User Guide | CG000685 | Rev B

Visium HD Spatial Gene Expression Reagent Kits

For use with:

Visium HD Reagent Kit, Small, PN-1000668

Visium Human Transcriptome Probe Kit v2 - Small, PN-1000466

Visium Mouse Transcriptome Probe Kit v2 - Small, PN-1000667

Visium HD Cassettes, 6.5 mm, 4 rxns, PN-1000669

Visium HD Slide, 6.5 mm, 2 rxns, PN-1000670

Visium CytAssist Reagent Accessory Kit, PN-1000499

Dual Index Kit TS Set A, 96 rxns PN-1000251

Manufactured under license from Centrillion

10xgenomics.com

Notices

Document Number

CG000685 | Rev B

Legal Notices

Instrument & Licensed Software Updates Warranties

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

Support

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Document Revision Summary

Document Number

CG000685

Title

Visium HD Spatial Gene Expression Reagent Kits User Guide

Revision

Rev B

Revision Date

September 10, 2024

Description of Changes

- Added additional tips on ensuring Visium HD slide is dry to Visium HD Slide Handling on page 23.
- Added new image demonstrating reagent removal to Reagent Addition to & Removal from Wells on page 25.
- Added additional information on Visium CytAssist alignment guides in Instrument Loading Guidelines on page 34.
- Added Fresh Frozen and Fixed Frozen documentation to Workflow Overview on page 13.
- Added Fresh Frozen and Fixed Frozen information to Sample Preparation on page 43.
- Adjusted time a Visium HD Slide may remain in buffer prior to instrument run in 3.1 Visium HD Slide Wash on page 59.
- Added new visual overview in 4.1 CytAssist-Enabled Probe Release & Capture on page 65.
- Added information on color balance to Sample Indices on page 97.
- Added background on recommended sequencing depth in Sequencing Depth on page 90.
- Added NovaSeq X plus to Illumina Sequencer Compatibility on page 97 and Sequencing Metrics on page 99.
- Added note on consequences of not drying the Visium HD slide to 1. Bubbles Trapped During Visium CytAssist Run on page 103.
- Added information on high split-mapped or half-mapped reads in 5. High Split-Mapped or Half-Mapped Reads on page 110.
- Added information on data loss due to hydrogel damage in 6. Data Loss due to Hydrogel Damage on page 111.
- Added sequences to Oligonucleotide Sequences on page 122.
- Updated for general minor consistency of language and terms throughout.

Table of Contents

Contents

Table of Contents

Table of Contents

Introduction

Reagent Kits

Reagent Kits	Part Number	Components	Component Part Number
Visium HD, Human Transcriptome, 6.5 mm*	1000675	Visium HD Slide, 6.5 mm, 2 rxns	1000670
		Visium Human Transcriptome Probes v2 - small	1000466
		Visium HD Reagents, small	1000668
		Visium HD Cassettes, 6.5 mm, 4 rxns	1000669
Visium HD Mouse, Transcriptome, 6.5 mm*	1000676	Visium HD Slide, 6.5 mm, 2 rxns	1000670
		Visium Mouse Transcriptome Probes v2 - small	1000667
		Visium HD Reagents, small	1000668
		Visium HD Cassettes, 6.5 mm, 4 rxns	1000669

*Also available in a pack of 4 as a 16 rxn kit.

Visium HD Slide, 6.5 mm, 2 rxns PN-1000670

Visium HD Slide, 6.5mm
2 rxns PN-1000670
(store at −80°C)

	#	PN
Visium HD Slide, 6.5 mm	1	2000970

Visium HD Cassettes*, 6.5 mm, 4 rxns PN-1000669

Visium HD Cassettes, 6.5 mm
4 rxns PN-1000669
(store at ambient temperature)

	#	PN
Visium 2-port Cassette S3, 6.5 mm		
Visium Cassette 2-port gasket, 6.5 mm	2	3001831
Visium Cassette Bottom	2	3001830
Visium Tissue Slide Cassette S3, 6.5 mm		
Tissue Slide Cassette Top	4	3001826
Movable Tissue Gasket 6.5 mm (preassembled with translator)	4	3001828
Movable Tissue Gasket Translator (preassembled with gasket)	4	3001927
Tissue Slide Cassette Bottom	4	3001825
Visium Slide Seals, 12 pack	2	2000283

*The Visium HD Workflow is run with Visium Tissue Slide Cassettes S3 and Visium Cassettes S3. These are referred to as Tissue Slide Cassettes and Visium Cassettes in this document, respectively. Consult the Visium Cassette S3 Quick Reference Card (CG000730) for assembly and disassembly information.

Visium HD Reagent Kit – Small, PN-1000668

Visium HD Reagent Kit – Small
PN-1000668
(store at –20°C)

		#	PN
○	Amp Mix B	1	2000567
●	Extension Enzyme	1	2000389
●	Extension Buffer	1	2000409
●	RNase Enzyme	1	3000605
●	2X RNase Buffer	1	2000411
●	Perm Enzyme B	1	3000553
●	TS Primer Mix B	1	2000537
●	Decrosslinking Buffer B	1	2001094

Visium Human Transcriptome Probe Kit v2 - Small, PN-1000466

Visium Human Transcriptome Probe Kit v2 - Small
PN-1000466
(store at −20°C)

		#	PN
○	FFPE Hyb Buffer	1	2000423
○	FFPE Post-Hyb Wash Buffer	1	2000424
●	Human WT Probes v2 – RHS	1	2000657
●	Human WT Probes v2 – LHS	1	2000658
○	Probe Ligation Enzyme	1	2000425
○	2X Probe Ligation Buffer	1	2000445
○	Post Ligation Wash Buffer	1	2000419

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Visium Mouse Transcriptome Probe Kit v2- Small, PN-1000667

Visium Mouse Transcriptome Probe Kit v2 - Small
PN-1000667
(store at −20°C)

		#	PN
○	FFPE Hyb Buffer	1	2000423
○	FFPE Post-Hyb Wash Buffer	1	2000424
●	Mouse WT Probes v2 – RHS	1	2000913
●	Mouse WT Probes v2 – LHS	1	2000912
○	Probe Ligation Enzyme	1	2000425
○	2X Probe Ligation Buffer	1	2000445
○	Post Ligation Wash Buffer	1	2000419

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Dual Index Kit TS Set A, 96 rxns PN-1000251

Dual Index Kit TS Set A
96 rxns PN-1000251
(store at −20°C)

	#	PN
Dual Index Plate TS Set A	1	3000511

10x Genomics Accessories

Product	#	Part Number (Kit)	Part Number (Item)
10x Magnetic Separator	1	1000499 (Visium CytAssist Reagent Accessory Kit)	230003 or 2000431
Low Profile Thermocycler Adapter	2		3000823

Third-Party Items

Successful execution of the Visium HD 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 Visium HD Spatial Gene Expression Protocol Planner (CG000698, Rev B or later) 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.

Workflow Overview

1 Sample Preparation

Before starting this User Guide, consult these documents to prepare samples. Choose **one** handbook for guidance on tissue preparation and staining.

Visium HD FFPE Tissue Preparation Handbook
Prepare formalin fixed & paraffin embedded tissue blocks, section tissue onto slides, stain, and image.
Demonstrated Protocol CG000684

Visium HD FF Tissue Preparation Handbook
Prepare Fresh Frozen tissue blocks, section tissue onto slides, stain, and image.
Demonstrated Protocol CG000763

Visium HD FxF Tissue Preparation Handbook
Prepare Fixed Frozen tissue blocks, section tissue onto slides, stain, and image.
Demonstrated Protocol CG000764

Visium HD Spatial Applications Imaging Guidelines
Optimize imaging settings.
Technical Note CG000688

Visium Cassette S3 Quick Reference Card
Practice cassette assembly and disassembly.
Quick Reference Card CG000730

Visium HD Spatial Applications Protocol Planner
Information on third-party items.
Planner CG000698

Visium CytAssist Accessory Kit Quick Reference Card
Determine slide allowable areas.
Quick Reference Card CG000548

2 Library Construction

Visium HD Spatial Gene Expression Reagent Kits User Guide
Construct Visium HD Spatial Gene Expression - Probe-based Libraries.
User Guide CG000685

Consult the 10x Genomics support website for additional documents

Protocol Steps & Timing

Steps	Timing	Stop & Store
Day 1		
Step 1: Probe Hybridization (page 45)		
1.1 Probe Hybridization (page 47)	Overnight	
Day 2		
Step 2: Probe Ligation (page 50)		
2.1 Post-Hybridization Wash (page 52)	18 min	
2.2 Probe Ligation (page 54)	60 min	
2.3 Post-Ligation Wash (page 55)	12 min	STOP 4°C ≤24 h
Step 3: Visium HD Slide Preparation (page 57)		
3.1 Visium HD Slide Wash (page 59)	20 min	
4.1 CytAssist-Enabled Probe Release & Capture (page 65)		
4.1 CytAssist-Enabled Probe Release & Capture (page 65)	60 min	
4.2 Probe Extension (page 75)	60 min	STOP 4°C ≤24 h
4.3 Probe Elution (page 76)4.3 Probe Elution (page 76)	15 min	
Step 5: Pre-Amplification and SPRIselect (page 77)		
5.1 Pre-Amplification (page 79)	40 min	
5.2 Pre-Amplification Cleanup - SPRIselect (page 80)	30 min	STOP 4°C ≤72 h or -20°C ≤4 weeks
Day 3		
Step 6: Visium HD Spatial Gene Expression – Probe-based Library Construction (page 81)		
6.1 Cycle Number Determination – qPCR (page 83)	45 min	
6.2 Sample Index PCR (page 85)	40 min	
6.3 Post-Sample Index PCR Cleanup – SPRIselect (page 87)	30 min	STOP -20°C long-term
6.4 Post-Library Construction QC (page 88)	50 min	

Stepwise Objectives

The Visium HD Spatial Gene Expression assay is designed to analyze mRNA in tissue sections derived from formalin fixed & paraffin embedded (FFPE), fresh frozen (FF), or fixed frozen (FxF) tissue samples. It uses probes targeting the whole transcriptome. Each Visium HD Slide contains Capture Areas with barcoded squares that include oligonucleotides required to capture gene expression probes. Before the assay, tissue sections are processed as described in their relevant Demonstrated Protocol. See Workflow Overview for documentation references.

Human or mouse whole transcriptome probe panels, consisting of ~3 pairs of specific probes for each targeted gene are added to the tissue, enabling hybridization and ligation of each probe pair. Tissue slides and Visium HD Slides are loaded into the Visium CytAssist instrument, where they are brought into proximity with one another. Gene expression probes are released from the tissue upon CytAssist-Enabled Probe Release & Capture, enabling capture by the spatially-barcoded oligonucleotides present in a hydrogel on the Visium slide surface. The Visium HD Slide is removed from the Visium CytAssist for downstream library preparation. Gene expression libraries are generated from each tissue section and sequenced. Spatial Barcodes are used to associate the reads back to the tissue section images for spatial mapping of gene expression.

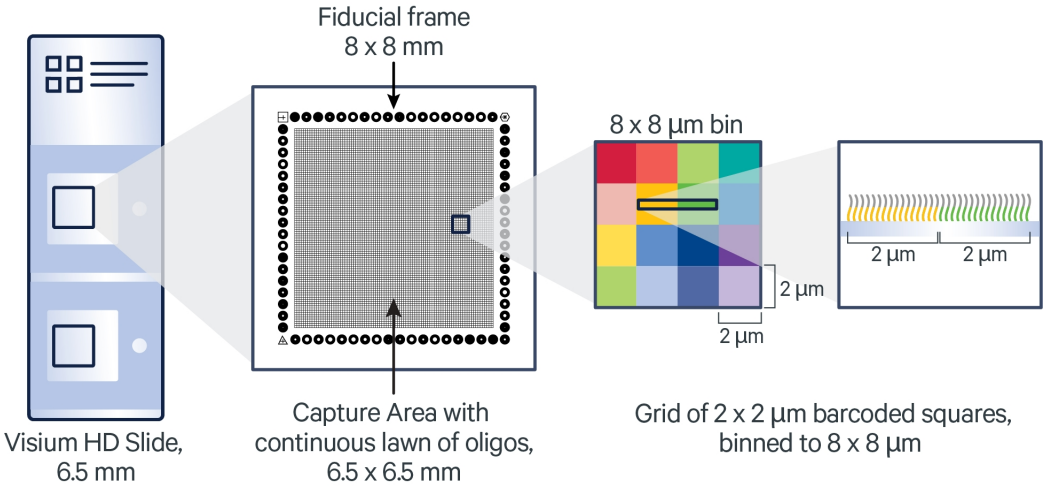
Visium HD Slides

The Visium HD Slide, 6.5 mm has 2 Capture Areas. Each Capture Area is 6.5 x 6.5 mm and is surrounded by a fiducial frame that is used for image alignment. The fiducial frame + Capture Area is 8 x 8 mm. The Capture Area is a continuous lawn of oligos comprised of 2 µm barcoded squares. Each barcoded square has oligos with the following composition:

- Illumina TruSeq partial read 1 sequencing primer, unique molecular identifier (UMI), Spatial Barcode, 30 nt poly(dT) sequence (captures ligation product).

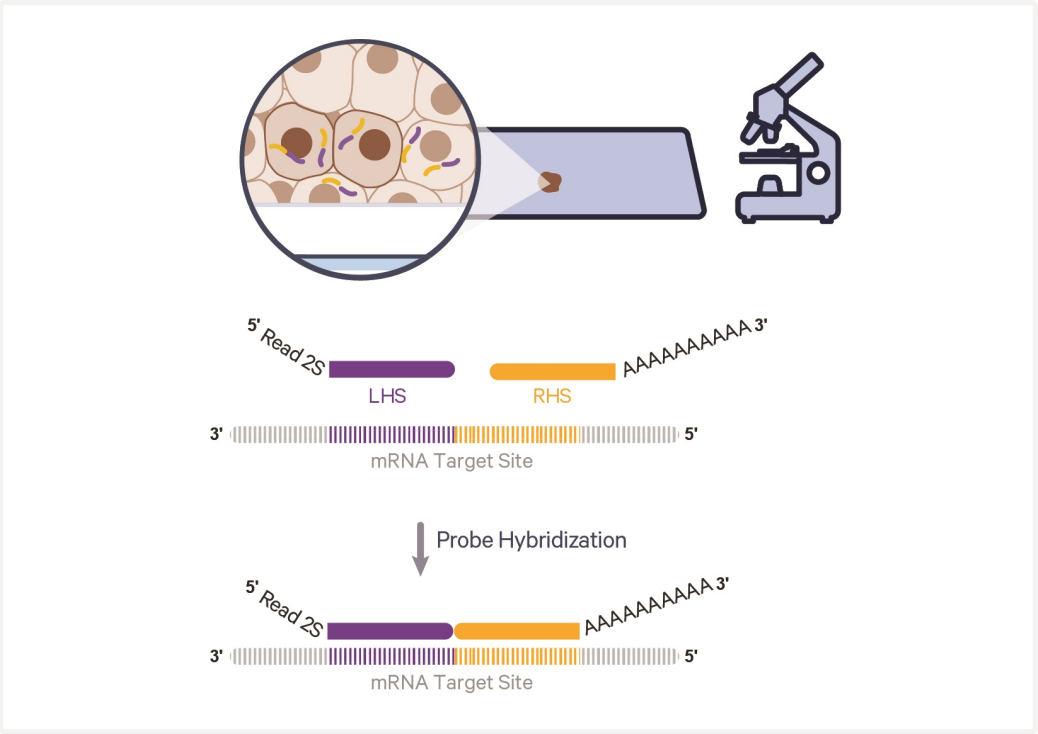
Each Capture Area is surrounded by a spacer. This spacer creates a reaction chamber that facilitates proper reagent addition.

The active surface of the slide is defined by an etched label that includes the serial number. The label should be legible. If the label appears reversed, the active surface is facing down. The slide should be loaded onto the instrument with the active surface facing up.



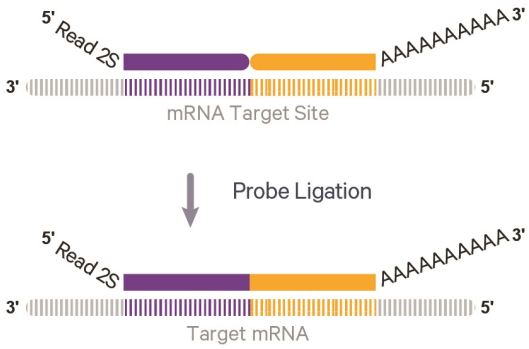
Step 1: Probe Hybridization

The human or mouse whole transcriptome probe panel, consisting of ~3 specific probes for each targeted gene, is added to the deparaffinized, destained, and decrosslinked tissues. Together, probe pairs hybridize to their complimentary target RNA.



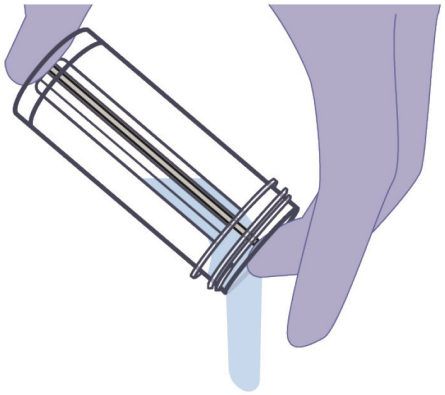
Step 2: Probe Ligation

After hybridization, a ligase is added to bridge the junction between the probe pairs that have hybridized to RNA, forming a ligation product.



Step 3: Visium HD Slide Preparation

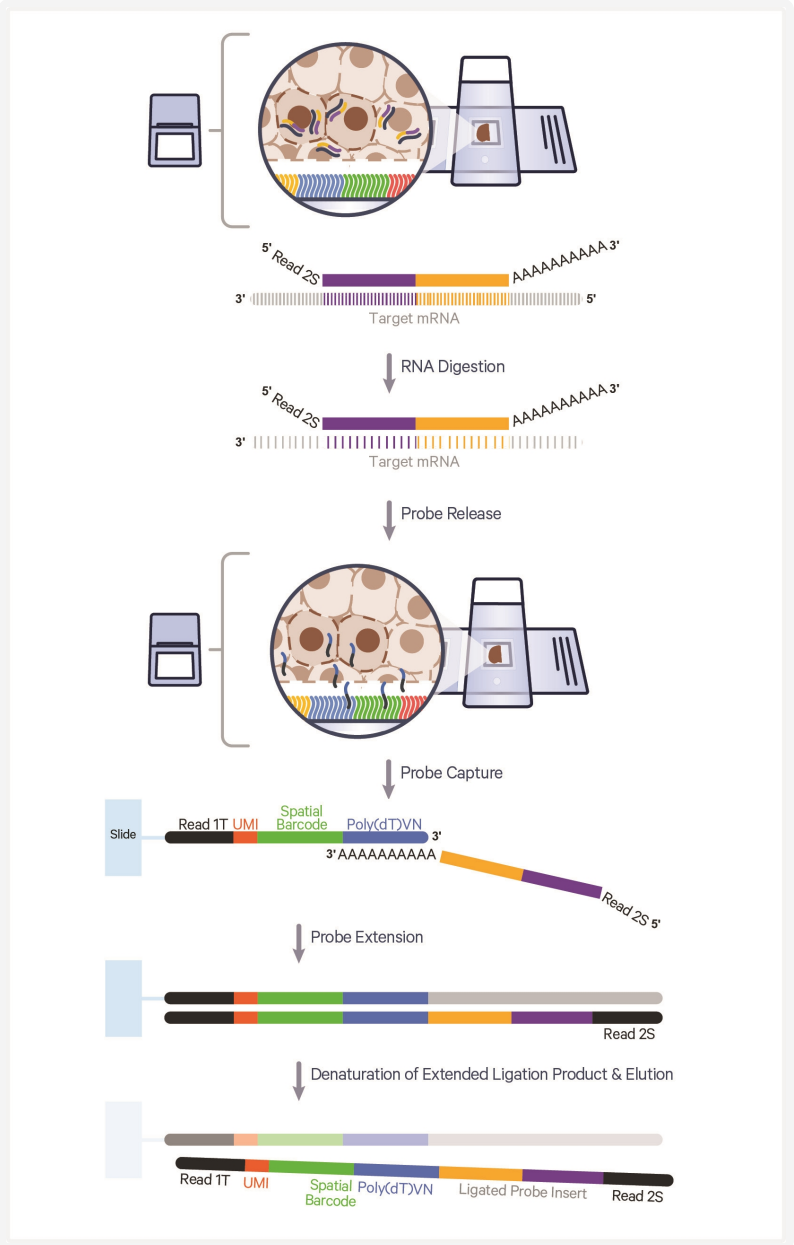
Visium HD Slides are thawed, washed, and equilibrated prior to placement on the Visium CytAssist instrument.



Step 4: Probe Release & Extension

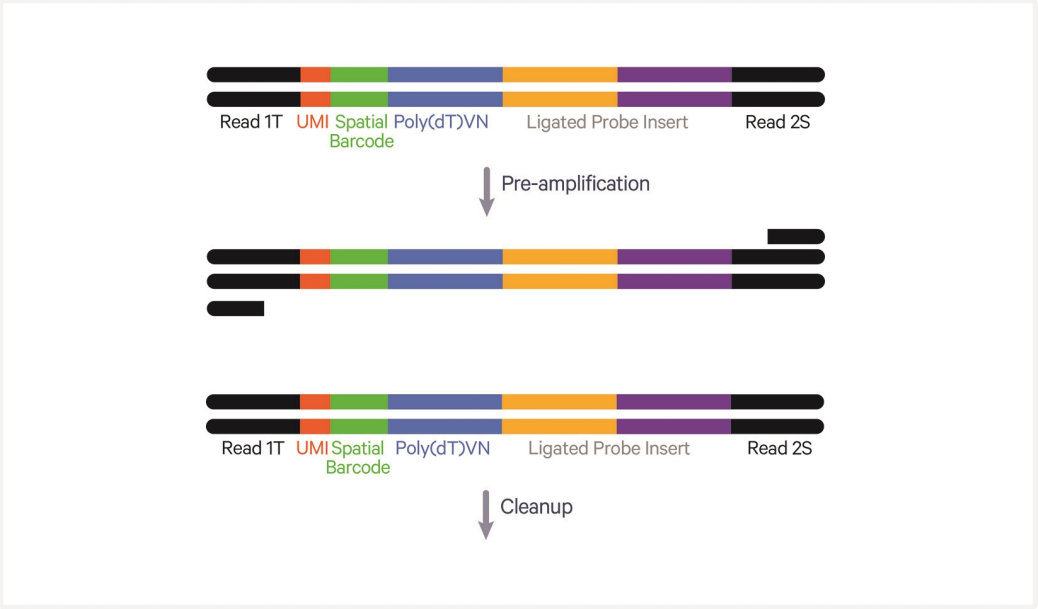
Probe release and capture occurs in the Visium CytAssist instrument. The single stranded ligation products are released from the tissue upon RNase treatment and captured on the Visium slide. Once ligation products are captured, the slides can be removed from the instrument.

Ligation products are extended by the addition of the Spatial Barcode, UMI, and partial Read 1 primer. This generates spatially-barcoded ligation products, which can then be carried forward for library preparation.



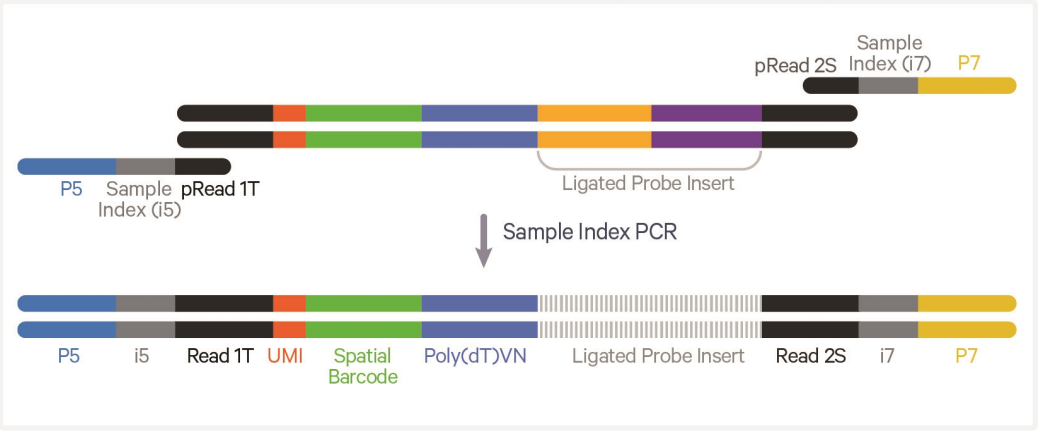
Step 5: Pre-amplification and SPRIselect

To generate ample material for library construction, barcoded ligation products are amplified. This pre-amplification is followed by SPRIselect cleanup.



Step 6: Visium HD Spatial Gene Expression - Probe-based Library Construction

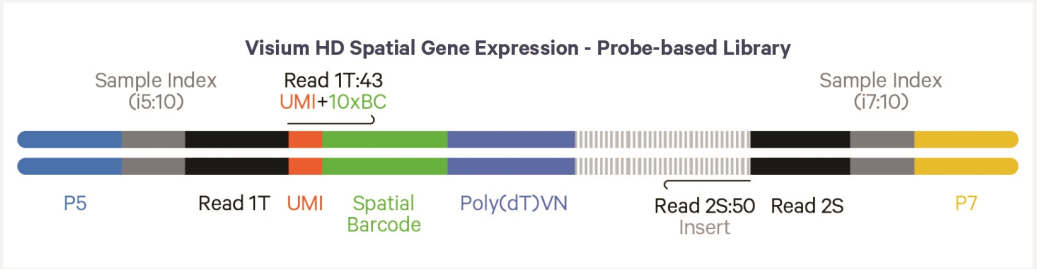
Pre-amplification material is collected for qPCR to determine sample index PCR cycle number for gene expression libraries. The amplified material then undergoes indexing via sample index PCR to generate final library molecules. The final libraries are cleaned up by SPRIselect, assessed on a Bioanalyzer or a similar instrument, quantified by qPCR, and then sequenced.



Sequencing

A Visium HD Spatial Gene Expression - Probe-based library comprises standard Illumina paired-end constructs, which begin and end with P5 and P7 adaptors. The 43 bp UMI and Spatial Barcode are encoded in TruSeq Read 1, while Small RNA Read 2 (Read 2S) is used to sequence the ligated probe product.

Illumina sequencer compatibility, sample indices, and library loading information are summarized in the Sequencing section.



See Oligonucleotide Sequences on page 122

Tips & Best Practices

TIPS

Icons

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

Indicates a version-specific update in volume, temperature, instruction, etc.

General Reagent Handling

- Fully thaw and thoroughly mix reagents before use.
- When pipette mixing reagents, unless otherwise specified, set pipette to 75% of total volume.

Visium Slide Storage

- Do not open the mylar bag containing the Visium Spatial slides until slides are ready to be used.

Pipette Calibration

- Follow manufacturer's calibration and maintenance schedules.
- Pipette accuracy is particularly important when using SPRIselect reagents.

