10x GENOMICS

User Guide | CG000805 | Rev B

Visium HD 3' Spatial Gene Expression

For use with:

Visium HD 3' Reagents - Kit A Small, PN-1000854

Visium HD 3' Reagents - Kit B Small, PN-1000855

Visium Slide Cassettes S3 6.5 mm, 2 pk, PN-1000847

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

Visium CytAssist Reagent Accessory Kit, PN-1000499

Visium CytAssist Alignment Aid Kit, 6.5 mm, PN-1000886

View videos for key techniques on slide preparation and use, slide drying, and more. To view, click here.

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

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Notices

Document Number

CG000805 | 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

CG000805

Title

Visium HD 3' Spatial Gene Expression User Guide

Revision

Rev B

Revision Date

August 25, 2025

Description of Changes

- Updated cap colors for Second Strand Enzyme, Second Strand Reagent, and Second Strand Primer in Reagent Kits on page 10, 4.0 Get Started on page 76, and 4.1 Second Strand Synthesis on page 77.
- Updated timing for cDNA amplification step in Protocol Steps & Timing on page 14.
- Added information on varying Visium HD Slide appearance to Visium HD Slides on page 16.
- Added information regarding best practice videos to cover page and Videos on page 23.
- Added information on lint-free laboratory wipes to Lint-free Laboratory Wipes on page 24
- Added additional guidance on adding and removing reagents from cassettes in Reagent Addition & Removal from Wells on page 27.
- Added tutorial for calculating SPRI dilutions and double-sided SPRI dilutions to SPRIselect Cleanup & Size Selection on page 33.
- Updated Visium HD Slide drying guidance in 1.1 Visium HD Slide Wash on page 51.

- Updated 0.1X SSC preparation volumes in 1.1 Visium HD Slide Wash on page 49.
- Added additional guidance if using a 50-ml centrifuge tube to 2.2 Destaining on page 59.
- Updated tissue slide drying and Visium HD Slide drying language in 3.1 CytAssist-Enabled Poly(A) RNA Capture on page 67.
- Added note to not flick the Visium HD slide after removing the slide from the cassette in 3.1 CytAssist-Enabled Poly(A) RNA Capture on page 65.
- Updated KOH preparation tables in 3.3 Denaturation on page 74 and 4.2 Second Strand Elution on page 78.
- Added note to clean instrument, export data, and review images to 4.1 Second Strand Synthesis on page 77.
- Updated cDNA example calculation in 5.3 cDNA QC & Quantification on page 86.
- Added note that if using TapeStation, only D5000 reagents/tape are recommended to 5.3 cDNA QC & Quantification on page 84.
- Added note that if using TapeStation, D5000 or D1000 reagents/tape can be used to 6.7 Post-Library Construction QC on page 97.
- Added TapeStation cDNA traces to Agilent TapeStation Traces on page 123.

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Introduction

Reagent Kits

Reagent Kits	Part Number	Components	Component Part Number	Quantity
Visium HD 3', 6.5 mm, 4 rxns*	1000857	Visium HD Slide, 6.5 mm, 2 rxns	1000670	2
		Visium HD 3' Reagents Kit A, Small	1000854	1
		Visium HD 3' Reagents Kit B, Small	1000855	1
		Visium Slide Cassettes S3, 6.5 mm, 2 pk	1000847	1

*Also available 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

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Visium Slide Cassettes S3*, 6.5 mm, 2 pk PN-1000847

Visium Slide Cassettes S3, 6.5 mm
2 pk PN-1000847
(store at ambient temperature)

	#	PN
Visium 2-port, 6.5 mm, Top, 2 pk	1	2001252 or 3002329
Visium Cassette Bottom, 2 pk	1	2001344 or 3002328
Visium Slide Seals, 12 pack	1	2000283

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*The Visium HD 3' Workflow is run with Visium Cassettes S3. These are referred to as Visium Cassettes in this document. Consult the Visium Cassette S3 Quick Reference Card (CG000730) for assembly and disassembly information. Visium Cassettes appearance may vary, but all Visium Cassettes have the same functionality and performance.

Visium HD 3' Reagents - Kit A Small PN-1000854

Visium HD 3' Reagents - Kit A Small PN-1000854
(store at −20°C)

		#	PN
○	Enhancer	1	2000482
○	Reducing Agent B	1	2000087
●	RNase Inhibitor B	2	2001400
●	Pre-equilibration Buffer	1	2001399
●	Perm Enzyme B	1	3000553
●	Perm Buffer	1	2001398

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Visium HD 3' Reagents - Kit B Small PN-1000855

Visium HD 3' Reagents - Kit B Small PN-1000855
(store at −20°C)

		#	PN
●	RT Reagent	1	2000086
●	RT Enzyme G	1	2001438
●	Template Switch Oligo B	1	2001027
●	Second Strand Enzyme	1	2000183
●	Second Strand Reagent	1	2000219
●	Second Strand Primer	1	2000217
●	cDNA Primers	1	2000089
○	Amp Mix	1	2000103
●	Fragmentation Enzyme	1	2000104
●	Fragmentation Buffer	1	2000091
●	DNA Ligase	1	220131
●	Ligation Mix	1	2001109

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Dual Index Kit TT Set A, 96 rxns PN-1000215

Dual Index Kit TT Set A
96 rxns PN-1000215
(store at −20°C)

	#	PN
Dual Index Plate TT Set A	1	3000431

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Visium CytAssist Alignment Aid Kit, 6.5 mm PN-1000886

Visium CytAssist Alignment Aid, 6.5 mm
PN-1000886
(store at ambient temperature)

	#	PN
Visium CytAssist Alignment Aid, 6.5 mm	1	3002814

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Visium CytAssist Reagent Accessory Kit PN-1000499

Visium CytAssist Reagent Accessory Kit
PN-1000499
(store at ambient temperature)

	#	PN
10x Magnetic Separator	1	2001212
Low Profile Thermocycler Adapter	2	3000823

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Third-Party Items

Successful execution of the Visium HD 3' 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 3' Spatial Gene Expression Protocol Planner (CG000803) 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.

Visium HD 3' Gene Expression Protocol Planner
Information on third-party items.
Planner CG000803

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

Visium HD 3' FF Tissue Preparation Handbook
Prepare fresh frozen tissue blocks, section tissue onto slides, stain, and image.
Demonstrated Protocol CG000804

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

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

2 Library Construction

Visium HD 3' Spatial Gene Expression User Guide
Construct Visium HD 3' Libraries.
User Guide CG000805

Consult the 10x Genomics support website for additional documents

Protocol Steps & Timing

Steps	Timing	Stop & Store
Step 1: Visium HD Slide & CytAssist Preparation (page 47)		
1.1 Visium HD Slide Wash (page 49)	60 min	
Step 2: Coverslip Removal & Destaining (page 55)		
2.1 Coverslip Removal (page 58)	10 min	
2.2 Destaining (page 59)	30 min	
Step 3: CytAssist-Enabled Poly(A) RNA Capture, Reverse Transcription, & Denaturation (page 61)		
3.1 CytAssist-Enabled Poly(A) RNA Capture (page 64)	50 min	
3.2 Reverse Transcription (page 73)	100 min	
3.3 Denaturation (page 74)	10 min	
Step 4: Second Strand Synthesis & Elution (page 75)		
4.1 Second Strand Synthesis (page 77)	55 min	STOP 4°C ≤24 h
4.2 Second Strand Elution (page 78)	10 min	
Step 5: cDNA Amplification and SPRIselect (page 79)		
5.1 cDNA Amplification (page 81)	55 min	STOP 4°C ≤24 h
5.2 cDNA Amplification Cleanup - SPRIselect (page 82)	20 min	STOP 4°C ≤72 h or -20°C ≤4 weeks
5.3 cDNA QC & Quantification (page 83)	30-60 min	
Step 6: Visium HD 3' Library Construction (page 87)		
6.1 Fragmentation, End Repair & A-tailing (page 90)	40 min	
6.2 Post Fragmentation End Repair & A-tailing Double Sided Size Selection – SPRIselect (page 91)	25 min	
6.3 Adaptor Ligation (page 92)	20 min	
6.4 Post-Ligation Cleanup – SPRIselect (page 93)	20 min	
6.5 Sample Index PCR (page 94)	30 min	STOP 4°C ≤72 h
6.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect (page 96)	25 min	STOP 4°C ≤72 h or -20°C long term
6.7 Post-Library Construction QC (page 97)	50 min	

Stepwise Objectives

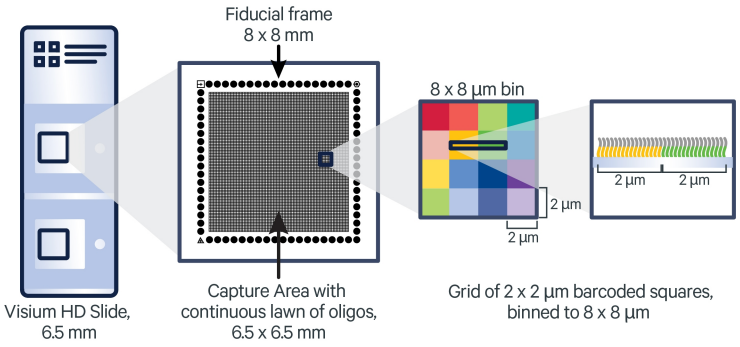
The Visium HD 3' Spatial Gene Expression assay is designed to analyze poly(A) RNA in tissue sections derived from fresh frozen (FF) tissue samples. Before the assay, FF tissue sections are processed as described in the Visium HD 3' Fresh Frozen Tissue Preparation Handbook (CG000804). See Workflow Overview on page 13 for documentation references.

Tissue slides and a Visium HD Slide are loaded into the Visium CytAssist instrument, where they are brought into proximity with one another. Tissue on the tissue slide is permeabilized, allowing for the release of poly(A) RNA and subsequent capture by the spatially-barcoded oligonucleotides present on the Visium HD 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.

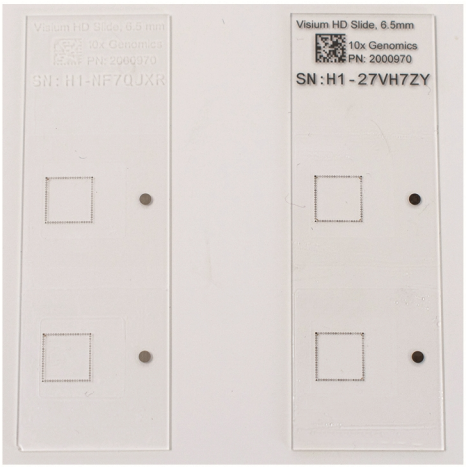
Visium HD Slides

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 an Illumina TruSeq partial read 1 sequencing primer, unique molecular identifier (UMI), Spatial Barcode, and 30 nt poly(dT) sequence (captures poly(A) RNA). 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 has 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.

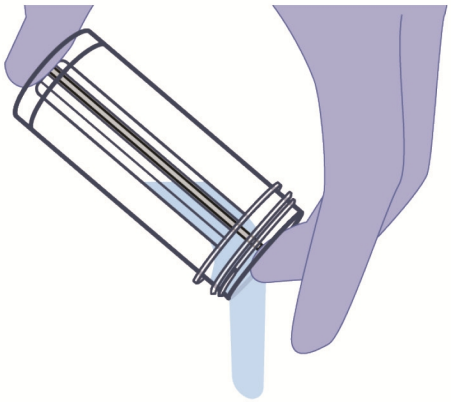


Visium HD Slide appearance may vary, but all Visium HD Slides have the same functionality and performance.



Step 1: Visium HD Slide & CytAssist Preparation

Visium HD Slides are thawed, washed, and equilibrated prior to placement on the Visium CytAssist instrument. The Visium CytAssist instrument is powered on and prepared for the experimental run.

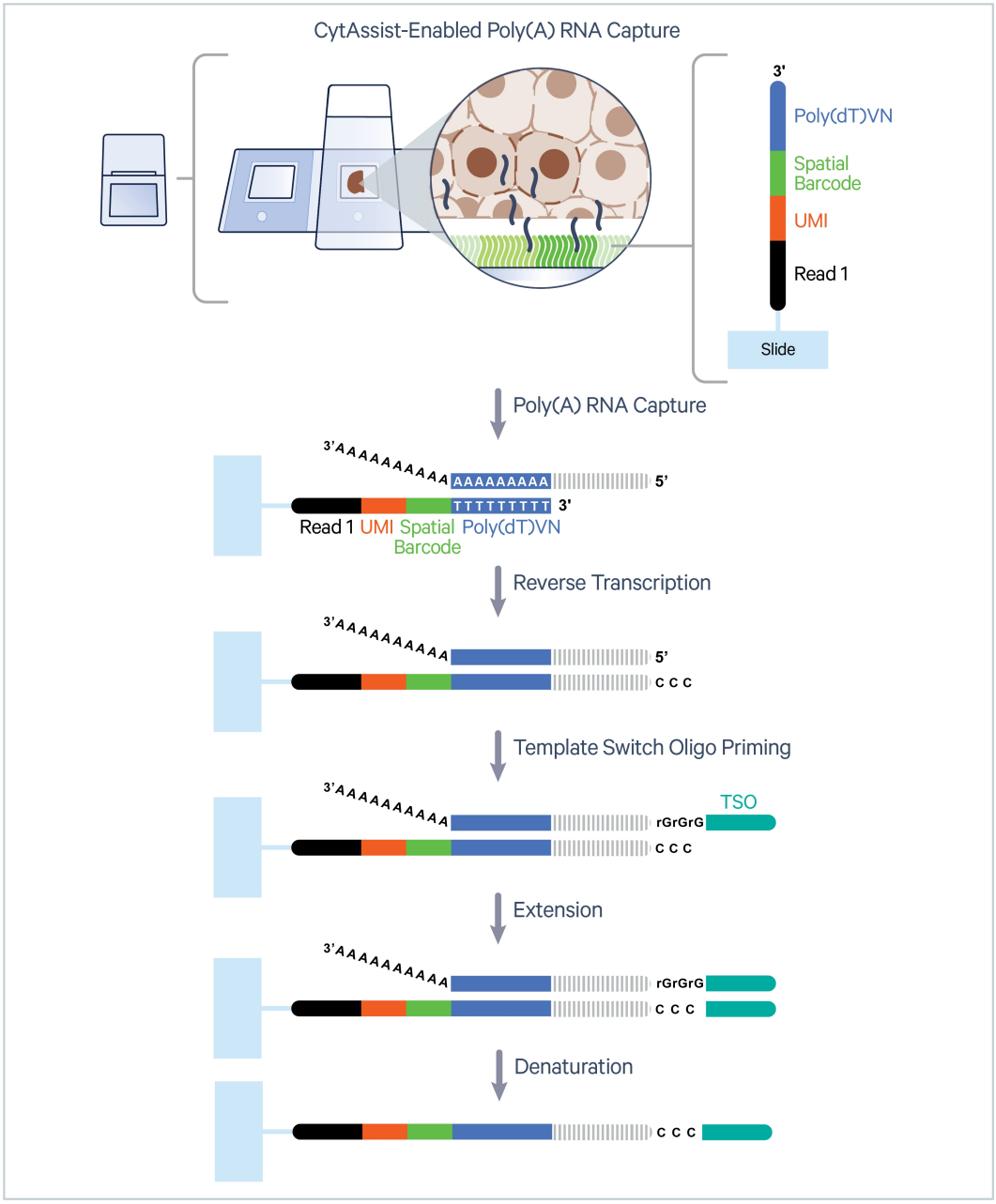


Step 2: Coverslip Removal & Destaining

Coverslips are removed from H&E-stained tissue slides by immersing the slides in water. After coverslip removal, tissue slides go through a destaining step.

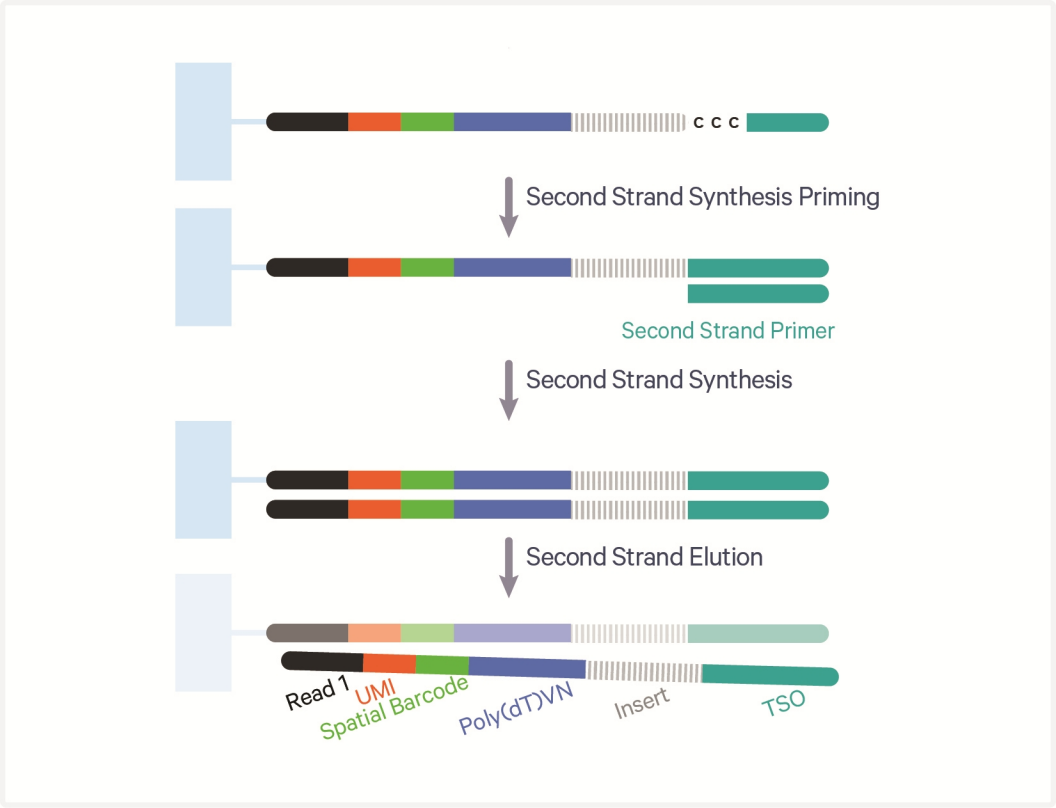
Step 3: CytAssist-Enabled poly(A) RNA Capture, Reverse Transcription, & Denaturation

Tissue slides are loaded onto the Visium CytAssist instrument, where fixed and stained tissues are permeabilized to allow for the release of poly(A) RNA. Oligos on the surface of the Visium HD Slide capture poly(A) RNA released from the overlying cells. RT Master Mix containing reverse transcription reagents is added to the Visium HD Slide. Incubation with the reagents produces spatially barcoded, full-length cDNA from poly(A) RNA on the slide.



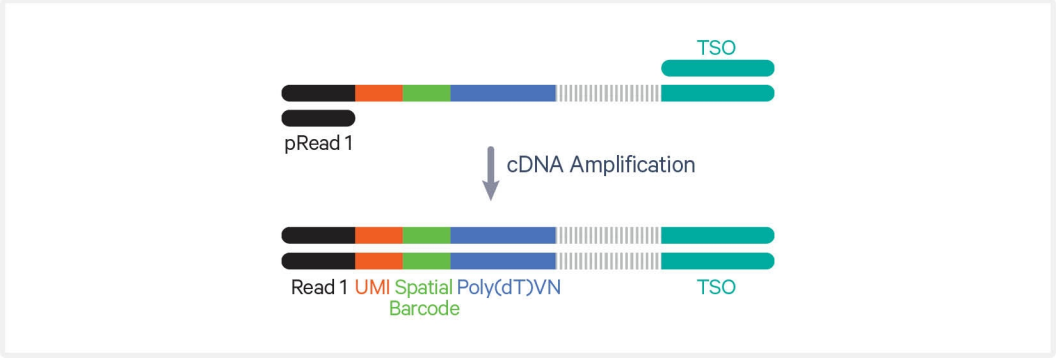
Step 4: Second Strand Synthesis & Elution

Second Strand Mix is added to the Visium HD Slide to initiate second strand synthesis. This is followed by elution and transfer of the cDNA from each Capture Area to a corresponding tube for amplification and library construction.



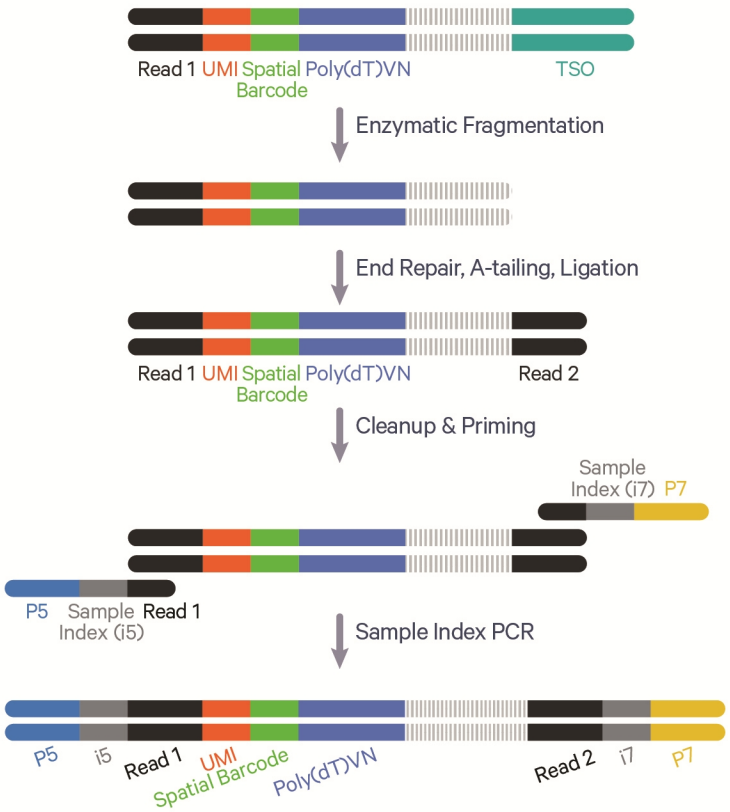
Step 5: cDNA Amplification and SPRIselect

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



Step 6: Visium HD 3' Library Construction

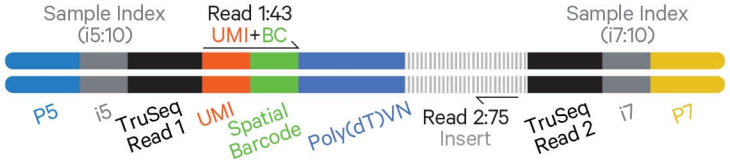
Enzymatic fragmentation and size selection are used to optimize the cDNA amplicon size. TruSeq Read 2 (read 2 primer sequence) is added during Adaptor Ligation. P5 and P7, as well as i7 and i5 sample indexes, are added during Sample Index PCR. The final libraries contain the P5 and P7 primers used in Illumina sequencing.



