10x GENOMICS

User Guide | CG000819 | Rev A

Xenium
In Situ Gene and Protein Expression

with Cell Segmentation Staining

For use with:

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

Xenium Decoding Consumables *1 run, 2 slides PN-1000487*

Xenium Pre-Designed Gene Expression Panel *2 rxns**

Xenium Custom Gene Panel,

Add-on, 4 & 16 rxns | Standalone, 4 & 16 rxns**

Xenium Cell Segmentation Staining Reagents, *2 rxns PN-1000661*

Xenium Protein Immunology Subpanel Kits, *2 rxns*

PN-1000833, 1000834, 1000846, 1000835, 1000836, 1000837 (each kit sold separately)

Xenium Instrument Accessory Kit Module A *PN-1000530*

Xenium Slide Kit

4 Slides PN-1000659 | 16 Slides PN-1000660

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

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

Notices

Document Number

CG000819 | 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

Email: support@10xgenomics.com
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Pleasanton, CA

Document Revision Summary

Document Number

CG000819

Title

Xenium In Situ Gene and Protein Expression with Cell Segmentation Staining User Guide

Revision

Rev A

Revision Date

July 22, 2025

Description of Changes

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Introduction

Reagent Kits

Xenium In Situ Gene Expression Reagent Kits

Refer to SDS for handling and disposal information.

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

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

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

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All items, except Xenium FFPE Tissue Enhancer (PN-2000798) and Perm Enzyme B (PN-3000553), are needed for this workflow.

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

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

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

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

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Only the Xenium Cassette Kit (2 cassettes + 16 lids) is needed for this workflow. Consult the Xenium Quick Reference Card for Slide Cassette Assembly (CG000623) for cassette assembly and removal instructions.

Xenium Pre-Designed Gene Expression Panel - (2 rxns)

Xenium Pre-Designed Gene Expression Panel
(2 rxns)
Shipped in dry ice
Store at −20°C

		#	PN
	Xenium Pre-Designed Gene Expression Probes*	1	-

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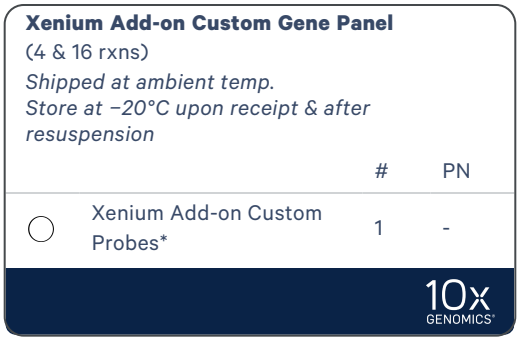
Example Xenium Pre-Designed Gene Expression Panel kit.

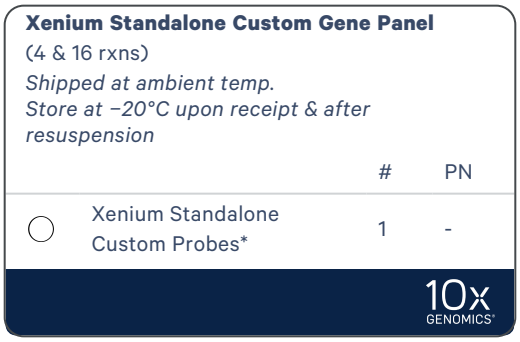
Xenium Add-on Custom Gene Panel - (4 & 16 rxns)



Example Xenium Add-on Custom Gene Panel kit.

Use TE Buffer, pH 8.0 to resuspend add-on custom probes. Relevant guidance is provided in the workflow steps.

Xenium Standalone Custom Gene Panel - (4 & 16 rxns)



Example Xenium Standalone Custom Gene Panel kit.

Use TE Buffer, pH 8.0 to resuspend standalone custom probes. Relevant guidance is provided in the workflow steps.

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

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

Xenium Cell Segmentation Staining Reagents
(2 rxns), PN-1000661
Shipped in dry ice
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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Xenium Protein Immunology Subpanel Kits

Each subpanel kit is sold separately

Xenium Protein Immunology Subpanel Kits*
(2 rxns)
**Each subpanel kit is sold separately*
Shipped in dry ice
Store at −20°C

	#	PN
Xenium Protein Immune Cell Subpanel A (2 rxns), Kit PN 1000833	1	2001380
Xenium Protein Immune Cell Subpanel B (2 rxns), Kit PN 1000834	1	2001381
Xenium Protein Immune Cell Subpanel C (2 rxns), Kit PN 1000846	1	2001426
Xenium Protein Proliferation & Differentiation Subpanel (2 rxns), Kit PN 1000835	1	2001382
Xenium Protein Immune Checkpoint Subpanel (2 rxns), Kit PN 1000836	1	2001383
Xenium Protein Tumor Subpanel (2 rxns), Kit PN 1000837	1	2001384

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Xenium Instrument Accessory Kit, Module A PN-1000530

Xenium Instrument Accessory Kit Module A
PN-1000530
Shipped at ambient temp.
Store at ambient temp.

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

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

Xenium Slide Kit, 4 Slides PN-1000659

Xenium Slide Kit, 4 slides
PN-1000659
Shipped in dry ice
Store at -20°C

	#	PN
Xenium Slides (2 pack)	2	3000941

Xenium Slide Kit, 16 Slides PN-1000660

Xenium Slide Kit, 16 slides
PN-1000660
Shipped in dry ice
Store at -20°C

	#	PN
Xenium Slides (2 pack)	8	3000941

Third Party Items

Successful execution of the Xenium In Situ Gene and Protein Expression workflow requires third-party reagents, kits, and equipment in addition to those provided by 10x Genomics. All third party reagents and consumables should be obtained prior to starting this workflow.

Consult the Xenium In Situ Gene Expression Protocol Planner (CG000601) for a list of the following third-party items:

- Additional reagents, kits, and equipment
- Tested pipette tips
- Tested thermal cyclers
- Tested blank slides

10x Genomics has tested all items listed in the Protocol Planner. Unless otherwise noted, 10x Genomics recommends using these items to ensure optimal assay performance. Substituting materials may adversely affect assay performance.

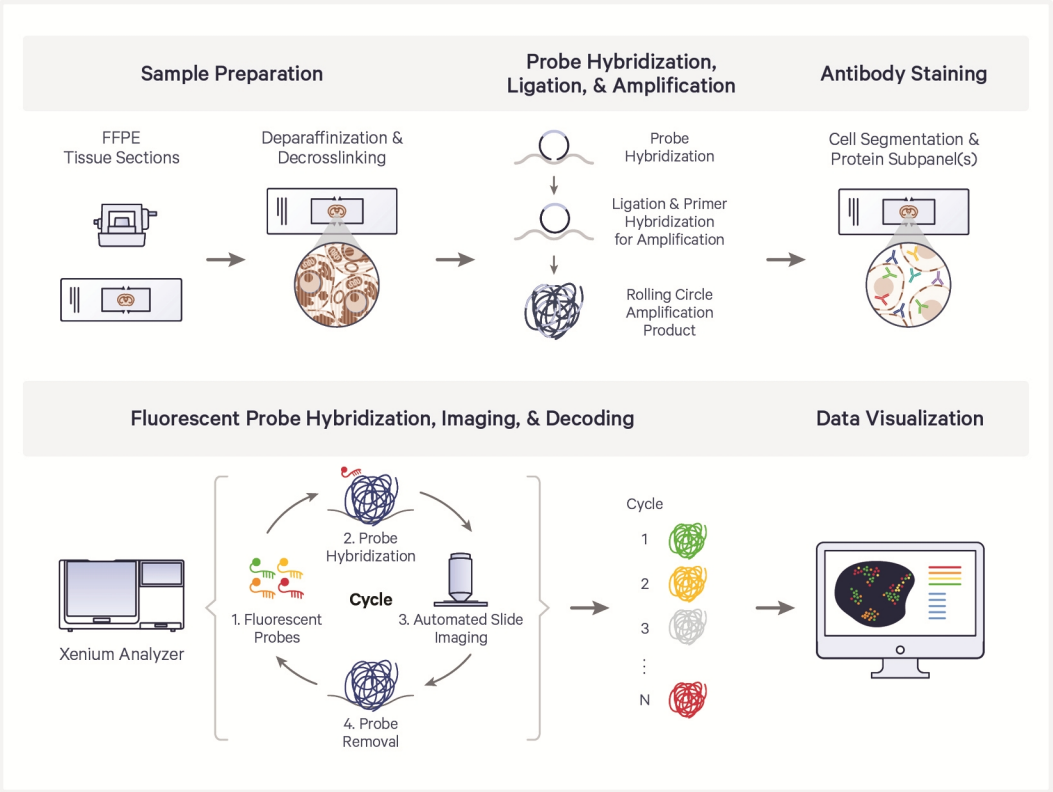
Protocol Steps & Timing

Steps	Timing	Stop & Store
Day 1		
Step 1: Probe Hybridization		
1.1 Buffer Preparation	20 min	
1.2 Custom Probe Preparation (optional)	10 min	
1.3 Probe Hybridization	16-24 h (overnight)	
Day 2		
Step 2: Post Hybridization Wash		
2.1 Post Hybridization Wash	35 min	
Step 3: Ligation		
3.1 Ligation	~2 h	
Step 4: Amplification		
4.1 Amplification	~2 h	
4.2 Post Amplification Wash	15 min	STOP 4°C ≤2 days
Step 5: Antibody & Cell Segmentation Staining		
5.1 Buffer Preparation	20 min	
5.2 Tissue Dehydration & Secondary Decrosslinking	40 min	
5.3 Block & Stain	~1 h + 16-24 h (overnight)	
Day 3		
5.4 Stain Enhancement	~1 h	
Step 6: Autofluorescence Quenching		
6.1 Autofluorescence Quenching	30 min	STOP 4°C overnight (in the dark)
6.2 Nuclei Staining	10 min	STOP 4°C ≤ 1 week (in the dark)

Storing slides for more than the recommended time risks a 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

Workflow Overview



Xenium In Situ Gene and Protein Expression assays RNA levels and detects protein in formalin fixed & paraffin embedded (FFPE) tissue sections. It uses targeted probes to assay RNA at the subcellular level and oligonucleotide conjugated antibodies grouped in subpanels for detecting immune cell protein markers. FFPE tissue sections placed on Xenium Slides are deparaffinized and decrosslinked as described in the Xenium In Situ - FFPE Tissue Preparation Handbook (CG000578).

Pre-designed, add-on custom, or standalone custom probe panels are then added to the tissue. Each circularizable DNA probe contains two regions that hybridize to the target RNA and a third region that encodes a gene-specific barcode. The two ends of the probes bind the target RNA and are ligated to generate a circular DNA probe. Following ligation, the circularized probe is enzymatically amplified, generating multiple copies of the gene-specific barcode for each RNA target. Tissue sections are stained with a mix of Xenium Protein antibody subpanels for identification of major immune cell types and subtypes, as well as immune checkpoint molecules. Cell segmentation reagents allow for labeling of cell nuclei, membranes, and interiors that are inputs for automated morphology-based cell segmentation analysis.

