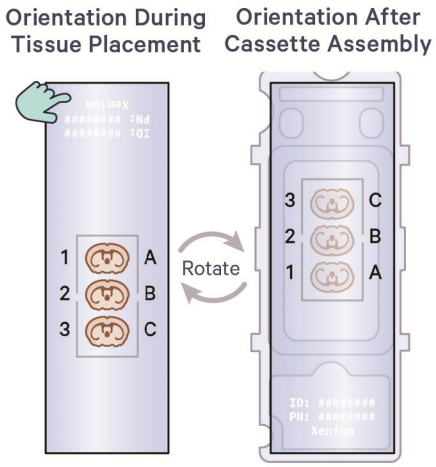
OCT embedded tissue blocks are removed from the **–80°C** storage and cryosectioned in a cryostat to generate appropriately sized sections for Xenium slides while keeping the samples frozen. Section as described in step 2.

Section Placement

Prior to section placement, a morphology check is performed to confirm that the sections are free of artifacts. Sections are placed within the frames of the

Sample Area on the Xenium slide. The Sample Area can accommodate two tissue sections of 1 cm^2 each, or more if the sections are smaller.

Slides are assembled into the Xenium Cassette with the etched label oriented towards the bottom. If holding the Xenium slide by the etched label for section placement, the slide will be rotated during cassette assembly and placement on the Xenium Analyzer. This results in an image that is rotated compared to initial section placement (refer to image below).

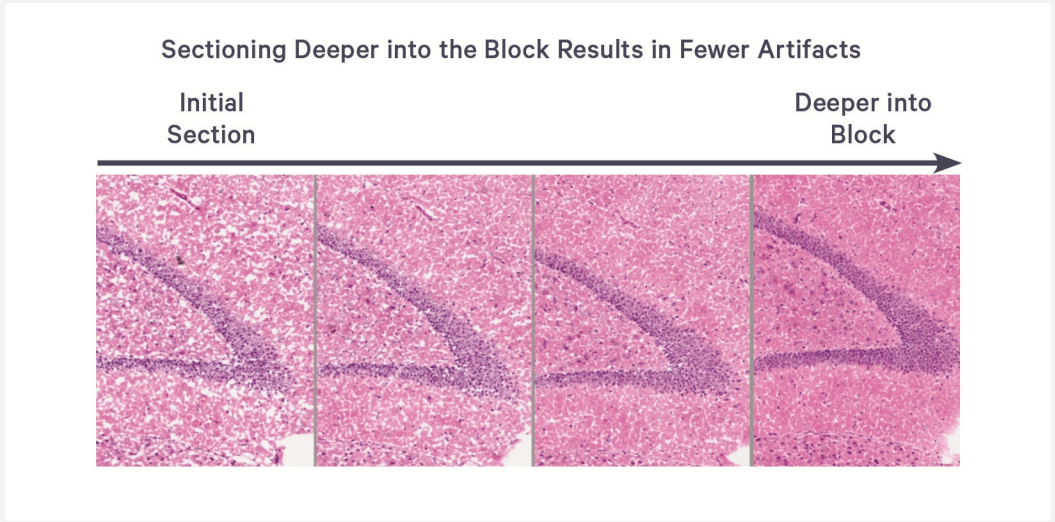


4.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Obtain				
☐	Xenium Slide	-	Slides should be chilled to cryostat temperature for **≥30 min**. Warm slides will lead to quick melting of the sections.	–20°C
☐	Blank Slide	-	Slides should be chilled to cryostat temperature for **≥30 min**. Warm slides will lead to quick melting of the sections.	Ambient
☐	Slide Mailer	-	Slide mailer should be chilled to cryostat temperature for **≥30 min**.	Ambient
☐	OCT-embeded Tissue Block	-	OCT embedded tissue block stored at **–80°C** must be equilibrated to cryostat chamber temperature for **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.	–80°C
☐	Specimen Chuck	-	Specimen chuck should be chilled to cryostat temperature for **≥5 min**.	Ambient
☐	Cryostat	-	Turn cryostat on to chill chamber. Recommended sectioning temperature is **–20°C** for cryostat blade and **–10°C** for the specimen head. Follow manufacturer's manual for detailed operations.	Ambient
☐	Razor Blade *(optional, if scoring)*	-	Chill to cryostat tempreature.	Ambient
☐	Anti-roll Plate *(optional)*	-	Anti-roll plate prevents rolling of tissue sections. Chill to cryostat temperature for **≥30 min**. Warm anti-roll plate will damage tissue during sectioning. 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.	Ambient
☐	Hematoxylin	-	-	Ambient

4.1 Morphology Check

- Repeated sectioning of tissue blocks may result in damage due to freezing artifacts. Prior to sectioning onto Xenium slides, tissue morphology should be checked for any undesired artifacts. This morphology check will determine if the block needs to be sectioned deeper to avoid artifacts.
- In the example below, the first section removed from the block displays "swiss cheese" morphology, with small spaces between each cell. Subsequent sections show fewer artifacts and are better sections for downstream analysis.



a. Acquire a tissue section as described in step 2 on a blank slide.

b. Remove slide from the cryostat and immerse in a slide mailer filled with hematoxylin for **30 sec**.

c. Immerse in a slide mailer filled with water for **20 sec**.

d. Wipe the back of the slide with a laboratory wipe.

e. Inspect tissue morphology under a microscope.

- If morphology is ideal, cut and discard 3 sections with the section thickness set to 10 µm. Proceed to step 4.2 Section placement on Xenium slides.
- If morphology is not ideal, repeat quality check with additional trimming of the block as needed.