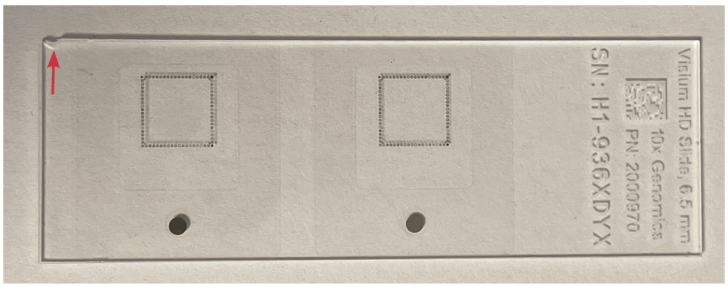
Visium HD Slide Handling

- Keep Visium HD Slide at –80°C until ready to use.
- Wipe Visium HD Slide Mailer with a lint-free laboratory wipe to ease in handling.
- Prior to loading the Visium HD Slide on the CytAssist instrument, Visium HD Slides will be thawed, washed, equilibrated, and dried. Using an external light source (like a flashlight) can help confirm that the Visium HD Slide is fully dried.
- Occasionally, small chips can arise on the edge of the Visium HD Slide. These are mainly cosmetic in nature and do not typically impact assay performance.

Sharp edges on the slide's corners may cause injury or damage if

mishandled. Handle with care. Wear proper PPE when handling and avoid contact with the chipped corner to prevent cuts or scratches.

Chipped Visium HD Slide



- Visium HD Slide preparation steps should be done gently to avoid damaging the slide.
- Visium HD Slides should only be washed if proceeding with a CytAssist instrument run.
- Always wear gloves when handling slides.
- Ensure that the active surface of a slide faces up and is never touched. The active surface is defined by a readable label. The image of the chipped Visium HD slide demonstrates the active surface facing up.
- Minimize exposure of the slides to sources of particles and fibers.
- When pipetting reagent onto a slide, avoid generating bubbles.
- If debris appears on the Visium HD Slide after performing slide washes, slide may be re-immersed in 0.1X SSC in the slider mailer to remove debris before proceeding. If performing an additional wash, ensure the slide is dried as described in the protocol.
- If necessary, unwashed, room temperature Visium HD Slides may be returned to –80°C. Protect from light. Do not exceed 3 h at room temperature. Do not exceed three freeze/thaw cycles.
- Do not allow the Visium HD Slide to dry, except where indicated in the protocol steps.

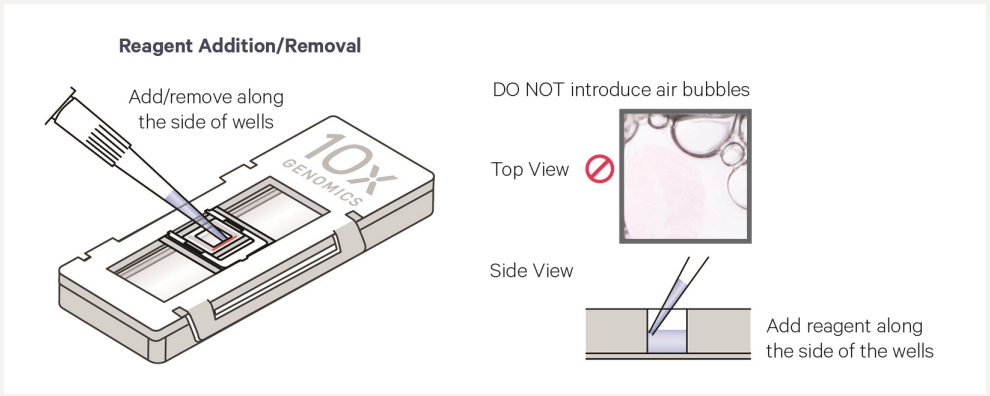
Tissue Slide Handling

- To ensure compatibility with the Visium CytAssist, tissue sections must be placed in specific areas on a blank slide. Tested slides, as well as appropriate tissue placement areas, are listed in the Visium HD Protocol Planner (CG000698).
- Always wear gloves when handling slides.

Reagent Addition to & Removal from Wells

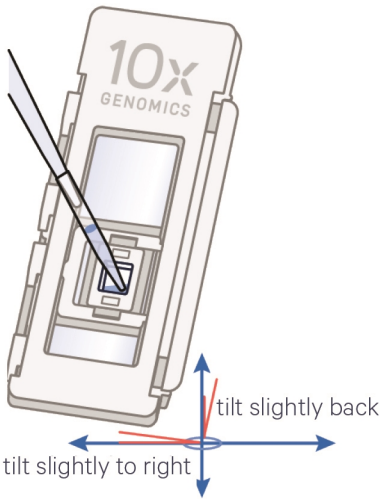
Reagent Addition

- Assemble slide into the cassette flat on a clean work surface.
- Dispense and remove reagents along the side of the wells without touching the slide surface, tissue sections (when applicable), and without introducing bubbles.
- DO NOT forcefully insert pipette tip into well corners to avoid damaging the Visium HD slide.
- If applicable, perform washes next to the thermal cycler to avoid significant changes in temperature during washes.
- When processing two or more Visium Tissue Slide Cassettes, remove and add reagent from the first cassette before proceeding to the next cassette. Ensure tissue sections are always covered with reagent in between removal and addition steps to avoid drying out of tissue samples.
- Always cover the Capture Area or tissue completely when adding reagents to the well. A gentle tap may help spread the reagent more evenly.



Reagent Removal from Wells

- Slightly tilt the cassette while removing the reagent.
- Place the pipette tip on the bottom edge of the wells.
- Remove reagents along the side of the wells without touching the tissue sections (when applicable) and without introducing bubbles.
- Remove all liquid from the wells in each step. To ensure complete removal, check the bottom of the well by tilting the cassette slightly. A meniscus at the bottom of the well will indicate the presence of liquid in the well. Repeat removal steps until no reagent remains.

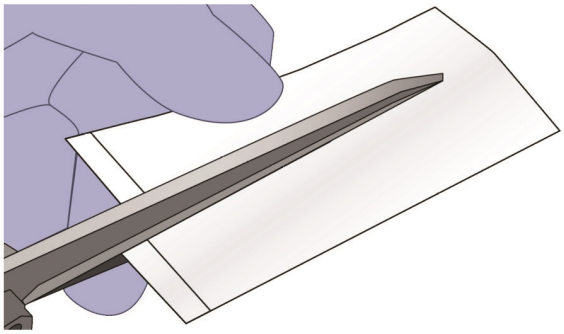


Visium Slide Seal Application & Removal

Application

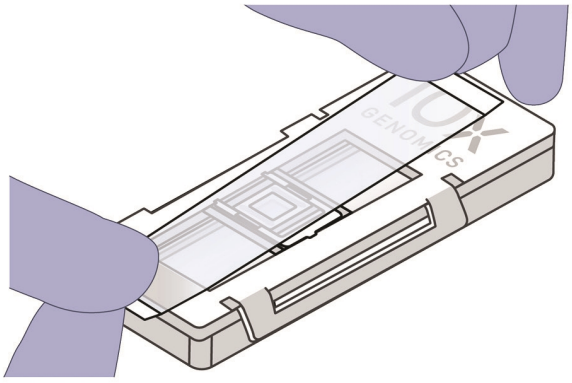
- If applying a Visium Slide Seal to a Tissue Slide Cassette, the seal must be cut in half lengthwise. Cut the seal as shown in the image below. Six pre-cut seals per tissue slide are necessary for this assay.

Cut Visium Slide Seal



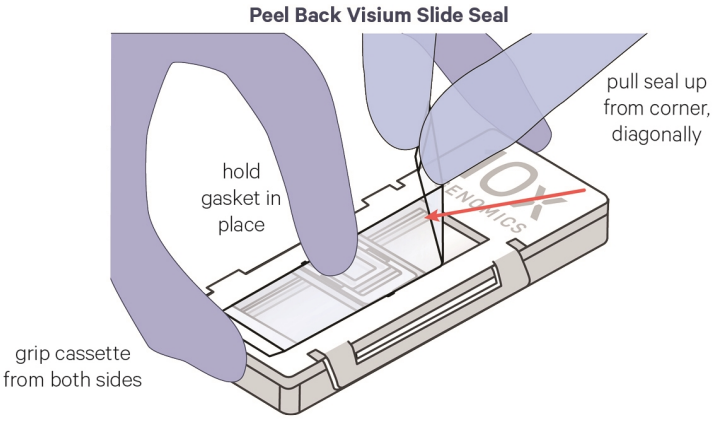
- Place the cassette flat on a clean work surface. Ensure surface of cassette is dry.
- Remove the back of the adhesive Visium Slide Seal.
- Align the Visium Slide Seal with the surface of the cassette and apply while firmly holding the cassette with one hand.
- Press on the Visium Slide Seal to ensure uniform adhesion.
- Use a fresh Visium Slide Seal when prompted during the protocol. Steps that do not require a new slide seal will specify that the slide seal should be pulled back and reapplied instead.

Apply Visium Slide Seal



Removal

- Place the cassette flat on a clean work surface.
- Grasp a Visium Slide Seal corner. Carefully pull on the Visium Slide Seal up and across the cassette while firmly holding the cassette. Ensure that no liquid splashes out of the wells. Movement of the gasket during slide seal removal could damage the tissue section.



Slide Incubation Guidance

Incubation at a Specified Temperature

- Position a Low Profile Thermocycler Adapter on a thermal cycler that is set at the incubation temperature.
- Ensure that the Low Profile Thermocycler Adapter is in contact with the thermal cycler block surface uniformly. Pre-equilibrate adapters at the HOLD temperature described in the protocol steps for at least five minutes.
- Open and close the thermal cycler lid gently. If the lid is adjustable, tighten lid only as much as necessary. Avoid overtightening. Image below is for demonstration purposes - thermal cycler lid should be closed when incubating the cassette.

Incubation at Room Temperature

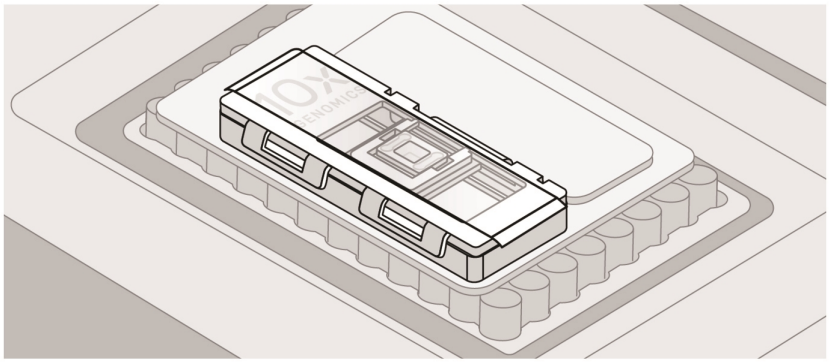
- Place the slide/cassette on a flat, clean, nonabsorbent work surface.

Cassette Incubation

Incubation using a Thermal Cycler:

- Position a Low Profile Thermocycler Adapter on a thermal cycler that is set at the incubation temperature. Low Profile Thermocycler Adapter must come to temperature before cassette placement.
- Ensure that the Low Profile Thermocycler Adapter is in contact with the thermal cycler surface uniformly. Consult the Visium HD Spatial Gene Expression Protocol Planner (CG000698) for information on thermal cycler compatibility.
- Ensure that the cassette sits flush with the raised portion of the Low Profile Thermocycler Adapter.
- Allow Low Profile Thermocycler Adapter to cool before removing from thermal cycler.

Ensure Cassette Sits Flush Against Thermocycler Adapter



Tissue Detachment on Tissue Slides

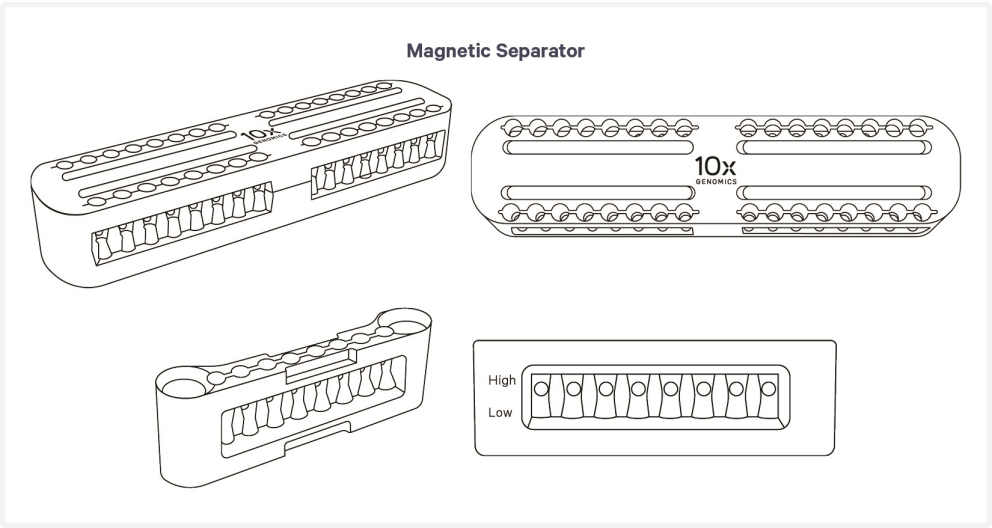
- Monitor section adhesion on tissue slides throughout the workflow.
- Ensure that tissue sections are on Superfrost Plus or positively charged slides. A list of tested slides can be found in the Visium HD Protocol Planner (CG000698).
- 10x Genomics recommends placing tissues with large amounts of connective tissue (like breast or colon) on Schott Nexterion Slide H - 3D Hydrogel Coated Slides (Schott North America, PN-1800434) to minimize risk of tissue detachment. Schott H slides may also be used if prior detachment has been observed with tissues of interest.

  - Follow manufacturer instructions for Schott Nexterion Slide H slides, which have specific requirements for storage and equilibration.

- Tissue detachment before the completion of Probe Release during the workflow can negatively impact performance. Ensure that all guidance in tissue preparation handbook is followed.
- For more information, see Troubleshooting on page 101.

10x Magnetic Separator

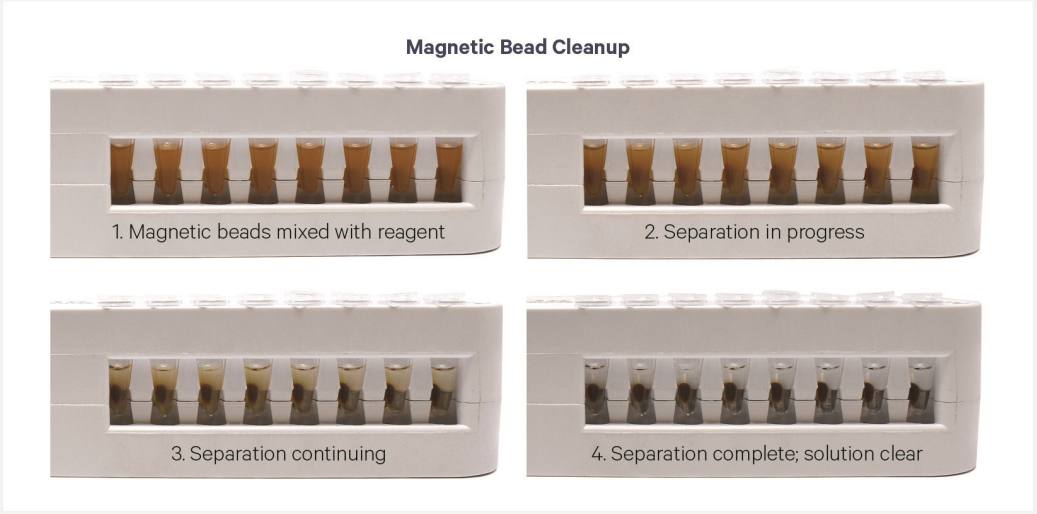
- Images below are illustrative - actual appearance of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.
- Offers two positions of the magnets (high and low) relative to a tube, depending on its orientation.
- Flip the magnetic separator over to switch between high (magnet•**High**) or low (magnet•**Low**) positions.
- If using MicroAmp 8-Tube Strips, use the high position (magnet•**High**) only throughout the protocol.



Magnetic Bead Cleanup Steps

- During magnetic bead-based cleanup steps that specify waiting “until the solution clears”, visually confirm clearing of solution before proceeding to the next step. See panel below for an example. If solution is not clear, leave on magnet until separation is complete.
- The time needed for the solution to clear may vary based on specific step, reagents, volume of reagents, etc.

- Images below are illustrative - actual appearance of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.



SPRIselect Cleanup & Size Selection

- After aspirating the desired volume of SPRIselect reagent, examine the pipette tips before dispensing to ensure the correct volume is transferred.
- Pipette mix thoroughly as insufficient mixing of sample and SPRIselect reagent will lead to inconsistent results.
- Use fresh preparations of 80% Ethanol.

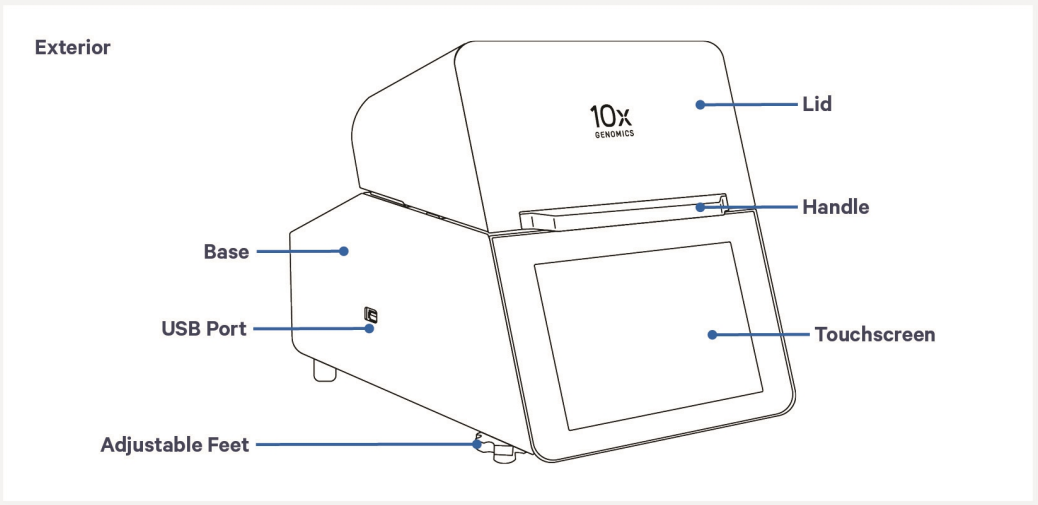
Sample Indices (i5/i7) in Sample Index PCR

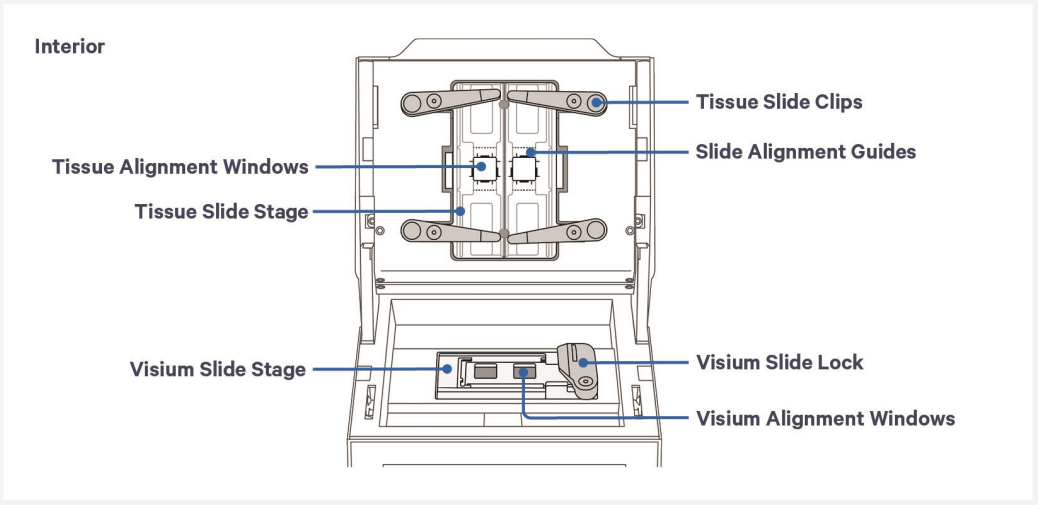
- Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run.
- Each well in the Dual Index Plate TS Set A contains a unique i7 and a unique i5 oligonucleotide.
- To avoid the risk of cross-contamination, use each plate well only once.
- Record the index plate well location for each sample.

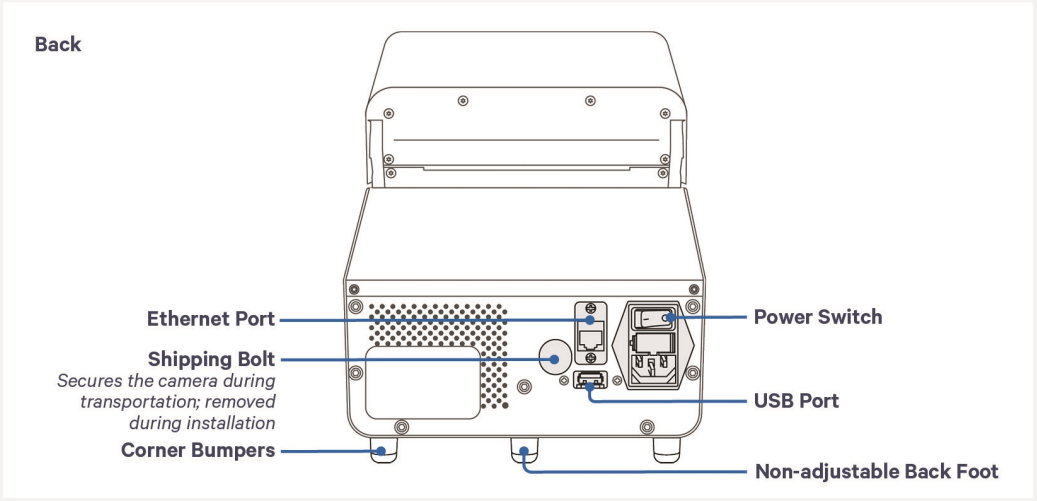
Visium CytAssist

Instrument Orientation

Firmware version 2.0.0 or higher is required in the Visium CytAssist used for this protocol.







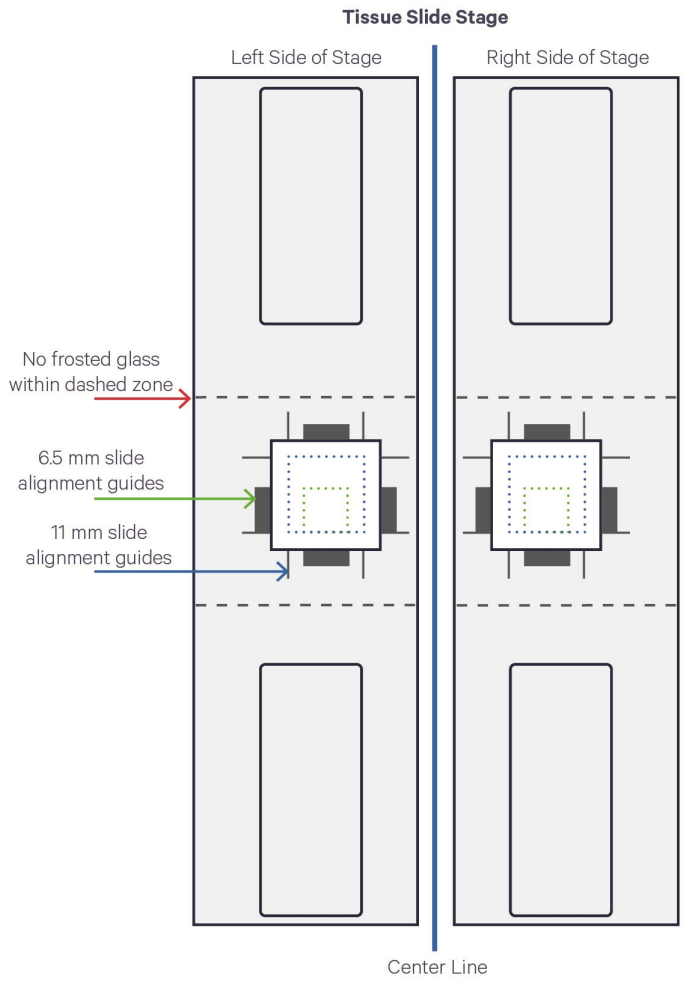
Instrument Loading Guidelines

- Ensure tissue section placement is compatible with Visium CytAssist by using the Visium CytAssist Accessory Kit Quick Reference Card (CG000548). A list of tested slide types can be found in the Visium HD Spatial Gene Expression Protocol Planner (CG000698).
- Consult the Visium CytAssist Training Kit UG (CG000549) for information on practicing tissue slide loading.
- Each tissue slide may be used for one Capture Area on a Visium HD Slide.

Tissue Slide Loading

a. Ensure the tissue on each slide fits within the alignment guides of the Tissue Slide Stage.

b. Align tissue within the center of the 6.5 mm slide alignment guides (rectangles) on either the left or right side of the stage.



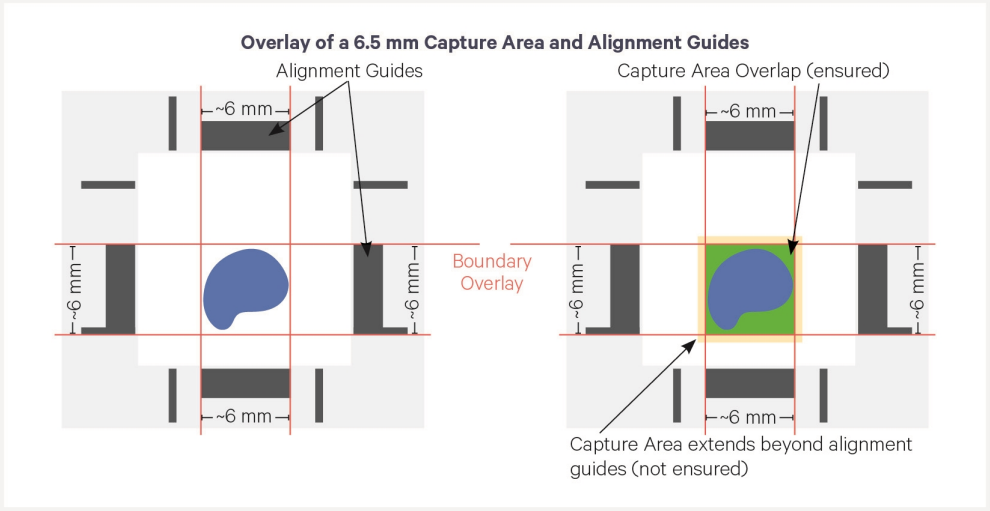
The dotted outlines of the Capture Areas that are shown in diagram are not present on the instrument's surface.
Slides should not overlap the center line.

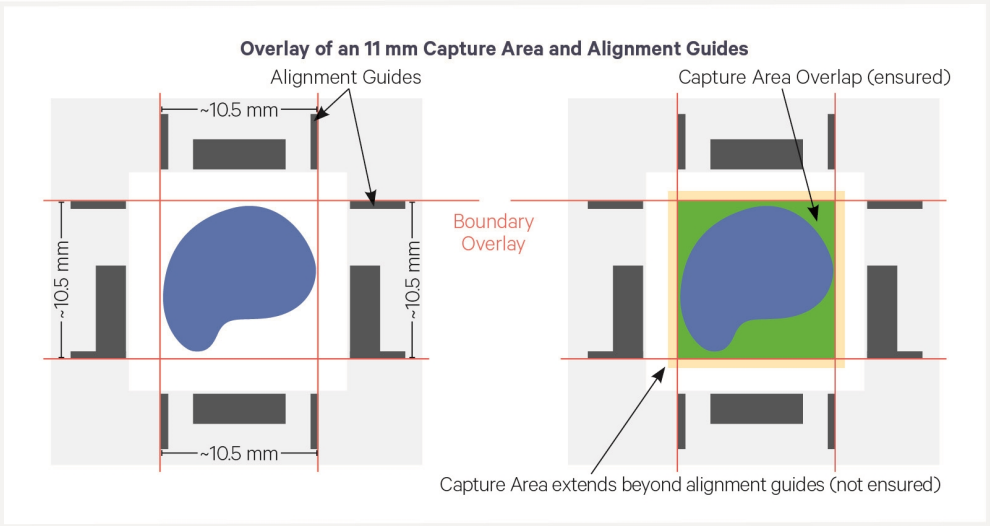
The **outside** of the alignment guides, shown in red, mark the inner boundaries of the fiducial frame on a Capture Area. They mark a distance **approximately** 6 mm (6.5 mm slides) or 10.5 mm (11 mm slides) wide on each side and surround the target region ensured to align within the fiducial frame. Though the Visium CytAssist instrument displays guides for 11 mm Capture Areas, the Visium HD Spatial Gene Expression Assay is only compatible with 6.5 mm Capture Areas.