Sequencing

A Visium HD 3' 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 and the cDNA insert is encoded in TruSeq Read 2. i7 and i5 sample index sequences are also incorporated as the index read. TruSeq Read 1 and TruSeq Read 2 are standard Illumina sequencing primer sites used in paired-end sequencing.

Visium HD 3' Library



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Tips & Best Practices

Icons

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

Indicates a video is available for the specified technique

Videos

- Videos describing key steps in the protocol can be found here.
- When a relevant video is available for a protocol step, it will be marked with a video icon.

General Reagent Handling

- Fully thaw and thoroughly mix reagents before use.
- When pipette mixing, unless otherwise specified, set pipette to 75% of total volume. Pipette until reagents are well combined unless a specific number of mixes is specified.

Visium HD Slide Storage

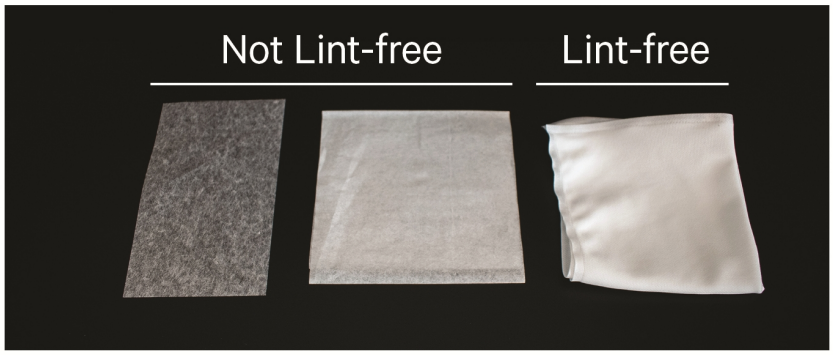
- Keep Visium HD Slide at –80°C until ready to use.
- Do not open the mylar bag containing the Visium HD slides until slides are ready to be used.

Pipette Calibration

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

Lint-free Laboratory Wipes

- All laboratory wipes used in the protocol must be 100% polyester and lint-free.
- Lens paper or non-lint free laboratory wipes are not suitable alternatives. See Visium HD 3' Spatial Gene Expression Protocol Planner (CG000803) for tested part numbers.

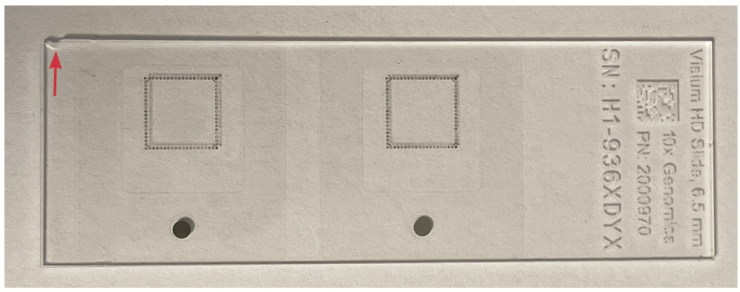


Visium HD Slide Handling

- Wipe Visium HD Slide Mailer with a lint-free laboratory wipe to ease in handling.
- Prior to beginning an instrument run, Visium HD Slides are 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 slide 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.
- If using a barcode scanner to read the serial number, ensure that the label is not wet.
- When disassembling the Visium HD Slide from the Visium Cassette, ensure that the cassette gaskets DO NOT touch any surfaces to avoid debris accumulation.

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 3' Spatial Gene Expression Protocol Planner (CG000803).
- Always wear gloves when handling slides.

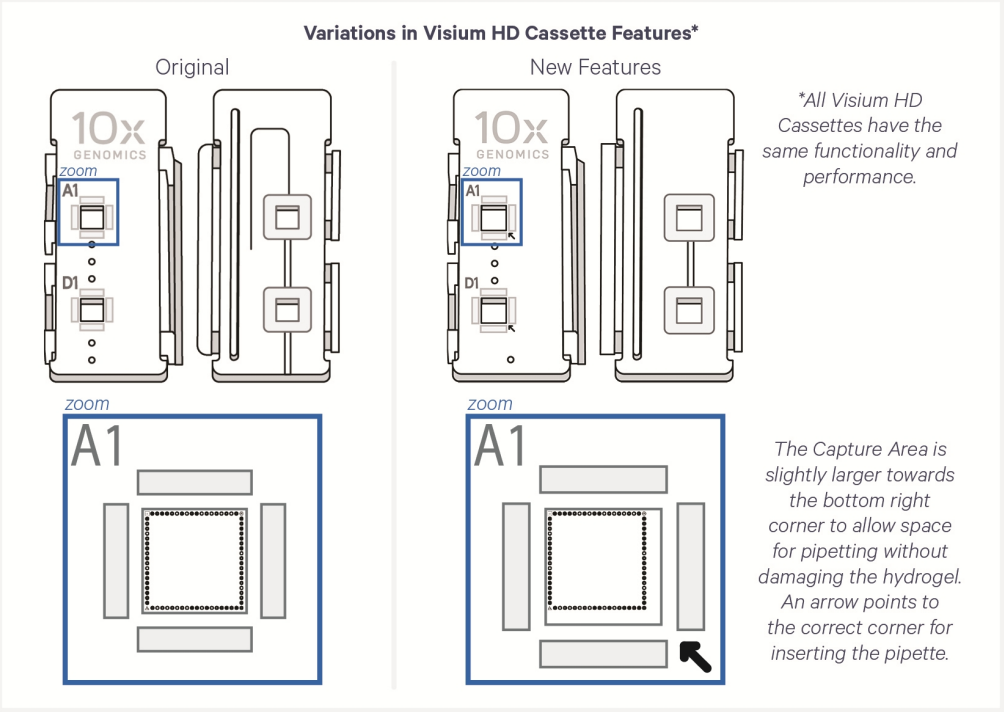
RNase-free Environment

- An RNase-free working environment is critical for optimal assay performance.
- An RNase decontamination solution should be used to clean workspaces and equipment.
- Clean workspaces and equipment every workday during the protocol.
- Use new plastic equipment (e.g. centrifuge tubes) and clean glassware as described in the protocol.

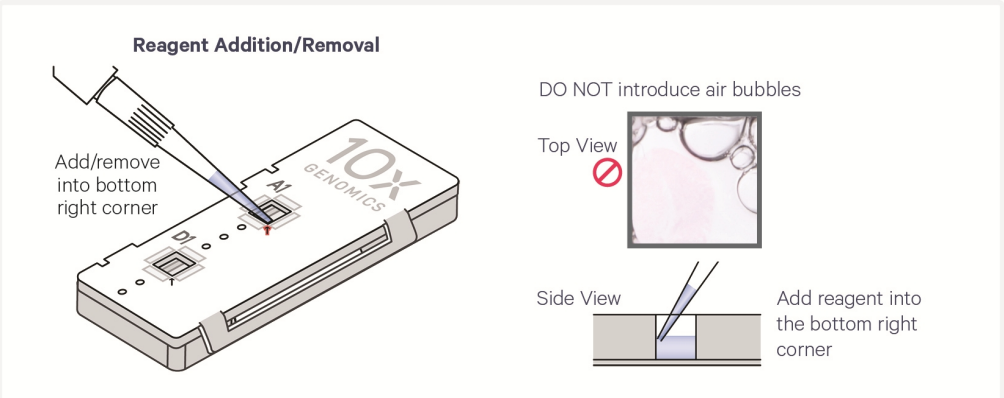
Reagent Addition & Removal from Wells

Reagent Addition

- Assemble slide into the cassette flat on a clean work surface. See Cassette Assembly Quick Reference Card (CG000730).

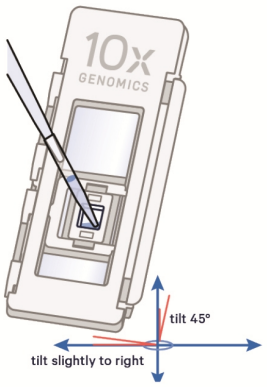


- Dispense and remove reagents along the side of the wells without touching the slide surface, tissue sections (when applicable), and without introducing bubbles.
- DO NOT forcefully insert pipette tip into well corners to avoid damaging the Visium HD Slide.
- Unless indicated otherwise, when adding and removing reagent from a cassette, do so one well at a time. For example, remove reagent from A1 and add the next reagent to A1, before moving into onto D1, to avoid drying the well.
- 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. DO NOT completely tilt the cassette at a 90 degree angle to avoid liquid from entering other grooves within the gasket. See image below for proper technique.
- 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

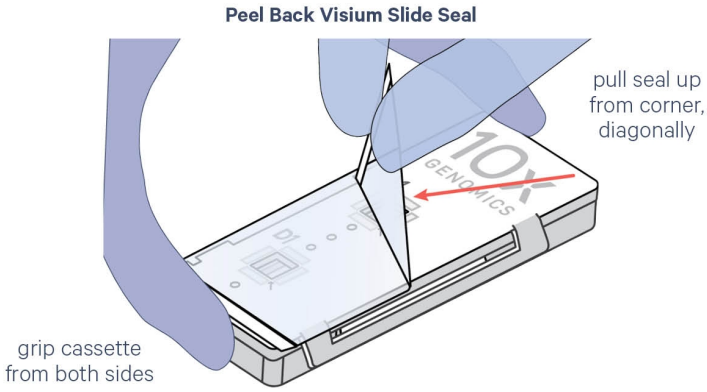
Application

- 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.

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.



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.

Incubation at Room Temperature

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

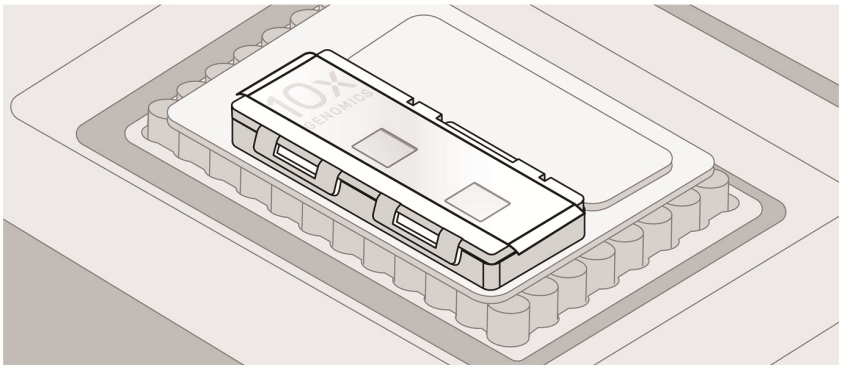
Cassette Incubation

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 3' Spatial Gene Expression Protocol Planner (CG000803) for information on thermal cycler compatibility.
- Ensure that the cassette sits flush with the raised portion of the Low Profile Thermocycler Adapter.

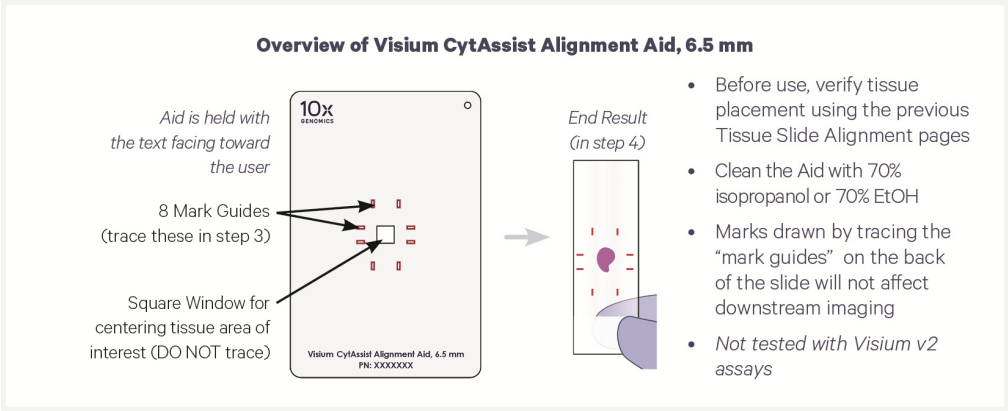
- Allow Low Profile Thermocycler Adapter to cool before removing from thermal cycler.

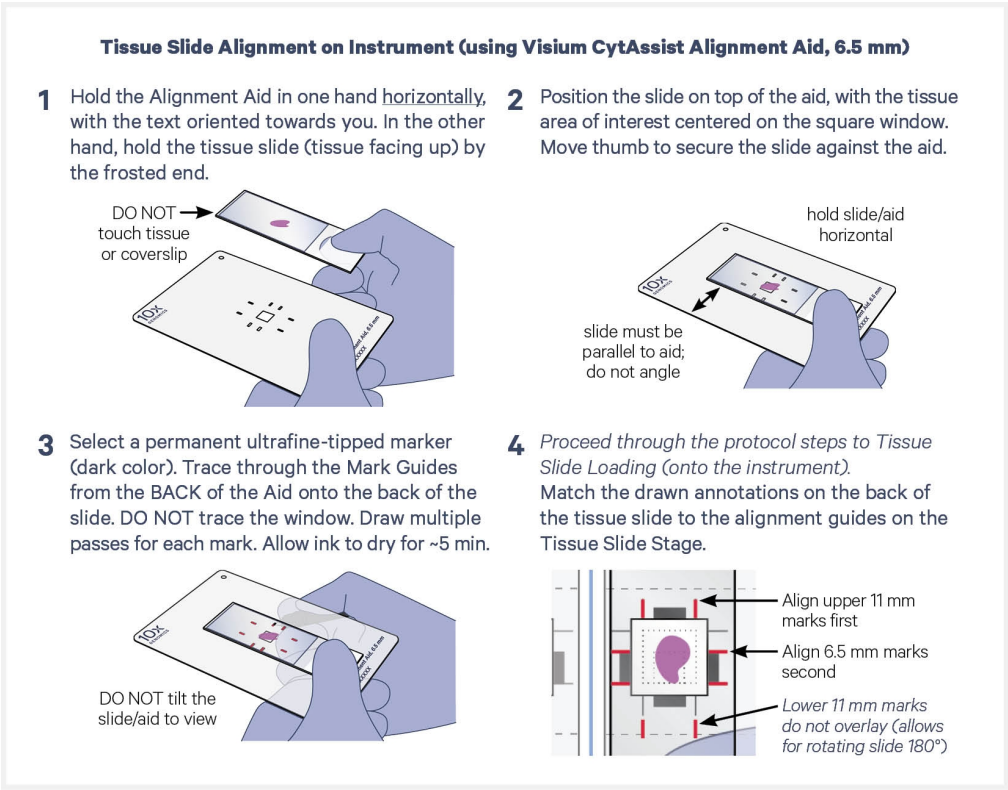
Ensure Cassette Sits Flush Against Thermocycler Adapter



Visium CytAssist Alignment Aid

- The Visium CytAssist Alignment Aid (6.5 mm, PN-1000886; available for purchase separately) is an optional accessory used to draw annotations onto the back of the tissue slide to assist with alignment onto the Visium CytAssist instrument.





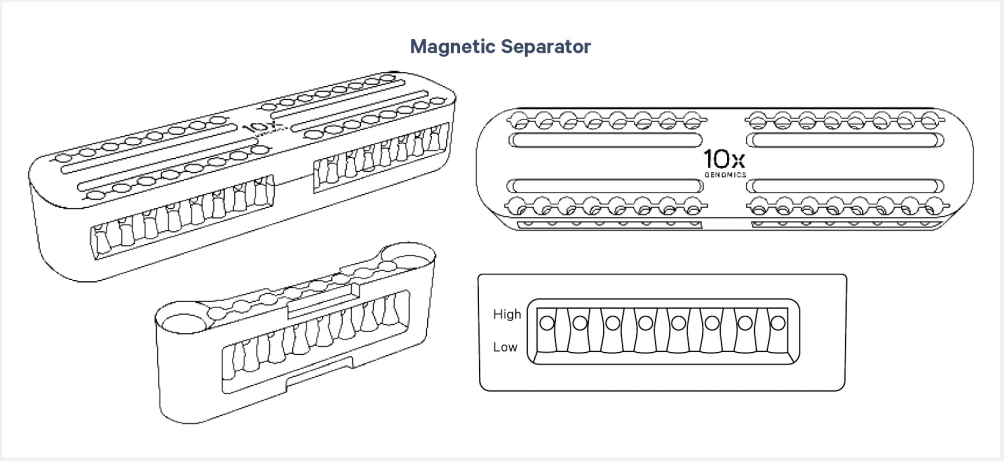
- Use aid when indicated in the protocol.
- Marks drawn by tracing the "mark guides" shown in the image above on the back of the slide using the aid will not affect downstream CytAssist

imaging.

- Before use:
  - Ensure back of tissue slide is dry.
  - If a coverslip is present, remove excess mounting medium by gently touching the slide to a lint-free laboratory wipe.
  - Clean aid with 70% isopropanol or 70% ethanol.
  - Obtain a permanent, ultrafine-tipped, dark-colored marker.
- During use:
  - If a coverslip is present, do not move coverslip.
  - Draw multiple passes for each mark.
  - Allow ink to dry for ~5 min.
- Once marks are drawn, avoid wiping the back of the slide vigorously to prevent mark removal. Instead, tap the back of the slide onto a lint-free laboratory wipe to remove excess moisture.

10x Magnetic Separator

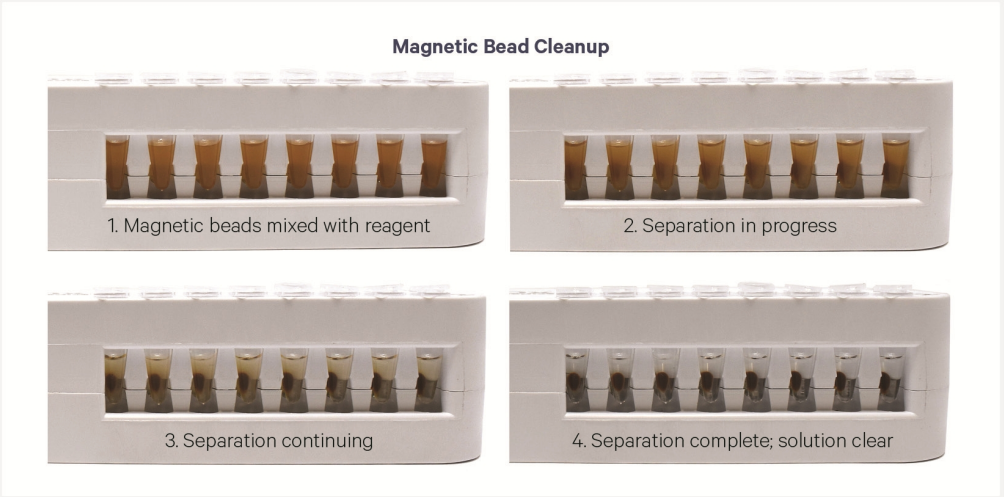
- Images below are illustrative - actual appearance of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.



- 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

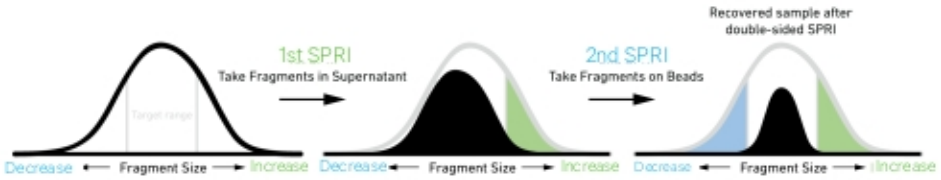
- Use fresh preparations of 80% Ethanol.
- Ensure SPRI beads are not expired and are thoroughly mixed before use.
- After aspirating the desired volume of SPRIselect reagent, examine the pipette tips before dispensing to ensure the correct volume is transferred.
- Pipette mix thoroughly as insufficient mixing of sample and SPRIselect reagent will lead to inconsistent results.
- Ensure solution is completely clear before removing supernatant.

Tutorial — SPRIselect Reagent: DNA Sample Ratios

SPRI beads selectively bind DNA according to the ratio of SPRIselect reagent (beads).

$$\text{Example Ratio: } \frac{\text{= Volume of SPRIselect reagent added to the sample}}{\text{Volume of DNA sample}} \quad \frac{\text{= 50 µl}}{\text{100 µl}} \quad \text{= } \mathbf{0.5X}$$

Schematic of Double-Sided Size Selection



After the first SPRI, supernatant is transferred for a second SPRI while larger fragments are discarded (green). After the second SPRI, fragments on beads are eluted and kept while smaller fragments are discarded (blue). Final sample has a tight fragment size distribution with reduced overall amount (black).

