User Guide | CG000760 | Rev A

Xenium Prime In Situ Gene Expression

with optional Cell Segmentation Staining

For use with:

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

Xenium Cassette Kit v2 (2 rxns) PN-1000723

Xenium Prime 5K Human Pan Tissue & Pathways Panel (2 rxns) PN-1000724

Xenium Prime 5K Mouse Pan Tissue & Pathways Panel (2 rxns) PN-1000725

Xenium Prime 5K Custom Add-On Panel (up to 50 genes) (12 rxns) PN1000731*

Xenium Prime 5K Custom Add-On Panel (51 to 100 genes) (12 rxns) PN1000766*

Xenium Cell Segmentation Staining Reagents (2 rxns) PN-1000661

Xenium Thermocycler Adaptor v2 PN-1000739

*Refer to the 10x Genomics Support Website for the most current list of available panels and part numbers.

Notices

Document Number

CG000760 | Rev A

Legal Notices

Instrument & Licensed Software Updates Warranties

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

Support

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Document Revision Summary

Document Number

CG000760

Title

Xenium Prime In Situ Gene Expression with optional Cell Segmentation Staining User Guide

Revision

Rev A

Revision Date

June 21, 2024

Specific Changes

General Changes

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Introduction

Reagent Kits

Xenium Prime In Situ Gene Expression Reagent Kits

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

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*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 Prep Reagents, Module B
(2 rxns), PN-1000720
Store at −20°C

		#	PN
○	Post Hybridization Wash Buffer*	1	2000395
●	Ligation Buffer B		2001233
●	Ligation Enzyme A*	1	2000397
●	Ligation Enzyme B*	1	2000398
●	Amplification Enhancer Buffer	1	2001234
●	Amplification Enhancer	1	2001235
●	Amplification Enhancer Wash Buffer	1	2001236
●	Amplification Mix*	1	2000392
○	Reducing Agent B		2000087
●	Autofluorescence Mix*	1	2000753
●	Nuclei Staining Buffer*	1	2000762

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*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 5K Human Pan Tissue & Pathways Panel - (2 rxns) PN-1000724

Xenium Prime 5K Human Pan Tissue & Pathway Panel
(2 rxns), PN-1000724
Store at −20°C

	#	PN
XeP 5K Hu PTP Panel Priming Oligos	1	2001224
XeP 5K Hu PTP Panel Probes	1	2001225

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Xenium Prime 5K Mouse Pan Tissue & Pathways Panel - (2 rxns) PN-1000725

Xenium Prime 5K Mouse Pan Tissue & Pathway Panel
(2 rxns), PN-1000725
Store at −20°C

	#	PN
XeP 5K Ms PTP Panel Priming Oligos	1	2001226
XeP 5K Ms PTP Panel Probes	1	2001227

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Xenium Prime 5K Custom Add-On Panel (up to 50 genes) - (12 rxns) PN-1000731

Xenium Prime 5K Custom Add-On Panel (up to 50 genes)
(12 rxns), PN-1000731
Store at −20°C

	#	PN
Xenium 5K Custom Add-On Priming Oligos (up to 50 genes)	1	3000xxx
Xenium 5K Custom Add-On Probes (up to 50 genes)	1	3000xxx

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Xenium Prime 5K Custom Add-On Panel (51 to 100 genes) - (12 rxns) PN-1000766

Xenium Prime 5K Custom Add-On Panel (51 to 100 genes)
(12 rxns), PN-1000766
Store at −20°C

	#	PN
Xenium 5K Custom Add-On Priming Oligos (51 to 100 genes)	1	3000xxx
Xenium 5K Custom Add-On Probes (51 to 100 genes)	1	3000xxx

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Refer to the 10x Genomics website for the most updated list of available panels.

Xenium Cell Segmentation Staining Reagents - (2 rxns) PN-1000661

Xenium Cell Segmentation Staining Reagents
(2 rxns), PN-1000661
Store at −20°C

	#	PN
Xenium Block and Stain Buffer	1	2001083
Xenium Multi-Tissue Stain Mix	1	2000991
Xenium Stain Enhancer	1	2000992
Xenium Cassette Insert	2	3001885
Slide Seals (2 pack)	1	2001124

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DO NOT use the Slide Seals with Xenium Cassette v2.

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

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

**Includes one Xenium Thermocycler Adaptor v2, PN-3002207*

Recommended 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

Additional Kits, Reagents & Equipment

10x Genomics has tested all items listed below. These items 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.

Item	Description	Supplier	Part Number (US)
Plastics			
1.5 ml tubes	DNA LoBind Tubes, 1.5 ml	Eppendorf	022431021
	Low DNA Binding Tubes, 1.5 ml	Sarstedt	72.706.700
15 ml tubes	15 ml PP Centrifuge Tubes *(or equivalent)*	Corning	430791
50 ml tubes	Self-Standing Polypropylene Centrifuge Tubes (50 ml), sterile *(or equivalent)*	Corning	430921
Pipette tips	Tips LTS 20UL Filter RT-L20FLR *(or equivalent)*	Rainin	30389226
	Tips LTS 200UL Filter RT-L200FLR *(or equivalent)*	Rainin	30389240
	Tips LTS 1ML Filter RT-L1000FLR *(or equivalent)*	Rainin	30389213
Kits & Reagents			
Nuclease-free Water	Nuclease-free Water (not DEPC treated)	Thermo Fisher Scientific	AM9937
TE Buffer	TE Buffer, TRIS-EDTA, 1X Solution, pH 8.0 *(nuclease-free)*	Fisher Scientific	BP24731
PBS	PBS - Phosphate Buffered Saline (10X) pH 7.4, RNase-free	Thermo Fisher Scientific	AM9624
SSC Buffer, 20X	SSC Buffer 20x Concentrate	Millipore Sigma	S6639
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
Ethanol	Ethyl alcohol, Pure (200 Proof, anhydrous)	Millipore Sigma	E7023-500ML
Equipment			
Pipettes	Pipet-Lite LTS Pipette L-20XLS+	Rainin	17014392
	Pipet-Lite LTS Pipette L-200XLS+	Rainin	17014391
	Pipet-Lite LTS Pipette L-1000XLS+	Rainin	17014382
Forceps	Fisherbrand Curved Medium Point General Purpose Forceps *(or any equivalent forceps)*	Fisher Scientific	16-100-110
Blank Slides	Superfrost Plus Slides *(optional, if practicing Xenium Cassette Insert assembly)*	Fisherbrand	12-550-15

Item	Description	Supplier	Part Number (US)
Mini centrifuge	VWR Mini Centrifuge *(or any equivalent mini centrifuge)*	VWR	76269-064
Refrigerated Microcentrifuge	Eppendorf Microcentrifuge *(or any equivalent microcentrifuge)*	Eppendorf	5425 R
Thermomixer	Eppendorf ThermoMixer C *(or any equivalent Thermomixer)*	Eppendorf	5382000023
Thermoblock	Eppendorf SmartBlock 2.0 mL *(or any equivalent Thermoblock)*	Eppendorf	5362000035
Additional Materials			
Waterbath *(bead bath untested)*			
Thermal Cycler *(see Recommended Thermal Cyclers)*			
Ice bucket			
Vortex			
Lens-cleaning Paper or Lint-free Laboratory Wipes *(for wiping thermal cycler adaptor)*			
Ultrapure/Milli-Q Water for Water Bath *(recommended), from Milli-Q Integral Ultrapure Water System or equivalent*			
Fume Hood *(optional; only if performing Probe Hybridization PBS-T washes in the hood)*			

This list may not include some standard laboratory equipment.

Protocol Steps & Timing

Steps	Timing	Stop & Store
Day 1		
Step 1: Priming Hybridization (page 42)		
1.1 Buffer Preparation (page 45)	20 min	
1.2 Custom Add-on Priming Oligo Preparation (optional) (page 46)	10 min	
1.3 Priming Hybridization (page 47)	~ 1.8 h	
1.4 Post Priming Hybridization Wash (page 52)	40 min	
Step 2: RNase Treatment & Polishing (page 54)		
2.1 RNase Treatment (page 56)	30 min	
2.2 Polishing (page 58)	70 min	
Step 3: Probe Hybridization (page 60)		
3.1 Custom Probe Preparation (optional) (page 62)	10 min	
3.2 Probe Hybridization (page 63)	16-24 h (overnight)	
Day 2		
Step 4: Post Hybridization Wash (page 66)		
4.1 Post Hybridization Wash (page 68)	25 min	
Step 5: Ligation (page 70)		
5.1 Ligation (page 72)	40 min	
Step 6: Amplification (page 74)		
6.1 Amplification Enhancement (page 76)	~2 h	
6.2 Post Amplification Enhancement Wash (page 77)	5 min	
6.3 Amplification (page 78)	~1.6 h	
6.4 Post Amplification Wash (page 79)	15 min	
Step 7: Cell Segmentation Staining (page 80) *optional*		
7.1 Buffer Preparation (page 82)	20 min	
7.2 Block and Stain (page 83)	~1 h + 16-24 h (overnight)	
Day 3		
7.3 Stain Enhancement (page 86)	~1 h	
Step 8: Autofluorescence Quenching (page 88)		
8.1 Autofluorescence Quenching (page 90)	45 min	
8.2 Nuclei Staining (page 93)	10 min	STOP 4°C overnight (in the dark) STOP 4°C overnight or 1 week (in the dark)

Storing slides for more than recommended time risks decrease in sensitivity. These risks are dependent on many factors, including input tissue quality and how nuclease-free and microbe-free the workflow is.

Stepwise Objectives

Xenium Prime In Situ Gene Expression assays RNA at the subcellular level by using targeted probes in formalin fixed & paraffin embedded (FFPE) or fresh frozen (FF) tissue sections. FFPE tissue sections placed on Xenium Slides are deparaffinized and decrosslinked as described in Xenium In Situ for FFPE - Deparaffinization & Decrosslinking (Demonstrated Protocol – CG000580). FF tissue sections placed on Xenium slides are fixed and permeabilized as described in Xenium In Situ for Fresh Frozen - Fixation & Permeabilization (Demonstrated Protocol – CG000581).

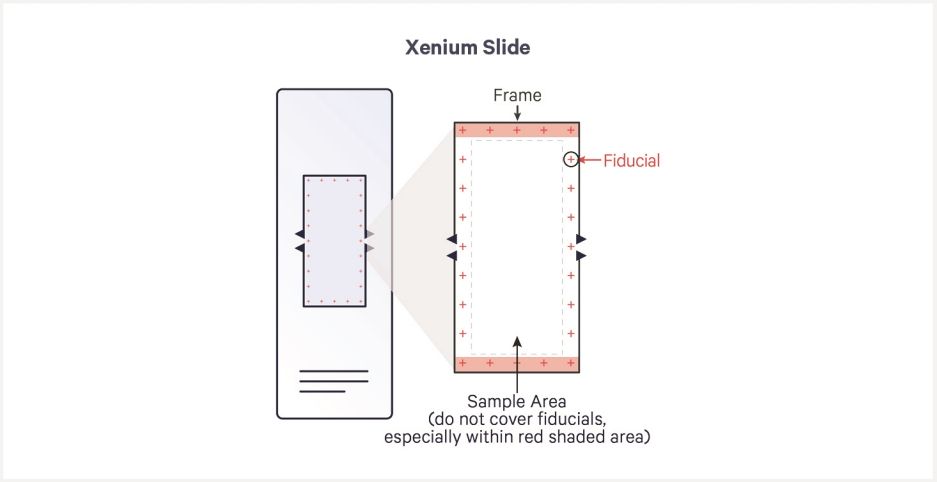
Xenium Prime pre-designed and add-on custom priming oligos are added to the tissue to hybridize with the target RNA. This is followed by RNase treatment to release the RNA strand from the hybridized priming oligos. A polishing step is performed followed by addition of Xenium Prime probes to the tissue for hybridization to the target RNA. The probe is ligated and the circularized probe is enzymatically amplified, generating multiple copies of the gene-specific barcode for each RNA target. Cell segmentation reagents added during an optional staining workflow allow for labeling of cell nuclei, membranes, and interiors that are inputs for automated morphology-based cell segmentation analysis.

Xenium slides containing FFPE or FF tissue sections are then loaded for imaging and analysis on the Xenium Analyzer instrument for high-throughput, automated in situ analysis. Fluorescently labeled oligos bind to the amplified DNA probes. Cyclical rounds of fluorescent probe hybridization, imaging, and removal generate optical signatures specific for each barcode, which are converted into a gene identity. Identified transcripts can be visualized using Xenium Explorer software.

This document outlines the protocol for generating Xenium Prime In Situ Gene Expression data from FFPE and FF tissue sections placed on Sample Areas of a Xenium slide.

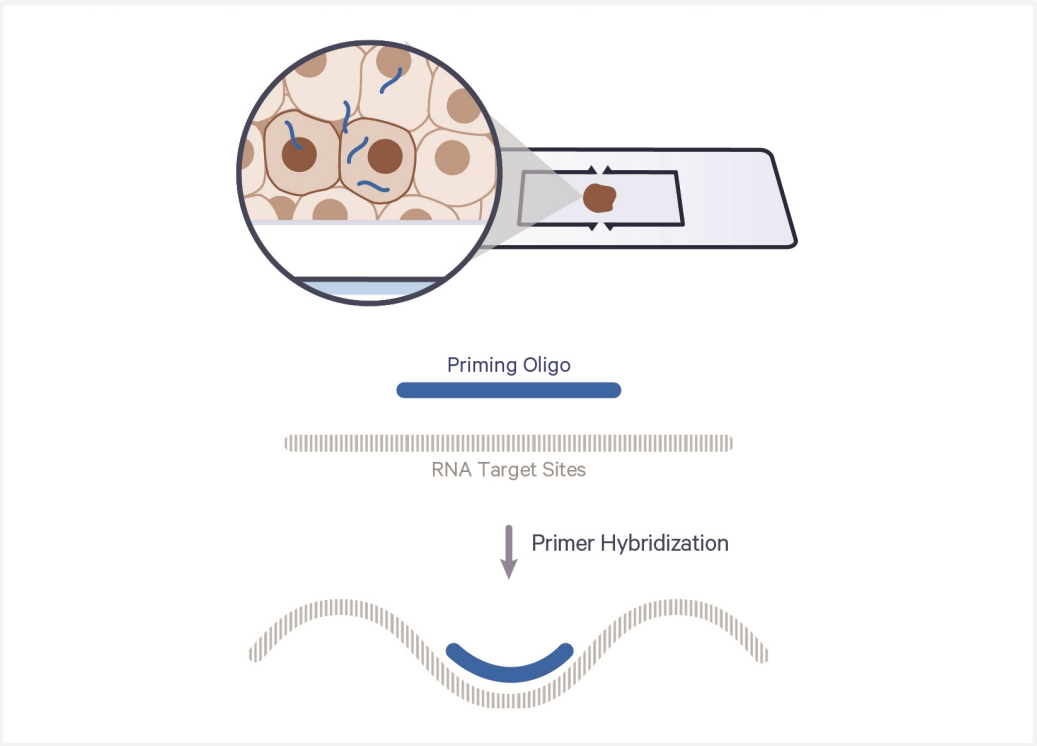
Xenium Slide

The Xenium slide has one Sample Area measuring 10.45 x 22.45 mm and is defined by a fiducial frame. The imageable area, measuring 12 mm x 24 mm, includes the area within the Sample Area + fiducial frame. FFPE or FF tissue sections are placed within the Sample Area for downstream processing and analysis.



