Xenium In Situ – FFPE Tissue Preparation Handbook

Introduction

The Xenium In Situ Gene Expression workflow is designed to measure mRNA in tissue sections derived from formalin fixed & paraffin embedded (FFPE) 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 FFPE Tissue Handbook provides guidance on:

- Tissue fixation and embedding
- Best practices for handling tissue samples and Xenium slides before and after sectioning
- DAPI and Hematoxylin and Eosin (H&E) staining to check tissue quality
- Sectioning and tissue placement practice
- Sectioning of tissue samples and placement of sections on Xenium slides
- Deparaffinization and decrosslinking 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), Xenium Protein, and Xenium Prime reagents and downstream assay workflows as specified in the table.

Compatible Reagent Kits & Downstream Workflows		
	Xenium v1 and Xenium Protein	**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)	
	Xenium In Situ Gene and Protein Expression with Cell Segmentation Staining (CG000819)	

*Contains Xenium Cassettes

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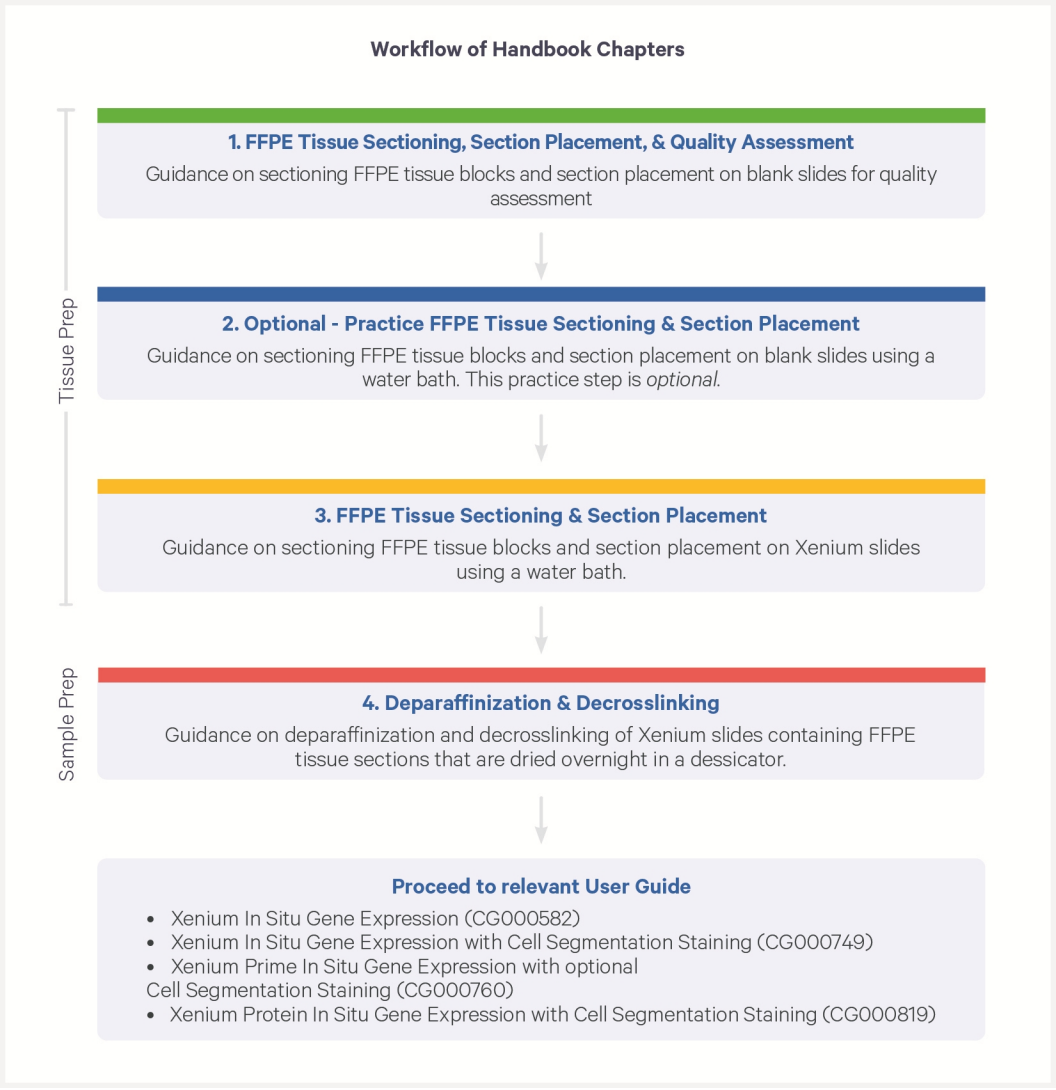
Document Revision Summary

Handbook Overview and Navigation

Overview

This handbook describes FFPE 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 and Xenium Protein workflows:

- Xenium In Situ Gene Expression (CG000582)
- Xenium In Situ Gene Expression with Cell Segmentation Staining (CG000749)
- Xenium In Situ Gene and Protein Expression with Cell Segmentation Staining (CG000819)

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.

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

	#	PN
Xenium Slides (2 pack)	1	3000941

10x GENOMICS

Xenium Prime Sample Prep Reagents, Module A
(2 rxns), PN-1000720
Shipped in dry ice
Store at –20°C

	#	PN
Perm Enzyme B	1	3000553
FFPE Tissue Enhancer*	1	2000798
Priming Hyb Buffer	1	2001228
Post-Priming Wash Buffer	1	2001229
RNase Enzyme	1	3000593
2X RNase Buffer	1	2000411
Polishing Enzyme	1	2001230
Polishing Buffer	1	2001231
Probe Hyb Buffer B	1	2001232

10x GENOMICS

*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 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.

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

	#	PN
Xenium Slides (2 pack)	1	3000941

10x GENOMICS

Xenium Prime Sample Prep Reagents, Module A
(2 rxns), PN-1000720
Shipped in dry ice
Store at –20°C

	#	PN
Perm Enzyme B	1	3000553
FFPE Tissue Enhancer*	1	2000798
Priming Hyb Buffer	1	2001228
Post-Priming Wash Buffer	1	2001229
RNase Enzyme	1	3000593
2X RNase Buffer	1	2000411
Polishing Enzyme	1	2001230
Polishing Buffer	1	2001231
Probe Hyb Buffer B	1	2001232

10x GENOMICS

*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.

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.

Tissue Handling, Fixation, and Embedding Guidelines

Tissue Handling

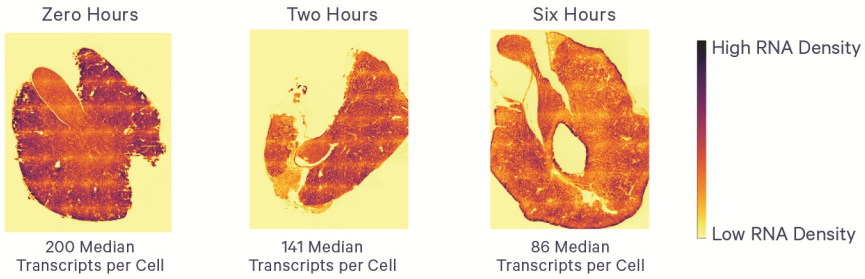
Prior to fixation and embedding, tissues should be handled according to the following guidelines to maximize RNA quality and prevent degradation.

Gentle Handling

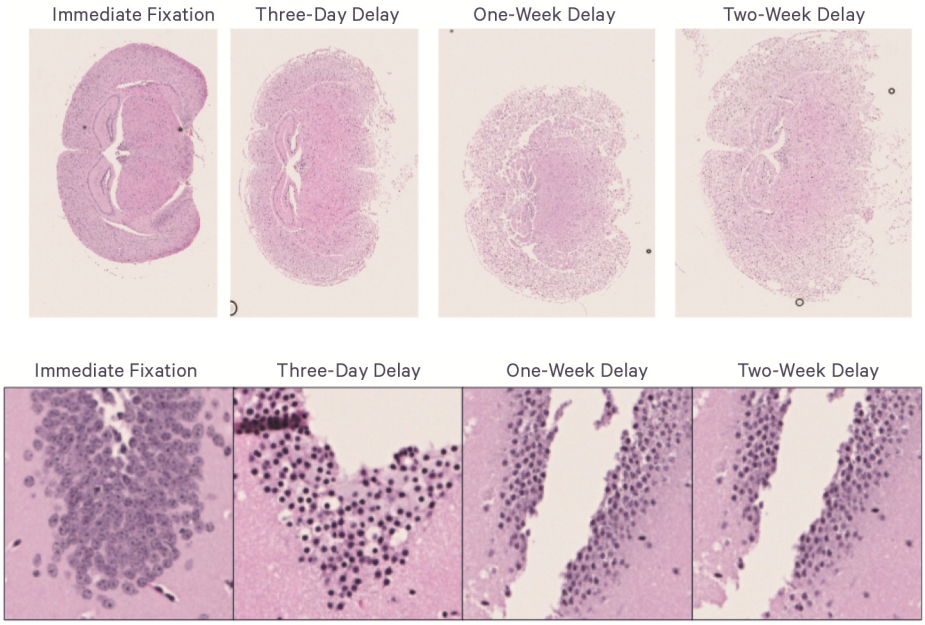
Tissues should be handled gently to avoid mechanical stress. Mechanical stress may damage tissue structure and prevent proper fixation. Examples of processes that introduce mechanical stress include ischemia, coagulative necrosis from electrocautery, and haemorrhages from surgical trauma.

Minimizing Ischemia/Post Mortem Interval (PMI) and Fixation Timing

Prolonged ischemia and PMI can negatively affect tissue quality. If processing delays occur, store tissues in an isotonic solution and avoid exceeding four hours between tissue resection and fixation, though this time may be tissue-dependent. The images below show the negative effect of prolonged ischemia on RNA density.



Delayed tissue fixation may lead to autolysis, degrading tissue and negatively impacting results. Tissue samples should be fixed immediately after resection.



Tissue Storage

Store tissues in a cool and moist environment to prevent tissue drying and degradation. Tissues can be kept in an isotonic solution (e.g. RNase-free PBS) if tissue cannot be fixed immediately. Keeping tissues in isotonic solutions is not suitable for long-term storage. Avoid exceeding four hours. 10x Genomics cannot provide specific guidance on isotonic solutions or RNA preservation products.

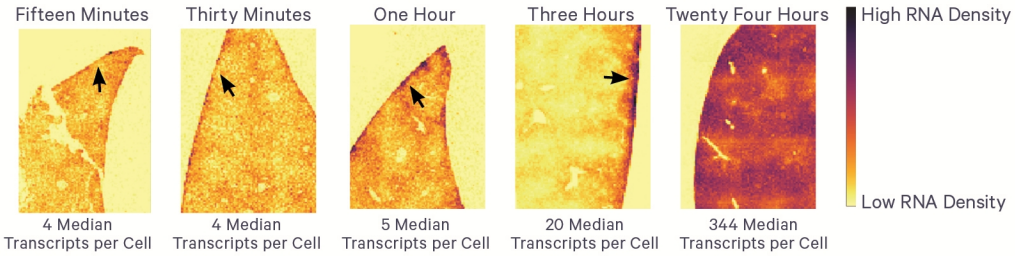
Tissue Fixation and Embedding Guidelines

Prior to sectioning, tissues should be fixed and embedded according to the following guidelines to maximize RNA quality and prevent degradation. These steps generate a formalin fixed & paraffin embedded block that is ready for sectioning.

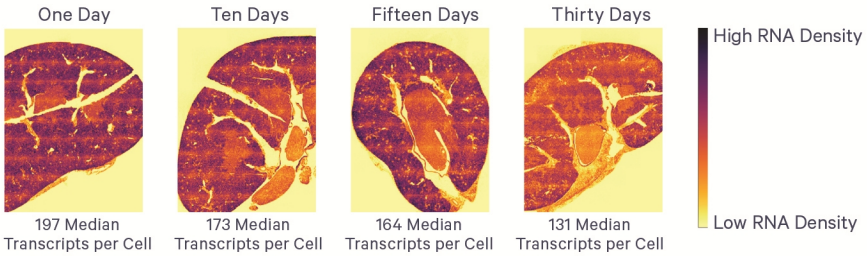
Optimizing Fixation Time

Under or overfixation of tissues can negatively impact assay performance. Fixation time may need optimization for specific organisms, tissue types, and disease states.

Underfixing tissues may result in the continued activities of some enzymes after tissue resection, which may cause degradation of proteins, nucleic acids, and lipids. RNA may also not be properly preserved in underfixed tissues. Lastly, underfixation can lead to tissue artifacts such as irregular chromatin patterns, overstained cytoplasm (eosin), and common autolysis artifacts such as separation of the epithelium from connective tissue. The images below show the negative impacts of underfixation in mouse liver on RNA density. Note the RNA density gradient that begins at the edge of the tissue.



Overfixation may lead to a loss of structural integrity of certain molecular features, lipid oxidation, excessive crosslinking, and decreased antigenicity. Additionally, overfixation may make sectioning more challenging due to tissue hardening. Overfixation can lead to tissue artifacts such as irregularly shaped/smaller cells or hyperchromatic nuclei staining. The images below show structural effects of overfixation in mouse kidney, as we well as the negative impact of overfixation on RNA density.



To avoid under or overfixation, consider the following:

Tissue Size

The thickness of the tissue significantly affects fixative penetration. Tissue sections should be no larger than a standard tissue cassette and be between 5–10 mm.

Fixative and Fixative to Tissue Ratio

The use of 10% Neutral Buffered Formalin (NBF) at a fixative to tissue ratio of 20:1 is recommended for optimal performance.

Fixation Time and Temperature

Fixation should be carried out at 4°C for ~16-24 h to ensure proper fixative penetration. Fixation time may be tissue-dependent and require optimization. Ensure tissue is completely submerged and introduce light agitation.

Optimizing Embedding

Dehydration

After fixation, tissues are dehydrated in an ethanol series to displace any water remaining in the tissue. Inadequate dehydration may result in poor preservation of tissue structure and damage during sectioning.

Clearing

Tissues are "cleared" using a solvent to remove any remaining ethanol. Inadequate clearing may lead to poor wax infiltration, which can result in hard and brittle blocks that are difficult to section.

Infiltration

After clearing, wax is applied to the tissue and allowed to infiltrate. Inadequate wax infiltration may result in holes or gaps in the final block, which may lead to sectioning difficulties. Typically, wax is melted at 60–62°C prior to adding to the tissue. It is then allowed to cool to 20°C. Avoid excessive heat and check temperatures regularly during embedding. Use a high-quality wax with a low melting temperature.

