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 In Situ Gene Expression protocols. Fixed and permeabilized tissue sections are inputs for the downstream Xenium In Situ Gene Expression - Probe Hybridization, Ligation & Amplification workflow.

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. After completing this Demonstrated Protocol (CG000581), proceed with the Xenium In Situ Gene Expression - Probe Hybridization, Ligation & Amplification User Guide (CG000582).

Contents

Xenium In Situ Gene Expression Reagent Kits

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	1	3000949
	Xenium Objective Wetting Consumable	1	2000749
●	Deionized Water	1	3001198
●	Xenium Sample Wash Buffer A	1	3001199
●	Xenium Sample Wash Buffer B	1	3001200
●	Xenium Probe Removal Buffer	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

10x GENOMICS

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

10x GENOMICS

Purchase the Xenium Cassette Kit (2 cassettes) (PN-1000566) for additional cassettes as needed.

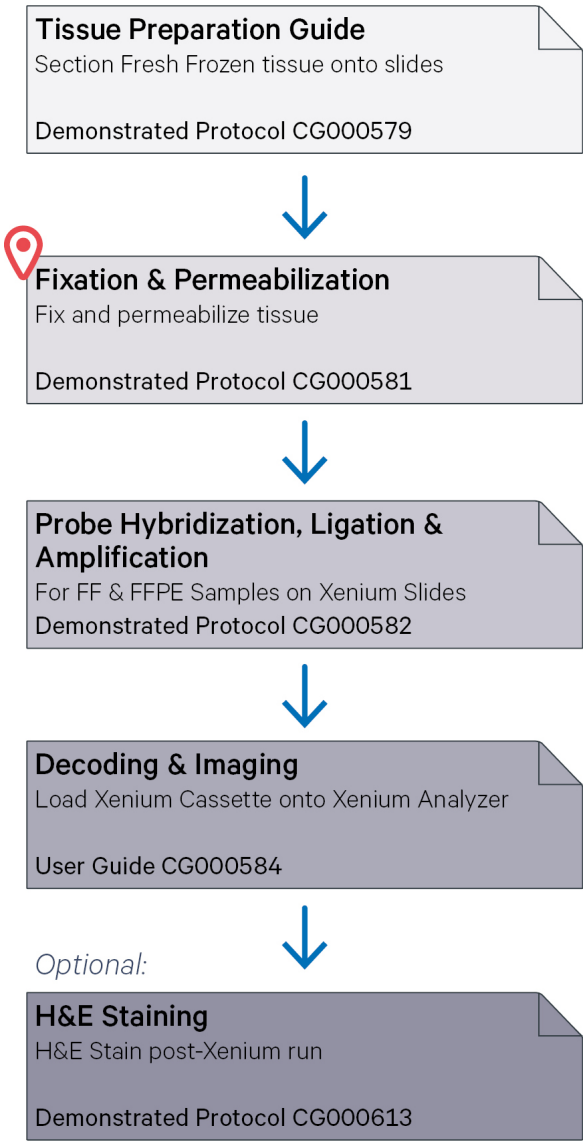
Recommended Thermal Cyclers

Supplier	Description	Part Number
Bio-Rad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module	1851197

Thermal cycler requirements if reactions are performed on a slide:

- Temperature-controlled lid
- 96 deep-well block or 0.2 ml block configuration
- The thermal cycler must be able to accommodate the Xenium Thermocycler Adaptor:
  - Well depth: 4.5 mm
  - Distance between block and heated lid: 12 mm
  - Reaction block dimensions: 95.5 x 73 mm

Workflow Overview



Visit the 10x Genomics Support website for the most current documentation.

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 or Paraformaldehyde	Formaldehyde (37% by Weight/Molecular Biology)	Thermo Fisher Scientific	BP531-500
		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)	Thermo Fisher Scientific	28320
☐	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 *(C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module, Bio-Rad, 1851197)*			
☐	Slide drying rack			
☐	Fume Hood			

For FF Tissue Sections: Fixation & Permeabilization	
☐	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.