Tutorial — Double-Sided Size Selection

Step a –First SPRIselect: Add 60 µl SPRIselect reagent to **100 µl** sample (**0.6X**).

$$\text{Ratio:} \quad \frac{\text{= Volume of SPRIselect reagent added to the sample}}{\text{Volume of DNA sample}} \quad \frac{\text{= 60 µl}}{\text{100 µl}} \quad \text{= 0.6X}$$

Step b – Second SPRIselect: Add **20 µl** SPRIselect reagent to supernatant from step a (**0.8X**).

$$\text{Ratio:} \quad \frac{\text{= Total Volume of reagent added to the sample (step a + b)}}{\text{Original Volume of DNA sample}} \quad \frac{\text{= 60 µl + 20 µl}}{\text{100 µl}} \quad \text{= } \mathbf{0.8X}$$

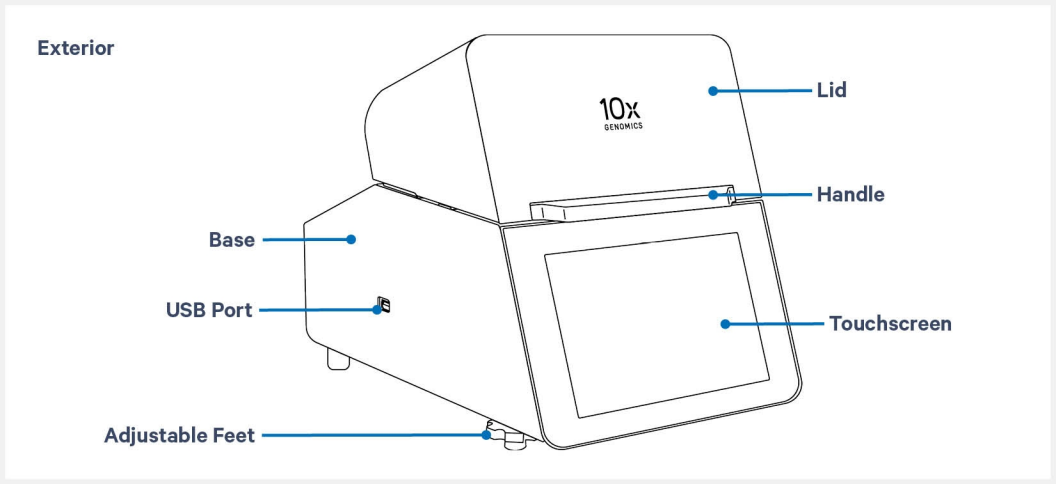
Sample Indices (i5/i7) in Sample Index PCR

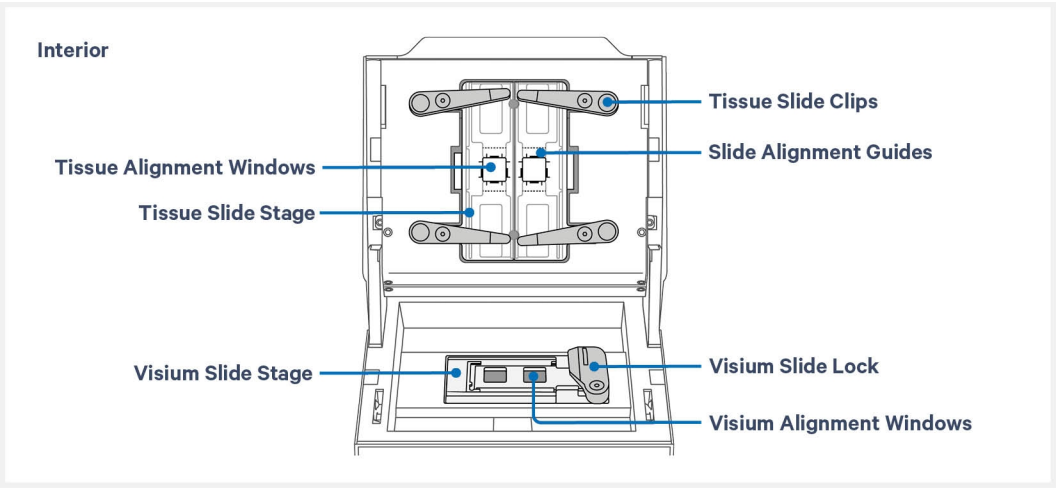
- Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run.
- Each well in the Dual Index Plate contains a unique i7 and a unique i5 oligonucleotide.
- Record the index plate well location for each sample.

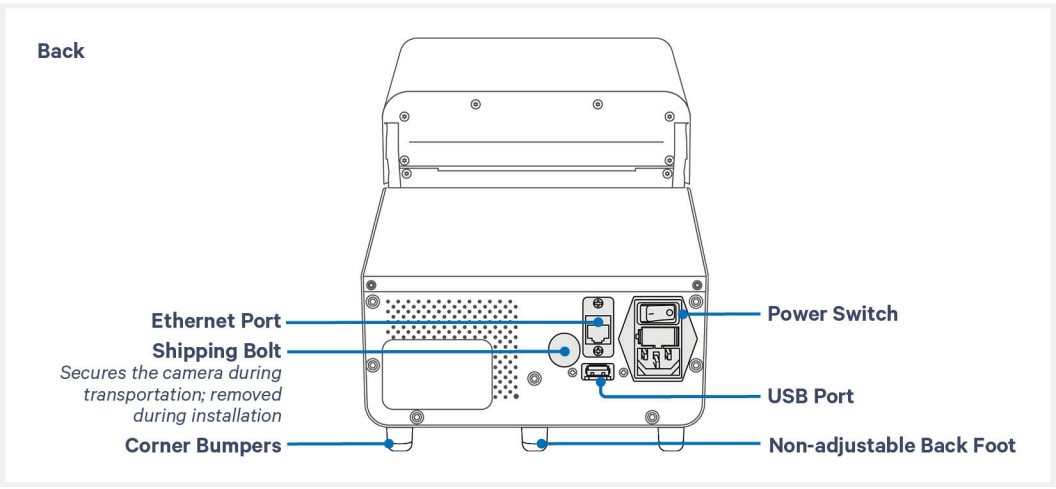
Visium CytAssist

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 3' Spatial Gene Expression Protocol Planner (CG000803).
- 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.

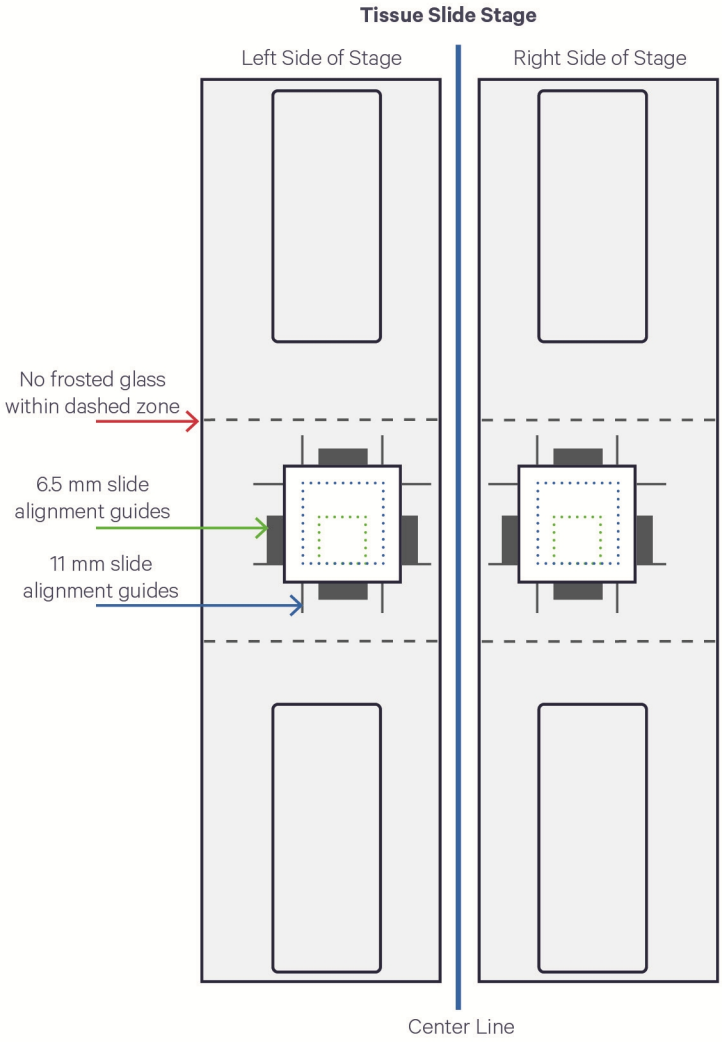
Visium CytAssist Alignment Aid

- The Visium CytAssist Alignment Aid is an optional accessory used to draw annotations onto the back of the tissue slide to assist with alignment onto the Visium CytAssist instrument. See Visium CytAssist Alignment Aid on page 31 for instructions.

Determine Slide Placement

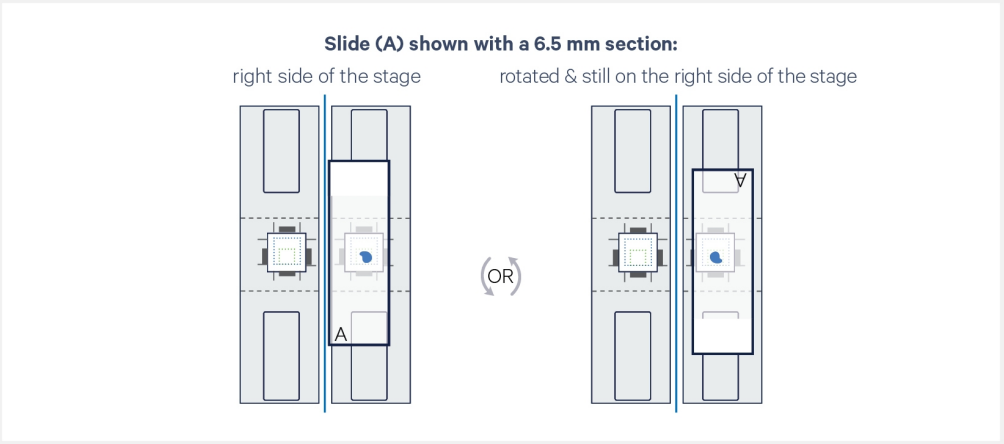
a. On the diagram below, align tissue to the center of the alignment guides for 6.5 mm (rectangles) or 11 mm (lines) Capture Areas on either the left or right side of the stage. Align the center of the tissue region of interest to the center of the Capture Area, instead of aligning the edge of the region to the edge of the Capture Area. If the Alignment Aid was used, use the annotations to assist with aligning. See Visium CytAssist Alignment Aid on page 31 for instructions.

If printing the image, select "actual size" or "100%" to print to scale. The blue Capture Area should measure 11 mm and the green capture area should measure 6.5 mm.



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.

b. 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. Frosted glass should not be within the dashed zone of the tissue slide stage.

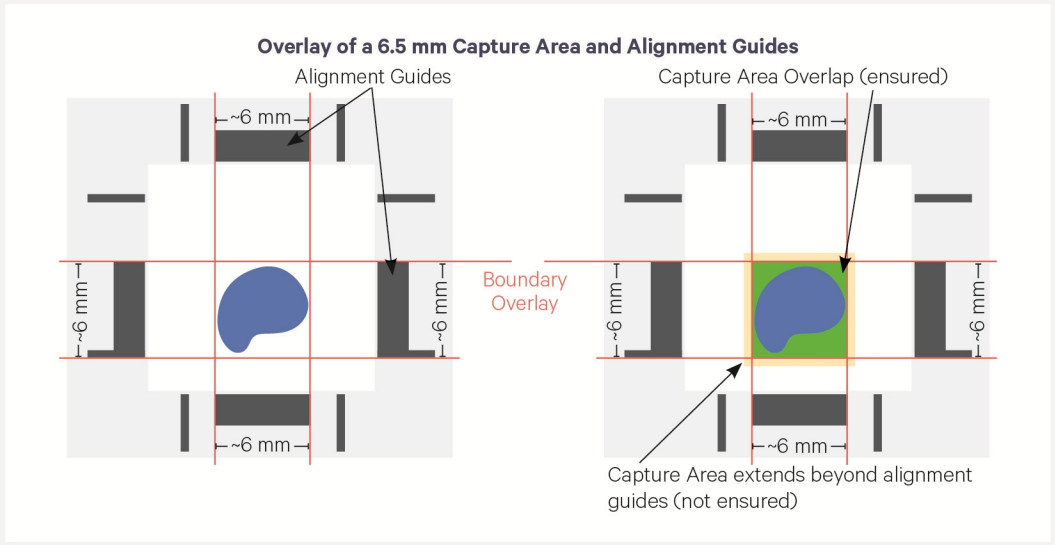


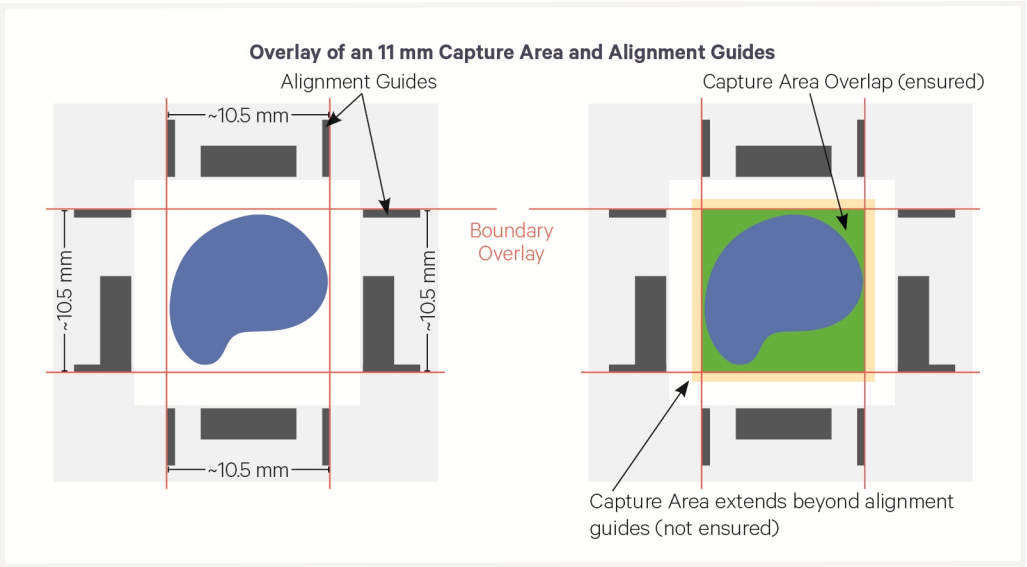
Fine Adjustments

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 3' Spatial Gene Expression Assay is only compatible with 6.5 mm Capture Areas.

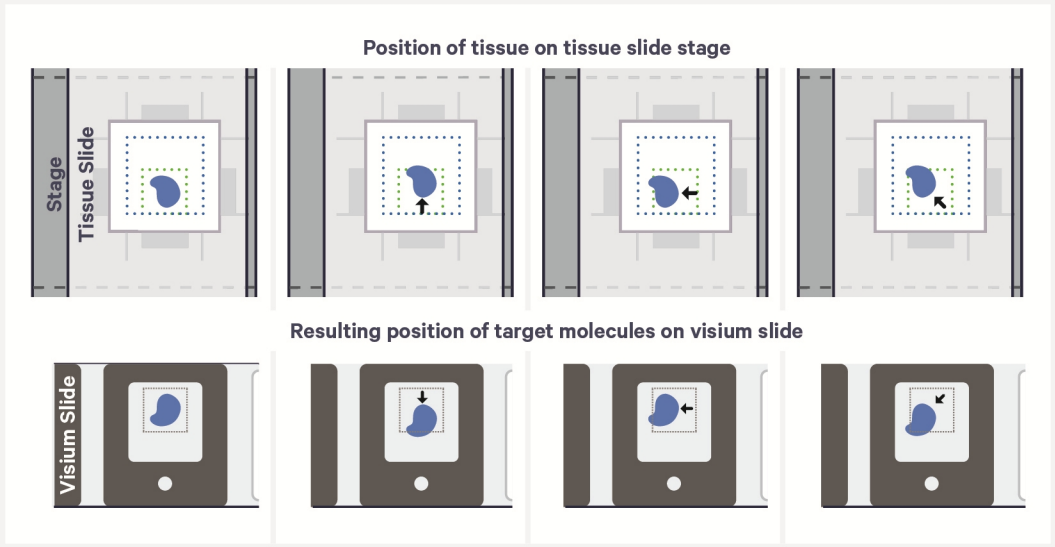
When placing a tissue slide on the Tissue Slide Stage, center the tissue or area of interest within the area inside the outer edges of the alignment guides (area in green in the images below).

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 below), but it is not ensured. 10x Genomics recommends placing high-priority tissue areas within the alignment guides.





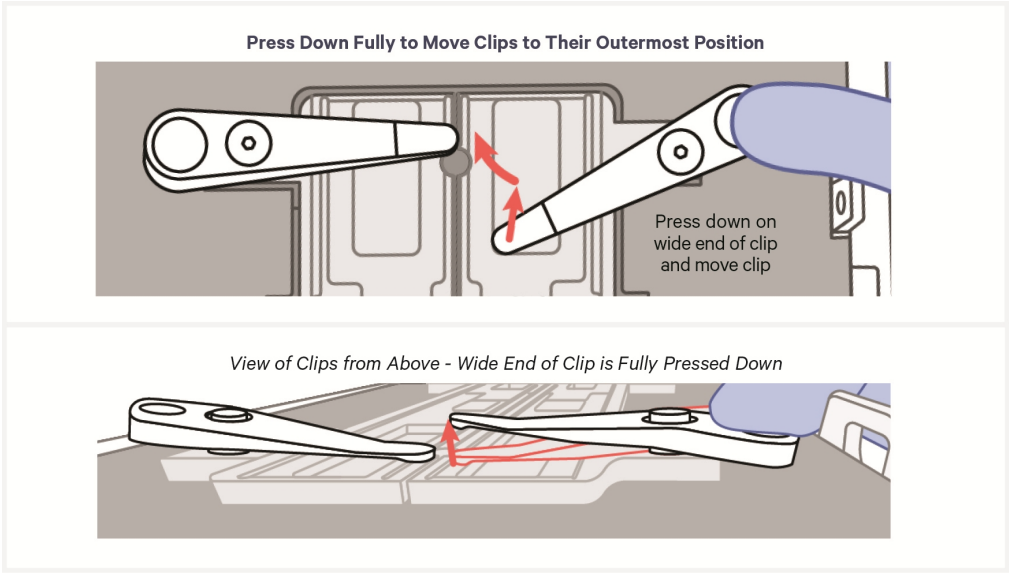
The image below demonstrates how movement of the Tissue Slides affects where target molecules will end up on the Visium HD Slide.



Tissue Slide Loading

a. Press down fully on the wide end of each clip to lift and rotate the narrow end to its outermost position.

Cradle the instrument lid for increased stability and fully press down on the clips with the thumb. See image below for extent of clip rotation.



b. Lay the tissue slide flat on the stage surface and against the center line. If necessary, rotate the slide 180° to better place off-center tissues closer to the center line. Slides should not cross/overlap the center line.

DO NOT touch the tissue section.

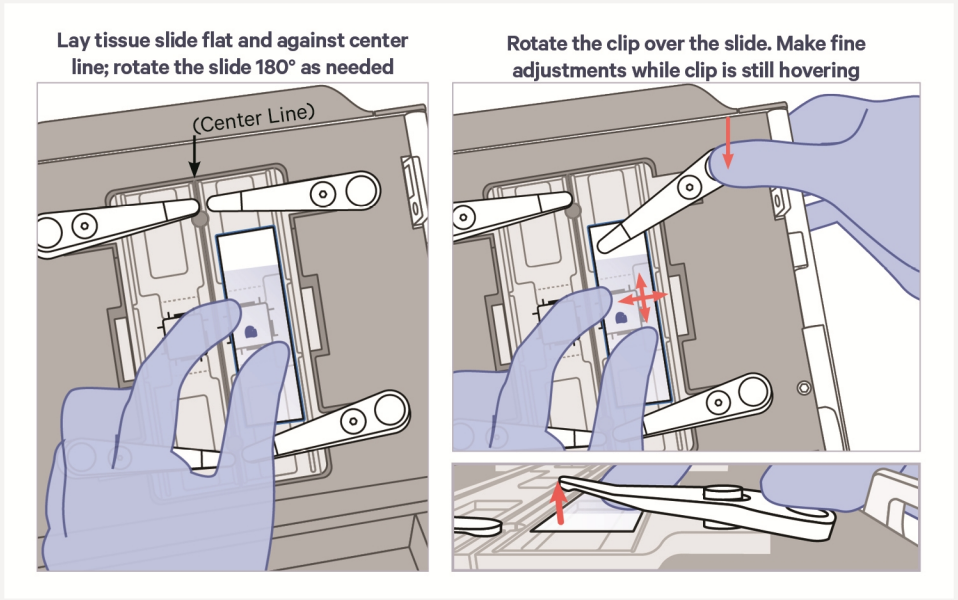
c. Use one hand to hold slide in place with two fingers, one on either side of the tissue. Use the other to both cradle the instrument lid and fully press down on the first clip. Rotate the clip over the slide.

d. While clip is hovering over the slide, make fine adjustments to align the tissue or area of interest to the center of the alignment guides. Gently release when adjustments are complete. If annotations were drawn onto the back of the tissue slide using the Visium CytAssist Alignment Aid, these annotations should be lined up with the relevant alignment guides on the Tissue Slide Stage. See instructions at the beginning of this section for more information.

DO NOT adjust slide while tissue clip is touching the slide, which may result in slide damage. DO NOT touch the tissue section.

DO NOT place clips on the edge of the slide, which may result in slide damage.