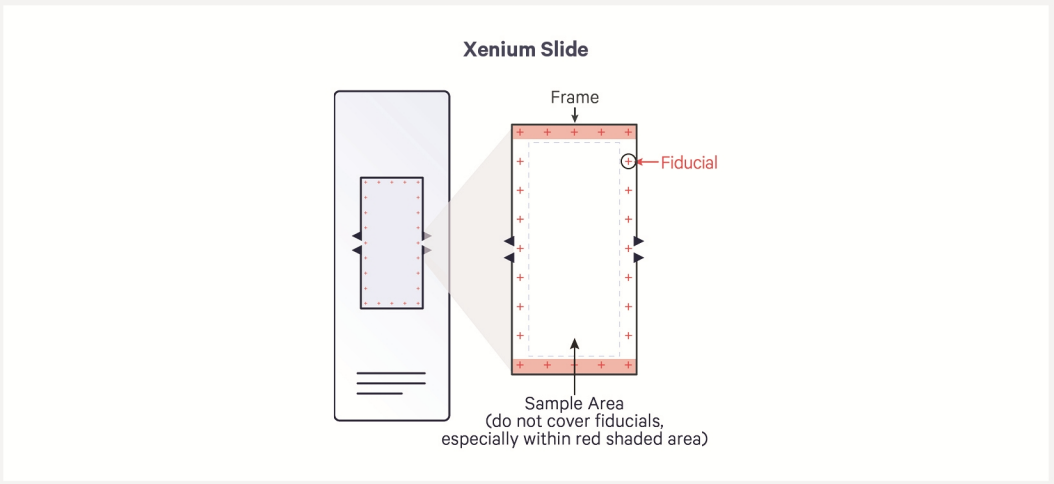
Xenium slides containing FFPE tissue sections are then loaded for imaging and analysis on the Xenium Analyzer instrument for high-throughput, automated in situ analysis. Fluorescently labeled oligos bind to the amplified DNA probes. Cyclical rounds of fluorescent probe hybridization, imaging, and removal generate optical signatures specific for each barcode, which are converted into a gene identity. Fluorescently labeled oligos specific to protein detection bind to oligonucleotide conjugated antibodies, revealing the target protein stain. Xenium Explorer software allows for the simultaneous visualization and analysis of gene and protein expression data on a per-cell basis.

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

A high-level overview of each step in this User Guide is provided in the following sections.

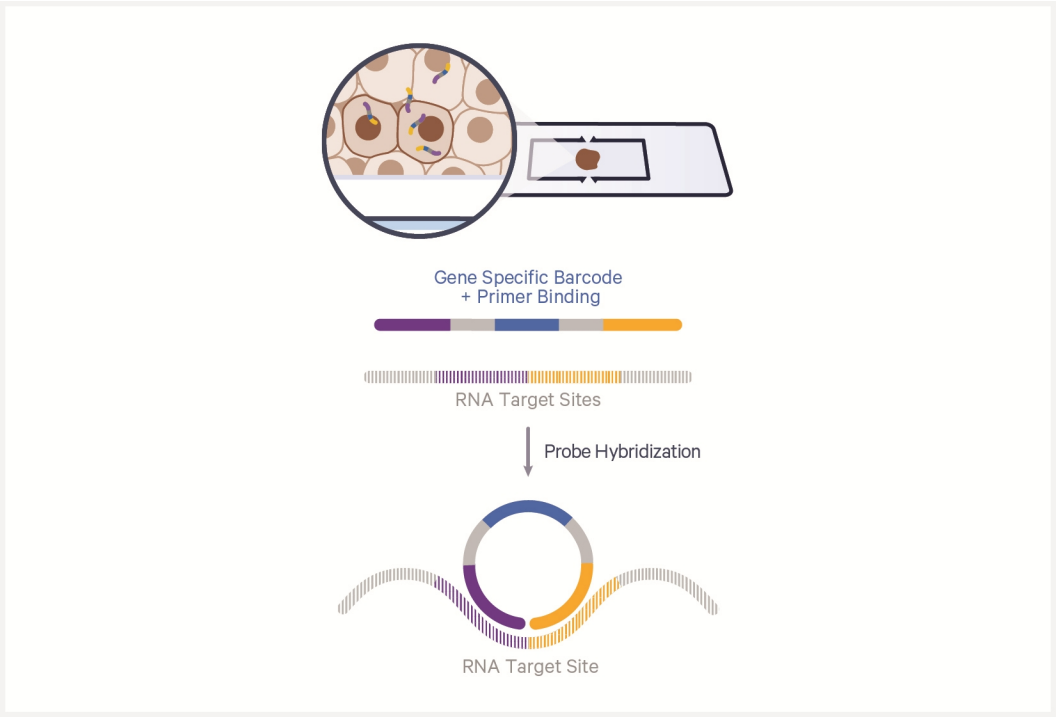
Xenium Slide

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 tissue sections are placed within the Sample Area for downstream processing and analysis.



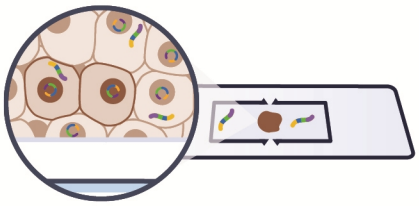
Step 1: Probe Hybridization

Pre-designed, add-on custom, or standalone custom probe panels are added to the FFPE tissue sections. The DNA probes are flanked by two regions that independently hybridize to the target RNA and also contain a gene-specific barcode sequence. The probes hybridize to their complementary target RNA in an overnight incubation.



Step 2: Post Hybridization Wash

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



Step 3: Ligation

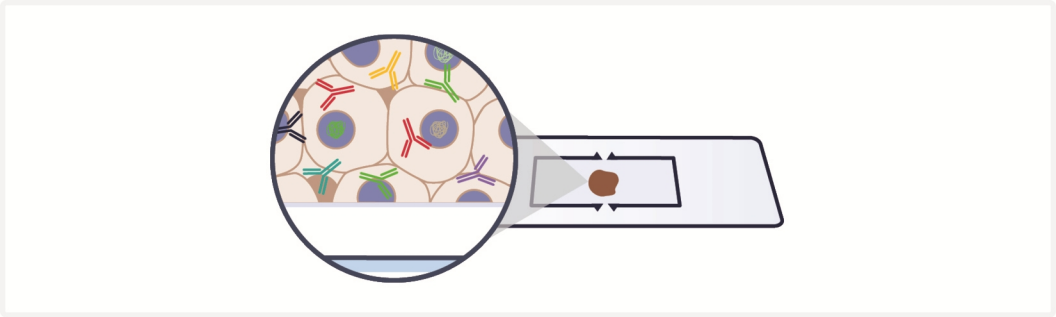
After removal of unbound probes, a ligase is added to seal the junction between the probe regions that have hybridized to RNA. Ligation of the probe ends on the targeted RNA region generates a circular DNA probe. This ligation ensures a unique level of probe specificity to the target region.

Step 4: Amplification

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

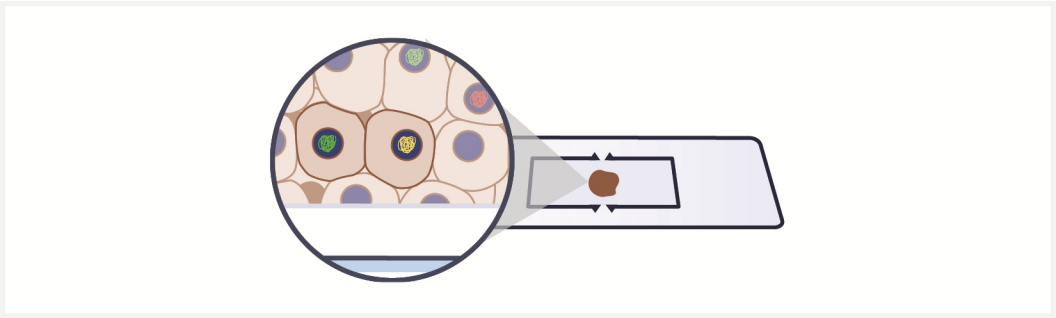
Step 5: Antibody & Cell Segmentation Staining

Following dehydration and secondary decrosslinking steps, blocking reagent is added to the tissue sections to reduce non-specific binding during staining. A mix of Xenium Protein subpanel and 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 6: Autofluorescence Quenching

Autofluorescence Quenching diminishes unwanted autofluorescence and enhances signal-to-noise ratio in the treated FFPE 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 are loaded into the Xenium Analyzer for imaging and decoding.



Tips & Best Practices

TIPS

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.

Probe Panel Handling

- 10x Genomics provides the following types of probe panels: pre-designed add-on custom, and standalone custom. Add-on custom panels are used to supplement pre-designed panels. Standalone custom probe panels are used alone and do not require pre-designed panels.
- Pre-designed probes are good for two Xenium slides. Add-on and Standalone custom probes are good for either four or sixteen Xenium slides.
- Add-on and Standalone custom probes are delivered lyophilized at room temperature and should be stored at -20°C upon resuspension.
- Custom probes must be resuspended prior to use. See Custom Probe Preparation (optional) on page 44 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.

Gene Probe Panel Storage & Shipping

- Pre-designed 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 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 and Standalone custom probe panels are lyophilized and are shipped at room temperature. Store at -20°C upon receipt.
- Add-on and Standalone custom 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, pH 8.0 at -20°C or at kit expiration date (whichever comes first).
- Custom probes must be resuspended prior to use in TE Buffer, pH 8.0. After resuspension, they are stable for short-term at room temperature (<8 hours). Resuspended custom probes should be discarded if left at room temperature for more than 8 hours.
- Custom 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.

Protein Subpanel Handling & Resuspension

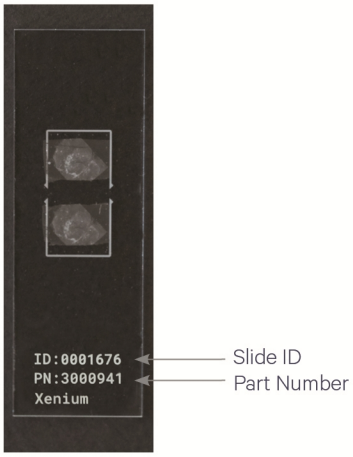
- Choose subpanel tubes based on experimental needs. Subpanels can be resuspended in any order.
- The same subpanels must be added to both slides in an experiment.
- Centrifuge subpanel tubes before resuspension to move lyophilized material to the bottom of the tube. Failure to perform this centrifugation step may alter antibody concentration and weaken staining.
- Pipette from bottom of tube during serial resuspension steps to avoid loss of liquid.
- When pipetting from subpanel tube to the 1.5 ml eppendorf tube to combine with cell segmentation markers, the entire volume should be transferred.
- Avoid bubble formation during handling and resuspension.