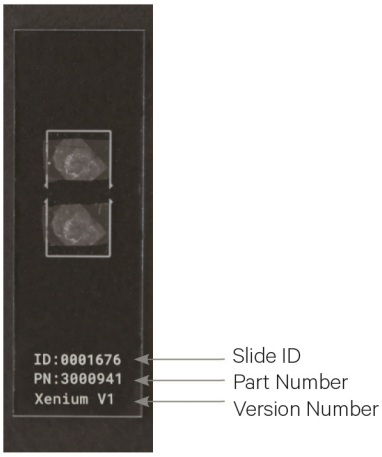
Pipette Calibration

- Follow manufacturer's calibration and maintenance schedules.

Xenium Slide

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

Xenium Slide with Tissue Sections



Slide Storage

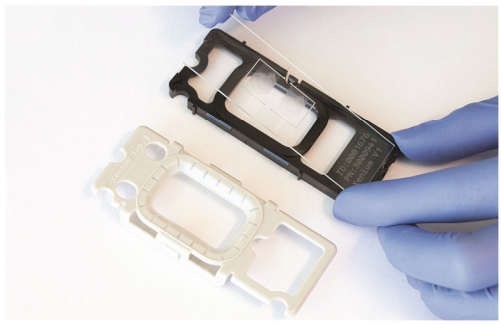
- Always store unused slides at -20°C in their original packaging and keep sealed. Once opened, slides should remain at room temperature in a dessicator and be used within one week.
- After tissue placement, store slides in a sealed container. If using an unsealed slide mailer, store in a secondary sealed 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.

Xenium Slide Handling



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

Xenium Cassette

- 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.

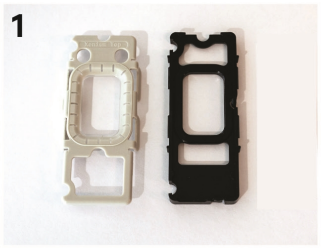
- The etched slide label is visible in the label window when properly assembled.
- Ensure that the Xenium Cassette and gasket are free of debris prior to assembly. If placing the top half of the cassette on a surface, ensure the gasket faces up so it does not collect debris.
- Visually inspect the gasket to ensure it is seated properly. If the gasket appears warped, the Xenium Cassette is still safe to use as long as the cassette can fully close.

Xenium Cassette Assembly

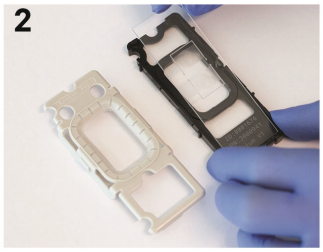
Ensure slide is completely dry before assembling into cassette.

Exercise caution when handling slide edges to prevent injury.

Place top and bottom halves of cassette on bench

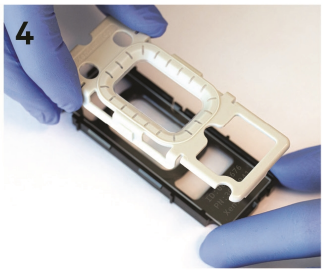


Place Xenium slide with tissue side facing upwards into bottom half of cassette; ensure label is toward bottom of cassette

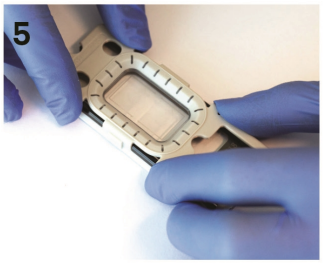


Press slide down into grooves of the bottom half of the cassette until it sits firmly in place

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



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

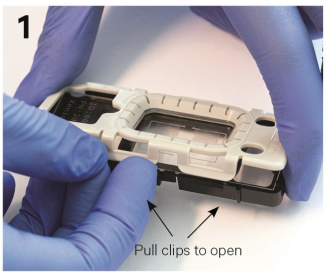


Slides in images are representative.

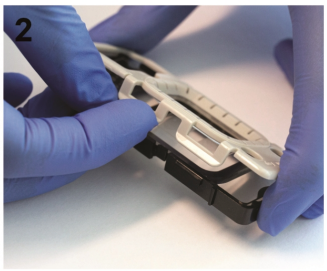
TIPS Once cassette is assembled, DO NOT remove slide until after Xenium Analyzer imaging and decoding for optional H&E staining step.

