Xenium In Situ for Fresh Frozen Tissues – Fixation & Permeabilization

Introduction

Xenium In Situ for Fresh Frozen Tissues is designed to measure mRNA in fresh frozen tissue sections and requires a Xenium slide with intact tissue sections as input. This protocol outlines fixation and permeabilization of fresh frozen tissue for use with 10x Genomics Xenium protocols. Fixed and permeabilized tissue sections are inputs for these downstream Xenium workflows:

- 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)

Additional Guidance

Consult the Xenium In Situ for Fresh Frozen - Tissue Preparation Guide (Document CG000579) for complete information on sectioning fresh frozen tissue and placing sections on Xenium slides. Process the slides with the tissue sections as described in this protocol (CG000581). This protocol is compatible with both Xenium In Situ (referred to as Xenium v1) and Xenium Prime In Situ reagents and downstream assay workflows as specified in the table. Follow any specific deviations indicated in the protocol for Xenium v1 versus Xenium Prime.

Compatible Reagent Kits & Downstream Workflows		
	Xenium v1	**Xenium Prime**
Reagent Kits	Xenium Decoding Consumables PN-1000487	Xenium Cassette Kit v2 PN-1000723
	Xenium Instrument Accessory Kit Module A PN-1000530	Xenium Thermocycler Adaptor v2 PN-1000739
	Xenium Cassette Kit PN-1000566	
Assay Workflows	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)

Items needed from each kit while executing this protocol are listed in the following sections.

Contents

Troubleshooting

Document Revision Summary

Xenium In Situ Gene Expression Reagent Kits

Compatible only with the following Xenium v1 workflows:

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

Refer to SDS for handling and disposal information.

Xenium Decoding Consumables (1 Run, 2 Slides) PN-1000487

Xenium Decoding Consumables
(1 run, 2 slides), PN-1000487
Store at ambient temperature

	#	PN
Xenium Cassette Kit (2 cassettes + 16 lids)	1	1000566
Extraction Tip	1	2000757
Pipette Tips	1	3000866
Xenium Buffer Cap	4	3000949
Xenium Objective Wetting Consumable	1	2000749
Deionized Water (bottle)	1	3001198
Xenium Sample Wash Buffer A (bottle)	1	3001199
Xenium Sample Wash Buffer B (bottle)	1	3001200
Xenium Probe Removal Buffer (bottle)	1	3001201

10x GENOMICS

Only the Xenium Cassette Kit (2 cassettes + 16 lids) (PN-1000566) is needed for this workflow.

Xenium Instrument Accessory Kit Module A PN-1000530

Xenium Instrument Accessory Kit Module A
PN-1000530
Store at ambient temperature

	#	PN
Waste Bottle	1	3000955
Xenium Waste Tip Tray	1	3000957
Xenium Thermocycler Adaptor	1	3000954

Only the Xenium Thermocycler Adaptor (PN-3000954) is needed for this workflow.

Xenium Cassette Kit - (2 Cassettes) PN-1000566

Xenium Cassette Kit (2 cassettes)
PN-1000566
Store at ambient temperature

	#	PN
Xenium Cassette lids (16 ct)	1	3001046
Xenium Cassettes (2 pack)	1	3000951

Purchase the Xenium Cassette Kit (2 cassettes) (PN-1000566) for additional cassettes 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 - (2 rxns) PN-1000720

Xenium Prime Sample Prep Reagents, Module A
(2 rxns), PN-1000720
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

Only FFPE Tissue Enhancer (PN-2000798) and Perm Enzyme B (PN-3000553) are needed in 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 Cassette Kit v2 - (2 cassettes) PN-1000723

Xenium Prime Cassettes and Inserts
PN-1000723
Store at ambient temperature

	#	PN
Xenium Cassette Top v2	2	3002205
Xenium Cassette Bottom v2	2	3002223
Xenium Cassette Lid v2	8	3002206
Xenium Cassette Insert	4	3001885

Xenium Cassette Insert is not needed in this workflow.

Xenium Thermocycler Adaptor v2- (1 adaptor) PN-1000739

Recommended Thermal Cyclers

Xenium v1 validated thermal cyclers:

Supplier	Description	Part Number
Bio-Rad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (discontinued)	1851197
Analytik Jena	Biometra TAdvanced 96 SG with 96-well block (silver, 0.2 mL) and gradient function	846-x-070-241 *(where x=2 for 230 V; 4 for 115 V; 5 for 100 V, 50-60 Hz)*
VWR	Gradient thermal cycler, XT^{96} Gradient, with 96-well gradient block and standard lid	76452-153
Marshall Scientific	MJ Research PTC-200 Thermal Cycler (discontinued)	05434-05

Xenium Prime validated thermal cyclers:

Supplier	Description	Part Number
Bio-Rad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (discontinued)	1851197
Bio-Rad	PTC Tempo Deepwell Thermal Cycler	12015392
Analytik Jena	Biometra TAdvanced 96 SG with 96-well block (silver, 0.2 mL) and gradient function	846-x-070-241 *(where x=2 for 230 V; 4 for 115 V; 5 for 100 V, 50-60 Hz)*
ThermoFisher Scientific	VeritiPro 96-well Thermal Cycler	A48141

Specific Reagents & Consumables

Fresh Frozen Tissue Sections: Fixation & Permeabilization

The listed items have been tested by 10x Genomics and perform optimally with the assay. **Substituting materials may adversely affect system performance.** For items with multiple options listed, choose option based on availability and preference. Refer to the manufacturer's website for regional part numbers.