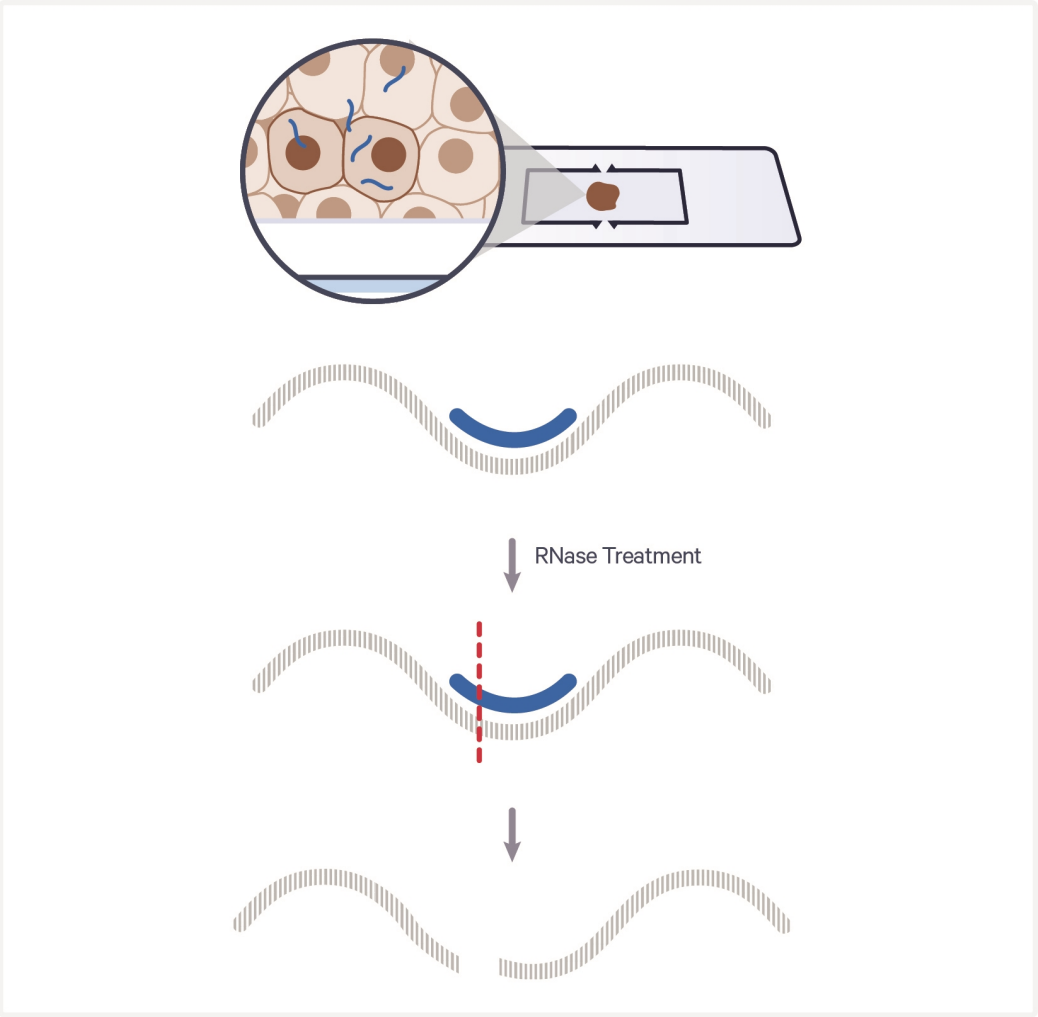
Step 1: Priming Hybridization

Xenium Prime 5K pre-designed and add-on custom priming oligos are added to the FFPE or FF tissue sections. The primimg oligos hybridize to target RNA. Excess, unbound priming oligos are washed away in the post priming hybridization wash step.



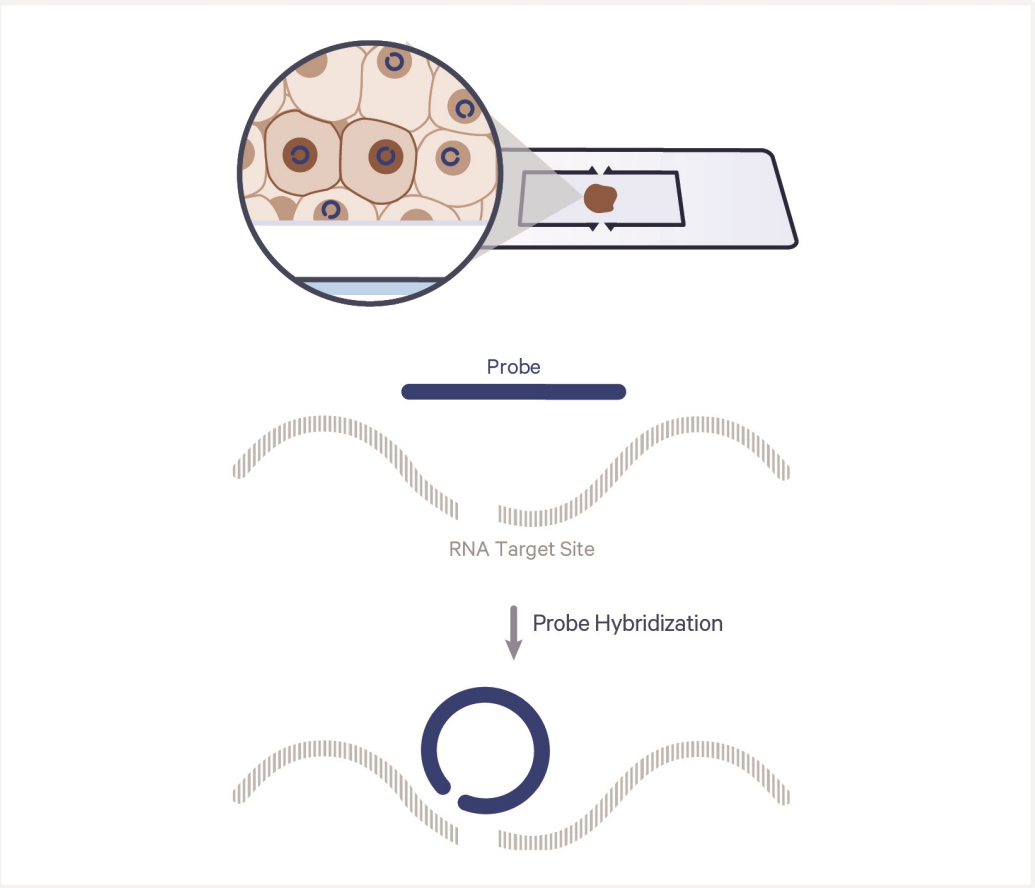
Step 2: RNase Treatment & Polishing

RNase treatment releases the RNA strand from the hybridized priming oligos. This is followed by a Polishing step that prepares the RNA template for the next step.



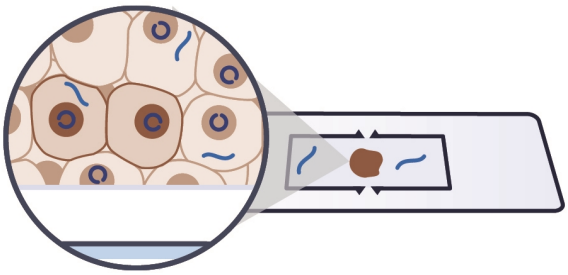
Step 3: Probe Hybridization

Xenium Prime 5K pre-designed and add-on custom probe panels are added to the FFPE or FF tissue sections. The DNA probes hybridize to the target RNA.



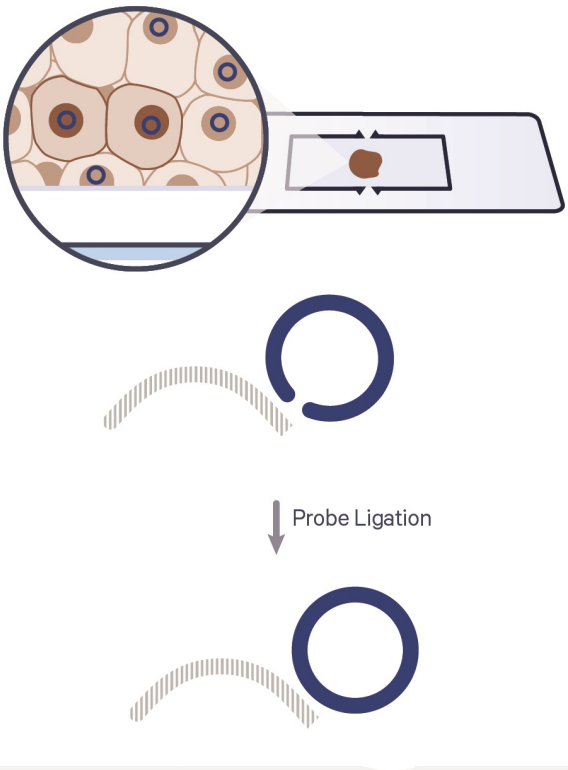
Step 4: Post Hybridization Wash

Excess, unbound probes are washed away in the post hybridization wash step.



Step 5: Ligation

After removal of unbound probes, a ligase is added to seal the junction between the probe regions that have hybridized to RNA. Ligation of the probe ends on the targeted RNA region generates a circular DNA probe. This ligation ensures a unique level of probe specificity to the target region.

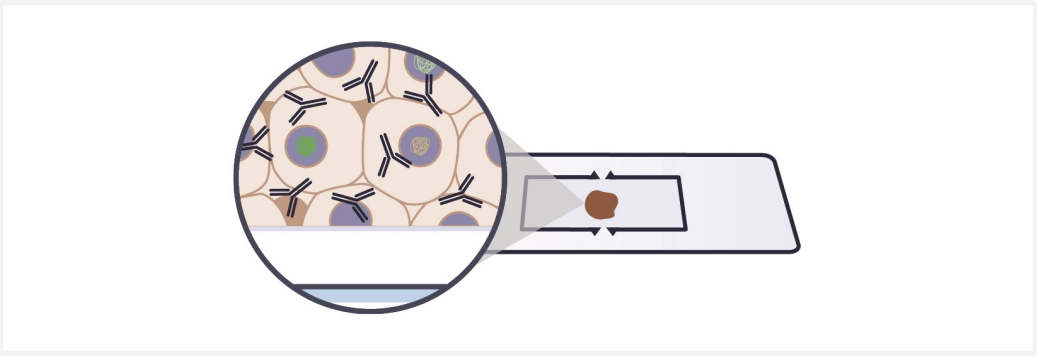


Step 6: Amplification Enhancement & Amplification

The ligation products are enzymatically amplified. Hundreds of copies of the gene-specific barcode are generated during the amplification process.

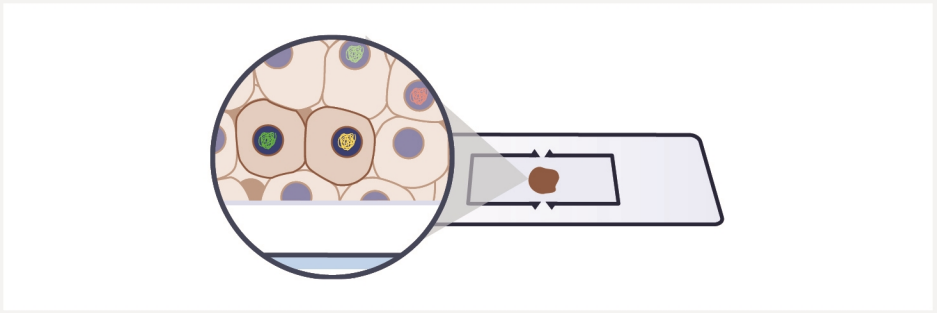
Step 7: Cell Segmentation Staining *(optional)*

Following a series of ethanol washes, blocking reagent is added to the tissue sections to reduce non-specific binding during staining. Cell segmentation antibodies bind their antigens in an overnight incubation, while excess antibodies are washed away in a post-incubation wash. Stain is then enhanced by the addition of Xenium Staining Enhancer reagent.



Step 8: Autofluorescence Quenching

Autofluorescence Quenching diminishes unwanted autofluorescence and enhances signal-to-noise ratio in the treated FFPE and FF tissue sections. Next, nuclei are stained with DAPI (derived from Xenium Nuclei Staining Buffer) to assist in identification of tissue or regions of interest during an instrument overview scan. Finally, tissue sections on Xenium slides assembled into Xenium Cassettes v2 are loaded into the Xenium Analyzer for imaging and decoding.



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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.
- Keep all enzymes and Master Mixes on ice during setup and use, unless otherwise stated.
- Promptly move reagents back to the recommended storage.

Pipette Calibration

- Follow manufacturer's calibration and maintenance schedules.

Priming Oligo and Probe Panel Handling

- 10x Genomics provides the following types of priming oligos and probe panels for the Xenium Prime workflow: pre-designed and add-on custom. Add-on custom panels are used to supplement pre-designed panels. Refer to the 10x Genomics website for the most current list of available panels.
- Only Xenium Prime oligos and probe panels are compatible with the Xenium Prime workflow.
- Pre-designed oligos and probes are good for two Xenium slides. Add-on custom oligos and probes are good for twelve Xenium slides.
- Add-on custom oligos and probes are delivered lyophilized at room temperature and should be stored at -20°C upon resuspension.
- Add-on custom oligos and probes must be resuspended prior to use. See Custom Probe Preparation (optional) on page 62 for more details.
- Record the Custom Panel Design ID and Slide Number before starting the

workflow. This information is critical for identifying the correct electronic decode file when setting up the Xenium Analyzer in downstream steps.

Priming Oligo and Probe Panel Storage & Shipping

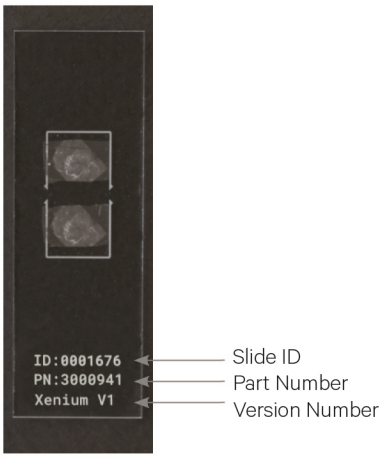
- Pre-designed oligos and probe panels are ready to use and are shipped on dry ice. Pre-designed probes should be stored at -20°C upon receipt. The expiration date is listed on the kit label.
- Pre-designed oligos and probes are stable short-term at room temperature (<8 hours). Pre-designed probes should be discarded if left at room temperature for more than 8 hours.
- Add-on custom oligos and probe panels are lyophilized and are shipped at room temperature. Should be stored at -20°C upon receipt.
- Add-on custom oligos and probes expire three years from the manufacture date in lyophilized form when stored at -20°C. Alternatively, custom probes expire one year from time of resuspension if stored in TE Buffer at -20°C or at kit expiration date (whichever comes first).
- Add-on custom oligos and probes Custom probes must be resuspended prior to use in TE Buffer, pH 8.0. After resuspension, they are stable short-term at room temperature (<8 hours). Resuspended custom oligos and probes should be discarded if left at room temperature for more than 8 hours.
- Add-on custom oligos and probes (in lyophilized form) accidentally left at room temperature for an extended period of time are likely stable. Contact support@10xgenomics.com if further assistance is needed.

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.

Xenium Slide with Tissue Sections

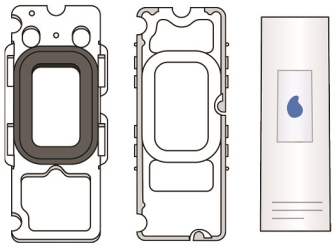


Xenium Cassette Assembly

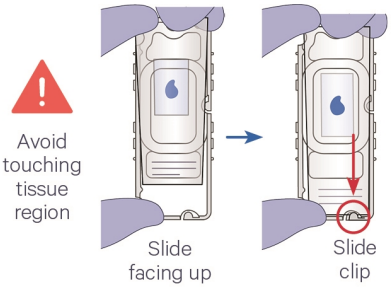
Xenium Cassette v2 Assembly

Exercise caution when handling slide edges to prevent injury.

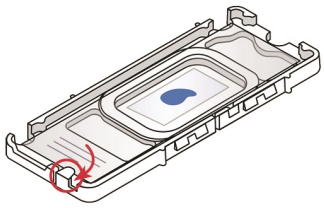
1 Place top and bottom halves of cassette on bench with the top cassette facing down and bottom cassette facing up.



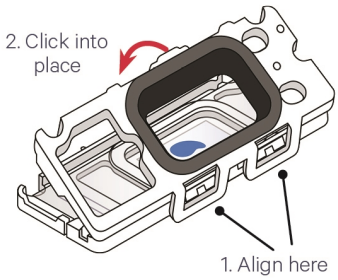
2 Place Xenium slide with label at bottom and tissue facing up into bottom half of cassette. Slip slide under slide clip located at bottom right of cassette.