Xenium Cassette Removal

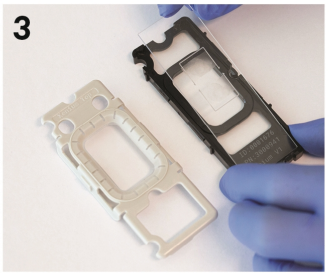
Pull inner clips from inner tabs to detach top and bottom halves of cassette



Open cassette by continuing to lift inner clips upward



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



Slides in images are representative.

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 Addition

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.

Reagent Removal

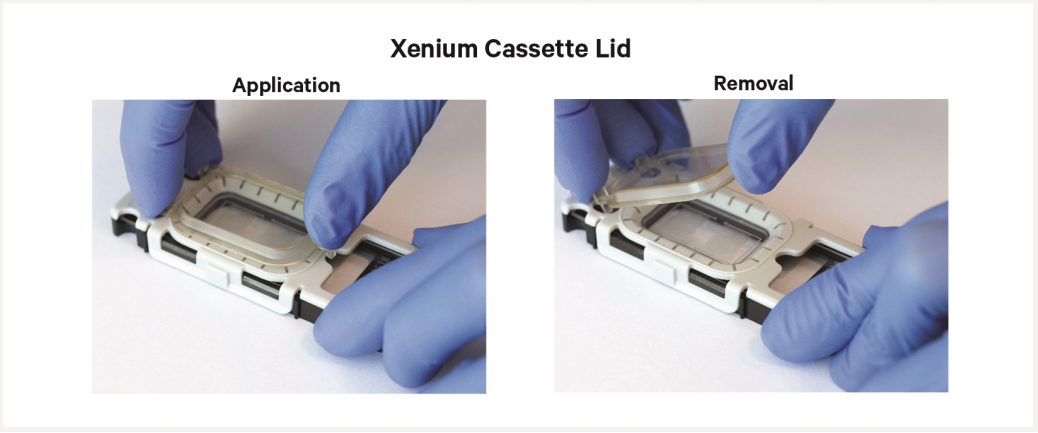
Xenium Cassette Lid Application & Removal

Application

- Place the Xenium Cassette flat on a clean work surface.
- Hold the Xenium Cassette Lid with index and middle finger on two upper tabs and thumb on the lower clip.
- Align the Xenium Cassette 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.

Removal

- Place the Xenium Cassette flat on a clean work surface.
- Push on the top of the two upper tabs with index and middle fingers.
- Use thumb to push in on the lower clip.
- While maintaining inward pressure, pull upward with thumb until the lower clip disengages.
- Ensure that no liquid splashes out of the well.



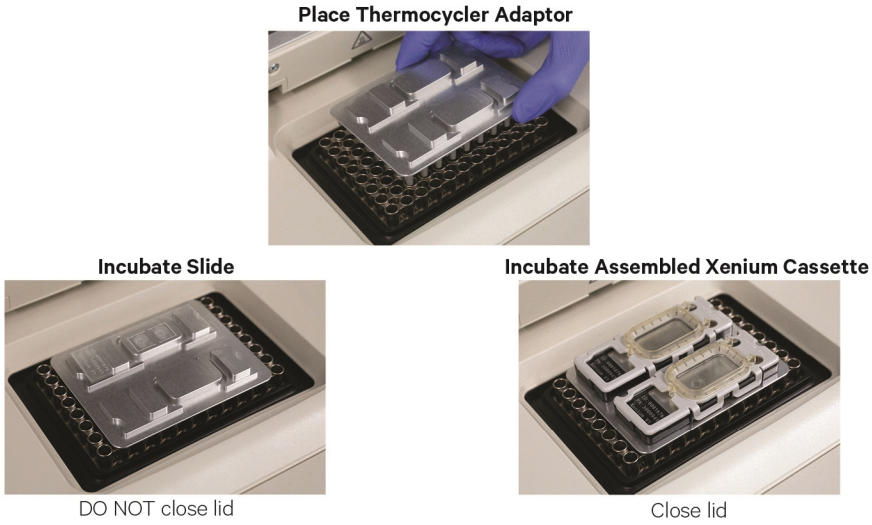
Note that Xenium Cassette Lids are a single use item and should be discarded after each use.