For FF Tissue Sections: Fixation & Permeabilization				
	Item	**Description**	**Vendor**	**Part Number**
☐	PBS	PBS - Phosphate Buffered Saline (10X) pH 7.4, RNase-free	Thermo Fisher Scientific	AM9624
☐	Nuclease-free Water	Nuclease-free water (not DEPC-treated)	Thermo Fisher Scientific	AM9932/ AM9937
☐	Formaldehyde	Formaldehyde (37% by Weight/Molecular Biology)	Thermo Fisher Scientific	BP531
	or	Formaldehyde Solution	Millipore Sigma	252549, F8775, or 47608
	Paraformaldehyde	Paraformaldehyde 16% Aqueous Solution, EM Grade	Electron Microscopy Sciences	15710
☐	Ethanol	Ethyl Alcohol, 200 Proof, anhydrous	Millipore Sigma	E7023
		Ethanol absolute ≥99.5%, TechniSolv, pure (Europe Only)	VWR	83813.360DP
☐	10% Tween-20	Tween 20 Surfact-Amps Detergent Solution (10% solution) *(not 100% Tween diluted to 10%)*	Thermo Fisher Scientific	28320
		10% Tween-20	Bio-Rad	1662404
☐	Methanol	Methanol, for HPLC	Millipore Sigma	34860
☐	SDS	Sodium dodecyl sulfate solution (for molecular biology, 10% in H2O)	Millipore Sigma	71736
☐	Forceps	Tweezers, 4" Wafer Handling	Excelta Corp	491P-SA-PI
☐	Slide Mailers	Sim port Scientific LockMailer Tamper Evident Slide Mailer	Fisher Scientific	22-038-399
	Additional Materials			
☐	Dry Ice			
☐	Thermal Cycler *(see Recommended Thermal Cyclers)*			
☐	Slide drying rack			

For FF Tissue Sections: Fixation & Permeabilization	
☐	Fume Hood
☐	Vortex
☐	Ice bucket

This list may not include some standard laboratory equipment.

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.

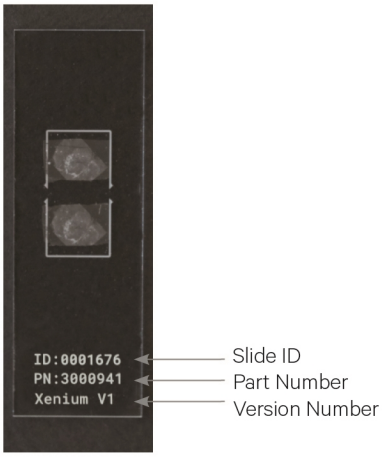
The instructions apply to both Xenium v1 and Xenium Prime assays. The consumables are referred to generically without always citing the version suffix. Based on the assay choice, always ensure that either Xenium v1 or Xenium Prime specific reagents and consumables are used.