Embedding

Finally, tissues are embedded in paraffin. Record orientation of the tissue during paraffin embedding to ensure proper spatiality for downstream region selection and analysis.

Additional Tips and Best Practices

- Regularly replace reagents used during the fixation and embedding process.
- DO NOT store or leave sample in fixative for an extended period of time, which will lead to overfixation
- Proceed immediately from fixation to dehydration, clearing, and embedding. If proceeding to dehydration immediately is not possible, transfer tissue from fixative to 70% ethanol for short-term storage.
- Store FFPE blocks properly to maintain integrity. Avoid exposure to high temperatures or humidity. For optimal storage, store at 4°C.

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.

Sample Preparation

- Store FFPE tissue blocks at 4°C.

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.

Section Thickness

- Recommended section thickness is 5 µm.

Optional Tissue Trimming & Scoring

FFPE tissue blocks can be trimmed or scored to fit multiple sections onto the Sample Area.

- **FFPE Tissue Block is smaller than Sample Area:** Paraffin around the embedded tissue in the FFPE tissue block should not be trimmed, and the section will fit the Sample Area.
- **FFPE Tissue Block is larger than Sample Area, but tissue is smaller than Sample Area:** Paraffin around the embedded tissue in the FFPE tissue block can be trimmed for the section to fit the Sample Area.
- **FFPE Tissue Block & Tissue are larger than Sample Area:** Paraffin around the embedded tissue in the FFPE tissue block can be trimmed, and the actual tissue can be scored to generate smaller sections to fit the Sample Area.
- Use a razor blade for trimming and scoring the tissue Block. See Optional Trimming/Scoring the Block on page 86 for details.
- Alternatively, tissue sections may be sectioned and trimmed on a cutting mat. See Alternative Trimming or Scoring of FFPE Block & Tissue on page 89 for details.
- Once the tissue has been trimmed and scored, use extra care during sectioning and section handling.

Water Bath Temperature & Section Floating Time

- Optimal water bath temperature and section floating time are critical for tissue section expansion. 42ºC is the recommended water bath temperature for most tissues. However, water bath temperature may need optimization based on the tissue type. Optimization should occur before utilization of a Xenium slide.
- Determine optimal water bath conditions before tissue placement on the Xenium slides by practicing section placement on a blank slide.
- If the tissue is taking too long to expand, turn the water bath temperature up by 1 or 2 degrees and let the section float for longer.
- If the tissue is expanding too quickly and dissociating, turn the water bath temperature down by 1 or 2 degrees and shorten the floating time.

Tissue Section Quality Control

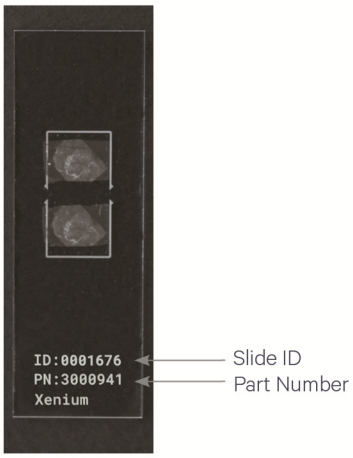
- After section placement on blank slides, sections should be DAPI and hematoxylin and eosin (H&E) stained to examine tissue morphology.
- Use the H&E staining to look for signs of over or underfixation and sampling artifacts. Use this information to determine if scoring is needed to select the area of interest.

The instructions apply to both Xenium v1/Xenium Protein 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/Xenium Protein 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 wax) 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



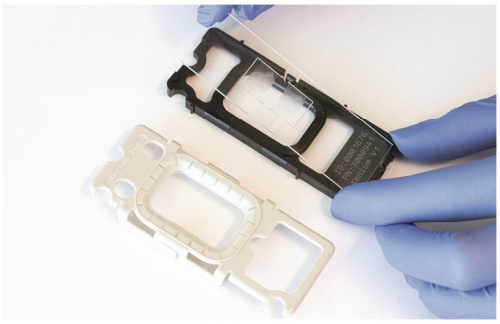
General Xenium Slide Handling

- 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 **-20°C**.
- DO NOT touch the surface of the slide.
- Prior to sectioning, slides should be equilibrated to room temperature for **30 min**.
- Once opened, the slide can remain at room temperature in a desiccator for up to one week.

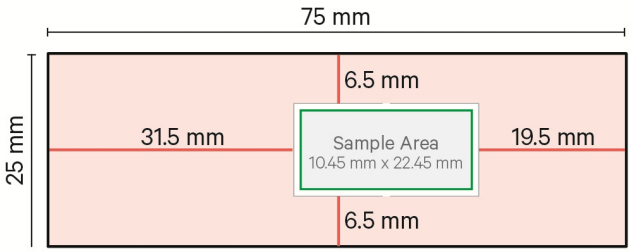
Handling Xenium Slides Containing Tissue Sections

- Slides containing tissue sections that have been incubated at **42°C** for **3h** and dried overnight at room temperature in a desiccator can be stored for up to **4 weeks** at room temperature in a desiccator.

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.

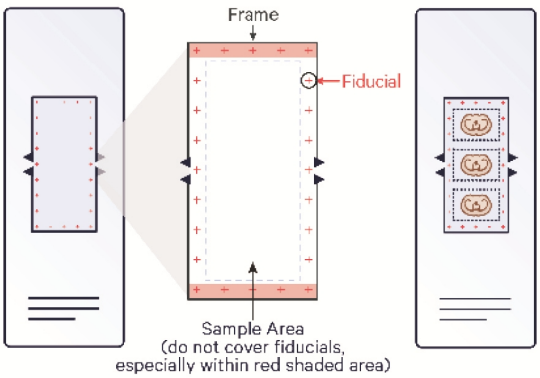
Images are to scale if scaling settings are not modified (select "actual size" or "100%" to print to scale).



- 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 wax 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

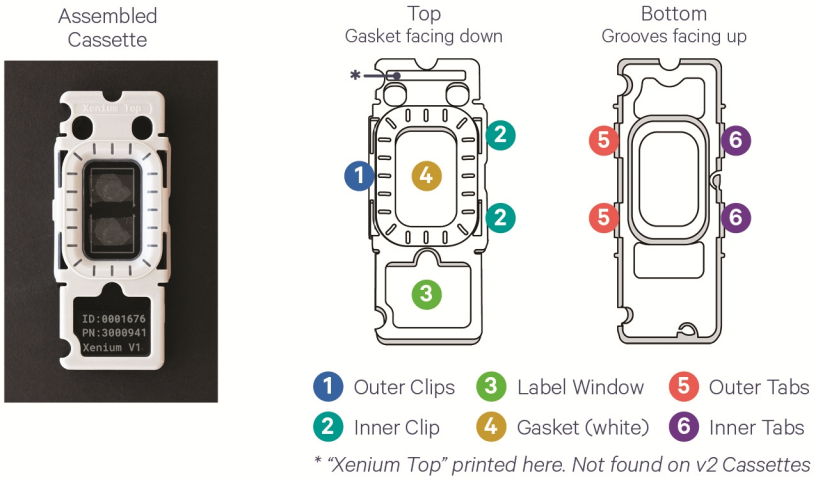
- Monitor section adhesion on Xenium slides throughout the workflow.
- Tissue detachment during the workflow can negatively impact performance. If observed, contact support@10xgenomics.com.
- For more information, refer to Troubleshooting.

Slide Storage

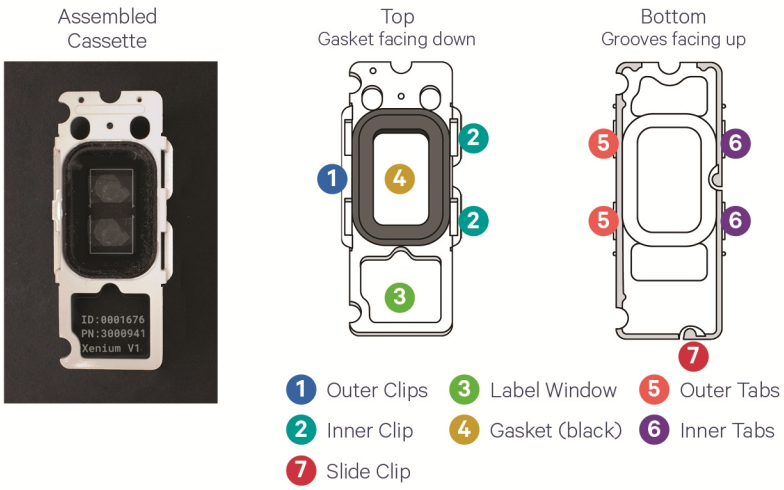
- Always store unused slides at -20°C in their original packaging and keep sealed. Once opened, slides should remain at room temperature in a desiccator and be used within one week to place tissue sections.
- After tissue sections are placed on the slide, store slides at room temperature in a desiccator for up to four weeks (not in a sealed container).

Xenium Cassette

Xenium Cassette
(Use with Xenium v1 Assays)



Xenium Cassette v2
(Use with Xenium Prime Assays)

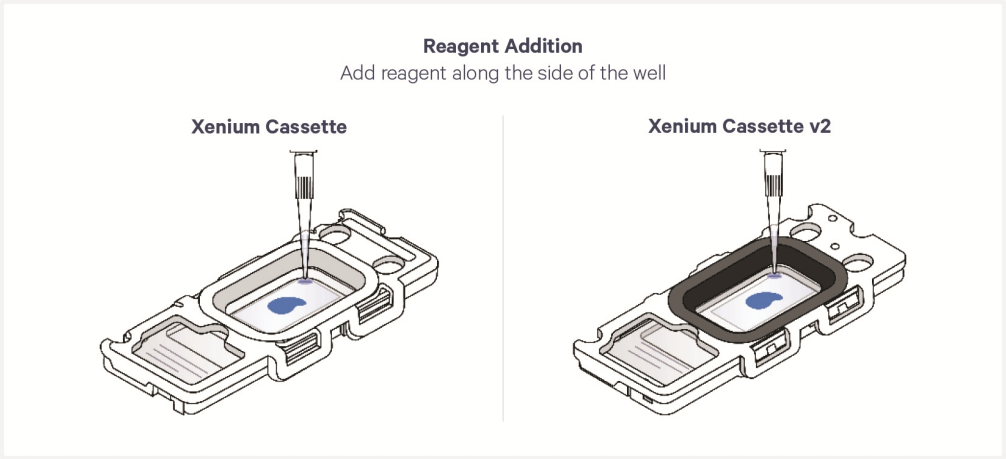


The following guidance applies to both Xenium Cassette and Xenium Cassette v2

- 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.

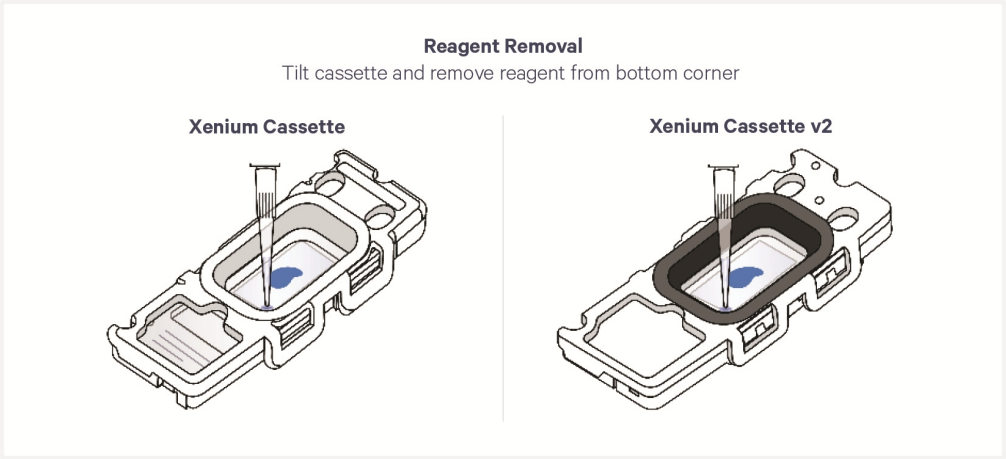
Reagent Addition to Wells

- 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.



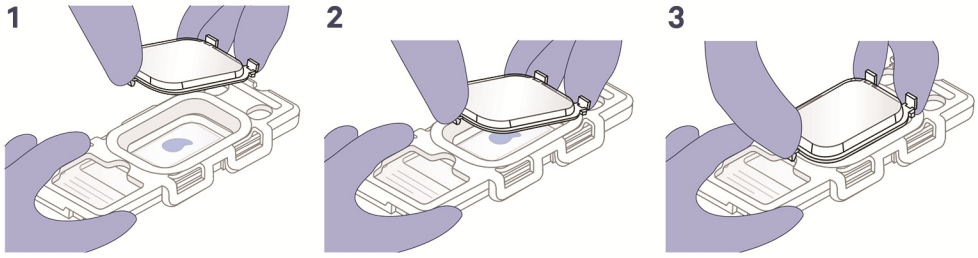
Xenium Cassette Lid Application & Removal

- 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

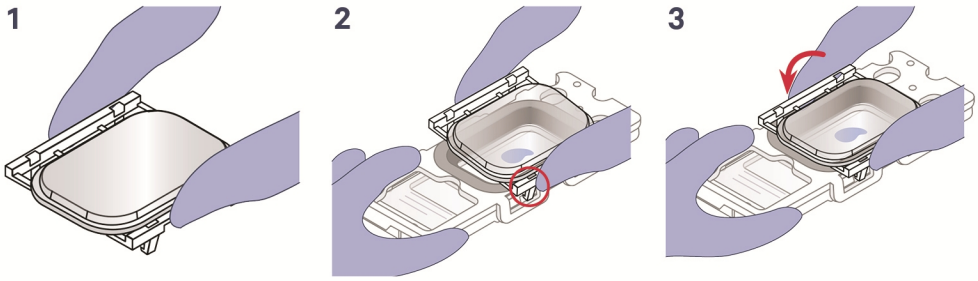


Hold the Xenium Cassette Lid with index and middle finger on two upper tabs and thumb on the lower clip.