General Xenium Slide Handling

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

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

Xenium Slide with Tissue Sections



Processing a Single Xenium Slide

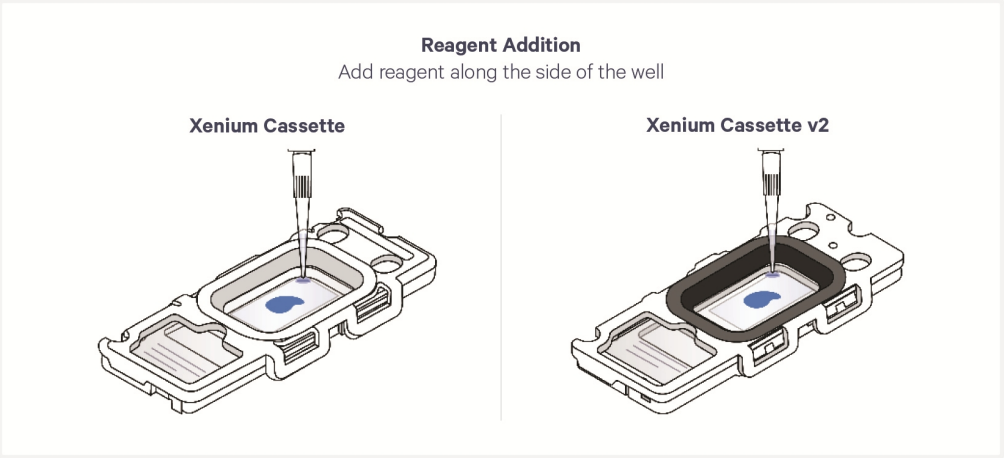
- Xenium reagent kits are sufficient for two reactions, and for optimal Xenium Analyzer throughput, two slides should be run at the same time.
- It is possible to perform the Xenium In Situ Gene Expression workflow with a single slide. To do this, ensure the following best practices are followed for optimal assay performance:
  - Assemble a mock Xenium Cassette using a blank slide and a cassette from the Xenium Cassette Kit (2 cassettes), PN-1000566.
  - Insert the blank slide into the Xenium Cassette. Cassettes should be assembled following the instructions in Troubleshooting for Cassette Assembly Failure on page 93.
  - Attach a Xenium Cassette Lid from the Xenium Cassette Kit (2 cassettes), PN-1000566 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.

Reagent Addition to Wells

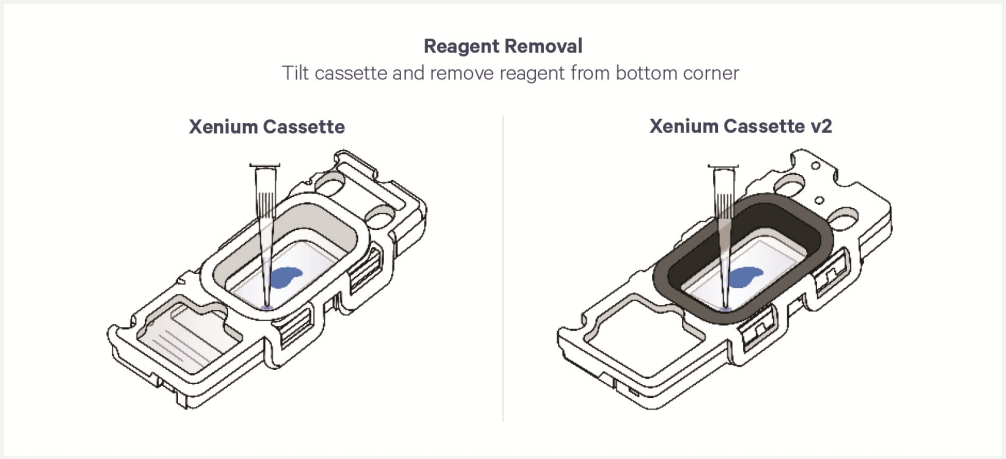
- Place assembled cassette flat on a clean work surface.
- Dispense and remove reagents along the side of the well without touching the tissue sections and without introducing bubbles.
- Always cover the Sample Area completely when adding reagents to the well. A gentle tap may help spread the reagent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

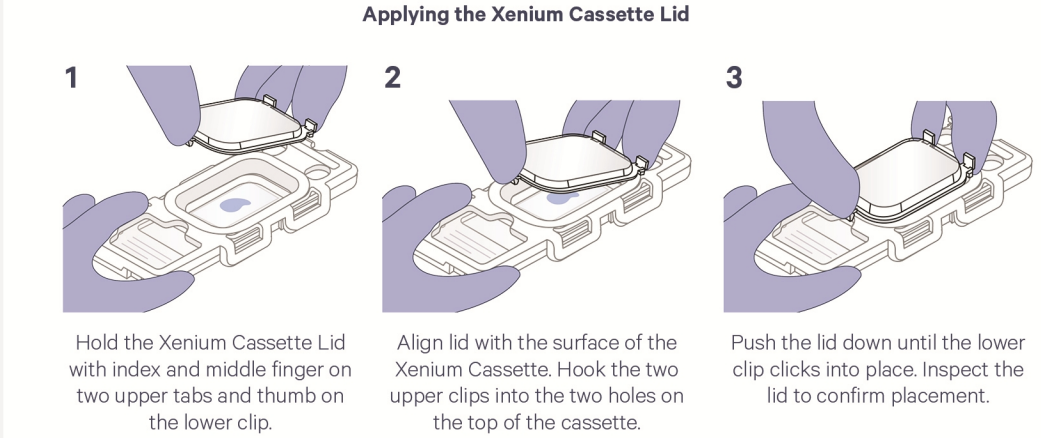
- Remove all liquid from the well in each step.



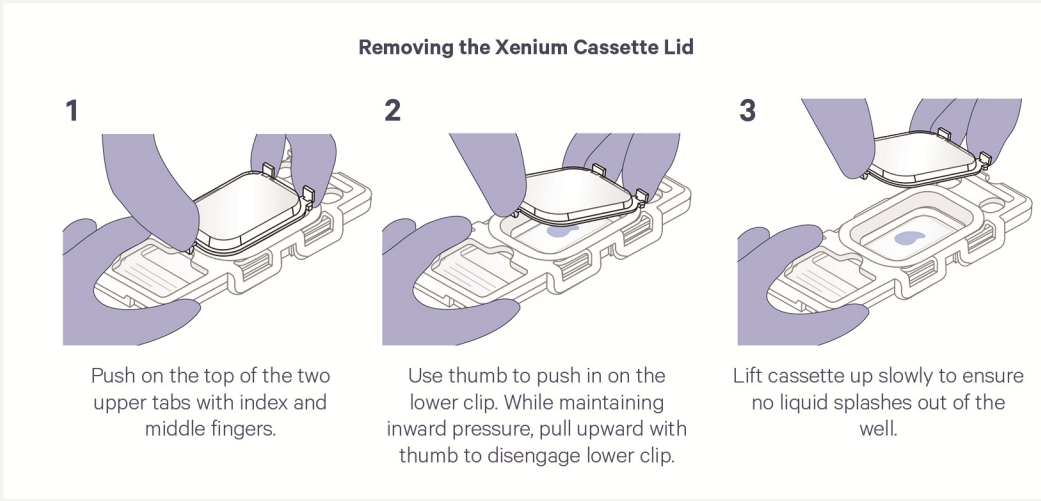
Xenium Cassette Lid Application & Removal

- Work on a clean surface.

Application



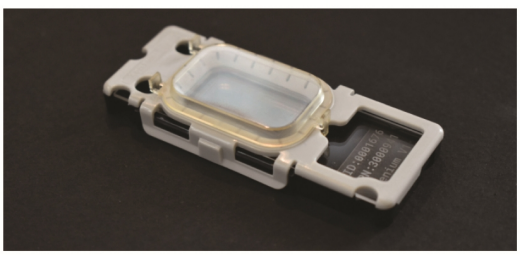
Removal



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

Xenium Cassette Storage

- Store an assembled Xenium cassette with slide by applying a Xenium Cassette Lid or slide seal at the indicated stopping points listed throughout the protocol and as outlined in the Protocol Steps & Timing on page 13.
- Cassettes should always be stored hydrated with recommended reagent and stored at the recommended temperature.



- **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 or slide seal applied to prevent evaporation.
- **Long-term Storage (1 week – 1 month):**
  - Remove all PBS-T from the cassette well.
  - Add **1,000 µl** 70% ethanol, incubate for **2 min** at **room temperature**, remove the ethanol.
  - Add **1,000 µl** 100% ethanol, incubate for **2 min** at **room temperature**, remove the ethanol.
  - Add **1,000 µl** 100% ethanol, incubate for **2 min** at **room temperature**, remove the ethanol.
  - Remove slide from the cassette and place in a slide mailer containing **10 ml** or more cryoprotectant (30% glycerol prepared in PBS) to fully submerge the slide. Clean the cassette as described in Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning on page 94. Save for a subsequent instrument run.
  - Store at **-20°C** for up to **1 month**.

    When ready to use:

- Equilibrate the mailer with the slide to room temperature (takes ~30 min).
- Once completely thawed, rinse the mailer 3X with **10 ml** PBS-T.
- Remove the slide from the mailer, assemble in the cassette (as described on Cassette Assembly Failure on page 93).
- Add **1,000 µl** PBS-T to the cassette well.

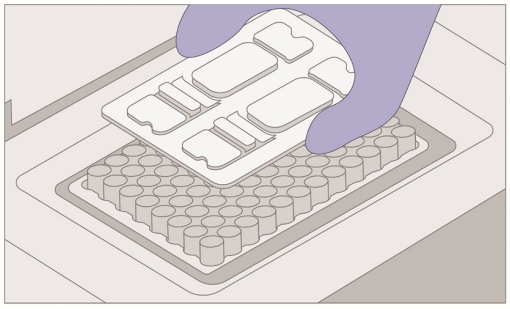
- The above storage guidelines can also be used for storing Xenium cassettes with slides after a Xenium Analyzer run (or in the event of a run failure). Contact support@10xgenomics.com for more information.

Slide Incubation Guidance

Incubation at a specified temperature

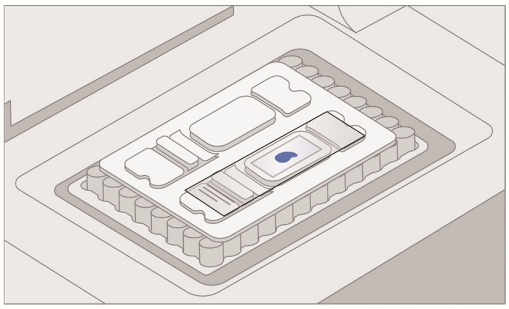
- Position a Xenium Thermocycler Adaptor on a thermal cycler that is set at the incubation temperature. Ensure thermal cycler has reached appropriate temperature prior to starting incubation.

Place Thermocycler Adaptor



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

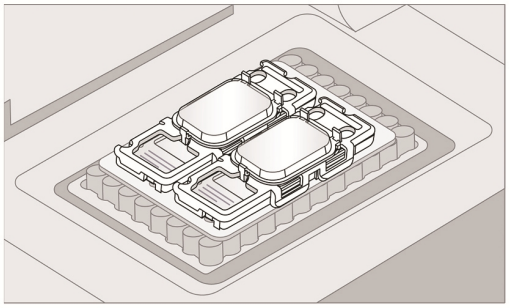
Incubate Slide



- Ensure the Sample Area is aligned with the corresponding area on the Thermocycler Adaptor. DO NOT close the lid.
- When incubating a slide encased in a cassette, place the assembled unit on the Thermocycler Adaptor with the well facing up. Ensure the cassette is in complete contact with the Thermocycler Adaptor. The cassette should

always be sealed with a Xenium Cassette Lid when on the Thermocycler Adaptor unless indicated otherwise.

Incubate Assembled Xenium Cassette



- 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

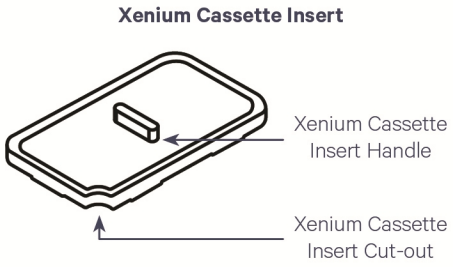
- Thermal cycler lid contact with the Xenium Cassette Lid is critical for assay performance.
- For thermal cyclers with adjustable lids, tighten the lid until an audible click is heard. Tightening past the click risks breaking the slide.