Xenium Slide

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

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

Xenium Slide with Tissue Sections



Slide Storage

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

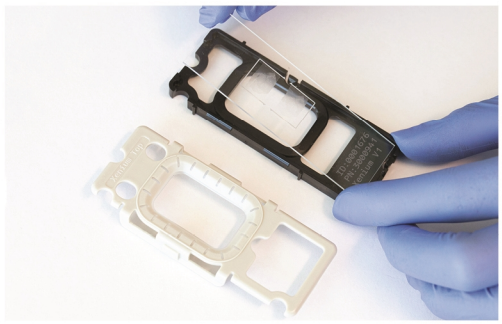
Store Slides in a Sealed Container

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

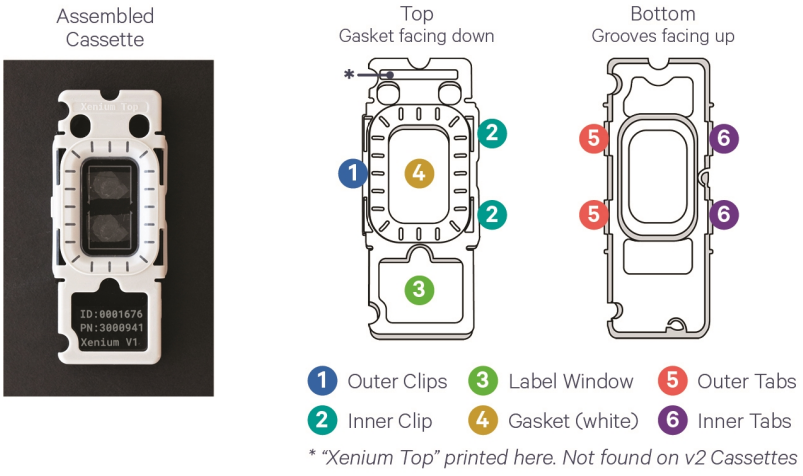
Xenium Slide Handling



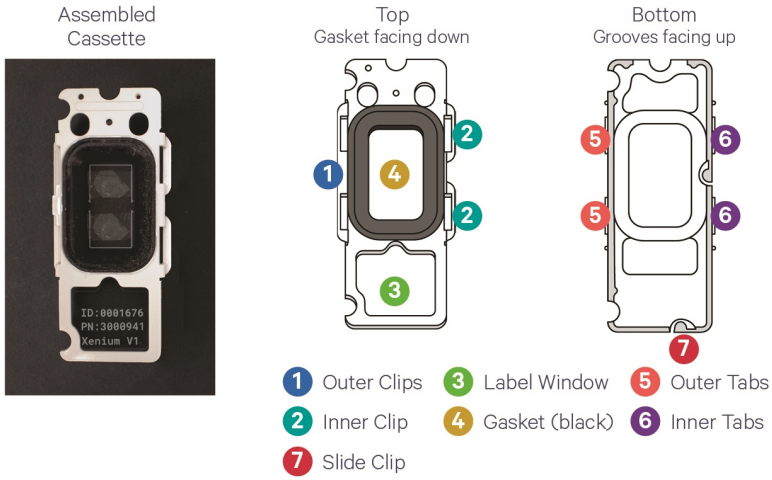
Note the Slide ID and Part numbers on the slide label will be required for downstream analysis.

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 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 is assembled manually. See Xenium Cassette Assembly & Removal instructions for details.
- 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.

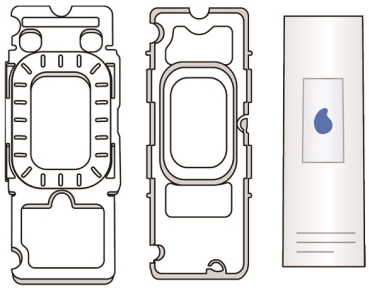
Xenium Cassette Assembly

Visually inspect cassette and gasket before cassette assembly.

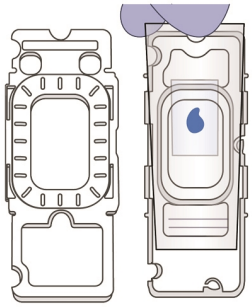
Xenium Cassette Assembly (for Xenium v1)

Exercise caution when handling slide edges to prevent injury.

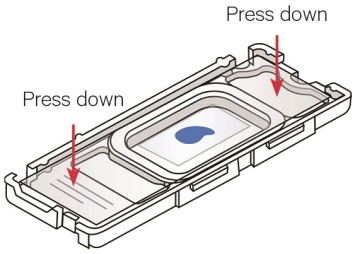
1 Place top and bottom halves of cassette on bench with the top cassette facing down and bottom cassette facing up.



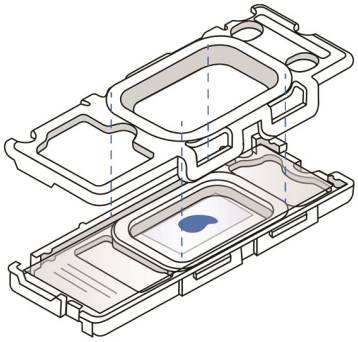
2 Place Xenium slide with tissue side facing upwards into bottom half of cassette; ensure label is toward bottom of cassette.



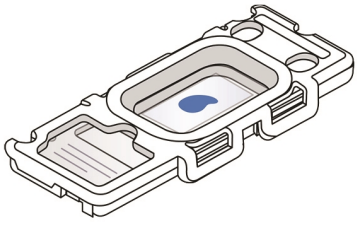
3 Press slide down into grooves of the bottom half of the cassette until it sits firmly in place.



4 Secure clips of top half with tabs of bottom half (on both sides).



5 Apply even pressure on top of cassette until all clips click shut. Verify that clips are completely secured over tabs.



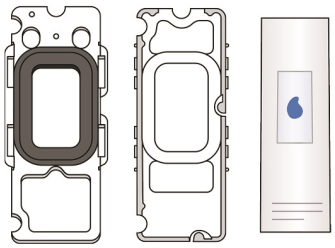
TIPS

Once cassette is assembled, DO NOT remove slide until after Xenium Analyzer imaging and decoding for optional H&E staining step.