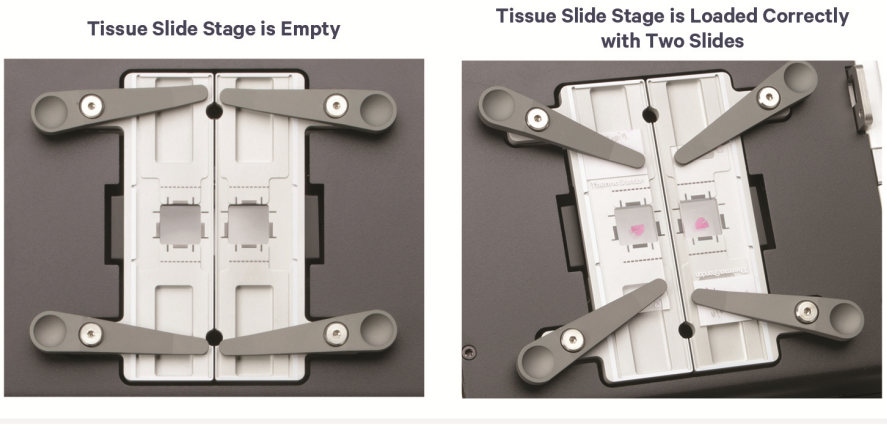
Reference images below for steps b-d.

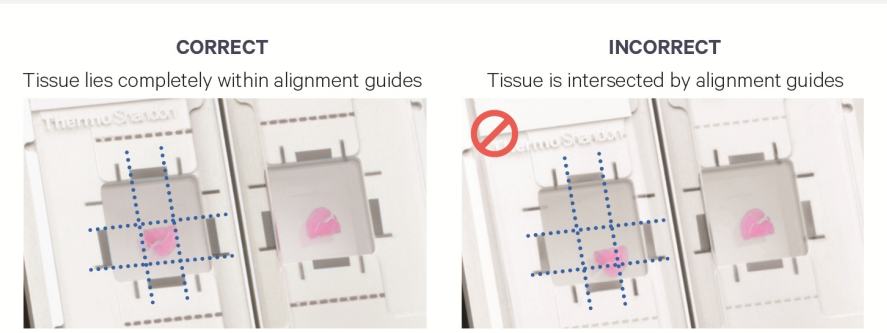


e. Continue to hold the slide in place while pressing down fully on the wide end of the second clip. Rotate the second clip onto the slide and gently release.

Use both clips whenever possible. The second clip may be omitted if the tissue slide is beyond its reach.

f. 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

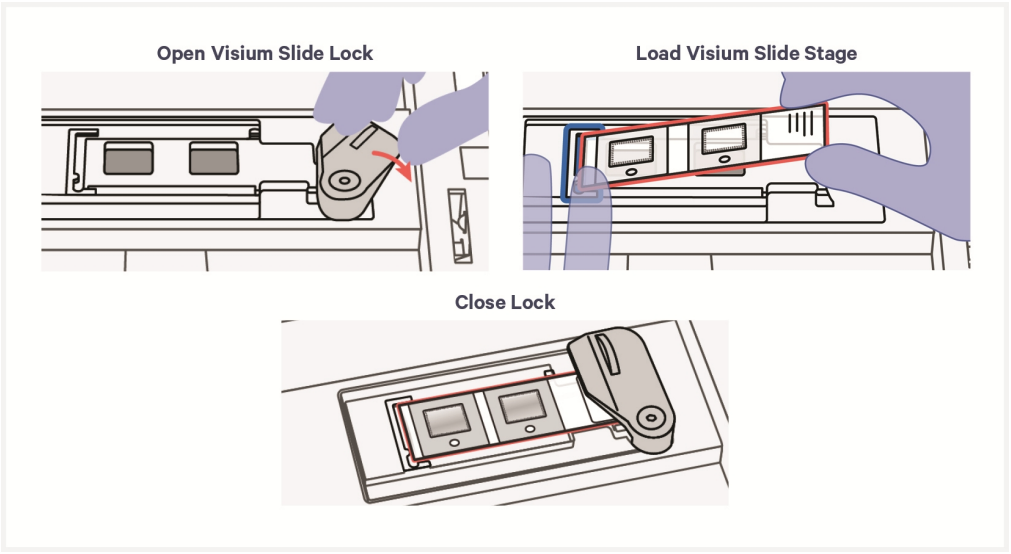
Visium HD Slide Loading

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).

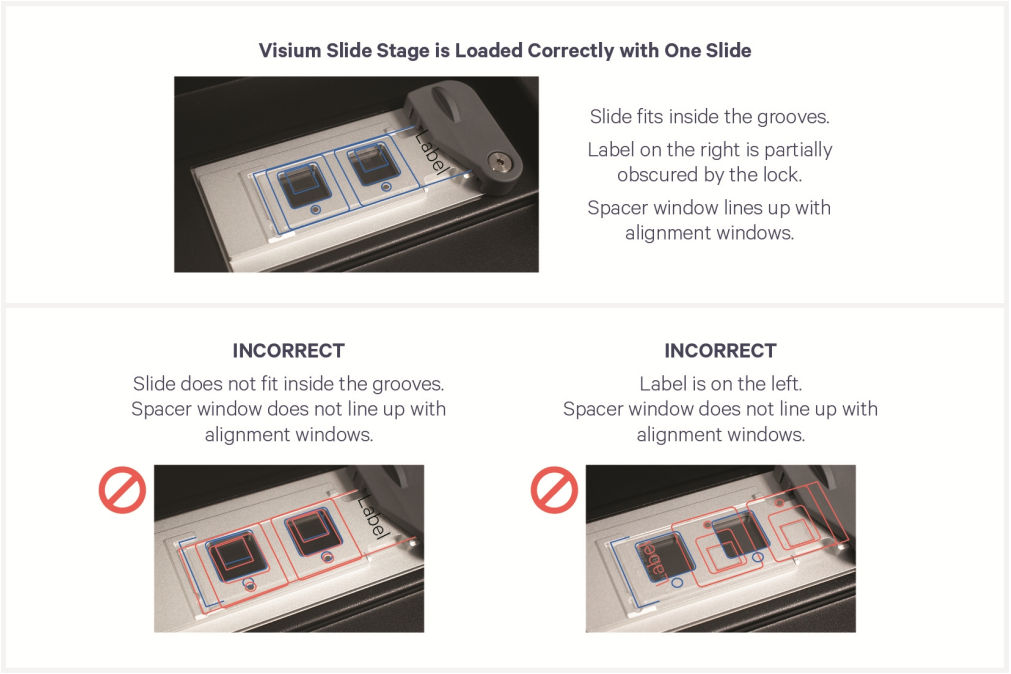
a. Open the Visium Slide Lock by using one finger to pivot.

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).



10x GENOMICS

Sample Preparation & Staining Guidelines

Sample Preparation

Proper tissue handling and preparation techniques preserve the morphological quality of the tissue sections and the integrity of RNA 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 documentation as described in the Workflow Overview on page 13. Listed below are key considerations described in the tissue preparation documentation.

Key Considerations for FF Samples	
Freezing and Embedding	
☐	Perform either separate or simultaneous tissue freezing & embedding.
☐	Store frozen samples in a sealed container at -80°C for long-term storage.
Slide Handling	
☐	Equilibrate slides to cryostat temperature before cryosectioning.
Cryosectioning	
☐	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.
☐	Assess RNA quality of the tissue block.
☐	Optional - assess tissue morphology via H&E staining.
Tissue Slide Handling	
☐	Keep slides cold and transport slides on dry ice.
☐	Store tissue slides in a slide mailer at -80°C for up to four weeks.
Tissue Slide Processing	
☐	After preparing tissue slides, follow the remaining steps in the Visium HD 3' FF Tissue Preparation Handbook (CG000804). The handbook contains information on tissue slide staining and imaging.

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Step 1:

Visium HD Slide & CytAssist Preparation

1.0 Get Started

The Visium CytAssist instrument requires firmware version 2.0.0 or higher for this protocol.

Clean workspaces, equipment, and gloves with an RNase decontamination solution before beginning the assay.

Items		10x PN	Preparation & Handling	Storage
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	20X SSC	-	-	Ambient
☐	Visium Cassette	Component: 2001252 or 3002329/2001344 or 3002328 Kit: 1000847	See Cassette Assembly Quick Reference Card (CG000730)	Ambient
Equilibrate to Room Temeprature				
☐ ●	Pre-equilibration Buffer	Tube: 2001399 Kit: 1000854	Thaw at room temperature, vortex, centrifuge briefly.	–20°C

1.1 Visium HD Slide Wash

a. 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 min - 3 h**. Prepare one Visium HD Slide at a time. For information on processing more than two Visium HD Slides, see Appendix on page 120.

DO NOT touch Visium HD Slide spacer during slide washes.

b. Prepare 0.1X SSC Buffer according to the table below in **two** 50-ml centrifuge tubes for a total of 80 ml of 0.1X SSC. Vortex. Maintain at room temperature. 0.1X SSC prepared at this step is sufficient for remaining steps in the protocol.

0.1X SSC Buffer	Stock	Final	1 Visium HD Slide + 15% (ml)*
Nuclease-free Water	-	-	39.8
SSC	20X	0.1X	0.2
Total	**-**		**40.0**

**Volumes are in ml instead of µl*

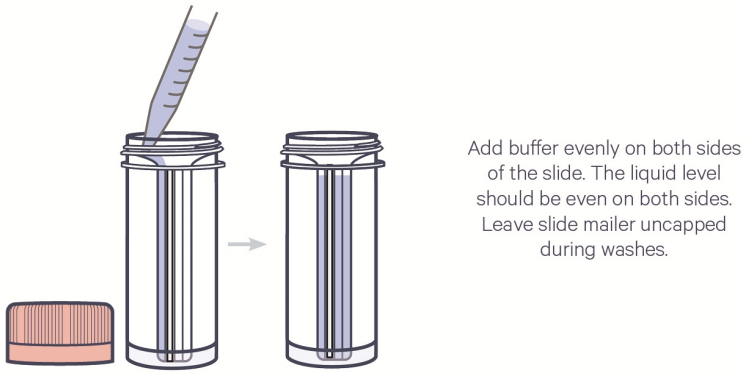
c. Prepare Pre-equilibration Mix according to the table below. Pipette mix and centrifuge briefly. Maintain at room temperature.

	Pre-equilibration Mix	10x PN	1 Visium HD Slide + 10% (µl)
	Nuclease-free Water	-	55
●	Pre-equilibration Buffer	2001399	55
	Total	**-**	**110**

d. Open slide mailer.

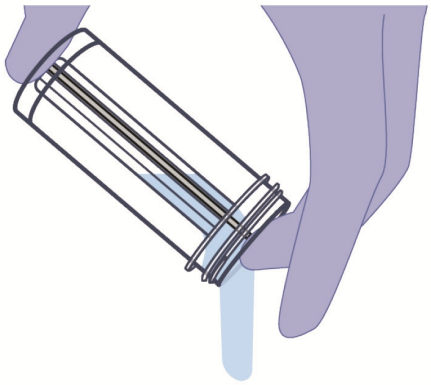
e. Add **7 ml** 0.1X SSC Buffer slowly along the side of the mailer on one side of the slide.

f. 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.



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

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



Three 0.1X SSC 5 min washes:

i. **5 Min Wash 1:**

- Add **7 ml** 0.1X SSC Buffer slowly along the side of the mailer on one side of the slide.
- 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.
- Incubate at **room temperature** for **5 min**.
- Remove 0.1X SSC Buffer by securing slide in place with one finger and pouring SSC Buffer from mailer.

j. 5 Min Wash 2:

- Add **7 ml** 0.1X SSC Buffer slowly along the side of the mailer on one side of the slide.
- 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.
- Incubate at **room temperature** for **5 min**.
- Remove 0.1X SSC Buffer by securing slide in place with one finger and pouring SSC Buffer from mailer.

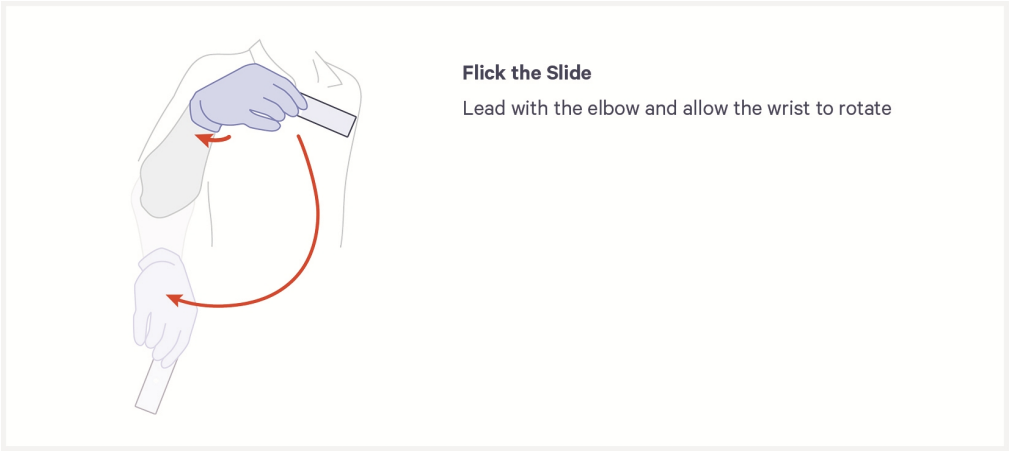
k. 5 Min Wash 3:

- Add **7 ml** 0.1X SSC Buffer slowly along the side of the mailer on one side of the slide.
- 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.
- Incubate at **room temperature** for **5 min**.
- Remove Visium HD Slide from mailer.

During the last wash, save SSC Buffer in the mailer in case Visium HD Slide needs additional immersion.

l. Inspect the Visium HD Slide for debris. If debris is visible, immerse the slide back in the mailer containing SSC and quickly remove.

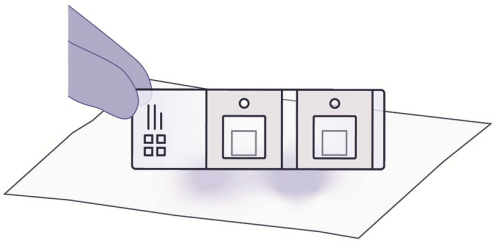
m. Flick the Visium HD Slide.



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

o. Place a fresh, lint-free laboratory wipe on the work surface.

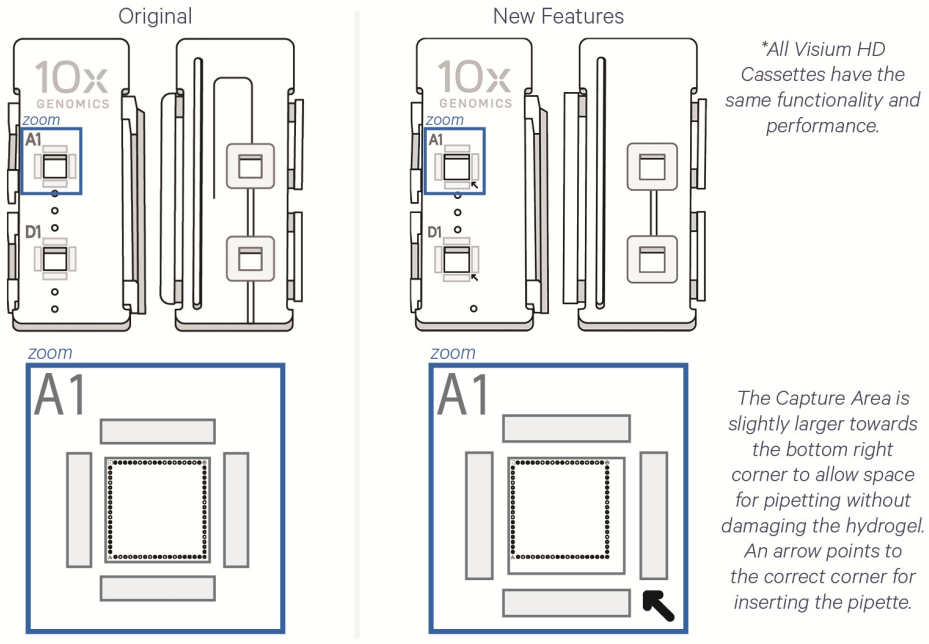
p. Gently touch long edge of Visium HD Slide on a lint-free laboratory wipe 5x to remove excess SSC buffer.



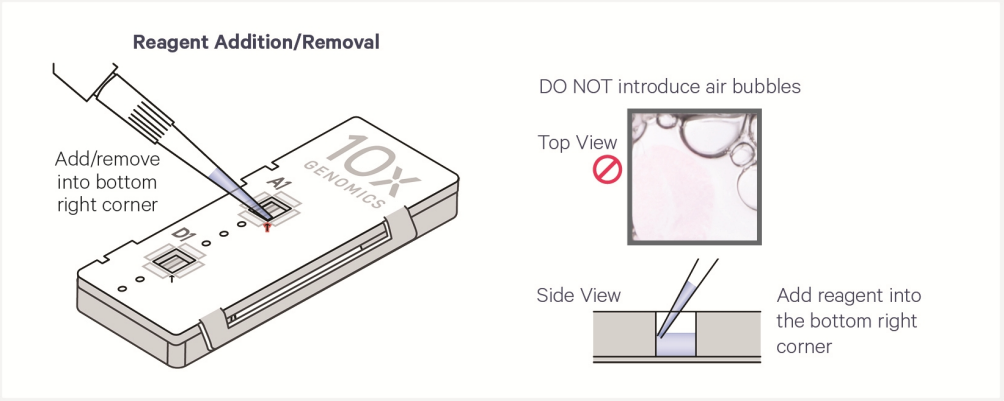
q. Inspect the Visium HD Slide and ensure it is free of particulate matter. If debris is visible, immerse the slide back in the mailer containing SSC and quickly remove. Repeat flicking and wiping as previously described.

r. Record Visium HD Slide serial number.

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



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



u. Remove 0.1X SSC from each well in the cassette containing the Visium HD Slide.

v. Repeat 0.1X SSC removal to ensure all 0.1X SSC is removed from the cassette.

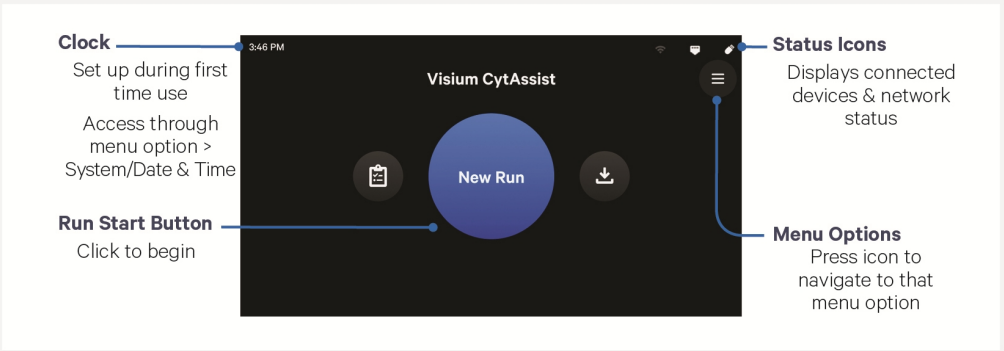
w. Add **50 µl** Pre-equilibration Mix to each well in the cassette.

DO NOT exceed 60 min before proceeding with a CytAssist run.

x. Apply a new Visium Slide Seal on the Visium Cassette.

y. 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.



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

aa. 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.

A barcode scanner may be used to scan the Visium HD Slide for automatic serial number input prior to cassette assembly. See Visium HD 3' Spatial Gene Expression Protocol Planner (CG000803) for compatible part numbers. The barcode scanner cannot scan the Visium HD Slide after cassette assembly, as the QR code will not be visible.

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

ab. Enter the following sample information:

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

ac. Proceed immediately to Step 2: Coverslip Removal & Destaining on page 55.

10x GENOMICS

Step 2:

Coverslip Removal & Destaining

2.0 Get Started

Items		10x PN	Preparation & Handling	Storage
Obtain				
☐	0.1 N HCl	-	If necessary, prepare 0.1 N HCl using nuclease-free water. Dispense 10 ml 0.1 N HCl into HCl mailer. Label slide mailer as HCl mailer. If using an alternate container, ensure volume will cover tissue. Volume is enough for two tissue slides. DO NOT reuse HCl.	Ambient
☐	1X PBS	-	If necessary, prepare 1X PBS fresh using nuclease-free water. Dispense 10 ml 1X PBS into PBS mailer. If using an alternate container, ensure volume will cover tissue. Volume is enough for two tissue slides. DO NOT reuse 1X PBS.	
☐	Nuclease-free Water	-	-	Ambient
☐	Low TE Buffer	-	-	Ambient
☐	1 L Beaker	-	Beaker will be cleaned per instructions within protocol.	Ambient
☐	RNase Decontamination Solution	-	-	Ambient
☐	70% Ethanol or 70% Isopropanol	-	-	Ambient
Equilibrate to room temperature				
☐	Perm Buffer	Tube: 2001398 Kit: 1000854	Thaw, vortex, verify no precipitate, centrifuge briefly prior to use.	–20°C

Before proceeding to the next step, prepare the reagents below that are required for 3.1 CytAssist-Enabled Poly(A) RNA Capture on page 64.

Items			10x PN	Preparation & Handling	Storage
Equilibrate to room temperature					
☐	○	Reducing Agent B	Tube: 2000087 Kit: 1000854	Thaw at room temperature, vortex, verify no precipitate, centrifuge briefly prior to use. Reducing Agent B is added to the Permeabilization Mix immediately before running the CytAssist instrument.	–20°C
☐	●	RT Reagent	Tube: 2000086 Kit: 1000855	Thaw at room temperature, vortex, verify no precipitate. After RT Reagent is thawed, move to ice. Centrifuge briefly prior to use.	–20°C
Place on ice					
☐	●	Perm Enzyme B	Tube: 3000553 Kit: 1000854	Pipette mix, centrifuge briefly prior to use. Perm Enzyme B is added to the Permeabilization Mix immediately before running the CytAssist instrument.	–20°C
☐	●	RT Enzyme G	Tube: 2001438 Kit: 1000855	Pipette mix, centrifuge briefly prior to use.	–20°C
☐	●	Template Switch Oligo B	Tube: 2001027 Kit: 1000855	Centrifuge briefly, resuspend in 65 µl **Low TE** Buffer. Vortex 15 sec at maximum speed, centrifuge briefly prior to use. Resuspended solution can be used immediately. After resuspension, store at –80°C. Thaw on ice for ≥30 minutes in subsequent uses.	–20°C

2.1 Coverslip Removal

If the Visium CytAssist Alignment Aid has not been used, use the Aid to draw alignment marks on the tissue slide prior to coverslip removal (if desired). See Visium CytAssist Alignment Aid on page 31 for more information.

a. Clean a 1 L beaker.