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.

Cassette Incubation

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. Consult the Xenium In Situ Gene Expression Protocol Planner (CG000601) for recommended forceps.
- Reagents are loaded onto the sample by pipetting into the Xenium Cassette Insert Cut-out. The well is sealed with the application of Slide Seal.
- The insert is assembled manually into the cassette. See Xenium Cassette Insert Application & Removal on page 36 for more details.

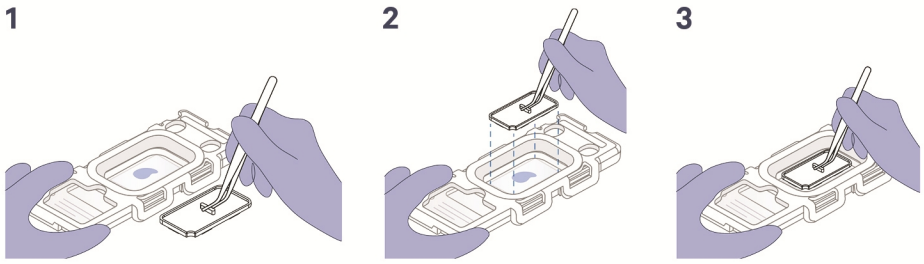
Xenium Cassette Insert Application & Removal

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

Application

Applying Xenium Cassette Insert

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

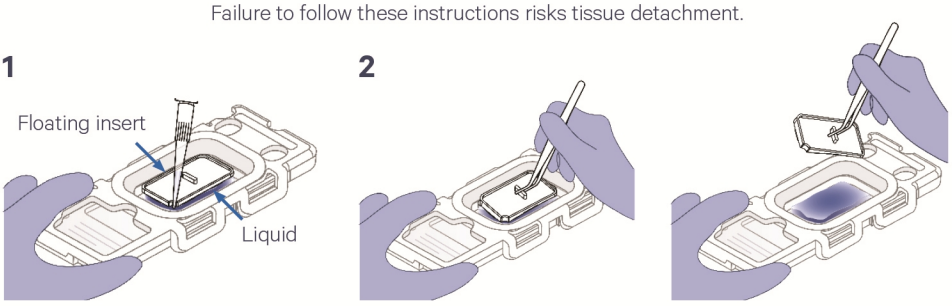


1. Place the Xenium Cassette flat on a clean work surface. Ensure cassette is free of debris. Use forceps to pick up Xenium Cassette Insert by Cassette Insert Handle.
2. Align the Xenium Cassette Insert with the Sample Area well of the Xenium Cassette. Feet and sides should not come into contact with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

Removal

Removing Xenium Cassette Insert

Failure to follow these instructions risks tissue detachment.

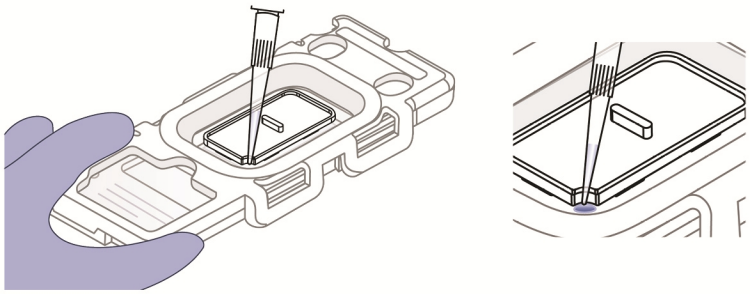


1. Place the Xenium Cassette flat on a clean work surface. Pipette 200 µl of PBS-T to float the Xenium Cassette Insert.
2. Use forceps to pick up Xenium Cassette Insert by Cassette Insert Handle. Carefully remove the insert from the Sample Area well to ensure no liquid splashes out of the well. Remove residual liquid splashed on cassette with a lint-free laboratory wipe if necessary.

Reagent Addition to Xenium Cassette Insert

- 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

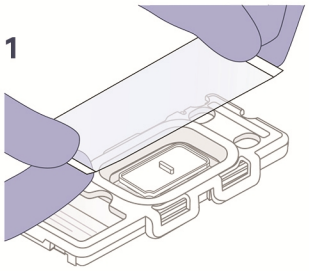


Slide Seal Application & Removal

Application

Slide Seal Application
Use a fresh Slide Seal when prompted

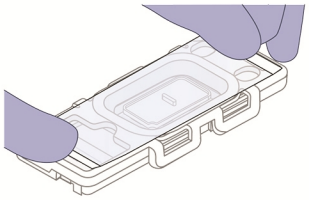
1



Place the cassette flat on a clean work surface. Ensure surface of cassette is dry. Remove liquid with a lint-free laboratory wipe.

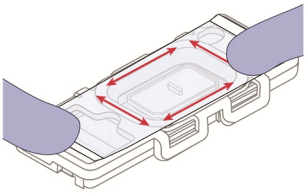
Remove the back of the Slide Seal adhesive.

2



Align the Slide Seal with the surface of the cassette and apply while firmly holding the cassette with both hands.

3



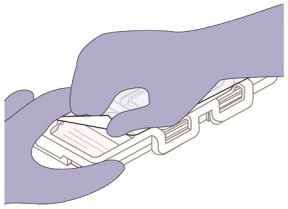
Press on the Slide Seal around the Sample Area to ensure uniform adhesion (red arrows).

DO NOT press on the Slide Seal in the middle of the Sample Area. This will damage tissue.

Removal

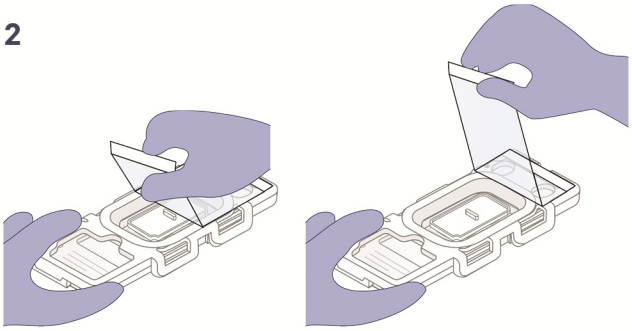
Slide Seal Removal

1



Place the cassette flat on a clean work surface. Hold cassette down firmly with one hand.

2



Carefully pull on the Slide Seal from the edge while continuing to firmly hold the cassette. Move slowly to ensure that no liquid splashes out of the well.

Xenium Cassette Insert Practice

Listed below are the practice steps that may be performed prior to using the Xenium Cassette Insert for Antibody & Cell Segmentation Staining on page 61.

Obtain the following items:

- Xenium Cassette
- Superfrost Plus Slide
- Xenium Cassette Insert
- Slide Seal
- PBS-T

TIPS

Cassettes may be obtained from previous runs and washed according to Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning on page 94 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.	Consult the Xenium Quick Reference Card for Slide Cassette Assembly (CG000623) for cassette assembly and removal instructions.
2. Place Insert Place the Xenium Cassette Insert gently onto the Xenium Cassette using forceps.	Xenium Cassette Insert Application & Removal on page 36
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 page 37
4. Apply Slide Seal Apply PCR film to seal the well. Press down around the Sample Area for uniform adhesion.	Slide Seal Application & Removal on the previous page
5. Remove Slide Seal Remove the PCR film carefully	Slide Seal Application & Removal on the previous page
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 36

10x GENOMICS

Step 1:

Probe Hybridization

1.0 Get Started

Items			10x PN	Preparation & Handling	Storage
Equilibrate to room temperature					
☐	●	Probe Hybridization Buffer	2000390	Thaw at room temperature for 15 min or until completely thawed. Check for precipitate and invert until clear. Maintain at room temperature after thawing.	–20°C
☐	●	Xenium Pre-Designed Gene Expression Probes*	-	Thaw at room temperature. See Probe Hybridization for additional handling instructions.	–20°C
☐	○	Xenium Add-on Custom Probes*	-	Resuspend add-on custom probes according to Custom Probe Preparation. For additional handling instructions, see Probe Hybridization. *Xenium v1 assay is not compatible with Xenium Prime add-on custom priming oligos.*	–20°C
☐	○	Xenium Standalone Custom Probes*	-	Resuspend standalone custom probes according to Custom Probe Preparation. For additional handling instructions, see Probe Hybridization. *Xenium v1 assay is not compatible with Xenium Prime standalone custom priming oligos.*	–20°C
Obtain					
☐		Assembled cassettes containing FFPE tissue samples	-	Consult Xenium in Situ for FFPE - Deparaffinization & Decrosslinking (Demonstrated Protocol CG000580).	-
☐		Nuclease-free Water	-	-	Ambient
☐		10X PBS, pH 7.4	-	-	Ambient
☐		10% Tween-20	-	-	Ambient
☐		Heatblock or waterbath	-	Preheat to 95°C.	Ambient
☐		Xenium Cassette Lids (16 ct)	3001046	See Tips & Best Practices.	Ambient
☐		Xenium Thermocycler Adaptor	3000954	See Tips & Best Practices.	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

*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.

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

1.1 Buffer Preparation

Prepare the following buffers fresh before starting the Xenium 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+10% (ml)	2X+10% (ml)
Nuclease-free Water	-	-	22.5	45.0
10X PBS, pH 7.4	10X	1X	2.5	5.0
Total	**-**	**-**	**25.0**	**50.0**

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+10% (ml)	2X+10% (ml)
1X PBS (prepared at Step 1.1a)	-	-	14.93	29.85
10% Tween-20	10%	0.05%	0.07	0.15
Total	**-**	**-**	**15.0**	**30.0**

1.2 Custom Probe Preparation (optional)

Proceed to Probe Hybridization, step 1.3, directly if using pre-designed probes only. Add-on and standalone custom probes are delivered lyophilized and must be resuspended before use. Resuspend add-on or standalone custom probes according to the instructions below before proceeding with Probe Hybridization.

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

a. Centrifuge custom probe panel tube briefly.

b. Resuspend lyophilized custom probes in TE Buffer, pH 8.0 according to the following table.

Custom Probe Resuspension	10x PN	TE Buffer, pH 8.0 (µl)
4 reactions/kit		
Xenium Add-on	varies	140
Xenium Standalone	varies	140
16 reactions/kit		
Xenium Add-on	varies	700
Xenium Standalone	varies	700

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

Custom probes resuspended in TE Buffer, pH 8.0 can be stored at -20°C until the expiration date specified on the kit.

1.3 Probe Hybridization

Before starting this protocol, ensure that tissue sections have been appropriately deparaffinized and decrosslinked if working with FFPE tissues. Consult Xenium In Situ - FFPE Tissue Preparation Handbook (CG000578) for more information.

TIPS *During reagent removal steps, ensure that **ALL the liquid is removed** from the wells. See Reagent Addition to Wells on page 27 for guidance.*

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

a. 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 (**34 µl**/ slide).

b. 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.

c. Prepare Probe Hybridization Mix according to the options below. Preparation instructions for each option can be found on the following page.