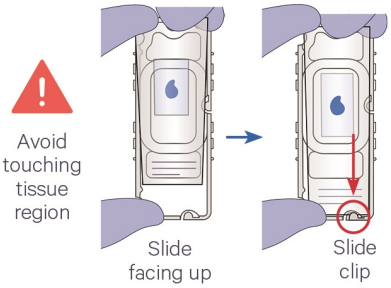
Xenium Cassette v2 Assembly (for Xenium Prime)

Exercise caution when handling slide edges to prevent injury.

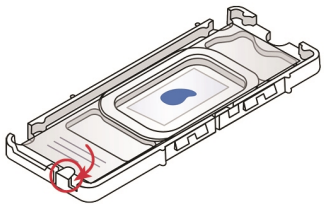
1 Place top and bottom halves of cassette on bench with the top cassette facing down and bottom cassette facing up.



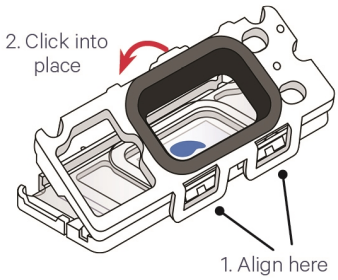
2 Place Xenium slide with label at bottom and tissue facing up into bottom half of cassette. Slip slide under slide clip located at bottom right of cassette.



3 After slide is safely underneath slide clip, place opposite side down into carrier. Ensure slide is sitting flat.

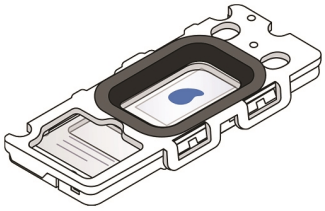


4 Align inner clips of top cassette to inner tabs of bottom cassette. Bring opposite side down until 2 audible clicks are heard.



5 Verify that clips are completely secured over tabs. Top cassette should sit flat and no gaps should be visible around entire cassette.*

**Consult Xenium Prime In Situ Gene Expresssion with optional Cell Segmentation Staining User Guide (CG000760) for cassette assembly failures.*

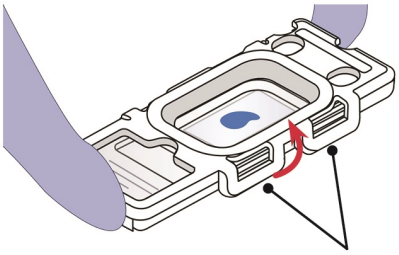


Xenium Cassette Removal

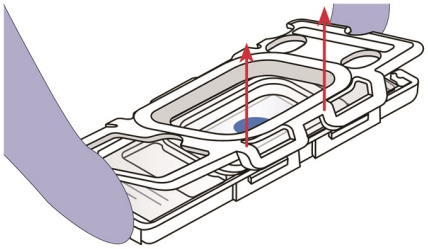
Xenium Cassette Removal (for Xenium v1)

Exercise caution when handling slide edges to prevent injury.

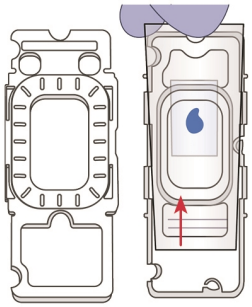
1 Pull inner clips from inner tabs to detach top and bottom halves of cassette.



2 Open cassette by continuing to lift inner clips upward.



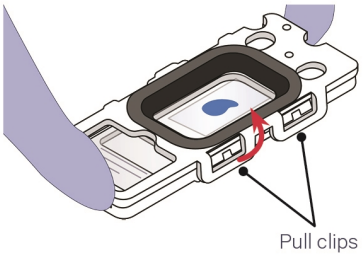
3 Hold slide by the label and lift slide out from bottom half.



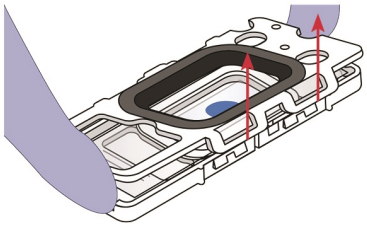
Xenium Cassette v2 Removal (for Xenium Prime)

Exercise caution when handling slide edges to prevent injury.

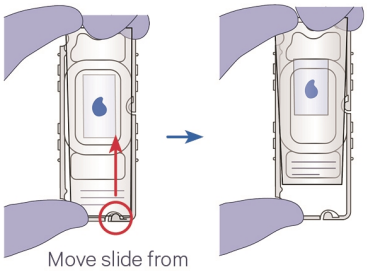
1 Pull inner clips from inner tabs to detach top and bottom halves of cassette.