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

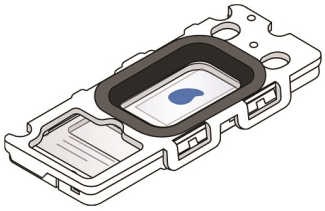


4 Align inner clips of top cassette to inner tabs of bottom cassette. Bring opposite side down until 2 audible clicks are heard.



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.*



TIPS

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

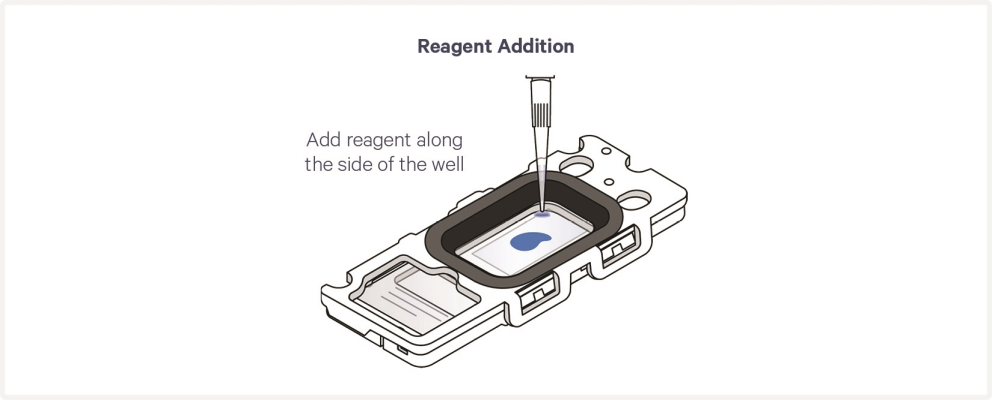
Processing a Single Xenium Slide

- Xenium reagent kits are sufficient for two reactions, and for optimal Xenium Analyzer throughput, two slides should be run at the same time.
- It is possible to perform the Xenium Prime workflow with a single slide. To do this, ensure the following best practices are followed for optimal assay performance:
    - Assemble a mock Xenium Cassette v2 using a blank slide and a cassette top and bottom from the Xenium Cassette Kit v2, PN-1000723. Assemble as described in Xenium Cassette Assembly on page 30.
    - Insert the blank slide into the Xenium Cassette v2. Cassettes should be assembled following the instructions in Troubleshooting for Cassette Assembly Failure on page 100.
    - Attach a Xenium Cassette Lid v2 from the Xenium Cassette Kit v2, PN-1000723 to the cassette containing the blank slide.

      When handling an assembled cassette with the lid applied, always hold from the bottom of the cassette and not the lid.
    - For all incubation steps with the thermal cycler lid closed, ensure the mock slide cassette is placed alongside the Xenium slide cassette containing tissue on the Thermocycler Adaptor v2.

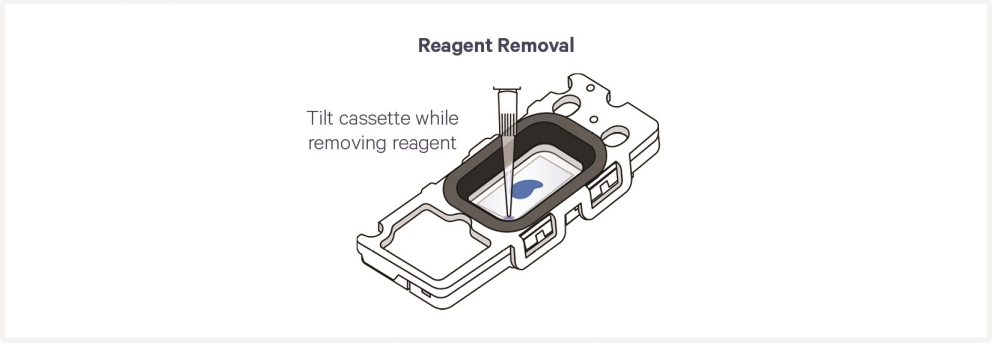
Reagent Addition to Wells

- 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.



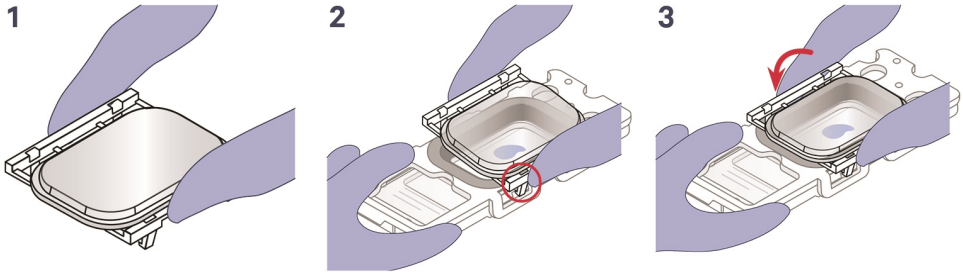
Xenium Cassette Lid Application & Removal

- 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

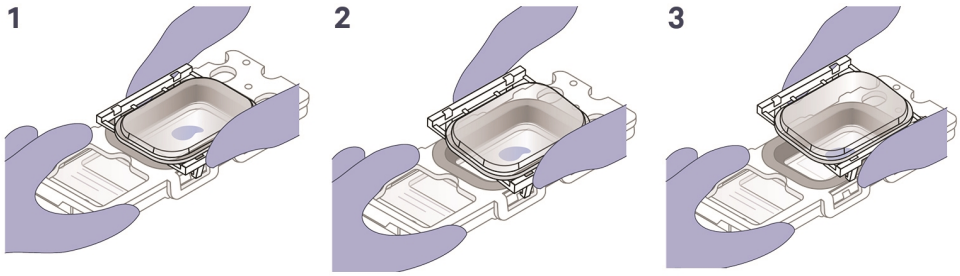
Applying the Xenium Cassette Lid v2



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 v2



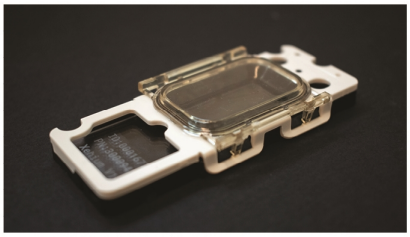
1. Apply even pressure on both sides of the lid using thumb and index finger.
2. While maintaining inward pressure, pull upward with index finger to unhook the side feet from the cassette.
3. Slowly lift cassette lid up to unhook the remaining lid feet. Ensure no liquid splashes out of the well

- When saving the lid is specified, wipe it with a lint-free laboratory wipe, place on a clean surface, and reuse in the next indicated step.

Xenium Cassette Storage

- Store an assembled Xenium cassette with slide by applying a Xenium Cassette Lid v2 at the indicated stopping points listed throughout the protocol and as outlined in the Protocol Steps & Timing on page 16.
- Cassettes should always be stored hydrated with recommended reagent and stored at the recommended temperature.

Assembled Cassette with Lid



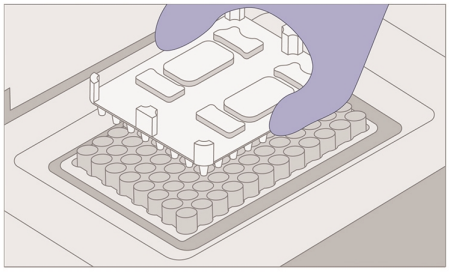
- **Short-term Storage (≤ 1 week):**
  - Store in **1,000 µl** PBS-T at **4°C** in the dark. Ensure that the slide is stored in microbe-free and nuclease-free conditions, with a Xenium Cassette Lid v2 applied to prevent evaporation.
- The above storage guidelines can also be used for storing Xenium cassettes with slides after a Xenium Analyzer run (or in the event of a run failure). Contact support@10xgenomics.com for more information.

Slide Incubation Guidance

Incubation at a specified temperature

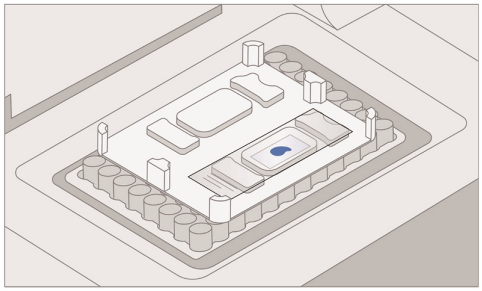
- Position a Xenium Thermocycler Adaptor v2 on a thermal cycler that is set at the incubation temperature. Ensure thermal cycler has reached appropriate temperature prior to starting incubation.

Place Thermocycler Adaptor v2



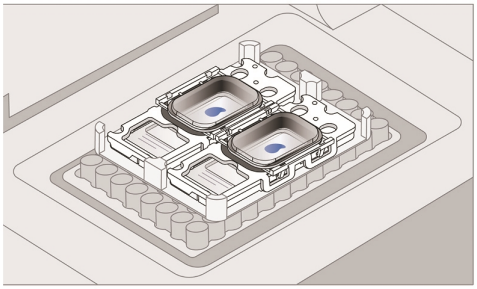
- Ensure that the Thermocycler Adaptor v2 is fully inserted into the thermal cycler and is in contact with the thermal cycler block surface uniformly.
- When incubating a slide, position the slide on the Thermocycler Adaptor v2 with the tissue side facing up.

Incubate Slide on Thermocycler Adaptor v2



- Ensure the Sample Area is aligned with the corresponding area on the Thermocycler Adaptor v2. DO NOT close the lid.
- When incubating a slide encased in a cassette, place the assembled unit on the Thermocycler Adaptor v2 with the well facing up. Ensure the cassette is in complete contact with the Thermocycler Adaptor v2. The cassette should always be sealed with a Xenium Cassette Lid when on the Thermocycler Adaptor unless indicated otherwise.

Incubate Assembled Xenium Cassette v2



- For steps not using a Xenium Cassette Insert, where indicated, after thermal cycler incubation, at least **400 µl** should be aspirated from cassette well. If less than **400 µl** is recovered, contact support@10xgenomics.com.
- Before each incubation step, always inspect the adaptor and wipe it using a lint-free laboratory wipe. If debris or liquid is noticed, wipe the adaptor using 70% isopropanol, followed by wiping with a lint-free laboratory wipe. Failing to do so could result in slide cracking.

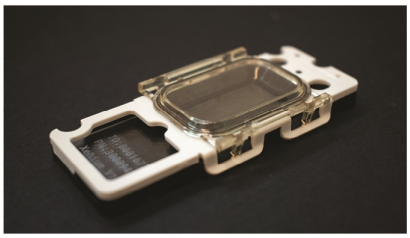
Tightening the thermal cycler lid

- Thermal cycler lid contact with the Xenium Cassette Lid v2 is critical for assay performance.
- For thermal cyclers with adjustable lids, tighten the lid until an audible click is heard. Tightening past the click risks breaking the slide.

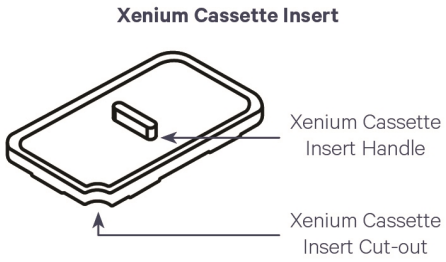
Incubation at room temperature

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

Assembled Cassette with Lid



Xenium Cassette Insert



- The Xenium Cassette Insert sits in the Sample Area of the assembled Xenium Cassette and creates a flow cell in the well for adding reagents.
- Feet on the bottom of the insert help to prevent damage to the tissue sample by creating a gap between the sample and cassette.
- Place the insert in the cassette only when specified.
- Pick up the insert by using forceps on the Xenium Cassette Insert Handle. Forceps are needed for all handling steps. See Additional Kits, Reagents & Equipment on page 14 for recommended forceps.
- Reagents are loaded onto the sample by pipetting into the Xenium Cassette Insert Cut-out.
- The insert is assembled manually into the cassette. See Xenium Cassette Insert Application & Removal on page 38 for more details.

Xenium Cassette Insert Application & Removal

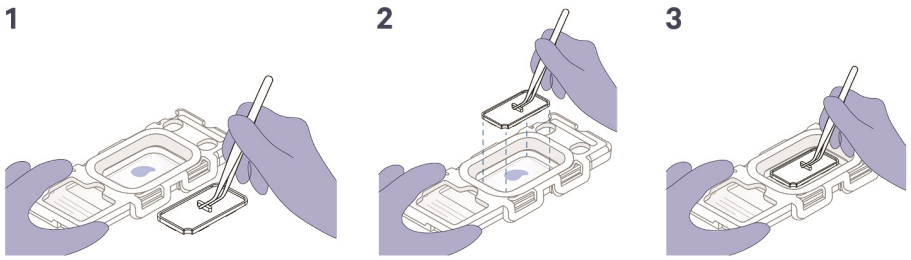
- Work quickly during application and removal steps to avoid drying out of tissue sections.

The instructions apply to both Xenium Cassette and Xenium Cassette v2. The illustrations show a Xenium Cassette.

Application

Applying Xenium Cassette Insert

Improper placement of the insert may cause scratching or damage to tissue sections.



Place the Xenium Cassette flat on a clean work surface. Ensure cassette is free of debris. Use forceps to pick up Xenium Cassette Insert by Cassette Insert Handle.

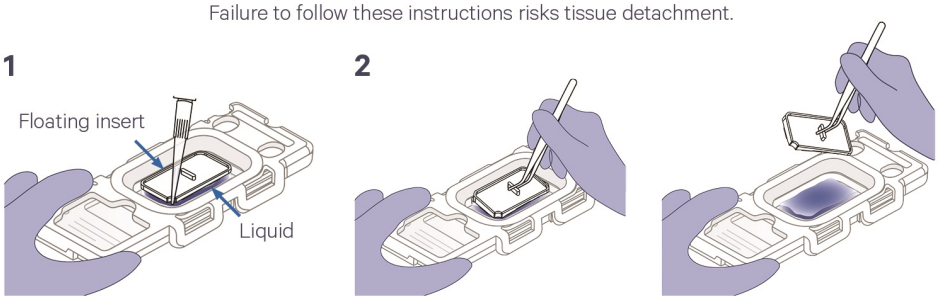
Align the Xenium Cassette Insert with the Sample Area well of the Xenium Cassette. Feet and sides should not come into contact with tissue

Carefully place insert within the well. Visually inspect for proper seating.

Removal

Removing Xenium Cassette Insert

Failure to follow these instructions risks tissue detachment.



Place the Xenium Cassette flat on a clean work surface. Pipette 200 µl of PBS-T to float the Xenium Cassette Insert.

Use forceps to pick up Xenium Cassette Insert by Cassette Insert Handle. Carefully remove the insert from the Sample Area well to ensure no liquid splashes out of the well. Remove residual liquid with a lint-free laboratory wipe if necessary.

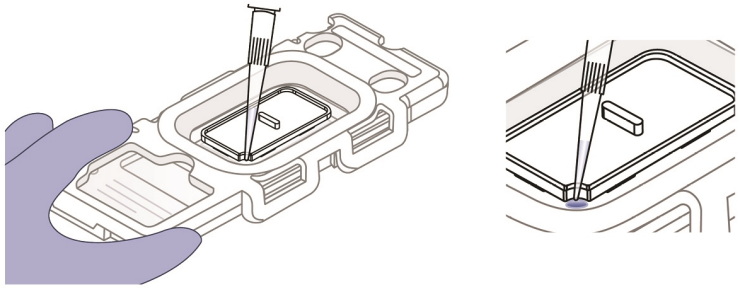
Reagent Addition to Xenium Cassette Insert

The instructions apply to both Xenium Cassette and Xenium Cassette v2. The illustrations show a Xenium Cassette.

- Place the assembled cassette with insert flat on a clean work surface.
- Before executing the steps requiring an insert, retrieve all items and reagents needed for the steps to minimize chances of tissue drying.
- Slowly dispense reagents under insert by pipetting into the Xenium Cassette Insert Cut-out along the side of the well.
- Avoid dispensing reagent too quickly as this may lead to bubble formation.
- Gently support the pipette tip while dispensing reagent to provide more stability during this step.
- Allow reagent to fill the bottom of the insert.
- DO NOT dispense past first stop of the pipette to avoid introducing bubbles.