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

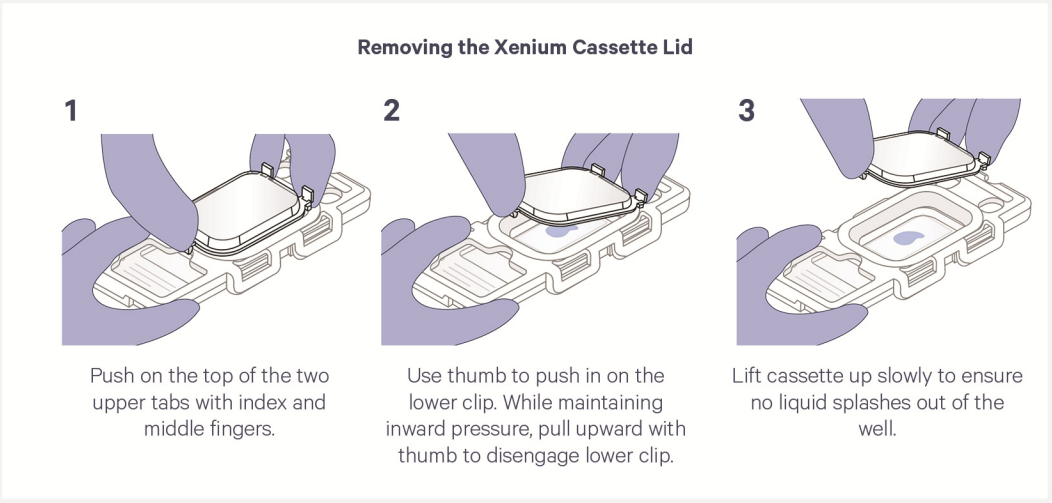


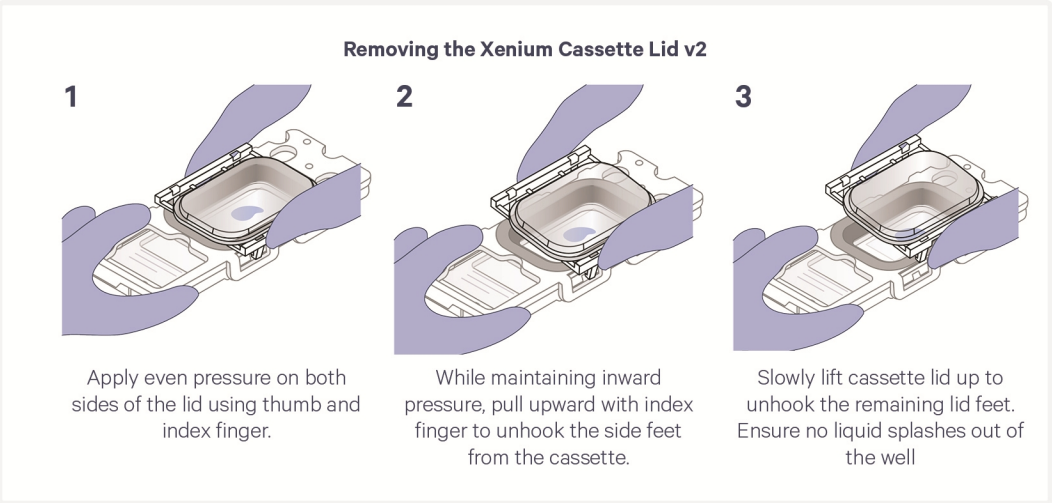
Hold the Xenium Cassette Lid v2 with thumb and index finger on the long sides.

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





Xenium Cassette Lids are a single use item and should be discarded after each use unless otherwise indicated. PBS-T washes DO NOT require sealing of the cassette with a lid.

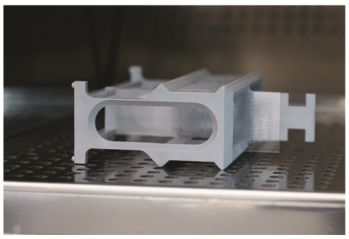
Xenium Slide Incubation

- After sectioning, slides with tissue sections must be dried with either a section dryer oven (preferred) or a thermocycler.

Incubation using a Section Dryer Oven

- If using a section dryer oven after tissue sectioning, ensure slides have been dried with a fan at room temperature to remove water trapped on top of and under the section prior to using the dryer oven.
- Place slides in a slide drying rack sideways to minimize paraffin wax from entering neighboring tissue.

Place in a Rack



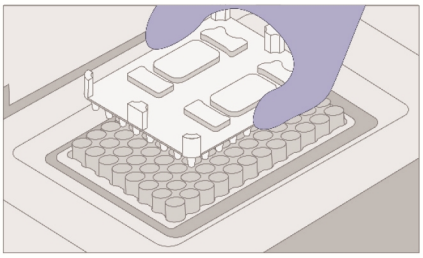
- Close the lid when incubating the slide in the oven.

Incubation on a Thermal Cycler

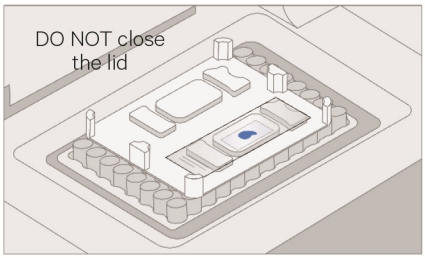
- Position a Xenium Thermocycler Adapter on a thermal cycler that is set at the incubation temperature.
  - Ensure thermal cycler has reached appropriate temperature before starting incubation.
  - Ensure that the Xenium Thermocycler Adapter is in contact with the thermal cycler surface uniformly.
- Place the slide on the Xenium Thermocycler Adapter with the tissue facing up.
  - Ensure that the entire bottom surface of the slide is in contact with Xenium Thermocycler Adapter.

    DO NOT close the thermal cycler lid when incubating the slide due to the risk of touching the tissue.
- 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 in a Thermal Cycler
Xenium Thermocycler Adaptor v2 shown for illustrative purposes



Place Thermocycler Adaptor



Incubate Slide

Tightening the thermal cycler lid

- Thermal cycler lid contact with the Xenium Cassette Lid is critical for assay performance.
- Tighten the thermal cycler lid until an audible click is heard.
- Tightening past the click risks breaking the slide.

Incubation at room temperature

- Place the slide/Xenium Cassette on a flat, clean, non-absorbent work surface.
- Ensure that no absorbent surface is in contact with the reagents on the slide during incubation.

Processing a Single Xenium Slide

The following guidance applies to both Xenium v1 and Xenium Prime workflows.

- Xenium reagent kits are sufficient for two reactions, and for optimal Xenium Analyzer throughput, two slides should be run at the same time.
- When pairing Xenium v1 or Xenium Prime workflows with Cell Segmentation Staining, process two slides per run. The Xenium Stain Enhancer (PN-2000992) cannot be stored once resuspended. Therefore, the 10x reagents for Xenium v1 and Xenium Prime Cell Segmentation workflows can only be used for one round, regardless of whether one or two slides are processed in a single run.

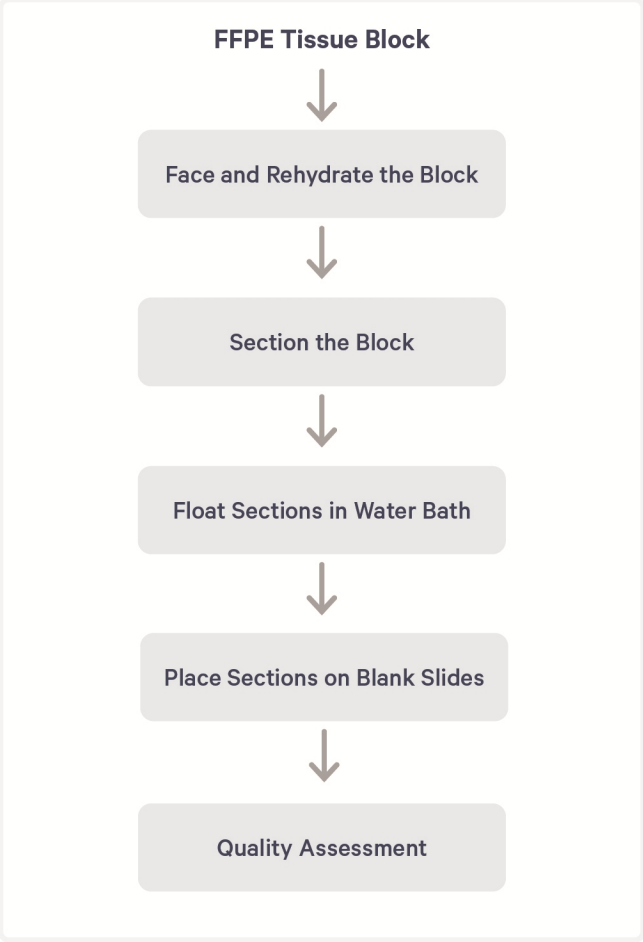
- It is possible to perform the Xenium workflow with a single slide. To do this, ensure the following best practices are followed for optimal assay performance:
  - Assemble a mock Xenium Cassette using a blank slide and a cassette from the Xenium Cassette Kit.
  - Insert the blank slide into the Xenium Cassette. Cassettes should be assembled following the instructions in Tips & Best Practices for Xenium Cassette Assembly.
  - Attach a lid from the Xenium Cassette Kit to the cassette containing the blank slide following Tips & Best Practices for Xenium Lid Application. It is not necessary to add liquid to the slide well before adding the lid.
  - For all incubation steps with the thermal cycler lid closed, ensure the mock slide cassette is placed alongside the Xenium Cassette containing tissue on the Thermocycler Adaptor.

1. FFPE Tissue Sectioning, Section Placement, and Quality Assessment

Overview

This section provides guidance on choosing a quality assessment method, sectioning FFPE tissue blocks, section placement on blank slides using a water bath, and quality assessment. Ensure that tissue sections have been processed according to guidelines in Tissue Handling, Fixation, and Embedding Guidelines on page 12.

Xenium slides are not used for the quality assessment. Sectioning and placement on Xenium slides for the full workflow occur in 3.1 Section Placement on Xenium Slides on page 51.



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.

DV200

DV200 assesses RNA integrity, where the DV200 score indicates the percentage of RNA fragments over 200 nucleotides in length. A DV200 score of less than 30% is correlated with low transcript density.

Face the Block and Rehydrate

FFPE tissue block is placed in a microtome and cut to face the tissue. The block is rehydrated in an ice bath.

Sectioning

The tissue block is sectioned using a microtome. A section may be saved at this step for optional DV200 assessment. See DV200 Performance and Recommendations on page 44 for more information.

Section Placement

Sections are collected in a water bath and are allowed to float on the water surface until they are flat and free from wrinkles and folds. The optimal floating time depends upon the specific tissue type and should be empirically determined when working with new tissue types. Expanded tissue sections are placed on a blank slide for H&E staining.

Quality Assessment

Tissue sections on slides are deparaffinized prior DAPI and H&E staining. A deparaffinization protocol is provided in the Appendix. Perform DAPI staining according to any preferred protocol. A DAPI protocol is provided in the Appendix. After DAPI staining and imaging, perform H&E staining according to any preferred protocol. An H&E protocol is provided in the Appendix. DAPI and H&E may be performed on the same tissue section or on serial sections.

DV200 analysis: RNA is extracted from tissue sections and analyzed as described in RNA Quality Assessment of FFPE Tissue Block on page 90.

1.0 Get Started

Items		10x PN	Preparation & Handling	Storage
Obtain				
☐	Mounting Media	-	-	Ambient
☐	Milli-Q Water	-	-	Ambient
☐	Water Bath	-	Equilibrate to optimal temperature.	Ambient
☐	Blank Slides	-		Ambient
☐	Brushes	-		Ambient
☐	Microtome	-		Ambient
☐	Microtome blade	-		Ambient
☐	Fan *(optional, but recommended)*	-		Ambient
☐	Section Dryer Oven *(optional, but recommended)*	-	Equilibrate to desired temperature.	Ambient
☐	Marker	-		Ambient
☐	Coverslips	-		Ambient
☐	Wide-bore pipettes	-		Ambient

1.1 Facing the Block

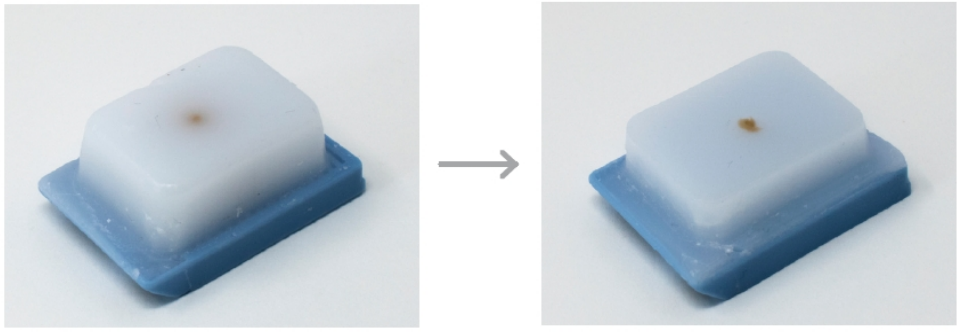
Before starting, wipe down all the surfaces and work areas with RNase Zap RNase decontaminating solution. If necessary, rewipe the area with 100% ethanol to quickly dry the surface.

a. Remove tissue blocks from storage.

b. Set the microtome to the 15 µm setting.

c. Cut the tissue block at 15 µm until all of the edges of the tissue are exposed or until the region of interest is exposed. The block should be at **room temperature** during cutting.

Exposing the Tissue Block



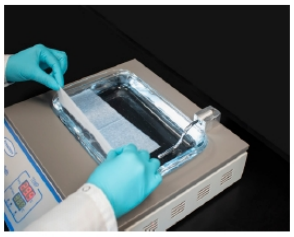
1.2 Sectioning

a. Fill up a water bath with Milli-Q water and ensure that the temperature is set at **42°C** and free from bubbles & particulates by gliding a laboratory wipe over the water surface. Repeat this step between sectioning as and when necessary.

TIPS

42ºC is the recommended water bath temperature for most tissues. However, water bath temperature may need optimization based on the tissue type. Determine optimal water bath conditions before tissue placement on the Xenium slides by practicing section placement on a blank slide. See Tips & Best Practices for guidance on optimizing water bath temperature. To better visualize the tissue sections, a Lighted Tissue Floating Bath with LED illumination can be used instead of standard water bath.

Remove Bubbles



b. Place a clean pipette tip box on ice.

Ice should be from filtered water. If not available, freeze Milli-Q water ahead of tissue sectioning.

c. Fill pipette tip box with 4°C Milli-Q or filtered water.

d. Place blocks in the cold water, ensuring that the tissue/sectioning face is fully submerged.

For optimal hydration, using an ice bath is preferred to an ice block.

e. Incubate on the ice bath for **10-30 min**. The incubation time depends upon the tissue type and the extent of dehydration. Extent of dehydration depends on processing method and age of tissue block.

Monitor the exposed tissue every 5-10 min during the ice bath incubation. The tissue surface should be smooth and shiny and free of bumps at the end of the incubation. Sectioning compression and shattering are usually due to insufficient hydration. For more information on tissue hydration, see

Troubleshooting section.