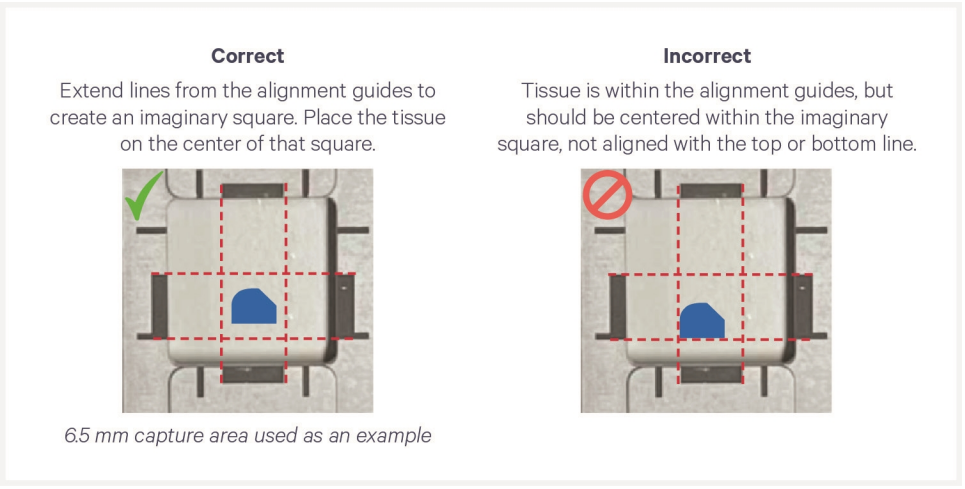
When placing a tissue slide on the Tissue Slide Stage, center the tissue or area of interest within the area inside the outer edges of the alignment guides (area in green in the images).

Alignment guide dimensions and locations are designed with a high tolerance to minimize data loss. Tissue within the alignment guides is

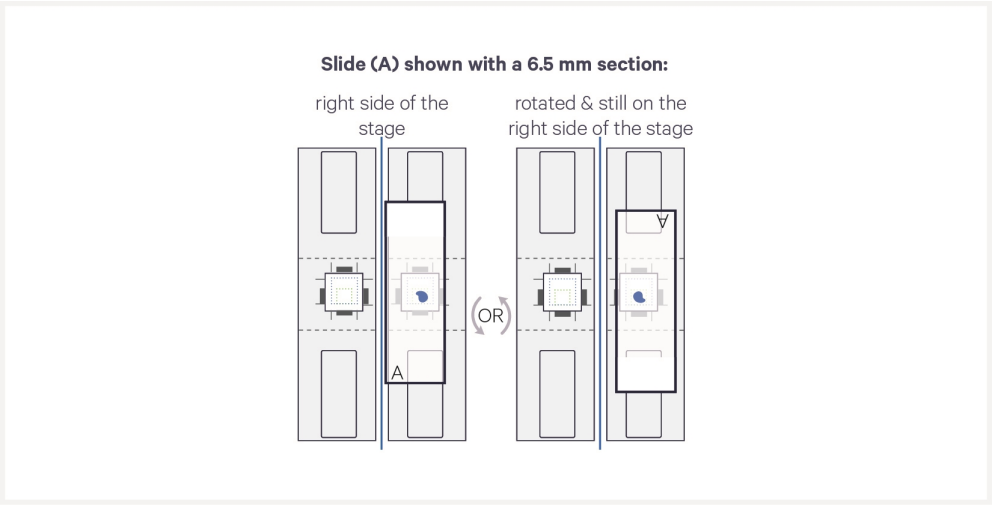
ensured to overlap with the Capture Area during a successful run. Tissue outside of these guides may be captured (area in yellow in the images), but it is not ensured. 10x Genomics recommends placing high-priority tissue areas within the alignment guides.



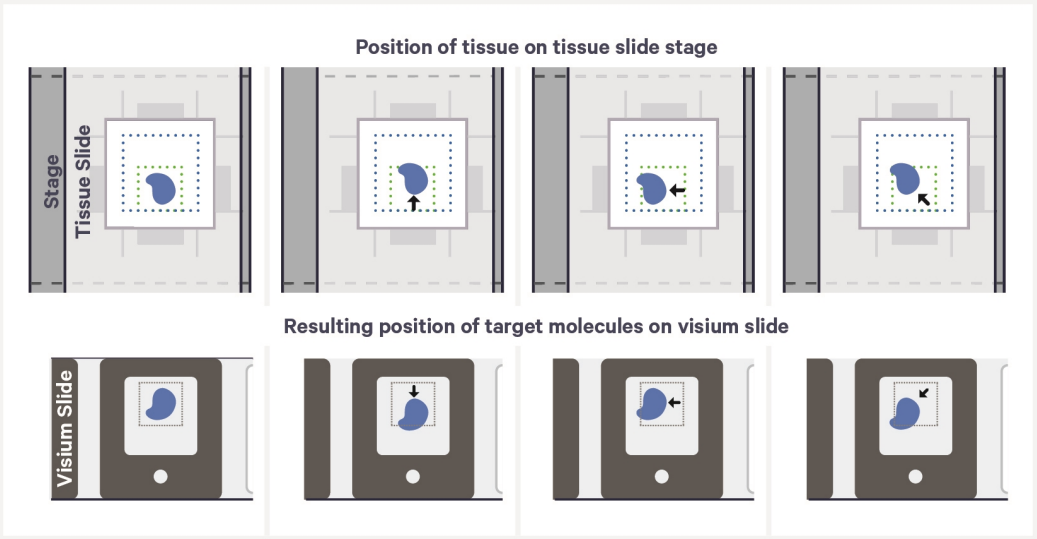




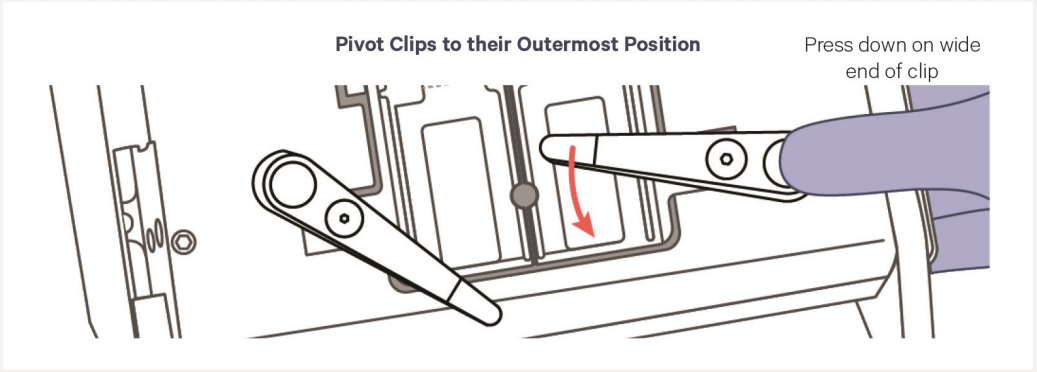
c. If necessary, rotate the slide 180° as shown. Place off-center tissues closer to the center line (shown as a blue line in the image below). Slides should not overlap the center line.



The image below demonstrates how movement of the Tissue Slides affects where target molecules will end up on the Visium slide.

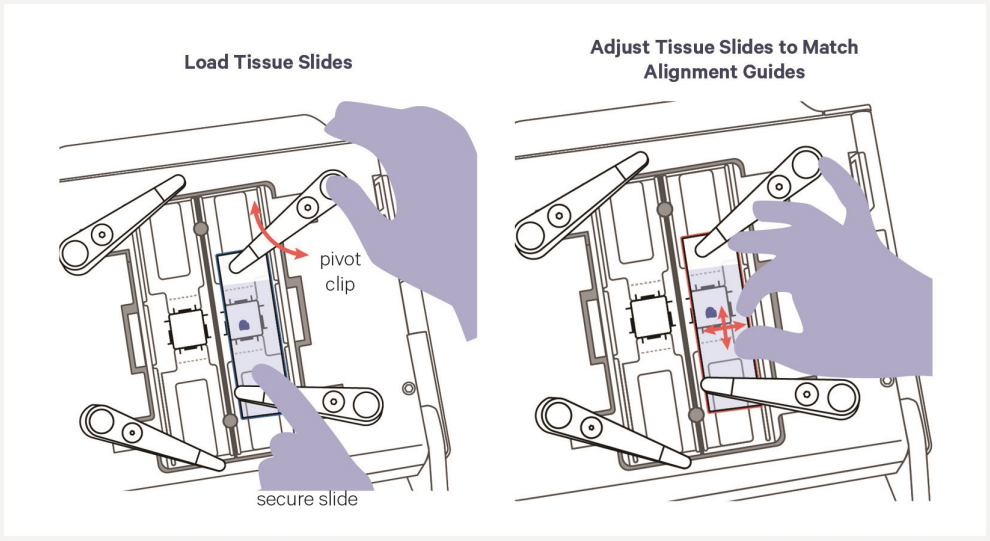


d. Press down on the wide end of clip to lift and pivot the narrow end of the clip. Pivot the clips to their outermost position.

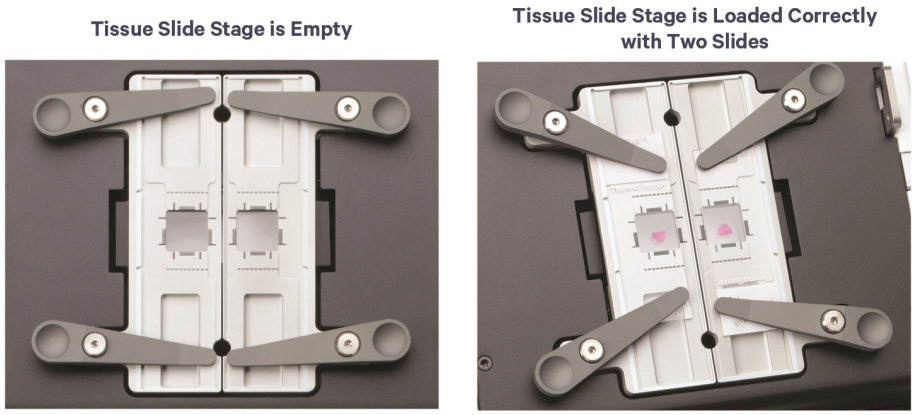


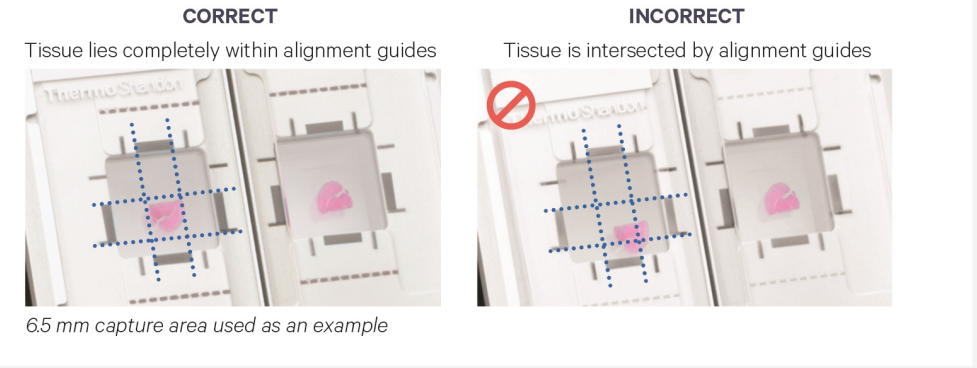
e. Lay the tissue slide flat against the stage surface. Both hands needed: use one hand to hold the slide in place and the other to pivot the clips and overlap the slide. Ensure that at least one clip secures the slide before adjusting the position further.

f. Use fingers to finely adjust the position of the tissue within the alignment guides.



g. If only one tissue on one slide will be analyzed, use a blank slide for the second position on the Tissue Slide Stage. If using only one tissue slide, the unused Capture Area on the Visium HD Slide cannot be used in another instrument run.





6.5 mm capture area used as an example

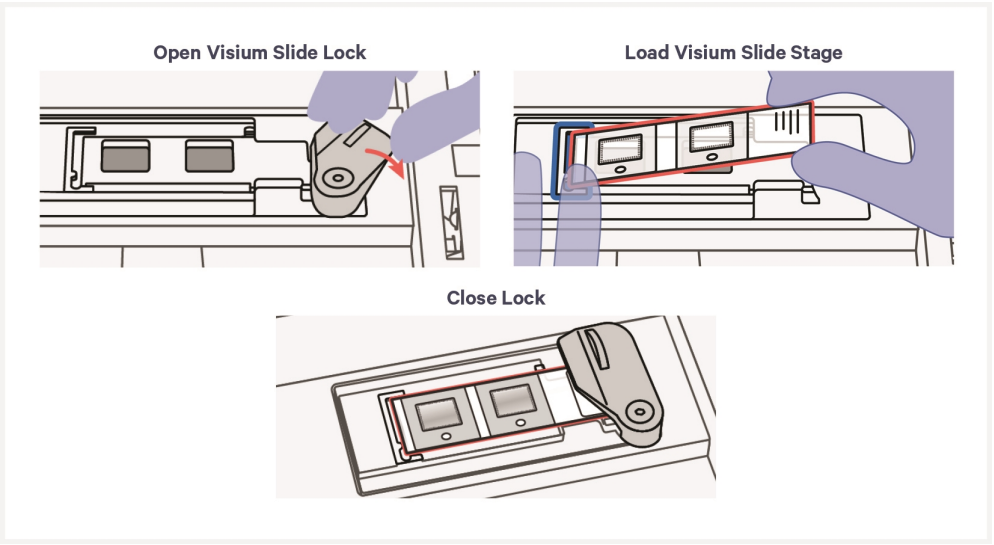
Visium HD Slide Loading

Before loading, record the slide serial number and note which tissue is placed in which Capture Area. Note which Capture Area will be placed on which side of the Visium Slide Stage (Left = D1, Right = A1).

a. Open the Visium Slide Lock by using one finger to pivot.

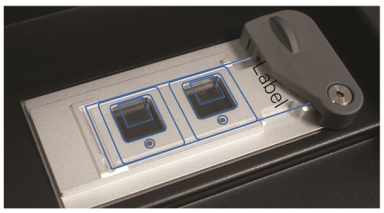
b. Line up the slide with the label face up and oriented to the right.

c. Fit the slide within the raised grooves on the left, top, and bottom.



d. Hold slide in place with one hand while slowly closing the Visium Slide Lock. Lock will partially obscure the slide label when correctly secured. Close lock gently to avoid slide damage. In the images below, the slide (red outline) does not line up with the stage (blue outline).

Visium Slide Stage is Loaded Correctly with One Slide



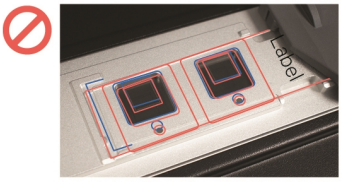
Slide fits inside the grooves.

Label on the right is partially obscured by the lock.

Spacer window lines up with alignment windows.

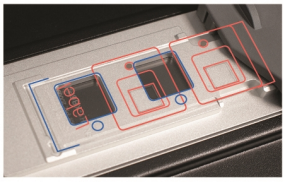
INCORRECT

Slide does not fit inside the grooves.
Spacer window does not line up with alignment windows.



INCORRECT

Label is on the left.
Spacer window does not line up with alignment windows.



Sample Preparation & Staining Guidelines

Sample Preparation

Proper tissue handling and preparation techniques preserve the morphological quality of the tissue sections and the integrity of mRNA transcripts. Maintaining high-quality RNA is critical to assay performance.

Before performing the protocol steps in this User Guide, ensure that all samples have followed the appropriate Tissue Preparation protocols as described in the Workflow Overview. Listed below are key considerations described in these tissue preparation protocols.

Key Considerations	
FFPE Tissue Sectioning & Section Placement	
☐	Assess RNA quality of the FFPE tissue block or from archived sections.
☐	Practice sectioning and section placement with a nonexperimental block before placing sections on slides.
☐	Section the tissue block and place sections on compatible blank slides using a water bath.
☐	Place tissue sections on the allowable area on the slide. Allowable area may vary depending on slide choice.
Tissue Slide Handling	
☐	Ensure slides are completely dried after tissue placement, using a fan if necessary.
☐	Store the slides containing FFPE sections for up to six months in a desiccator at room temperature.
☐	After preparing tissue slides, stain sections according to the desired staining protocol in the Visium HD FFPE Tissue Preparation Handbook (CG000684).
Tissue Slide Processing	
☐	After preparing tissue slides, process sections according to the desired staining protocols in the Visium HD FFPE Tissue Preparation Handbook (CG000684). The handbook contains information on tissue slide staining and imaging.

Key Considerations for FF Samples	
Freezing and Embedding	
☐	Snap freeze samples in a bath of isopentane and liquid nitrogen.
☐	Embed frozen samples in OCT.
☐	Store frozen samples in a sealed container at -80°C for long-term storage.
Slide Handling	
☐	Equilibrate slides to cryostat temperature before cryosectioning.
Cryosectioning	
☐	Assess RNA quality of the tissue block.
☐	Optional - assess tissue morphology via DAPI and H&E staining.

Key Considerations for FF Samples	
☐	Practice sectioning and section placement with a nonexperimental block before placing sections on slides.
☐	Equilibrate OCT tissue block to the cryostat chamber temperature for 30 min.
☐	Section the tissue block and place sections on compatible blank slides.
Tissue Slide Processing	
☐	After preparing tissue slides, process sections according to the desired staining protocols in the Visium HD FF Tissue Preparation Handbook (CG000763). The handbook contains information on tissue slide staining and imaging.
Tissue Slide Handling	
☐	Maintain slides containing sections in a low moisture environment.
☐	Keep slides cold and transport slides on dry ice.
☐	Store tissue slides in a slide mailer at -80°C for up to two months.

Key Considerations for FxF Samples	
Fixation, Embedding, and Freezing	
☐	Fix samples in 4% PFA or formaldehyde
☐	Cryopreserve samples in a sucrose gradient.
☐	Embed fixed samples in OCT and freeze.
Sample Block Storage	
☐	Store frozen samples in a sealed container at -80°C for long-term storage.
Slide Handling	
☐	Equilibrate slides to cryostat temperature before cryosectioning.
Cryosectioning	
☐	Assess RNA quality of the tissue block.
☐	Optional - assess tissue morphology via DAPI and H&E staining.
☐	Practice sectioning and section placement with a nonexperimental block before placing sections on slides.
☐	Equilibrate OCT tissue block to the cryostat chamber temperature for 30 min.
☐	Section the tissue block and place sections on compatible blank slides.
Tissue Slide Processing	
☐	After preparing tissue slides, process sections according to the desired staining protocols in the Visium HD FxF Tissue Preparation Handbook (CG000764). The handbook contains information on tissue slide staining and imaging.
Tissue Slide Handling	
☐	Maintain slides containing sections in a low moisture environment.
☐	Keep slides cold and transport slides on dry ice.
☐	Store tissue slides in a slide mailer at -80°C for up to two months.

Step 1:

Probe Hybridization

1

1.0 Get Started

Each 10x Genomics reagent tube is enough for two 6.5 mm Visium HD Slides (four Tissue Slides). Number of reactions in reagent preparation tables refers to the number of Tissue Slides.

Items			10x PN	Preparation & Handling	Storage
Equilibrate to room temperature					
☐	○	FFPE Hyb Buffer	Tube: 2000423 Kit: 1000466 or 1000667	Thaw at room temperature. If precipitate persists, heat at 37°C until dissolved. Avoid vortexing to prevent bubble formation. Pipette mix 10x. Keep the buffer at room temperature after thawing and while performing the workflow.	–20°C
☐	●	Human WT Probes v2 - RHS	Tube: 2000657 Kit: 1000466	Thaw at room temperature. Vortex and centrifuge briefly. Maintain at room temperature.	–20°C
☐	●	Human WT Probes v2 - LHS	Tube: 2000658 Kit: 1000466	Thaw at room temperature. Vortex and centrifuge briefly. Maintain at room temperature.	–20°C
☐	●	Mouse WT Probes v2 - RHS	Tube: 2000913 Kit: 1000667	Thaw at room temperature. Vortex and centrifuge briefly. Maintain at room temperature.	–20°C
☐	●	Mouse WT Probes v2 - LHS	Tube: 2000912 Kit: 1000667	Thaw at room temperature. Vortex and centrifuge briefly. Maintain at room temperature.	–20°C
Obtain					
☐		Nuclease-free Water	-	-	Ambient
☐		10X PBS, pH 7.4	-	-	Ambient
☐		Visium Slide Seals	Component: 2000284 Kit: 1000669	See Tips & Best Practices. 3 pre-cut slide seals are necessary for the complete workflow.	Ambient
☐		10% Tween-20	-	-	Ambient

1.1 Probe Hybridization

TIPS

During reagent removal steps, ensure that **ALL liquid is removed** from wells. Use a P20 pipette to remove any remaining liquid. See Tips & Best Practices for guidance on Reagent Removal.

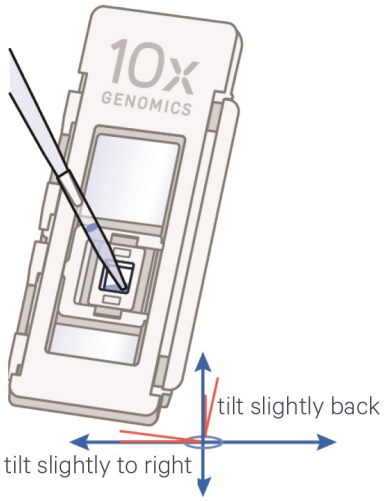
a. Prepare Pre-Hybridization Mix according to the table shortly before use. Add reagents in the order listed. Vortex and centrifuge briefly. Maintain at **room temperature**.

Pre-Hybridization Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
Nuclease-free Water	-	134.2	295.3	590.6
10X PBS, pH 7.4	-	15.0	33.0	66.0
10% Tween-20	-	0.8	1.7	3.4
Total	**-**	**150.0**	**330.0**	**660.0**

b. Retrieve Tissue Slide Cassettes containing H&E stained or IF stained sections.

c. Peel back Visium Slide Seals.

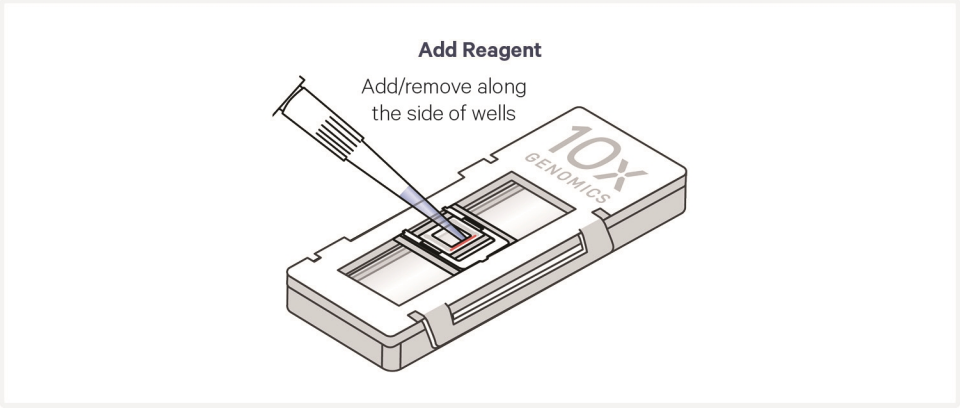
d. Using a pipette, remove all buffer from each well at well corners. Use a P20 pipette to remove any remaining liquid. See image below for proper liquid removal technique.



e. Add **150 µl** Pre-Hybridization Mix along the side of each well to uniformly cover tissue sections, without introducing bubbles.

f. Re-apply Visium Slide Seal on each Tissue Slide Cassette.

g. Incubate for **15 min** at **room temperature**.



TIPS

h. Place a Low Profile Thermocycler Adapter in the thermal cycler. Prepare thermal cycler with the following incubation protocol and start the program. See Tips & Best Practices for more information on cassette incubation in a thermal cycler.

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

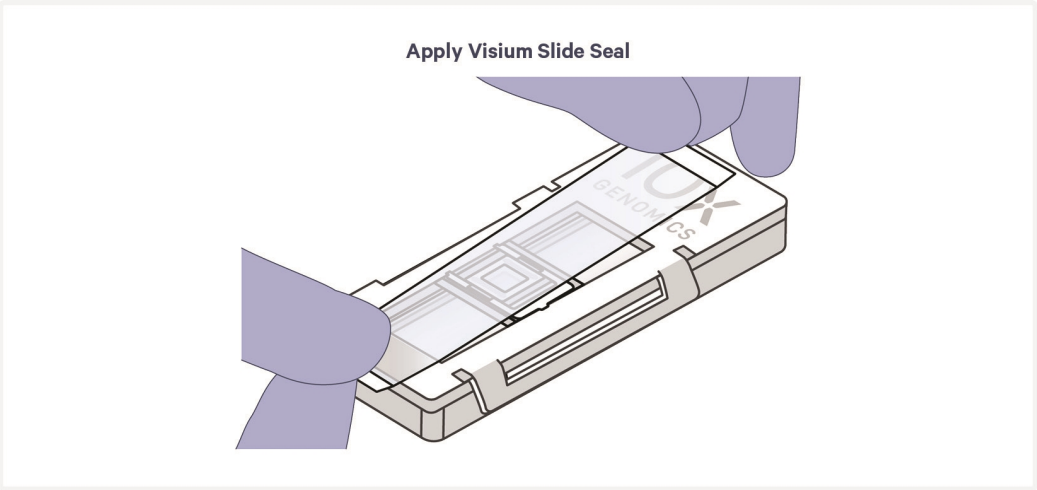
i. Prepare Probe Hybridization Mix according to the table shortly before use. Add reagents in the order listed. Keep at **room temperature**. Pipette mix 10x and centrifuge briefly.

	Probe Hybridization Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
	Nuclease-free Water	-	10.0	22.0	44.0
○	FFPE Hyb Buffer	2000423	70.0	154.0	308.0
●	Human WT Probes v2 - RHS or Mouse WT Probes v2 - RHS	2000657 or 2000913	10.0	22.0	44.0
●	Human WT Probes v2 - LHS or Mouse WT Probes v2 - LHS	2000658 or 2000912	10.0	22.0	44.0
	Total	**-**	**100.0**	**220.0**	**440.0**

j. Remove all Pre-Hybridization Mix from each well.

k. Add **100 µl** room temperature Probe Hybridization Mix to each well.

l. Apply a new pre-cut Visium Slide Seal to each Tissue Slide Cassette and place on the Low Profile Thermocycler Adapter on the thermal cycler. Close the thermal cycler lid.



m. Skip Pre-equilibrate step to initiate Hybridization.

Step 2:

Probe Ligation

2

2.0 Get Started

Each 10x Genomics reagent tube is enough for two 6.5 mm Visium HD Slides (four Tissue Slides). Number of reactions in reagent preparation tables refers to the number of Tissue Slides.