Reagent Addition to Xenium Cassette Insert

Use Insert cut-out to pipette liquid



Xenium Cassette Insert Practice

Listed below are the practice steps that may be performed prior to using the Xenium Cassette Insert.

Obtain the following items:

- Xenium Cassette v2
- Superfrost Plus Slide
- Xenium Cassette Insert
- Xenium Cassette Lid v2
- PBS-T

TIPS

Cassettes may be obtained from previous runs and washed according to Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning on page 103 instructions. Xenium Cassette Inserts can also be cleaned as per these instructions and reused. If an unused Xenium Cassette Insert is available, that can also be used for the practice.

Practice Steps	Guidance
1. Assemble Xenium Cassette Assemble a Xenium Cassette and blank slide.	Cassette Assembly Failure on page 100
2. Place Insert Place the Xenium Cassette Insert gently onto the Xenium Cassette using forceps.	Xenium Cassette Insert Application & Removal on page 38
3. Add Reagent Add **100 µl** PBS-T through the Xenium Cassette Insert Cut-out to uniformly cover the Sample Area, without introducing bubbles.	Reagent Addition to Xenium Cassette Insert on the previous page
4. Apply Lid	Xenium Cassette Lid Application & Removal on page 33
5. Remove Lid	Xenium Cassette Lid Application & Removal on page 33
6. Remove Insert Add **200 µl** PBS-T into Xenium Cassette Insert Cut-out to float the insert. Remove the insert using forceps.	Xenium Cassette Insert Application & Removal on page 38

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.

10x GENOMICS

Step 1:

Priming Hybridization

1.0 Get Started

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	Priming Hyb Buffer	2001228	Thaw in a thermomixer (300 rpm shaking) at 37°C for 5 min or until completely thawed/clear. Check for precipitate and invert until clear. Maintain at room temperature after thawing.	–20°C
☐	Post-Priming Wash Buffer	2001229	Thaw at 37°C for 5 min or until completely thawed/clear. Vortex and centrifuge briefly. Maintain at room temperature after thawing.	–20°C
☐	Xenium 5K Hu PTP Priming Oligos or Xenium 5K Ms PTP Priming Oligos	2001224 or 2001226	Thaw at room temperature. See 1.3 Priming Hybridization on page 47 for additional handling instructions.	–20°C
☐	Xenium 5K Add-on Priming Oligos*	-	Resuspend add-on priming oligos according to Custom Add-on Priming Oligo Preparation (optional) on page 46. For additional handling instructions, see 1.3 Priming Hybridization on page 47 *Xenium Prime assay is not compatible with Xenium v1 add-on probes.*	
Obtain				
☐	Assembled cassettes containing FFPE or FF tissue samples	-	Consult Xenium in Situ for FFPE - Deparaffinization & Decrosslinking (Demonstrated Protocol CG000580) or Xenium in Situ for Fresh Frozen - Fixation & Permeabilization (Demonstrated Protocol CG000581), respectively.	-
☐	Nuclease-free Water	-	-	Ambient
☐	TE Buffer, TRIS-EDTA, 1X Solution, pH 8.0 (nuclease-free)	-	The pH of the stock solution should be 8.0. Readjusting the pH is NOT recommended.	Ambient
☐	10X PBS, pH 7.4	-	-	Ambient
☐	20X SSC	-	-	Ambient
☐	10% Tween-20	-	-	Ambient

Items		10x PN	Preparation & Handling	Storage
☐	Heatblock or thermal cycler	-	Preheat to 95°C.	Ambient
☐	Forceps	-	-	Ambient
☐	Xenium Cassette Lids v2	3002206	One new lid.	Ambient
☐	Xenium Thermocycler Adaptor v2	3002207	See Tips & Best Practices.	Ambient
☐	Xenium Cassette Insert	3001885	Remove from −20°C and equilibrate to room temperature.	−20°C

*Thaw appropriate priming oligos based on experimental needs. Refer to the 10x Genomics Support Website for the most updated list of available priming oligos and part numbers.

Pre-heat appropriate equipment to: 37°C & 95°C
Program a thermal cycler with Priming Hybridization & Post Priming Hybridization Wash incubation protocol.

1.1 Buffer Preparation

Prepare the following buffers fresh before starting the Xenium Prime In Situ Gene Expression workflow. The volumes of each buffer are sufficient for washes in all subsequent steps.

a. Prepare 1X PBS according to the table below before use and maintain at **room temperature.** Add reagents in the order listed and mix.

1X PBS	Stock	Final	1X (ml)	2X (ml)
Nuclease-free Water	-	-	45.0	90.0
10X PBS, pH 7.4	10X	1X	5.0	10.0
Total	**-**	**-**	**50.0**	**100.0**

b. Using 1X PBS from step 1.1a, prepare PBS-Tween Buffer (PBS-T) according to the table below before use and maintain at **room temperature.** Add reagents in the order listed. Invert gently to mix.

PBS-T	Stock	Final	1X (ml)	2X (ml)
1X PBS (prepared at Step 1.1a)	-	-	24.88	49.75
10% Tween-20	10%	0.05%	0.125 (= 125 µl)	0.250 (= 250 µl)
Total	**-**	**-**	**25.0**	**50.0**

c. Prepare 0.5X SSC-T according the table below and maintain at **room temperature**. Add reagents in the order listed and mix well.

0.5X SSC-T	Stock	Final	1X (ml)	2X (ml)
Nuclease-free Water	-	-	1.6	3.2
SSC Buffer, 20X	20X	0.5X	0.0412 (=41.2 µl)	0.0825 (=82.5 µl)
10% Tween-20	10%	0.05%	0.0082 (= 8.2 µl)	0.0165 (= 16.5 µl)
Total	**-**	**-**	**1.65**	**3.3**

1.2 Custom Add-on Priming Oligo Preparation (optional)

Proceed to Priming Hybridization directly if using pre-designed priming oligos only. Custom add-on priming oligos are delivered lyophilized and must be resuspended before use. Resuspend oligos according to the instructions below before proceeding with Priming Hybridization.

a. Centrifuge lyophillized priming oligos tube briefly.

b. Resuspend lyophilized priming oligos in TE Buffer according to the following table.

Add-on Priming Oligo Resuspension	10x PN	TE Buffer (µl)
12 reactions/kit		
Xenium Prime 5K Add-on Priming Oligos	varies	140.0

c. Replace the cap firmly. Vortex twice for **15 sec** each. Maintain at **room temperature** for **5 min**.

d. Centrifuge tube briefly and maintain at **room temperature**.

e. If priming oligos are already resuspended, thaw at **room temperature** prior to starting Priming Hybridization.

Resuspended oligos in TE Buffer can be stored at -20°C until the expiration date specified on the kit.

1.3 Priming Hybridization

Before starting this protocol, ensure that tissue sections have been appropriately deparaffinized and decrosslinked if working with FFPE tissues. Ensure that tissue sections have been appropriately fixed and permeabilized if working with fresh frozen tissues. Consult Xenium In Situ for FFPE - Deparaffinization & Decrosslinking Demonstrated Protocol (CG000580) or Xenium In Situ for Fresh Frozen - Fixation & Permeabilization Demonstrated Protocol (CG000581), respectively, for more information.

TIPS

*During every reagent removal step in the protocol, ensure that **ALL the liquid is removed** from the wells. See Reagent Addition to Wells on page 32 for guidance.*

a. Obtain oligos that have been thawed or equilibrated to room temperature. Briefly centrifuge the probe tube and remove an aliquot appropriate for the number of desired Xenium slides (**52.5 µl**/slide).

b. Preheat oligos by incubating at **95°C** for **2 min** in a heatblock or thermal cycler, followed by **1 min** on **ice**. Maintain on ice.

c. Prepare Priming Hybridization Mix shortly before use and maintain at **room temperature**. Add reagents in the order listed. Pipette mix 10X and centrifuge briefly.

Option 1: Priming Hybridization Mix (pre-designed priming oligos)

	Priming Hybridization Mix *(pre-designed priming oligos only)*	10x PN	1X+5% (µl)	2X+5% (µl)
	Priming Hyb Buffer	2001228	94.5	189.0
	TE Buffer	-	10.5	21
	Xenium 5K Hu PTP Panel Priming Oligos or Xenium 5K Ms PTP Panel Priming Oligos	2001224 or 2001226	52.5	105.0
	Total	**-**	**157.5**	**315.0**

Option 2: Priming Hybridization Mix (add-on custom priming oligos used with pre-designed priming oligos)

	Priming Hybridization Mix *(add-on custom priming oligos used with pre-designed priming oligos)*	10x PN	1X+5% (µl)	2X+5% (µl)
●	Priming Hyb Buffer	2000390	94.5	189.0
	Xenium 5K Hu PTP Panel Priming Oligos or Xenium 5K Ms PTP Panel Priming Oligos	2001224 or 2001226	52.5	105.0
	Xenium Prime 5 K Add-on Priming Oligos*†	varies	10.5	21.0
	Total	**-**	**157.5**	**315.0**

*Refer to the 10x Genomics Support Website for the most updated list of available panels and part numbers.

†Priming Oligos resuspended in TE Buffer can be stored at -20°C until the expiration date specified on the kit.

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

This thermal cycler protocol includes programming for both Priming Hybridization and the Post Priming Hybridization Wash steps.

Lid Temperature	Reaction Volume	Run Time
50°C	100 µl	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	50°C	Hold
Priming Hybridization	50°C	01:30:00
Hold	50°C	Hold
Post Priming Hybridization Wash	50°C	00:30:00
Hold	50°C	Hold

Final hold should not exceed 2-3 min

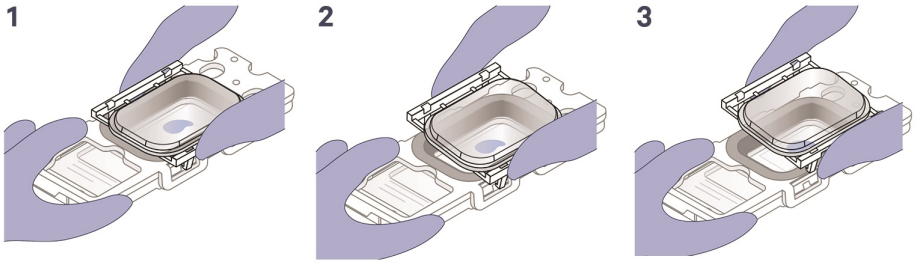
e. Retrieve the assembled Xenium Cassette v2 containing FFPE or fresh frozen (FF) tissue sections.

Record the Custom Panel Design ID and Slide Number before starting workflow. This information is critical for identifying the correct electronic decode file when setting up the Xenium Analyzer in downstream steps.

f. Obtain forceps, Xenium Cassette Insert, and all other items required for steps g-j before executing the steps.

g. Remove the Xenium Cassette Lid v2. **Save the lid** for the next step.

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

Remove all the buffer from FFPE or FF tissues (prepared according to CG000580 or CG000581 Demonstrated Protocols, respectively).

TIPS

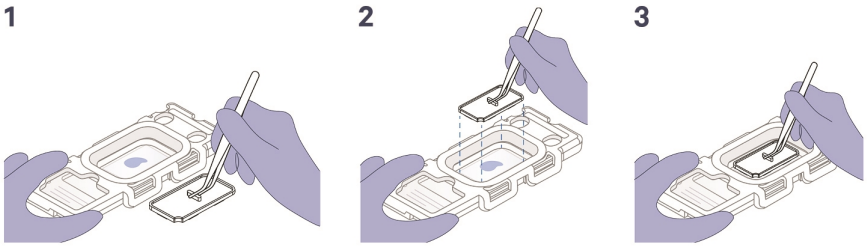
If processing two slides at a time, remove and add reagent from first slide before proceeding to second slide. Ensure tissue sections are always covered with reagent in between removal and addition steps to avoid drying out of tissue samples.

h. Gently place a Xenium Cassette Insert onto the Xenium Cassette v2 using forceps.

The instructions apply to both Xenium Cassette and Xenium Cassette v2. The illustrations show a Xenium Cassette.

Applying Xenium Cassette Insert

Improper placement of the insert may cause scratching or damage to tissue sections.

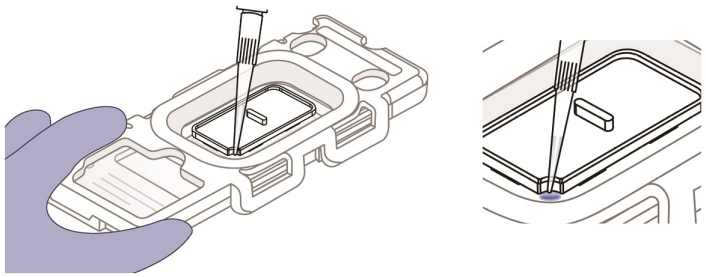


1. Place the Xenium Cassette flat on a clean work surface. Ensure cassette is free of debris. Use forceps to pick up Xenium Cassette Insert by Cassette Insert Handle.
2. Align the Xenium Cassette Insert with the Sample Area well of the Xenium Cassette. Feet and sides should not come into contact with tissue
3. Carefully place insert within the well. Visually inspect for proper seating.

i. Add **150 µl** Priming Hybridization Mix along to the Xenium Cassette Insert using the cut-out to pipette solution under the insert to uniformly cover the tissue sections, without introducing bubbles as described in Xenium Cassette Insert on page 37.

Refer to Bubbles under Xenium Cassette Insert on page 98 for guidance on resetting the cassette insert if bubbles are present.

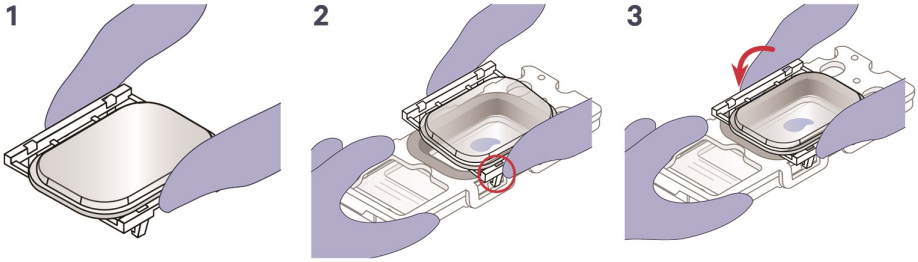
Reagent Addition to Xenium Cassette Insert
Use Insert cut-out to pipette liquid



j. Apply a new Xenium Cassette Lid v2 on the cassette and place on the Xenium Thermocycler Adaptor v2 on the pre-heated thermal cycler. Tightly close the thermal cycler lid until an audible click is heard.

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

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.

TIPS

Audible click will be heard in thermal cyclers with an adjustable lid (i.e. Bio-Rad C1000 Touch Thermal Cycler).

k. Skip Pre-equilibrate step to initiate Priming Hybridization.

TIPS

Start thawing Post-Priming Wash Buffer as outlined in Post Priming Hybridization Wash on page 52.

l. After Priming Hybridization is complete, **immediately** proceed to next step.

1.4 Post Priming Hybridization Wash

a. Retrieve Post-Priming Wash Buffer and incubate at **37°C** for **5 min** or until thawed completely. Vortex and centrifuge briefly. Maintain at **room temperature**.

b. Remove the cassette from the Thermocycler Adaptor v2 and place on a flat, clean work surface.

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

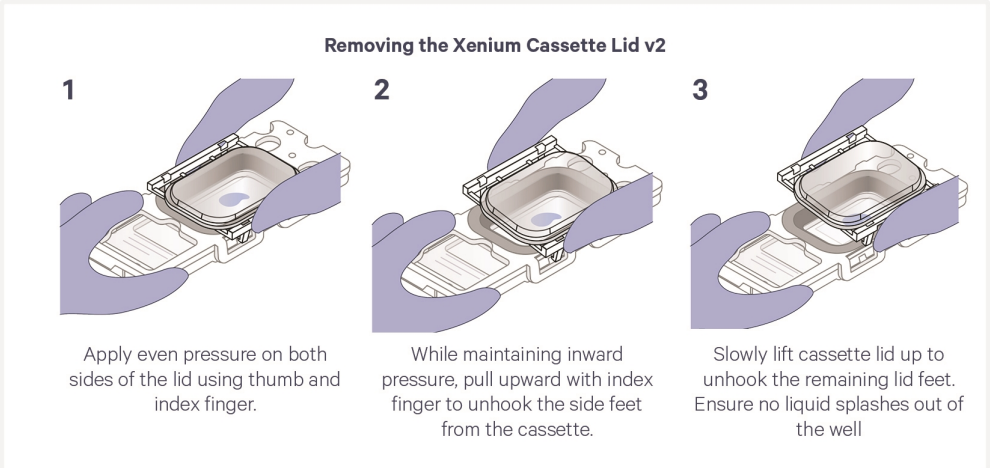
Bubbles on the surface of the slide are normal and unlikely to compromise assay performance. DO NOT aspirate or pop bubbles, as this can lead to detachment or scratching of the tissue.