4.2 Section Placement on Xenium slides

Confirm		
Item	**Preparation & Handling**	
Section thickness setting	Recommended section thickness is **10 µm**.	
Anti-roll plate is in place (optional)	Anti-roll plate prevents rolling of tissue sections. Chill to cryostat temperature for **≥30 min**. Warm anti-roll plate will damage tissue during sectioning. 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.	 Position of Anti-roll Plate
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.	

Optional: Before placing sections on Xenium slides, trace the Sample Area onto the back of the slide with a marker. Using an unsupported marker may compromise assay performance. See Xenium Slide Template on page 16 for Xenium slide layout.

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

b. Place the section within the Sample Area on the pre-equilibrated Xenium slide by gently touching the section with the active surface of the slide.

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

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

Ensure that the entire tissue section is fully adhered to the slide and the slide is inside the cryostat chamber throughout section placement. DO NOT remove the slide from the cryostat chamber at any point during

sectioning and tissue placement.

d. Immediately place the slide with tissue section on the cryobar to freeze the section. Continue transferring sections onto the Sample Area.

e. Transfer the slide containing tissue sections to a slide mailer pre-equilibrated in the cryostat.

f. Store slides at **–80°C** for up to **4 weeks** or immediately proceed to Xenium protocols.

- Store up to two slides in a sealed container. Ensure slides are separated by a space to prevent damage. If using an unsealed slide mailer, store in a secondary sealed container, such as a resealable bag.

- Maintain slides containing sections in a cold and low moisture environment. DO NOT expose slides to room temperature as the resulting condensation will cause tissue disintegration.

See General Xenium Slide Handling on page 14 for information on slide handling.

Slide Shipping

- If needed, slides containing tissue sections can be shipped. See Appendix for detailed Shipping Guidelines.

Leftover Tissue Block Storage

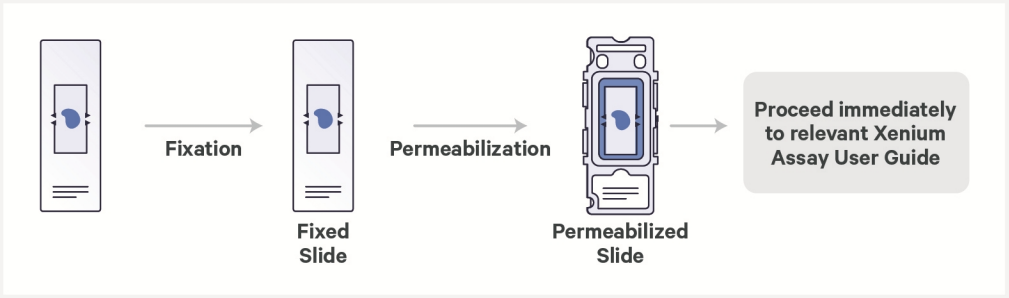
- Remove leftover tissue block attached to the specimen stage from the cryostat's specimen head and place onto cryobar.
- Cover the exposed tissue with a thin layer of chilled OCT and allow to freeze.
- To separate the frozen tissue block from the stage, lift the tissue block and the stage from the cryobar and lightly warm the stage with hands or an aluminum block at room temperature

  DO NOT let the block and tissue to fully melt, as this will severely damage the tissue. Separation of the tissue block from the specimen stage is optional. The frozen tissue block can be stored attached to the specimen stage in a sealed container at –80°C.
- Immediately place the tissue block in dry ice. Ensure that the melted areas have refrozen.
- Store in a sealed container at **–80°C** for **long-term** storage.

5. Fixation & Permeabilization

Overview

This chapter provides guidance on fixation and permeabilization of Xenium slides containing fresh frozen tissue sections.