Items			10x PN	Preparation & Handling	Storage
Equilibrate to room temperature					
☐	◯	FFPE Post-Hyb Wash Buffer	Tube: 2000424 Kit: 1000466 or 1000667	Thaw at room temperature. If precipitate persists, heat at 37°C until dissolved. Vortex briefly. Flick tube until liquid settles at the bottom of the tube.	–20°C
☐	◯	2X Probe Ligation Buffer	Tube: 2000445 Kit: 1000466 or 1000667	Thaw at room temperature until no precipitate remains. Vortex and centrifuge briefly.	–20°C
☐	◯	Post-Ligation Wash Buffer	Tube: 2000419 Kit: 1000466 or 1000667	Thaw at room temperature. If precipitate persists, heat at 37°C until dissolved. Vortex.	–20°C
Place on ice					
☐	◯	Probe Ligation Enzyme	Tube: 2000425 Kit: 1000466 or 1000667	Centrifuge briefly. Maintain on ice.	–20°C
Obtain					
☐		Nuclease-free Water	-	-	Ambient
☐		20X SSC Buffer	-	-	Ambient

2.1 Post-Hybridization Wash

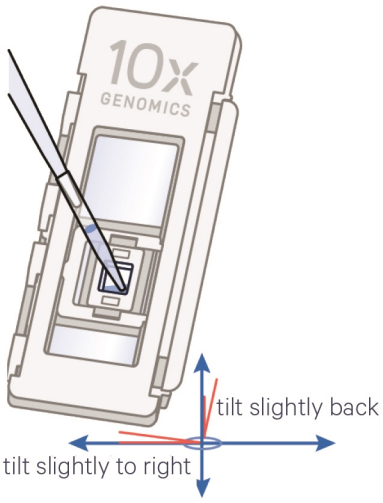
a. Aliquot FFPE Post-Hyb Wash Buffer (**495 µl/per 6.5 mm sample**) and pre-heat to **50°C** in a water bath or thermomixer. Maintain pre-heated wash buffer at **50°C** throughout washes.

b. Prepare 2X SSC Buffer according to the table. Add reagents in the order listed. Maintain at **room temperature**. DO NOT discard excess buffer, as it will be used for subsequent washes in the protocol.

SSC Buffer	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
SSC	20X	2X	150	315	615
Nuclease-free Water	-	-	1,350	2,835	5,535
Total	**-**		**1,500***	**3,150***	**6,150***

c. Remove Visium Tissue Slide Cassettes from Low Profile Thermocycler Adapter and place on a flat, clean work surface.

TIPS **d.** Peel back Visium Slide Seals and using a pipette, remove all Probe Hybridization Mix from each well. See Tips and Best Practices for slide seal removal guidance.



e. **Immediately** add **150 µl** pre-heated FFPE Post-Hyb Wash Buffer to each well. Avoid well drying or cooling to room temperature. Removal and addition of buffers should be done quickly.

TIPS **f.** Re-apply Visium Slide Seal on each Tissue Slide Cassette and place on Low Profile Thermocycler Adapter on pre-heated thermal cycler. Close thermal

cycler lid. See Tips & Best Practices for more information on cassette incubation on a thermal cycler.

Ensure that Tissue Slide Cassette sits snugly on the Low Profile Thermocycler Adapter for all wash steps.

g. Skip the Hybridization step on thermal cycler and initiate Post-Hybridization Wash.

h. Incubate Visium Tissue Slide Cassettes in thermal cycler at **50°C** for **5 min**.

i. Remove Tissue Slide Cassettes from Low Profile Thermocycler Adapter and place on a flat, clean work surface. Exercise caution, as slide is hot.

j. Peel back Visium Slide Seals and using a pipette, remove all FFPE Post-Hyb Wash Buffer from each well.

k. **Immediately** add **150 µl** pre-heated FFPE Post-Hyb Wash Buffer to well. Avoid well drying or cooling to room temperature. Removal and addition of buffers should be done quickly.

l. Re-apply Visium Slide Seal on each Tissue Slide Cassette and place on Low Profile Thermocycler Adapter on pre-heated thermal cycler. Close thermal cycler lid.

m. Incubate in thermal cycler at **50°C** for **5 min**.

n. **Repeat** steps i-m one more time for a total of three washes.

o. Peel back Visium Slide Seals and remove FFPE Post-Hyb Wash Buffer from each well.

p. Add **150 µl** 2X SSC Buffer to each well and re-apply Visium Slide Seal.

q. Let the Tissue Slide Cassettes cool to **room temperature** (**~3 min**) before proceeding to the next step.

2.2 Probe Ligation

a. Place a Low Profile Thermocycler Adapter onto a thermal cycler (if not already placed), prepare with the following incubation protocol, and start the program.

Lid Temperature	Reaction Volume	Run Time
37°C (lid may be turned off if the instrument does not enable 37°C)	100 µl	1 h
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	37°C	Hold
Ligation	37°C	01:00:00
Hold	4°C	Hold

b. Prepare Probe Ligation Mix according to the table shortly before use. Add reagents in the order listed. Pipette mix 10x and centrifuge briefly. DO NOT vortex. Maintain on ice.

	Probe Ligation Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
	Nuclease-free Water	-	24.0	52.8	105.6
◯	2X Probe Ligation Buffer	2000445	30.0	66.0	132.0
◯	Probe Ligation Enzyme	2000425	6.0	13.2	26.4
	Total	**-**	**60.0**	**132.0**	**264.0**

c. Remove Visium Slide Seals from each Tissue Slide Cassette and remove all 2X SSC Buffer from each well.

d. Add **60 µl** Probe Ligation Mix directly to tissue sections in each well, without introducing bubbles. Tap Tissue Slide Cassettes gently to ensure uniform coverage.

e. Apply a new pre-cut Visium Slide Seal on each Tissue Slide Cassette and place on the Low Profile Thermocycler Adapter on the pre-heated thermal cycler. Close the thermal cycler lid.

f. Skip Pre-equilibrate step to initiate Ligation.

2.3 Post-Ligation Wash

Use room temperature Post-Ligation Wash Buffer at first wash step (step 2.3f). Use pre-heated Post-Ligation Wash Buffer at second wash step (step 2.3k).

a. If the stopping point after the Post-Ligation Wash will not be used, remove Visium HD slide mailer from –80°C. Remove slide mailer from mylar bag. Keep slide mailer upright, capped, and thaw at **room temperature** for **30–60 min**. Two Visium HD slides may be prepared at the same time.

b. Pre-heat Post-Ligation Wash Buffer (**110 µl/sample**) to **57°C**. Only **100 µl** per 6.5 mm sample is needed.

c. Remove the Tissue Slide Cassettes from the Low Profile Thermocycler Adapter and place on a flat, clean work surface.

d. Immediately prepare thermal cycler with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
57°C	100 µl	-
Step	**Temperature**	**Time**
Incubate	57°C	Hold

e. Remove Visium Slide Seals from each Tissue Slide Cassette and using a pipette, remove all Probe Ligation Mix from each well.

f. Immediately add **100 µl room temperature** Post-Ligation Wash Buffer to each well. Removal and addition of buffers should be done quickly.

g. Apply a new pre-cut Visium Slide Seal on each Tissue Slide Cassette and place on Low Profile Thermocycler Adapter on pre-heated thermal cycler. Close thermal cycler lid. See Tips & Best Practices for more information on cassette incubation in a thermal cycler.

Ensure that Tissue Slide Cassette sits snugly on the Low Profile Thermocycler Adapter for all wash steps.

h. Incubate at **57°C** for **5 min**.

i. Remove Tissue Slide Cassettes from the Low Profile Thermocycler Adapter and place on a flat, clean work surface.

j. Peel back Visium Slide Seals and using a pipette, remove all Post-Ligation Wash Buffer from each well.

k. Add **100 µl pre-heated** Post-Ligation Wash Buffer to each well.

l. Re-apply Visium Slide Seal on Tissue Slide Cassette and place on Low Profile Thermocycler Adapter on pre-heated thermal cycler. Close thermal cycler lid.

m. Incubate at **57°C** for **5 min**.

n. Remove Tissue Slide Cassettes from Low Profile Thermocycler Adapter and place on a flat, clean work surface.

o. Peel back Visium Slide Seals and using a pipette, remove all Post Ligation Wash Buffer from each well.

p. Add **150 µl** 2X SSC Buffer prepared in 2.1 Post-Hybridization Wash on page 52 to each well.

q. Remove all 2X SSC Buffer from each well.

r. Add **150 µl** 2X SSC Buffer to each well.

s. Re-apply Visium Slide Seals on each Tissue Slide Cassette.

t. Allow Tissue Slide Cassettes to come to room temperature for **5 min**.

STOP

u. Store at **4°C** for up to **24 h** or proceed to next step.

Step 3:

Visium HD Slide Preparation

3

3.0 Get Started

Each 10x Genomics reagent tube is enough for two 6.5 mm Visium HD Slides. Number of reactions in reagent preparation tables refers to the number of Visium HD slides.

Firmware version 2.0.0 or higher is required in the Visium CytAssist used for this protocol.

Items		10x PN	Preparation & Handling	Storage
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	20X SSC	-	-	Ambient
☐	Visium Cassette	Component: 3001830/ 3001831 Kit: 1000669	See Cassette Assembly Quick Reference Card (CG000577)	Ambient

3.1 Visium HD Slide Wash

a. If Visium HD Slide has not been thawed, remove Visium HD slide mailer from –80°C. Remove slide mailer from mylar bag. DO NOT uncap slide mailer. Keep slide mailer upright and thaw at **room temperature** for **30–60 min**. Two Visium HD slides may be prepared at the same time.

DO NOT touch spacer during slide washes.

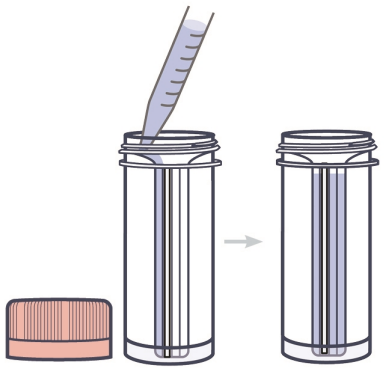
b. Prepare 0.1X SSC Buffer according to the table below. Pipette mix. Maintain at room temperature.

0.1X SSC Buffer	Stock	Final	1 Visium HD Slide (ml)	2 Visium HD Slides (ml)
Nuclease-free Water	-	-	59.7	119.4
SSC	20X	0.1X	0.3	0.6
Total	**-**		**60.0**	**120.0**

c. Open slide mailer.

d. Add **7 ml** 0.1X SSC Buffer slowly along the side of the mailer on one side of the slide.

e. Add **7 ml** 0.1X SSC Buffer slowly along the side of the mailer on the other side of the slide, so that both sides of the slide are immersed in 0.1X SSC Buffer.



Add buffer evenly on both sides of the slide. The liquid level should be even on both sides. Leave slide mailer uncapped during washes.

f. Incubate at **room temperature** for **1 min**. DO NOT close the mailer.

g. Remove 0.1X SSC Buffer by securing slide in place with one finger and pouring SSC Buffer from mailer.

h. Add **7 ml** 0.1X SSC Buffer slowly along the side of the mailer on one side of the slide.

i. Add **7 ml** 0.1X SSC Buffer slowly along the side of the mailer on the other side of the slide, so that both sides of the slide are immersed in 0.1X SSC Buffer.

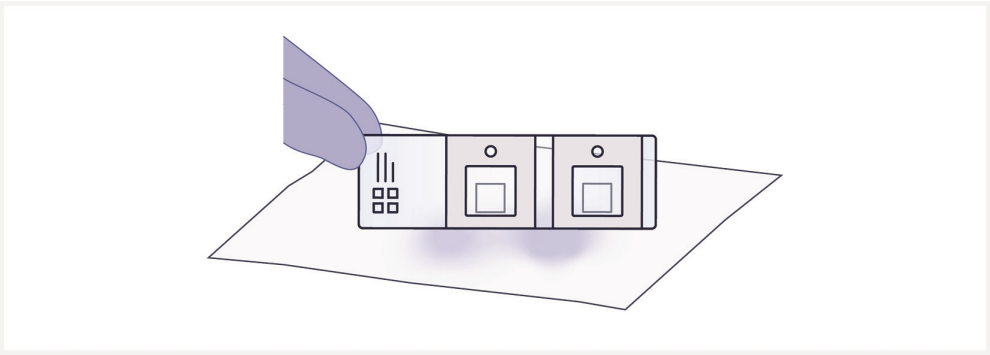
j. Incubate at **room temperature** for **5 min**.

k. Remove 0.1X SSC Buffer by securing slide in place with one finger and pouring SSC Buffer from mailer.

l. Repeat h-k twice more for a total of three **5 min** washes. During the last wash, save SSC Buffer in the mailer in case Visium HD Slide needs additional wash.

m. Ensure back (side without spacers) of Visium HD Slide is dry. If necessary, remove excess moisture from the back of the slide with a lint-free laboratory wipe.

n. Gently touch long edge of Visium HD slide on a lint-free laboratory wipe 3–5x to remove excess SSC buffer



TIPS

o. Ensure Visium HD Slide is free of particulate matter. If necessary, wash

Visium HD slide by immersing in slide mailer with 0.1X SSC buffer from step l.

Record slide serial number before placing Visium HD slide into cassette.

p. Place Visium HD Slide in a new 6.5 mm Visium Cassette. See Visium Cassette S3 Quick Reference Card (Document CG000730) for assembly instructions.

q. Add **100 µl** 0.1X SSC to each well in the cassette.

r. Apply a new pre-cut Visium Slide Seal on the Visium Cassette.

s. Leave 0.1X SSC Buffer in the Visium Cassette at **room temperature** until Visium HD Slide equilibration. DO NOT exceed **2 h** before proceeding with a CytAssist run. Keep Visium Cassette and Visium HD slide free from debris.

Step 4:

Probe Release & Extension

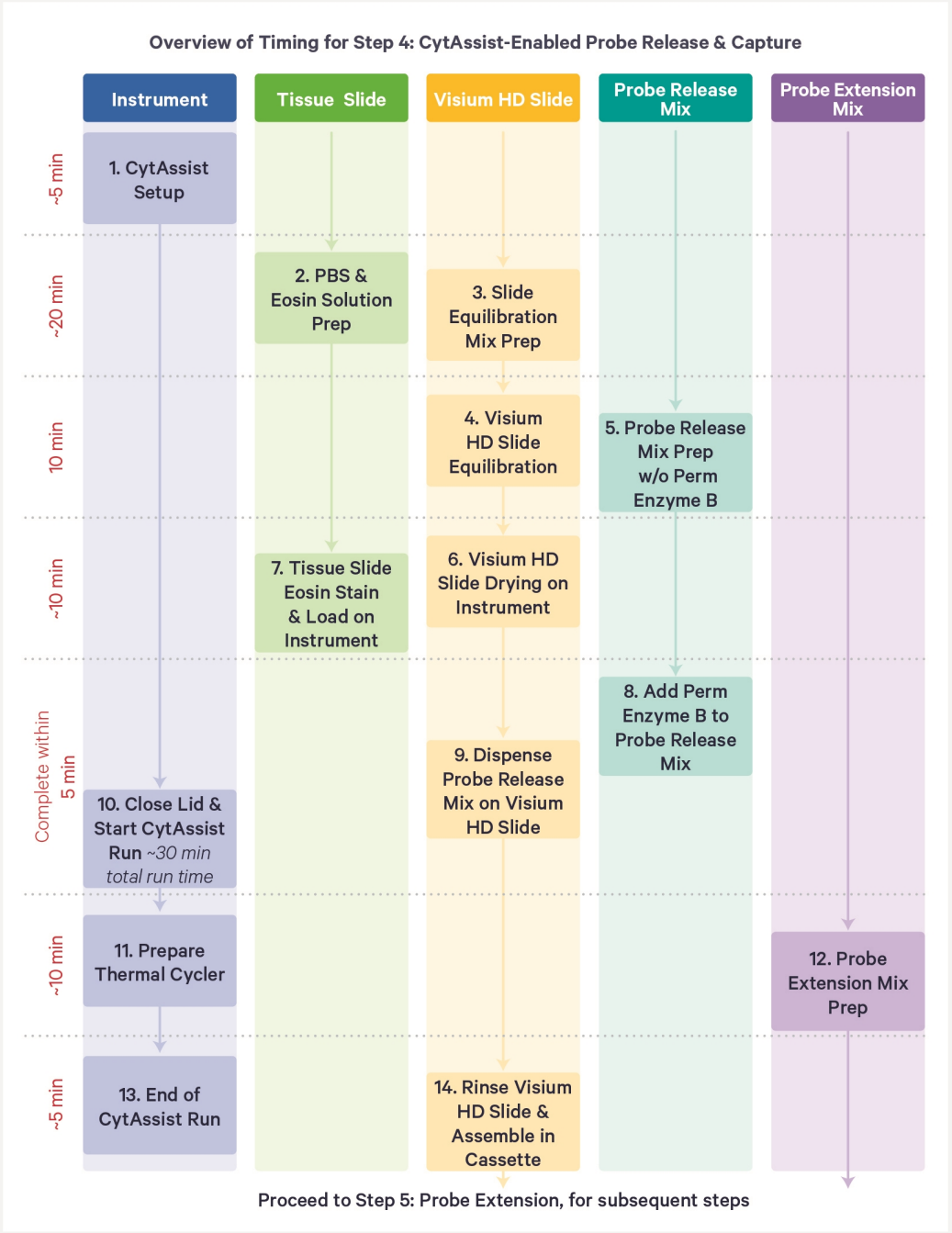
4

4.0 Get Started

Each 10x Genomics reagent tube is enough for two 6.5 mm Visium HD Slides (four tissue slides). Number of reactions in reagent preparation tables refers to the number of Tissue Slides. After instrument run, number of reactions refers to the number of wells in a Visium Cassette.

Ensure that the Visium CytAssist is powered on, running firmware version 2.0.0 or higher, is clean, and ready to perform an experimental run.

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	2X RNase Buffer	Tube: 2000411 Kit: 1000668	Thaw at room temperature. Pipette mix slowly and thoroughly. DO NOT vortex.	–20°C
☐	Extension Buffer	Tube: 2000409 Kit: 1000668	Thaw at room temperature, vortex, centrifuge briefly.	–20°C
☐	Perm Enzyme B	Tube: 3000553 Kit: 1000668	Pipette mix, centrifuge briefly. Maintain at room temperature until ready to use. Perm Enzyme B is added to the Probe Release Mix immediately before running the CytAssist instrument.	–20°C
Place on ice				
☐	RNase Enzyme	Tube: 3000605 Kit: 1000668	Pipette mix, centrifuge briefly. Maintain on ice until ready to use.	–20°C
☐	Extension Enzyme	Tube: 2000389 Kit: 1000668	Pipette mix, centrifuge briefly. Maintain on ice until ready to use.	–20°C
Obtain				
☐	Visium HD Slide	-	Obtain washed Visium HD Slide.	Ambient
☐	Nuclease-free Water	-	-	Ambient
☐	Tris 1 M, pH 8.0 (Tris-HCl)	-	Manufacturer's recommendations.	Ambient
☐	Alcoholic Eosin	-	Manufacturer's recommendations.	Ambient
☐	10X PBS	-	Use 10X PBS stock to prepare 1X PBS.	Ambient
☐	8 M KOH Solution	-	Manufacturer's recommendations.	Ambient
☐	Qiagen Buffer EB	-	Manufacturer's recommendations.	Ambient
☐	Visium Slide Seals	Component: 2000283 Kit: 1000669	See Tips & Best Practices.	Ambient

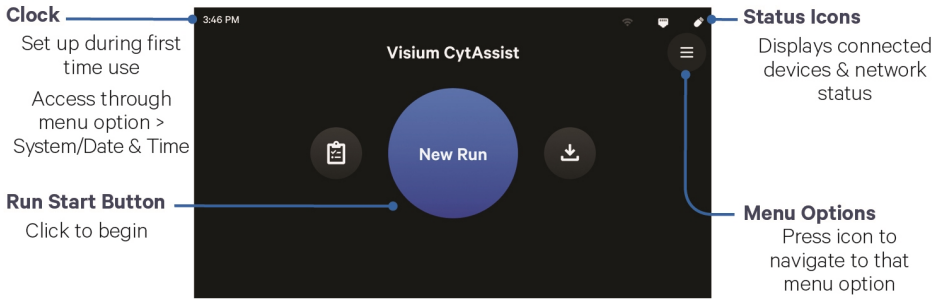

Overview of Timing for Step 4: CytAssist-Enabled Probe Release & Capture
Instrument
Tissue Slide
Visium HD Slide
Probe Release Mix
Probe Extension Mix
~5 min
~20 min
10 min
~10 min
Complete within 5 min
~10 min
~5 min
1. CytAssist Setup
2. PBS & Eosin Solution Prep
3. Slide Equilibration Mix Prep
4. Visium HD Slide Equilibration
5. Probe Release Mix Prep w/o Perm Enzyme B
6. Visium HD Slide Drying on Instrument
7. Tissue Slide Eosin Stain & Load on Instrument
8. Add Perm Enzyme B to Probe Release Mix
9. Dispense Probe Release Mix on Visium HD Slide
10. Close Lid & Start CytAssist Run ~30 min total run time
11. Prepare Thermal Cycler
12. Probe Extension Mix Prep
13. End of CytAssist Run
14. Rinse Visium HD Slide & Assemble in Cassette
Proceed to Step 5: Probe Extension, for subsequent steps

4.1 CytAssist-Enabled Probe Release & Capture

If processing more than two tissue slides, keep remaining tissue slides at **4°C** with 2X SSC buffer. For more guidance, see Appendix on page 113.

a. Ensure that Visium CytAssist is powered on, clean, and ready for an experimental run. Visium CytAssist firmware version 2.0.0 or higher is required for this protocol.

The home screen is the most common state of the instrument. There are several key functions accessible directly from the home screen.



b. Press blue New Run button on the touchscreen to initiate run.

c. Enter the following assay configuration information:

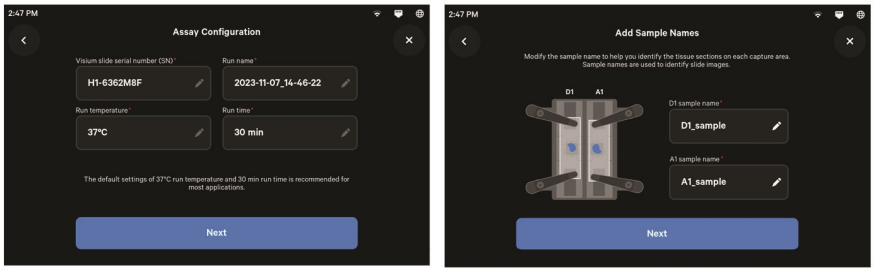
- Visium Slide serial number. **Ensure serial number is accurate.** If serial number is entered in the wrong format, the check mark button to proceed will be grayed out. Record serial number for future use.

  A slide scanner may be used to scan the Visium HD slide for automatic serial number input. See Visium HD Spatial Gene Expression Protocol Planner (CG000698) for compatible part numbers. The slide scanner may be used to scan the bottom of the cassette if the Visium HD slide is already assembled into the cassette.

- Custom run name, temperature, and time (**37°C** for **30 min** is recommended for most applications)

d. Enter the following sample information:

- Sample names and the locations of each sample on the instrument (A1 for right side, D1 for left side)



e. Prepare 1X PBS. Maintain at room temperature.

1X PBS	10x PN	2 Tissue Slides (µl) (includes overage)	4 Tissue Slides (µl) (includes overage)
Nuclease-free Water	-	5,940	11,880
10X PBS	-	660	1,320
Total	**-**	**6,600**	**13,200**

f. Prepare 10% Eosin. Vortex and centrifuge briefly. Eosin should be prepared fresh for each CytAssist instrument run.

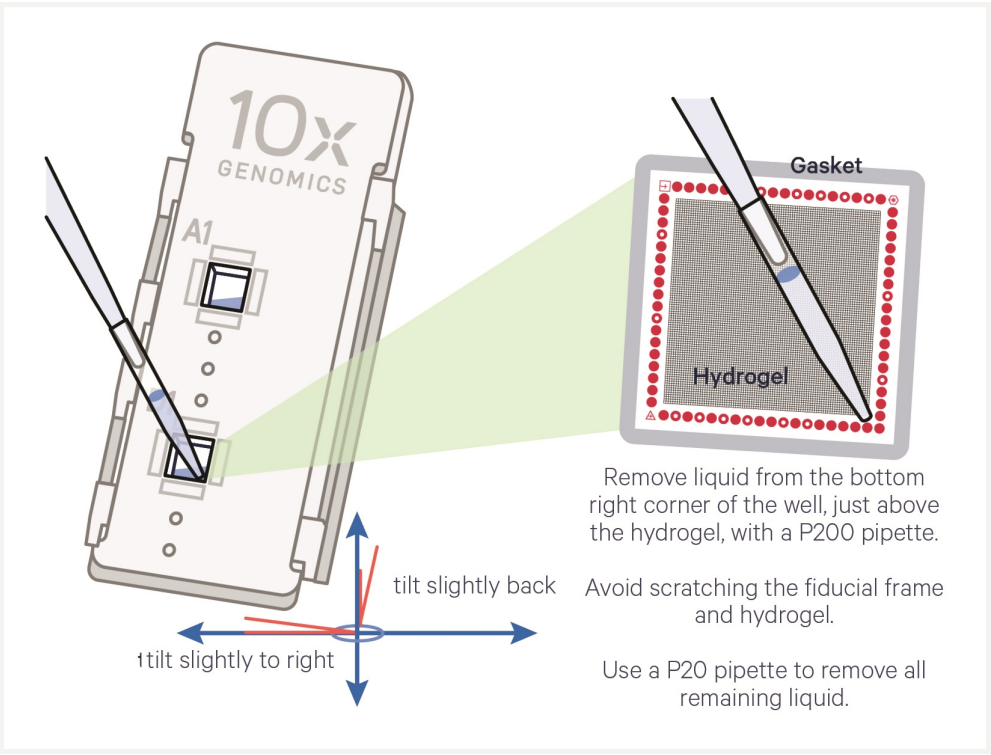
10% Eosin	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
Alcoholic Eosin	-	15	33	66
1X PBS	-	135	297	594
Total	**-**	**150**	**330**	**660**

g. Prepare Slide Equilibration Mix according to the table below. Pipette mix and centrifuge briefly. Maintain on ice.

	Slide Equilibration Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
	Nuclease-free Water		44.0	96.8	193.6
●	2X RNase Buffer	2000411	50.0	110.0	220.0
●	RNase Enzyme	3000605	6.0	13.2	26.4
	Total	**-**	**100.0**	**220.0**	**440.0**

h. Retrieve Visium Cassette with Visium HD Slide.

i. Remove Visium Slide Seal and using a P200 pipette, remove 0.1X SSC from the right corner of each well in the cassette without scratching the fiducial frame or hydrogel. Use a P20 pipette to remove any remaining liquid. See image below for proper liquid removal technique.



j. Add **100 µl** Slide Equilibration Mix to each well in the cassette. Gently tap to ensure uniform coverage of the Capture Area.

k. Apply a new uncut Visium Slide Seal on the Visium Cassette and incubate at **room temperature** for **10 min**.

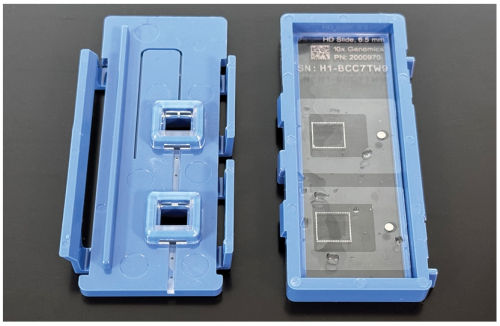
l. Prepare Probe Release Mix during Visium HD Slide equilibration. DO NOT prepare Probe Release Mix for more than two tissue slides at a time. Pipette mix thoroughly until solution is homogenous. Avoid generating bubbles. Maintain on ice.