Fluid on the Thermocycler Adaptor may indicate a reagent leak from the cassette. See Troubleshooting for more details.

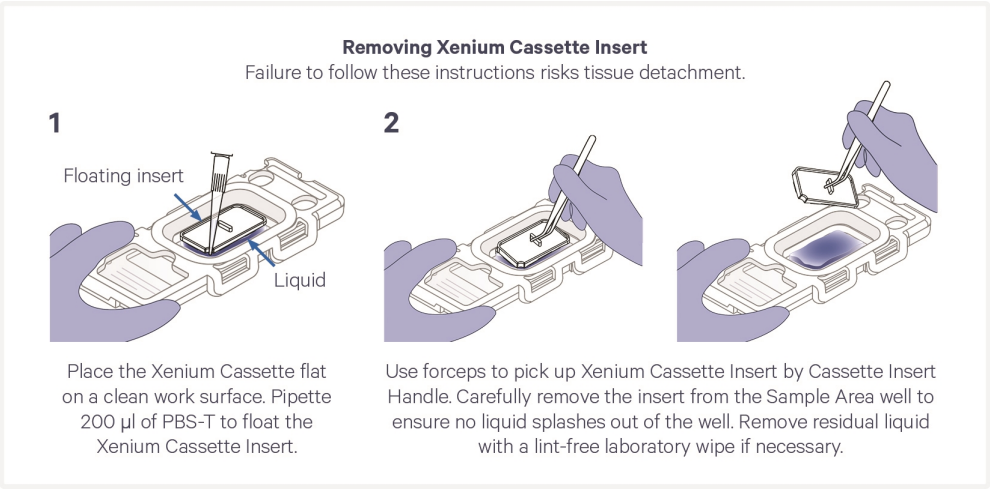
c. Keep that the thermal cycler programmed as specified on page 47 with the Post Priming Hybridization Wash incubation protocol.

DO NOT let the cassette cool down before proceeding to PBS-T washes.

d. Remove the Xenium Cassette Lid v2. Carefully pipette **200 µl** PBS-T into Xenium Cassette Insert Cut-out to float the insert. **Save the lid** for use in following indicated steps.



e. Carefully remove Xenium Cassette Insert with forceps. **Discard** the insert.



f. Using a pipette, remove all the buffer from well corners.

TIPS

If processing two slides at a time, remove and add reagent from first slide before proceeding to second slide. Ensure tissue sections are always covered with reagent in between removal and addition steps to avoid drying out of tissue samples.

Two PBS-T washes (PBS-T prepared at step 1.1):

TIPS

Always add along the side of the well to uniformly cover the tissue sections, without introducing bubbles. Removal and addition of buffers should be done quickly to prevent drying of tissue sections.

g. **Wash 1:** Add **500 µl** PBS-T. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

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

i. Add **500 µl** Post-Priming Wash Buffer to the well.

j. Apply the previously used Xenium Cassette Lid v2 on the cassette and place on the Thermocycler Adaptor v2 on the pre-heated thermal cycler. Close the thermal cycler lid.

k. Skip Pre-equilibrate step to initiate Post Priming Hybridization Wash.

TIPS

Start thawing 2X RNase Buffer and Polishing Buffer at room temperature as outlined in *Get Started on page 55*.

l. After the Post Priming Hybridization Wash is complete, **immediately** proceed to the next step.

The hold time after Post Priming Hybridization Wash incubation should not exceed 2-3 min.

Step 2:

RNase Treatment & Polishing

2

2.0 Get Started

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	2X RNase Buffer	2000411	Thaw at room temperature. Vortex and centrifuge briefly. Maintain at room temperature after thawing.	–20°C
☐	Polishing Buffer	2001231	Thaw at room temperature. Vortex and centrifuge briefly. Maintain at room temperature after thawing.	–20°C
Maintain on ice				
☐	RNase Enzyme	3000593	Pipette mix, centrifuge briefly. Maintain on ice until ready to use.	–20°C
☐	Polishing Enzyme	2001230	Pipette mix, centrifuge briefly. Maintain on ice until ready to use.	–20°C
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	Forceps	-	-	Ambient
☐	Xenium Cassette Lids v2	3002206	One new lid.	Ambient
☐	Xenium Thermocycler Adaptor v2	3002207	See Tips & Best Practices.	Ambient
☐	Xenium Cassette Insert	3001885	Remove from –20°C and equilibrate toroom temperature.	–20°C

TIPS

Program a thermal cycler with two different incubation protocols:

- RNase Treatment incubation protocol
- Polishing incubation protocol

2.1 RNase Treatment

a. Prepare RNase Mix on ice.

RNase Mix *(add reagents in the order listed, pipette mix 10X; maintain on ice)*	10x PN	1X+10% (µl)	2X+10% (µl)
Nuclease-free Water	2001228	269.5	539.0
2X RNase Buffer	2000411	275.0	550.0
RNase Enzyme	3000953	5.5	11.0
Total	**-**	**550.0**	**1100.0**

b. Remove the Xenium Cassette v2 from the Thermocycler Adaptor v2 and place on a flat, clean work surface.

c. Remove the cassette lid. Using a pipette, remove all the buffer from the cassette. **Save the lid** for use in following indicated steps.

Three PBS-T washes (PBS-T prepared at step 1.1):

d. **Wash 1: Immediately** add **500 µl** PBS-T. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

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

f. **Wash 3:** Add **500 µl** PBS-T. Incubate for **1 min** at **room temperature**.

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

Lid Temperature	Reaction Volume	Run Time
37°C (lid may be set to the lowest temperature if the instrument does not enable 37°C)	100 µl	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	37°C	Hold
RNase Treatment	37°C	00:20:00
Hold	37°C	Hold

h. Remove all PBS-T to complete wash 3.

i. Add **500 µl** RNase Mix to the well.

j. Apply the previously used Xenium Cassette Lid v2 on the cassette and place on the Thermocycler Adaptor v2 on the pre-heated thermal cycler.

Close the thermal cycler lid.

k. Skip Pre-equilibrate step to initiate RNase Treatment.

l. After the RNase Treatment is complete, **immediately** proceed to the next step.

2.2 Polishing

a. Prepare Polishing Reaction Mix on ice.

Polishing Reaction Mix *(add reagents in the order listed, pipette mix 10X; maintain on ice)*	10x PN	1X+10% (µl)	2X+10% (µl)
Polishing Buffer	2001231	495.0	990.0
Nuclease-free Water	-	27.5	55.0
Polishing Enzyme	2001230	27.5	55.0
Total	**-**	**550.0**	**1100.0**

b. Remove the Xenium Cassette v2 from the Thermocycler Adaptor v2 and place on a flat, clean work surface.

c. Remove the cassette lid. Using a pipette, remove all the buffer from the cassette. **Discard** the lid.

Three 0.5X SSC-T washes (SSC-T prepared in step 1.1):

*The following wash steps use **0.5X SSC-T**.*

d. **Wash 1: Immediately** add **500 µl** 0.5X SSC-T. Incubate for **1 min** at **room temperature**. Remove all SSC-T.

e. **Wash 2:** Add **500 µl** 0.5X SSC-T. Incubate for **1 min** at **room temperature**. Remove all SSC-T.

f. **Wash 3:** Add **500 µl** 0.5X SSC-T. Incubate for **1 min** at **room temperature**.

g. Prepare a thermal cycler with the following incubation protocol.

Lid Temperature	Reaction Volume	Run Time
37°C (lid may be set to the lowest temperature if the instrument does not enable 37°C)	100 µl	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	37°C	Hold
Polishing	37°C	01:00:00
Hold	37°C	Hold

h. Remove all 0.5X SSC-T to complete wash 3.

i. Add **500 µl** Polishing Reaction Mix to the well.

j. Apply **a new** Xenium Cassette Lid v2 on the Xenium Cassette v2 and place on the Thermocycler Adaptor v2 on the pre-heated thermal cycler. Close the thermal cycler lid.

k. Skip Pre-equilibrate step to initiate the Polishing Protocol.

TIPS

Start thawing Probe Hybridization reagents (~10-15 min before the Polishing incubation ends) as outlined in *Get Started on page 61**.*

l. After the Polishing Protocol is complete, **immediately** proceed to the next step.

Step 3:

Probe Hybridization

3

3.0 Get Started

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	Probe Hyb Buffer B	2001232	Thaw at 37°C for 5 min or until completely thawed. Check for precipitate and invert until clear. Maintain at room temperature after thawing.	–20°C
☐	Xenium 5K Hu PTP Panel Probes or Xenium 5K Ms PTP Panel Probes	2001225 or 2001227	Thaw at room temperature. See Probe Hybridization on page 63 for additional handling instructions.	–20°C
☐	Xenium 5K Add-on Custom Probes*	-	Resuspend add-on custom probes according to Custom Probe Preparation (optional) on page 62. For additional handling instructions, see Probe Hybridization on page 63. *Xenium Prime assay is not compatible with Xenium v1 add-on probes.*	
Obtain				
☐	Heatblock or thermal cycler	-	Preheat to 95°C.	Ambient
☐	Forceps	-	-	Ambient
☐	TE Buffer, TRIS-EDTA, 1X Solution, pH 8.0 (nuclease-free)	-	The pH of the stock solution should be 8.0. Readjusting the pH is NOT recommended.	Ambient
☐	Xenium Cassette Lids v2	3002206	Previously used.	Ambient
☐	Xenium Thermocycler Adaptor v2	3002207	See Tips & Best Practices.	Ambient
☐	Xenium Cassette Insert	3001885	Remove from –20°C and equilibrate to room temperature.	Ambient
☐	Fume Hood *(optional; only if performing PBS-T washes in the hood)*	-	-	-

*Thaw appropriate probe panels based on experimental needs. Refer to the 10x Genomics Support Website for the most updated list of available panels and part numbers.

TIPS

Pre-heat appropriate equipment to: 37°C & 95°C
Program a thermal cycler with the Probe Hybridization incubation protocol

3.1 Custom Probe Preparation (optional)

Proceed to Probe Hybridization on page 63 directly if using pre-designed probes only. Add-on probes are delivered lyophilized and must be resuspended before use. Resuspend add-on probes according to the instructions below before proceeding with Probe Hybridization.

Confirm the number of reactions provided for the add-on probes prior to resuspension.

a. Centrifuge custom probe panel tube briefly.

b. Resuspend lyophilized probes in TE Buffer according to the following table.

Custom Add-On Probe Resuspension	10x PN	TE Buffer (µl)
12 reactions/tube		
Xenium 5K Add-on Custom Probes*	varies	140.0

c. Replace the cap firmly. Vortex twice for **15 sec** each. Maintain at **room temperature** for **5 min**.

d. Centrifuge custom probe panel tube briefly and maintain at **room temperature**.

e. If probes are already resuspended, thaw at **room temperature** prior to starting Probe Hybridization.

Probes resuspended in TE Buffer can be stored at -20°C until the expiration date specified on the kit.

3.2 Probe Hybridization

a. Remove the Xenium Cassette v2 from the Thermocycler Adaptor v2 and place on a flat, clean work surface.

b. Remove the cassette lid. Using a pipette, remove all the buffer from the cassette. **Save the lid** for use in following indicated steps.

Three PBS-T washes (PBS-T prepared in step 1.1):

These PBS-T washes can either be performed inside a biosafety hood or on the laboratory bench. If working in a biosafety hood, ensure that the remaining protocol steps can still be accomplished within the required time.

c. **Wash 1:** Add **500 µl** PBS-T. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

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

e. **Wash 3:** Add **500 µl** PBS-T. Incubate for **1 min** at **room temperature**.

The samples can continue to be in PBS-T while the following steps are being executed.

f. Obtain probes that have been thawed or equilibrated to **room temperature**. Briefly centrifuge the probe tube and remove an aliquot appropriate for the number of desired Xenium slides (base Panel Probes - **52.5 µl**/slide; if using add-on Custom Probes -**10.5 µl**/slide).

g. Immediately before use, preheat probes by incubating at **95°C** for **2 min** in a heatblock or thermal cycler, followed by **1 min** on **ice**. Maintain on ice.

h. Prepare Probe Hybridization Mix according to the options below.

- Pre-designed probe panels only
- Add-on custom probe panels used with pre-designed probe panels

Option 1: Probe Hybridization Mix (pre-designed probe panels only)

	Probe Hybridization Mix *(pre-designed probe panels only)*	10x PN	1X+5% (µl)	2X+5% (µl)
●	Probe Hyb Buffer B	20001232	94.5	189.0
	TE Buffer	-	10.5	21.0
	Xenium 5K Hu PTP Panel Probes or Xenium 5K Ms PTP Panel Probes	2001225 or 2001227	52.5	105.0
	Total	**-**	**157.5**	**315.0**

Option 2: Probe Hybridization Mix (add-on custom probe panels used with pre-designed probe panels)

	Probe Hybridization Mix *(add-on custom probe panels used with pre-designed probe panels)*	10x PN	1X+5% (µl)	2X+5% (µl)
●	**Probe Hyb Buffer B**	20001232	94.5	189.0
	Xenium 5K Hu PTP Panel Probes or Xenium 5K Ms PTP Panel Probes	2001225 or 2001227	52.5	105.0
	Xenium 5K Add-on Custom Probes*†	varies	10.5	21.0
	Total	**-**	**157.5**	**315.0**

*Refer to the 10x Genomics Support Website for the most updated list of available panels and part numbers.

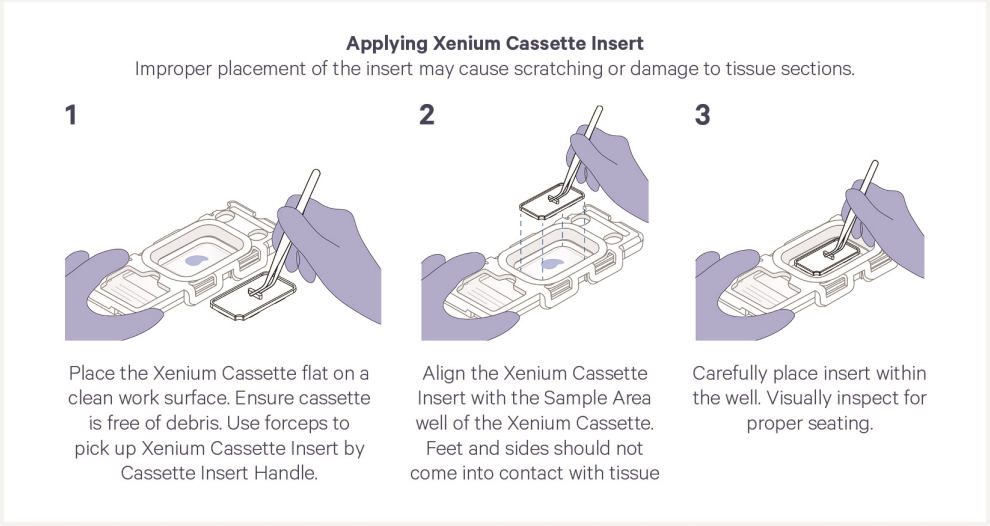
†Custom probes resuspended in TE Buffer can be stored at -20°C until the expiration date specified on the kit.