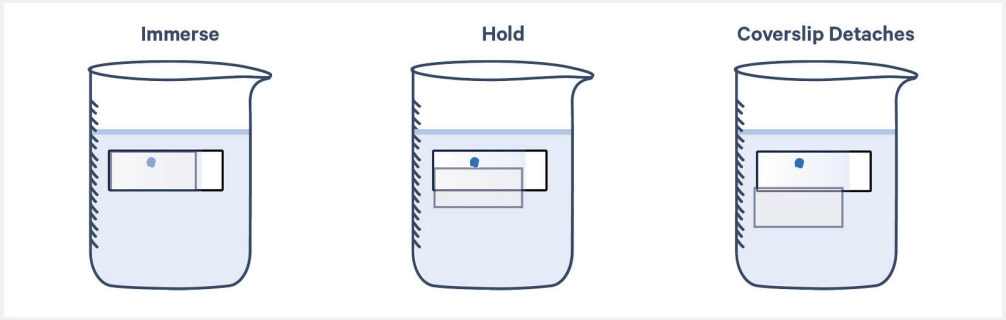
- Spray with RNase decontamination solution; leave for **10 sec** to **1 min**.
- Spray with 70% isopropanol or 70% ethanol.
- Rinse with Milli-Q water.

b. Dispense **800 ml** Milli-Q water in a beaker. Up to 10 slides may be processed using this beaker.

c. Immerse slides **sideways** in the beaker containing **800 ml** water with coverslipped surface fully sideways to prevent coverslip from dragging across tissue.

d. Hold slides in water until the coverslip slowly separates away from the slide.

To avoid tissue section damage or detachment, DO NOT move slide up and down, shake forcibly, or manually move coverslip.



e. Gently immerse slides 30x in water to ensure all Mounting Medium is removed.

f. Proceed **immediately** to Destaining.

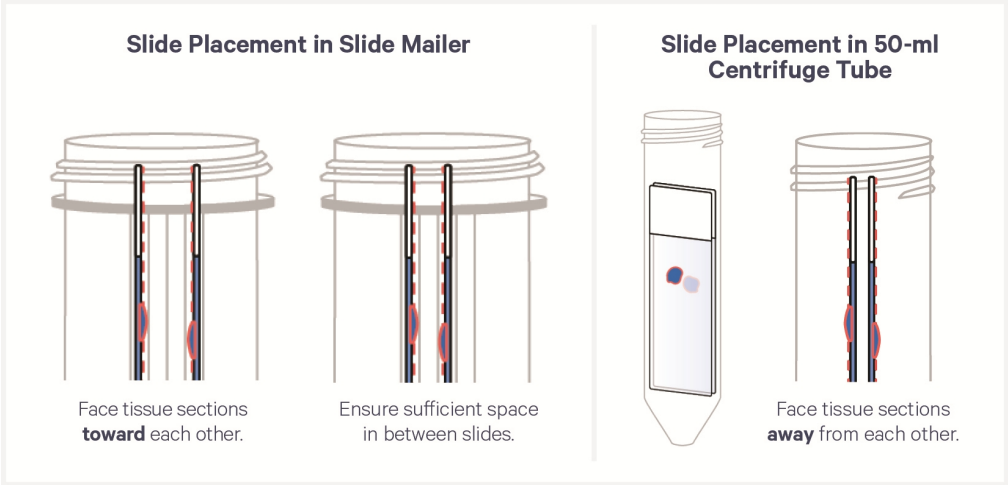
DO NOT allow slides to dry.

2.2 Destaining

a. Immerse slides 10x in 0.1 N HCl mailer.

TIPS

When immersing slides in solutions, ensure that tissue sections are completely submerged. If needed, use forceps to hold the slides. A 50-ml centrifuge tube may also be used for this step. Ensure that no more than two slides are processed in the centrifuge tube and that slides are placed back to back, such that tissue sections face away from each other. Ensure volume completely covers tissue sections.



b. Immerse slides in 0.1 N HCl mailer, close the mailer, and incubate for **15 min** at **room temperature**.

c. Prepare Permeabilization Mix during 0.1 N HCl incubation. Pipette mix thoroughly until solution is homogenous. Avoid generating bubbles. Maintain at room temperature.

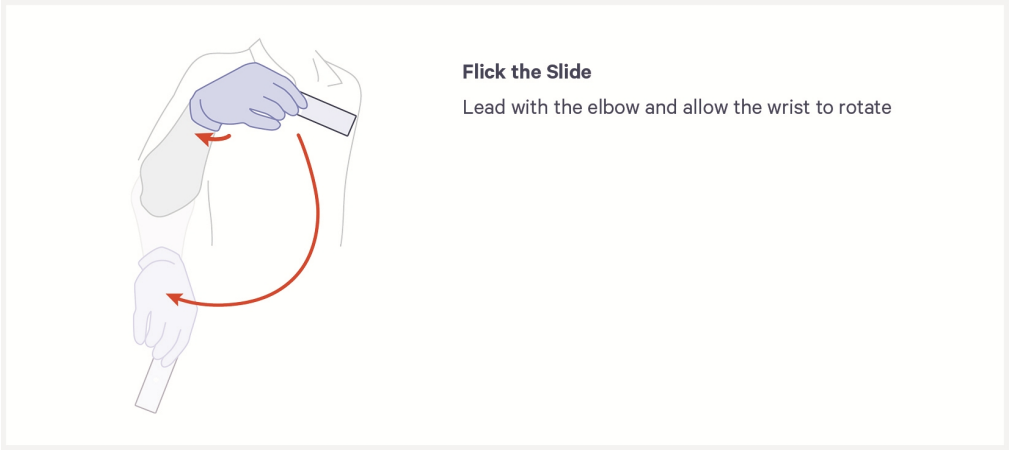
Permeabilization Mix will also require Perm Enzyme B and Reducing Agent B, which will be added at step 3.1k.

	Permeabilization Mix	10x PN	2 Tissue Slides (µl) (includes overage)
	Nuclease-free Water	-	10.4
●	Perm Buffer	2001398	20.0
	Total	**-**	**30.4**

d. Immerse slides 10x in 1X PBS mailer.

e. Immerse slides in 1X PBS mailer and incubate for **5 min** at **room temperature**.

f. Remove tissue slides from 1X PBS mailer and immediately flick to remove excess PBS.



g. Inspect the slide for the presence of any large droplets. If present, repeat flicking.

h. Once the slide has no liquid droplets on the tissue surface, remove any excess PBS with a lint-free laboratory wipe in areas outside of the tissue, without touching the tissue sections.

Lint-free laboratory wipes are strongly recommended to ensure no debris is introduced. See the Visium HD 3' Spatial Gene Expression Protocol Planner (CG000803) for a list of tested part numbers.

i. Place a fresh lint-free laboratory wipe on the work surface.

j. Gently tap the back of tissue slides onto a lint-free laboratory wipe.

k. Place slides on a lint-free laboratory wipe with tissue sections facing up on a flat, clean work surface.

l. Proceed immediately to 3.0 Get Started on page 62

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Step 3:

CytAssist-Enabled Poly(A) RNA Capture, Reverse Transcription, & Denaturation

3.0 Get Started

Some reagents required for this step should have been prepared in the prior section, 2.0 Get Started on page 56. Ensure that all reagents are prepared and ready.

Items	10x PN	Preparation & Handling	Storage
Obtain			
☐ Nuclease-free Water	-	-	Ambient
☐ 8M KOH	-	-	Ambient
☐ 20X SSC	-	-	Ambient
☐ Qiagen Buffer EB	-	Manufacturer's Recommendation	Ambient
☐ Visium Slide Seals	Component 2000283 Kit: 1000847	See Tips & Best Practices.	Ambient

Overview of Timing for Step 3: CytAssist-Enabled Poly(A) RNA Capture, Reverse Transcription & Denaturation *(follow the instructions in numerical order)*

	Slides	Reagents	Instruments
~15 min	**1.** Transfer Visium HD Slide to Dry on CytAssist		
	2. Load Tissue Slides on CytAssist	**3.** Complete Perm Mix	
Complete within 5 min	**4.** Dispense Perm Mix on Visium HD Slide		**5.** Close Lid & Start CytAssist Run *~30 min total run time*
~5 min		**7.** Prepare RT Master Mix 1	**6.** Prepare Thermal Cycler for RT 1
~5 min	**9.** Five SSC Washes for Visium HD Slide		**8.** CytAssist Run Complete *Wait until safe stopping point to unload & clean CytAssist*
~50 min	**10.** Add RT MM 1 to Visium HD Slide in Cassette		**11.** Start Thermal Cycler for RT 1
		13. Prepare RT Master Mix 2	**12.** RT 1 Complete
~40 min	**15.** Add RT MM 2 to Visium HD Slide in Cassette		**14.** Prepare Thermal Cycler for RT 2
		17. Prepare KOH	**16.** Start Thermal Cycler for RT 2
	19. Denaturation using KOH		**18.** RT 2 Complete

Proceed to Step 4: Second Strand Synthesis & Elution, for subsequent steps

3.1 CytAssist-Enabled Poly(A) RNA Capture

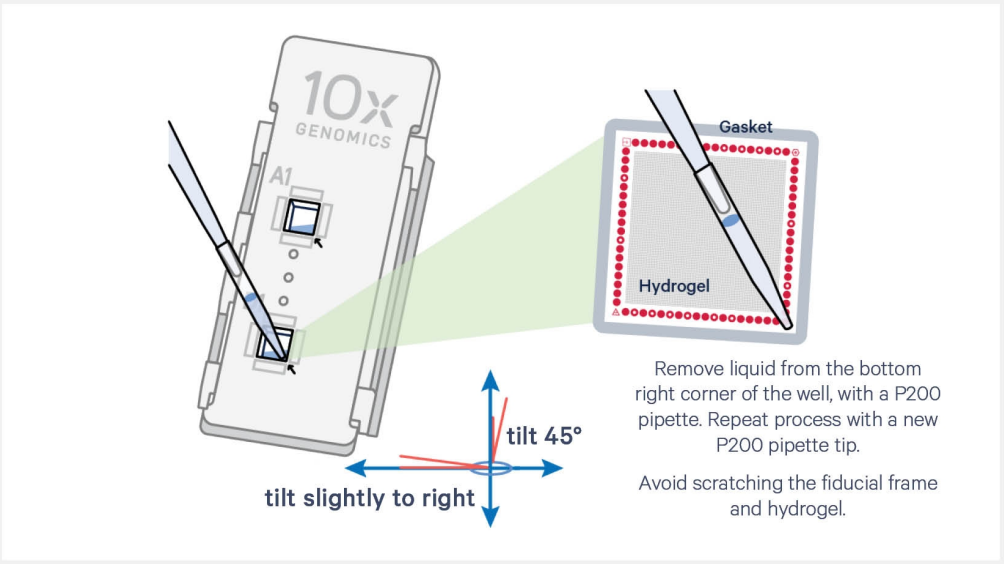
Visium HD Slide Steps

a. Retrieve Visium Cassette with Visium HD Slide.

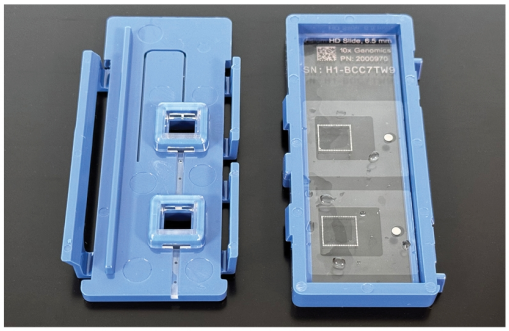
b. Remove Visium Slide Seal and perform the following:

- Hold the cassette at a 45º angle slightly tilted to the right so that the buffer pools to the lower right hand corner of the well.
- Set a P200 pipette to 200 µl.
- Place the pipette tip at the bottom right corner of the well without scratching the fiducial frame or hydrogel.
- Remove Pre-equilibration Mix from the well.
- Repeat this process using a new P200 tip.
- Once all the liquid has been removed, repeat for the remaining well.
- See image below for proper liquid removal technique.

Failure to remove Pre-equilibration Mix completely may delay Visium HD Slide drying and result in reduced assay performance.



c. 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 up, as shown in the image below. This minimizes the risk of introducing debris on the top half of the cassette after disassembly. Avoid introducing debris to the cassette after disassembly. See Visium Cassette S3 Quick Reference Card (CG000730) for more information on cassette disassembly.

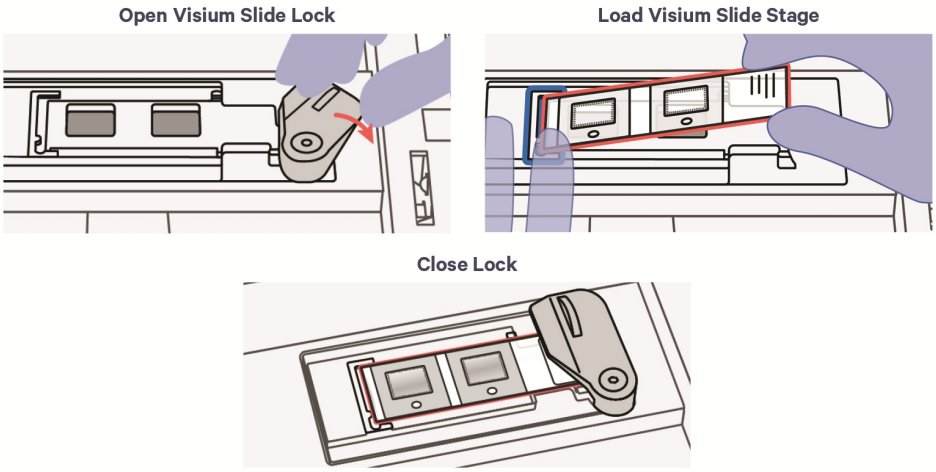


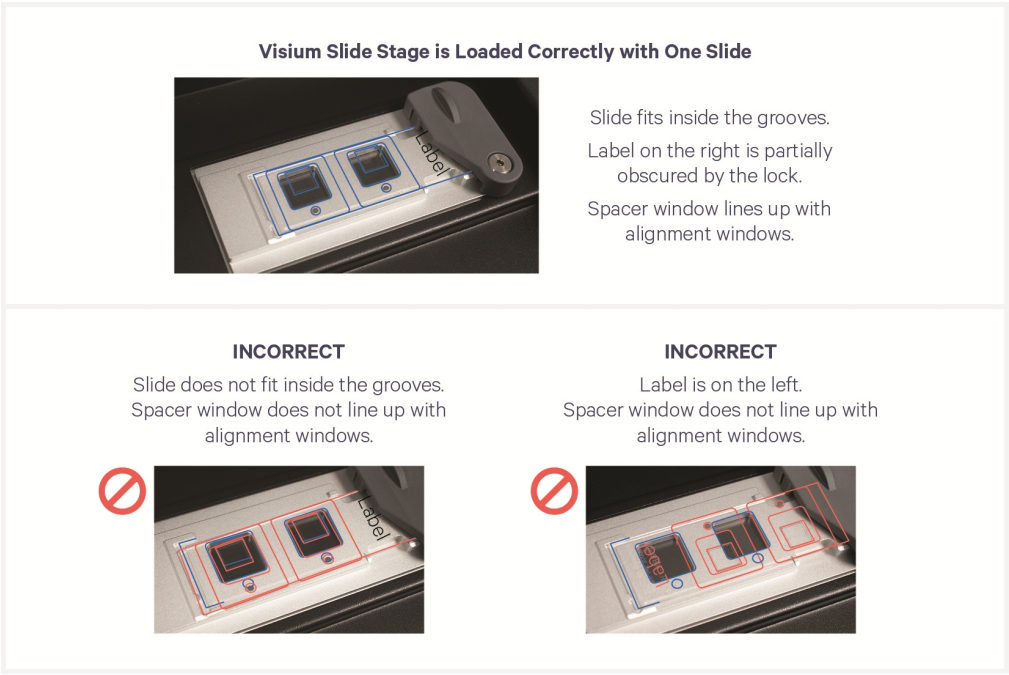
d. Remove Visium HD Slide from Visium Cassette and inspect the slide (avoid touching active surface). If necessary, remove excess moisture from the back of the slide with a lint-free laboratory wipe.

DO NOT flick the slide.

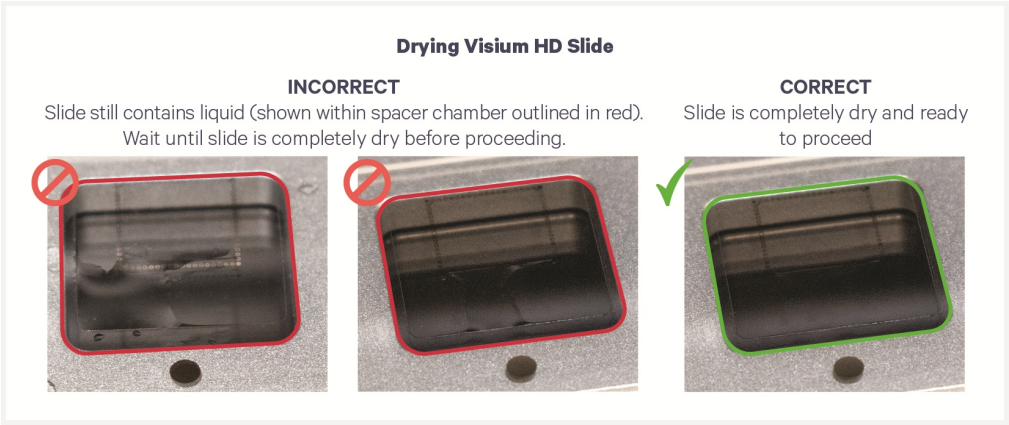
Save Visium Cassette for use after the instrument run, keeping all parts free from debris. Ensure back of Visium HD Slide is dry.

e. 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.





f. Allow Visium HD Slide to dry on the Visium Slide Stage for **10 min.** Inspect entire spacer chamber. Look at the slide at different angles to confirm the presence of liquid. If liquid remains on the slide or anywhere in the spacer chamber, continue drying and proceed immediately when no liquid remains within the spacer chamber. Ensure area around the Visium CytAssist instrument is free from debris.



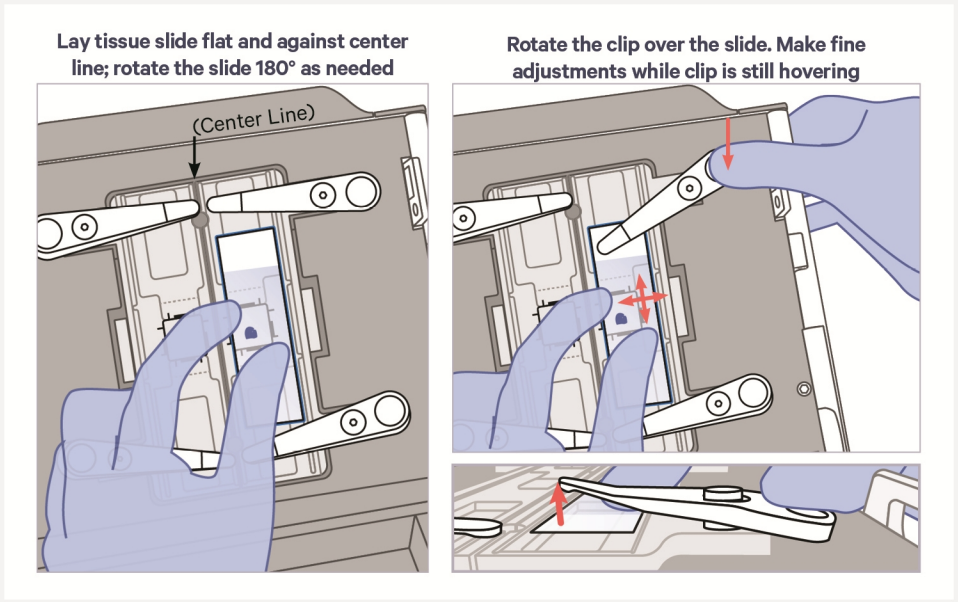
While the Visium HD Slide is drying, prepare and align tissue slides as described in the next section.

Tissue Slide Steps

g. Retrieve tissue slides.

h. Gently tap the back of tissue slides onto a lint-free laboratory wipe. If the Alignment Aid was used, avoid vigorously wiping the back of the slide to prevent removal of marks. Instead, tap the back of the slide onto a lint-free laboratory wipe to remove excess moisture.

i. Load tissue slides into the Visium CytAssist instrument, ensuring that the frosted areas of the tissue slide are not within the dashed zone on the tissue slide stage. Ensure tissue section is completely dry prior to instrument run. Examine the aligned tissue slide from multiple angles to ensure that tissue is completely dry. See Instrument Loading Guidelines on page 37 for more information.



***Before proceeding to next step**, ensure that Visium HD slide and tissue slides are completely dry.*

Reagent Addition & Instrument Run Initialization

j. Pipette mix Perm Enzyme B (PN-3000553) and vortex Reducing Agent B (PN-2000087). Centrifuge both briefly.

k. Add **1.6 µl** of Reducing Agent B and **8 µl** of Perm Enzyme B to **30.4 µl** of Permeabilization Mix to complete Permeabilization Mix. Pipette mix 15x with pipette set to 30 µl. Avoid generating bubbles. Centrifuge for **5 sec**.