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

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

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 Hybridization Buffer	2000390	315.0	630.0
	TE Buffer, pH 8.0	-	177.0	354.0
●	Xenium Pre-Designed Gene Expression Probes*	-	33.0	66.0
	Total	**-**	**525.0**	**1,050.0**

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 Hybridization Buffer	2000390	315.0	630.0
	TE Buffer, pH 8.0	-	144.0	288.0
●	Xenium Pre-Designed Gene Expression Probes*	varies	33.0	66.0
○	Xenium Add-on Custom Probes*†	varies	33.0	66.0
	Total	**-**	**525.0**	**1,050.0**

Option 3: Probe Hybridization Mix (standalone custom probe panels only)

	Probe Hybridization Mix *(standalone custom probe panels only)*	10x PN	1X+5% (µl)	2X+5% (µl)
●	Probe Hybridization Buffer	2000390	315.0	630.0
	TE Buffer, pH 8.0	-	177.0	354.0
○	Xenium Standalone Custom Probes*†	varies	33.0	66.0
	Total	**-**	**525.0**	**1,050.0**

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

†Custom probes resuspended in TE Buffer, pH 8.0 can be stored at -20°C until the expiration date specified on the kit.
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.

d. 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

e. Retrieve the assembled Xenium Cassette and remove all the buffer.

f. Add **500 µl** room-temperature Probe Hybridization Mix along the side of the well to uniformly cover the tissue sections, without introducing bubbles.

TIPS *Before reusing the lid, it can be cleaned as described in* *Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning on page 94*.

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

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

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

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

Step 2:

Post Hybridization Wash

2.0 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

TIPS *Program a thermal cycler with Post Hybridization Wash incubation protocol.*

2.1 Post Hybridization Wash

a. Remove the Xenium Cassette 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.

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

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

b. Remove the Xenium Cassette Lid. **Save the lid** for use in the following indicated step. Using a pipette, remove all Probe Hybridization Mix from well corners.

c. 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. At least 400 µl of liquid should be aspirated from each slide.

Two 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**.

f. 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
Post Hybridization Wash	37°C	00:30:00
Hold	37°C	Hold

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

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

TIPS *Before reusing the lid, it can be cleaned as described in* *Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning on page 94*.

i. Apply the previously used Xenium Cassette Lid on the cassette and place on the Thermocycler Adaptor 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 53*.

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

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Step 3:

Ligation

3.0 Get Started

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	Ligation Buffer	2000391	Thaw at room temperature for 15 min or until completely thawed. Pipette mix 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				
☐	PBS-T	-	Prepared at Step 1.1.	Ambient

TIPS *Program a thermal cycler with Ligation incubation protocol.*

3.1 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	2000391	481.2	962.5
●	Ligation Enzyme A	2000397	13.8	27.5
●	Ligation Enzyme B	2000398	55.0	110.0
	Total	**-**	**550.0**	**1,100.0**

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

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

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. At least 400 µl of liquid should be aspirated from each slide.

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 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

Lid Temperature	Reaction Volume	Run Time
Ligation	37°C	02:00:00
Hold	37°C	Hold

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

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

TIPS *Before reusing the lid, it can be cleaned as described in* *Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning on page 94*.

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

k. Skip Pre-equilibrate step to initiate Ligation.

TIPS *Start thawing Amplification reagents (except enzymes) during Ligation incubation as outlined in* *Get Started on page 57*.

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

Step 4:

Amplification

4.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Place on ice				
☐	Amplification Mix	2000392	Immediately after initiating Ligation step, thaw on ice. Vortex and centrifuge briefly. Ensure completely thawed before use, with no precipitate remaining.	–20°C
☐	Amplification Enzyme	2000399	Transfer to ice before use. Pipette mix 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

4.1 Amplification

a. Prepare Amplification Master Mix on ice shortly before use. 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, vortex, centrifuge briefly before use)*	2000392	495.0	990.0
●	Amplification Enzyme	2000399	55.0	110.0
	Total	**-**	**550.0**	**1,100.0**

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

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

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. At least 400 µl of liquid should be aspirated from each slide.

Three PBS-T washes (PBS-T prepared in 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: Immediately** add **500 µl** PBS-T. Incubate for **1 min** at **room temperature**. Remove all PBS-T.

f. **Wash 3: Immediately** 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 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)	100 µl	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	30°C	Hold
Amplification	30°C	02:00:00

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

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

TIPS *Before reusing the lid, it can be cleaned as described in* *Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning on page 94*.

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

k. Skip pre-equilibrate step to initiate Amplification.

TIPS *Start thawing Block and Stain Buffer during Amplification incubation as outlined in* *Get Started on page 62*.

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

4.2 Post Amplification Wash

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

b. Remove the Xenium Cassette Lid. Using a pipette, remove all Amplification Mix from the well. **Save the lid** for use in the following indicated step.

Three TE Buffer, pH 8.0 Washes:

c. **Wash 1:** Add **500 µl** TE Buffer, pH 8.0 to the well. Incubate **1 min** at **room temperature**. Remove all TE buffer.

d. **Wash 2:** Add **500 µl** TE Buffer, pH 8.0 to the well. Incubate **1 min** at **room temperature**. Remove all TE buffer.

e. **Wash 3:** Add **500 µl** TE Buffer, pH 8.0 to the well (buffer removal will be in next step to complete wash 3).

TIPS *Before reusing the lid, it can be cleaned as described in* *Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning on page 94*.

STOP **f.** Store the slides in TE Buffer, pH 8.0 for **≤2 days** at **4°C** with the previously used lid applied. Alternatively, proceed directly to the next step. Maintain the cassette with the TE Buffer at room temperature (previously used lid applied) while preparing buffers for the next step.

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Step 5:

Antibody & Cell Segmentation Staining

5.0 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 68. Maintain on ice.	–20°C
Equilibrate to room temperature			
☐ Xenium Stain Enhancer	2000992	Prepare **next day** according to Stain Enhancement on page 74.	–20°C
☐ Xenium Cassette Insert	3001885	Remove from –20°C and equilibrate to room temperature.	–20°C
☐ Slide Seals	2001124	Remove from –20°C and equilibrate to room temperature.	–20°C
☐ Tris EDTA Buffer, pH 9.0 (1X)	See TE Buffer table on the following page for details		-
Obtain			
Xenium Protein Subpanel tubes are strictly single-use, regardless of whether one or two slides are processed. Only retrieve the subpanel tube needed for the experiment. The same subpanel needs to be used for both slides in the same instrument run. Running different subpanel selections in the same instrument run is not supported.			
☐ Xenium Protein Immune Cell Subpanel A	2001380	Retrieve immediately before use.	–20°C
☐ Xenium Protein Immune Cell Subpanel B	2001381	Retrieve immediately before use.	–20°C
☐ Xenium Protein Immune Cell Subpanel C	2001426	Retrieve immediately before use.	–20°C
☐ Xenium Protein Proliferation & Differentiation Subpanel	2001382	Retrieve immediately before use.	–20°C
☐ Xenium Protein Immune Checkpoint Subpanel	2001383	Retrieve immediately before use.	–20°C
☐ Xenium Protein Tumor Subpanel	2001384	Retrieve immediately before use.	–20°C
☐ Xenium Cassette Lids	3001046	New	Ambient
☐ Nuclease-free	-	-	Ambient

Item		10x PN	Preparation & Handling	Storage
	Water			
☐	Ethanol	-	-	Ambient
☐	PBS-T	-	Prepared at Step 1.1.	Ambient
☐	Forceps	-	-	Ambient
☐	Ice bucket	-	-	Ambient
☐	1X PBS	-	Prepared at Step 1.1. To be obtained **next day** for Stain Enhancement on page 74.	Ambient

TE Buffer		Stock	Preparation & Handling	
Choose one based on preference & availability Refer to 5.1 Buffer Preparation on the next page for buffer preparation guidance. Using TE Buffer, pH 8.0 instead of TE Buffer, pH 9.0 for Secondary Decrosslinking may cause weaker staining than anticipated. Confirm correct pH of TE Buffer before performing Secondary Decrosslinking.				
Genemed (10-0046)	Tris EDTA Buffer pH 9.0, 1000 ml	1X	Remove from 4°C and equilibrate to room temperature. Store according to manufacturer recommendations.	4°C
Biolegend (422703)	Tris-EDTA pH 9.0 Antigen Retrieval Buffer, 100 ml	10X	Dilute to 1X with nuclease-free water before use. Store according to manufacturer recommendations.	Ambient
Biolegend (422704	Tris-EDTA pH 9.0 Antigen Retrieval Buffer, 500 ml	10X		
Abcam (ab93684)	Tris-EDTA, pH 9.0	100X		
Novus (NB900-62085)	Tris-EDTA, pH 9.0	10X		

Refer to 5.1 Buffer Preparation on the next page for TE Buffer preparation.

TIPS

Pre-chill centrifuge to 4°C.

5.1 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.

100% Ethanol			
Items	**Stock**	**Final**	**Total Amount (µl)**
Nuclease-free Water	-	-	-
100% Ethanol	100	100	4,400
Total	**-**	**-**	**4,400**

70% Ethanol			
Items	**Stock**	**Final**	**Total Amount (µl)**
Nuclease-free Water	-	-	660
100% Ethanol	100	70	1,540
Total	**-**	**-**	**2,200**

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

1X Diluted Xenium Block and Stain Buffer (for 2 slides)					
Items		**10x PN**	**Stock**	**Final**	**Total Amount (µl)**
	Nuclease-free Water	-	-	-	1,031.3
●	Xenium Block and Stain Buffer	2001083	4X	1X	343.8
	Total	**-**	**-**	**-**	**1,375**

d. If necessary, dilute TE Buffer, pH 9.0 stock solutions to 1X (for two slides) in a centrifuge tube. Add reagents in the order listed. Vortex and centrifuge briefly. Maintain at room temperature.

Choose one based on availability.

TE Buffer, pH 9.0 (Genemed, PN 10-0046)*			
Items	**Stock**	**Final**	**Total Amount (µl)**
Nuclease-free Water	-	-	-
TE Buffer, pH 9.0	1X	1X	1,100
Total	**-**	**-**	**1,100**

TE Buffer, pH 9.0 (Biolegend, PN 422703 or PN 422704)			
Items	**Stock**	**Final**	**Total Amount (µl)**
Nuclease-free Water	-	-	990
TE Buffer, pH 9.0	10X	1X	110
Total	**-**	**-**	**1,100**

TE Buffer, pH 9.0 (Abcam, PN ab93684)			
Items	**Stock**	**Final**	**Total Amount (µl)**
Nuclease-free Water	-	-	1,089
TE Buffer, pH 9.0	100X	1X	11
Total	**-**	**-**	**1,100**

TE Buffer, pH 9.0 (Novus, PN NB900-62085)			
Items	**Stock**	**Final**	**Total Amount (µl)**
Nuclease-free Water	-	-	990
TE Buffer, pH 9.0	10X	1X	110
Total	**-**	**-**	**1,100**

5.2 Tissue Dehydration & Secondary Decrosslinking

a. Prepare a thermal cycler with the following incubation protocol. Place a Thermocycler Adaptor 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	0:05:00

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

c. Remove the Xenium Cassette Lid. Using a pipette, remove all **TE Buffer, pH 8.0** from the well. Discard old cassette lids.

If processing two slides at a time, remove and add reagent from first slide before proceeding to second slide.

DO NOT let tissue dry out during the ethanol wash steps and between removal of ethanol and addition of PBS-T.

Three Ethanol Washes:

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

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

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

g. Place the Xenium Cassette **without lid** on the Thermocycler Adaptor on the pre-heated thermal cycler. DO NOT close the thermal cycler lid.

h. Skip pre-equilibrate step to initiate Drying.