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

Lid Temperature	Reaction Volume	Run Time
50°C	100 µl	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	50°C	Hold
Probe Hybridization	50°C	Overnight (16 - 24 h)
Hold	50°C	Hold

j. Remove all PBS-T to complete wash 3.

k. Gently place a Xenium Cassette Insert onto the Xenium Cassette v2 using forceps.



l. Add **150 µl** room-temperature Probe Hybridization Mix along to the Xenium Cassette Insert using the cut-out to pipette solution under the insert to uniformly cover the tissue sections, without introducing bubbles as described in Xenium Cassette Insert on page 37.

m. Apply the previously used Xenium Cassette Lid v2 on the cassette and place on the Thermocycler Adaptor v2 on the pre-heated thermal cycler. Tightly close the thermal cycler lid until an audible click is heard.

n. Skip Pre-equilibrate step to initiate Probe Hybridization.

o. After Probe Hybridization is complete, proceed to the next step.

Step 4:

Post Hybridization Wash

4

4.0 Get Started

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐ ◯	Post Hybridization Wash Buffer	2000395	Thaw at room temperature for 30 min or until thawed completely. Vortex and centrifuge briefly. Keep the buffer at room temperature after thawing.	–20°C
Obtain				
☐	PBS-T	-	Prepared at Step 1.1.	Ambient
☐	Xenium Cassette Lids v2	3002206	Previously used.	Ambient
☐	Xenium Cassette Insert	3001885	Remove from –20°C and equilibrate to room temperature.	Ambient
☐	Forceps	-	-	Ambient

TIPS

Program a thermal cycler with Post Hybridization Wash incubation protocol.

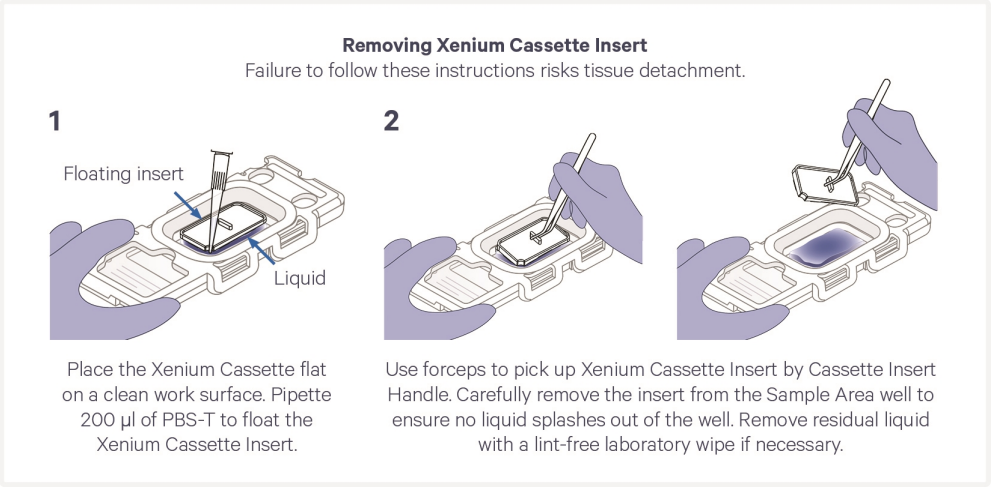
4.1 Post Hybridization Wash

a. Remove the Xenium Cassette v2 from the Thermocycler Adaptor and place on a flat, clean work surface.

DO NOT let the cassette cool down before proceeding to PBS-T washes.

b. Remove the Xenium Cassette Lid v2. Carefully pipette **200 µl** PBS-T into Xenium Cassette Insert Cut-out to float the insert. **Save lid** for use in following indicated steps.

Carefully remove Xenium Cassette Insert with forceps. Discard used Xenium Cassette Inserts.



c. Using a pipette, remove all the buffer from well corners.

Two PBS-T washes (PBS-T prepared at step 1.1):

d. **Wash 1: Immediately** add **500 µl**. Incubate for **1 min** at **room temperature.** Remove all PBS-T.

e. **Wash 2:** Add **500 µl** PBS-T. Incubate for **1 min** at **room temperature**.

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

Lid Temperature	Reaction Volume	Run Time
37°C	100 µl	-
(lid may be set to the lowest temperature if the instrument does not enable 37°C)		
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	35°C	Hold
Post Hybridization Wash	35°C	00:15:00
Hold	35°C	Hold

Hold should not exceed 2-3 min

g. Remove all PBS-T to complete wash 2.

h. Add **500 µl** Post Hybridization Wash Buffer B to the well.

i. Apply the previously used Xenium Cassette Lid v2 on the cassette and place on the Thermocycler Adaptor v2 on the pre-heated thermal cycler. Close the thermal cycler lid.

j. Skip Pre-equilibrate step to initiate Post Hybridization Wash.

TIPS

Start thawing Ligation reagents during Post Hybridization Wash incubation as outlined in *Get Started on page 71*.

k. After the Post Hybridization Wash is complete, **immediately** proceed to the next step.

The hold time after Post Hybridization Wash incubation should not exceed 2-3 min.

Step 5:

Ligation

5

5.0 Get Started

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	Ligation Buffer B	2001233	Thaw at room temperature for 15 min or until completely thawed. Vortex and centrifuge briefly. Maintain at room temperature after thawing.	–20°C
Place on ice				
☐	Ligation Enzyme A	2000397	Pipette mix and centrifuge briefly. Maintain on ice until ready to use.	–20°C
☐	Ligation Enzyme B	2000398	Pipette mix and centrifuge briefly. Maintain on ice until ready to use.	–20°C
Obtain				
☐	Xenium Cassette Lids v2	3002206	Previously used.	Ambient
☐	Xenium Cassette Insert	3001885	Remove from –20°C and equilibrate to room temperature.	Ambient
☐	PBS-T	-	Prepared at Step 1.1.	Ambient

TIPS Program a thermal cycler with Ligation incubation protocol.

5.1 Ligation

a. Prepare Ligation Mix shortly before using. Add reagents in the order listed. Pipette mix 10X and centrifuge briefly. Maintain on ice.

	Ligation Mix	10x PN	1X+10% (µl)	2X+10% (µl)
●	Ligation Buffer B	2001233	481.5	963.0
●	Ligation Enzyme A	2000397	13.5	27.0
●	Ligation Enzyme B	2000398	55.0	110.0
	Total	**-**	**550.0**	**1,100.0**

b. Remove the Xenium Cassette v2 from the Thermocycler Adaptor v2 and place on a flat, clean work surface.

c. Remove the Xenium Cassette Lid v2. Using a pipette, remove all the buffer from the well. **Save the lid** for use in the following indicated steps.

Three PBS-T washes (PBS-T prepared at step 1.1):

d. **Wash 1: Immediately** add **500 µl** PBS-T. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

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

f. **Wash 3:** Add **500 µl** PBS-T. Incubate for **1 min** at **room temperature**.

g. Prepare a thermal cycler with the following incubation protocol. Place a Thermocycler Adaptor v2 on the thermal cycler and start the program.

Lid Temperature	Reaction Volume	Run Tie
42°C (lid may be set to the lowest temperature if the instrument does not enable 42°C)	100 µl	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	42°C	Hold
Ligation	42°C	00:30:00
Hold	42°C	Hold

h. Remove all PBS-T to complete wash 3.

i. Add **500 µl** Ligation Mix to the well.

j. Apply the previously used Xenium Cassette Lid v2 on the cassette and place on the Thermocycler Adaptor v2 on the pre-heated thermal cycler.

Close the thermal cycler lid.

k. Skip Pre-equilibrate step to initiate Ligation.

Start thawing Amplification Enhancement and Amplification reagents (except Amplification Enhancer) during Ligation incubation as outlined in *Get Started on page 75*.

l. After Ligation is complete, **immediately** proceed to next step.

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Step 6:

Amplification

6.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	Amplification Enhancer Buffer	2001234	Thaw at 37°C for 5 min. Vortex and centrifuge briefly. Maintain at room temperature.	–20°C
☐	Amplification Enhancer Wash Buffer	2001236	Thaw at 37°C for 5 min. Vortex and centrifuge briefly. Maintain at room temperature.	–20°C
☐	Amplification Mix	2000392	Thaw at room temperature. Vortex and centrifuge briefly. Maintain at room temperature until ready to use.	–20°C
Place on ice (immediately before use)				
☐	Amplification Enhancer	2001235	Transfer to ice immediately before use. Pipette mix 10X (pipette set to 75 µl) and centrifuge briefly. Maintain on ice until ready to use.	–20°C
Obtain				
☐	PBS-T	-	Prepared at Step 1.1.	Ambient
☐	TE Buffer, TRIS-EDTA, 1X Solution, pH 8.0 (nuclease-free)	-	-	Ambient
☐	Xenium Cassette Lids v2	3002206	Previously used.	Ambient

TIPS

Pre-heat appropriate equipment to: 37°C
Program a thermal cycler with two different incubation protocols:

- Amplification Enhancement incubation protocol
- Amplification incubation protocol

6.1 Amplification Enhancement

a. Prepare Amplification Enhancer Master Mix on ice. Add reagents in the order listed. Pipette mix 10X and centrifuge briefly. Maintain on ice.

	Amplification Enhancer Master Mix	10x PN	1X +10% (µl)	2X +10% (µl)
●	Amplification Enhancer Buffer	2001234	495.0	990.0
●	Amplification Enhancer	2001235	55.0	110.0
	Total	**-**	**550.0**	**1,100.0**

b. Remove the Xenium Cassette v2 from the Thermocycler Adaptor v2 and place on a flat, clean work surface.

c. Remove the Xenium Cassette Lid v2. Using a pipette, remove all the buffer from the well. **Save lid** for use in the following indicated steps.

Three PBS-T Washes (PBS-T prepared in step 1.1):

d. **Wash 1: Immediately** add **500 µl** PBS-T. Incubate at **room temperature** for **1 min**. Remove all PBS-T.

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

f. **Wash 3:** Add **500 µl** PBS-T. Incubate at **room temperature** for **1 min**.

g. Prepare a thermal cycler with the following incubation protocol. Place a Thermocycler Adaptor v2 on the thermal cycler and start the program.

Lid Temperature	Reaction Volume	Run Time
Lid Temperature Off	100 µl	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	4°C	Hold
Amplification Enhancer	4°C	02:00:00
Hold	4°C	Hold

h. Remove all PBS-T to complete wash 3.

i. **Immediately** add **500 µl** Amplification Enhancement Master Mix to the well.

j. Apply the previously used Xenium Cassette Lid v2 on the cassette and place on the Thermocycler Adaptor v2 on the thermal cycler. Close the thermal cycler lid.

k. Skip pre-equilibrate step to initiate Amplification Enahncer.

Prepare ahead:

l. Prepare Amplification Master Mix on ice (to be used in step 6.3 Amplification on the next page). Add reagents in the order listed. Pipette mix 10X and centrifuge briefly. Maintain on ice.

	Amplification Master Mix	10x PN	1X +10% (µl)	2X +10% (µl)
●	Amplification Mix *(Thaw completely before use)*	2000392	495.0	990.0
	Nuclease-free Water	-	55.0	110.0
	Total	**-**	**550.0**	**1,100.0**

m. After Amplification Enhancement is complete, **immediately** proceed to next step.

6.2 Post Amplification Enhancement Wash

a. Remove the Xenium Cassette v2 from the Thermocycler Adaptor v2 and place on a flat, clean work surface.

b. Remove the Xenium Cassette Lid v2. Using a pipette, remove all the buffer from the well. **Save the lid** for the following indicated steps.

c. Add **500 µl** Amplification Enhancer Wash Buffer to the well.

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

e. Proceed **immediately** to the next step.

6.3 Amplification

a. Prepare a thermal cycler with the following incubation protocol. Place a Thermocycler Adaptor v2 on the thermal cycler and start the program.

Lid Temperature	Reaction Volume	Run Time
30°C (lid may be set to the lowest temperature if the instrument does not enable 30°C)	-	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	30°C	Hold
Amplification	30°C	01:30:00
Hold	30°C	Hold

Hold should not exceed 2-3 min

Using a pipette, remove all the buffer from the well.

b. **Immediately** add **500 µl** Amplification Master Mix (prepared in step 6.1) to the well.

c. Apply the previously used Xenium Cassette Lid v2 on the Xenium Cassette and place on the Thermocycler Adaptor v2 on the thermal cycler. Close the thermal cycler lid.

d. Skip pre-equilibrate step to initiate Amplification.

TIPS

If performing Cell Segmentation Staining, start thawing Block and Stain Buffer during incubation as outlined in *Get Started on page 81*.

If NOT performing Cell Segmentation Staining, start thawing Autofluorescence Quenching reagents during incubation as outlined in *Get Started on page 89*. *DO NOT add the ethanol until right before use.*

e. After Amplification is complete, **immediately** proceed to the next step.

The hold time after Amplification incubation should not exceed 2-3 min.

6.4 Post Amplification Wash

a. Remove the Xenium Cassette v2 from the Thermocycler Adaptor v2 and place on a flat, clean work surface.

b. Remove the Xenium Cassette Lid v2. Using a pipette, remove all Amplification Mix from the well. **Save the lid** for the next step.

Three TE Washes:

c. **Wash 1:** Add **500 µl** TE Buffer to the well. Incubate **1 min** at **room temperature**. Remove all TE buffer.

d. **Wash 2:** Add **500 µl** TE Buffer to the well. Incubate **1 min** at **room temperature**. Remove all TE buffer.

e. **Wash 3:** Add **500 µl** TE Buffer to the well (buffer removal will be in next step to complete wash 3).

f. Proceed to either to Cell Segmentation Staining on page 80 or directly to Autofluorescence Quenching on page 88 if not performing cell segmentation staining.

Step 7:

Cell Segmentation Staining

Optional

7

7.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Maintain on Ice				
☐	Xenium Block and Stain Buffer	2001083	Thaw at room temperature for 30 min. Vortex and centrifuge briefly. Maintain on ice.	–20°C
☐	Xenium Multi-Tissue Stain Mix	2000991	Prepare according to Block and Stain on page 83. Maintain on ice.	–20°C
☐	Xenium Stain Enhancer	2000992	To be prepared **next day** according to Stain Enhancement on page 86. Maintain on ice.	–20°C
Equilibrate to room temperature				
☐	Xenium Cassette Insert	3001885	Remove from –20°C and equilibrate to room temperature.	–20°C
Obtain				
☐	Xenium Cassette Lids v2	3002206	Previously used.	Ambient
☐	Nuclease-free Water	-	-	Ambient
☐	Ethanol	-	-	Ambient
☐	1X PBS	-	Prepared at Step 1.1. To be obtained **next day** for Stain Enhancement.	Ambient
☐	PBS-T	-	Prepared at Step 1.1.	Ambient
☐	Forceps	-	-	Ambient
☐	Ice bucket	-	-	Ambient

7.1 Buffer Preparation

a. Retrieve PBS-Tween (PBS-T) prepared in step 1.1.

b. Prepare ethanol dilutions in 15-ml conical tubes using Nuclease-free Water. Vortex and centrifuge briefly. Maintain at room temperature.

For Ethanol Washes		
Items		**Preparation & Handling**
☐	100% Ethanol	Label one 15-ml conical tube as 100% ethanol. Add 15 ml 100% ethanol.
☐	70% Ethanol	Label one 15-ml conical tube as 70% ethanol. Add 10.5 ml 100% ethanol and 4.5 ml Nuclease-free Water.

c. Prepare 1X Diluted Xenium Block and Stain Buffer (for 2 slides) in a centrifuge tube for both blocking and staining steps. Add reagents in the order listed. Vortex and centrifuge briefly. Maintain on ice.

1X Diluted Xenium Block and Stain Buffer (for 2 slides)				
Items		**Stock**	**Final**	**Total Amount (µl)**
☐	Nuclease-free Water	-	-	990.0
☐	Xenium Block and Stain Buffer	4X	1X	330.0
	Total	**-**	**-**	**1,320.0**

7.2 Block and Stain

a. Retrieve the Xenium Cassette from the previous step and place on a flat, clean work surface.

b. Remove the Xenium Cassette Lid v2. Using a pipette, remove all TE Buffer from the well. **Save the lid** for the next indicated steps.

DO NOT let tissue dry out during the ethanol wash steps and between removal of ethanol and addition of PBS-T. Left over ethanol volumes may be used for ethanol wash steps in *Autofluorescence Quenching on page 90*.