FFPE Tissue Block in Ice Bath

f. Carefully wipe off the excess oils from a 35X Ultra disposable blade using a laboratory wipe with 100% ethanol. Let the ethanol evaporate before proceeding. Always use new blades for sectioning.

g. Secure the blade in the disposable blade holder of the microtome and place the knife guard over the blade to minimize injury. Ensure that the clearance angle is set to 10°. Set the microtome to 5 µm (or desired cutting thickness) for sectioning.

h. After hydration is complete, place the tissue block in the specimen clamp and align it with the blade. For tissue blocks with exposed tissue, discard the first few sections and start collection on subsequent sections.

i. To collect sections, place the paintbrush tip slightly above and parallel to the blade. Lift section by lightly touching the edge with the paintbrush while rotating the wheel handle.

j. With the help of the brush, pick section up. Immediately place it on the water surface of the water bath, making sure that the brush tip goes underneath and away from the section.

k. Proceed directly to Section Placement.

1.3 Section Placement on Blank Slides

a. Allow sections to float for previously determined optimal time.

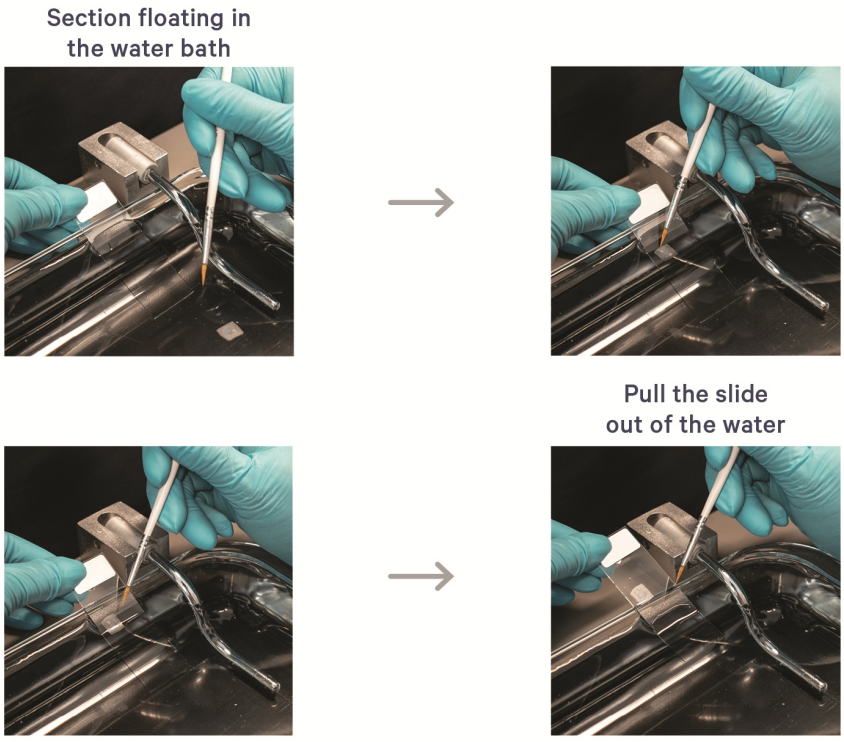
b. Insert the slide into the water.

c. Using the paintbrush or the probe, maneuver the section to the slide.

If sections float away from the slide, the slide can also be dipped into the water bath before section placement.

d. Pull the slide up and out of the water, ensuring there are no air bubbles trapped underneath and set aside.

If sections require repositioning and the sections have not dried onto the slide, repeat section placement by resubmerging the slide and refloating sections in the water bath. Ensure that minimal water is trapped beneath the section and that the section does not disintegrate from extended floating in the water bath.



e. Repeat, if needed, for additional sections, ensuring that the previous sections do not get re-submerged in the water.

f. Dry tissue sections upright in a drying rack at room temperature until tissue is opaque and no water remains on top of or under the section. A fan may be used to assist in drying.

g. Place the slides in a slide drying rack and incubate for **10 min** at **42°C** in an oven or thermal cycler.

TIPS

See Tips & Best Practices for guidance on slide incubation.

STOP

h. Proceed to Deparaffinization & Quality Assessment.

1.4 Deparaffinization & Quality Assessment

Tissue sections on slides must be deparaffinized prior to quality assessment. A deparaffinization protocol is provided in the Appendix.

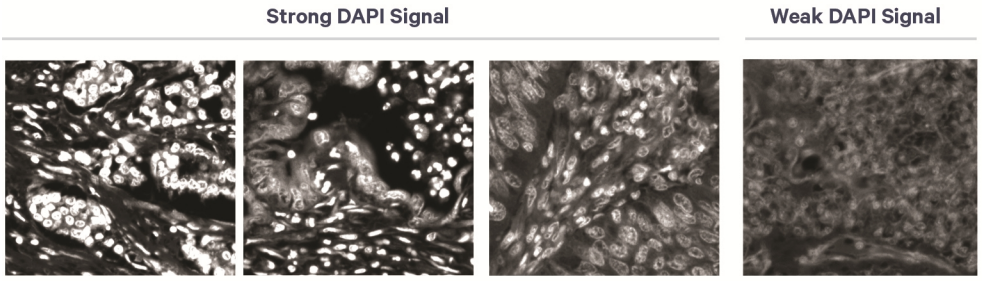
Quality assessment is composed of two parts: DAPI staining and H&E staining. Perform DAPI staining according to any preferred protocol. A DAPI protocol is provided in the Appendix. After DAPI staining and imaging, perform H&E staining according to any preferred protocol. An H&E protocol is provided in the Appendix. DAPI and H&E may be performed on the same tissue section or on serial sections.

1.5 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 3. FFPE Tissue Sectioning & Section Placement on page 48 for placement of tissue sections on Xenium slides or proceed with additional quality assessment methods.

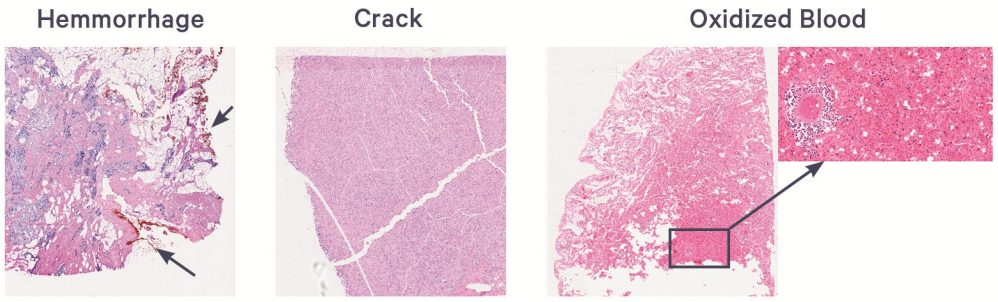


1.6 H&E Tissue Quality Assessment

Review the H&E image thoroughly to assess tissue quality and select area(s) of interest. If scoring the block or section is necessary, refer to Optional Trimming/Scoring the Block on page 86.

Ensure that the tissue section is free from tissue processing and sectioning artifacts as shown in Troubleshooting on page 69. Tissue processing artifacts may include improper fixation, squeeze/crush artifacts, and hemorrhaging. Examples are shown in the images below. If H&E staining was performed on the same tissue section used for DAPI quality assessment (see previous section), ensure that DAPI staining overlaps well with nuclei seen during H&E imaging.

If imaging reveals satisfactory tissue morphology, proceed with 2. Optional - Practice FFPE Tissue Sectioning & Section Placement on page 46 if additional sectioning practice is needed, 3. FFPE Tissue Sectioning & Section Placement on page 48 for placement of tissue sections on Xenium slides, or any additional quality assessment methods.



DV200 Performance and Recommendations

Assess DV200 on a serial section according to preferred method. A DV200 method is provided in the Appendix. 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

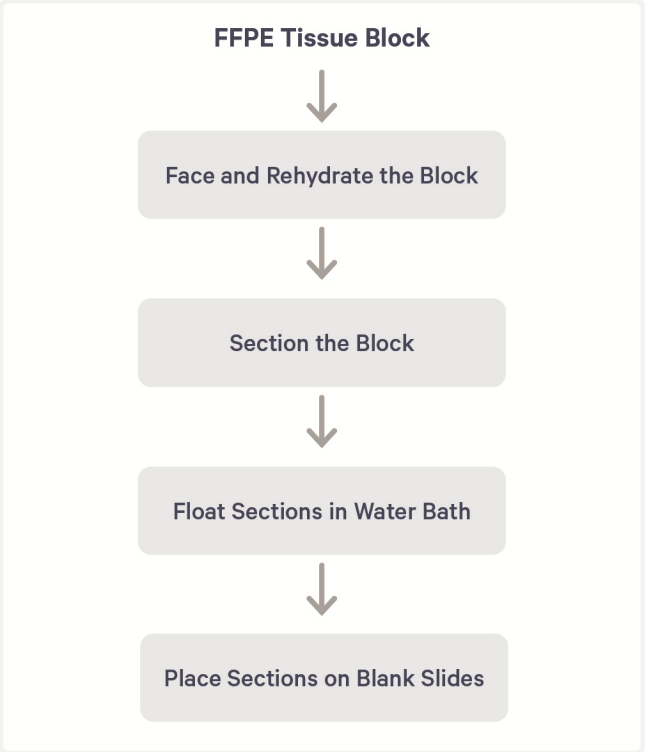
- Fixation method
- Miscellaneous upstream tissue handling and processing

10x Genomics recommends that the tissues used in the Xenium In Situ assay should have a DV200 of >30%.

2. Optional - Practice FFPE Tissue Sectioning & Section Placement

Overview

This section provides guidance on sectioning FFPE tissue blocks and section placement on blank slides using a water bath. This practice step is optional. After examining the H&E tissue section, if necessary, score of the block to isolate the region of interest.



Face the Block and Rehydrate

FFPE tissue block is placed in a microtome and cut to face the tissue. Facing is only necessary for new, unused blocks. For tissue blocks with exposed tissue, discard the first few sections and start collection on subsequent sections. The block is rehydrated in an ice bath. Perform as described in step 1.

Sectioning

The tissue block is sectioned using a microtome to generate appropriately sized sections for blank slides. Perform as described in step 1.

Section Placement

Draw the template shown in Xenium Slide Template on page 22 onto a blank slide to simulate the Sample Area on the Xenium slide. Sections are collected in a water bath and are allowed to float on the water surface until they are flat and free from wrinkles and folds. The optimal floating time depends upon the specific tissue type and should be empirically determined when working with new tissue types.

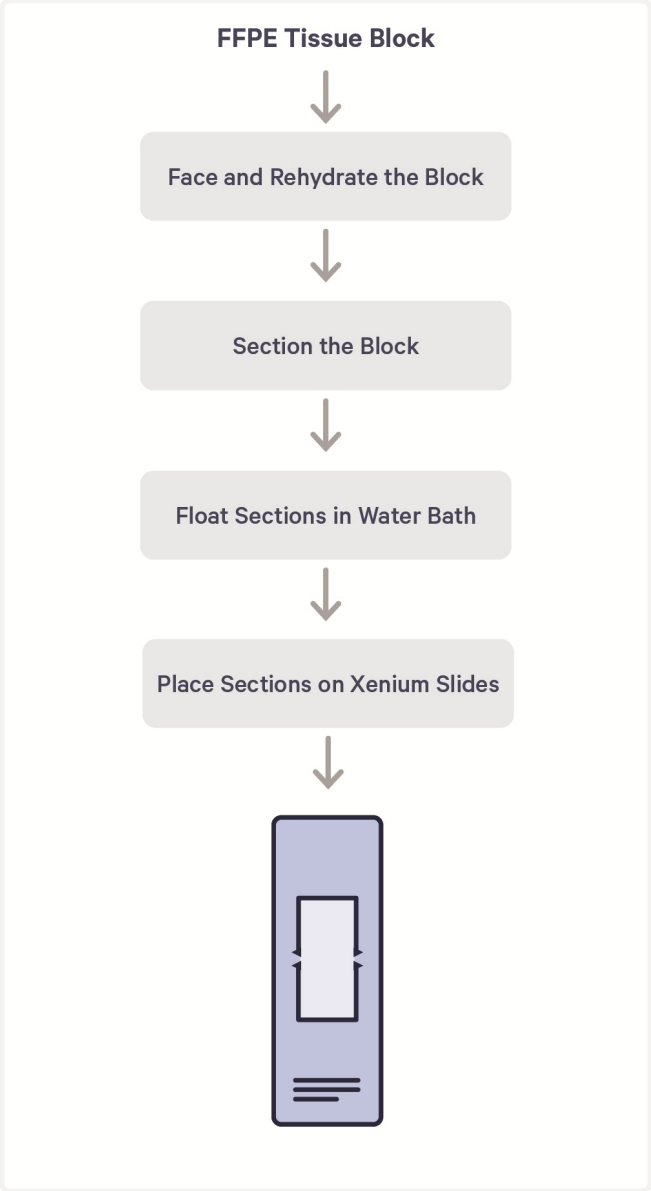
2.0 Get Started

Items		10x PN	Preparation & Handling	Storage
Obtain				
☐	Milli-Q Water	-	-	Ambient
☐	Water Bath	-	Equilibrate to optimal temperature.	Ambient
☐	Blank Slides	-		Ambient
☐	Brushes	-		Ambient
☐	Microtome	-		Ambient
☐	Microtome blade	-		Ambient
☐	Fan *(optional, but recommended)*	-		Ambient
☐	Section Dryer Oven *(optional, but recommended)*	-	Equilibrate to desired temperature.	Ambient
☐	Marker	-		Ambient
☐	Coverslips	-		Ambient

3. FFPE Tissue Sectioning & Section Placement

Overview

This section provides guidance on sectioning FFPE tissue blocks and section placement on the Xenium slides using a water bath.



Face the Block and Rehydrate

FFPE tissue block is placed in a microtome and cut to face the tissue. Facing is only necessary for new, unused blocks. For tissue blocks with exposed tissue, discard the first few sections and start collection on subsequent sections. The block is rehydrated in an ice bath. Perform as described in step 1.

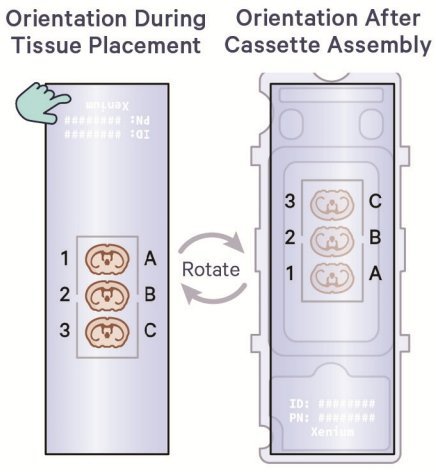
Sectioning

The tissue block with exposed tissue is optionally trimmed or scored to make it compatible in size to the Sample Area on Xenium slides. The tissue block is then sectioned by a microtome to generate appropriately sized sections for Xenium slides. Perform as described in step 1.