Optional: photograph the slide on the thermal cycler. This image can be used for comparison purposes to identify tissue detachment downstream in the workflow. See Troubleshooting for more details.

i. **Immediately** remove the Xenium Cassette from the Thermocycler Adaptor and place on a flat, clean work surface.

j. Add **1,000 µl** PBS-T. Incubate for **1 min** at **room temperature**.

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

Lid Temperature	Reaction Volume	Run Time
80°C	100 µl	-
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	22°C	Pre-equilibrate
Secondary Decrosslinking	80°C	0:20:00
Cooling	22°C	0:10:00
Hold	22°C	Hold*

**DO NOT leave Xenium Cassette in thermal cycler on Hold at 22°C for an extended amount of time.*

Skipping or changing the recommended Secondary Decrosslinking conditions (duration or temperature) may impact protein or RNA background, sensitivity, specificity, and stain intensity.

l. Remove all PBS-T.

m. Add **500 µl TE Buffer, pH 9.0**.

Visually inspect TE Buffer to confirm that it is the correct reagent (TE Buffer, pH 9.0). DO NOT use TE Buffer pH 8.0.

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

o. Skip Pre-equilibrate step to initiate Secondary Decrosslinking.

p. After Secondary Decrosslinking is complete, **immediately** proceed to the next step.

5.3 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. Using a pipette, carefully remove all **TE Buffer, pH 9.0** from the well. **Save the lid** for use in the following indicated step.

If processing two slides at a time, remove and add reagent from first slide before proceeding to second slide.

Two PBS-T washes:

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

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

e. Add **500 µl** 1X Diluted Xenium Block and Stain Buffer (from 5.1 Buffer Preparation on page 64) to Xenium Cassette for blocking.

Keep remaining volume for later indicated steps.

TIPS

Before reusing the lid, it can be cleaned as described in Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning on page 94.

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

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

h. Add **125 µl** 1X Diluted Xenium Block and Stain Buffer (from 5.1 Buffer Preparation on page 64) to the Xenium Multi-Tissue Stain Mix tube.

i. Pipette mix 15X (pipette set to 100 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	125.0

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

Prepare Antibody Mix

During Xenium Multi-Tissue Stain Mix incubation, prepare Antibody Mix. Xenium Protein Subpanel tubes are strictly single-use, regardless of whether one or two slides are processed. Only retrieve the subpanel tube needed for the experiment. The same subpanel needs to be used for both slides in the same instrument run. Running different subpanel selections in the same instrument run is not supported.

	Antibody Mix	10x PN
	Xenium Multi-Tissue Stain Mix	2000991
	Xenium Protein Immune Cell Subpanel A	2001380
	Xenium Protein Immune Cell Subpanel B	2001381
	Xenium Protein Immune Cell Subpanel C	2001426
	Xenium Protein Immune Checkpoint Subpanel	2001383
	Xenium Protein Proliferation & Differentiation Subpanel	2001382
	Xenium Protein Tumor Subpanel	2001384

- Centrifuge Xenium Protein Subpanel tubes for **5 sec**.
- Remove the cap of each subpanel tube and inspect the tube walls across the whole tube to locate the lyophilized pellet. If pellet is not located on the sidewall, assume that the pellet is located at the bottom of the tube (will likely be visible at the bottom of the tube). See examples below.

TIPS

The pellet is easier to locate in the tube after the cap is removed.

Locating Lyophilized Pellet Prior to Resuspension
Pellet may be located on the side of the tube

Bottom of tube

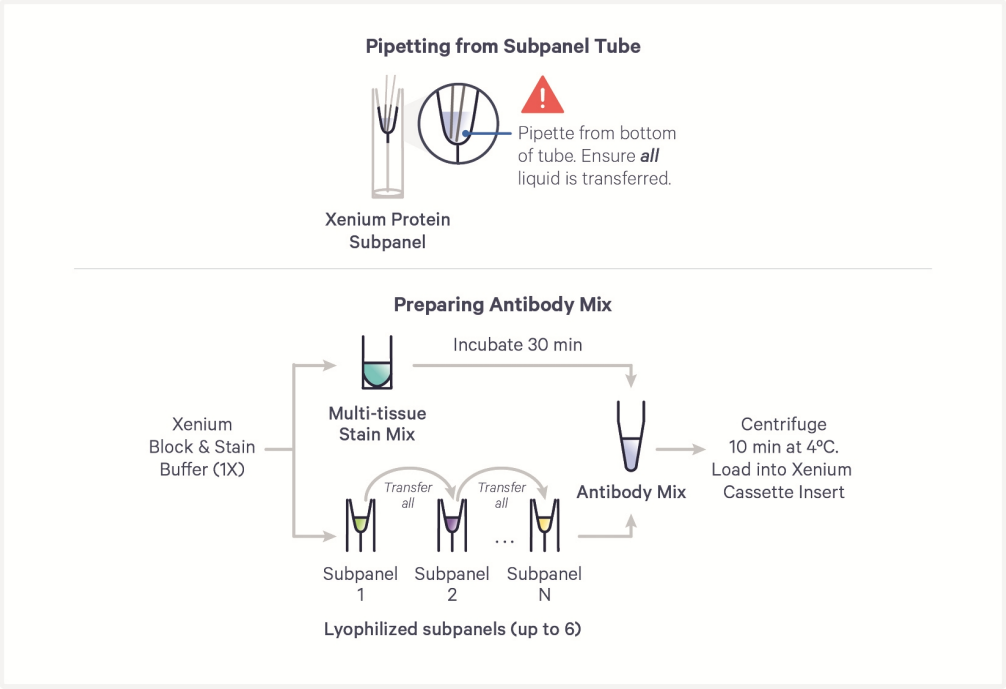
Top down view

Side of tube

Side view

Top down view

Example Antibody Mix resuspension (if using all subpanels):



When preparing Antibody Mix, the subpanels can be added in any order.

k. Pipette **125 µl** 1X Diluted Xenium Block and Stain Buffer directly on the pellet in subpanel tube 1. Pipette mix 15X or until pellet is resuspended. Pulse centrifuge briefly.

Use the same pipette tip for resuspending and transferring across all the subpanel tubes. Switching pipette tips may impact stain intensity.

Pipette from bottom of subpanel tube to ensure entire volume is transferred to next tube. Avoid generating bubbles.

TIPS

The subpanels may be added in any order.

l. Transfer the entire volume from subpanel tube 1 directly on the pellet in subpanel tube 2. Pipette mix 15X or until pellet is resuspended. Pulse centrifuge briefly.

m. Transfer entire volume from subpanel tube 2 directly on the pellet in subpanel tube 3. Pipette mix 15X or until pellet is resuspended. Pulse centrifuge briefly.

n. Transfer entire volume from subpanel tube 3 directly on the pellet in subpanel tube 4. Pipette mix 15X or until pellet is resuspended. Pulse centrifuge briefly.

o. Transfer entire volume from subpanel tube 4 directly on the pellet in subpanel tube 5. Pipette mix 15X or until pellet is resuspended. Pulse centrifuge briefly.

p. Transfer entire volume from subpanel tube 5 directly on the pellet in subpanel tube 6. Pipette mix 15X or until pellet is resuspended. Pulse centrifuge briefly.

q. Pipette **110 µl** from subpanel tube 6 into a **new 1.5 ml**-Eppendorf tube (low-bind). Maintain on ice.

*Some volume loss is expected. If **≥100 µl** is retrieved, proceed with assay workflow. If **<100 µl** is retrieved, staining intensity may be decreased. See Incorrect Antibody Resuspension on page 90 for additional guidance.*

Replace the pipette tip with a new one in the following steps.

r. Pipette **110 µl** Xenium Multi-Tissue Stain Mix into the **same** Eppendorf tube. Pipette mix 15X (pipette set to 180 µl).

s. Centrifuge eppendorf tube containing resuspended Antibody Mix and Xenium Multi-Tissue Stain Mix for **10 min** at **14,000 rcf** at **4°C**. Maintain on ice.

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

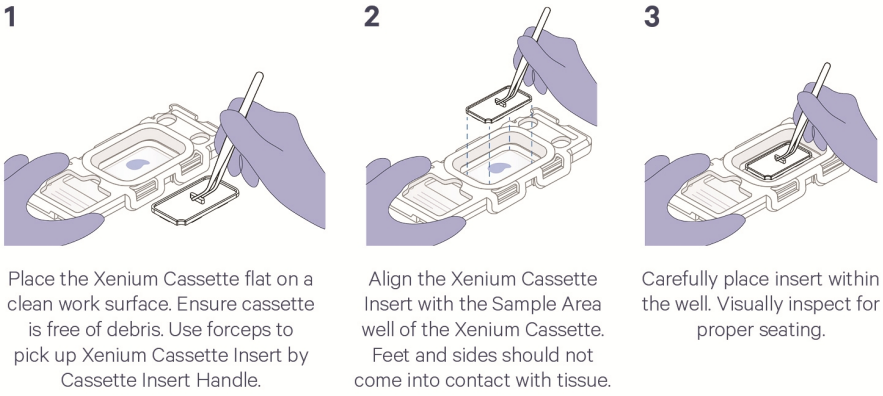
u. Remove the Xenium Cassette Lid and using a pipette, remove 1X Diluted Xenium Block and Stain Buffer from well corners. **Save the lid** for the **next day**. DO NOT let the tissue sections dry out.

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

Leave second slide with 1X Diluted Xenium Block and Stain Buffer until the first cassette has been assembled with insert and Antibody Mix has been added.

Applying Xenium Cassette Insert

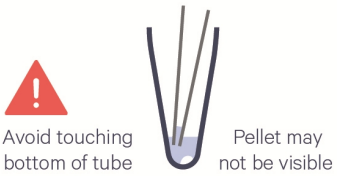
Improper placement of the insert may cause scratching or damage to tissue sections.



w. Using a pipette along the side of the tube, withdraw **100 µl** Antibody Mix and add to the Xenium Cassette Insert using the cut-out to pipette solution under the Xenium Cassette Insert.

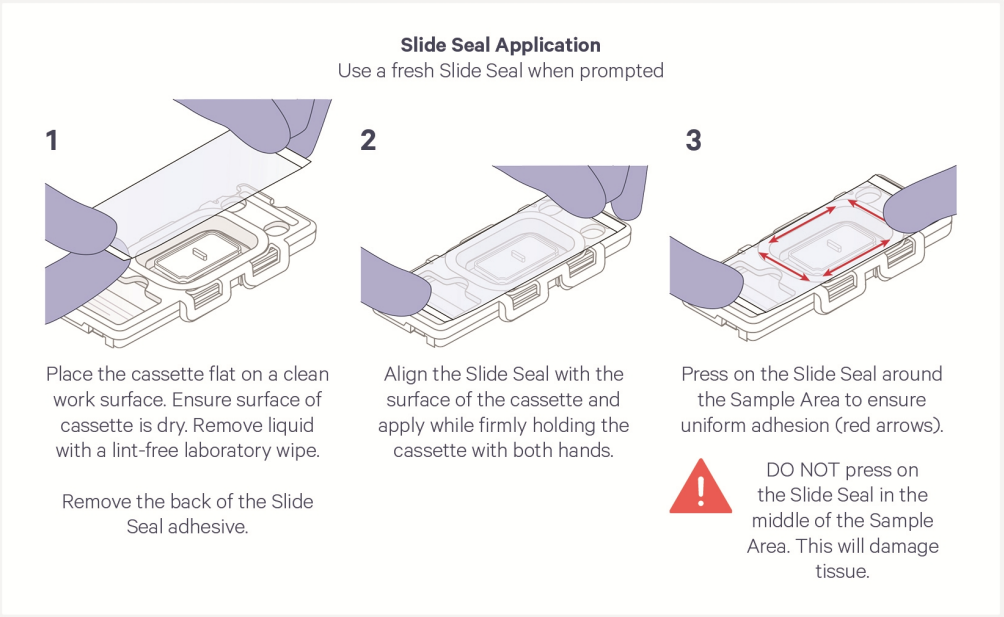
Pipette from top of Antibody Mix solution without touching bottom of tube.