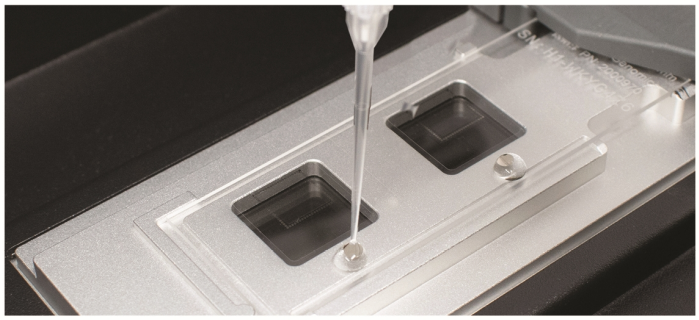
*The time between adding Perm Enzyme B and Reducing Agent B to Permeabilization Mix and starting the Visium CytAssist instrument run should be less than **5 min**.*

	Permeabilization Mix	10x PN	2 Tissue Slides (µl) (includes overage)
	Nuclease-free Water	-	**Added during Destaining**
●	Perm Buffer	2001398	**Added during Destaining**
○	Reducing Agent B	2000087	1.6
●	Perm Enzyme B	3000553	8
	Total	**-**	**40**

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

m. Slowly dispense **17 µl** of Permeabilization 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

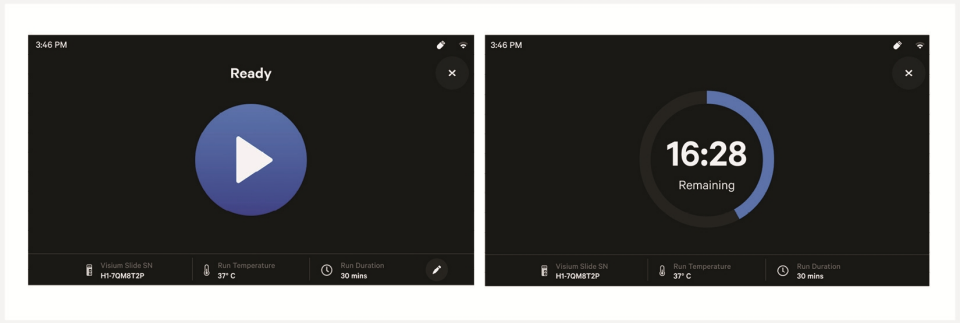


n. Close lid and press Next.

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

o. 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.



- A yellow warning sign before a run begins indicates a user-recoverable error. If an error occurs, follow on-screen prompts.

p. Place a Low Profile Thermocycler Adapter in the thermal cycler. Prepare a thermal cycler with the following incubation protocol for Reverse Transcription 1 and start the program.

Reverse Transcription 1		
Lid Temperature	**Reaction Volume**	**Run Time**
53°C	100 µl	48 min
Step	**Temperature**	**Time**
Pre-equilibrate	53°C	Hold
Reverse Transcription 1	53°C	00:45:00
Cool	4°C	00:03:00
Hold	4°C	Hold

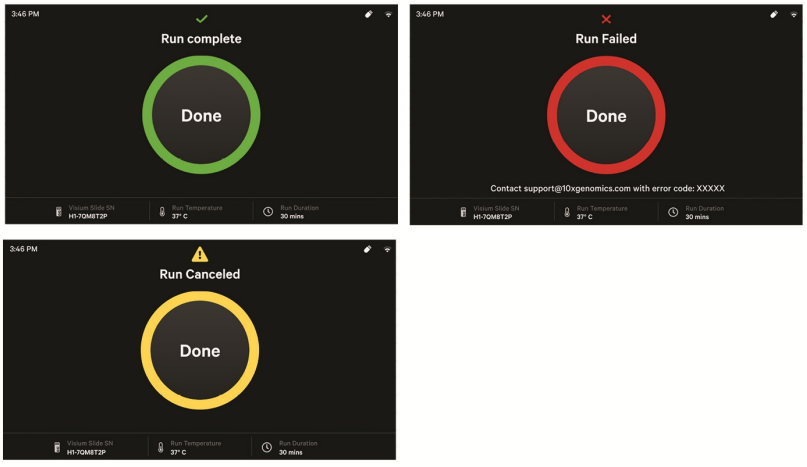
q. Prepare RT Master Mix 1 on ice. Pipette mix 10x and centrifuge briefly. Maintain on ice.

A separate master mix will be prepared for Reverse Transcription 2 at a later time using the same stock reagents.

	RT Master Mix 1 *Add reagents in the order listed.*	PN	1X (µl)	1 Visium Slide (µl) (two reactions, includes overage)
	Nuclease-free Water	-	41.7	91.7
●	RT Reagent	2000086	17.5	38.5
○	Reducing Agent B	2000087	1.4	3.1
●	Template Switch Oligo B	2001027	4.9	10.8
●	RT Enzyme G	2001438	4.5	9.9
	Total	**-**	**70.0**	**154.0**

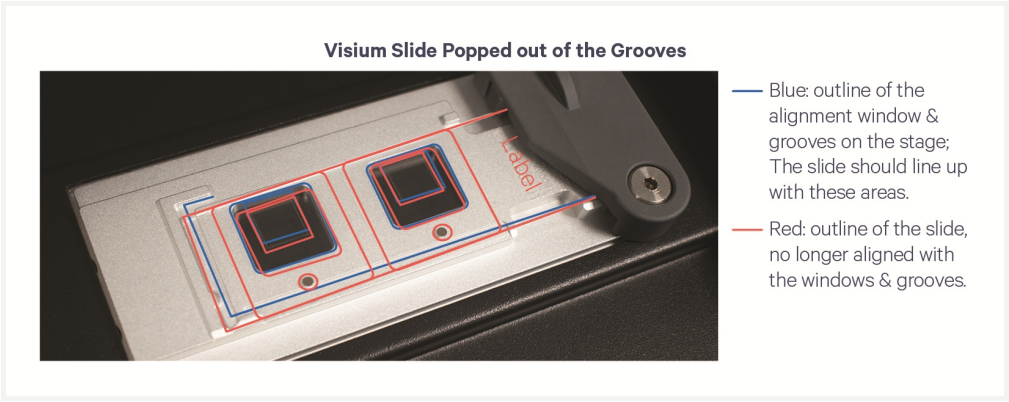
r. At the end of a run, the button will display "Done" and a "Run Info" tab at the bottom of the screen. DO NOT allow sample to sit in the Visium CytAssist after run completion. **Immediately** move to next step.

- 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).



s. 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 HD

Slide to move after opening instrument.

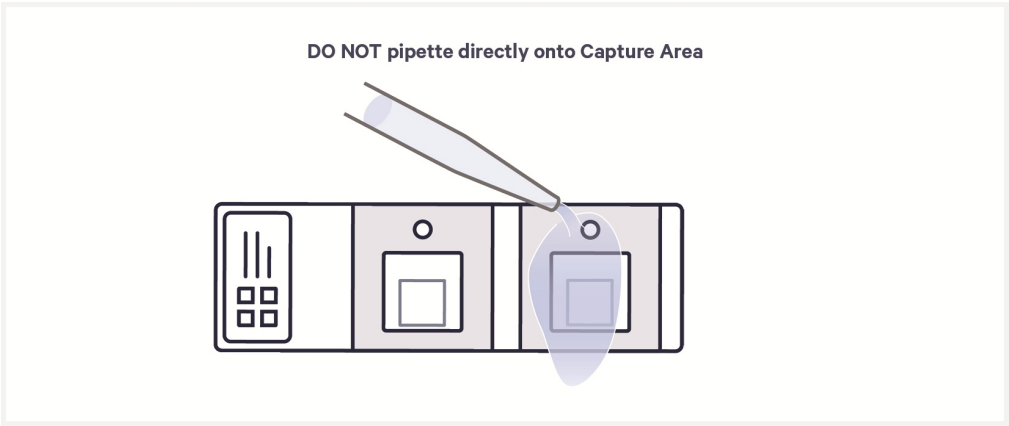


t. **Immediately** remove Visium HD Slide. It is normal if tissue or stain remains on tissue slides after run completion.

Five 0.1X SSC Washes:

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

u. **Wash 1:** While holding Visium HD Slide over liquid waste container, rinse each Capture Area with **1 ml** 0.1X SSC. DO NOT pipette directly onto Capture Areas, which are surrounded by the fiducial frames.



v. **Wash 2:** While holding Visium HD Slide over liquid waste container, rinse each Capture Area with **1 ml** 0.1X SSC. DO NOT pipette directly onto Capture Areas, which are surrounded by the fiducial frames.

w. **Wash 3:** While holding Visium HD Slide over liquid waste container, rinse each Capture Area with **1 ml** 0.1X SSC. DO NOT pipette directly onto Capture Areas, which are surrounded by the fiducial frames.

x. **Wash 4:** While holding Visium HD Slide over liquid waste container, rinse each Capture Area with **1 ml** 0.1X SSC. DO NOT pipette directly onto

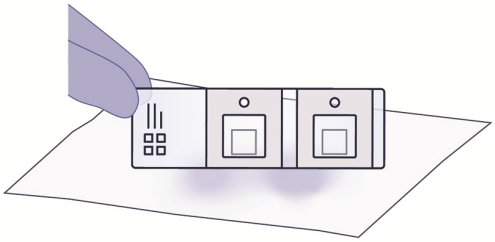
Capture Areas, which are surrounded by the fiducial frames.

y. **Wash 5:** While holding Visium HD Slide over liquid waste container, rinse each Capture Area with **1 ml** 0.1X SSC. DO NOT pipette directly onto Capture Areas, which are surrounded by the fiducial frames.

If any tissue remains, repeat 0.1X SSC washes until pink stain is removed.

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

DO NOT touch the Capture Area.



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

Some moisture remaining on the Visium HD Slide is normal.

ab. Proceed immediately to 3.2 Reverse Transcription on the next page. CytAssist instrument should only be cleaned and data transferred at a safe stopping point.

3.2 Reverse Transcription

a. Remove any residual 0.1X SSC from the wells.

b. Add **70 µl** RT Master Mix 1 to each well.

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

d. Skip Pre-equilibrate step to initiate Reverse Transcription 1.

e. When Reverse Transcription 1 is complete, prepare RT Master Mix 2 on ice. Pipette mix 10x and centrifuge briefly. Maintain on ice.

	RT Master Mix 2 *Add reagents in the order listed.*	PN	1X (µl)	1 Visium Slide (µl) (two reactions, includes overage)
	Nuclease-free Water	-	35.3	77.6
●	RT Reagent	2000086	17.5	38.5
○	Reducing Agent B	2000087	1.4	3.1
●	Template Switch Oligo B	2001027	4.9	10.8
●	RT Enzyme G	2001438	10.9	24.0
	Total	**-**	**70.0**	**154.0**

f. Remove the Visium Cassette from the Low Profile Thermocycler Adapter and place on a flat, clean work surface after the Reverse Transcription 1 is complete.

g. Prepare a thermal cycler with the following incubation protocol for Reverse Transcription 2 and start the program.

Reverse Transcription 2		
Lid Temperature	**Reaction Volume**	**Run Time**
42°C (lid may be left at 50°C if the instrument does not enable 42°C)	100 µl	33 min
Step	**Temperature**	**Time**
Pre-equilibrate	42°C	Hold
Reverse Transcription 2	42°C	00:30:00
Cool	4°C	00:03:00
Hold	4°C	Hold

h. Remove the Visium Slide Seal and using a pipette, remove all Reverse Transcription 1 Mix from the wells.

i. Add **100 µl** 0.1X SSC to each well.

j. Remove all 0.1X SSC buffer from the wells.

k. Add **70 µl** RT Master Mix 2 to each well.

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

m. Skip Pre-equilibrate step to initiate Reverse Transcription 2.

3.3 Denaturation

a. Prepare 0.08 M KOH shortly before use, adding reagents in the order listed. Vortex and centrifuge briefly. Maintain at room temperature. Discard after use.

0.08 M KOH	Stock	Final	2X + Overage* (µl)
Nuclease-free Water	-	-	495.0
KOH	8 M	0.08 M	5.0
Total	**-**	**-**	**500.0**

**Significant overage is provided to avoid pipetting small volumes*

b. At the end of Reverse Transcription 2 incubation, remove the Visium Cassette from the Low Profile Thermocycler Adapter and place on a flat, clean work surface.

c. Remove the Visium Slide Seal and using a pipette, remove all Reverse Transcription 2 Mix from the wells.

d. Add **75 µl** 0.08 M KOH (diluted from stock) to each well.

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

f. Using a pipette, remove 0.08 M KOH from the wells.

g. Add **100 µl** Buffer EB to each well.

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Step 4:

Second Strand Synthesis & Elution

4.0 Get Started

Items		10x PN	Preparation & Handling	Storage
Equilibrate to Room Temperature				
☐	Second Strand Reagent	Tube: 2000219 Kit: 1000855	Thaw, vortex, centrifuge briefly.	–20°C
☐	Second Strand Primer	Tube: 2000217 Kit: 1000855	Thaw, vortex, centrifuge briefly.	–20°C
Place on ice				
☐	Second Strand Enzyme	Tube: 2000183 Kit: 1000855	Pipette mix, centrifuge briefly.	–20°C
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	Qiagen Buffer EB	-	-	Ambient
☐	Tris-HCl 1 M, pH 7.0	-	-	Ambient
☐	8 M KOH	-		Ambient
☐	Visium Slide Seals	Component: 2000283 Kit: 1000874	See Tip & Best Practices.	Ambient

4.1 Second Strand Synthesis

a. Leave the Low Profile Thermocycler Adapter on the thermal cycler. Prepare the thermal cycler with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
53°C	100 µl	48 min
Step	**Temperature**	**Time**
Pre-equilibrate	53°C	Hold
Second Strand Synthesis	53°C	00:45:00
Cool	4°C	00:03:00
Hold	4°C	Hold

b. Prepare Second Strand Mix on ice. Pipette mix and centrifuge briefly. Maintain on ice.

	Second Strand Mix *Add reagents in the order listed*	PN	1X (µl)	2X +10% (µl)
●	Second Strand Reagent	2000219	69.5	152.9
●	Second Strand Primer	2000217	4.0	8.8
●	Second Strand Enzyme	2000183	1.5	3.3
	Total	-	**75.0**	**165.0**

c. Using a pipette, remove Buffer EB from the wells.

d. Add **75 µl** Second Strand Mix to each well.

e. Apply a new Visium Slide Seal on the Visium 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 Second Strand Synthesis.

g. Clean CytAssist instrument. Consult Visium CytAssist Instrument User Guide for more information.

h. Export run data and review CytAssist images during Second Strand Synthesis. Consult Visium CytAssist Instrument User Guide (CG000542) for more information on exporting run data. If images look abnormal, contact support@10xgenomics.com.

STOP **i.** Proceed to Second Strand Elution or store at **4°C** for up to **24 h**.

4.2 Second Strand Elution

a. Prepare 0.08 M KOH shortly before use, adding reagents in the order listed. Vortex and centrifuge briefly. Maintain at room temperature. Discard after use.

0.08 M KOH	Stock	Final	2X + Overage* (µl)
Nuclease-free Water	-	-	495.0
KOH	8 M	0.08 M	5.0
Total	**-**	**-**	**500.0**

**Significant overage is provided to avoid pipetting small volumes*

b. At the end of Second Strand Synthesis incubation, remove the Visium Cassette from the Low Profile Thermocycler Adapter and place on a flat, clean work surface.

c. Remove the Visium Slide Seal and using a pipette, remove all Second Strand Mix from the wells.

d. Add **100 µl** Buffer EB to each well.

e. Using a pipette, remove Buffer EB from the wells.

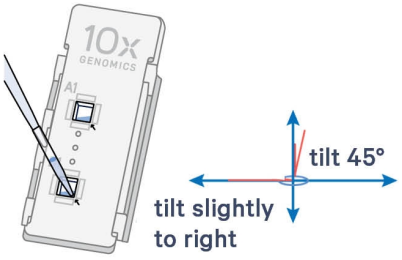
Failure to remove all solution may impact assay performance.

f. Add **35 µl** 0.08 M KOH (diluted from stock) to each well.

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

h. Add **5 µl** Tris-HCl 1 M, pH 7.0 to a tube in an 8-tube strip for each sample.

i. Using a pipette set to 35 µl, transfer all solution for each sample to a tube in an 8-tube strip. See image below for proper removal technique.



j. Inspect cassette wells. If there is any liquid remaining in the wells, return to the cassette wells and pipette again to ensure all solution is removed. Replace the tip with a new pipette tip and collect any remaining solution.

k. Vortex 8-tube strip, centrifuge briefly, and place on ice.

10x GENOMICS

Step 5:

cDNA Amplification and SPRIselect

5.0 Get Started

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

Item		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	cDNA Primers	Tube: 2000089 Kit: 1000855	Thaw at room temperature, vortex, and centrifuge briefly.	–20°C
☐	Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendations.	-
Place on ice				
☐	Amp Mix	Tube: 2000103 Kit: 1000855	Vortex, centrifuge briefly.	–20°C
Obtain				
☐	Qiagen Buffer EB	-		Ambient
☐	10x Magnetic Separator	Component: 2001212 Kit: 1000499	See Tips & Best Practices.	Ambient
☐	80% Ethanol	-	Prepare fresh. Prepare two 1.5-centrifuge tubes with 1000 µl, for a total of 2000 µl. Store at room temperature.	

80% Ethanol Store at room temperature	Stock	Final	1000 µl 1X
100% Ethanol	100%	80%	800 µl
Nuclease-free Water	-	-	200 µl

5.1 cDNA Amplification

a. Prepare cDNA Amplification Mix on ice. Add reagents in the order listed. Vortex and centrifuge briefly. Maintain on ice.

	cDNA Amplification Mix	PN	1X (µl)	2X + 10% (µl)
○	Amp Mix	2000103	50	110
●	cDNA Primers	2000089	15	33
	Total	**-**	**65**	**143**

b. Add **65 µl** cDNA Amplification Mix to each tube from 4.2 Second Strand Elution on page 78. 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	~45 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	00:01:00
5	Go to Step 2, repeat 14X for a total of 15 cycles	
6	72°C	00:01:00
7	4°C	00:03:00
8	4°C	Hold

STOP

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

5.2 cDNA 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 **60 µl** SPRIselect reagent (0.6X) to each pre-amplification reaction in an 8-tube strip (105 µl) and pipette mix 15x (pipette set to 130 µl).

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

c. Place on the magnet•**High** for **3 min**. Verify solution is clear.

d. Remove supernatant.

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

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 **40.5 µl** Buffer EB. Pipette mix 15x (pipette set to 30 µl).

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

m. Place tube strip on magnet •**Low** for **3 min**. Verify solution is clear.

n. Transfer **40 µl** sample to a new tube strip on ice.

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

5.3 cDNA QC & Quantification

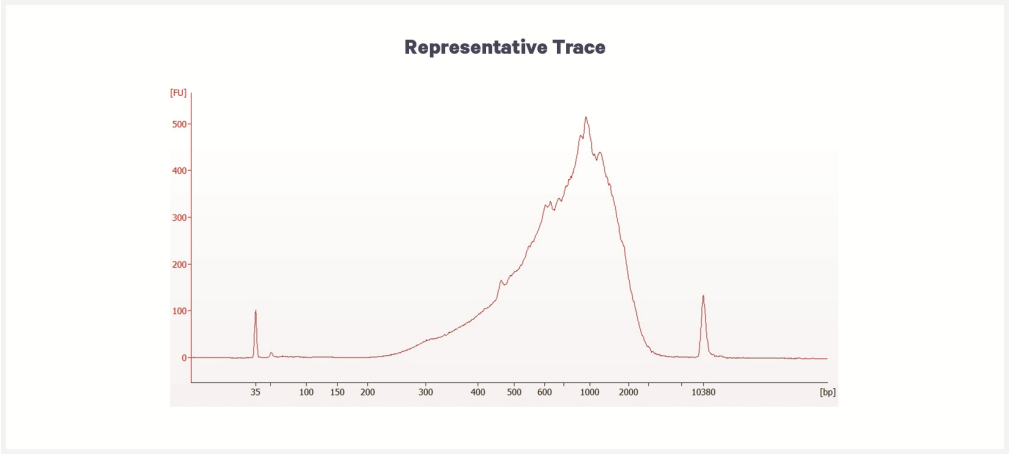
a. Dilute sample (1:5 dilution, i.e **1 µl** sample in **4 µl** of EB buffer) until it is at an appropriate concentration for the Bioanalyzer.

Quantification of sample prior to dilution may be necessary to calculate the optimal dilution.

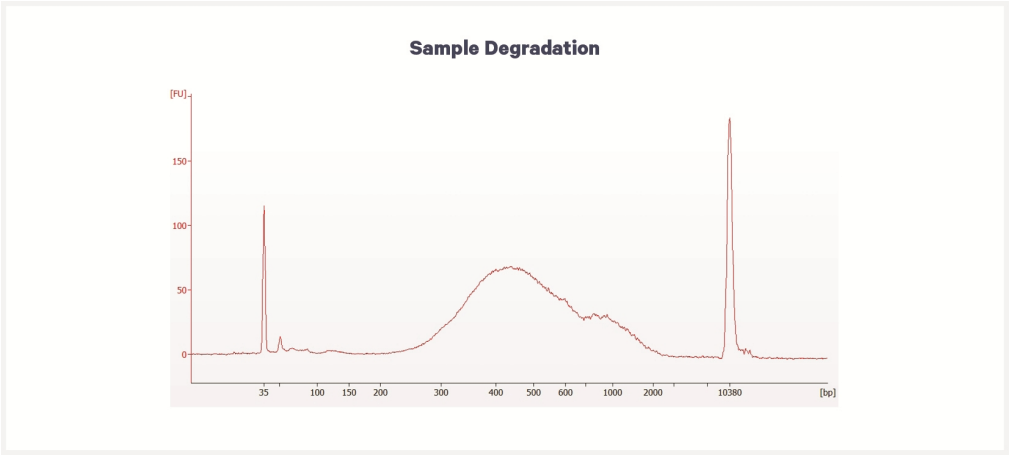
b. Run **1 µl** of diluted sample on an Agilent Bioanalyzer High Sensitivity chip. If peak is too small or flat, retry with a lower dilution.

cDNA profile may vary depending on tissue type and quality.