Protocol Steps & Timing

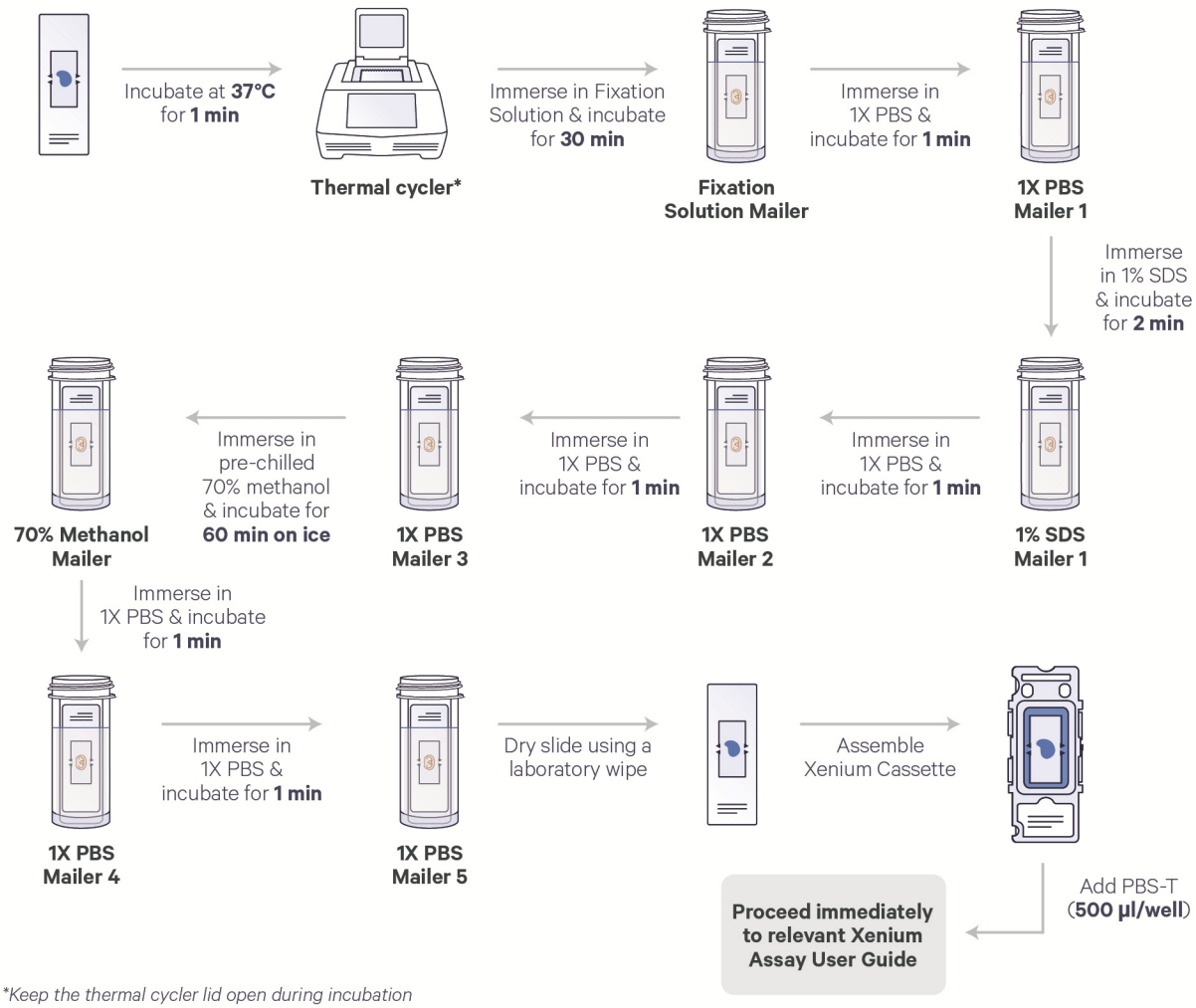
~2.5 hours

Steps		Timing	Stop & Store
Step 1 – Fixation & Permeabilization			
1.1	Preparation - Buffers	30 min	
1.2	Slide Preparation	5 min	
1.3	Fixation	30 min	
1.4	Permeabilization	65 min	
1.5	Cassette Assembly	10 min	

Note there are no safe stopping points during this workflow.

Protocol Overview

Fixation & Permeabilization ~100 min



**Keep the thermal cycler lid open during incubation*

5.0 Get Started

Each 10x Genomics reagent tube is good for two Xenium Slides.

Fixation & Permeabilization Items			10x PN	Preparation & Handling	Storage
Obtain					
☐		Nuclease-free Water	-	-	Ambient
☐		10X PBS	-	-	Ambient
☐		Formaldehyde or Paraformaldehyde	-	-	Ambient
☐		10% SDS	-	-	Ambient
☐		Methanol	-	-	Ambient
☐		10% Tween-20	-	-	Ambient
☐		Slide Mailers	-	-	Ambient
☐		Forceps	-	-	Ambient
☐		Xenium Slides (2 pack) with fresh frozen tissue sections	3000941	Prepared according to Xenium In Situ for Fresh Frozen - Tissue Preparation Guide (CG000579).	-80°C
	Xenium v1	Thermocycler Adaptor Xenium Cassette v1	3000954 3000951	See Tips & Best Practices	Ambient
	OR				
☐	Xenium Prime	Thermocycler Adaptor v2 Xenium Cassette Top v2 Xenium Cassette Bottom v2	3002207 3002205 3002223	See Tips & Best Practices	Ambient

The instructions apply to both Xenium v1 and Xenium Prime assays. The consumables are referred to generically without always citing the version suffix. Based on the assay choice, always ensure that either Xenium v1 or Xenium Prime specific reagents and consumables are used.

5.1 Preparation - Buffers

Prepare all buffers fresh according to the tables below before retrieving tissue sections from **-80°C.**

TIPS *Prepare buffers in appropriate sized conical tube or bottle and transfer carefully to corresponding slide mailer.*

a. Prepare 1X PBS. Add reagents in the order listed. Invert gently to mix. Maintain at room temperature. This volume of 1X PBS is sufficient for washes in all subsequent steps of this Demonstrated Protocol.

1X PBS				
Items		**Stock**	**Final**	**Total Amount (ml)**
	Nuclease-free water	-	-	63.0
	RNase free PBS, pH 7.4	10X	1X	7.0
	Total	**-**	**-**	**70.0**

b. Using 1X PBS from step 1.1a, prepare Fixation Solution using either Formaldehyde or Paraformaldehyde according to the appropriate table below. Add reagents in the order listed. Invert gently to mix. Maintain at room temperature.

Fixation Solution *(using Formaldehyde)*				
Items		**Stock**	**Final**	**Total Amount (ml)**
	1X PBS	-	-	9.0
	Formaldehyde	37%	3.7%	1.0
	Total	**-**	**-**	**10.0**

OR

Fixation Solution *(using Paraformaldehyde)*				
Items		**Stock**	**Final**	**Total Amount (ml)**
	1X PBS	-	-	7.5
	Paraformaldehyde	16%	4%	2.5
	Total	**-**	**-**	**10.0**

c. Prepare 1% Sodium dodecyl sulfate (SDS). Verify no precipitate in SDS before use. Add reagents in the order listed. Invert gently to mix. Maintain at room temperature.

1% SDS				
Items		**Stock**	**Final**	**Total Amount (ml)**
	Nuclease-free water	-	-	9.0
	SDS *(verify no precipitate)*	10%	1%	1.0
	Total	**-**	**-**	**10.0**

d. Prepare 70% Methanol. Add reagents in the order listed. Invert gently to mix.

Pre-chill 70% Methanol on ice for **30 min** before starting Fixation protocol. Cap mailer and submerge in the ice up to the lower part of the pink cap.