Slide Incubation Guidance

Incubation at a specified temperature

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 prior to 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 slide(s) 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 the last 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 slide(s) 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 the first or last position 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

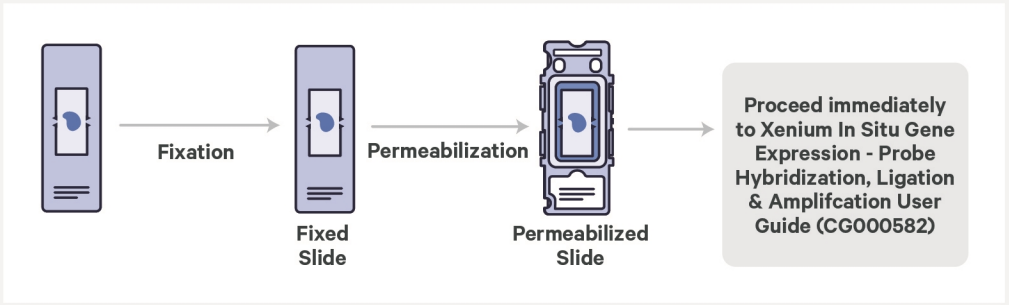
Steps		Timing	Stop & Store
Step 1 – Fixation & Permeabilization			
1.1	Preparation - Buffers	30 min	
1.2	Slide Preparation	5 min	
1.3	Fixation	30 min	
1.4	Permeabilization	65 min	
1.5	Cassette Assembly	10 min	

Note there are no safe stopping points during this workflow.