Four ethanol washes:

c. **Wash 1:** Add **1,000 µl** 70% ethanol. Incubate for **2 min** at **room temperature**. Remove the ethanol.

d. **Wash 2:** Add **1,000 µl** 100% ethanol. Incubate for **2 min** at **room temperature**. Remove the ethanol.

e. **Wash 3:** Add **1,000 µl** 100% ethanol. Incubate for **2 min** at **room temperature**. Remove the ethanol.

f. **Wash 4:** Add **1,000 µl** 70% ethanol. Incubate for **2 min** at **room temperature**. Remove the ethanol.

g. **Immediately** add **1,000 µl** PBS-T. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

h. Add **500 µl** 1X Diluted Xenium Block and Stain Buffer (from 7.1 Buffer Preparation on the previous page) to cassette for blocking.

Keep remaining volume for later indicated steps.

i. Apply the previously used Xenium Cassette Lid v2 on the Xenium Cassette and incubate for **1 hour** at **room temperature**.

j. During incubation, prepare Xenium Multi-Tissue Stain Mix. Centrifuge tube for **5 sec**.

k. Add **220 µl** 1X Diluted Xenium Block and Stain Buffer (from 7.1 Buffer Preparation on the previous page) to the Xenium Multi-Tissue Stain Mix tube.

l. Pipette mix 15X with (pipette set to 175 µl) and centrifuge briefly. Avoid bubbles if possible.

		Xenium Multi-Tissue Stain Mix	10x PN	1X Diluted Xenium Block and Stain Buffer (µl)
☐	●	Xenium Multi-Tissue Stain Mix	2000991	220.0

DO NOT add any additional antibodies to the Xenium Multi-Tissue Stain Mix as that can compromise assay performance.

m. Incubate resuspended Xenium Multi-Tissue Stain Mix for **30 min** at **room temperature**.

n. Centrifuge Xenium Multi-Tissue Stain Mix for **10 min** at **14,000 rcf** at **4°C**. Maintain on ice.

o. Obtain forceps and Xenium Cassette Insert to prepare for application of the insert onto the cassette

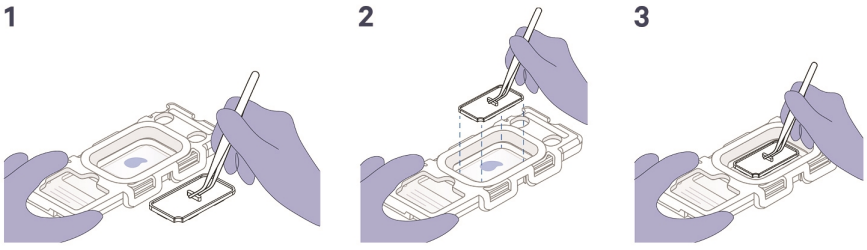
p. Remove the Xenium Cassette Lid v2 and using a pipette, remove 1X Diluted Xenium Block and Stain Buffer from well corners. **Save the lid** for the next indicated steps. DO NOT let the tissue sections dry out.

q. Gently place Xenium Cassette Insert onto the Xenium Cassette using forceps.

TIPS *Leave second slide with 1X Diluted Xenium Block and Stain Buffer until the first cassette has been assembled with insert and Xenium Multi-Tissue Stain Mix has been added.*

Applying Xenium Cassette Insert

Improper placement of the insert may cause scratching or damage to tissue sections.



Place the Xenium Cassette flat on a clean work surface. Ensure cassette is free of debris. Use forceps to pick up Xenium Cassette Insert by Cassette Insert Handle.

Align the Xenium Cassette Insert with the Sample Area well of the Xenium Cassette. Feet and sides should not come into contact with tissue

Carefully place insert within the well. Visually inspect for proper seating.

r. Using a pipette along the side of the tube (avoid touching pellet), withdraw **100 µl** Xenium Multi-Tissue Stain Mix and add to the Xenium Cassette Insert using the cut-out to pipette solution under the Xenium Cassette Insert.

Pipette slowly into cut-out to avoid introducing bubbles as bubbles may impact staining. See Bubbles under Xenium Cassette Insert on page 98 for guidance on resetting the cassette insert if bubbles are present.

s. Apply the previously used Xenium Cassette Lid v2 on the cassette.

t. Incubate the Xenium Cassette **overnight (16-24 h)** at **4°C** (in a refrigerator or incubator).

7.3 Stain Enhancement

a. Following overnight incubation, prepare Xenium Stain Enhancer. Thaw at **room temperature** for **10 min.** Centrifuge briefly. Visually inspect the tube to ensure white powder is at the bottom of the tube before opening. Add **1,100 µl** 1X PBS. Pipette mix 5X and centrifuge for **5 sec**.

		Xenium Stain Enhancer	10x PN	1X PBS (µl)
☐	●	Xenium Stain Enhancer	2000992	1,100

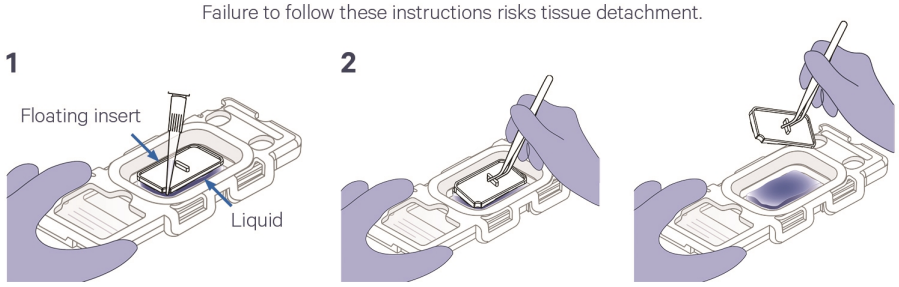
b. Remove the Xenium Cassette Lid v2. Carefully pipette **200 µl** PBS-T into Xenium Cassette Insert Cut-out to float the insert. **Save the lid** for use in the following indicated step.

c. Carefully remove Xenium Cassette Insert with forceps. **Discard** used Xenium Cassette Inserts.

TIPS

Removing Xenium Cassette Insert

Failure to follow these instructions risks tissue detachment.



Place the Xenium Cassette flat on a clean work surface. Pipette 200 µl of PBS-T to float the Xenium Cassette Insert.

Use forceps to pick up Xenium Cassette Insert by Cassette Insert Handle. Carefully remove the insert from the Sample Area well to ensure no liquid splashes out of the well. Remove residual liquid with a lint-free laboratory wipe if necessary.

d. Using a pipette, remove all Xenium Multi-Tissue Stain Mix from well corners.

Three PBS-T Washes (PBS-T prepared in step 1.1):

e. **Wash 1:** Add **500 µl** PBS-T. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

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

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

h. Add **500 µl** resuspended Xenium Stain Enhancer to the well.

i. Apply the previously used Xenium Cassette Lid v2 on the cassette. Incubate at **room temperature** for **20 min**.

TIPS

Start thawing Autofluorescence Quenching reagents during Stain Enhancement as outlined in *Get Started on page 89*.

j. Remove the Xenium Cassette Lid v2. Using a pipette, remove all Xenium Stain Enhancer from well corners. **Save the lid** for the next step.

Two PBS-T Washes (PBS-T prepared in step 1.1):

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

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

m. Add **500 µl** PBS-T.

n. After Cell Segmentation Staining is complete, proceed **immediately** to the next step.

Step 8:

Autofluorescence Quenching

8

8.0 Get Started

Items			10x PN	Preparation & Handling	Storage
Equilibrate to room temperature					
☐	●	Xenium Autofluorescence Mix	2000753	Thaw in a thermomixer (with 2.0-ml thermoblock) for 15 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 waterbath for 15 min at 37°C. Cool to room temperature for 5 min. Vortex for 30 sec and centrifuge briefly.*	–20°C
☐	○	Reducing Agent B	2000087	Thaw at room temperature. Vortex and centrifuge briefly.	–20°C
☐	●	Xenium Nuclei Staining Buffer	2000762	Thaw at room temperature. Vortex and centrifuge briefly. Keep in the dark until ready to use.	–20°C
Obtain					
☐		Nuclease-free Water	-	-	Ambient
☐		1X PBS	-	Prepared at Step 1.1.	Ambient
☐		PBS-T	-	Prepared at Step 1.1.	Ambient
☐		100% Ethanol	-	-	Ambient
☐		Xenium Cassette Lids v2	3002206	Previously used (use new lid if storing slides).	Ambient

*If processing only one slide, remove the volume required (specified in the following section), and refreeze the remaining reagent in the tube at –20°C.

TIPS

Pre-heat appropriate equipment to: 37°C

8.1 Autofluorescence Quenching

a. Prepare the following for Autofluorescence Quenching:

i. Prepare diluted Reducing Agent B. Add reagents in the order listed and vortex to mix. Maintain at room temperature.

	Diluted Reducing Agent B	10x PN	Stock	Final	1X+10% (µl)	2X+10% (µl)
	1X PBS (prepared at Step 1.1)	-	-	-	544.5	1,089.0
◯	Reducing Agent B	2000087	-	-	5.5	11.0
	Total	**-**	-	-	**550.0**	**1,100.0**

ii. Prepare 70% ethanol. Add reagents in the order listed and vortex to mix. Maintain at room temperature.

70% Ethanol	10x PN	Stock	Final	1X+10% (µl)	2X+10% (µl)
Nuclease-free Water	-	-	-	330.0	660.0
100% Ethanol	-	100%	70%	770.0	1,540.0
Total	**-**	-	-	**1,100.0**	**2,200.0**

iii. Prepare Autofluorescence Solution using thawed Xenium Autofluorescence Mix prepared according to step 8. Add reagents in the order listed and vortex to mix. Maintain at room temperature in the dark until ready to use.

If processing only one slide, remove the Xenium Autofluorescence Mix volume required for 1X+10% (specified in the table below), and refreeze the remaining reagent at −20°C. Once diluted with ethanol, the Xenium Autofluorescence Mix should not be refrozen.

	Autofluorescence Solution	10x PN	Stock	Final	1X+10% (µl)	2X+10% (µl)
	100% Ethanol	-	100%	-	544.5	1,089.0
●	Xenium Autofluorescence Mix	2000753	-	-	5.5	11.0
	Total	**-**	-	-	**550.0**	**1,100.0**

b. Retrieve the Xenium Cassette v2 from either after Post Amplification Wash on page 79 or after Stain Enhancement on page 86 and place on a flat, clean work surface.

c. Using a pipette, remove all the buffer from the well. **Save the lid** for the following indicated step.

Skip the next three PBS wash steps d-f if Cell Segmentation Staining was done.

d. **Wash 1:** Skip if cell segmentation was done. Add **1,000 µl** 1X PBS prepared at step 1.1 to the well and incubate for **1 min** at **room temperature**. Remove all 1X PBS.

e. **Wash 2:** Skip if cell segmentation was done. Add **1,000 µl** 1X PBS prepared at step 1.1 to the well and incubate for **1 min** at **room temperature**. Remove all 1X PBS.

f. **Wash 3:** Skip if cell segmentation was done. Add **1,000 µl** 1X PBS prepared at step 1.1 to the well and incubate for **1 min** at **room temperature**. Remove all 1X PBS.

g. Add **500 µl** Diluted Reducing Agent B prepared at step 8.1ai to the well.

h. Apply the previously used a new Xenium Cassette Lid v2 on the cassette, and incubate for **10 min** at **room temperature**.

i. Remove the Xenium Cassette Lid v2. Using a pipette, remove all Diluted Reducing Agent B from the well. **Save the lid** for use in following indicated steps.

Three ethanol washes:

j. **Wash 1:** Add **1,000 µl** 70% ethanol. Incubate for **1 min** at **room temperature**. Remove the ethanol.

k. **Wash 2:** Add **1,000 µl** 100% ethanol. Incubate for **1 min** at **room temperature**. Remove the ethanol.

l. **Wash 3:** Add **1,000 µl** 100% ethanol. Incubate for **1 min** at **room temperature**. Remove the ethanol.

m. Pipette mix Autofluorescence Solution prepared at step 8.1aiii thoroughly before dispensing onto sample to prevent settling of reagent. Add **500 µl** Autofluorescence Solution to the well.

n. Reapply Xenium Cassette Lid v2 on the cassette, and incubate for **10 min** at **room temperature in the dark**.

o. Prepare a thermal cycler with the following incubation protocol. Place a Thermocycler Adaptor v2 on the thermal cycler and start the program.

Lid Temperature	Reaction Volume	Run Time
37°C (lid may be set to the lowest temperature if the instrument does not enable 37°C)	100 µl	-

Step	Temperature	Time *hh:mm:ss*
Pre-equilibrate	37°C	Hold
Drying	37°C	00:05:00

p. Remove the Xenium Cassette Lid v2. Using a pipette, remove all the buffer from the well. **Save the lid** for use in following indicated step.

Three ethanol washes:

q. **Wash 1:** Add **1,000 µl** 100% ethanol. Incubate for **2 min** at **room temperature**. Remove the ethanol.

r. **Wash 2:** Add **1,000 µl** 100% ethanol. Incubate for **2 min** at **room temperature**. Remove the ethanol.

s. **Wash 3:** Add **1,000 µl** 100% ethanol. Incubate for **2 min** at **room temperature**. Remove the ethanol.

t. Place Xenium Cassette v2 **without lid** on the Thermocycler Adaptor v2 on the thermal cycler to dry. DO NOT close the thermal cycler lid.

u. Skip pre-equilibrate step to initiate Drying.

v. **Immediately** remove the cassette from the Thermocycler Adaptor and place on a flat, clean work surface.

w. Add **1,000 µl** 1X PBS prepared at step 1.1 to rehydrate the tissue and incubate for **1 min** at **room temperature in the dark**.

x. Remove all 1X PBS.

y. Add **1,000 µl** PBS-T and incubate for **2 min** at **room temperature in the dark**.

Optional: photograph the slide against a white background. This image can be used for comparison purposes to identify tissue detachment downstream in the workflow. See Troubleshooting for more details.

z. Store slides **16-24 h** (overnight) at **4°C in the dark** with a Xenium Cassette Lid applied on the Xenium Cassette. If storing slides, DO NOT discard the lid; instead save for Nuclei Staining. Alternatively, proceed to the next step.

8.2 Nuclei Staining

a. Retrieve thawed Xenium Nuclei Staining Buffer prepared as outlined in 8.0 Get Started on page 89.

b. Retrieve the Xenium Cassette v2 from the previous step and place on a flat, clean work surface.

c. If stored, remove the Xenium Cassette Lid v2. Using a pipette, remove all PBS-T from the well. **Save the lid** for use in following indicated steps.

d. Add **500 µl** Xenium Nuclei Staining Buffer and incubate **1 min** at **room temperature in the dark**.

e. Remove all Nuclei Staining Buffer.

Three PBS-T washes (PBS-T prepared at step 1.1):

f. **Wash 1:** Add **1,000 µl** PBS-T. Incubate for **1 min** at **room temperature in the dark**. Remove all PBS-T.

g. **Wash 2:** Add **1,000 µl** PBS-T. Incubate for **1 min** at **room temperature in the dark**. Remove all PBS-T.

h. **Wash 3:** Add **1,000 µl** PBS-T. Incubate for **1 min** at **room temperature in the dark**. Remove all PBS-T

i. Add **1,000 µl** PBS-T.

j. Store slides (as specified below) or alternatively, proceed directly to the Xenium Analyzer User Guide (CG000584).

Xenium Analyzer v3.0 (XA v3.0) or higher is required on the instrument for running the Xenium Prime workflow. When executing the workflow on the instrument, note that the Xenium Prime workflow is compatible only with Xenium Prime panels and reagents.

Short-term storage for **≤1 week** at **4°C in the dark** with a new Xenium Cassette Lid v2 applied on the cassette.

Ensure that the slide is stored in microbe-free and nuclease-free conditions, with a new Xenium Cassette Lid v2 applied to prevent evaporation. Storing slides for more than recommended time risks decrease in sensitivity. These risks are dependent on many factors, including input tissue quality and how nuclease-free and microbe-free the workflow is.