Section Placement

Sections are collected in a water bath and are allowed to float on the water surface until they are flat and free from wrinkles and folds. The optimal floating time depends upon the specific tissue type and should be empirically determined when working with new tissue types. Expanded tissue sections are placed on the slide of choice. Prior to placing tissue sections on a Xenium slide, practice section placement on a blank slide. Do not obstruct the fiducials while placing tissue.

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).



3.0 Get Started

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	Xenium Slides	3000941	Equilibrate at room temperature for 30 min prior to sectioning.	–20°C
Obtain				
☐	Water Bath	-	Equilibrate to optimal temperature.	Ambient
☐	Brushes	-		Ambient
☐	Microtome	-		Ambient
☐	Microtome blade	-		Ambient
☐	Fan *(optional, but recommended)*	-		Ambient
☐	Section Dryer Oven *(optional, but recommended)*	-	Equilibrate to desired temperature.	Ambient
☐	Marker	-		Ambient

3.1 Section Placement on Xenium Slides

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 22 for slide layout.

Prior to section placement, face, rehydrate, and section the block as described in step 1.

a. Allow sections to float for previously determined optimal time.

b. Insert the slide into the water.

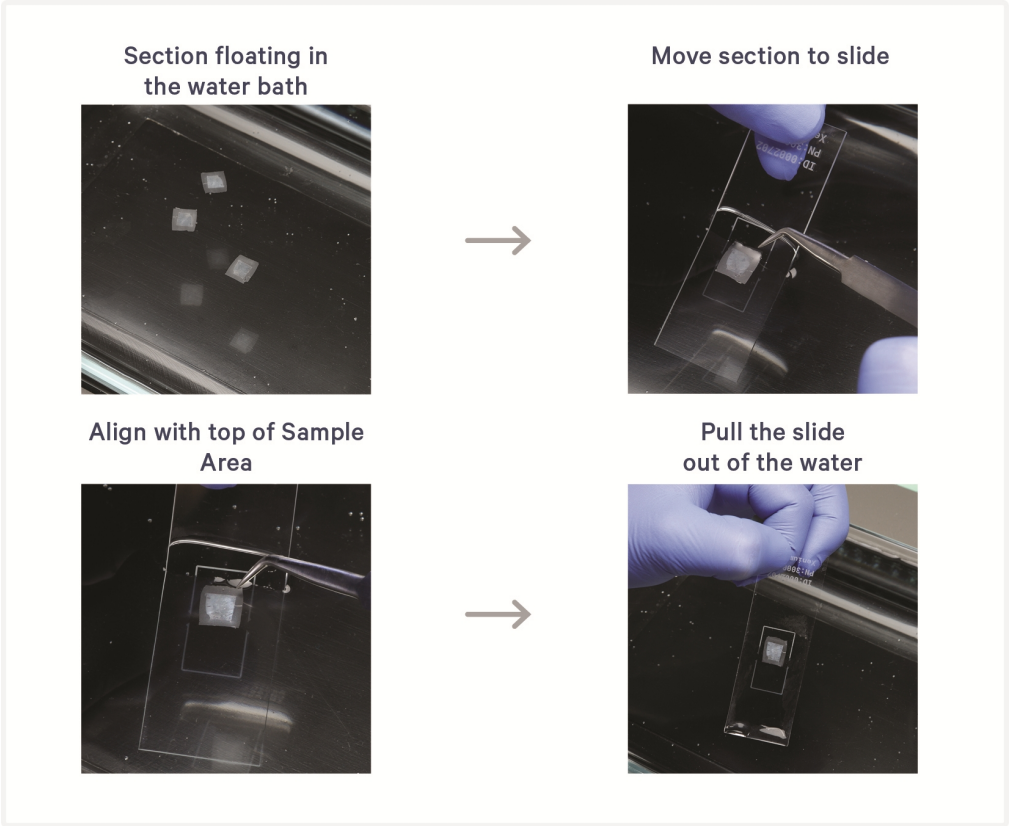
c. Using the paintbrush or the probe, maneuver the section to the top of the Sample Area on the slide.

If sections float away from the slide, Xenium slide can also be dipped into the water bath before section placement.

d. Pull the slide up and out of the water, ensuring there are no air bubbles trapped underneath and set aside. Avoid covering the fiducial frame.

If sections require repositioning and the sections have not dried onto the slide, it may be possible to repeat section placement by resubmerging the slide and refloating sections in the water bath. Ensure that minimal water is trapped beneath the section and that the section does not disintegrate from extended floating in the water bath.

If sections are placed incorrectly, contact support@10xgenomics.com to troubleshoot.



e. Repeat for the remaining sections, ensuring that the previous sections do not get re-submerged in the water.

f. Dry tissue sections upright in a drying rack at room temperature until tissue is opaque and no water remains on top of or under the section. A fan may be used to assist in drying. If no fan is used, slides may require extended drying time for up to **30 min** at **room temperature** to ensure no water remains under the section or until dry (inspect visually, do not touch tissue).

g. Place the slides in a slide drying rack and incubate for **3 h** at **42°C** in an oven or thermal cycler.

TIPS *See Tips & Best Practices for guidance on slide incubation.*

h. *Optional, but recommended* - Place slides in a desiccator and keep overnight at **room temperature** to ensure proper drying. A lack of proper drying may result in tissue detachment or artifacts.

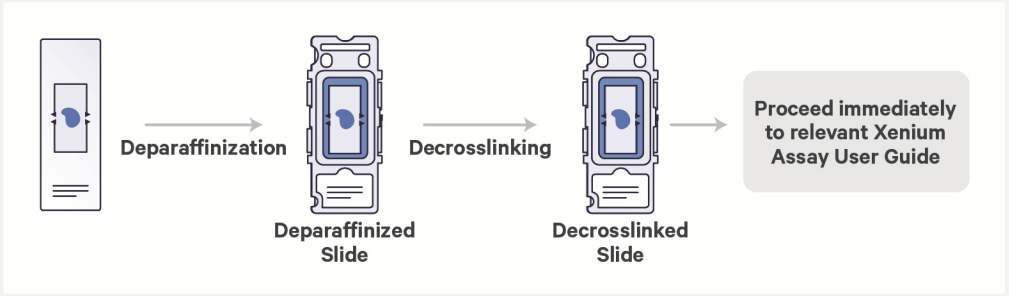
STOP **i.** After drying, proceed to 4. Deparaffinization & Decrosslinking on page 53 or store the slide containing dry tissue sections at room temperature in a desiccator for up to **4 weeks**.

4. Deparaffinization & Decrosslinking

Overview

This chapter provides guidance on deparaffinization and decrosslinking of Xenium slides containing FFPE tissue sections that are dried overnight in a desiccator. After paraffin is removed from the tissue sections during the deparaffinization process, tissues are rehydrated and decrosslinked to release the sequestered RNA from the tissue. Following deparaffinization and decrosslinking, proceed immediately to the relevant downstream assay 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)
- Xenium In Situ Gene and Protein Expression with Cell Segmentation Staining (CG000819)



Protocol Steps & Timing

~4.5 hours*

Steps		Timing	Stop & Store
Deparaffinization & Decrosslinking			
4.1	Buffer Preparation - Deparaffinization & Decrosslinking	30 min	
4.2	Deparaffinization	• Xenium v1 - 3 h (includes 2 h incubation at 60°C) • Xenium Prime - 1.5 h (includes 30 min incubation at 60°C)	
4.3	Cassette Assembly	10 min	
4.4	Decrosslinking	1 h	

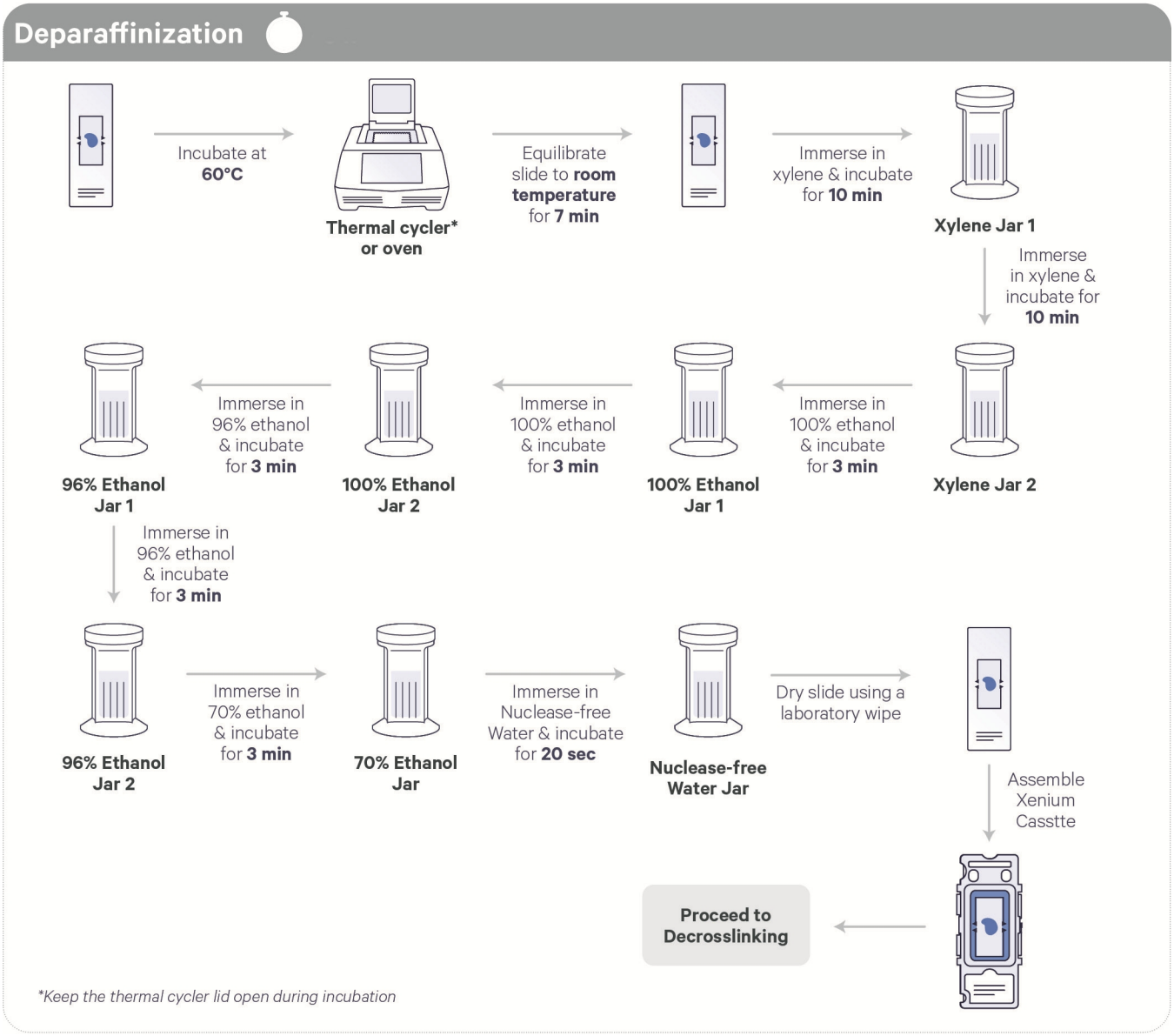
*If using Xenium Prime reagents, the total protocol time is ~3 h

Note there are no safe stopping points during this workflow.

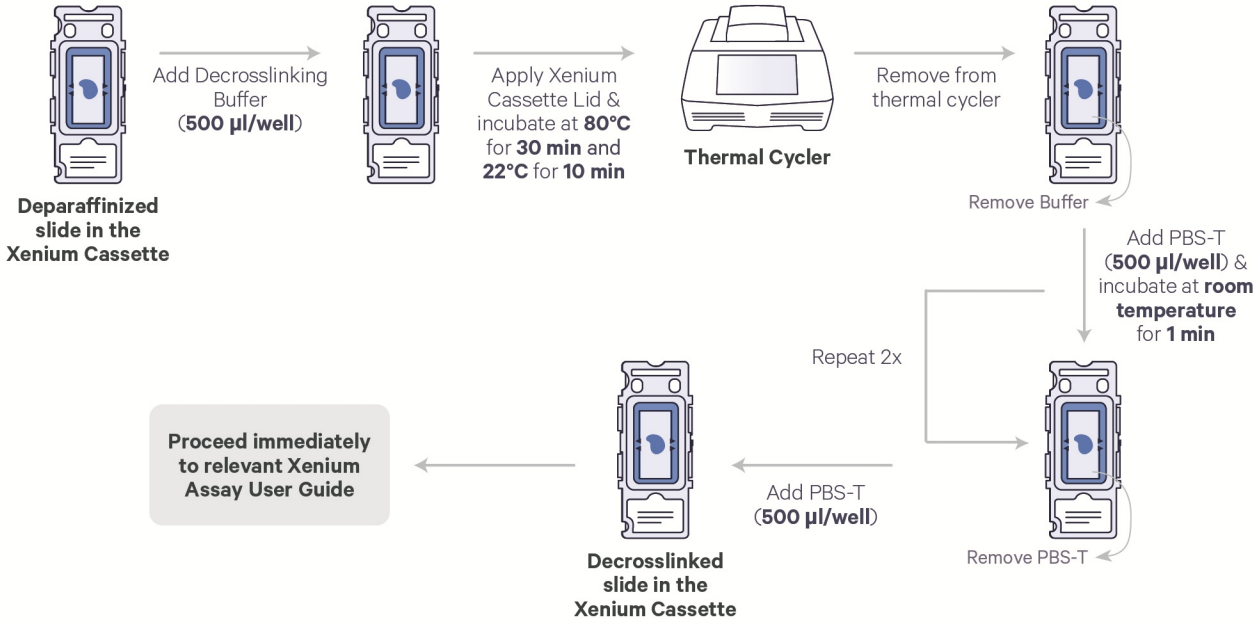
Protocol Overview

During Deparaffinization, the Xenium Slide incubation time at 60°C for:

- Xenium v1 is **2 h** (Deparaffinization time is **~3 h**)
- Xenium Prime is **30 min** (Deparaffinization time is **~1.5 h**)



Decrosslinking Sections ~1 h



4.0 Get Started - Deparaffinization & Decrosslinking

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

Deparaffinization Items			10x PN	Preparation & Handling	Storage
Obtain					
☐		Xylene	-	-	Ambient
☐		Ethanol	-	Prepare Ethanol dilutions using Nuclease-free water.	Ambient
☐		Nuclease-free Water	-	-	Ambient
☐		10X PBS	-	-	Ambient
☐		Forceps	-	-	Ambient
☐		Slide Rack	-	-	Ambient
☐		Coplin jars/Staining dishes	-	-	Ambient
☐		Xenium Slides with FFPE tissue sections	3000941	Prepared according to Xenium In Situ for FFPE - Tissue Preparation Guide (CG000578).	Room temperature in a dessicator
	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/Xenium Protein 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/Xenium Protein or Xenium Prime specific reagents and consumables are used.