70% Methanol				
Items		**Stock**	**Final**	**Total Amount (ml)**
	Methanol	100%	70%	7.0
	Nuclease-free water	-	-	3.0
	Total	**-**	**-**	**10.0**

e. Using 1X PBS from step 1.1a, prepare PBS-Tween (PBS-T). Add reagents in the order listed. Invert gently to mix. Maintain at room temperature. This volume of PBS-T is sufficient for washes in all subsequent steps of this Demonstrated Protocol.

PBS-T				
Items		**Stock**	**Final**	**Total Amount (µl)**
	1X PBS	-	-	1,990
	Tween-20	10%	0.05%	10
	Total	**-**	**-**	**2,000**

TIPS *Pipette Tween-20 slowly to fully dispense from pipette tip and to avoid formation of air bubbles.*

f. Prepare eight total slide mailers for fixation.

For Fixation & Permeabilization		
Items (from 1.1a-1.1d)		**Preparation & Handling**
	Fixation Solution	Label one slide mailer as Fixation Solution Mailer. Dispense 10 ml Fixation Solution.
	1X PBS	Label five slide mailers as 1X PBS Mailer 1, 1X PBS Mailer 2, 1X PBS Mailer 3, 1X PBS Mailer 4, and 1X PBS Mailer 5. Dispense 10 ml 1X PBS in each.
	1% SDS	Label one slide mailer as 1% SDS Mailer. Dispense 10 ml 1% SDS solution.
	70% Methanol	Label one slide mailer as 70% Methanol Mailer. Dispense 10 ml 70% Methanol. Pre-chill 70% Methanol on ice for 30 min.

5.2 Slide Preparation

The instructions apply to both Xenium v1 and Xenium Prime assays. The consumables are referred to generically without always citing the version suffix. Based on the assay choice, always ensure that either Xenium v1 or Xenium Prime specific reagents and consumables are used.

a. Place Xenium Thermocycler Adaptor in thermal cycler set to incubate at **37°C.** DO NOT close the lid.

Ready the Fixation Solution Mailer and a timer set to 1 min, which are needed in the following steps.

b. Prepare an ice bucket of dry ice.

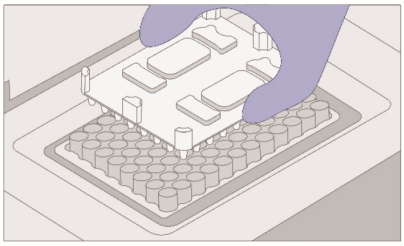
c. Remove slide mailer containing stored fresh frozen tissue slides from **-80°C** and bury into the dry ice.

TIPS

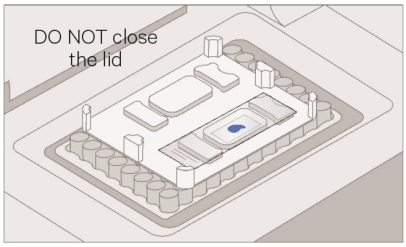
Alternatively, submerge an uncapped empty slide mailer in dry ice and incubate for **5 min**. *Remove slides from* **-80°C** *storage with a pair of forceps and immediately place in pre-chilled empty slide mailer on dry ice.*

d. Using a pair of slide forceps, move slides from dry ice to the **37°C** pre-heated thermal cycler for **1 min**. Place slides on the Thermocycler Adaptor with the tissue side facing up. Ensure the Sample Area is aligned with the corresponding area on the Thermocycler Adaptor. DO NOT close the lid.

Incubation in a Thermal Cycler
Xenium Thermocycler Adaptor v2 shown for illustrative purposes



Place Thermocycler Adaptor



Incubate Slide for 1 min at 37°C

5.3 Fixation

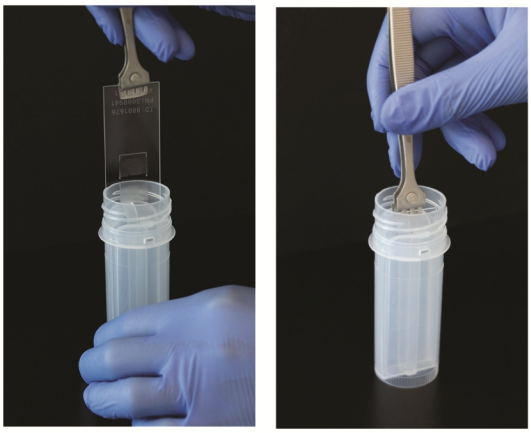
The instructions apply to both Xenium v1 and Xenium Prime assays. The consumables are referred to generically without always citing the version suffix. Based on the assay choice, always ensure that either Xenium v1 or Xenium Prime specific reagents and consumables are used.

a. **Immediately** remove slide from thermal cycler following incubation. Gently immerse slide in the Fixation Solution Mailer using slide forceps and incubate for **30 min** at **room temperature**.

Formaldehyde and Paraformaldehyde should be handled in a biosafety hood due to their hazardous nature. Transfer slides immediately to Fixation Solution following removal from thermal cycler to prevent formation of freezing artifacts on the slides.

See Tips & Best Practices for guidance on properly immersing slides into mailers.

Slide Immersion



TIPS

Ensure 70% Methanol Mailer is pre-chilled on ice before proceeding to next step.

5.4 Permeabilization

TIPS *Start thawing reagents for Probe Hybridization during Permeabilization as indicated in the Xenium In Situ Gene Expression - Probe Hybridization, Ligation & Amplification User Guide (CG000582).*

a. Gently immerse slide in the 1X PBS Mailer 1 and incubate for **1 min** at **room temperature**.