	Probe Release Mix	10x PN	2 Tissue Slides (µl) (includes overage)
●	2X RNase Buffer	2000411	20.0
●	RNase Enzyme	3000605	17.5
	Total	**-**	**37.5**

m. Remove Visium Slide Seal and using a P200 pipette, remove Slide Equilibration Mix from the right corner of each well in the cassette without scratching the fiducial frame or hydrogel. Use a P20 pipette to remove any remaining liquid. See image in step i for proper liquid removal technique.

Failure to remove Slide Equilibration Mix completely may result in reduced assay performance.

n. Remove top half of Visium Cassette, leaving Visium HD Slide resting in bottom half of Visium Cassette. Rest top half of Visium Cassette such that the gaskets face away from work surface, as shown in the image below. See Visium Cassette S3 Quick Reference Card (CG000730) for more information on cassette disassembly.

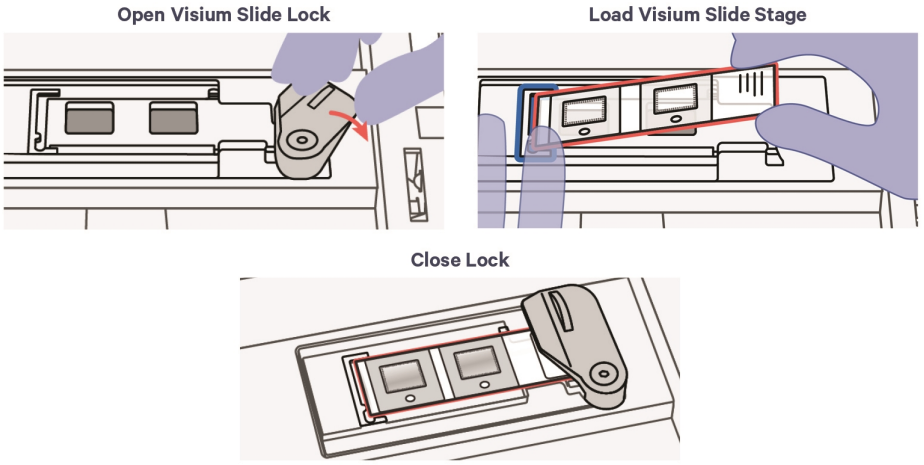


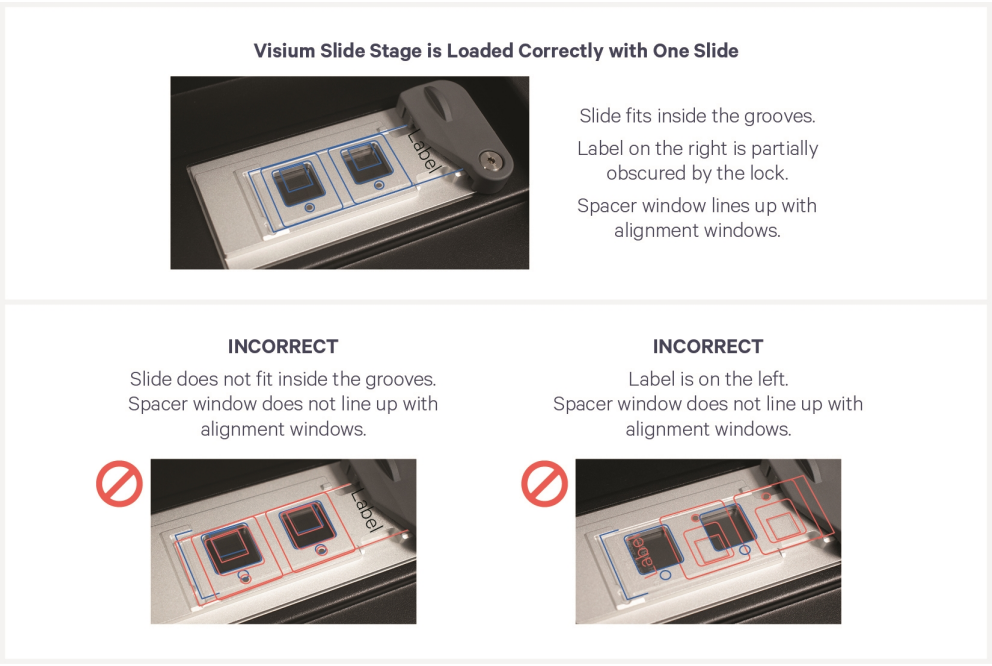
o. Remove Visium HD Slide from Visium Cassette. Avoid touching active surface. If necessary, remove excess moisture from the back of the slide with a lint-free laboratory wipe.

The time between completing **step o** and starting **step q** should not exceed **5 min**.

Save Visium Cassette for use after the instrument run. Ensure back of Visium HD Slide is dry.

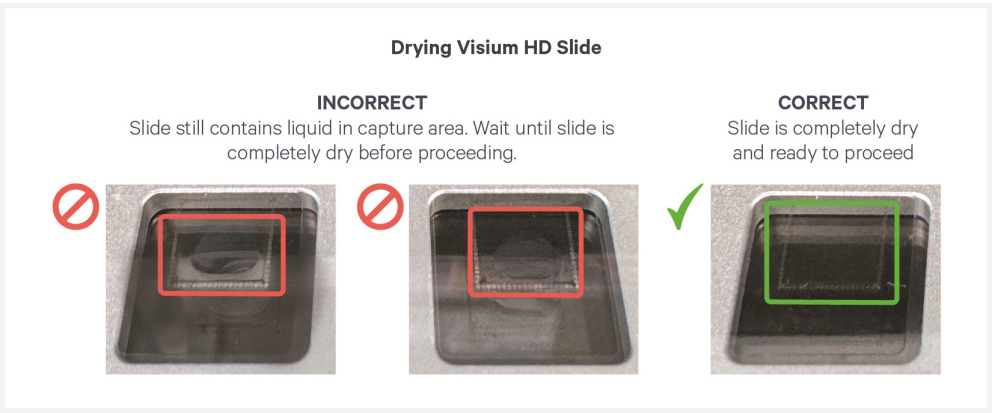
p. Load Visium HD Slide against the grooves of Visium Slide Stage, using one hand to load the slide and the other to close the Visium Slide Lock.





q. Allow Visium HD Slide to dry on the Visium Slide Stage for **10 min.** Inspect entire well. If liquid remains on the slide or anywhere in the well, continue drying and proceed immediately when no liquid remains within the well.

Ensure area around the Visium CytAssist instrument is free from debris. While the Visium HD Slide is drying, prepare Tissue Slides as described in steps r-aa.



r. Retrieve Tissue Slide Cassettes.

s. Using a pipette, remove all 2X SSC Buffer from each well of the Tissue Slide Cassettes.

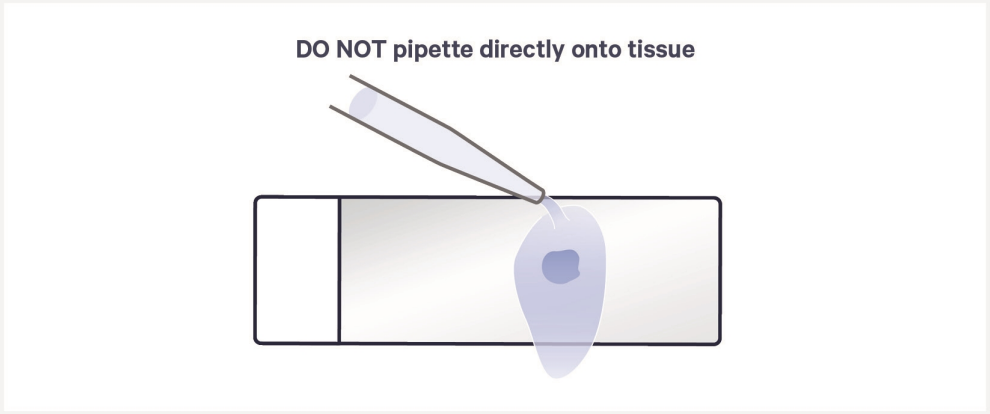
t. Remove tissue slides from Tissue Slide Cassettes.

u. Add **100 µl** 10% Eosin to uniformly cover each tissue section per slide.

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

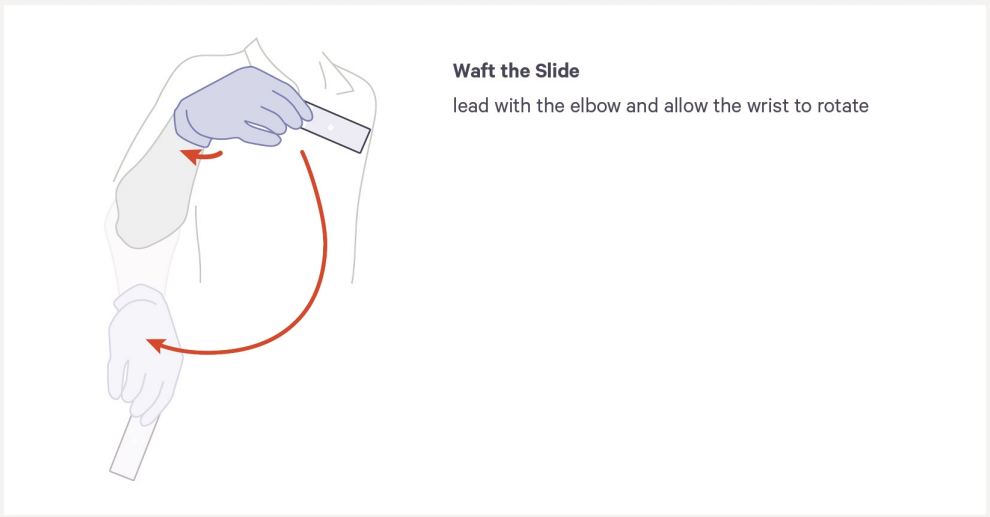
w. Remove 10% Eosin by holding slide at an angle over a liquid waste container.

x. While holding the slide over the liquid waste container, rinse with **1 ml** 1X PBS. DO NOT pipette directly onto tissue.



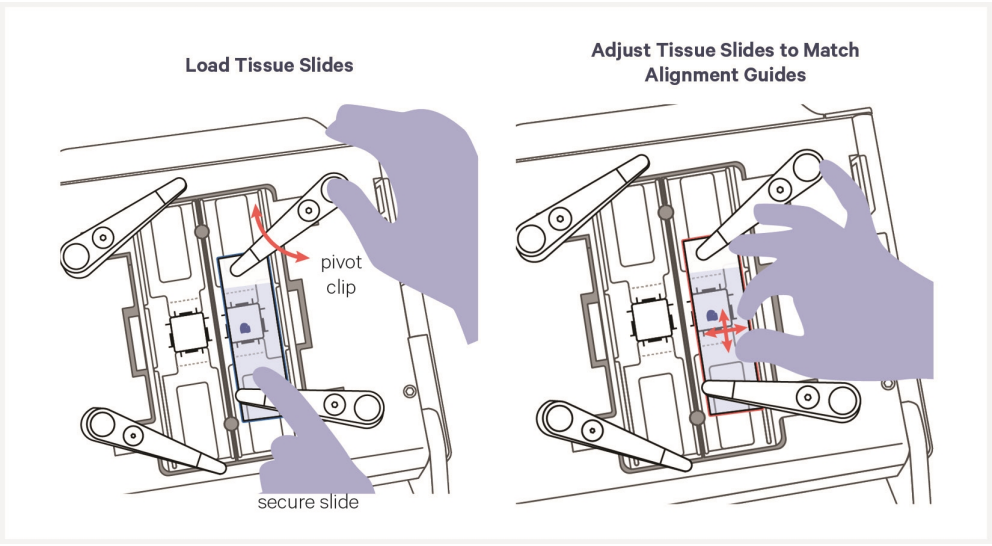
y. Repeat step x two more times for a total of three washes.

z. Gently waft/flick slide back and forth to remove excess PBS. Remove any excess PBS with a lint-free laboratory wipe in areas outside of the tissue, without touching the tissue sections.



aa. Wipe back of tissue slides with a lint-free laboratory wipe and load into Visium CytAssist. Ensure tissue section is completely dry prior to instrument run. If necessary, repeat slide wafting. See Instrument Loading Guidelines on page 34 for more information.

Complete tissue slide alignment before Visium HD slide is done drying.



Before proceeding to next step, ensure that Visium HD slide is completely dry. See step q.

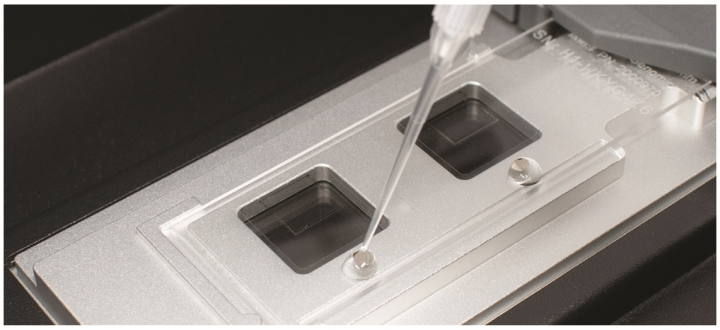
ab. Pipette mix Perm Enzyme B (PN-3000553) and centrifuge briefly. Remove Probe Release Mix from ice. Add **2.5 µl** of Perm Enzyme B to **37.5 µl** of Probe Release Mix (prepared at step 4.1h). Pipette mix 15x with pipette set to 30 µl. Avoid generating bubbles. Centrifuge for **5 sec**.

The time between adding Perm Enzyme B to Probe Release Mix and starting the Visium CytAssist instrument run should be less than **5 min**.

ac. Slowly aspirate **17 µl** of Probe Release Mix and inspect the pipette tip. Ensure that no bubbles were drawn up into the pipette tip.

ad. Slowly dispense **17 µl** of Probe Release Mix into the center of each spacer well on the Visium HD Slide, using a fresh pipette tip for each dispense. Do not push plunger beyond first stop to avoid generating bubbles.

Dispense Reagent into Center of each Spacer Well - Some Reagent Spreading is Normal

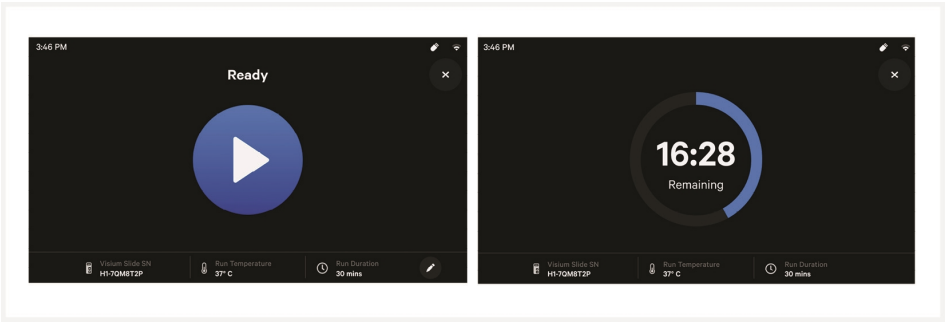


ae. Close lid and press Next.

The home screen will now display a play symbol and run information along the bottom of the screen.

af. Press play button to start the run. **37°C** for **30 min** is recommended for most applications.

- Midrun progress bar will show the time remaining in the run.



- Yellow before a run begins indicates a user-recoverable error. If an error occurs, follow on-screen prompts.

ag. Place Low Profile Thermocycler Adapter in the thermal cycler. Prepare thermal cycler with the following incubation protocol and start the program.

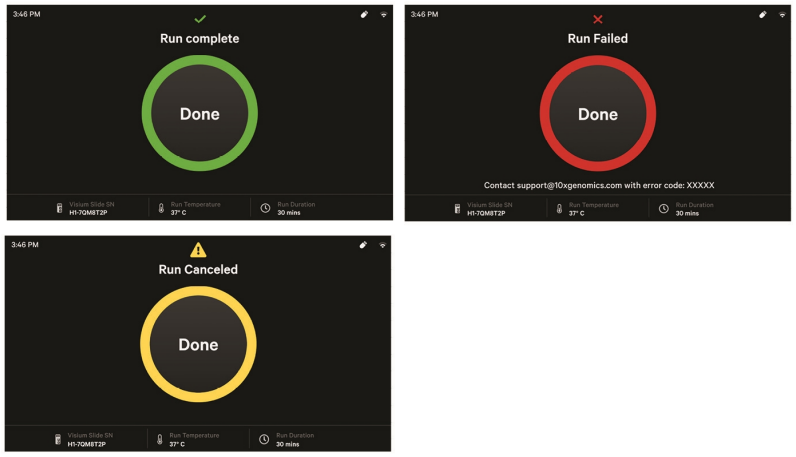
Lid Temperature	Reaction Volume	Run Time
53°C (lid may be turned off if the instrument does not enable 53°C)	100 µl	60 min
Step	**Temperature**	**Time**
Pre-equilibrate	53°C	Hold
Probe Extension 1	53°C	00:30:00
Cool Down	4°C	00:03:00
Hold	4°C	Hold
Probe Extension 2	53°C	00:30:00
Cool Down	4°C	00:03:00
Hold	4°C	Hold

ah. Prepare Probe Extension Mix. Pipette mix. Centrifuge briefly. Maintain on ice.

	Probe Extension Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
	Extension Buffer	2000409	147.0	323.4	646.8
	Extension Enzyme	2000389	3.0	6.6	13.2
	Total	**-**	**150.0**	**330.0**	**660.0**

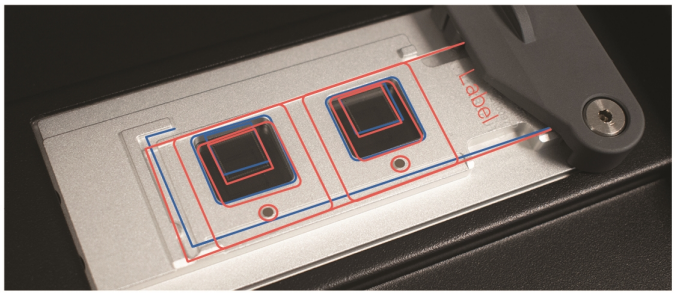
ai. At the end of a run, the button will display "Done" and a "Run Info" tab at the bottom of the screen. DO NOT allow sample to sit in the Visium CytAssist after run completion. **Immediately** move to next step.

- Green indicates a successfully completed run.
- Red indicates a failed run/error
- Yellow at the end of a run indicates an incomplete run.
- For more information on errors, consult the Visium CytAssist Instrument User Guide (CG000542).



aj. Click "Done" button and open lid. DO NOT power off the instrument at this time, as it needs to process support data. It is normal for the Visium slide to move after opening instrument.

Visium Slide Popped out of the Grooves

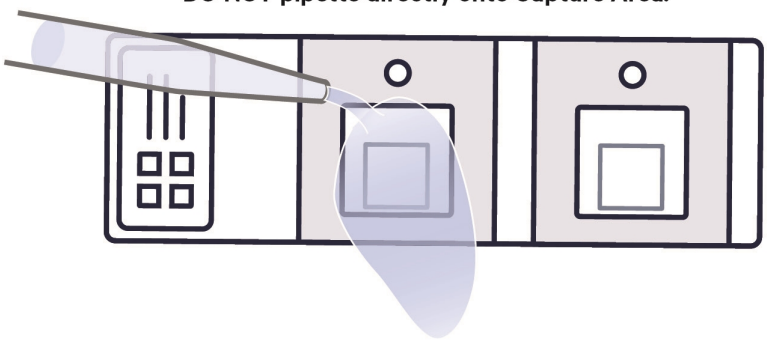


ak. Immediately remove Visium HD Slide. It is normal if tissue remains of tissue slides after run completion.

al. While holding Visium HD Slide over liquid waste container, rinse each Capture Area with **1 ml** Buffer EB. DO NOT pipette directly onto Capture Areas, which are surrounded by the fiducial frames.

Rinse slide near Visium CytAssist instrument to ensure prompt washing of Capture Areas.

DO NOT pipette directly onto Capture Area.



am. Repeat step al two more times for a total of three washes per Capture Area.

an. Place Visium HD Slide in the same Visium Cassette from earlier in this step.

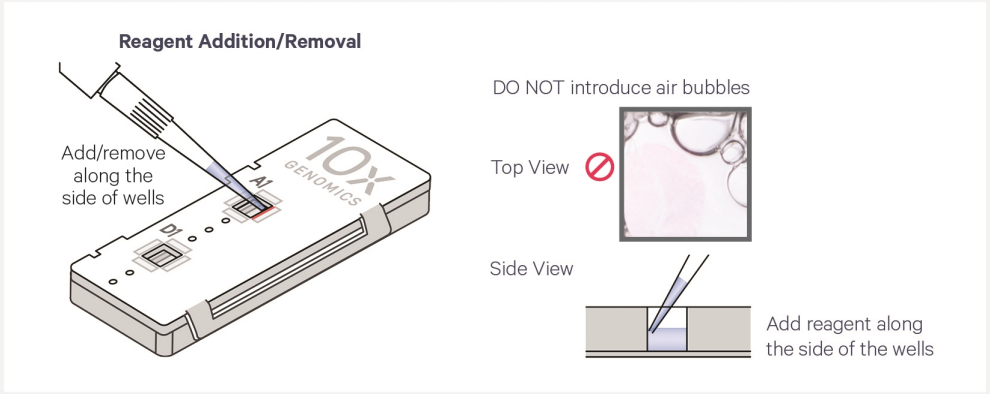
Some moisture remaining on the Visium HD Slide is normal.

ao. Proceed immediately to Probe Extension. CytAssist instrument should only be cleaned and data transferred at a safe stopping point.

4.2 Probe Extension

Probe Extension occurs over two rounds, Probe Extension 1 and Probe Extension 2.

a. Add **75 µl** Probe Extension Mix to each well. Gently tap Visium Cassette to ensure uniform coverage of Capture Area. Keep remaining Probe Extension Mix on ice.



b. Apply new uncut Visium Slide Seal on Visium Cassette and place on the Low Profile Thermocycler Adapter on pre-heated thermal cycler. Close thermal cycler lid.

c. Skip Pre-equilibrate step to initiate Probe Extension 1.

d. After the first Cool Down, open thermal cycler lid and remove cassette from Low Profile Thermocycler Adapter,

e. Peel back Visium Slide Seal and remove Probe Extension Mix from each well.

f. Add **75 µl** Probe Extension Mix to each well.

Gently tap Visium Cassette to ensure uniform coverage of Capture Area.

g. Re-apply Visium Slide Seal and place cassette on Low Profile Thermocycler Adapter. Close thermal cycler lid.

h. Skip Hold step to initiate Probe Extension 2.

STOP

i. Sample may remain at **4°C** in the thermal cycler for up to **24 h**.

j. Clean CytAssist instrument. Consult Visium CytAssist Instrument User Guide (CG000542) for more information.

4.3 Probe Elution

a. Prepare 0.08 M KOH Mix according to the appropriate table shortly before use, adding reagents in the order listed. Vortex and centrifuge briefly. Maintain at **room temperature**.

6.5 mm Slides KOH Mix	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
Nuclease-free Water	-	-	49.5	108.9	217.8
KOH	8 M	0.08 M	0.5	1.1	2.2
Total	**-**	**-**	**50.0**	**110.0**	**220.0**

b. Remove Visium Cassette from Low Profile Thermocycler Adapter and place on a flat, clean work surface after Probe Extension is complete.

c. Remove Visium Slide Seal and using a pipette, remove all Probe Extension Mix from wells.

d. Add **150 µl** Buffer EB to each well.

e. Remove all Buffer EB from wells.

f. Add **50 µl** 0.08 M KOH Mix to each well. Gently tap Visium Cassette to ensure uniform coverage of Capture Area.

g. Incubate at **room temperature** for **10 min**.

h. Add **3 µl** 1 M Tris-HCl pH 8.0 to a tube in an 8-tube strip for each sample.

i. Transfer all solution for each sample containing the probes to a tube in an 8-tube strip containing 1 M Tris-HCl. Vortex, centrifuge briefly, and place on ice. DO NOT leave behind any solution in the wells. *See Tips & Best Practices for reagent removal instructions.*

Step 5:

Pre-Amplification and SPRIselect

5

5.0 Get Started

Number of reactions in reagent preparation tables refers to the number of samples from Probe Elution.

Item		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐ ●	TS Primer Mix B	Tube: 2000537 Kit: 1000688	Thaw at room temperature, vortex, and centrifuge briefly.	−20°C
☐	Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendations.	-
Place on ice				
☐ ○	Amp Mix B	Tube: 2000567 Kit: 1000668	Vortex, centrifuge briefly.	−20°C
Obtain				
☐	Qiagen Buffer EB	-		Ambient
☐	10x Magnetic Separator	Component: 230003 or 2000431 Kit: 1000499	See Tips & Best Practices.	Ambient
☐	80% Ethanol	-	Prepare fresh. Prepare 1 ml per reaction.	Ambient

5.1 Pre-Amplification

a. Prepare Pre-Amplification Mix on ice according to the appropriate table. Add reagents in the order listed. Pipette mix and centrifuge briefly. Maintain on ice.

	6.5 mm Slides Pre-Amplification Mix	PN	1X (µl)	2X + 10% (µl)	4X + 10% (µl)
	Nuclease-free Water		19.5	42.9	85.8
○	Amp Mix B	2000567	25.0	55.0	110.0
●	TS Primer Mix B	2000537	2.5	5.5	11.0
	Total	**-**	**47.0**	**103.4**	**206.8**

b. Add **47 µl** Pre-Amplification Mix to each tube from 4.3 Probe Elution on page 76. Pipette mix and centrifuge briefly.

c. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:03:00
2	98°C	00:00:15
3	63°C	00:00:20
4	72°C	00:00:30
5	Go to Step 2, repeat 9X for a total of 10 cycles	
6	72°C	00:01:00
7	4°C	Hold

5.2 Pre-Amplification Cleanup - SPRIselect

If using MicroAmp 8-Tube Strips, use the high position (magnet•**High**) only throughout the protocol.

a. Vortex to resuspend SPRIselect reagent. Add **120 µl** SPRIselect reagent (1.2X) to each pre-amplification reaction in an 8-tube strip (100 µl) and pipette mix 15x (pipette set to 175 µl).

b. Incubate **5 min** at **room temperature**.

c. Place on the magnet•**High** until solution clears.

d. Remove supernatant.

e. Add **300 µl** 80% ethanol to pellet. Wait **30 sec**. Pipette carefully as **300 µl** is at tube limit.

f. Remove ethanol.

g. Add **200 µl** 80% ethanol to pellet. Wait **30 sec**.

h. Remove ethanol.

i. Centrifuge briefly and place on magnet•**Low**.

j. Remove any remaining ethanol without disturbing the beads. Air dry for **2 min**. DO NOT exceed **2 min** as this will decrease elution efficiency.

k. Remove from magnet. Add **105 µl** Buffer EB. Pipette mix 15x (pipette set to 100 µl).

l. Incubate **2 min** at **room temperature**.

m. Place tube strip on magnet •**High** until solution clears.

n. Transfer **100 µl** sample to a new tube strip.

o. Store at **4°C** for up to **72 h**, **-20°C** for up to **4 weeks**, or proceed to next step.