2 Open cassette by continuing to lift inner clips of top cassette upward.

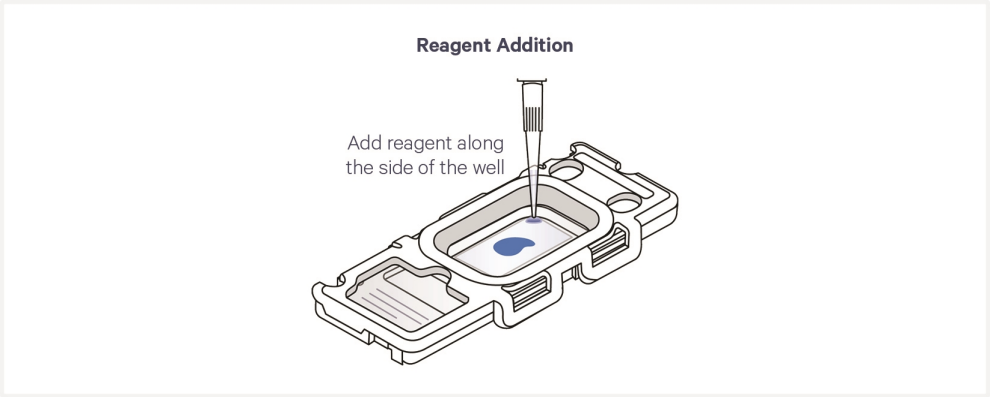


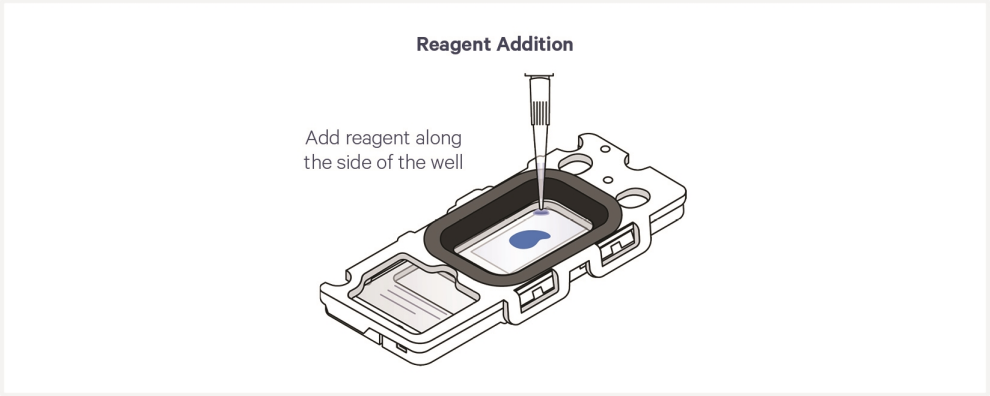
3 Hold top of slide and slowly lift slide out from underneath the slide clip. Then slowly remove from bottom cassette



Reagent Addition to Wells

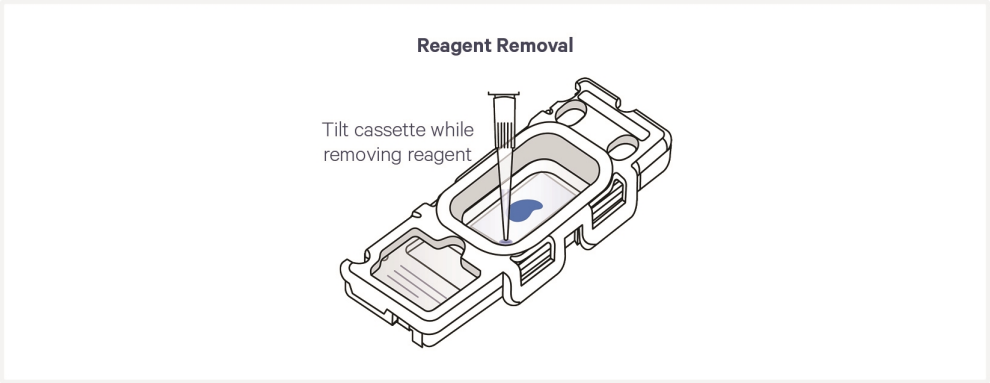
- Place assembled cassette flat on a clean work surface.
- Dispense and remove reagents along the side of the well without touching the tissue sections and without introducing bubbles.
- Always cover the Sample Area completely when adding reagents to the well. A gentle tap may help spread the reagent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

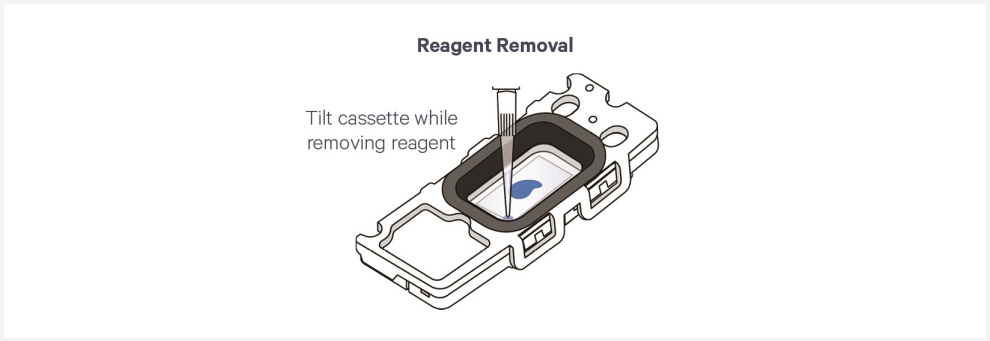




Reagent Removal from Wells

- 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.