1. Fixation & Permeabilization

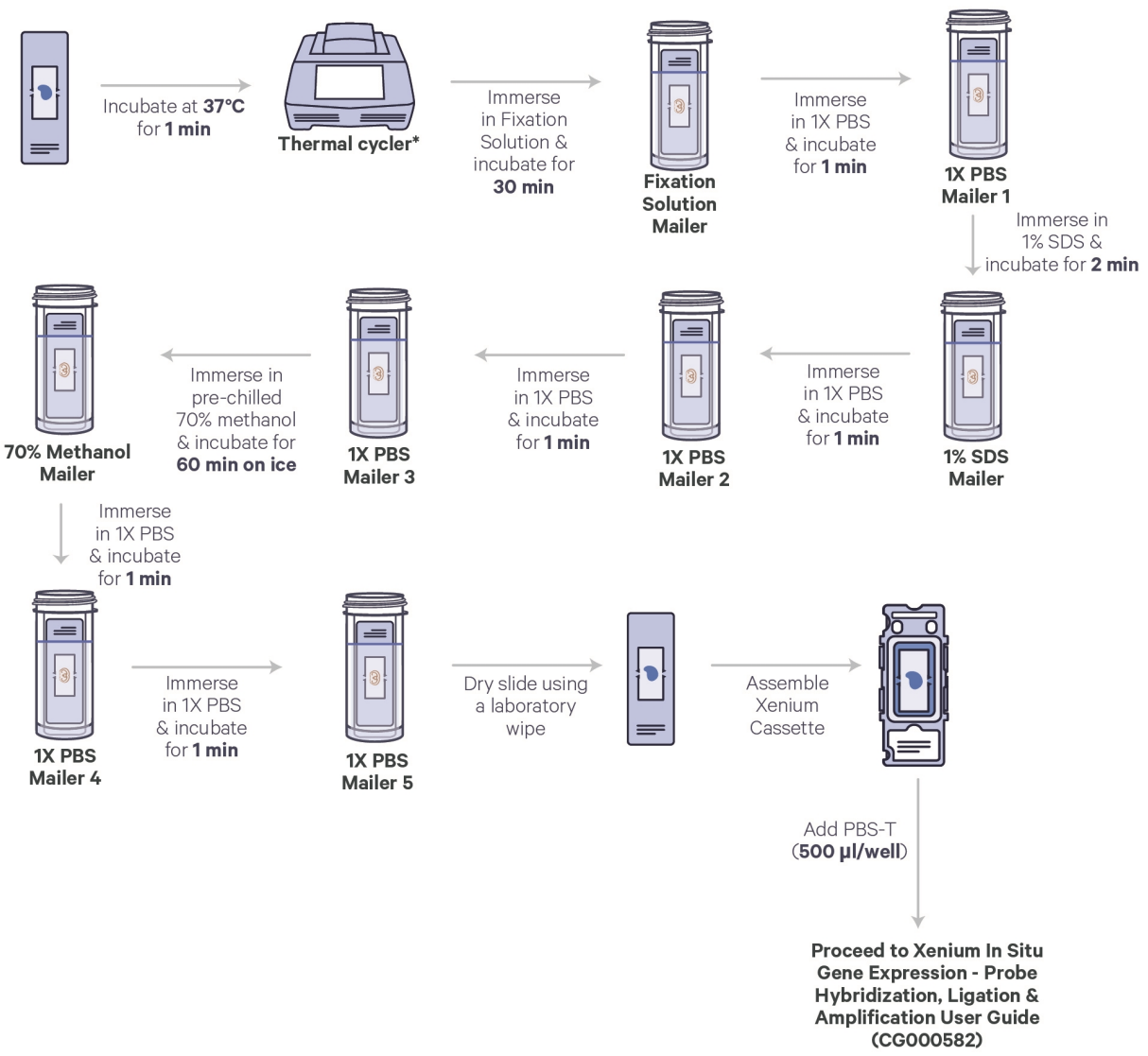
1.0 Overview

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

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.*

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	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). Mix SDS by vortexing stock thoroughly before making dilution. Verify no precipitate. 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 *(vortex stock, 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	-	-	1,990.0
☐	Tween-20	10%	0.05%	10.0
☐	**Total**	**-**	**-**	**2,000.0**

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

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.

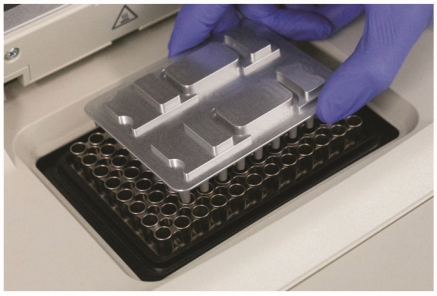
c. Remove slide mailer containing stored fresh frozen tissue slide(s) from **-80°C** and bury into the dry ice.

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 the slide(s) from dry ice to the **37°C** pre-heated thermal cycler for **1 min**. Place 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 the lid.

Incubation in a Thermal Cycler



Place Thermocycler Adaptor

Incubate Slide for 1 min at 37°C

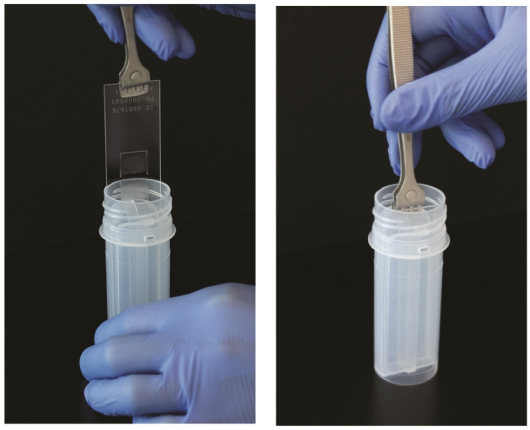
1.3 Fixation

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

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

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

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

TIPS *Refer to Tips & Best Practices for guidance on Xenium Cassette Assembly.*

b. Add **500 µl** 1X PBS-T.

c. Proceed **immediately** to Xenium In Situ Gene Expression - Probe Hybridization, Ligation & Amplification User Guide (CG000582).

Assembled Xenium Cassette

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.

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

Cassette Assembly Failure

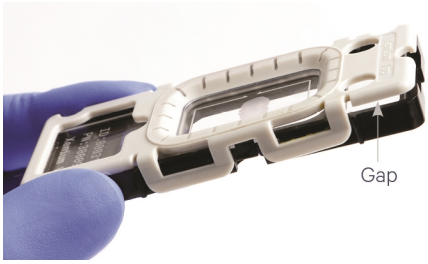
Incorrect assembly of the Xenium Cassette with a Xenium slide can negatively impact assay performance. Always dry the front and the back of the slide completely using a lint-free laboratory wipe while avoiding touching or damaging of the tissue sections. Inspect the slide carefully to ensure it is seated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

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
	

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