Step 6:

Visium HD Spatial Gene Expression – Probe-based Library Construction

6

6.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐ ●	TS Primer Mix B	Tube: 2000537 Kit: 1000688	Thaw at room temperature, vortex, and centrifuge briefly.	−20°C
☐	Dual Index Plate TS Set A	Tube: 3000511 Kit: 1000251	Thaw at room temperature, vortex, and centrifuge briefly.	−20°C
☐	Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendations.	-
☐	Agilent TapeStation Screen Tape and Reagents If used for QC		Manufacturer's recommendations.	-
☐	Agilent Bioanalyzer High Sensitivity DNA kit If used for QC	-	Manufacturer's recommendations.	-
Place on ice				
☐ ○	Amp Mix B	Component: 2000567 Kit:	Vortex, centrifuge briefly. Maintain on ice.	−20°C
☐	KAPA SYBR Fast qPCR Master Mix	-	Manufacturer's recommendations.	-
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	Qiagen Buffer EB	-		Ambient
☐	10x Magnetic Separator	Component: 230003 or 2000431 Kit: 1000499	See Tips & Best Practices.	Ambient
☐	80% Ethanol	-	Prepare fresh. Prepare 400 µl per reaction.	Ambient

6.1 Cycle Number Determination – qPCR

a. Dilute TS Primer Mix B 1:10 in nuclease-free water. The amount of diluted primer mix required to generate the qPCR mixes is described in the table below.

b. Prepare qPCR Mix on ice according to the table below. Add reagents in the order listed. Vortex and centrifuge briefly. Maintain on ice.

A passive reference dye, such as ROX, may be required. Consult the Roche KAPA SYBR FAST qPCR Kit website or the appropriate qPCR system manufacturer for guidance.

Gene Expression qPCR Mix	Stock	Final	1X (µl)	3X* + 10% (µl)	5X* + 10% (µl)
KAPA SYBR FAST qPCR Master Mix Minimize light exposure	2X	1X	5.0	16.5	27.5
Diluted TS Primer Mix B	-	-	1.0	3.3	5.5
Nuclease-free Water	-	-	3.0	9.9	16.5
Total			**9.0**	**29.7**	**49.5**

*Includes one negative control

c. Add **9 µl** qPCR Mix to one well per sample in a qPCR plate (a well for negative control may be included).

d. Dilute **2 µl** sample from Pre-Amplification Cleanup - SPRIselect in **8 µl** nuclease-free water. Pipette mix, centrifuge briefly.

e. Transfer **1 µl** diluted sample from Pre-Amplification Cleanup - SPRIselect to each qPCR plate well containing qPCR Mix. For the negative control, add **1 µl** nuclease-free water to the corresponding well. Pipette mix.

f. Apply seal and centrifuge briefly. Record which sample is in which well of the qPCR plate.

g. Prepare a qPCR system with the following protocol, place the plate in the thermal cycler, and start the program.

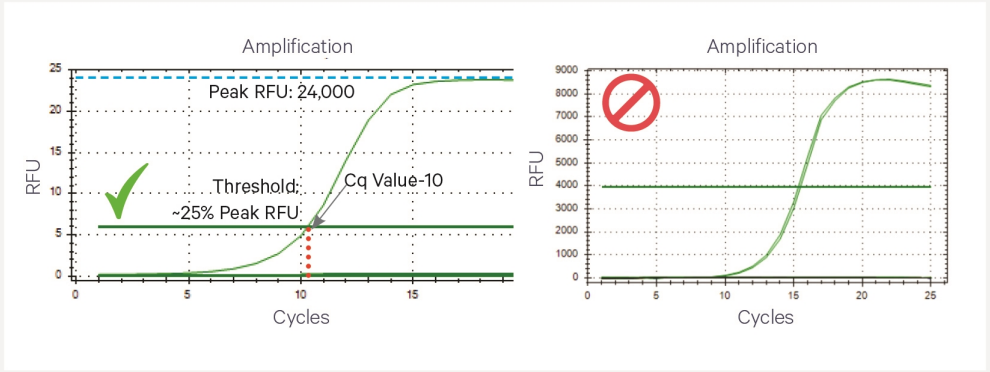
Lid Temperature	Reaction Volume	Run Time
105°C	10 µl	35 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:03:00
2	98°C	00:00:05
3	63°C Read signal	00:00:30
4	Go to step 2, 24x (total of 25 cycles)	-

h. Record the Cq value for each sample.

Set the y-axis to a linear scale. Plot RFU on the y-axis if not using a reference dye, or ΔRn if using a reference dye. The threshold for determining the Cq value should be set along the exponential phase of the amplification plot, at ~25% of the peak fluorescence value.

If the Cq value is > 20 cycles, refer to Troubleshooting on page 101 or contact support@10xgenomics.com before proceeding.

Representative qPCR Amplification Plots



6.2 Sample Index PCR

a. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x Sample Index name (PN-3000511 Plate TS Set A well ID) used.

b. Prepare Amplification Master Mix and pipette mix 10x shortly before use.

	Amplification Master Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
	Nuclease-free Water	-	45	99	198
◯	Amp Mix B	2000567	25	55	110
	Total	**-**	**70**	**154**	**308**

c. Add **70 µl** Amplification Master Mix to a tube in an 8-tube strip for each sample.

d. Add **25 µl** of each sample from pre-amplification to a separate tube previously aliquoted with Amplification Master Mix.

e. Add **5 µl** of an individual Dual Index TS Set A to each tube and record the well ID used. Pipette mix and centrifuge briefly.

f. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	Variable
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:03:00
2	98°C	00:00:15
3	63°C	00:00:20
4	72°C	00:00:30
5	Go to step 2, use the Cq Value +1 as the total # of cycles. See table below for total # of cycle examples.	
6	72°C	00:01:00
7	4°C	Hold

Round Cq values up to the nearest whole number and add one cycle (examples below). Samples within ±1 cycle numbers can be combined in a single SI-PCR run at the higher cycle number.

Do not combine samples if the cycle number difference is greater than 1 to avoid library overamplification.

Example Cycle Numbers

Cq Value from qPCR	+1	Total Cycles
7.2	+1	9
8.5	+1	10
13.7	+1	15

Example Batched Cycles

Cq Value from qPCR	+1	Total Cycles	Batched Cycles
7.2	+1	9	10
9.5	+1	11	
13.7	+1	15	16
14.6	+1	16	

Any remaining pre-amplification material can be stored at **4°C** for up to **72 h** or at **-20°C** for up to **4 weeks** for generating additional libraries.

6.3 Post-Sample Index PCR Cleanup – SPRIselect

If using MicroAmp 8-Tube Strips, use the high position (magnet•**High**) only throughout the protocol

a. Vortex to resuspend SPRIselect Reagent. Add **85 µl** SPRIselect Reagent (**0.85X**) to each sample. Pipette mix 15x (pipette set to 175 µl).

b. Incubate **5 min** at **room temperature**.

c. Place on magnet•**High** until solution clears.

d. Remove supernatant.

e. Add **200 µl** 80% ethanol to pellet. Wait **30 sec**.

f. Remove ethanol.

g. **Repeat** steps e and f for a total of two washes.

h. Centrifuge briefly. Place on the magnet•**Low**.

i. Remove any remaining ethanol. Air dry for **2 min**. DO NOT exceed **2 min** as this will decrease elution efficiency.

j. Remove from magnet. Add **27 µl** Buffer EB. Pipette mix 15x.

k. Incubate **2 min** at **room temperature**.

l. Place on the magnet•**Low** until solution clears.

m. Transfer **25 µl** sample to a new tube strip.

STOP

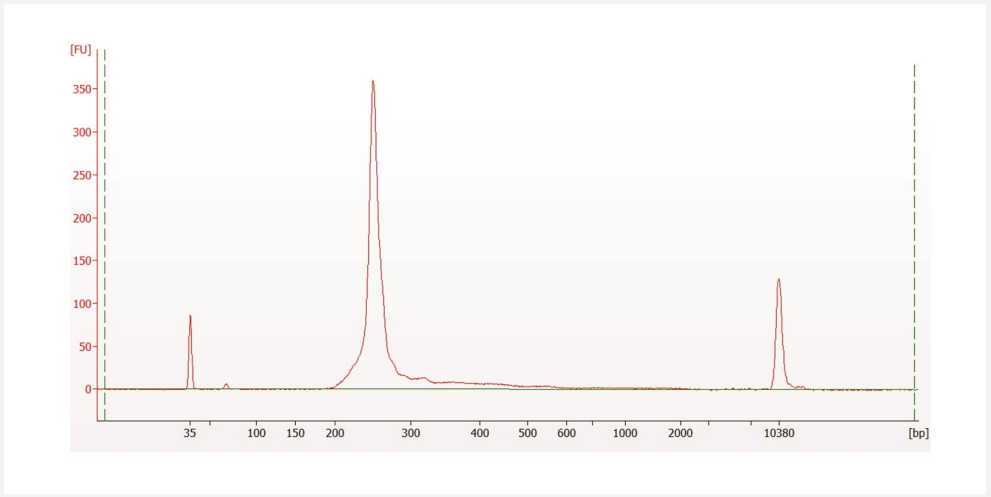
n. Store at **-20°C** for **long-term** storage.

6.4 Post-Library Construction QC

a. Dilute sample (1:50 dilution, i.e **1 µl** sample in **49 µl** of EB buffer) until it is at an appropriate concentration for the Bioanalyzer.

b. Run **1 µl** of sample on an Agilent Bioanalyzer High Sensitivity chip. If peak is too small or flat, retry with a lower dilution. See Troubleshooting on page 101 for more information.

Representative Trace



Determine the average fragment size from the Bioanalyzer trace. The expected average fragment size is 250 bp. This will be used as the insert size for library quantification.

Alternate QC Methods:

- Agilent TapeStation
- LabChip

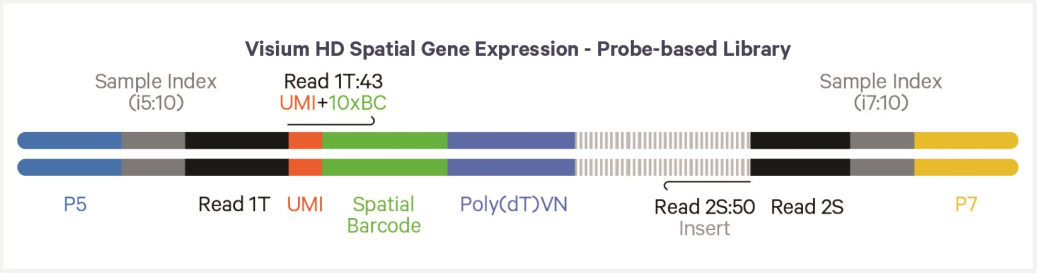
See Appendix on page 113 for representative trace

See Post Library Construction Quantification on page 119

Sequencing

Sequencing Libraries

Libraries contain standard Illumina paired-end constructs, which begin with P5 and end with P7. Spatial Barcodes are encoded at the start of TruSeq Read 1 (Read 1T), while i7 and i5 sample index sequences are incorporated as the index read. Read 1T, and Small RNA Read 2 (Read 2S) are standard Illumina sequencing primer sites used in paired-end sequencing. Read 1T are used to sequence the 43 bp Spatial Barcode and UMI. Read 2S is used to sequence the Ligated Probe Insert. Sequencing these libraries produces a standard Illumina BCL data output folder.



Sequencing Depth

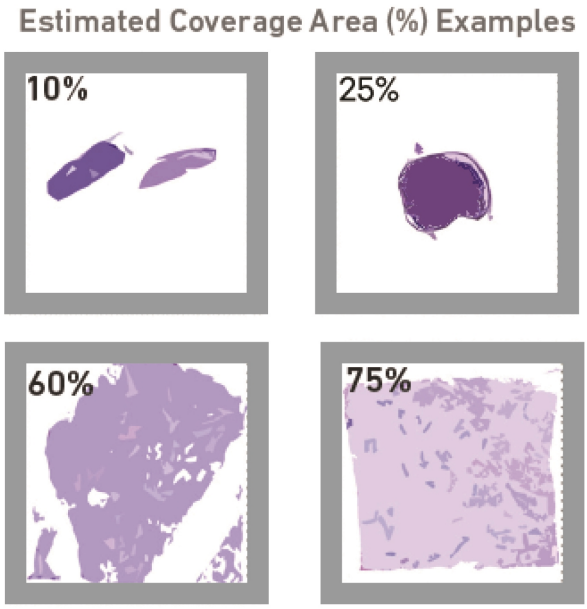
The minimum sequencing depth for Visium HD is 275 million read pairs per fully-covered Capture Area. The recommended minimum sequencing depth was chosen because it achieved >50% sequencing saturation for >50% formalin fixed, paraffin embedded (FFPE) tissues tested at 10x Genomics. To achieve >50% saturation for >50% of fresh frozen (FF) tissues tested by 10x Genomics, 700 million read pairs per fully-covered Capture Area were required. To achieve >50% saturation for >50% of the Fixed Frozen (FxF) tissues tested by 10x Genomics, 500 million read pairs per fully-covered Capture Area were required.

10x Genomics recommends calculating the minimum sequencing depth for libraries by multiplying the percentage of the Capture Area covered by tissue by 275 million read pairs.

For example, a Capture Area that is 60% covered tissue would require at least 165 million read pairs (0.60 X 275,000,000).

Example: Sequencing Depth for a Sample

- **Estimate the approximate Capture Area (%)** covered by the tissue section.
- **Calculate total sequencing depth=** (Coverage Area x 275,000,000 read pairs
- **Example calculation for 60% coverage:** 0.60 x 275,000,000 read pairs = 165,000,000 total read pairs for that sample



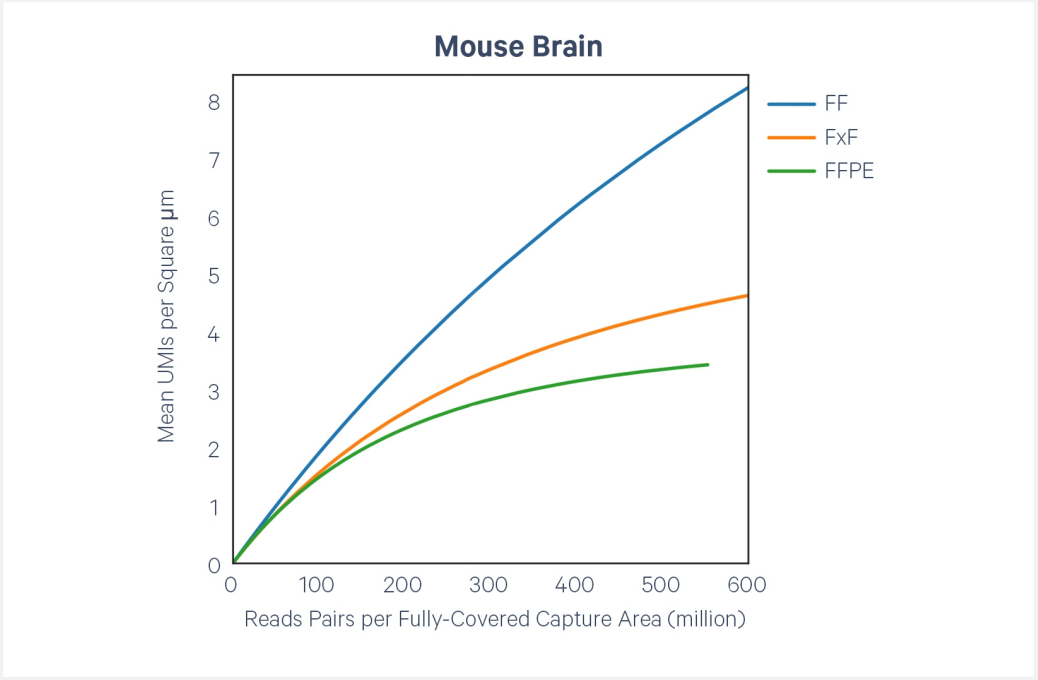
Sequencing saturation, at a particular sequencing depth, is driven by the complexity of the library and the percent usable read pairs. To achieve saturation with high complexity libraries (unique UMIs), more sequencing is required to read all unique UMIs present in the library. Saturation is achieved more efficiently (with less read pairs) for libraries with a high percentage of usable read pairs. The main driver of saturation for Visium HD libraries is library complexity.

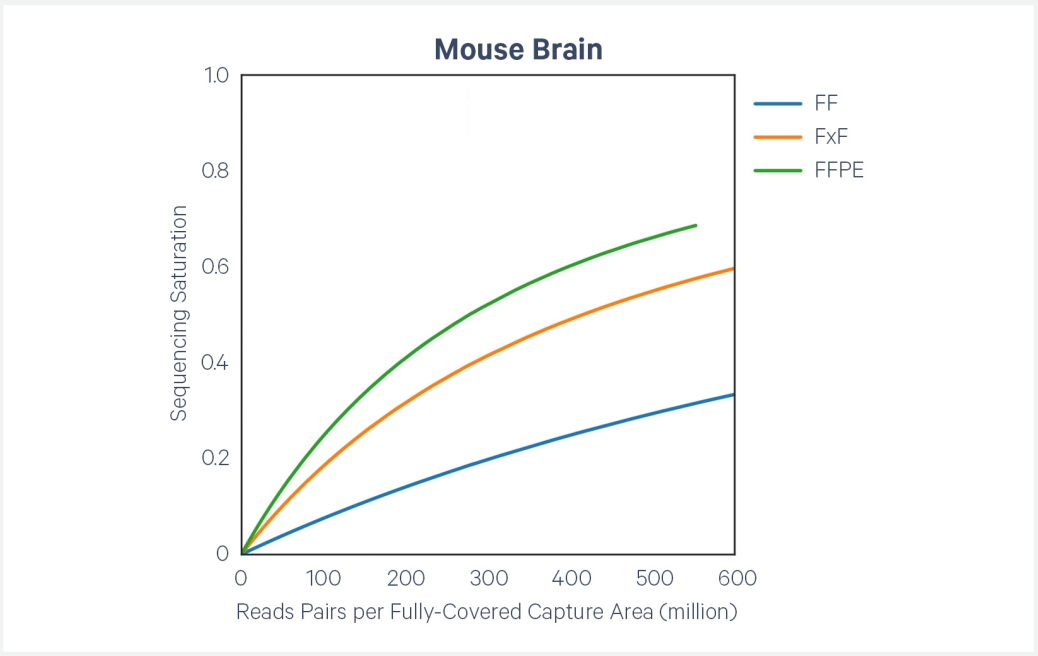
Visium HD library complexity, or the UMIs recovered from a tissue sample, is dependent on fixation method, tissue composition, tissue type, and RNA quality. Visium HD FF libraries will typically be more complex compared to equivalent FFPE tissue libraries. At a particular sequencing depth, a FF tissue run with Visium HD will typically result in a higher number of UMIs per square μm and a lower sequencing saturation compared to an equivalent FFPE sample of the same tissue type.

The figures below show the relationship between sequencing depth, sequencing saturation, and mean UMIs per square μm across different tissue preservation methods. The first graph shows the relationship between mean UMIs per square μm and sequencing depth. At the same sequencing depth, FF with good RNA quality will typically have higher UMIs per square μm compared to FFPE. Around the recommended sequencing depth, the slope of the line representing the FFPE samples becomes smaller, indicating faster saturation and smaller increases in UMIs per square μm with additional sequencing.

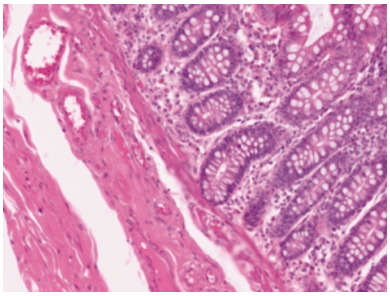
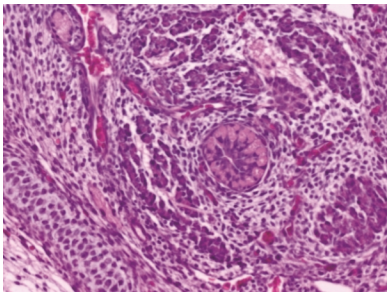
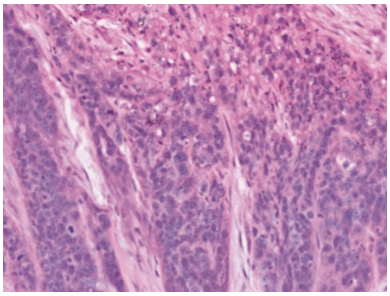
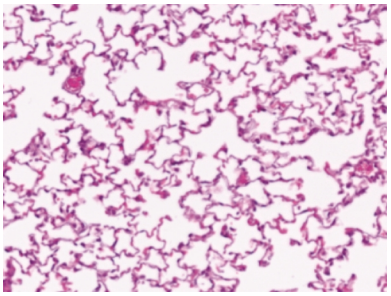
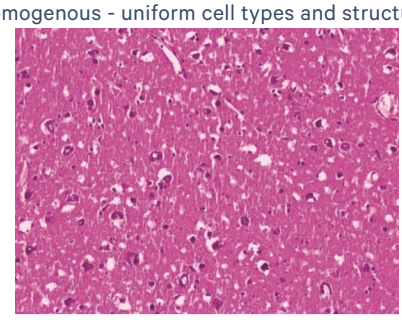
The second graph shows the relationship between sequencing depth and sequencing saturation for the same samples shown in the first graph. At the

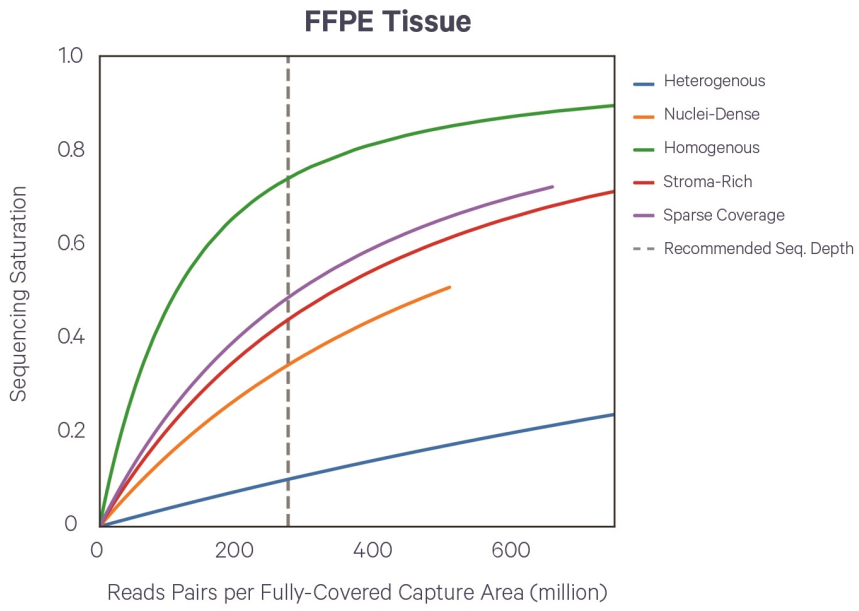
same sequencing depth, FF with good RNA quality will typically have lower saturation compared to FFPE due to higher complexity. In these cases, additional sequencing is often desirable.



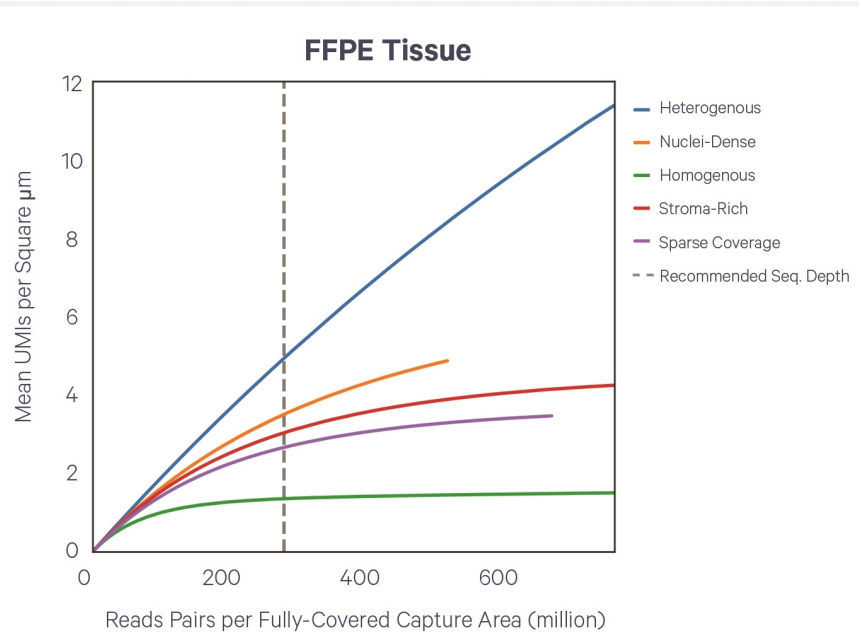


Tissue composition can also influence the ideal sequencing depth for a sample. The figures below demonstrate the relationship between sequencing depth and mean UMIs per square μm for a variety of sample types to help guide selection of sequencing depth. All H&E images below were taken at 20X magnification.

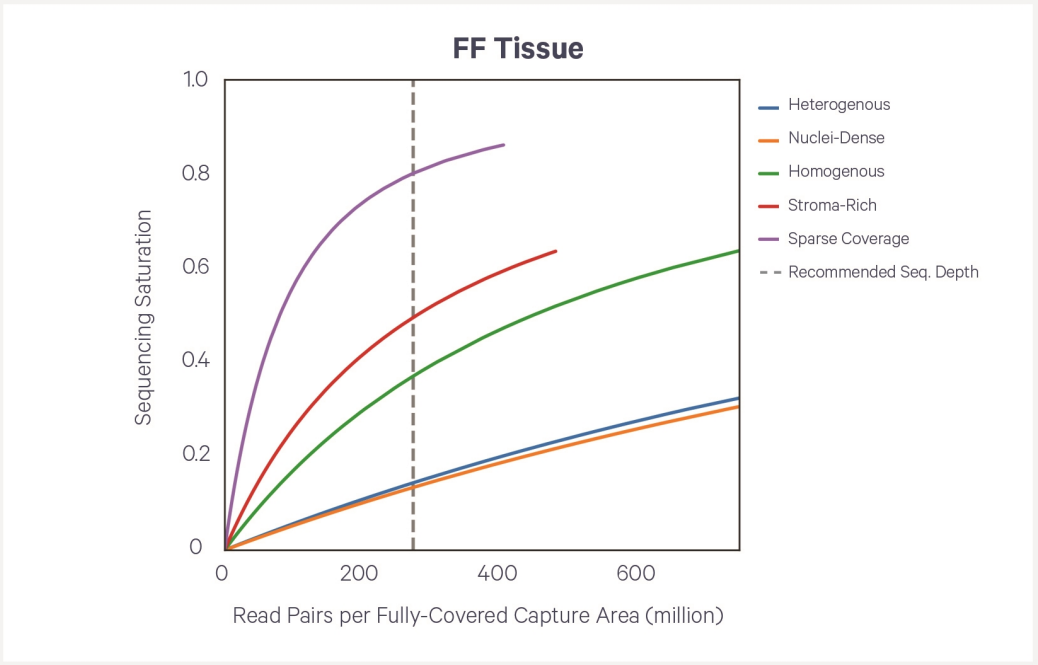
Tissue Compositions Tested	
Stroma-Rich - extracellular matrix, connective tissue, or adipose tissue 	Heterogenous - variety of cell types and structures 
Nuclei-Dense - cells are very compact 	Sparse Coverage - cells are separated by large spaces 
Homogenous - uniform cell types and structures 	



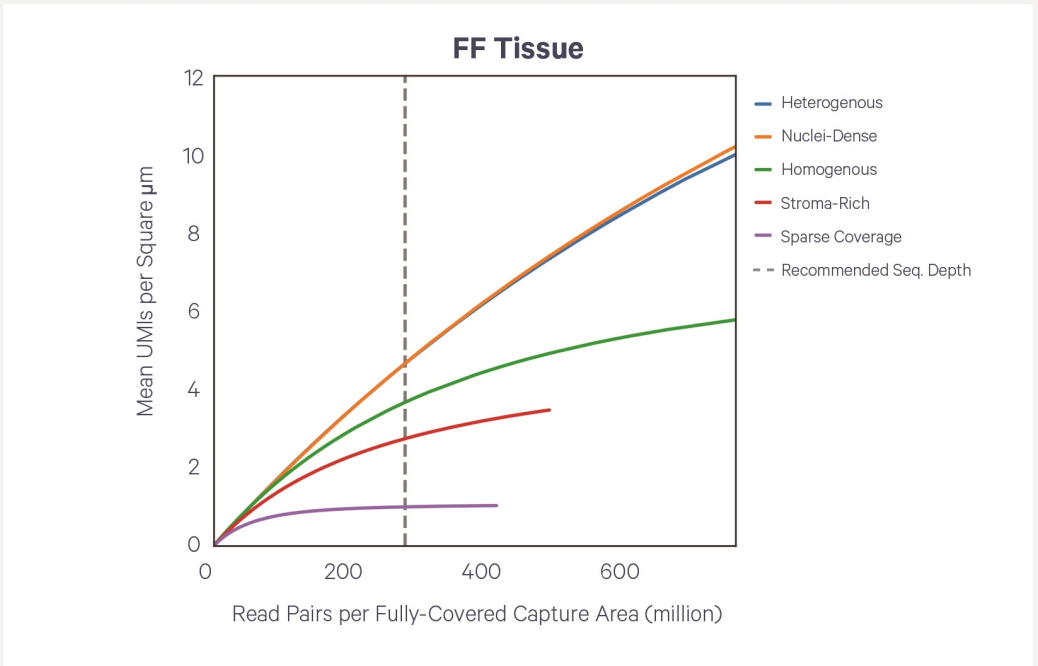
The above graph shows the relationship between sequencing saturation per sample and sequencing depth per sample.