Remove any marker annotations on slide using a lint-free laboratory wipe and 100% Ethanol.

b. Gently immerse slide in the 1% SDS Mailer and incubate for **2 min** at **room temperature**.

c. Gently immerse slide in the 1X PBS Mailer 2 and incubate for **1 min** at **room temperature**.

d. Gently immerse slide in the 1X PBS Mailer 3 and incubate for **1 min** at **room temperature**.

e. Gently immerse slide in the pre-chilled 70% Methanol Mailer and incubate for **60 min** on **ice**. Cap mailer and fully submerge in the ice up to the lower part of the pink cap.

Methanol Incubation

f. Gently immerse slide in the 1X PBS Mailer 4 for **1 min** at **room temperature**.

g. Gently immerse slide in the 1X PBS Mailer 5 for **1 min** at **room temperature**.

h. Remove slide from the 1X PBS Mailer 5.

5.5 Cassette Assembly

The instructions apply to both Xenium v1 and Xenium Prime assays. The consumables are referred to generically without always citing the version suffix. Based on the assay choice, always ensure that either Xenium v1 or Xenium Prime specific reagents and consumables are used.

a. Remove any remaining 1X PBS from the slide using a lint-free laboratory wipe. Dry back of slide and front of slide outside of Sample Area completely without touching or damaging the tissue sections. Place the slide in the cassette.

TIPS

Consult the Xenium Quick Reference Card for Slide Cassette Assembly (CG000623) for guidance on Xenium Cassette Assembly. Work quickly to avoid drying out of tissue sections.

b. Add **500 µl** 1X PBS-T.

Optional: photograph the slide against a black background. This image can be used for comparison purposes to identify tissue detachment downstream in the workflow. Work quickly as this is not a safe stopping point. See Troubleshooting on page 1 for more details.

c. Proceed **immediately** to the relevant user guide:

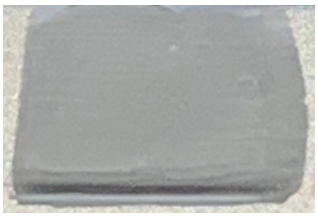
- Xenium In Situ Gene Expression (CG000582)
- Xenium In Situ Gene Expression with Cell Segmentation Staining (CG000749)
- Xenium Prime In Situ Gene Expression with optional Cell Segmentation Staining (CG000760)

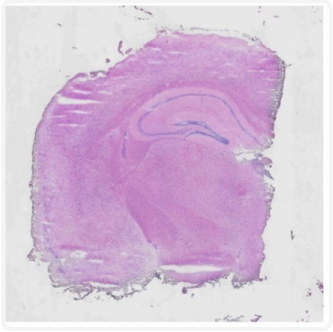
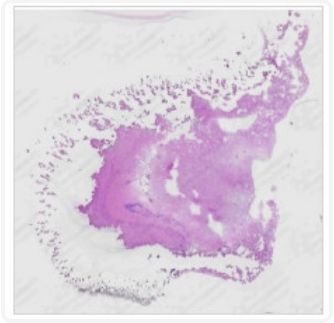
Assembled Xenium Cassette

Xenium Cassette

Xenium Cassette v2

Troubleshooting

Impact of Cryostat Specimen Head Temperatures on Tissue Tearing			
-10°C	-14°C	-20°C	-30°C
			
Normal Section			Tissue section has significant tearing.

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.

Tissue Detachment on Xenium Slides

Tissue sections may detach from Xenium slides during on-slide workflows. Tissue adhesion to the slide is impacted both by tissue processing and tissue quality.

Careful tissue preparation is critical for adhesion in on-slide workflows. Consult Xenium In Situ for Fresh Frozen - Tissue Preparation Guide (Document CG000578) for tissue QC and sectioning best practices. Below are some additional best practices for minimizing detachment during on-slide workflows:

- Do not pipette directly onto the tissue.
- Gently add and remove reagents from the well. Forceful addition or removal of reagents can agitate tissue and lead to detachment.
- Avoid touching in and around the Sample Area of the Xenium slide.
- Work quickly and carefully during reagent addition and removal.

In addition to following best practices, it is possible to monitor section adhesion on Xenium slides throughout the workflow. Taking a photograph of the slide at the beginning of the on-slide workflow and comparing with post-assay workflow images can help identify whether tissue shape has changed significantly, an indication of detachment. Steps when slide photos can be taken are noted in the protocol. These QC images can be compared with the DAPI overview scan as part of the Web Summary file to see whether tissue morphology has changed in the workflow.

If tissue detachment occurs, send pictures to support@10xgenomics.com for further assistance.

Cassette Assembly Failure

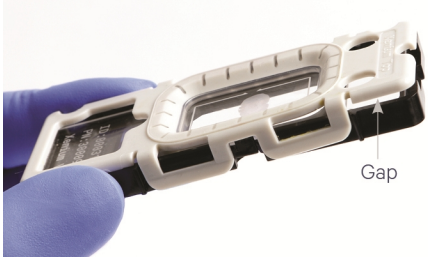
Incorrect assembly of the Xenium Cassette with a Xenium slide can negatively impact assay performance. Always dry the front and the back of the slide completely using a lint-free laboratory wipe while avoiding touching or damaging of the tissue sections. Inspect the slide carefully to ensure it is seated fully within the cassette before assembly. Additionally, inspect gasket before assembly to ensure it is not damaged or leaking.

Example scenarios that may indicate incorrect Xenium Cassette assembly are described below:

- If a gap appears between the two halves of the cassette after assembly.
- If the cassette does not click shut or appears domed after assembly.
- If the Xenium Thermocycler Adaptor is wet following removal from the thermal cycler, indicating reagent leakage from the cassette.

If the cassette is incorrectly assembled, disassemble and reassemble the cassette as instructed in Tips & Best Practices.