Decrosslinking Items			10x PN	Preparation & Handling	Storage
Equilibrate to room temperature					
☐		Perm Enzyme B	3000553	Maintain at room temperature. Pipette mix, centrifuge briefly. DO NOT vortex.	-20°C
☐		Xenium FFPE Tissue Enhancer*	2000798	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. Perform thaw and cool steps in the dark.**	-20°C
Obtain					
☐		Nuclease-free Water	-	-	Ambient
☐		1X PBS	-	Prepared at Step 1.1a.	Ambient
☐		10% Tween-20	-	-	Ambient
☐		Urea	-	-	Ambient
☐	Xenium v1	Thermocycler Adaptor Xenium Cassette Lid	3000954 3001046	See Tips & Best Practices	Ambient
		OR			
	Xenium Prime	Thermocycler Adaptor v2 Xenium Cassette Lid v2	3002207 3002206	See Tips & Best Practices	Ambient

**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.*

TIPS **Pre-heat thermomixer or water bath to 37°C in advance of intended use.

4.1 Preparation - Buffers

For Deparaffinization:

Prepare all buffers fresh according to the tables below.

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	-	-	9.0
	RNase free PBS, pH 7.4	10X	1X	1.0
	Total	**-**	**-**	**10.0**

b. Prepare eight total coplin jars for deparaffinization steps. Prepare Ethanol dilutions using Nuclease-free water.

For Deparaffinization		
Items		**Preparation & Handling**
	Xylene	Label two coplin jars as Xylene Jar 1 and 2. Fill to capacity with xylene in each.
	100% Ethanol	Label two coplin jars as 100% Ethanol Jar 1 and 2. Fill to capacity with 100% ethanol.
	96% Ethanol	Label two coplin jars as 96% Ethanol Jar 1 and 2. Fill to capacity with 96% ethanol.
	70% Ethanol	Label one coplin jar as 70% Ethanol Jar. Fill to capacity with 70% ethanol.
	Nuclease-free water	Label one coplin jar as Nuclease-free Water Jar. Fill to capacity with Nuclease-free 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. Prepare fresh reagents after every 20 slides or every week (whichever comes first).*

For Decrosslinking:

Prepare all buffers fresh according to the tables below.

c. 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	-	-	4,975.0
	Tween-20	10%	0.05%	25.0
	Total	**-**	**-**	**5,000.0**

d. Prepare Diluted Perm Enzyme B using 1X PBS prepared from step 1.1a. 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				
Items		**Stock**	**Final**	**Total Amount (µl)**
	1X PBS	1X	-	998.0
	Perm Enzyme B *(Maintain at room temperature. Pipette mix, centrifuge briefly. DO NOT vortex).*	-	-	2.0
	Total	**-**	**-**	**1,000.0**

e. Prepare Decrosslinking Buffer using Diluted Perm Enzyme B prepared at step 1.1d. Add reagents in the order listed. Pipette mix thoroughly. Maintain at room temperature.

Decrosslinking Buffer					
Items		**Stock**	**Final**	**1 slide+10% (µl)**	**2 slides+10% (µl)**
	Xenium FFPE Tissue Enhancer *(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. Perform thaw and cools steps in the dark).*	-	-	508.8	1,017.5
	Urea	8 M	0.5 M	34.4	68.8
	Diluted Perm Enzyme B	-	-	6.9	13.8
	Total	**-**	**-**	**550.0**	**1,100.0**

4.2 Deparaffinization

Deparaffinization 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.

The instructions apply to both Xenium v1/Xenium Protein 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/Xenium Protein or Xenium Prime specific reagents and consumables are used.

a. Retrieve the slide with tissue sections from the desiccator after overnight drying.

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

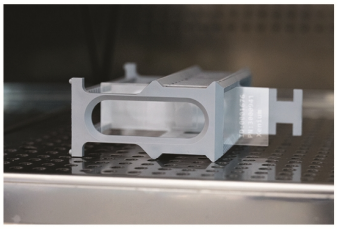
b. Place slide in a Section Dryer Oven and incubate uncovered at **60°C**:

- Xenium v1 for **2 h**
- Xenium Prime for **30 min**

Keep the oven lid closed during incubation.

Incubation in a Section Dryer Oven

Place slide in a rack



Place slide sideways and keep the oven door closed during incubation

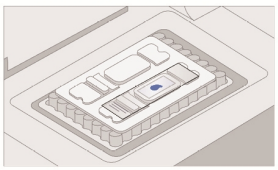
Alternatively, place a Thermocycler Adaptor on a thermal cycler set at **60°C.** Place slide on the Thermocycler Adaptor with the tissue side facing up and incubate at **60°C**:

- Xenium v1 for **2 h**
- Xenium Prime for **30 min**

  DO NOT close the thermal cycler lid.

Place Thermocycler Adaptor

Incubate Slide at 60°C

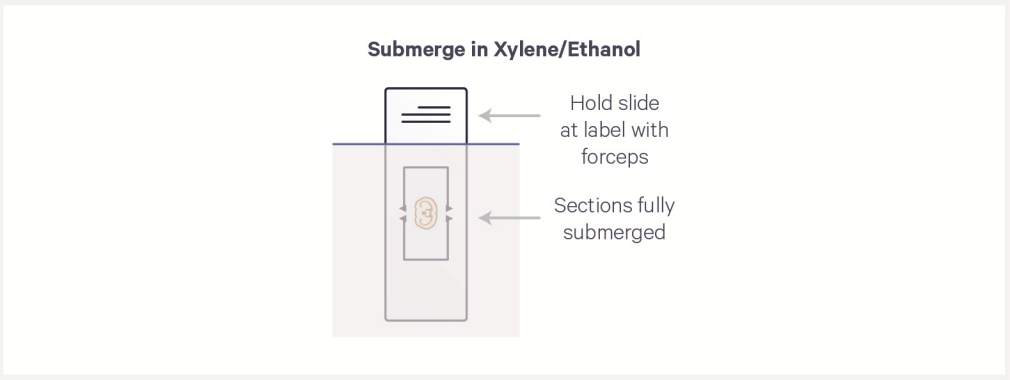


DO NOT close lid

c. Remove from the oven or thermal cycler and allow the slide to cool down to **room temperature** for **7 min**.

Optional: photograph the slide against a black background during 7 min cool down at room temperature. 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 for more details.

d. Gently immerse slide in the Xylene Jar 1. Secure the jar cap to prevent xylene loss.



Hold slide at label with forceps for xylene immersion steps. When immersing slides in xylene, ensure that the tissue sections are completely submerged.

e. Incubate for **10 min**.

f. Gently immerse slide in the Xylene Jar 2 and incubate for **10 min**.

g. Gently immerse slide in the 100% Ethanol Jar 1 for **3 min**.

Hold slide at label with forceps for ethanol immersion steps. When immersing slides in ethanol, ensure that the tissue sections are completely submerged.

h. Gently immerse slide in the 100% Ethanol Jar 2 for **3 min**.

i. Gently immerse slide in the 96% Ethanol Jar 1 for **3 min**.

j. Gently immerse slide in the 96% Ethanol Jar 2 for **3 min**.

k. Gently immerse slide in the 70% Ethanol Jar for **3 min**.

l. Gently immerse slide in the Nuclease-free water Jar for **20 sec**.

4.3 Cassette Assembly

The instructions apply to both Xenium v1/Xenium Protein 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/Xenium Protein or Xenium Prime specific reagents and consumables are used.

a. Remove any remaining nuclease-free water 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.

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 1for more details.

Assembled Xenium Cassette

Xenium Cassette

Xenium Cassette v2

4.4 Decrosslinking

The instructions apply to both Xenium v1/Xenium Protein 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/Xenium Protein or Xenium Prime specific reagents and consumables are used.

Reagent addition and removal should be done carefully. Remove reagents along the side of the well. Do not touch tissue sections or introduce bubbles.

a. Place a Xenium Thermocycler Adaptor in the thermal cycler.

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

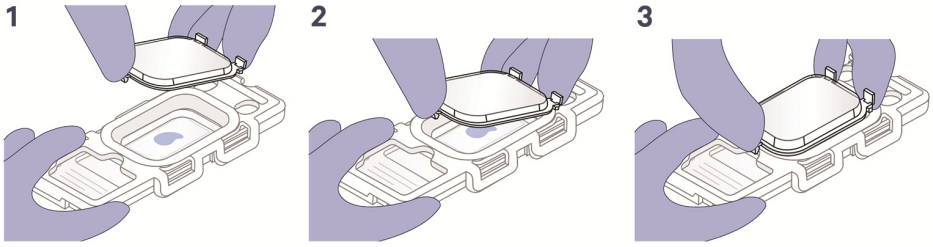
Lid Temperature	Reaction Volume	Run Time
80°C	100µL	-
Step	**Temperature**	**Time**
Hold	22°C	Hold
Decrosslinking	80°C	00:30:00
Re-equilibrate	22°C	00:10:00
Hold	22°C	Hold

c. Remove 1X PBS from step 1.3b.

d. Add **500 µl** Decrosslinking Buffer along the side of the well to uniformly cover the tissue sections, without introducing bubbles. Tap Xenium Cassette gently to ensure uniform coverage.

e. Apply **a new** Xenium Cassette Lid on the Xenium Cassette and place the cassette on the Thermocycler Adaptor at **22°C.** Close the thermal cycler lid.

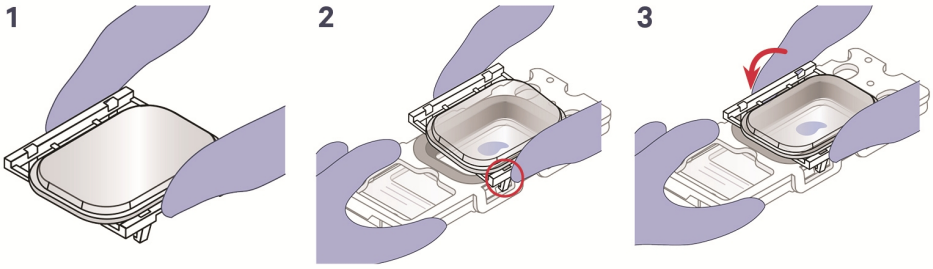
Applying the Xenium Cassette Lid



1. Hold the Xenium Cassette Lid with index and middle finger on two upper tabs and thumb on the lower clip.
2. Align lid with the surface of the Xenium Cassette. Hook the two upper clips into the two holes on the top of the cassette.
3. Push the lid down until the lower clip clicks into place. Inspect the lid to confirm placement.

OR

Applying the Xenium Cassette Lid v2



1. Hold the Xenium Cassette Lid v2 with thumb and index finger on the long sides.
2. 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.
3. Slowly push down on the other side of the lid until that side clicks into place. Inspect the lid to confirm placement.

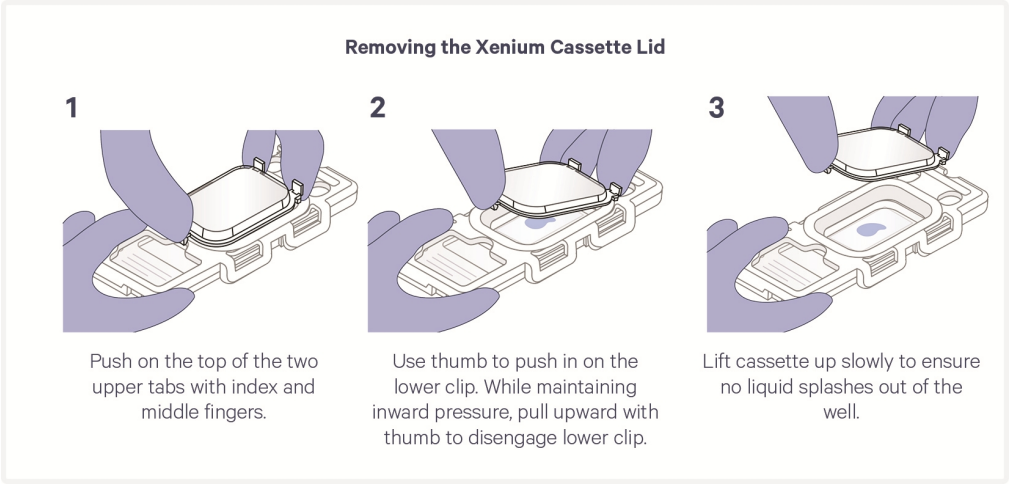
f. Skip Hold step and initiate Decrosslinking.

TIPS *Start thawing reagents for the relevant downstream workflow.*

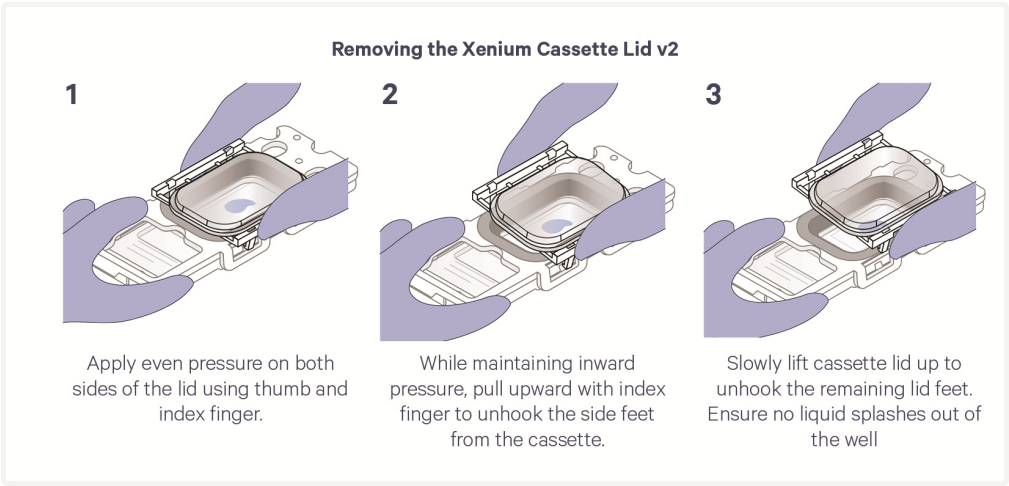
g. Remove Xenium Cassette from the thermal cycler and place on a flat, clean work surface.