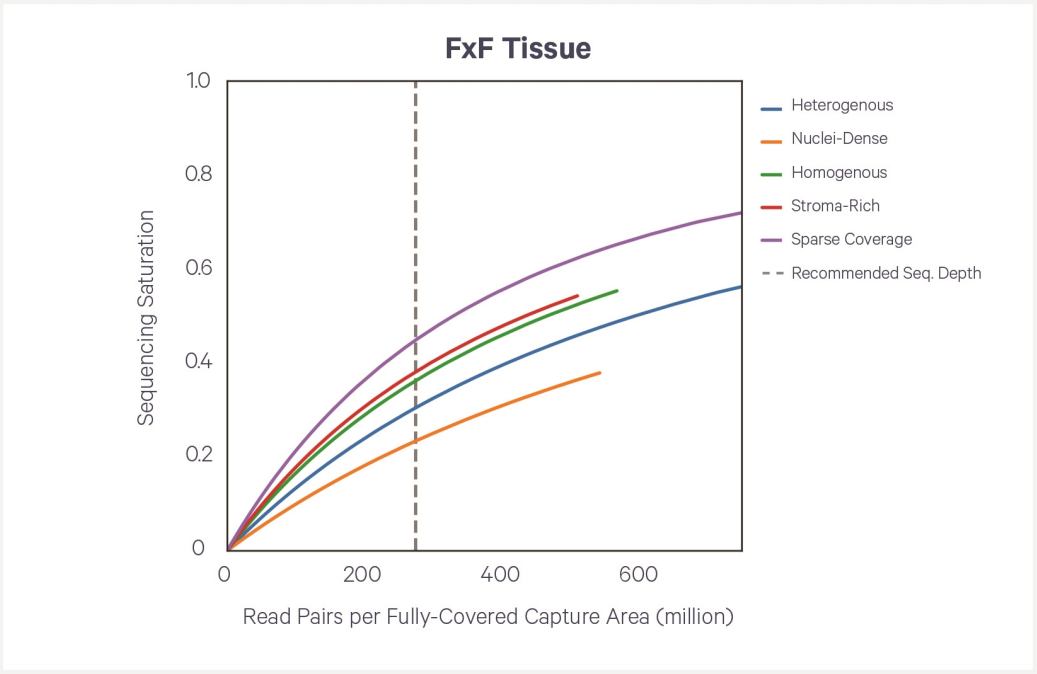
The above graph shows the relationship between mean UMIs per square µm of the Capture Area covered by tissue and sequencing depth per sample.



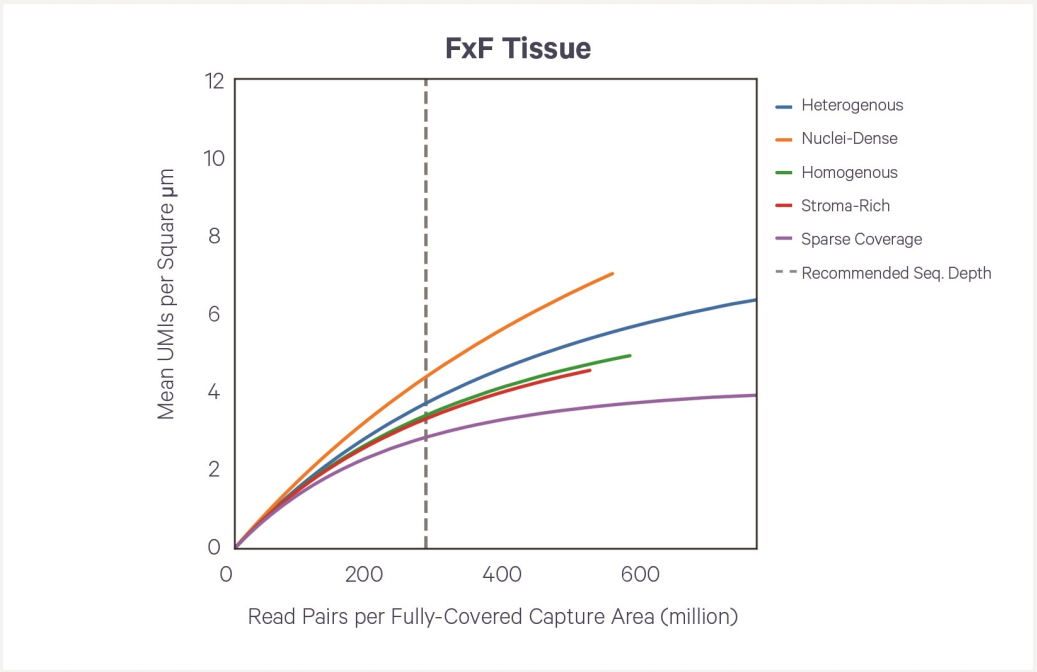
The above graph shows the relationship between sequencing saturation per sample and sequencing depth per sample.



The above graph shows the relationship between mean UMIs per square µm of the Capture Area covered by tissue and sequencing depth per sample.



The above graph shows the relationship between sequencing saturation per sample and sequencing depth per sample.



The above graph shows the relationship between mean UMIs per square µm of the Capture Area covered by tissue and sequencing depth per sample.

Sequencing Type & Run Parameters

Use the sequencing run type and parameters indicated.

Visium HD Spatial Gene Expression - Probe-based Library
Paired-end, dual indexed sequencing
Read 1: 43 cycles i7 Index: 10 cycles i5 Index: 10 cycles Read 2S: 50 cycles

Illumina Sequencer Compatibility

The compatibility of the listed sequencers has been verified by 10x Genomics. Some variation in assay performance is expected based on sequencer choice.

- NextSeq 2000
- NovaSeq 6000
- NovaSeq X Plus

Sample Indices

Each well of the Dual Index Kit TS Set A (PN-1000251) contains a mix of one unique i7 and one unique i5 sample index. If multiple samples are pooled in a sequence lane, the sample index name (i.e. the Dual Index TS Set A plate well ID, SI-TS-) is needed in the sample sheet used for generating FASTQs with "spaceranger mkfastq". Samples utilizing the same sample index should not be pooled together or run on the same flow cell lane, as this would not enable correct sample demultiplexing.

Color balance in the index read is important for optimal quality and demultiplexing results. When pooling samples on the NovaSeq X Series instruments, Illumina recommends avoiding index combinations which only have signals from A bases, G bases, or G+A bases in any given cycle. Refer to Illumina's guidance on index color balancing.

Library Pooling

Visium HD libraries derived from samples prepared using different methods (such as FFPE, FF, FxF) may be pooled for sequencing, taking into account differences in tissue covered spot on a Capture Area and per-spot read depth requirements. Pooling Visium HD libraries with other 10x libraries for sequencing is not recommended.

Library Loading

Library quantification should be done with the KAPA DNA Quantification Kit using the average insert size determined by Agilent Bioanalyzer, Perkin Elmer LabChip, or Agilent Tapestation QC. Alternate methods to KAPA qPCR for final library concentration may result in underquantification and consequently overloading.

Once quantified and normalized, libraries should be denatured and diluted as recommended for Illumina sequencing platforms. Consult Illumina documentation for denaturing and diluting libraries.

Instrument	Loading Concentration (pM)	PhiX (%)
NextSeq 2000	650	1
NovaSeq 6000 Standard	100-150	1
NovaSeq 6000 XP Workflow	150-200	1
NovaSeq X Plus	400	1

Sequencing Metrics

FFPE samples were processed according to documentation listed in the Workflow Overview on page 13. Libraries were pooled and sequenced at the indicated run parameters to generate the % Base and % ≥Q30 plots shown in the following figures. These same libraries were used to illustrate sequence compatibility. All libraries were quantified with the KAPA DNA Quantification Kit and sequenced with 1% PhiX. Though only FFPE libraries are shown in this section, libraries from fresh frozen (FF) and fixed frozen (FxF) tissue sections are expected to perform similarly. All libraries followed the following sequencing configuration and run parameters:

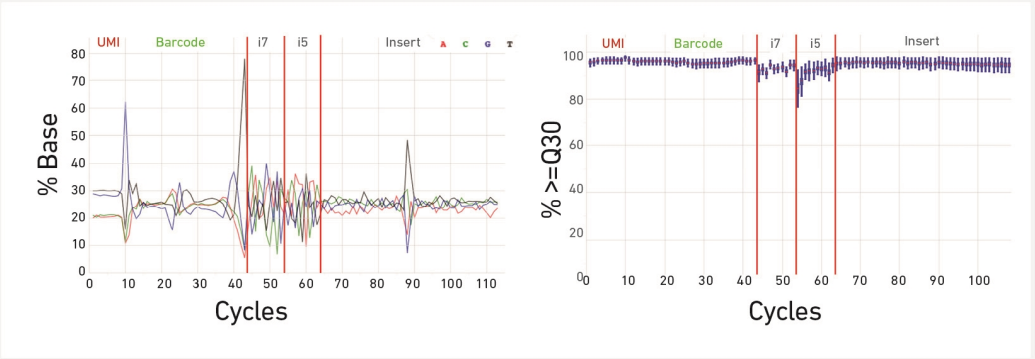
- Targeted sequencing depth: Minimum of 275 million read pairs multiplied by fraction Visium HD slide tissue coverage.
- Paired-end, dual indexing: Read 1: 43 cycles; i7 Index: 10 cycles; i5 Index: 10 cycles; Read 2: 50 cycles

% Base by cycle and % ≥Q30 Quality Score distribution showed highly consistent profiles for all sequencing platforms tested. The data serve as guidelines for assessing the quality of library sequencing. Additional factors that may contribute to overall success of a sequencing run and impact downstream application performance metrics include:

- Starting with a high-quality tissue block.
- Generating sequencing data from sections that remain adhered to the slide.
- Final libraries with fragment lengths within the expected size range for each library type.
- Reliable and accurate library quantification using the KAPA qPCR DNA Quantification Kit for Illumina Platforms (Roche, KK4824) and the average insert size determined by Agilent Bioanalyzer, Perkin Elmer LabChip, or Agilent TapeStation QC. Alternative methods to KAPA qPCR, such as Qubit, for final library quantification may result in underquantification and result in overloading flow cells.
- Sequencing platform loading concentrations follow recommendations described in Library Loading on the previous page, which are based on KAPA qPCR quantification. Overloading/over-clustering may result in poor run performance, decrease sequencing quality, and lower total data output as compared to optimally loaded runs. The loading recommendations for an individual sequencer are listed as general guidance and additional optimization may be required.

Probe-based Libraries

Representative % Base and % ≥Q30 plots are from a pool of sixteen Probe-based libraries sequenced on a NovaSeq 6000 SP flow cell.



Libraries were sequenced on the NextSeq 2000, NovaSeq 6000 and NovaSeq X Plus. "Data by Cycle" plots from the Illumina SAV software displaying the percentage of base calls and Q30 quality scores are shown. Mapped Reads (%) refers to Reads Mapped Confidently to Probe Set for Probe-based (GEX).

Instr.	Load Conc. (pM)	% Occupancy	%PF	R1	R2	R1	i7	i5	R2	GEX
				Yield per Lane (Gb)		% ≥Q30				Mapped Reads (%)
NextSeq 2000	650	81.2	73.1	51.4	59.9	95.6	96.6	95.1	94.4	97.2
NovaSeq 6000 SP	300	93.2	82.8	135	160	95	92	89	93	92.1
NovaSeq X Plus	400	84.7	71.79	41.4	48.3	95.9	94.6	94.7	95.3	97.7

Troubleshooting

Before CytAssist Instrument Run

1. 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 targeted reads usable (see table below). A similar effect is observed when washing for less than the recommended 5 min, 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.

Wash	Number of Washes	Fraction Reads Usable (Mean)
Post Hybridization Wash	1	0.29
	2	0.41
	3	0.79
Post Ligation Washes	1	0.69
	2	0.75

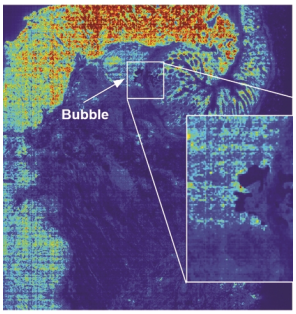
2. Inadequate Visium HD Slide Preparation

- Leaving Visium HD Slide in 0.1X SSC inside slide mailer longer than 4 h after Visium HD Slide washing may result in spacer detachment.
- Failure to remove excess liquid from the back of the Visium HD Slide before loading the slide onto the Visium CytAssist may result in distorted fiducial imaging, which can impact fiducial registration.
- Insufficient drying of Visium HD Slide prior to instrument run may result in bubble entrapment and uneven flow of Probe Release Mix.

During CytAssist Instrument Run

1. Bubbles Trapped During Visium CytAssist Run

Bubbles may Result in no Usable Sequencing Reads during the CytAssist Assay

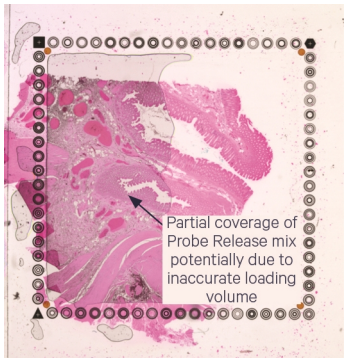
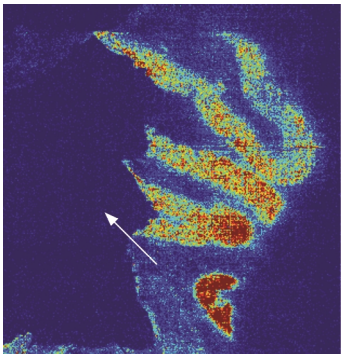
CytAssist Image	UMI Counts
	

Bubbles during a sample run are rare, but may result in a lack of useable reads in the tissue area where the bubbled occurred. The most common cause of bubbles is incomplete drying of the tissue slide and/or the Visium HD slide. Bubbles can also result from inaccurate dispensing volume of Probe Release Mix. Ensure that the entire well is inspected for bubbles, not just the Capture Area. Ensure that the entire well is also dry during the drying step, not just the Capture Area.

Avoid generating bubbles during reagent dispensing by pipetting slowly and avoiding expelling air from the pipette tip. Additionally, ensure that the frosted area of the tissue slides are outside of the alignment guide lines on the Tissue Slide Stage and that tissue slides are compatible with the CytAssist instrument. Consult the Visium HD Spatial Gene Expression Protocol Planner (CG000698) for information on tested slides.

2. Reagent Flow Failure

Reagent Flow Failure May Cause Loss of Data

CytAssist Image	UMI Count
	

Inappropriate flow of reagents during a Visium CytAssist experiment run may result in transcript mislocation. This may be caused by improper formulation of the Probe Release Mix or inaccurate loading volume onto the Visium HD Slide. If the UMI map appears abnormal (complete loss of UMIs from a significant portion of the tissue), contact support@10xgenomics.com.

Ensure that frosted areas of the tissue slide are not within the dashed zone on the Visium Tissue Slide Stage. Keep Visium slides and tissue slides free from physical damage, dust, and debris. After Eosin wash, remove as much PBS as possible by flicking the tissue slide and wiping any remaining droplets around tissue with a lint-free laboratory wipe.

3. Visium CytAssist Overheating

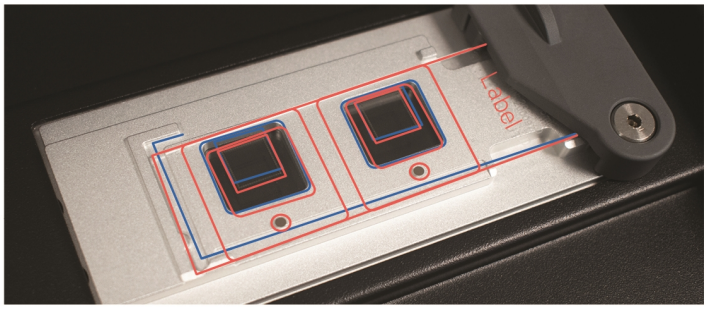
If the Visium CytAssist instrument overheats during an experiment run, the run will fail. A warning message will appear instructing users to carefully remove slides and contact support@10xgenomics.com.

After CytAssist Instrument Run

1. Slide Popped Off of Visium Slide Stage

Upon opening the lid after a successful run, the Visium slide may pop out of the grooves on the Visium Slide Stage. This does not impact assay performance or slide quality.

Visium Slide Popped out of the Grooves



2. Visium HD Slide Removal Delayed

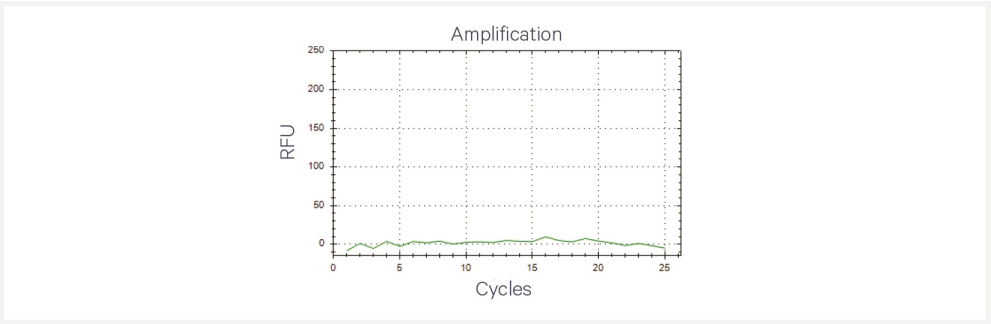
A delay in removing and processing the Visium HD Slide after run completion may impact data quality.

3. No qPCR Amplification

No amplification during Cycle Number Determination may be due to the following:

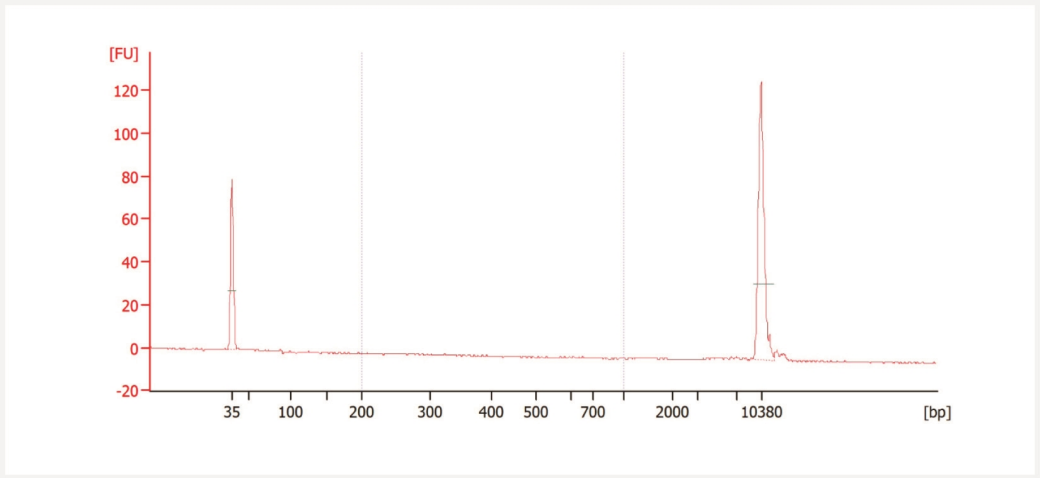
- Incorrect qPCR machine programming (i.e. detecting a fluorophore other than SYBR Green)
- Issues with pipetting small volumes
- Failure to neutralize KOH
- Failure to add primers to pre-amp or qPCR mix
- Unusually low recovery from tissue due to high percentage of connective tissue in Capture Area
- Incorrect preparation of Probe Release Mix
- Mistake in Probe Release Mix addition timing

- Leakage from the Visium CytAssist Tissue Slide Cassette during workflow



4. Flat Line in Bioanalyzer Library Trace

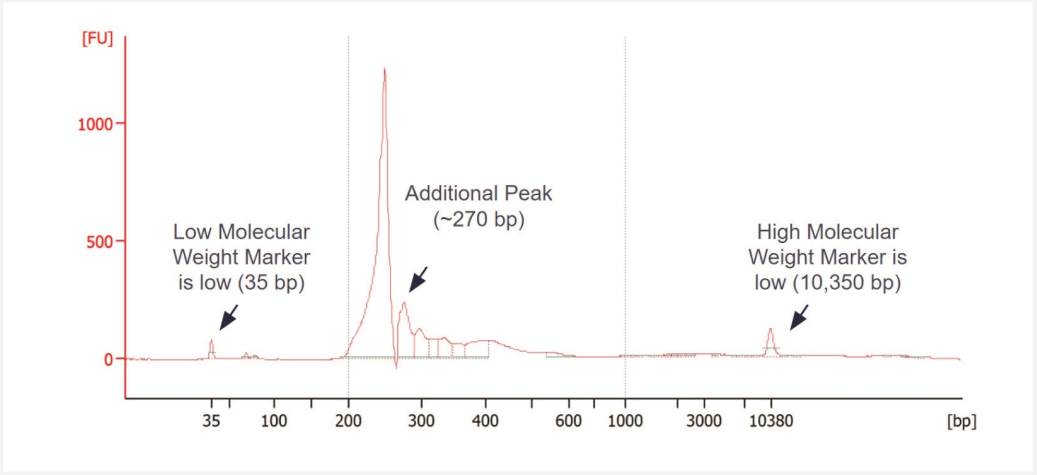
A normal qPCR output but no peak visible in the Bioanalyzer trace may be due to a mistake in the SI-PCR step, errors during SPRIselect cleanup, an overdilution of the final library, Bioanalyzer issue, or thermal cycler failure.



5. Overloaded or Overamplified Trace

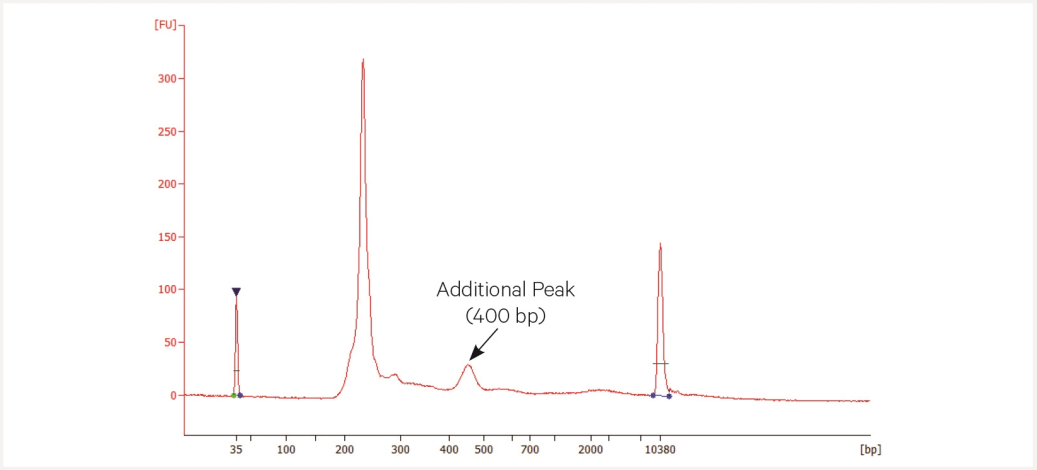
Overloaded Trace

The image below is an example of an overloaded trace. Note the double peak at around 270 bp. The low and high molecular weight markers are in low abundance compared to the sample. Ensure the library was diluted 1:50 prior to loading per protocol recommendations. If the library was diluted, ensure the library concentration is within the specification range of the automated electrophoresis kit being used. The library can be quantified using a Qubit and diluted further if appropriate.



Overamplified Trace

The image below is an example of an overamplified trace. Note peak at 400 bp.



Ensure interpretation of qPCR plot is correct. If necessary, batch samples into separate SI-PCR runs if Cq is > 1 cycle. If batching samples, the cycle number determined should be ±1. If needed, select the value in between.

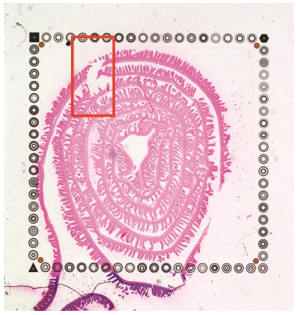
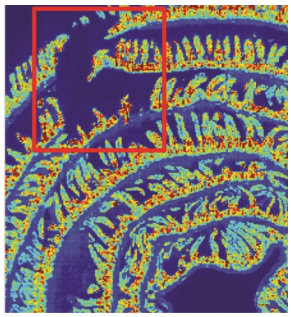
For example, a sample with Cq value of 7.5 should go through 9 cycles during SI-PCR (round up to 8 and add 1) and a sample with a Cq value of 9.2 should go through 11 cycles during SI-PCR (round up to 10 and add 1). If running together, samples should undergo 10 cycles during SI-PCR.

Issues Impacting Tissue Analysis

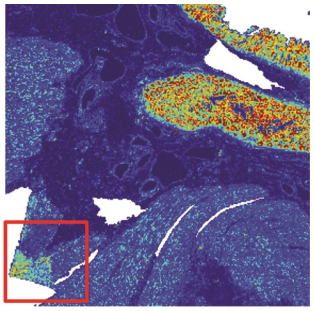
1. Tissue Detachment and Folding

Tissue detachment may result in a lack of Fraction Reads Usable in the region where detachment occurred. If the tissue has folded on itself, this may also cause elevated UMI counts in the overlapping areas or a loss of spatiality due to ineffective permeabilization. Tissue folding may also cause lower UMI capture. Inspect images carefully to identify these areas. Ensure that slides tested by 10x Genomics were used for tissue placement. For a list of tested slides, refer to the Visium HD Spatial Gene Expression Application Protocol Planner (CG000698). For more information, consult the sample preparation documentation described in Workflow Overview.