Incorrect cassette assembly as indicated by a gap between the two halves of the cassette

Correct cassette assembly	Incorrect cassette assembly
	

Appendix

Fixation and Quality Assessment

Tissue sections should be fixed before quality assessment (DAPI and H&E staining)

Fixation Items		10x PN	Preparation & Handling	Storage
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	10X PBS	-	-	Ambient
☐	Formaldehyde or Paraformaldehyde	-	-	Ambient
☐	Slide Mailers	-	-	Ambient
☐	Forceps	-	-	Ambient

DAPI Staining Items		10x PN	Preparation & Handling	Storage
Obtain				
☐	DAPI Solution	-	Use any preferred DAPI stock solution. Dilute DAPI stock solution to 5 µg/ml in PBS-T to generate DAPI solution. 1.1 ml of DAPI solution is enough for two slides.	Ambient
☐	10X PBS	-	Prepare 1X PBS using nuclease-free water.	Ambient
☐	Slide Mailer	-	-	Ambient
☐	Glycerol Mounting Medium	-	The dilution below is not necessary if stock glycerol is already at 85%. Invert to mix. Briefly centrifuge to remove bubbles.	

Glycerol Mounting Medium ***1 slide = 1 Tissue Slide***	**Stock**	**Final**	**1 Slide (µl)**	**2 Slides +15% (µl)**	**4 Slides +15% (µl)**
Glycerol	100%	85%	127.5	293.3	586.5
Nuclease-free Water	-	-	22.5	51.7	103.5
Total	**-**	**-**	**150.0**	**345.0**	**690.0**

H&E Staining Items		10x PN	Preparation & Handling	Storage
Obtain				
☐	Hematoxylin	-	-	Ambient
☐	Eosin	-	-	Ambient
☐	Bluing Reagent	-	-	Ambient
☐	Mounting Media	-	-	Ambient
☐	Xylene	-	-	Ambient
☐	Ethanol	-	Prepare Ethanol dilutions using Milli-Q water.	Ambient
☐	Forceps	-	-	Ambient
☐	Coplin Jars/Staining Dishes	-	-	Ambient
☐	Milli-Q Water	-	-	Ambient

Preparation - Buffers

Prepare all buffers fresh according to the tables below before retrieving tissue sections from **-80°C.**

TIPS *Prepare buffers in appropriate sized conical tube or bottle and transfer carefully to corresponding slide mailer.*

a. Prepare 1X PBS. Add reagents in the order listed. Invert gently to mix. Maintain at room temperature. This volume of 1X PBS is sufficient for washes in all subsequent steps of this Demonstrated Protocol.

1X PBS				
Items		**Stock**	**Final**	**Total Amount (ml)**
	Nuclease-free water	-	-	9
	RNase free PBS, pH 7.4	10X	1X	1
	Total	**-**	**-**	**10**

b. Using 1X PBS from step 1.1a, prepare Fixation Solution using either Formaldehyde or Paraformaldehyde according to the appropriate table below. Add reagents in the order listed. Invert gently to mix. Maintain at

room temperature.

Fixation Solution *(using Formaldehyde)*				
Items		**Stock**	**Final**	**Total Amount (ml)**
	1X PBS	-	-	9.0
	Formaldehyde	37%	3.7%	1.0
	Total	**-**	**-**	**10.0**

OR

Fixation Solution *(using Paraformaldehyde)*				
Items		**Stock**	**Final**	**Total Amount (ml)**
	1X PBS	-	-	7.5
	Paraformaldehyde	16%	4%	2.5
	Total	**-**	**-**	**10.0**

c. Dispense 10 ml Fixation Solution into one slide mailer.

Slide Preparation

The instructions apply to both Xenium v1 and Xenium Prime assays. The consumables are referred to generically without always citing the version suffix. Based on the assay choice, always ensure that either Xenium v1 or Xenium Prime specific reagents and consumables are used.

a. Place Xenium Thermocycler Adaptor in thermal cycler set to incubate at **37°C.** DO NOT close the lid.

Ready the Fixation Solution Mailer and a timer set to 1 min, which are needed in the following steps.

b. Prepare an ice bucket of dry ice.

c. Remove slide mailer containing stored fresh frozen tissue slides from **-80°C** and bury into the dry ice.

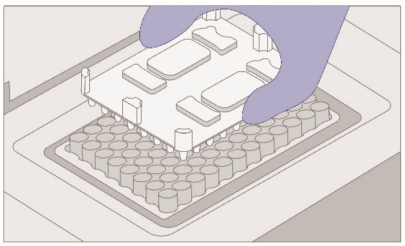
TIPS

Alternatively, submerge an uncapped empty slide mailer in dry ice and incubate for **5 min**. *Remove slides from* **-80°C** *storage with a pair of forceps and immediately place in pre-chilled empty slide mailer on dry ice.*

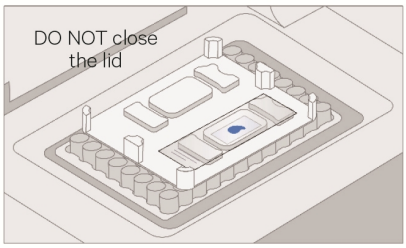
d. Using a pair of slide forceps, move slides from dry ice to the **37°C** pre-heated thermal cycler for **1 min**. Place slides on the Thermocycler Adaptor with the tissue side facing up. Ensure the Sample Area is aligned with the

corresponding area on the Thermocycler Adaptor. DO NOT close the lid.