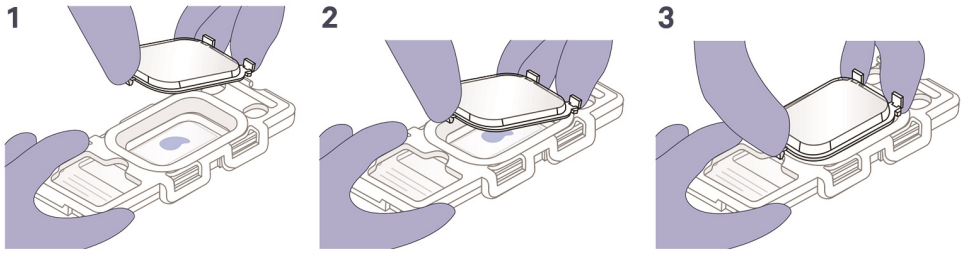
Xenium Cassette Lid Application & Removal

- 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.

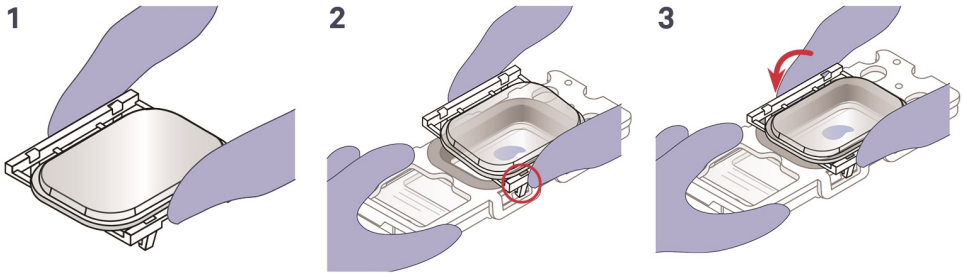
Application

Applying the Xenium Cassette Lid



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.

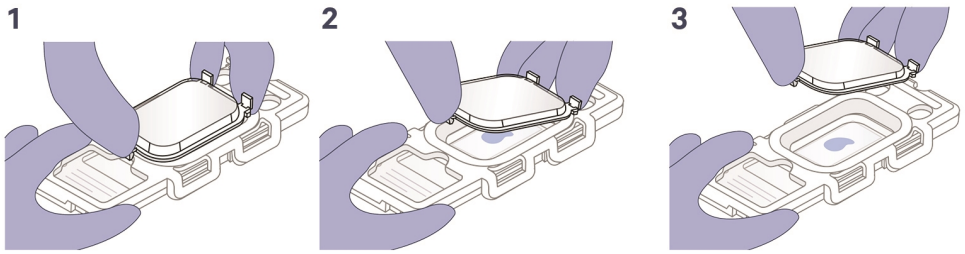
Applying the Xenium Cassette Lid v2



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.

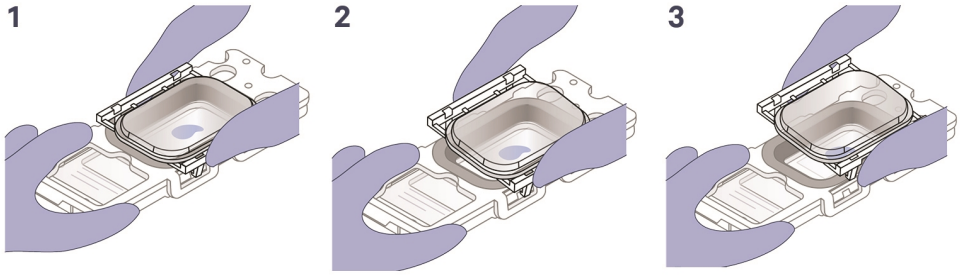
Removal

Removing the Xenium Cassette Lid



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

Removing the Xenium Cassette Lid v2



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

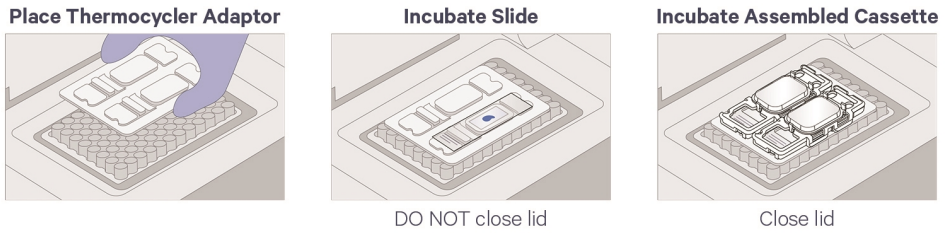
Slide Incubation Guidance

Incubation at a Specified Temperature

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

Incubation using a Thermal Cycler:

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



Incubation on Ice

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

Incubation at room temperature

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

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.