Lower molecular weight product (35–150 bp) may be present. This is normal and does not affect sequencing or application performance.



Though the trace below is a result of sample degradation, it is considered within expectations. For abnormal cDNA traces that would prohibit proceeding to library construction, see 4. Abnormal cDNA and Final Library Traces on page 115.



Alternate Quantification Methods:

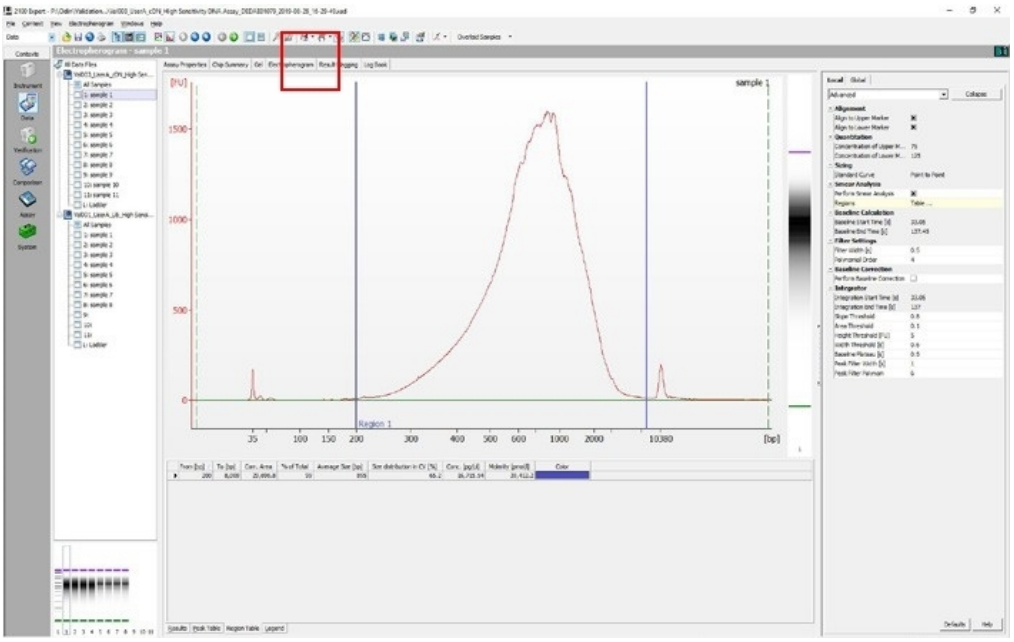
- Agilent TapeStation *(only D5000 reagents/tape are recommended)*
- Perkin Elmer LabChip

See Perkin Elmer LabChip Traces on page 122 and Agilent TapeStation Traces on page 123 for representative traces.

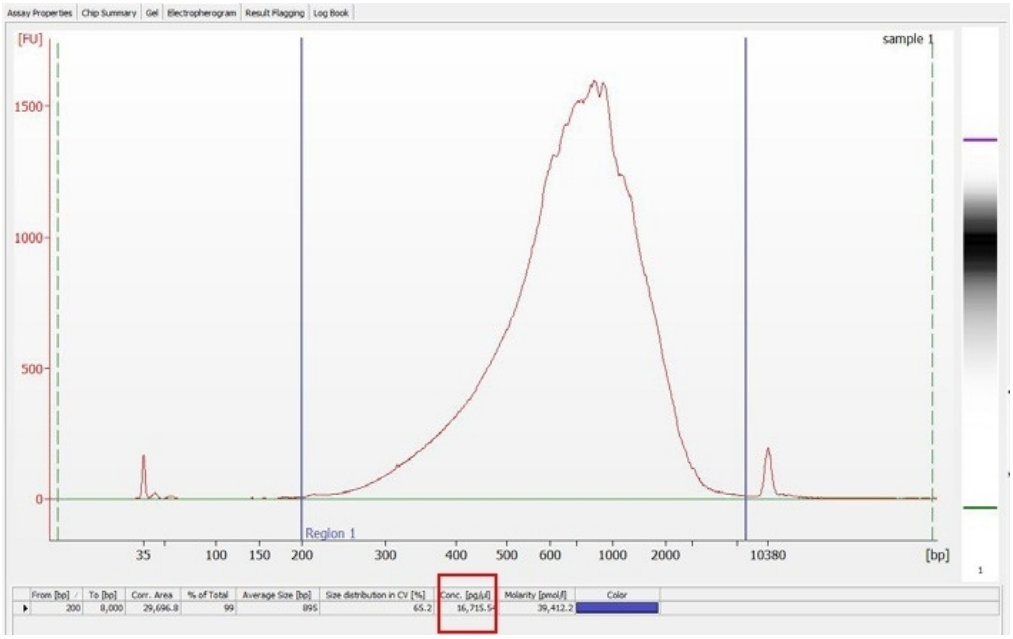
EXAMPLE CALCULATION

i. Select Region

Under the “Electropherogram” view choose the “Region Table”. Manually select the region of ~200 – ~7,000 bp.



ii. Note Concentration [pg/µl]



iii. Calculate

Multiply the cDNA concentration [pg/µl] reported via the Agilent 2100 Expert Software by the elution volume (40 µl) of the Post cDNA Amplification Reaction Clean Up sample and by the dilution factor. Then divide by 1,000 to obtain the total cDNA yield in ng.

Example Calculation of cDNA Total Yield

Concentration: 16,715.54 pg/µl
Elution Volume: 40

Example Calculation of cDNA Total Yield

Concentration: **16,715.54 pg/µl** Elution volume: 40 µl; Dilution Factor: 5

Total cDNA Yield

$$= \frac{\text{Conc'n (pg/µl) x Elution Vol. (µl) x Dilution Factor}}{\text{1000 (pg/ng)}}$$

$$= \frac{\text{16,715.54 x 40 x 5}}{\text{1000 (pg/ng)}}$$

=3342.8 ng

Carrying Forward ONLY 25% of total cDNA yield

=Total cDNA x 0.25 =3,342.8 ng x 0.25

=835.7 ng

See step 6.5 Sample Index PCR on page 94 for appropriate number of Sample Index PCR cycles based on carry forward cDNA/input mass.

10x GENOMICS

Step 6:

Visium HD 3' Library Construction

6.0 Get Started

		Item	10x PN	Preparation & Handling	Storage
Equilibrate to room temperature					
☐	●	Fragmentation Buffer	Tube: 2000091 Kit: 1000855	Vortex, verify no precipitate. Centrifuge briefly.	–20°C
☐	●	Ligation Mix	Tube: 2001109 Kit: 1000855	Vortex, verify no precipitate. Centrifuge briefly.	–20°C
☐		Dual Index Plate TT Set A	Component: 3000431 Kit: 1000215	Thaw at room temperature, vortex, and centrifuge briefly.	–20°C
☐		Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendations.	-
☐		Library QC Reagents		Use Agilent Bioanalyzer, Perkin Elmer LabChip, or Agilent TapeStation for QC. Obtain appropriate reagents based on method chosen.	
Place on ice					
☐	●	Fragmentation Enzyme *Ensure that Fragmentation Buffer and Fragmentation Enzyme from the same kit are used together. Lots are matched for optimal performance.*	Tube: 2000104 Kit: 1000855	Pipette mix, centrifuge briefly before using.	–20°C
☐	●	DNA Ligase	Tube: 220131 Kit: 1000855	Pipette mix, centrifuge briefly before using.	–20°C
☐	○	Amp Mix	Tube: 2000103 Kit: 1000855	Vortex, centrifuge briefly.	–20°C
Obtain					
☐		Nuclease-free Water	-	-	Ambient
☐		Qiagen Buffer EB	-		Ambient

<table>
<tr><th>Item</th><th></th><th>10x PN</th><th>Preparation & Handling</th><th>Storage</th></tr>
<tr><td>☐</td><td>10x Magnetic Separator</td><td>Component: 2001212
Kit: 1000499</td><td>See Tips & Best Practices.</td><td>Ambient</td></tr>
<tr><td>☐</td><td>80% Ethanol</td><td>-</td><td>Prepare fresh. Prepare two 1.5-centrifuge tubes with 1250 µl, for a total of 2500 µl. Store at room temperature.

80% Ethanol Store at room temperature | Stock | Final | 1250 µl 1X
100% Ethanol | 100% | 80% | 1000 µl
Nuclease-free Water | - | - | 250 µl</td><td></td></tr>
</table>

6.1 Fragmentation, End Repair & A-tailing

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

Lid Temperature	Reaction Volume	Run Time
65°C	50 µl	~35 min
Step	**Temperature**	**Time**
Pre-cool block Pre-cool block prior to preparing the Fragmentation Mix	4°C	Hold
Fragmentation	32°C	00:05:00
End Repair & A-tailing	65°C	00:30:00
Hold	4°C	Hold

b. Prepare Fragmentation Mix on ice. Pipette mix and centrifuge briefly. Maintain on ice.

	Fragmentation Mix *Add reagents in the order listed*	PN	1X (µl)	2X + 10% (µl)
●	Fragmentation Buffer	2000091	5	11
●	Fragmentation Enzyme	2000104	10	22
	Total	**-**	**15**	**33**

c. Add **25 µl** Buffer EB to separate tubes in a tube strip (maintained on ice) for each sample from 5.2 cDNA Amplification Cleanup - SPRIselect on page 82.

d. Add **15 µl** Fragmentation Mix to each tube containing Buffer EB.

e. Transfer **10 µl** purified cDNA from each sample from cDNA Amplification - Cleanup to separate tubes in a tube strip containing Buffer EB and Fragmentation Mix.

f. Pipette mix 15x (pipette set to 35 µl) **on ice**. Centrifuge briefly.

g. Transfer into the pre-cooled thermal cycler (**4°C**).

h. Skip pre-cool block step to initiate Fragmentation.

6.2 Post Fragmentation End Repair & A-tailing Double Sided Size Selection – SPRIselect

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

a. Vortex to resuspend SPRIselect reagent. Add **30 µl** SPRIselect **(0.6X)** reagent to each sample. Pipette mix 15x (pipette set to 75 µl).

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

c. Place on the magnet •**High** for **3 min**. Verify solution is clear.

DO NOT discard supernatant.

d. Transfer **75 µl** supernatant to a new tube strip.

e. Vortex to resuspend SPRIselect reagent. Add **10 µl** SPRIselect reagent **(0.8X)** to each sample. Pipette mix 15x (pipette set to 80 µl).

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

g. Place on the magnet•**High** for **3 min**. Verify solution is clear.

h. Remove 80 µl supernatant. DO NOT discard any beads.

i. Add **125 µl** 80% ethanol to the pellet. Wait **30 sec**.

j. Remove the ethanol.

k. Add **125 µl** 80% ethanol to the pellet. Wait **30 sec**.

l. Remove the ethanol.

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

n. Remove remaining ethanol. DO NOT over dry to ensure maximum elution efficiency.

o. Remove from the magnet. Add **50.5 µl** Buffer EB to each sample. Pipette mix 15x.

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

q. Place on the magnet•**High** for **3 min**. Verify solution is clear.

r. Transfer **50 µl** sample to a new tube strip on ice.

6.3 Adaptor Ligation

a. Prepare Adaptor Ligation Mix. Pipette mix and centrifuge briefly. Maintain on ice.

	Adaptor Ligation Mix *Add reagents in the order listed*	PN	1X (µl)	2X + 10% (µl)
●	Ligation Mix	2001109	40	88
●	DNA Ligase	220131	10	22
	Total	**-**	**50**	**110**

b. Add **50 µl** Adaptor Ligation Mix to **50 µl** sample (**maintained on ice**). Pipette mix 15x (pipette set to 90 µl) on ice. Centrifuge briefly.

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

Lid Temperature	Reaction Volume	Run Time
30°C (lid may be turned off if the instrument does not enable 30°C)	100 µl	15 min
Step	**Temperature**	**Time**
Pre-equilibrate	20°C	Hold
Adaptor Ligation	20°C	00:15:00
Hold	4°C	Hold

6.4 Post-Ligation 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 **80 µl** SPRIselect Reagent (**0.8X**) to each sample. Pipette mix 15x (pipette set to 150 µl).

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

c. Place on magnet•**High** for **3 min**. Verify solution is clear.

d. Remove supernatant.

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

f. Remove ethanol.

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

h. Remove ethanol.

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

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

k. Remove from magnet. Add **30.5 µl** Buffer EB. Pipette mix 15x.

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

m. Place on the magnet•**Low** for **3 min**. Verify solution is clear.

n. Transfer **30 µl** sample to a new tube strip on ice.

6.5 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-3000431 Dual Index Plate TT Set A well ID) used.

b. Add **50 µl** Amp Mix (PN-2000103) to each tube from 6.4 Post-Ligation Cleanup – SPRIselect on the previous page in an 8-tube strip on ice.

c. Add **20 µl** of an individual Dual Index Plate TT Set A to each tube and record the well ID used. Pipette mix and centrifuge briefly.

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

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~30 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	54°C	00:00:30
4	72°C	00:00:20
5	Go to step 2, see below for # of cycles	
6	72°C	00:01:00
7	4°C	00:03:00
7	4°C	Hold

Select total cycles based upon carrying forward 25% cDNA input (cDNA yield/4) input calculated during cDNA QC. An example calculation is provided in 5.3 cDNA QC & Quantification on page 83.

cDNA Input	Total Cycles
1–10 ng	15
10–50 ng	14
50–100 ng	13
100–250 ng	12
250-350 ng	11
350-600 ng	10
600-800 ng	9
800-1,100 ng	8
1,100-1,300 ng	7
1,300-1,500 ng	6
>1,500 ng	5

Samples within ±1 cycle numbers can be combined in a single SI-PCR run at the higher cycle number.

e. Store at **4°C** for up to **72 h** or proceed to the next step.

6.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect

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

a. Vortex to resuspend the SPRIselect reagent. Add **60 µl** SPRIselect Reagent **(0.6X)** to each sample. Pipette mix 15x (pipette set to 150 µl).

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

c. Place on the magnet •**High** for **3 min**. Verify solution is clear.

DO NOT discard supernatant.

d. Transfer **150 µl** supernatant to a new tube strip.

e. Vortex to resuspend the SPRIselect reagent. Add **20 µl** SPRIselect Reagent **(0.8X)** to each sample. Pipette mix 15x (pipette set to 150 µl).

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

g. Place the magnet•**High** for **3 min**. Verify solution is clear.

h. Remove **165 µl** supernatant. DO NOT discard any beads.

i. With the tube still in the magnet, add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.

j. Remove the ethanol.

k. With the tube still in the magnet, add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.

l. Remove the ethanol.

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

n. Remove remaining ethanol. DO NOT over dry to ensure maximum elution efficiency.

o. Remove from the magnet. Add **35.5 µl** Buffer EB. Pipette mix 15x.

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

q. Place on the magnet•**Low** for **3 min**. Verify solution is clear.

r. Transfer **35 µl** to a new tube strip on ice.

s. Store at **4°C** for up to **72 h** or at **−20°C** for **long-term** storage.

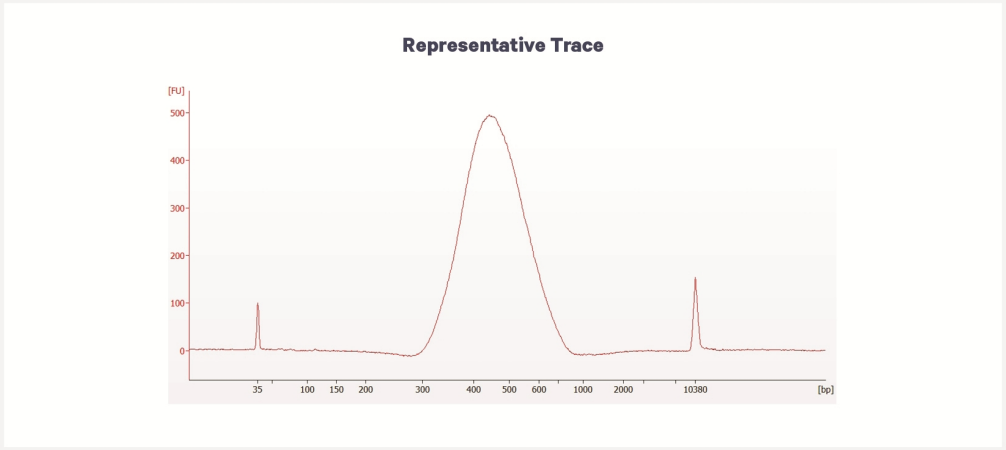
6.7 Post-Library Construction QC

a. Dilute sample (1:5 dilution, i.e **1 µl** sample in **4 µl** of EB buffer) until it is at an appropriate concentration for the Bioanalyzer.

Quantification of sample prior to dilution may be necessary to calculate the optimal dilution.

b. Run **1 µl** of diluted sample on an Agilent Bioanalyzer High Sensitivity chip. If peak is too small or flat, retry with a lower dilution.See 4. Abnormal cDNA and Final Library Traces on page 115 for more information.

Representative Trace



Determine the average fragment size from the Bioanalyzer trace. The expected average fragment size is 450 bp. This will be used as the insert size for library quantification.

Alternate QC Methods:

- Agilent TapeStation *(D1000 or D5000 reagents/tape are recommended)*
- Perkin Elmer LabChip

See Perkin Elmer LabChip Traces on page 122 and Agilent TapeStation Traces on page 123 for representative traces.

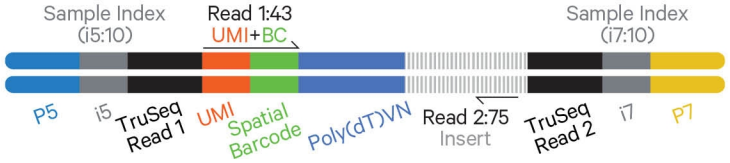
See Post Library Construction Quantification on page 121

Sequencing

Sequencing Libraries

A Visium HD 3' 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 TruSeq Read 2 is used to sequence the cDNA fragment. i7 and i5 sample index sequences are also incorporated as index reads. TruSeq Read 1 and TruSeq Read 2 are standard Illumina sequencing primer sites used in paired-end sequencing.

Visium HD 3' Library



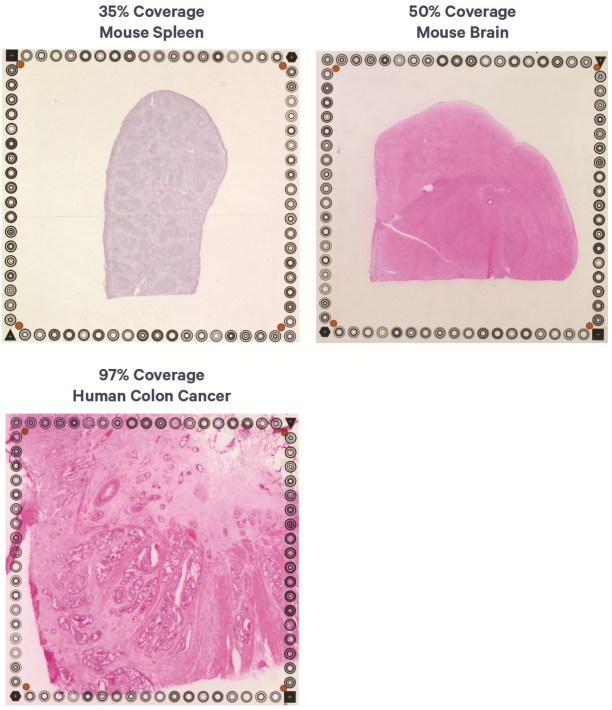
Sequencing Depth

The minimum sequencing depth for Visium HD 3' Spatial Gene Expression is 550 million read pairs per fully-covered Capture Area. The recommended minimum sequencing depth was chosen because it achieved >75% sequencing saturation for >50% of fresh frozen samples across a variety of species and tissue types.

Calculating sequencing depth requires estimating the approximate Capture Area (%) covered by tissue. This may be performed visually or by using the Visium Manual Alignment Wizard in Loupe Browser for a more accurate measurement. See examples below for estimating coverage area visually. If using Loupe Browser, the number of bins associated with tissue can be calculated during the “Identify Tissue” step. For more information, consult the 10x Genomics Support website.

Example: Sequencing Depth for a Sample

- **Estimate the approximate Capture Area (%)** covered by the tissue section.
- **Calculate total sequencing depth=** (Coverage Area x 550,000,000 read pairs
- **Example calculation for 50% coverage:** 0.50 x 550,000,000 read pairs = 275,000,000 total read pairs for that sample



Sequencing Type & Run Parameters

Use the sequencing run type and parameters indicated.

Visium HD 3' Library
Paired-end, dual indexed sequencing
TruSeq Read 1: 43 cycles i7 Index: 10 cycles i5 Index: 10 cycles TruSeq Read 2: 75 cycles

Sequencer Compatibility

10x Genomics libraries contain P5 and P7 adaptors, which can be used for Illumina sequencing. These libraries can also be modified to enable sequencing on various long and short-read sequencing platforms, with some platforms requiring third-party analysis tools. For a list of tested sequencers, consult the Sequencer Compatibility for Visium HD 3' Spatial Gene Expression page on the 10x Genomics support site.

Some variation in assay performance is expected based on sequencer choice. For more information on sequencing platform compatibility, refer to the 10x Genomics Compatible Products page at the 10x Genomics support website.