Incubation in a Thermal Cycler
Xenium Thermocycler Adaptor v2 shown for illustrative purposes



Place Thermocycler Adaptor



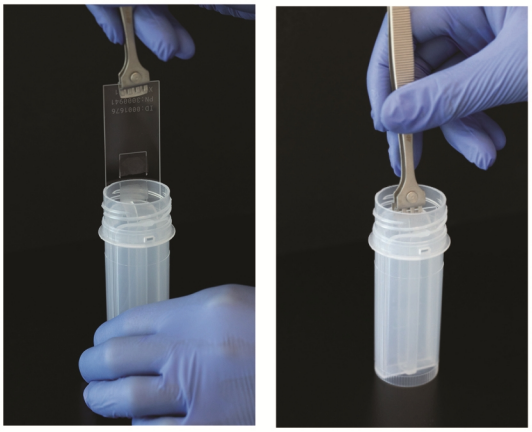
Incubate Slide for 1 min at 37°C

Fixation

a. **Immediately** remove slide from thermal cycler following incubation. Gently immerse slide in the Fixation Solution Mailer using slide forceps and incubate for **30 min** at **room temperature**.

Formaldehyde and Paraformaldehyde should be handled in a biosafety hood due to their hazardous nature. Transfer slides immediately to Fixation Solution following removal from thermal cycler to prevent formation of freezing artifacts on the slides.

Slide Immersion



b. Gently immerse slide in the 1X PBS Mailer 1 and incubate for **1 min** at **room temperature**.

Proceed directly to DAPI staining. Leave slide in PBS until DAPI reagents have been prepared.

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. Wipe excess liquid from the front and back of the slides without touching the tissue section.

Coverslipping

Before mounting the coverslip, ensure that the slide is dry. Moisture on the surface of the slide may result in faulty mounting. Wipe away any residual droplets with a lint-free laboratory wipe.

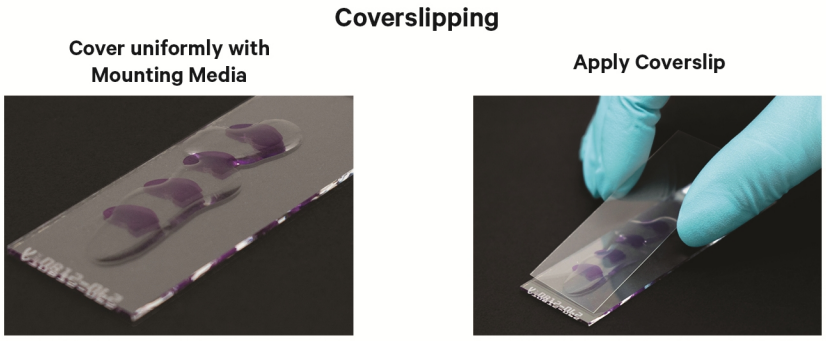
a. Place slide on a flat, clean, non-absorbent work surface.

b. Using a **wide-bore** pipette tip, add **150-200 µl** Glycerol Mounting Medium to uniformly cover all tissue sections on the slide.

c. Apply the coverslip at an angle on one end of the slide. Slowly lower the coverslip, without introducing bubbles. Allow mounting media to spread and settle.

d. If needed, remove any large excess of mounting media by carefully wicking away from the edge of the coverslip with a lint-free laboratory wipe. Be careful not to move coverslip and disturb the tissue.

e. Once coverslipping is complete, **immediately** proceed with imaging. See 2.3 DAPI Quality Assessment on page 42 for information on evaluating DAPI staining. Underexposure may result in inaccurate assessment of DAPI quality. Begin by overexposing samples, then reducing exposure until the resolution improves. If possible, process a control section in parallel from a block that has yielded good data.



Slides in images are representative

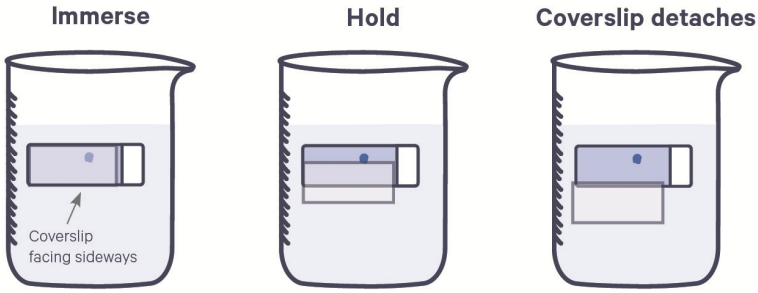
Coverslip Removal

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 the coverslipped surface fully sideways to prevent the coverslip from dragging across the 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 the slide up and down, shake forcibly, or manually move the coverslip.



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

e. Wipe the back of the slide with a laboratory wipe. Place on a flat, clean, nonabsorbent work surface and air dry for **5 min**.

f. Proceed with H&E Staining.

H&E Preparation

TIPS *Prepare buffers in appropriate sized conical tube or bottle and transfer carefully to corresponding coplin jar.*

a. Filter Hematoxylin & Eosin solutions using filter paper before starting H&E Staining protocol.

b. Prepare sixteen total coplin jars for H&E Staining steps.

For H&E Staining	
Items	**Preparation & Handling**
Hematoxylin Solution	Label one coplin jar as Hematoxylin Jar. Fill to capacity with Mayer's Hematoxylin Solution.
Bluing Solution	Label one coplin jar as Bluing Solution Jar. Fill to capacity with Bluing Solution.
70% Ethanol	Label one coplin jar as 70% Ethanol Jar. Fill to capacity with 70% ethanol.
95% Ethanol	Label three coplin jars as 95% Ethanol Jar 1, 2, and 3. Fill to capacity with 95% ethanol.
Eosin Solution	Label one coplin jar as Eosin Solution Jar. Fill to capacity with Eosin Solution.
100% Ethanol	Label two coplin jars as 100% Ethanol Jar 1 and 2. Fill to capacity with 100% Ethanol.
Xylene	Label two coplin jars as Xylene Jar 1 and 2. Fill to capacity with Xylene.
Milli-Q Water	Label five coplin jars as Milli-Q Water Jar 1, 2, 3, 4, and 5. Fill to capacity with Milli-Q Water.