Protocol Steps & Timing

~2.5 hours

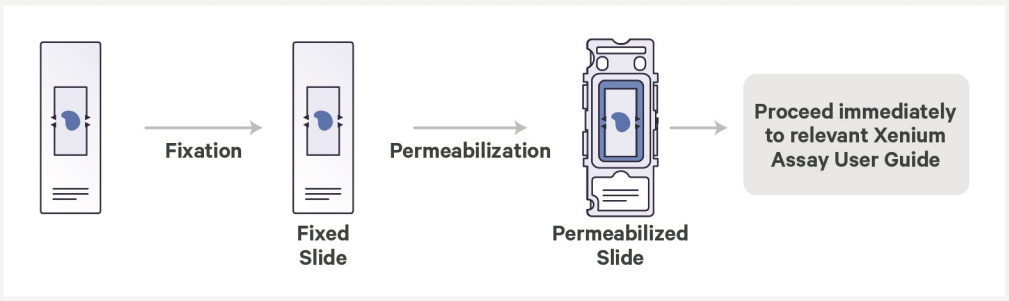
Steps		Timing	Stop & Store
Step 1 – Fixation & Permeabilization			
1.1	Preparation - Buffers	30 min	
1.2	Slide Preparation	5 min	
1.3	Fixation	30 min	
1.4	Permeabilization	65 min	
1.5	Cassette Assembly	10 min	

Note there are no safe stopping points during this workflow.