Pipetting Antibody Mix



Pipette slowly into cut-out to avoid introducing bubbles as bubbles may impact staining. See Bubbles under Xenium Cassette Insert on page 89 for guidance on resetting the cassette insert if bubbles are present.

x. Carefully seal the cassette well using Slide Seal. Press down around the cassette well edges to ensure complete sealing between cassette and Slide Seal.



y. Incubate the Xenium Cassette **overnight (16-24 h)** at **4°C**.

TIPS

If loading Xenium Analyzer instrument the next day, begin thaw of Xenium Protein Detection Reagent Module C and Decoding Module B. Consult the Xenium Analyzer User Guide (CG000584) for specific guidance.

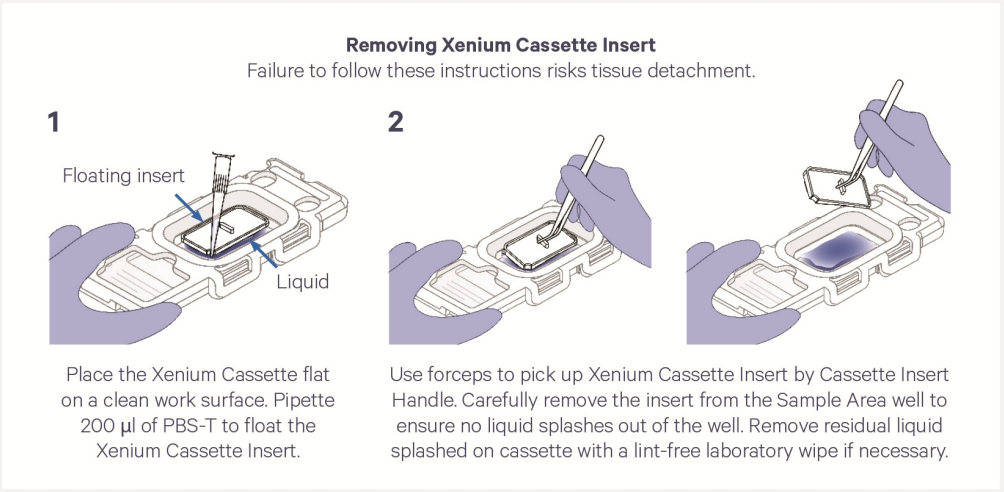
5.4 Stain Enhancement

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 Slide Seal from Xenium Cassette. Carefully pipette **200 µl** PBS-T into Xenium Cassette Insert Cut-out to float the insert.

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



d. Using a pipette, remove all Antibody 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.

TIPS *Before reusing the lid, it can be cleaned as described in* *Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning on page 94*.

i. Apply the previously used Xenium Cassette Lid 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 78*.

j. Remove the Xenium Cassete Lid. Using a pipette, remove all Xenium Stain Enhancer from well corners. **Save the lid** for use in the following indicated 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 Antibody & Cell Segmentation Staining is complete, proceed **immediately** to the next step.

10x GENOMICS

Step 6:

Autofluorescence Quenching

6.0 Get Started

Items			10x PN	Preparation & Handling	Storage
Equilibrate to room temperature					
☐	●	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
☐	●	Nuclei Staining Buffer	2000762	Thaw at room temperature. Vortex and centrifuge briefly.	–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

*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.

100% ethanol is needed for washes in addition to making 70% ethanol dilutions.

6.1 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 6.0. 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 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 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
●	Autofluorescence Mix	2000753	-	-	5.5	11.0
	Total	**-**	-	-	**550.0**	**1,100.0**

b. Retrieve the Xenium Cassette from step 5.4 and place on a flat, clean work surface.

c. Using a pipette, remove all PBS-T from the well.

d. Add **500 µl** Diluted Reducing Agent B prepared at step 6.1ai to the well.

TIPS *Before reusing the lid, it can be cleaned as described in* *Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning on page 94*.

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

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

Three ethanol washes:

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

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

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

j. Pipette mix Autofluorescence Solution prepared at step 6.1aiii thoroughly before dispensing onto sample to prevent settling of reagent. Add **500 µl** Autofluorescence Solution to the well.

TIPS *Before reusing the lid, it can be cleaned as described in* *Xenium Cassette, Xenium Cassette Insert, and Lid Cleaning on page 94*.

k. Apply the previously used Xenium Cassette Lid on the cassette, and incubate for **10 min** at **room temperature in the dark**.

l. Prepare a thermal cycler with the following incubation protocol. Place a Thermocycler Adaptor 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

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

Three ethanol washes:

Ethanol washes DO NOT need to be performed in the dark.

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

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

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

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

r. Skip pre-equilibrate step to initiate Drying.

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

t. 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**.

u. Remove all 1X PBS.

v. 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.

w. Store slides **16-24 h** (overnight) at **4°C in the dark** with the previously used Xenium Cassette Lid applied on the Xenium Cassette. If storing slides, DO NOT discard the lid; instead save for Step 6.2 Nuclei Staining. Alternatively, proceed to the next step.

6.2 Nuclei Staining

a. Retrieve thawed Nuclei Staining Buffer prepared as outlined in 6.0 Get Started on page 78.

b. Retrieve the Xenium Cassette from the previous step 6.1w and place on a flat, clean work surface.

c. If stored, remove the Xenium Cassette Lid. Using a pipette, remove all PBS-T from the well.

d. Add **500 µl** 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). Verify latest version of Xenium Analyzer User Guide before proceeding to instrument loading.

Slides from different Xenium assay workflows cannot be run together on the same instrument run.

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

Long-term storage for **(1 week – 1 month)** at **-20°C**. Perform serial ethanol washes to dehydrate, transfer the slide to a mailer, and store in **10 ml** cryoprotectant (30% Glycerol prepared in PBS), as described in Xenium Cassette Storage on page 30

Ensure that the slide is stored in microbe-free and nuclease-free conditions, with a new Xenium Cassette Lid or slide seal 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.

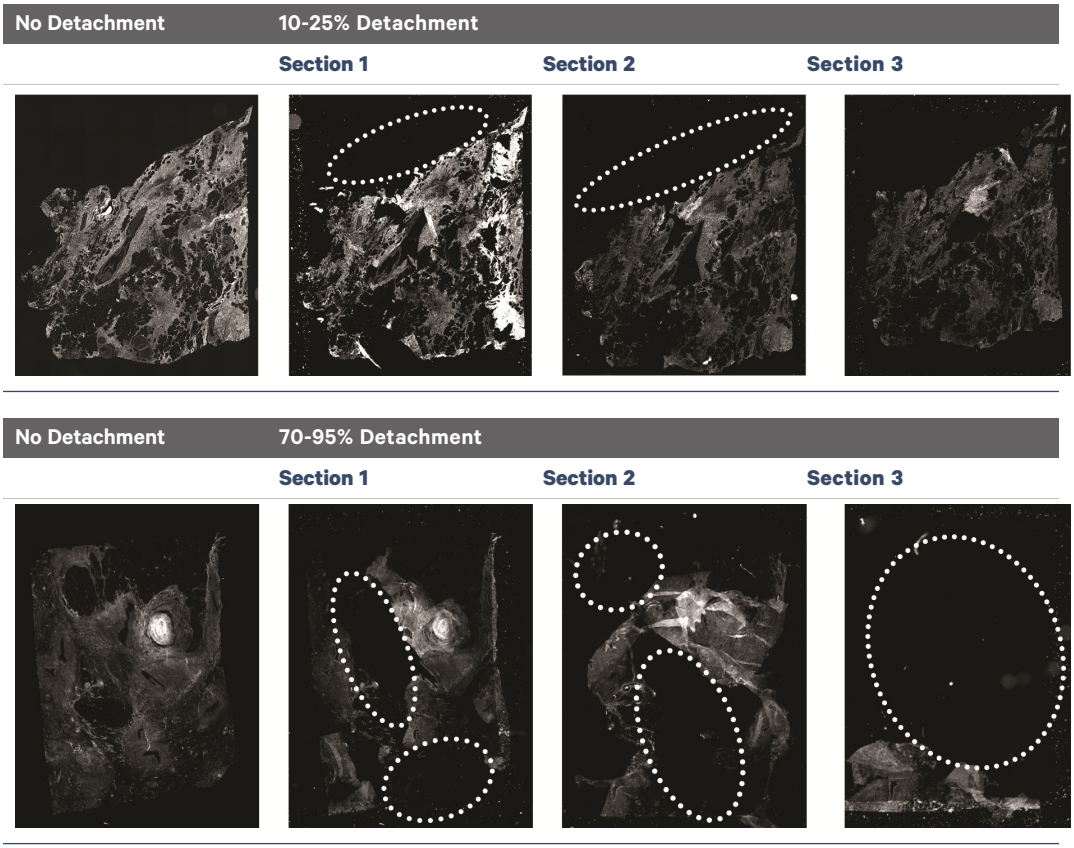
The above storage guidelines can also be used for storing Xenium cassettes with slides after Xenium Analyzer run (or in the event of a run failure). Contact support@10xgenomics.com for more information.

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



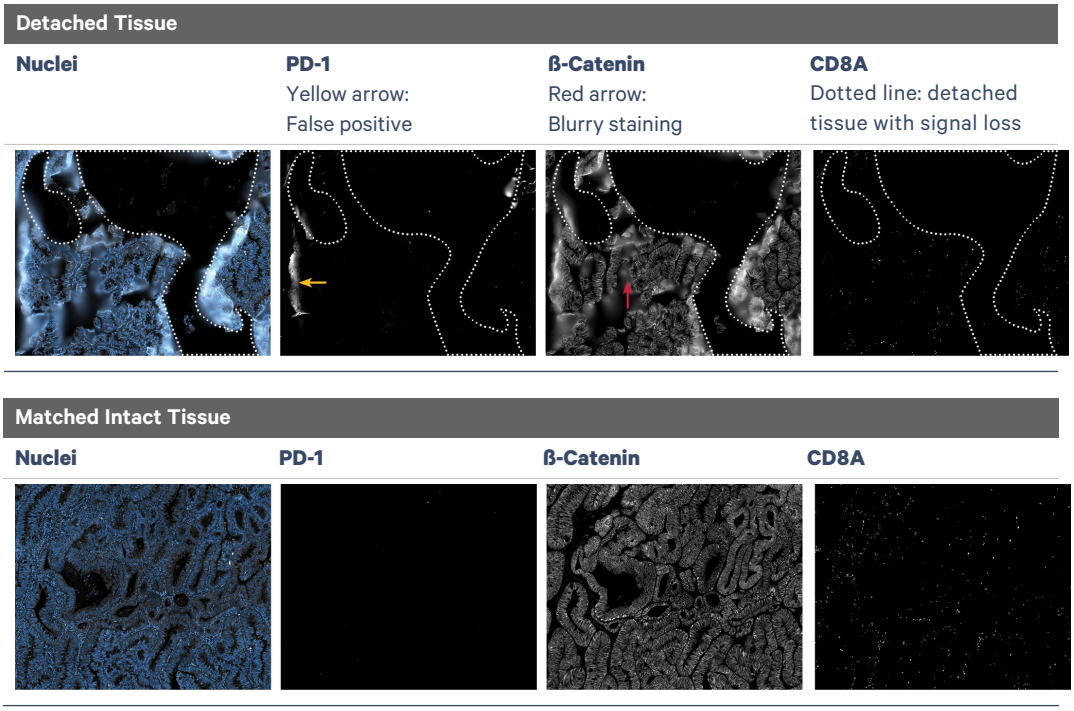
Percentages represent tissue detachment/"area that cannot be analyzed" at the end of the Xenium Analyzer workflow. White circles indicate areas of tissue detachment.