TIPS *Alternatively, a slide staining dish can be used in place of a coplin jar. Adjust volumes accordingly. Use xylene-resistent dishes, gloves, and forceps during workflow.*

H&E Staining

H&E Staining steps should be performed in a fume hood due to the hazardous nature of xylene. Xylene jars should be covered at all times to prevent evaporation.

a. Gently immerse slide in the Milli-Q Water Jar 1 for **2 min** at **room temperature**.

TIPS *Water immersions may be performed in glass beakers containing Milli-Q water, if preferred.*

b. Gently immerse slide in the Hematoxylin Solution Jar for **20 min** at **room temperature**.

Substitution of Hematoxylin with a brand other than the recommended option may lead to tissue staining that is darker than anticipated following 20 min incubation.

c. Gently immerse slide in the Milli-Q Water Jar 2 for **1 min** at **room temperature**.

d. Gently immerse slide in the Milli-Q Water Jar 3 for **1 min** at **room temperature**.

e. Gently immerse slide in the Milli-Q Water Jar 4 for **1 min** at **room temperature**.

f. Gently immerse slide in the Bluing Solution Jar for **1 min** at **room temperature**.

g. Gently immerse slide in the Milli-Q Water Jar 5 for **1 min** at **room temperature**.

h. Gently immerse slide in the 70% Ethanol Jar for **3 min** at **room temperature**.

i. Gently immerse slide in the 95% Ethanol Jar 1 for **3 min** at **room temperature**.

j. Gently immerse slide in the Eosin Solution Jar for **2 min** at **room temperature**.

k. Gently immerse slide in the 95% Ethanol Jar 2 for **30 sec** at **room temperature**.

l. Gently immerse slide in the 95% Ethanol Jar 3 for **30 sec** at **room temperature**.

m. Gently immerse slide in the 100% Ethanol Jar 1 for **30 sec** at **room temperature**.

n. Gently immerse slide in the 100% Ethanol Jar 2 for **30 sec** at **room temperature**.

o. Gently immerse slide in the Xylene Jar 1 for **3 min** at **room temperature**.

p. Gently immerse slide in the Xylene Jar 2 for **3 min** at **room temperature**.

Coverslipping

Before mounting the coverslip, ensure that the slide is dry by gently flicking the slide to remove any droplets. Moisture on the surface of the slide may

result in faulty mounting. Wipe away any residual droplets with a lint-free laboratory wipe.

The mounting medium used for mounting H&E-stained sections is different than what was used to mount slides after DAPI staining. Ensure that that the appropriate mounting media is used.

a. Place slide on a flat, clean, non-absorbent work surface.

b. Using a **wide-bore** pipette tip, add **150-200 µl** mounting media to uniformly cover all tissue sections on the slide.

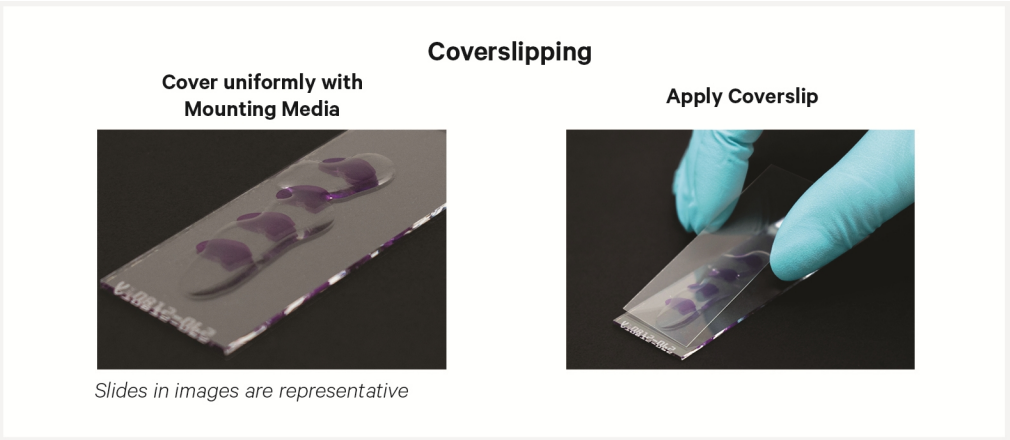
c. Apply the coverslip at an angle on one end of the slide. Slowly lower the coverslip, without introducing bubbles. Allow mounting media to spread and settle.

d. If needed, remove any large excess of mounting media by carefully wicking away from the edge of the coverslip with a lint-free laboratory wipe. Be careful not to move coverslip and disturb the tissue.

e. Dry the coverslipped slide for **30 min** at **room temperature**.

STOP **f.** Once coverslipping is complete, **immediately** proceed with imaging.

If imaging reveals satisfactory tissue morphology, proceed with 4. Cryosectioning & Section Placement on page 47.



Slides in images are representative

Shipping Guidance

- After sectioning, place up to two slides in a slide mailer.
- Place slide mailer in a sealed bag with a bag of desiccant at **-80°C** until ready for shipment.
- When ready for shipment, place mailer in a styrofoam box with dry ice.

Document Revision Summary

Document Number	CG000579
Title	Xenium In Situ – Fresh Frozen Tissue Preparation Guide
Revision	Rev F
Revision Date	August 2025
Description of Changes	• Added slide thickness to Xenium Slide Template on page 16. • Added Fixation and Permeabilization instructions to Fixation & Permeabilization on page 53. • Added QR code to Document Revision Summary. • Updated for general minor consistency of language, format, and terms throughout.

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

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