Tissue Detachment May Cause a Loss of Gene Expression

CytAssist Image	UMI Count
	

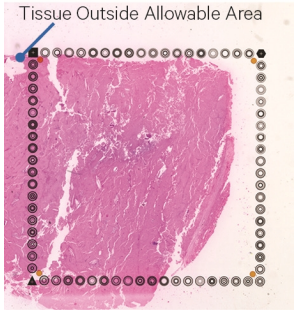
Tissue Folding May Cause Elevated UMI Counts

CytAssist Image	UMI Count
	

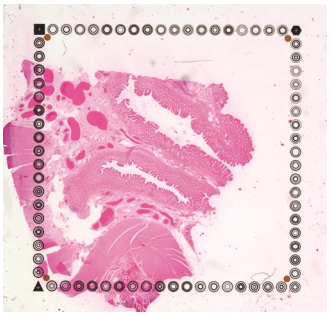
2. Tissue Not Within Allowable Area

Tissue Outside of Allowable Area is Not Analyzed

Tissue Larger than Capture Area



Tissue Not Properly Aligned

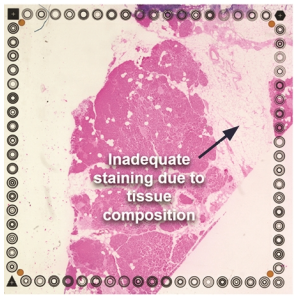


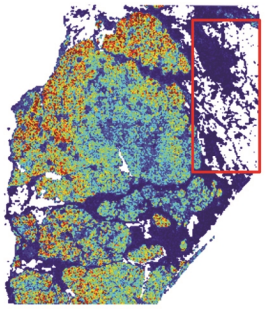
Tissues that are not placed within the allowable area on compatible glass slides will not be analyzed. This may occur if the tissue is larger than the Capture Area or if the tissue slide is not properly aligned when loading onto the Tissue Slide Stage. Consult the Visium HD Protocol Planner (CG000698) for information on tested glass slides.

3. Tissue Segmentation Failure

Examples of Scenarios that Lead to Tissue Segmentation Failure

Tissue Composition/Morphology May Result in Inadequate Staining



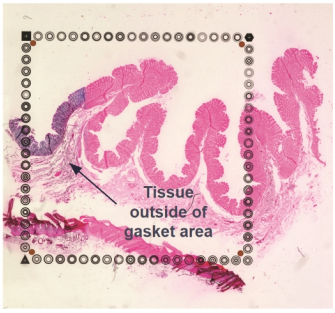
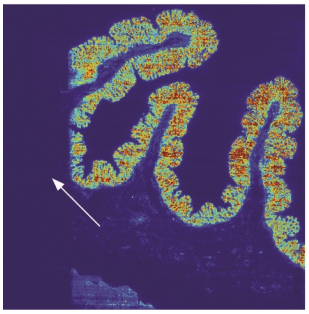


Space Ranger may fail to detect tissue for a variety of reasons, which may necessitate manual fiducial alignment and tissue detection via Loupe Browser. Tissue segmentation failure may result from:

- If the contrast between background and tissue is poor (due to inadequate staining, technical error, or tissue composition) tissue segmentation failure may occur.

4. Tissue within Capture Area Not Analyzed

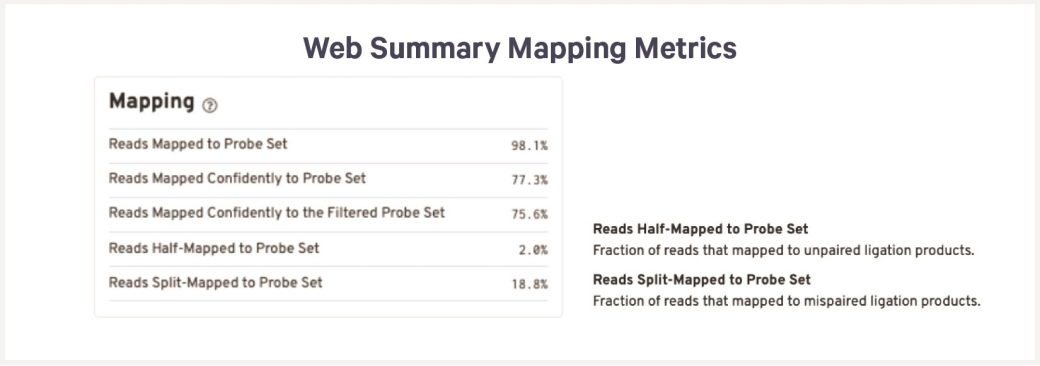
Gasket Covers Portion of Tissue within Capture Area

H&E Stained Tissue	UMI Counts
	

If the gasket covers a portion of the tissue within the Capture Area, that area will not be exposed to the appropriate reagents; thus, probes will not be captured. In the example above, a lack of destaining on the left side of the tissue implies that this area was obscured by the gasket. Though the area is within the Capture Area, probe capture will not occur due to this obstruction. Gasket obstruction may be caused by improper gasket assembly, resulting in the gasket being placed directly onto areas of interest. In Loupe Browser, manual alignment must be performed to exclude the tissue area obscured by the gasket. Ensure that the area of interest is centered within the gasket.

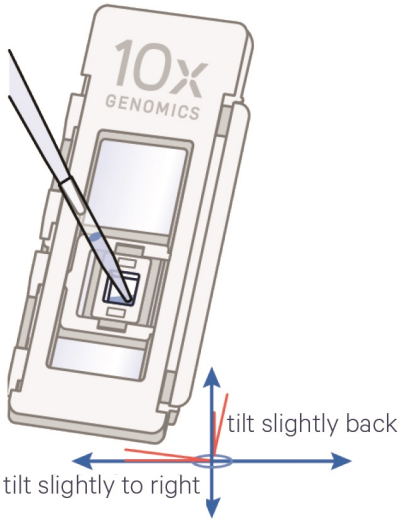
Additionally, tissue placed within the Capture Area may be excluded from the assay workflow due to improper tissue slide alignment when loading the tissue slide into the Visium CytAssist instrument.

5. High Split-Mapped or Half-Mapped Reads



High split-mapped or half-mapped reads can result from insufficient and/or inefficient washing of the tissue especially during the post-hybridization and post-ligation wash steps, as well as poor tissue quality. Follow all best practices, including completely removing residual liquid prior to proceeding to subsequent steps (e.g. using a P20 pipette to remove residual volume, tilting the cassette to collect the liquid at a corner, see illustration below),

performing the wash steps close to the thermal cycler, ensuring that the tissue section(s) do not dry out during the washes, checking that the cassette is fully seated on the Thermocycler Adapter between performing each wash, cassette is fully sealed when necessary to prevent evaporation, and ensuring that the thermal cycler lid is set to the appropriate temperature to prevent condensation during the incubation steps.

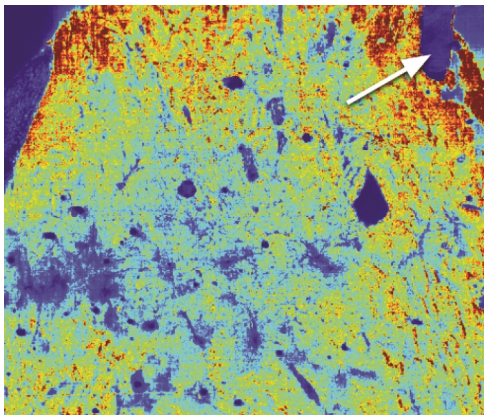


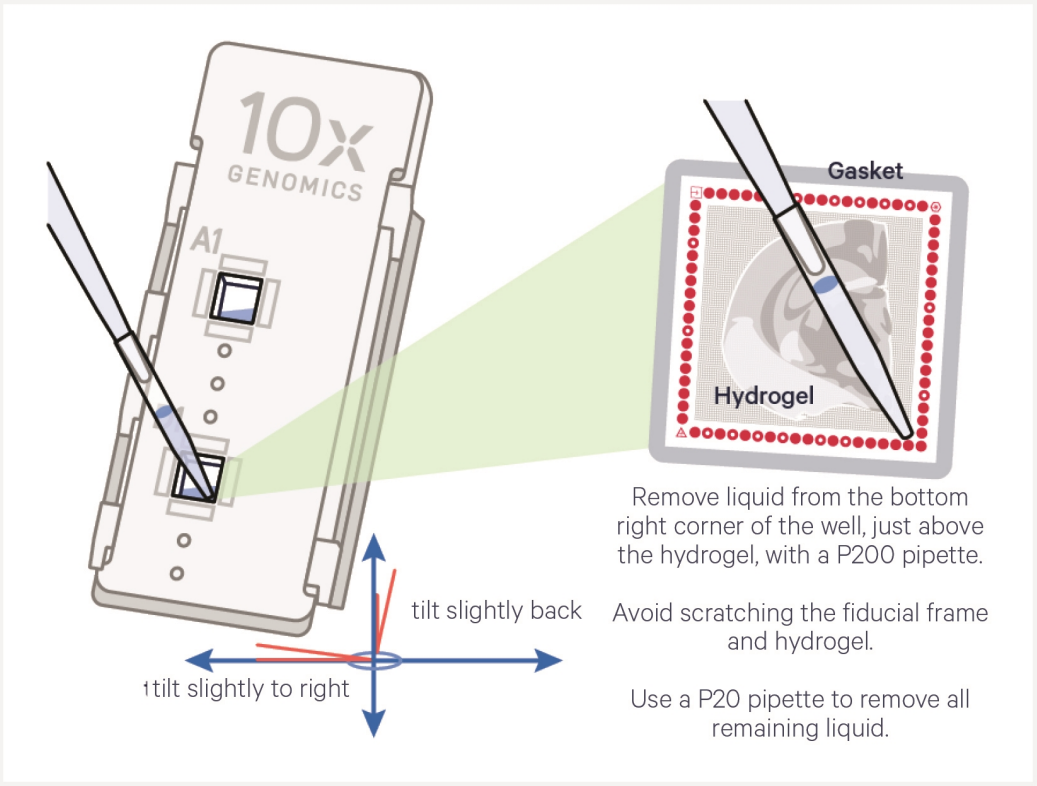
6. Data Loss due to Hydrogel Damage

Damage to the hydrogel may result in data loss. Avoid damaging the hydrogel by adhering to best practices when removing reagent from the well as described in Reagent Addition to & Removal from Wells on page 25. In the example below, a scratch on the hydrogel noted by the arrow has resulted in a loss of UMI count.

Hydrogel Damage May Result in Data Loss

UMI Count

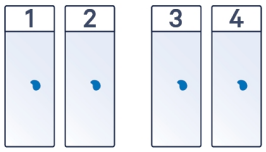
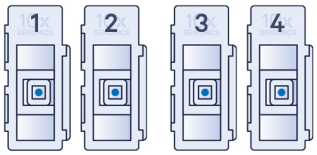
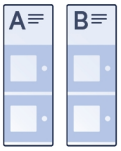
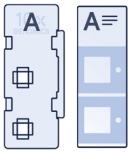
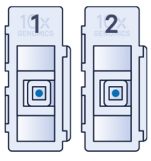
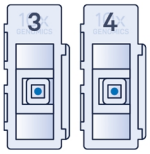
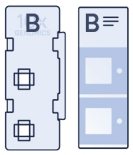


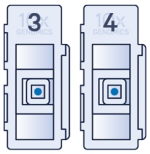
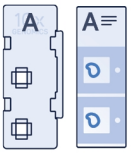
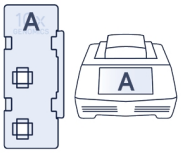
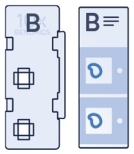
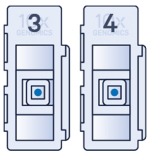
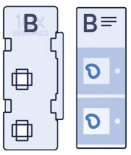
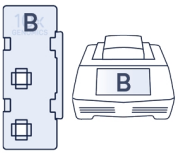

10x
GENOMICS
A1
tilt slightly back
tilt slightly to right
Gasket
Hydrogel
Remove liquid from the bottom right corner of the well, just above the hydrogel, with a P200 pipette.
Avoid scratching the fiducial frame and hydrogel.
Use a P20 pipette to remove all remaining liquid.

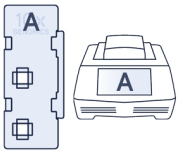
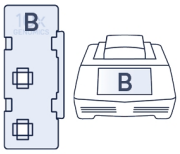
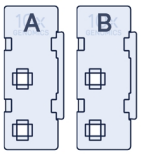
Appendix

HD Workflow Overview for Processing 4 Tissue Slides with 1 Visium CytAssist Instrument

To execute this workflow, two thermal cyclers are required. This table is meant as a reference only. Consult the detailed steps in this user guide for execution.

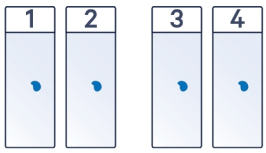
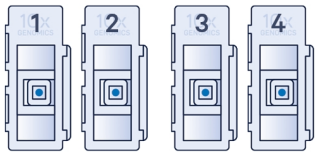
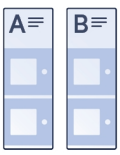
HD Workflow: 4 Slides, 1 CytAssist		
Step	**Run A**	**Run B**
Sample Prep		
Tissue slides 1 - 4 *at the same time*		• Prepare tissue slides via sectioning, staining and imaging • Destaining and Decrosslinking
Step 1: Probe Hybridization, Step 2: Probe Ligation		
Tissue slides 1 - 4 *at the same time*		• Probe Hybridization • Post-Hybridization Wash • Probe Ligation • Post-Ligation Wash
Step 3: Visium HD Sample Preparation		
3.1 Visium HD Slide Wash slides A & B *at the same time*		• Thaw and wash Visium HD slides • Place Visium slides in Visium cassettes
Step 4: Probe Release & Extension		
4.1 CytAssist-Enabled Probe Release & Capture for Visium slide A Start CytAssist (Run A) with tissue slides 1 & 2	   	• Prep CytAssist • Prep PBS, Eosin & Slide Equilib Mix • Add Equilib Mix to Visium Slide A • Incubate for 10 min (room temp) • Prep Probe Release Mix (no Perm Enzyme B) • Load Visium Slide on CytAssist to dry (10 min) • Stain tissue slides 1 & 2 with Eosin • Load tissue slides into CytAssist • Add Perm Enzyme B to Probe Release Mix, dispense onto Visium slide spacer wells • Close lid and start run • Prep thermal cycler A • Prep Probe Extension Mix
	  	• Store tissue slides 3 & 4 at 4°C with 2X SSC buffer in tissue slide cassettes • Store Visium HD slide B in final 0.1X SSC wash

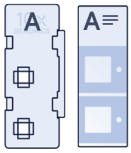
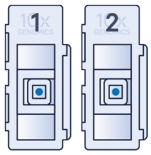
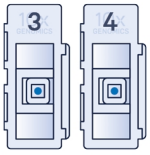
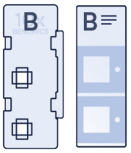
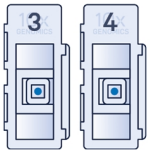
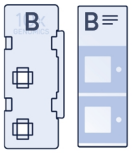
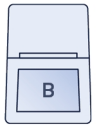
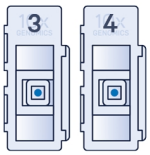
HD Workflow: 4 Slides, 1 CytAssist		
Step	**Run A**	**Run B**
Equilibration for tissue slides 3 & 4 *during Run A*	*Once CytAssist has completed the run and is cooling down, begin equilibration for slides 3 & 4.*	 • Remove slides 3 & 4 from storage • Equilibrate for 5 min (room temp.)
CytAssist (Run A) complete	 • Open CytAssist • Wash Visium slide A • Place in Visium Cassette	
4.2 Probe Extension 1 for Visium Slide A	 • Add Probe Extension Mix • Apply seal and place on thermal cycler A	
Ensure 20 min cool-down period after completion of Run A Start CytAssist (Run B) with tissue slides 3 & 4		  • Prep CytAssist • Prep PBS, Eosin & Slide Equilib Mix • Add Equilib Mix to Visium Slide B • Incubate for 10 min (room temp) • Prep Probe Release Mix (no Perm Enzyme B) • Load Visium Slide on CytAssist to dry (10 min)   • Stain tissue slides 3 & 4 with Eosin • Load tissue Slides into CytAssist • Add Perm Enzyme B to Probe Release Mix, dispense onto Visium slide spacer wells • Close lid and start run • Prep thermal cycler B • Prep Probe Extension Mix
CytAssist (Run B) complete		 • Open CytAssist • Wash Visium slide B • Place in Visium Cassette
4.2 Probe Extension 1 for Visium Slide B		 • Add Probe Extension Mix • Apply seal and place on thermal cycler B

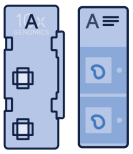
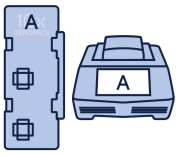
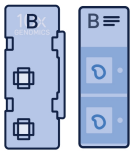
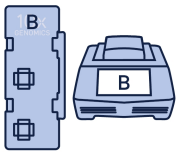
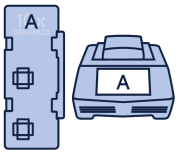
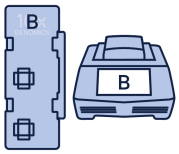
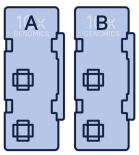
HD Workflow: 4 Slides, 1 CytAssist		
Step	**Run A**	**Run B**
4.2 Probe Extension 2 for Visium Slide A	 • Remove cassette A • Remove Probe Ext. Mix from wells • Add fresh Probe Extension Mix • Reapply seal and place on thermal cycler A • Skip the 4°C hold	
4.2 Probe Extension 2 for Visium Slide B		 • Remove cassette B • Remove Probe Ext. Mix from wells • Add fresh Probe Extension Mix • Reapply seal and place on thermal cycler B • Skip the 4°C hold
4.3 Probe Elution		*Proceed with Visium slides A & B*

HD Workflow Overview for Processing 4 Tissue Slides with 2 Visium CytAssist Instruments

To execute this workflow, two thermal cyclers are required. This table is meant as a reference only. Consult the detailed steps in this user guide for execution.

HD Workflow: 4 Slides, 2 CytAssists		
Step	**Run A, CytAssist A**	**Run B, CytAssist B**
Sample Prep		
Tissue slides 1 - 4 *at the same time*		• Prepare tissue slides via sectioning, staining and imaging • Destaining and Decrosslinking
Step 1: Probe Hybridization, Step 2: Probe Ligation		
Tissue slides 1 - 4 *at the same time*		• Probe Hybridization • Post-Hybridization Wash • Probe Ligation • Post-Ligation Wash
Step 3: Visium HD Sample Preparation		
3.1 Visium HD Slide Wash slides A & B *at the same time*		• Thaw and wash Visium HD slides • Place Visium slides in Visium cassettes

HD Workflow: 4 Slides, 2 CytAssists		
Step	**Run A, CytAssist A**	**Run B, CytAssist B**
Step 4: Probe Release & Extension		
4.1 CytAssist-Enabled Probe Release & Capture for Runs A & B		 • Prep CytAssist A • Prep PBS, Eosin & Slide Equilibration Mix for Runs A & B
4.1 CytAssist-Enabled Probe Release & Capture for Visium slide A Start CytAssist A with tissue slides 1 & 2	 • Add Equilib Mix to Visium Slide A • Incubate for 10 min (room temp) • Prep Probe Release Mix (no Perm Enzyme B) • Load Visium Slide on CytAssist to dry (10 min)  • Stain Tissue slides 1 & 2 with Eosin • Load Tissue Slides into CytAssist  • Add Perm Enzyme B to Probe Release Mix, dispense onto Visium slide spacer wells • Close lid and start run • Prep thermal cycler A • Prep Probe Extension Mix 	 • Store tissue slides 3 & 4 at 4°C with 2X SSC buffer in tissue slide cassettes   • Store Visium HD slide B in final 0.1X SSC wash with Visium Slide Seal
Equilibration for tissue slides 3 & 4		 • Remove slides 3 & 4 from storage • Equilibrate for 5 min (room temp.)
Start CytAssist B with tissue slides 3 & 4		 • Prep CytAssist B • Add Equilib Mix to Visium Slide B • Incubate for 10 min (room temp) • Prep Probe Release Mix (no Perm Enzyme B)  • Load Visium Slide on CytAssist to dry (10 min) • Stain tissue slides 3 & 4 with Eosin • Load tissue Slides into CytAssist  • Add Perm Enzyme B to Probe Release Mix, dispense onto Visium slide spacer wells • Close lid and start run • Prep thermal cycler B  • Prep Probe Extension Mix

HD Workflow: 4 Slides, 2 CytAssists		
Step	**Run A, CytAssist A**	**Run B, CytAssist B**
CytAssist (Run A) complete	 • Open CytAssist • Wash Visium slide A • Place in Visium Cassette	
4.2 Probe Extension 1 for Visium Slide A	 • Add Probe Extension Mix • Apply seal and place on thermal cycler A	
CytAssist (Run B) complete		 • Open CytAssist B • Wash Visium slide B • Place in Visium Cassette
4.2 Probe Extension 1 for Visium Slide B		 • Add Probe Extension Mix • Apply seal and place on thermal cycler B
4.2 Probe Extension 2 for Visium Slide A	 • Remove cassette A • Remove Probe Extension Mix from wells • Add fresh Probe Extension Mix • Reapply seal and place on thermal cycler A • Skip the 4°C hold	
4.2 Probe Extension 2 for Visium Slide B		 • Remove cassette B • Remove Probe Extension Mix from wells • Add fresh Probe Extension Mix • Reapply seal and place on thermal cycler B • Skip the 4°C hold
4.3 Probe Elution		*Proceed with Visium slides A & B*

Post Library Construction Quantification

a. Thaw KAPA Library Quantification Kit for Illumina Platforms.

b. Dilute **2 µl** sample with deionized water to appropriate dilutions that fall within the linear detection range of the KAPA Library Quantification Kit for Illumina Platforms. (For more accurate quantification, make the dilution(s) in duplicate).

c. Make enough Quantification Master Mix for the DNA dilutions per sample and the DNA Standards (plus 10% excess) using the guidance for 1 reaction volume below.

Quantification Master Mix	1X (µl)
SYBR Fast Master Mix + Primer	12
Water	4
Total	16

d. Dispense **16 µl** Quantification Master Mix for sample dilutions and DNA Standards into a 96 well PCR plate.

e. Add **4 µl** sample dilutions and **4 µl** DNA Standards to appropriate wells. Centrifuge briefly.

f. Prepare a qPCR system with the following protocol. Insert the plate and start the program.

Lid Temperature	Reaction Volume	Run Time
-	20 µl	35 min
Step	**Temperature**	**Time**
1	95°C	00:03:00
2	95°C	00:00:05
3	67°C Read signal	00:00:30
4	Go to Step 2, 29X (Total 30 cycles)	

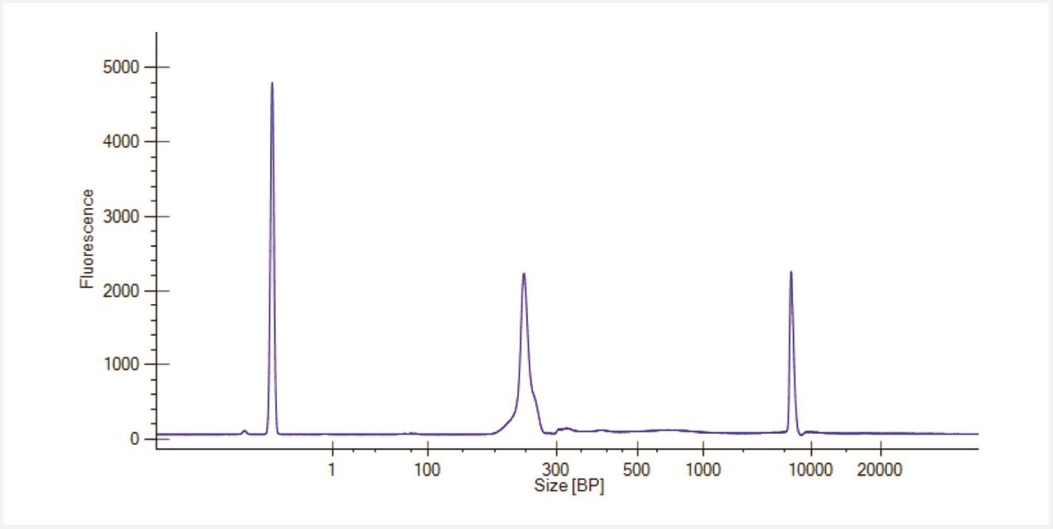
g. Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on insert size derived from the Bioanalyzer/TapeStation trace.

LabChip Traces

Protocol Step 6.3 - GEX Post Library Construction QC

Representative Trace

Run manufacturer's recommended volume of diluted sample (1:5 dilution)



DNA High Sensitivity Reagent Kit was used. Protocol steps correspond to the steps in this User Guide.

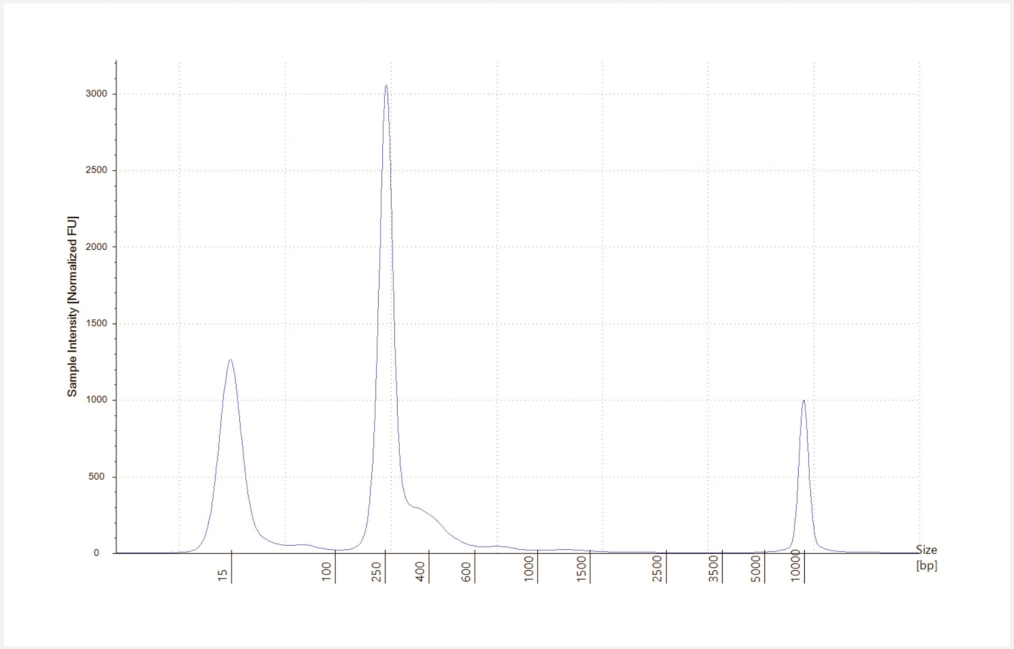
Agilent TapeStation Traces

Agilent TapeStation High Sensitivity D5000 ScreenTape was used. Protocol steps correspond to the steps in this User Guide.

Protocol Step 6.4 - GEX Post Library Construction QC

Representative Trace

Run manufacturer's recommended volume of diluted sample (1:20 dilution)



Oligonucleotide Sequences

Slide Primers

5'-TTTTTTTCCCTACACGACGCTCTTCCGATCT-UMI-SpatialBarcode-GVV-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'

Visium HD Spatial Gene Expression - Probe-based Library

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-UMI-SpatialBarcode-GVV-T30-VN-Ligated_Probe_Insert-TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'

3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-UMI-SpatialBarcode-GVV-T30-BN-Ligated_Probe_Insert-ACCTTAAGAGCCCACGGTTCCTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'