Protein Staining and Tissue Detachment

Tissue detachment can alter tissue morphology and arrangement, compromising the accuracy of protein staining and the reliability of images which can impact downstream analysis.

- In areas of tissue detachment, reduced protein staining intensity is observed making it difficult to achieve uniform staining and consistent imaging. If there is complete detachment, the slide retains no tissue, resulting in total signal loss.
- Partial or micro-detachment may shift or fold the tissue over itself during instrument run cycles. This may lead to irregularities in the staining pattern, potentially causing the imaging signal to appear artificially bright, blurry, or clustered in specific areas.

If protein staining in specific regions appears inconsistent, check the DAPI images for out-of-focus nuclei in those areas. This is often a strong indicator of tissue detachment. For accurate assessment, use high-resolution Xenium Explorer images rather than lower-resolution analysis summaries.



Tissue Detachment on Xenium Slides

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

Careful tissue preparation is critical for adhesion in on-slide workflows. Consult Xenium In Situ for FFPE - Tissue Preparation Guide (Document CG000579) for tissue QC and sectioning best practices. Below are some additional best practices for minimizing detachment during on-slide workflows:

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

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

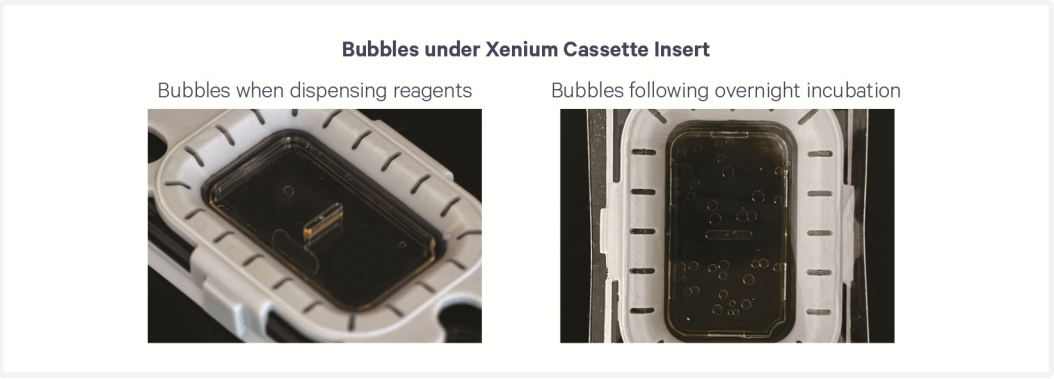
If tissue detachment occurs, send pictures to support@10xgenomics.com for further assistance.

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 can 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 when dispensing reagents

Bubbles may occur when dispensing Antibody Mix under the Xenium Cassette Insert.

If bubbles are observed, DO NOT aspirate or pop the bubbles as this can 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 at start of overnight Antibody Mix incubation

Bubbles present at the start of overnight incubation may locally change stain intensity in the region with the bubble.

Bubbles after overnight incubation

Bubbles can also appear after overnight incubation of the slide (Probe Hybridization or Antibody & Cell Segmentation Staining) due to bubble nucleation and are unlikely to impact staining or assay performance.

Incorrect pH TE Buffer

Secondary Decrosslinking

Using TE Buffer, pH 8.0 instead of TE Buffer, pH 9.0 for Secondary Decrosslinking may cause weaker staining than anticipated. Confirm correct pH of TE Buffer before performing Secondary Decrosslinking.

Probe Resuspension

Although not tested internally, using TE Buffer, pH 9.0 may potentially impact sensitivity or specificity of data. Confirm correct pH of TE Buffer before performing Probe Resuspension.

Amplification Wash

Using TE Buffer, pH 9.0 for Amplification Wash is likely low risk. However, this has not been tested internally and may potentially impact sensitivity or specificity of data. Confirm correct pH of TE Buffer before performing Amplification Wash.

Incorrect Antibody Resuspension

Exceeding the recommended volume of 1X Diluted Xenium Block and Stain Buffer during antibody resuspension can diminish staining intensity and compromise data quality. Pipette carefully and confirm correct volume and dilution of 1X Diluted Xenium Block and Stain Buffer is added to antibodies during resuspension.

Pipetting errors and bubble formation can lead to volume loss during transfer steps of serial resuspension and incorrectly concentrated antibodies. To prevent volume loss, ensure complete transfer of the antibody solution between tubes and minimize bubble generation by slow, controlled pipetting, avoiding air expulsion.

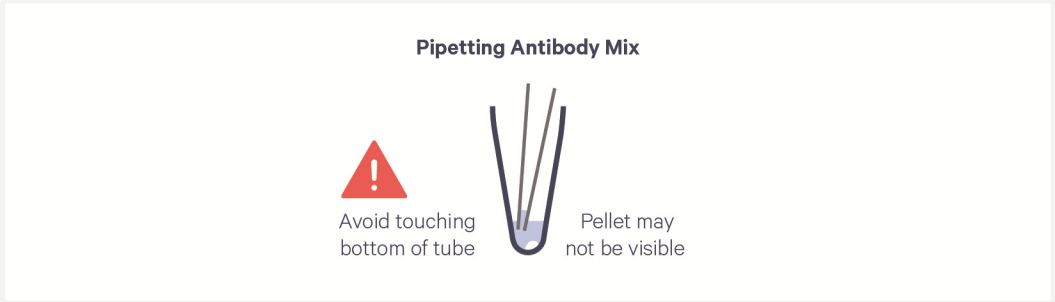
Some volume loss is expected. If **≥100 µl** is retrieved following transfer of combined antibodies into new eppendorf tube, proceed with assay workflow. If **<100 µl** is retrieved, staining intensity may be decreased.

Incorrect Antibody Preparation

Antibody aggregates

Antibody aggregates are clumps of antibodies that can form when Xenium Multi-Tissue Stain Mix is prepared incorrectly (i.e. not centrifuged or not used immediately after preparation) or if antibodies remain in 1X Diluted Xenium Block and Stain Buffer for extended periods of time. They resemble irregular shaped dots in a .tif image or in Xenium Explorer outputs.

Antibodies and cell segmentation markers are combined and centrifuged to remove aggregates that may form during the workflow. To avoid formation of antibody aggregates, ensure centrifugation steps are followed carefully for the recommended times in the User Guide. After centrifugation of Antibody Mix, avoid pipetting from the bottom of the eppendorf tube to prevent aspiration of potential antibody aggregates.



Number of Washes

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.

Samples Dry Out

Drying of tissue samples may lead to decreased decoding efficiency and unusable data and will impact overall assay performance and sensitivity. It may also decrease staining intensity. 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 13.

Cassette Assembly Failure

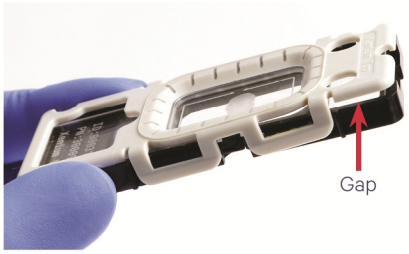
Incorrect assembly of the Xenium cassettes with a Xenium slide can negatively impact assay performance. Always dry the front and the back of the slide completely using a lint-free laboratory wipe while avoiding touching or damaging of the tissue sections. Inspect the slide carefully to ensure it is seated 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.
- 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. Consult the Xenium Quick Reference Card for Slide Cassette Assembly (CG000623) for cassette assembly and removal instructions. If cassette is still assembled incorrectly, proceed with a new cassette.

Cassette Assembly Failure



Cassette assembly with a gap will result in reagent leakage. If gap is visible, disassemble and reassemble cassette before proceeding.

Inspect gasket during cassette assembly. Incorrect cassette assembly or damaged parts can lead to assay failure.

*Leakage may also indicate a crack in the slide. DO NOT proceed with assay if slide is cracked or broken. Cracked or broken slides will result in assay failure.

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.

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.

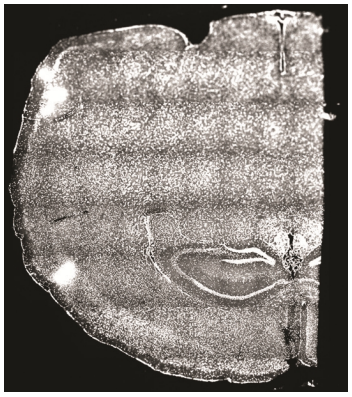
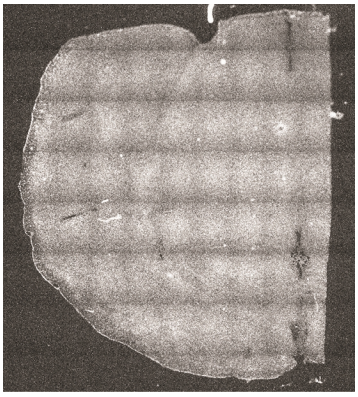
Incorrect or insufficient Autofluorescence Quenching may cause variation in staining of tissues ***(mouse brain pictured below)***

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 Slides & Sample Prep Reagents Kit - (2 slides, 2 rxns), PN-1000460. 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)*

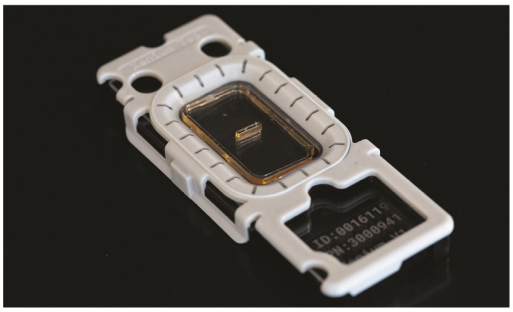
Correct Nuclei Staining Protocol	No Nuclei Staining Performed
	

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 39.

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.

Improper Placement of Xenium Cassette Insert



Appendix

Probe Panel Selection

Ensure that a compatible gene panel has been selected prior to executing the Xenium In Situ Gene Expression workflow. 10x Genomics provides the option of using pre-designed gene panels, pre-designed panels that are customized by adding genes of interest, and standalone custom gene panels.

Pre-designed Gene Panels

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

Custom Gene Panels

Contact your 10x Genomics Sales Executive for information about designing a standalone custom gene panel or add-on custom gene panels that are compatible with pre-designed panels. If you do not know your Sales Executive, please contact customerservice@10xgenomics.com.

If utilizing a custom panel, the Design ID on the label of the tube containing the custom panel should match with the first portion of the custom gene panel electronic file name.

Sample Shipping

Processed Xenium slides may be shipped within 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 30. 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.