DO NOT proceed with assay if slide is cracked or broken. Cracked or broken slides will result in assay failure.

h. Remove the Xenium Cassette Lid and using a pipette, remove all Decrosslinking Buffer from the well.



OR



Three PBS-T Washes:

PBS-T washes DO NOT require sealing of the cassette with a lid.

i. **Wash 1:** Add **500 µl** PBS-T to the well. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

j. **Wash 2:** Add **500 µl** PBS-T to the well. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

k. **Wash 3:** Add **500 µl** PBS-T to the well. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

l. Add **500 µl** PBS-T to the well.

Optional: photograph the slide against a black background. This image can be used for comparison purposes to identify tissue detachment downstream

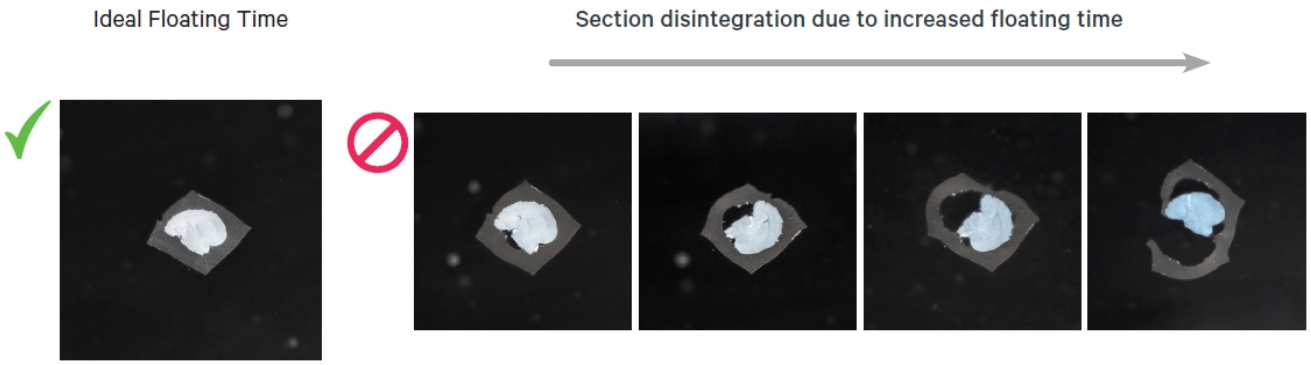
in the workflow. Work quickly as this is not a safe stopping point. See Troubleshooting for more details.

m. Proceed directly to the relevant user guide:
This is not a safe stopping point.

- 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)
- Xenium In Situ Gene and Protein Expression with Cell Segmentation Staining (CG000819)

Troubleshooting

Ideal Floating Time Determination

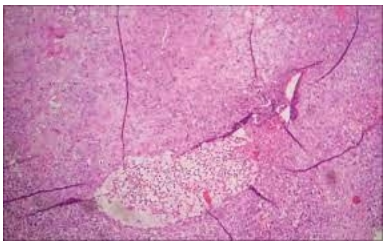


Common Issues when Processing FFPE Tissue Blocks

Section Phenotype	Tissue Characteristic	Guidance
Tissue is very dry	High Connective Tissue or High Cell Density Tissue	Soak block for an extended period of time. Re-soak tissue block every few sections.
Shattering, rapid expansion	High Adipose Tissue	If tissue is shattering, ensure tissue block is very cold by soaking the block in ice water for 10 min, re-soaking every few sections. If tissue expansion is occurring, lower water bath temperature to 37-39°C.
Rapid expansion	Low Cell Density Tissue	Lower water bath temperature to 37-39°C.

Common Artifacts that cause Detachment or Misleading Data

Wrinkles



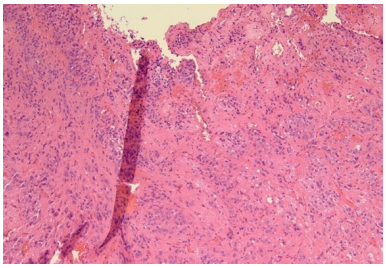
Causes

- Section compression (due to warm block or dull blade) during sectioning leads to wrinkle formation. These wrinkles become permanent when placed in the water bath.
- Accumulated wax or static electricity on microtome parts also contribute to section compression.

Troubleshooting

- Ensure that the block is well hydrated.
- Adjust temperature down and increase float time.
- Gently and gradually lay FFPE sections onto water bath surface, lengthwise.
- Utilize a new blade.
- Ensure microtome is cleaned with 100% ethanol to minimize static and section compression (bunching on blade).

Folds



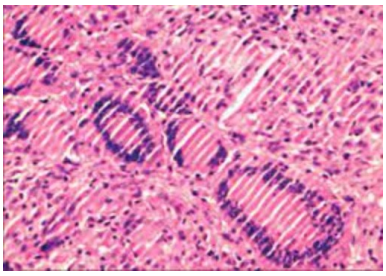
Causes

- Mostly happens when placing the section on the water bath especially when the section is uneven.
- If the fold is at the edge this most likely can happen during sectioning or mounting on the slide.

Troubleshooting

- Gently and gradually lay FFPE ribbons or sections onto water bath surface, lengthwise.
- If sections curl during sectioning, gently flatten them with a brush before floating.

Venetian Blinds or Shatter



Causes

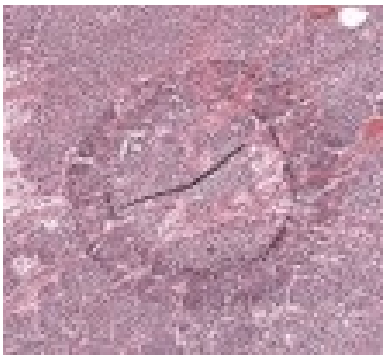
- Parallel lines in the section mostly appear due to dry tissue because of under- hydration of the block in the ice bath.
- Less likely due to dull blade or loose parts of the microtome.

Troubleshooting

- Increase incubation time of the block in ice bath.
- Tighten down components of microtome and make sure the blade is at a correct angle.

Common Artifacts that cause Detachment or Misleading Spatial Data

Air Bubbles



Causes

- Air bubbles from the bottom of the water bath can rise and stick under the section.

Troubleshooting

- Using a brush, gently remove the bubbles from the bottom of the water bath before floating the sections.

Waves

May be block-specific and easily observed under the microscope right after the section is picked up from the water bath. Minor waves can disappear after longer flotation in the water bath. Larger waves can create wrinkles or folds that are permanent after drying of the section.

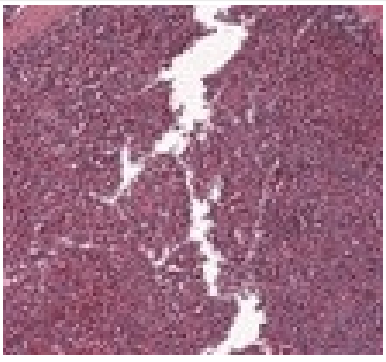
Causes

- Tissue incompletely infiltrated with wax absorbs water faster during hydration step. When sections from such blocks are floated, the parts that absorbed enough water will have difficulty flattening and become wavy.

Troubleshooting

- Chill the block on a cold block or on ice avoiding contact with water.
- Submerge the block for 5-15 min in the ice bath for gentle hydration.
- Increase flotation times and/or temperature of the water bath.

Cracks



Causes

- Dry and over-processed tissue can crack during sectioning.
- The cracks that are created before tissue embedding will be filled with wax when section is observed under the microscope after sectioning and wax will be washed away after deparaffinization or H&E staining.

Troubleshooting

- Prolonged hydration on the ice bath will most likely reduce the cracks.
- There is no solution for cracks created before tissue was embedded in wax.

Common Artifacts that cause Detachment or Misleading Spatial Data

Sweating

Causes

- Inadequate dehydration or under-processing of the tissue causes parts of the section to attract water and create this blistering effect. Such sections can disintegrate in the water bath.
- The blisters consist of:
  - Xylene or xylene substitutes if the cause is under-processing and insufficient removal of xylene or
  - Water droplets if the cause is inadequate dehydration.

Troubleshooting

- Be cautious about how long the block is kept in ice bath. Long incubation time in ice bath can impact section quality and thus should be avoided.
- Long flotation time in the water bath should be avoided.

Water Retention

Causes

- Sections from tissues that are under-processed or excessively hydrated before sectioning may retain water under the section mounted on a slide.
- Water will accumulate under section and cause uneven drying ultimately leading to detachment.

Troubleshooting

- Gently flicking the slide will help to get rid of the water. This is one of the most overlooked cause of detachment.

Disintegrating/Exploding Section

Causes

- Sections from tissues that are under-processed and not sufficiently infiltrated with wax can rapidly absorb water and explode or gradually disintegrate in the water bath.
- Tissues that are not sufficiently dehydrated can show similar phenotypes.
- Floating at high temperatures (42-50°C) can exacerbate the disintegration.

Troubleshooting

- Chilling of the block should be mostly performed on ice or cold block (30-60 min).
- Exposure to water in ice bath during chilling should be kept to a minimum (5-10 min).
- Flotation water bath temperature should be lowered to 38-40°C.

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 FFPE - Tissue Preparation Guide (Document CG000579) 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

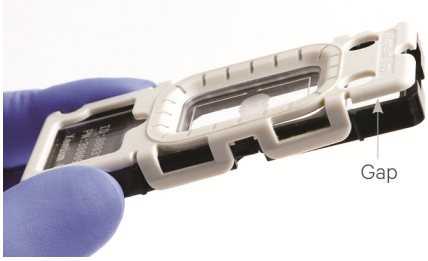
Incorrect assembly of the Xenium Cassette with a Xenium slide can negatively impact assay performance. Always dry the front and the back of the slide completely using a lint-free laboratory wipe while avoiding touching or damaging of the tissue sections. Inspect the slide carefully to ensure it is seated 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

Deparaffinization & Quality Assessment

Tissue slides should be deparaffinized before quality assessment (DAPI and H&E staining)

Deparaffinization Items		10x PN	Preparation & Handling	Storage
Obtain				
☐	Xylene	-	-	Ambient
☐	Ethanol	-	Prepare Ethanol dilutions using Nuclease-free water.	Ambient
☐	Nuclease-free Water	-	-	Ambient
☐	Forceps	-	-	Ambient
☐	Slide Rack	-	-	Ambient
☐	Coplin jars/Staining dishes	-	-	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

For Deparaffinization:

Prepare all buffers fresh according to the tables below.

a. Prepare eight total coplin jars for deparaffinization steps. Prepare Ethanol dilutions using Nuclease-free water.

For Deparaffinization	
Items	**Preparation & Handling**
Xylene	Label two coplin jars as Xylene Jar 1 and 2. Fill to capacity with xylene in each.
100% Ethanol	Label two coplin jars as 100% Ethanol Jar 1 and 2. Fill to capacity with 100% ethanol.
96% Ethanol	Label two coplin jars as 96% Ethanol Jar 1 and 2. Fill to capacity with 96% ethanol.
70% Ethanol	Label one coplin jar as 70% Ethanol Jar. Fill to capacity with 70% ethanol.
Nuclease-free water	Label one coplin jar as Nuclease-free Water Jar. Fill to capacity with Nuclease-free 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. Prepare fresh reagents after every 20 slides or every week (whichever comes first).*

Deparaffinization

Deparaffinization 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.

The instructions apply to both Xenium v1/Xenium Protein 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/Xenium Protein or Xenium Prime specific reagents and consumables are used.

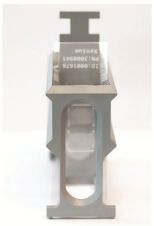
a. Retrieve the slide with tissue sections from the desiccator after overnight drying.

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

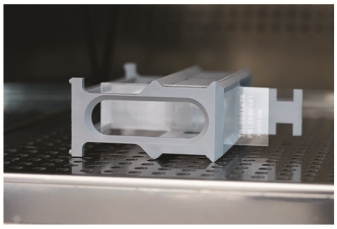
b. Place slide in a Section Dryer Oven and incubate uncovered at **60°C** for **30 min.**

Keep the oven lid closed during incubation.

Incubation in a Section Dryer Oven



Place slide in a rack

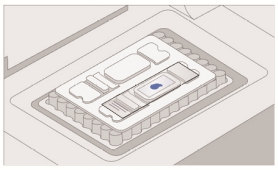


Place slide sideways and keep the oven door closed during incubation

Alternatively, place a Thermocycler Adaptor on a thermal cycler set at **60°C.** Place slide on the Thermocycler Adaptor with the tissue side facing up and incubate at **60°C** for **30 min**. DO NOT close the thermal cycler lid.

Place Thermocycler Adaptor

Incubate Slide for 30 min 60°C

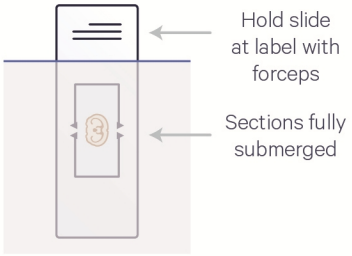


DO NOT close lid

c. Remove from the oven or thermal cycler and allow the slide to cool down to **room temperature** for **7 min**.

d. Gently immerse slide in the Xylene Jar 1. Secure the jar cap to prevent xylene loss.

Submerge in Xylene/Ethanol



Hold slide at label with forceps for xylene immersion steps. When immersing slides in xylene, ensure that the tissue sections are completely submerged.

e. Incubate for **10 min**.

f. Gently immerse slide in the Xylene Jar 2 and incubate for **10 min**.

g. Gently immerse slide in the 100% Ethanol Jar 1 for **3 min**.

Hold slide at label with forceps for ethanol immersion steps. When immersing slides in ethanol, ensure that the tissue sections are completely submerged.

h. Gently immerse slide in the 100% Ethanol Jar 2 for **3 min**.

i. Gently immerse slide in the 96% Ethanol Jar 1 for **3 min**.

j. Gently immerse slide in the 96% Ethanol Jar 2 for **3 min**.

k. Gently immerse slide in the 70% Ethanol Jar for **3 min**.

l. Gently immerse slide in the Nuclease-free water Jar for **20 sec**.

Proceed directly to DAPI staining. Leave slide in water 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.