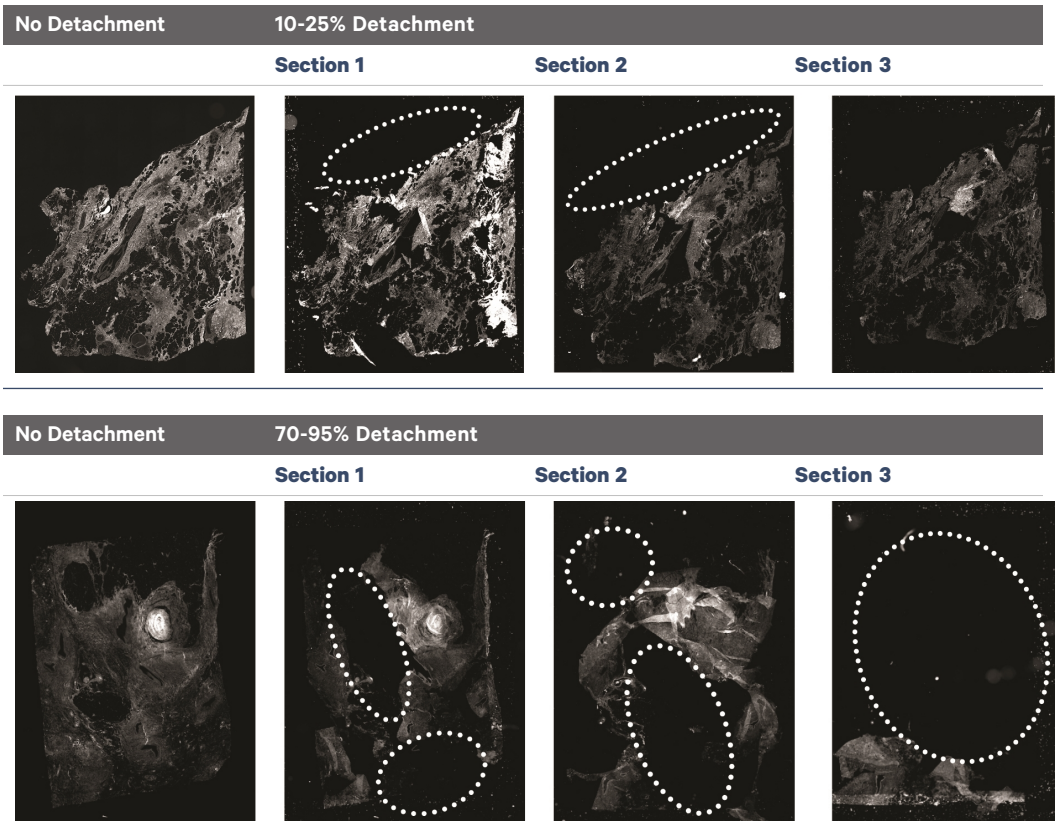
The above storage guidelines can also be used for storing Xenium cassettes with slides after Xenium Analyzer run (or in the event of a run failure). Contact support@10xgenomics.com for more information.

Troubleshooting

Tissue Detachment and Folding

Tissue detachment may result in a lack of decodable data in the region where detachment occurred. If the tissue has folded on itself, this may also cause elevated signal in the overlapping areas. Inspect images carefully to identify these areas. If tissue detachment is observed during this workflow, contact support@10xgenomics.com

Tissue Detachment in Human Breast as viewed on Xenium Analyzer Overview Scan



Percentages represent tissue detachment/"area that cannot be analyzed" at the end of the Xenium Analyzer workflow. White circles indicate areas of tissue detachment.

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) and 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.

Bubbles during Workflow

Bubbles may occur throughout the workflow, including during PBS-T washes. Bubbles floating on the surface of the slide are unlikely to compromise assay performance. However, bubbles that are in contact with the tissue during a Xenium Analyzer run may result in a lack of decodable data in the tissue area where the bubbles occurred.

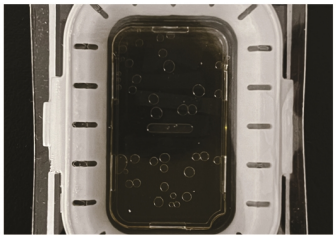
Avoid generating bubbles during reagent dispensing by pipetting slowly and avoiding expelling air from the pipette tip. Gently tap or rock the cassette after reagent dispension and inspect the cassette carefully to ensure liquid is fully covering the tissue. DO NOT aspirate or pop the bubbles as this could lead to tissue detachment or scratching of the tissue. Ensure there are no bubbles on the assembled cassette before loading it into the Xenium Analyzer.

Bubbles under Xenium Cassette Insert

Bubbles under Xenium Cassette Insert

Bubbles when dispensing reagents

Bubbles following overnight incubation



Bubbles when dispensing reagents

Bubbles may occur when dispensing Xenium Multi-Tissue Stain Mix under the Xenium Cassette Insert.

If bubbles are observed, DO NOT aspirate or pop the bubbles as this could lead to tissue detachment or scratching of the tissue. Instead, remove the insert and reapply the insert to expel these bubbles.

These bubbles may impact staining quality and can potentially impact assay performance.

- When dispensing a reagent: pipette slowly and avoid expelling any air from the pipette tip.
- After dispensing the reagent: visually inspect the cassette to ensure that the reagent is fully covering the tissue.
-
  - If bubbles are observed, remove the insert and reapply the insert to expel these bubbles.
  - Gently lift the insert peeling it off from one corner until the bubbles are released.
  - Place the insert back the way it was removed.

Bubbles after overnight incubation

Bubbles can also appear after overnight incubation of the slide with the Xenium Multi-Tissue Stain Mix due to bubble nucleation and are unlikely to impact staining or assay performance.

Number of Washes

Washes post-RNase treatment, post-Polishing, post-Hybridization and post-Ligation washes are critical for assay performance. Failure to perform the

correct number of washes can reduce the fraction of usable decodable data. A similar effect is observed when washing for less than the recommended time, or when reagent is carried over during the washes. Remove all liquid from the well when washing, and refer to User Guide for correct number of washes and incubation times.

As recommended in the protocol, use only 0.5X SSC-T for washes prior to Polishing incubation. Using any other buffer, such as PBS-T, will impact assay performance.

Samples Dry Out

Drying of tissue samples may lead to decreased decoding efficiency and unusable data and will impact overall assay performance and sensitivity. Work quickly and ensure reagents are dispensed evenly across tissues during incubation and wash steps throughout the workflow to prevent drying out of tissues. If processing two slides at a time, remove and add reagent from first slide before proceeding to second slide. Ensure tissue sections are always covered with reagent in between removal and addition steps. Note that there are no safe stopping points except for those described in the protocol and outlined specifically in the Protocol Steps & Timing on page 16.

Cassette Assembly Failure

Incorrect assembly of the Xenium cassettes 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.
- For Xenium Cassette v2, slide is not placed underneath the slide clip.
- 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 the following pages.

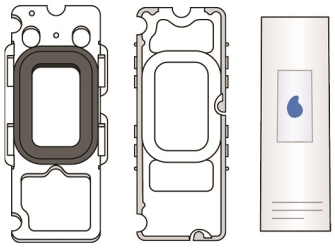
The guidance applies to both Xenium Cassette and Xenium Cassette v2. The image below shows a Xenium Cassette.

Inspect gasket during cassette assembly. Damaged gaskets can lead to leaks in the cassette.

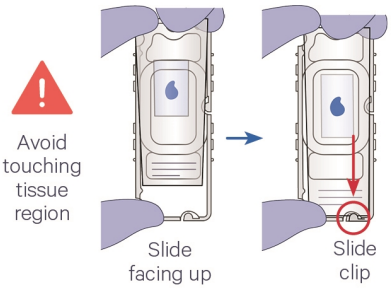
Xenium Cassette v2 Assembly

Exercise caution when handling slide edges to prevent injury.

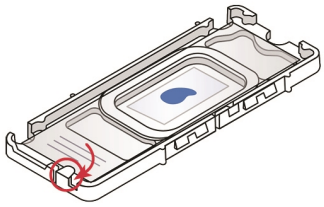
1 Place top and bottom halves of cassette on bench with the top cassette facing down and bottom cassette facing up.



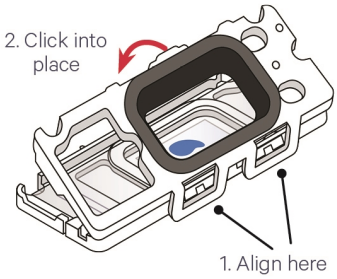
2 Place Xenium slide with label at bottom and tissue facing up into bottom half of cassette. Slip slide under slide clip located at bottom right of cassette.



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

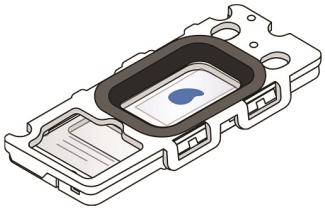


4 Align inner clips of top cassette to inner tabs of bottom cassette. Bring opposite side down until 2 audible clicks are heard.



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

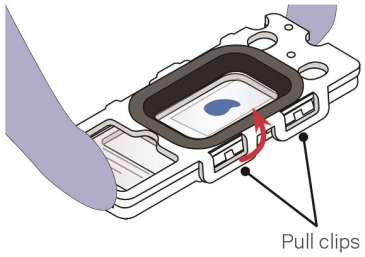
**Consult Xenium Prime In Situ Gene Expresssion with optional Cell Segmentation Staining User Guide (CG000760) for cassette assembly failures.*



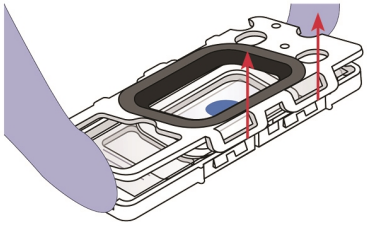
Xenium Cassette v2 Removal

Exercise caution when handling slide edges to prevent injury.

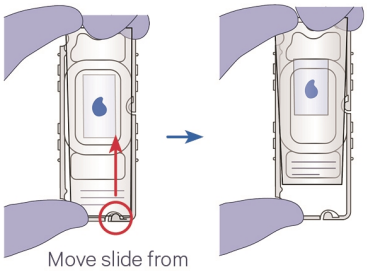
1 Pull inner clips from inner tabs to detach top and bottom halves of cassette.



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



Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning

Xenium Cassettes, Inserts, and Lids are single use items and are to be discarded after use (unless specified in the protocol step that the lid should be saved and reused). Cassettes (prior to cassette assembly) or lids that are accidentally dropped may be reused after thorough cleaning. Note that PBS-T washes DO NOT require sealing of the cassette.

The instructions apply to both Xenium Cassette and Xenium Cassette v2 and lids.

Cleaning Procedure:

- Rinse the lid under running Milli-Q Water
- Spray with 70% isopropanol
- Rinse under running Milli-Q Water
- Spray with 70% isopropanol a second time
- Rinse under running Milli-Q Water
- Air dry

Incorrect Autofluorescence Quenching

Variation in stain color is normal and tissue-type dependent in tissue sections correctly stained with Autofluorescence Solution. Incorrect staining scenarios are listed below:

- Uneven staining with Autofluorescence Solution may be visible as a non-uniform stain across a tissue section.
- Overquenching can cause tissue to overheat on the Xenium Analyzer, and data generated in the overheated spots may be compromised or missing.

Improper Autofluorescence Quenching risks lower transcript quality scores and reduced median transcripts per cell, but depends on the sample type. Underquenching is lower risk than overquenching.

Ensure Autofluorescence Solution is well mixed and dispensed uniformly across the tissue sections to avoid uneven staining. Autofluorescence Quenching has been optimized for a large number of tissue types and modifying the dilution listed in the User Guide is not recommended. Cassette should be sealed properly and firmly during incubation to prevent reagent evaporation.

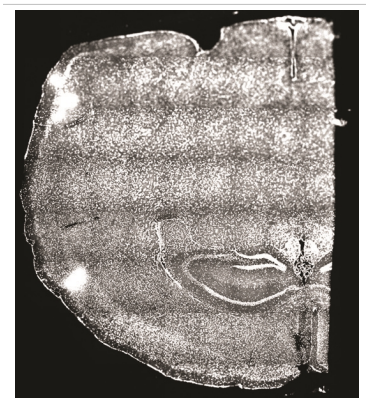
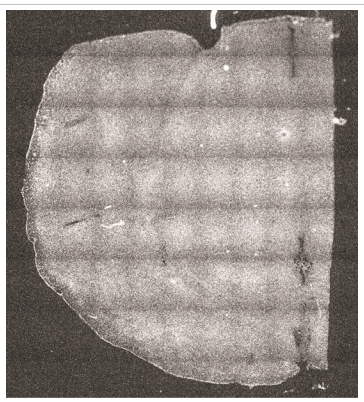
Incorrect or insufficient Autofluorescence Quenching may cause variation in staining of tissues *(mouse brain pictured below)*

Unstained	Normally Stained	Overstained	Understained
			

Incorrect Nuclei Staining

Incorrect staining of nuclei may lead to poor image quality and an inability to easily identify tissue or regions of interest when selecting areas to image during a Xenium Analyzer overview scan. Follow the Nuclei Staining protocol as instructed using the Xenium Nuclei Staining Buffer provided in the Xenium Prime Sample Prep Reagents - (2 rxns) PN-1000720. Confirm Xenium Nuclei Staining Buffer is well mixed and applied uniformly across tissue sections. All incubations with Xenium Nuclei Staining Buffer should be performed in the dark. If an alternate staining protocol or buffer is used, lower quality images may be obtained.

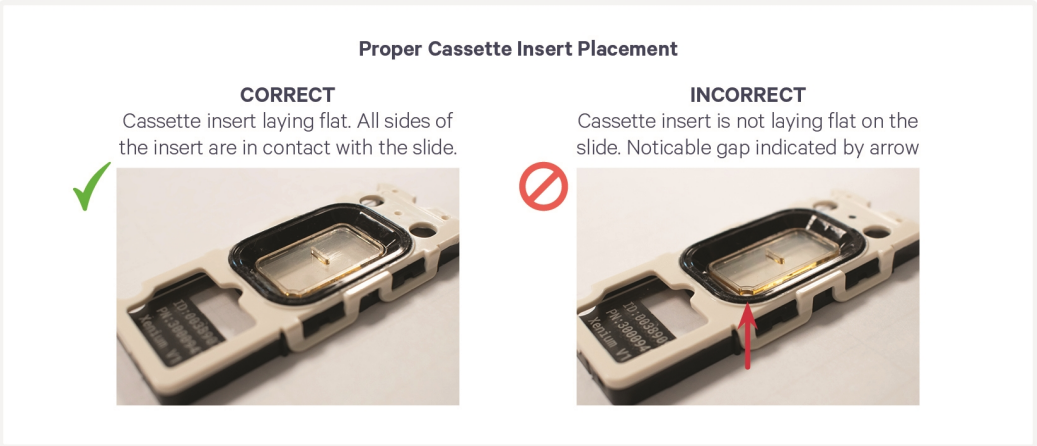
Incorrect or insufficient nuclei staining may impact image quality and region of interest selection *(as viewed on a Xenium Analyzer overview scan)*

Correct Nuclei Staining Protocol	No Nuclei Staining Performed
	

Improper Placement of Xenium Cassette Insert

Improper placement of the Xenium Cassette Insert in the Xenium Cassette can negatively impact assay performance and may cause damage to tissue sections. Inspect the insert carefully to ensure it is seated fully within the cassette. Practice placement of insert with a blank slide if necessary to avoid damage to experimental samples. Refer to Xenium Cassette Insert Practice on page 40.

Place the assembled cassette on a white surface to check insert placement. If the insert is incorrectly placed (see image below), remove and reapply the insert into the cassette. Gently lift the cassette insert by the handle using forceps. DO NOT lift the insert straight up as this may cause tissue detachment. Instead, gently peel back one corner and lift the insert. Finally, place insert back the way it was removed.



Appendix

Sample Shipping

Processed Xenium slides may be shipped with in 1 week of completing the Xenium In Situ assay workflow. After Nuclei Staining, remove all PBS-T, disassemble the Xenium Cassette, and place no more than two slides in a mailer. Ship using one of the two options:

- Fill the mailer (containing the slides) to capacity with PBS-T, place the mailer in a container with ice packs, and ship (overnight to 2 days).
- Dehydrate the slides by serial ethanol washes as described on Xenium Cassette Storage on page 34. Fill the mailer (containing the slides) to capacity with a cryoprotectant (30% Glycerol), place the mailer in a container with dry ice, and ship (overnight to 2 days).

Note that assay performance may be compromised post-shipping and handling.