1. Fixation & Permeabilization

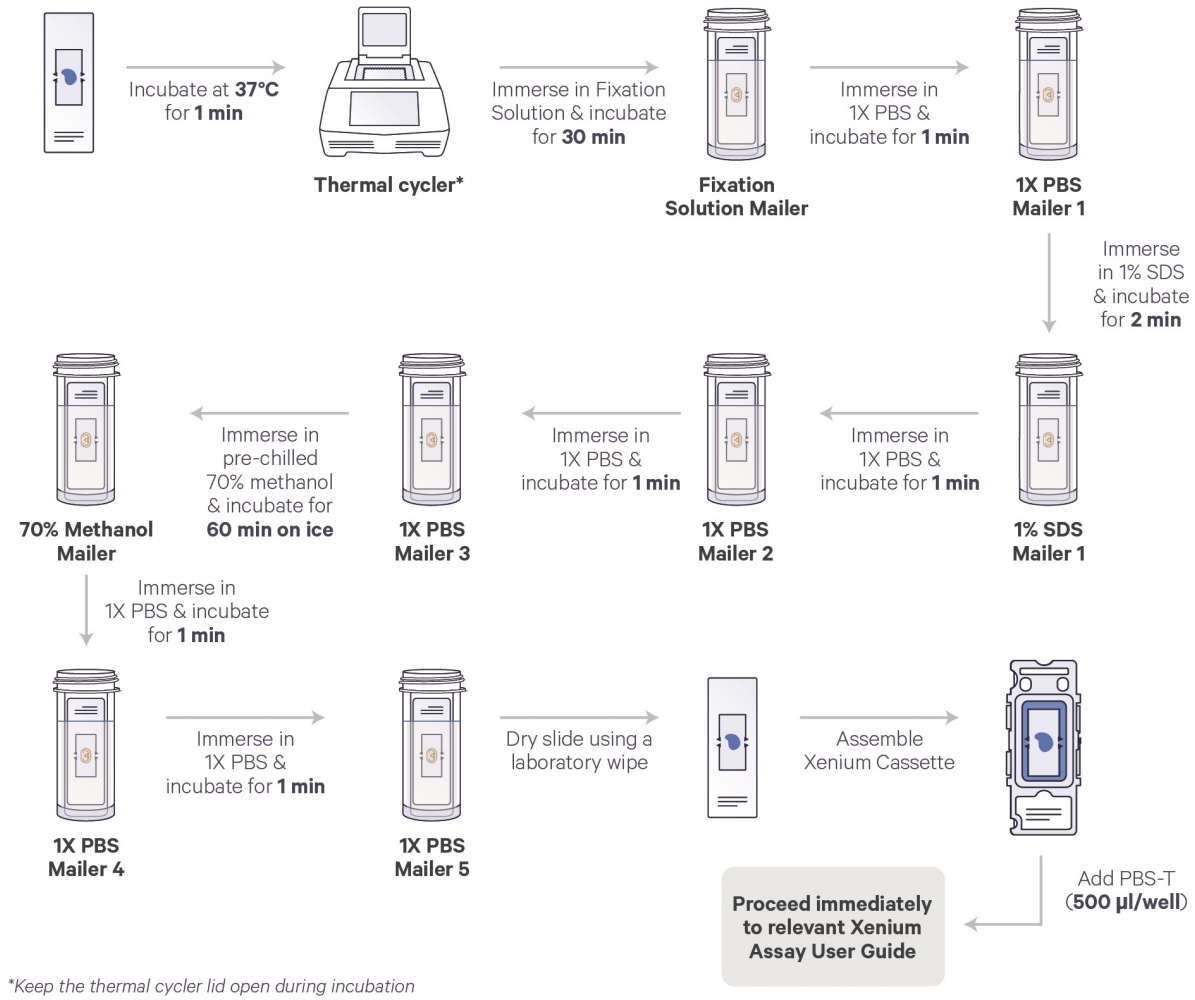
1.0 Overview

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



Protocol Overview

Fixation & Permeabilization ~100 min



Keep the thermal cycler lid open during incubation

Get Started - Fixation & Permeabilization

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

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

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

1.1 Preparation - Buffers

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

TIPS *Prepare buffers in appropriate sized conical tube or bottle and transfer carefully to corresponding slide mailer. Sale volume of buffer as per container size used.*

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

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

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

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

OR

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

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

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

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

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

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

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

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

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

f. Prepare eight total slide mailers for fixation.

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

1.2 Slide Preparation

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

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

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

b. Prepare an ice bucket of dry ice.

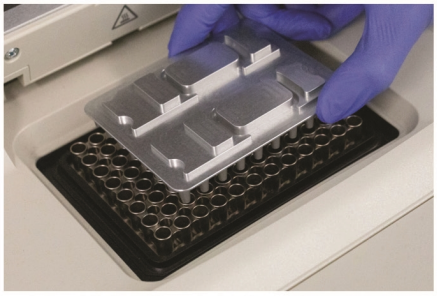
c. Remove slide mailer containing stored fresh frozen tissue slides from **-80°C** and bury into the dry ice.

TIPS

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

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

Incubation in a Thermal Cycler



Place Thermocycler Adaptor

Incubate Slide for 1 min at 37°C

1.3 Fixation

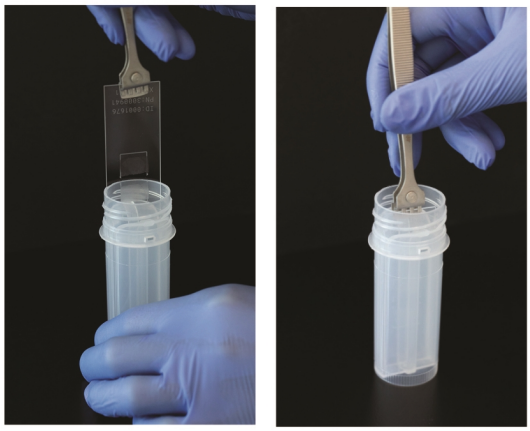
The instructions apply to both Xenium v1 and Xenium Prime assays. The consumables are referred to generically without always citing the version suffix. Based on the assay choice, always ensure that either Xenium v1 or Xenium Prime specific reagents and consumables are used.

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

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

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

Slide Immersion



TIPS

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

1.4 Permeabilization

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

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

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

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

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

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

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

Methanol Incubation

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

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

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

1.5 Cassette Assembly

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

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

TIPS

Refer to Cassette Assembly on page 37 for guidance on Xenium Cassette Assembly. Work quickly to avoid drying out of tissue sections.

b. Add **500 µl** 1X PBS-T.

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

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

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

Assembled Xenium Cassette

Xenium Cassette v1

Xenium Cassette v2

Troubleshooting

Tissue Detachment on Xenium Slides

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

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

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

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

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

Cassette Assembly Failure

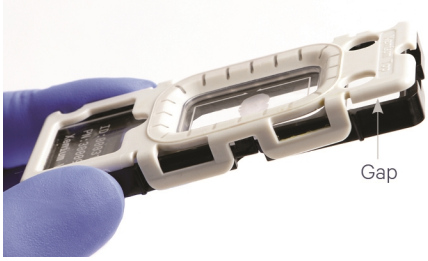
Incorrect assembly of the Xenium Cassette with a Xenium slide can negatively impact assay performance. Always dry the front and the back of the slide completely using a lint-free laboratory wipe while avoiding touching or damaging of the tissue sections. Inspect the slide carefully to ensure it is seated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

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

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

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

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

Correct cassette assembly	Incorrect cassette assembly
	

Document Revision Summary

Document Number	CG000581
Title	Xenium In Situ for Fresh Frozen – Fixation & Permeabilization Demonstrated Protocol
Revision	Rev D
Revision Date	June 2024
Specific Changes	Updated to include Xenium Prime: • workflow compatibility (page 1) • reagent & equipment compatibility (pages 6-8) • tips & best practices (page 16-26) • documents (pages 1, 38)
General Changes	Updated for general minor consistency of language and terms throughout.

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