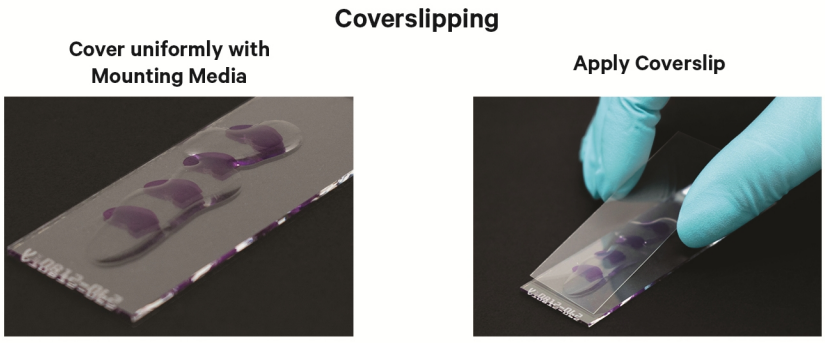
a. Place slide on a flat, clean, non-absorbent work surface.

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 1.5 DAPI Quality Assessment on page 43 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

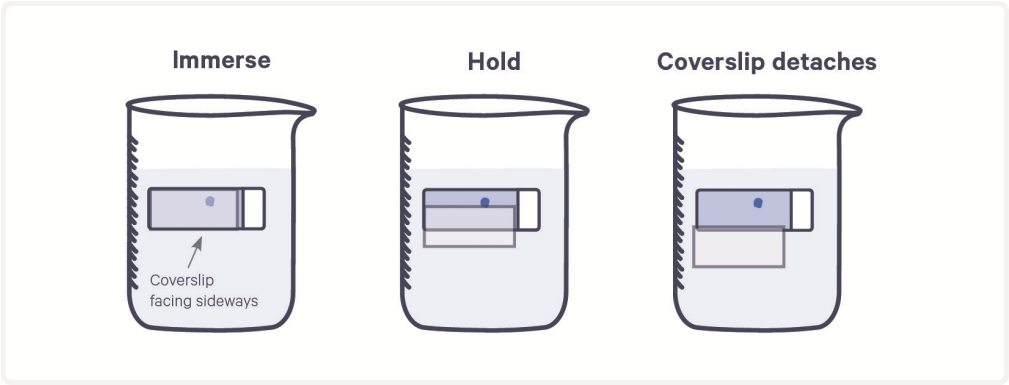
Coverslip Removal

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.

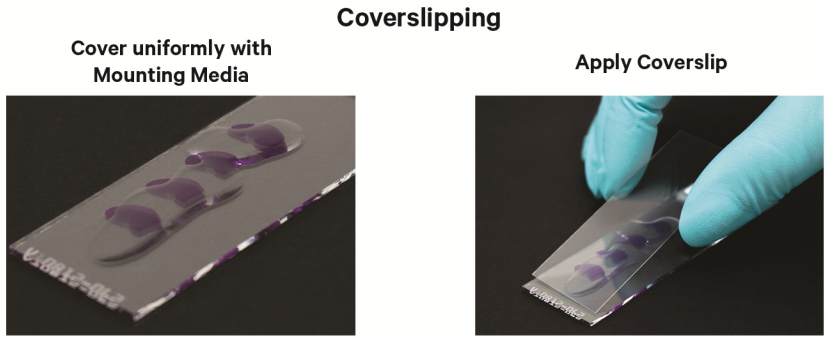
d. If needed, remove any large excess of mounting media by carefully wicking away from the edge of the coverslip with a lint-free laboratory wipe. Be careful not to move coverslip and disturb the tissue.

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 3. FFPE Tissue Sectioning & Section Placement on page 48 .



Slides in images are representative

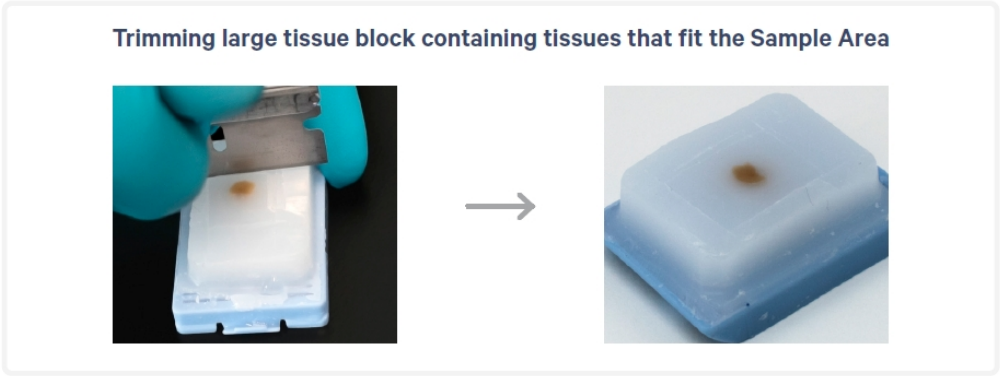
Optional Trimming/Scoring the Block

Before starting, wipe down all surfaces and work areas with RNaseZap RNase decontaminating solution. If necessary, rewipe the area with 100% ethanol to quickly dry the surface. Tissue sections placed on the Xenium slide should not obscure the fiducial frame. To ensure proper fit, the FFPE tissue block can be trimmed or scored as described below.

FFPE Tissue Block is larger than Sample Area, but tissue is smaller than Sample Area

For tissue samples that fit the Sample Area and are embedded in a large tissue block, the excess paraffin around the tissue can be trimmed to remove the excess and obtain smaller sections that fit the Sample Area.

- After facing, remove the tissue block from the microtome and start trimming.
- Using a razor blade, remove excess paraffin from around the tissue, so it is not larger than the Fiducial frame.
- Retain paraffin edges around the tissue samples to enhance tissue attachment on the slide.



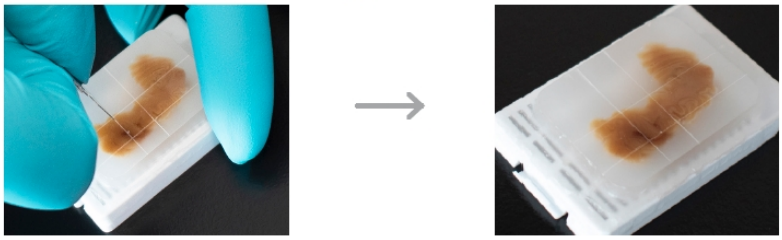
FFPE Tissue Block & Tissue are larger than Sample Area

Tissue samples larger than the Sample Area can be scored using a razor blade to generate smaller sections.

- Remove the tissue block from the microtome and start scoring.
- To score, lightly glide a razor blade over the surface of the tissue to introduce a shallow cut. This should yield approximately 10-15 trimmed

sections. A deep incision may lead to tissue damage and disintegration.

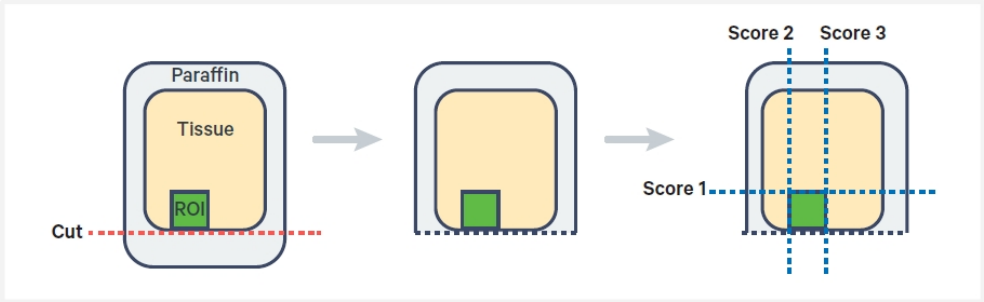
Scoring tissues larger than the Sample Area



Scoring Configurations

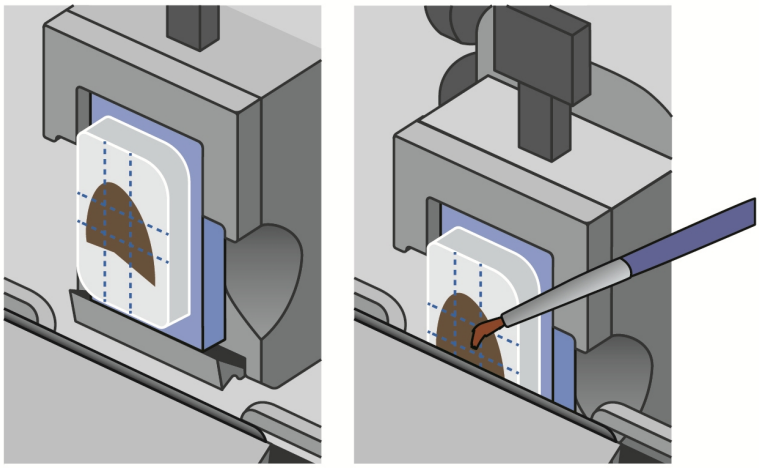
Region of interest (ROI) is at the Top or Bottom of the Tissue

- If the ROI is at the bottom of the tissue, remove excess wax such that only three scoring cuts are necessary.
- This configuration may prevent curling of the ROI, as the excess wax at the top of the block may curl before affecting the ROI
- Retain paraffin edges around the tissue samples to enhance tissue attachment on the slide.



ROI is in the Middle of the Tissue

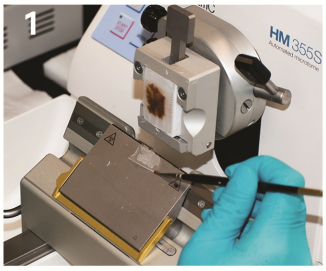
- If the ROI is in the middle of the tissue, make four scoring cuts as shown below.
- To prevent curling, place the brush over the edge of the ROI as the blade crosses the section. Avoid cutting the brush bristles on the blade.



Alternative Trimming or Scoring of FFPE Block & Tissue

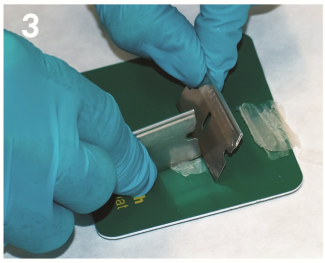
Keep all suface and materials RNase free. Avoid tearing of the FFPE sections during cutting. Keep the 5 µm FFPE sections from curling.

Set the microtome to 5 µm for tissue block and begin sectioning

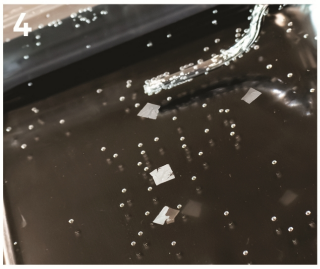


Collect sections with a paintbrush and place onto a cutting mat

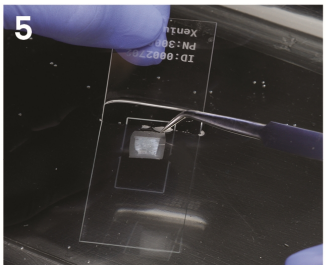
Cut tissue sections into to fit in the Sample Area of the Xenium slide



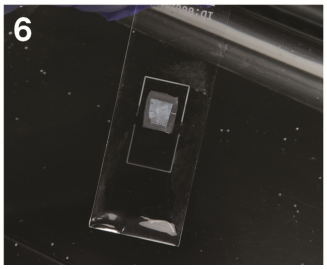
Place sections in the water bath



Align section with Sample Area



Pull the slide out of the water



Shipping Guidance

- After sectioning, slides can be stored at **room temperature** in a desiccator.
- When ready for shipment, place up to 2 slides in a slide mailer with desiccant in a sealed bag.
- Ship in a styrofoam or cooler box with cool packs.
- Upon arrival at destination, slides should be stored at **room temperature** in a desiccator.

RNA Quality Assessment of FFPE Tissue Block

This section provides guidance on determining the RNA quality of the tissue block by calculating DV200 of RNA extracted from freshly collected tissue sections.

Mean RNA fragment size is a reliable determinant of RNA quality. This requires a measurement of the percentage of total RNA fragments >200 nucleotides (DV200) for RNA quality assessment upstream of assay workflow. For more information on DV200, see DV200 Performance and Recommendations on page 44.

Before starting RNA extraction, apply RNaseZap to a paper towel and wipe work surface, centrifuge rotor and shafts of the pipettes. Rinse with RNase-free water and then wipe dry. The electrodes of the Bioanalyzer can also be cleaned with RNaseZap by following Agilent decontamination procedure.

a. Collect 10 µm tissue sections for RNA extraction during FFPE block sectioning. Discard the first few sections if the block was already exposed. The required number of sections depends upon tissue size. Consult RNA extraction kit manufacturer instructions to determine the appropriate number of sections. See below for guidance:

- Collect ~4 sections for smaller tissues (≤6.5 x 6.5 mm)
- Collect 1-2 sections for larger tissues (≥6.5 x 6.5 mm)

b. Place the sections inside a **pre-cooled**, RNase-free microcentrifuge tube. Sections may be stored at **–80°C** for **long-term** storage. For sections stored at **–80°C**, equilibrate to **room temperature** for **5 min** before adding the deparaffinization solution.

c. Proceed to RNA extraction using RNeasy FFPE Kit and follow manufacturer's instructions.

d. Using a Nanodrop or Qubit Fluorometer, measure RNA concentration to determine the appropriate dilution for DV200 evaluation using the RNA 6000 Pico Kit or TapeStation High Sensitivity Kit. RNA outside of the recommended concentration may lead to inaccurate DV200 evaluation.

e. Store purified RNA at **–80°C** for **long-term** storage or immediately proceed to DV200 evaluation using either the Agilent RNA 6000 Pico Kit or TapeStation High Sensitivity Kit. Follow manufacturer's instructions (Agilent) for DV200 evaluation.

Document Revision Summary

Document Number	CG000578
Title	Xenium In Situ – FFPE Tissue Preparation Handbook
Revision	Rev F
Revision Date	August 2025
Specific Changes	• Added information on Xenium In Situ Gene and Protein Expression to Additional Guidance on page 2 and Overview on page 5. • Added slide thickness to Xenium Slide Template on page 22. • Added deparaffinization and decrosslinking instructions to 4. Deparaffinization & Decrosslinking on page 53. • Added new preparation and handling guidance for Xenium FFPE Tissue Enhancer to 4.0 Get Started - Deparaffinization & Decrosslinking on page 57. • Added guidance about cracked slides to 4.4 Decrosslinking on page 65. • Updated Shipping Guidance on page 90. • Added QR code to Document Revision Summary • Updated for general minor consistency of language, format, and terms throughout.

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