Sample Indices

Each well of the Dual Index Kit TT Set A (PN-1000215) 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 Plate TT Set A plate well ID, SI-TT-) 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. For example, 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

10x Genomics has not tested pooling Visium HD 3' libraries with other 10x Genomics libraries; therefore, pooling is not recommended due to possible impact on assay performance.

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. For a list of sequencers and loading concentrations, consult the Sequencer Compatibility for Visium HD 3' Spatial Gene Expression page on the 10x Genomics support website.

Sequencing Metrics

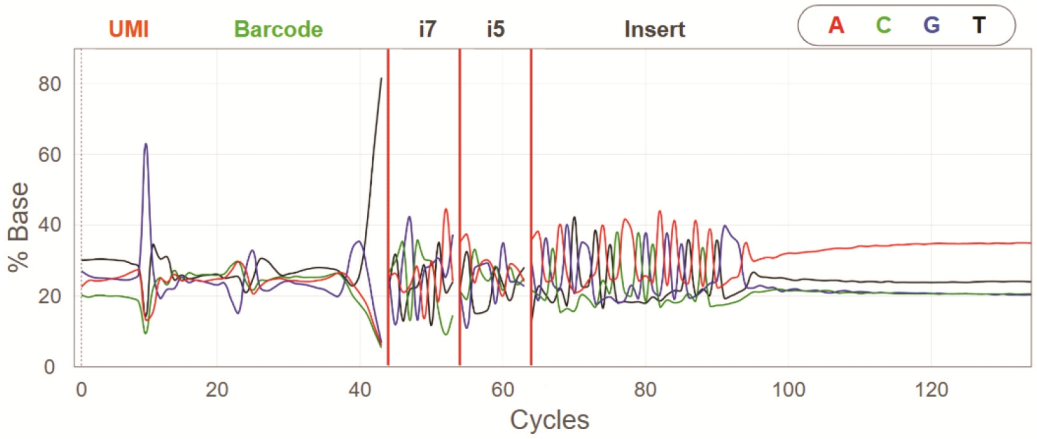
To determine sequencing metrics for a selected sequencing platform, Visium HD 3' Libraries were generated and sequenced at the indicated run parameters to generate the % Base and % ≥Q30 plots shown in the following figures. All libraries were quantified with the KAPA DNA Quantification Kit and sequenced with 1% PhiX.

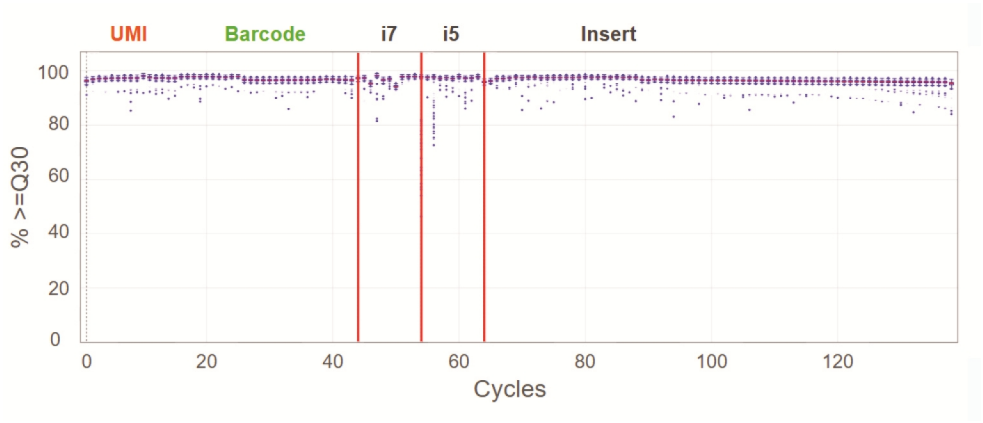
All libraries followed the following sequencing configuration and run parameters:

- Targeted sequencing depth: Minimum of 550 million read pairs multiplied by fraction Visium HD 3' Slide tissue coverage.
- Paired-end, dual indexing: TruSeq Read 1 (R1): 43 cycles; i7 Index: 10 cycles; i5 Index: 10 cycles; TruSeq Read 2 (R2): 75 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 and maintaining RNA quality.
- 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 the Sequencer Compatibility for Visium HD 3' Spatial Gene Expression page on the 10x Genomics support site, 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 general guidance and additional optimization may be required.





Libraries were sequenced on the NovaSeq X Plus. "Data by Cycle" plots from the Illumina SAV software displaying the percentage of base calls and Q30 quality scores are shown.

2 Sample Pool							
Instr.	Load Conc. (pM)	% Occupancy	%PF	R1	i7	i5	R2
				% ≥Q30			
NovaSeq X Plus	200	94.80	84.71	96.48	96.37	96.57	96.57

4 Sample Pool							
Instr.	Load Conc. (pM)	% Occupancy	%PF	R1	i7	i5	R2
				% ≥Q30			
NovaSeq X Plus	200	94.30	83.77	95.99	96.55	95.75	95.65

Troubleshooting

Before CytAssist Instrument Run

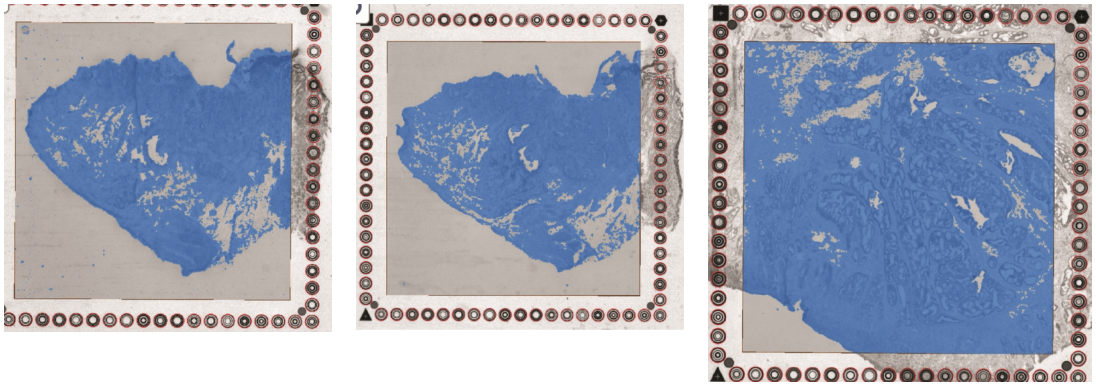
1. Inadequate Visium HD Slide Preparation

- 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 reagents.

2. Extended Tissue Slide PBS Incubation

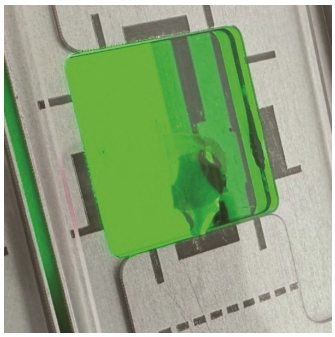
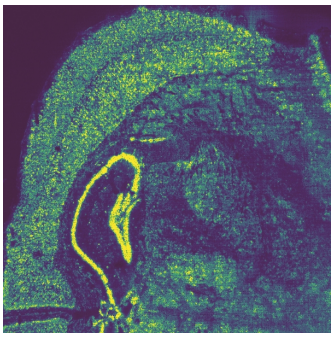
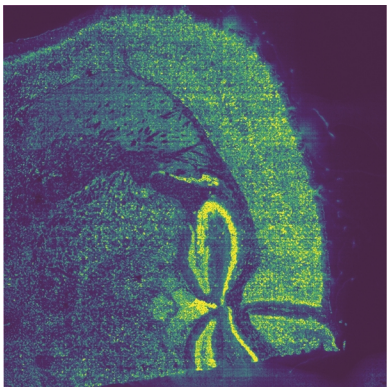
Extended Tissue Slide PBS Incubation

Tissue Detection Impacted due to Weak Staining



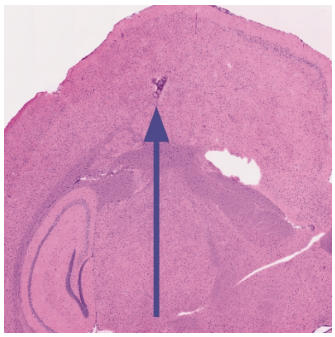
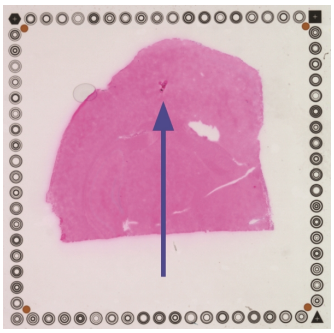
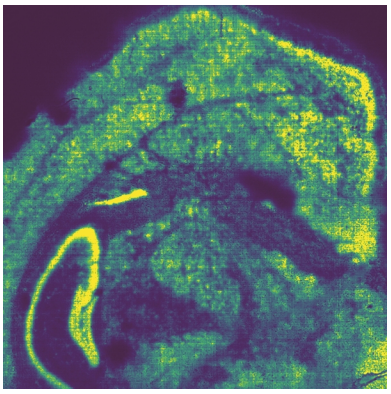
Extended PBS incubation beyond the 5 minute mark may result in increased destaining that causes automatic tissue detection to fail. If tissue detection is impacted, manual tissue detection may be required. This has no impact on sensitivity and data quality. Remove tissue slides at 5 min during the PBS incubation as described in the protocol.

3. PBS Droplets on Tissue Sections

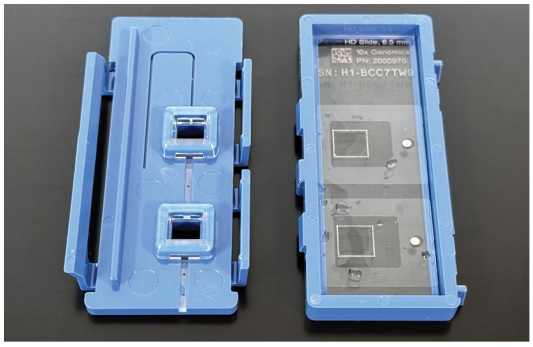
Large PBS Droplets on Tissue Sections during CytAssist Drying May Result in Blurry UMI Maps		
Large PBS Droplet on Tissue Section	**UMI Map - Blurry Within and Outside Tissue**	**UMI Map - Blurry Outside Tissue**
		

Large PBS droplets remaining on tissue sections may result in loss of spatial fidelity demonstrated by blurry post-sequencing UMI plots. Flick slides vigorously after PBS incubation until no large PBS droplets remain on tissue sections. Ensure tissue section is dry prior to starting an instrument run.

4. Debris on Tissue or Visium HD Slide

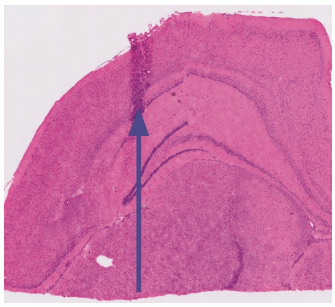
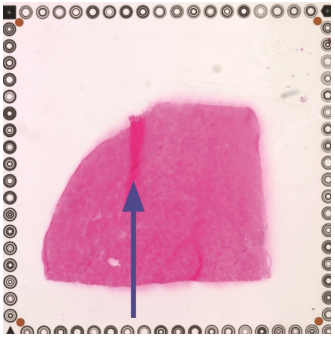
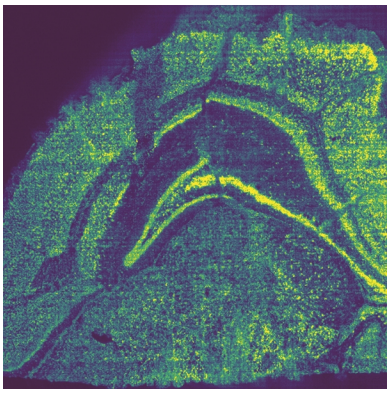
Debris on Slide may cause Blurry UMI Map		
Debris on Tissue Slide (Microscope Image)	**Debris on Tissue Slide (CytAssist Image)**	**UMI Map - Blurry Inside and Outside Tissue**
		

Debris on a Tissue Slide or Visium HD Slide may cause loss of spatial fidelity demonstrated by blurry post-sequencing UMI plots. Always work in a clean environment. When handling the Visium cassette, ensure that gaskets always face away from the work surface, as shown in the image below, to avoid debris collecting on the gasket.



5. Folded Tissue on Tissue Slide

Tissue Folding may cause Blurry UMI Map

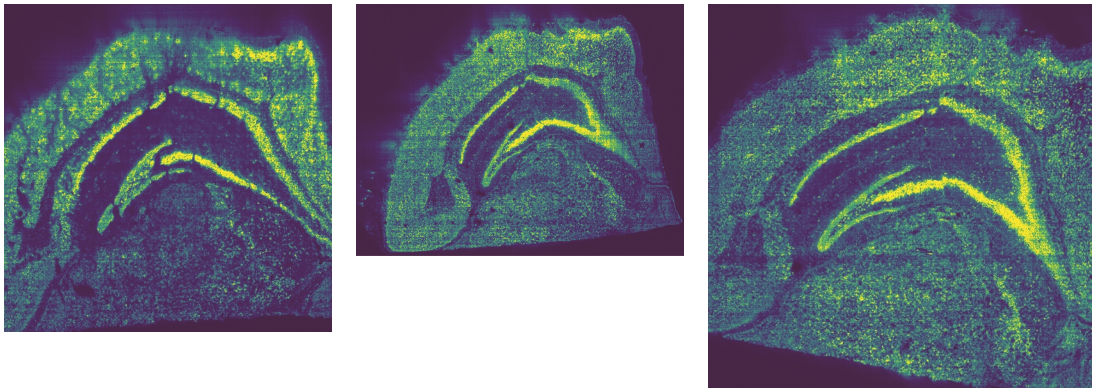
Folded Tissue (Microscope Image)	Folded Tissue (CytAssist Image)	UMI Map - Blurry Inside and Outside Tissue
		

Folded tissue may cause a loss of spatial fidelity demonstrated by blurry post-sequencing UMI plots. If possible, screen H&E images during H&E staining and imaging to select tissue sections without folds.

6. Incomplete Removal of Pre-equilibration Buffer

Incomplete Removal of Pre-equilibration Buffer may cause Blurry UMI Map

UMI Map - Blurry Inside and Outside Tissue



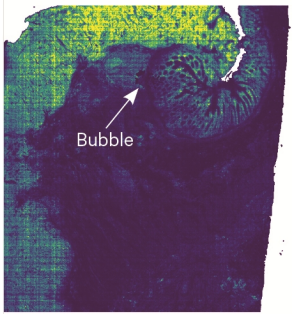
Incomplete removal of pre-equilibration buffer may result in blurry UMI maps. When removing Pre-equilibration Buffer, perform the following:

- Using a P200 pipette set at 200 µl, remove Pre-equilibration Mix from the right corner of **one** well in the cassette without scratching the fiducial frame or hydrogel.
- Replace P200 tip with a new P200 tip.
- With the pipette still set at 200 µl, remove Pre-equilibration Mix from the right corner of the **same** well in the cassette.
- Repeat process for remaining well.

During CytAssist Instrument Run

1. Bubbles Trapped During Visium CytAssist Run

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

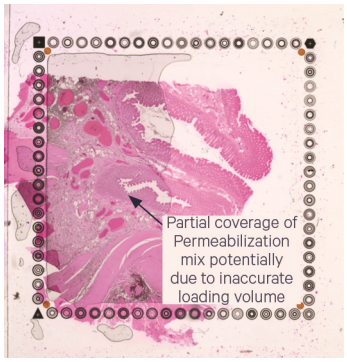
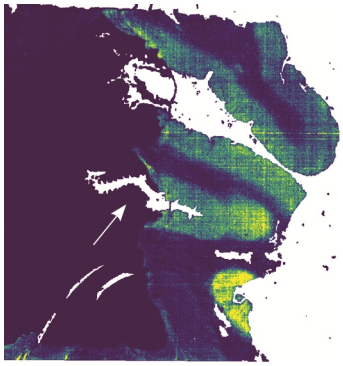
CytAssist Image	UMI Counts
	

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 reagents onto the slide. Ensure that the entire spacer chamber is inspected for bubbles, not just the Capture Area. Ensure that the entire spacer chamber 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 3' Spatial Gene Expression Protocol Planner (CG000803) for information on tested slides.

2. Reagent Flow Failure

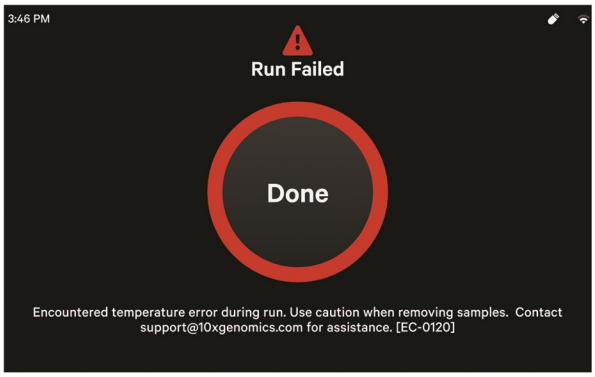
Reagent Flow Failure May Cause Loss of Data

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 loading of the Permeabilization Mix onto the Visium HD Slide or debris on 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. Both tissues slides and Visium HD slides should be completely dry. See Lint-free Laboratory Wipes on page 24 for more information.

3. Visium CytAssist Overheating



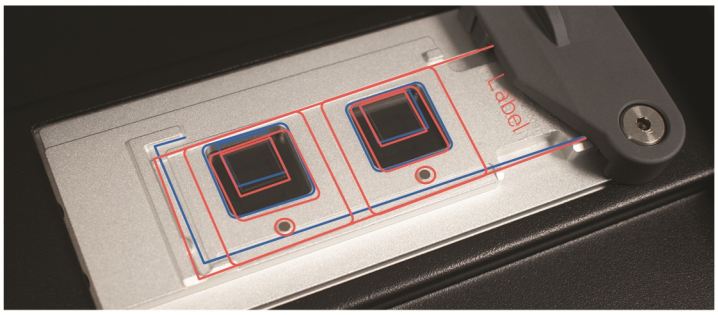
If the Visium CytAssist instrument overheats during an experiment run, the run will fail. A warning message will appear instructing users to carefully remove slides and contact support@10xgenomics.com.

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

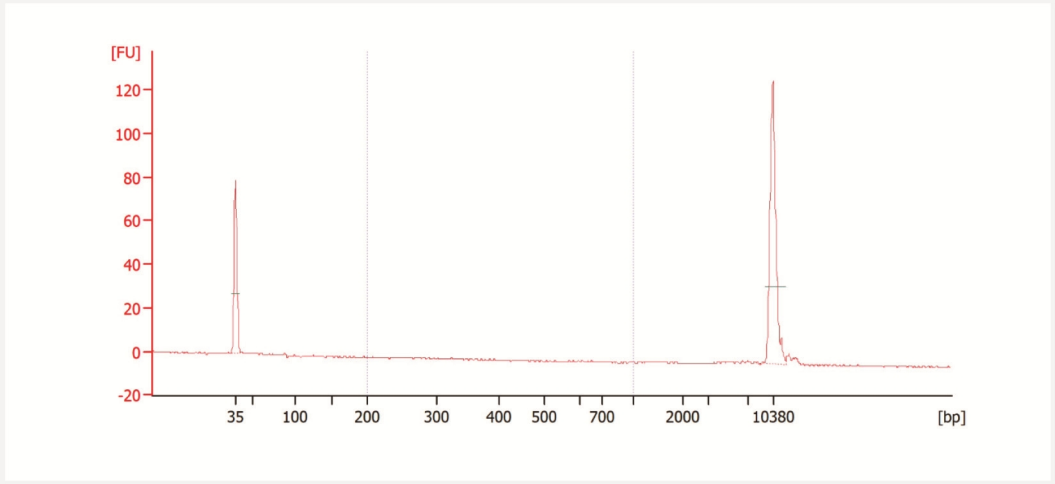


2. Visium HD Slide Removal Delayed

A delay in removing and processing the Visium HD Slide after run completion may impact data quality. Immediately proceed with slide washes while adjacent to the instrument.

3. No cDNA Amplification

No cDNA amplification may result on a flat cDNA trace.



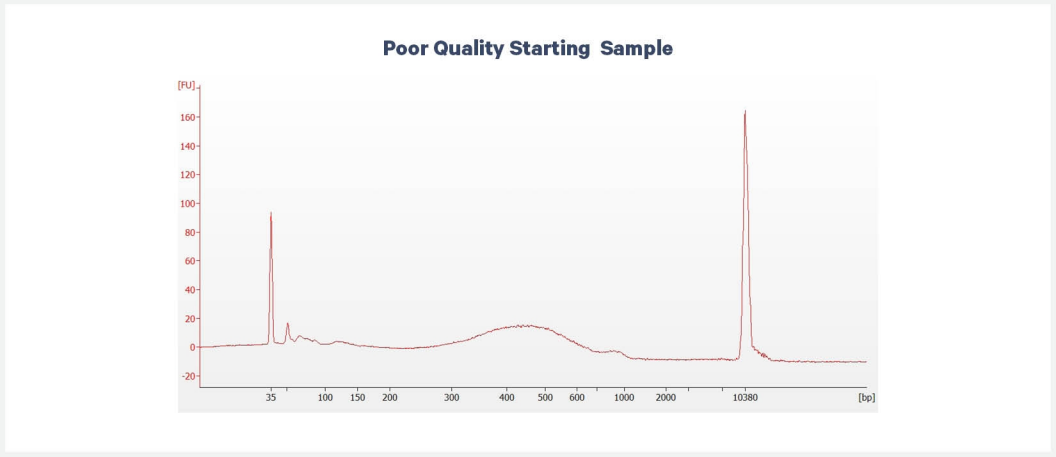
This may be due to the following:

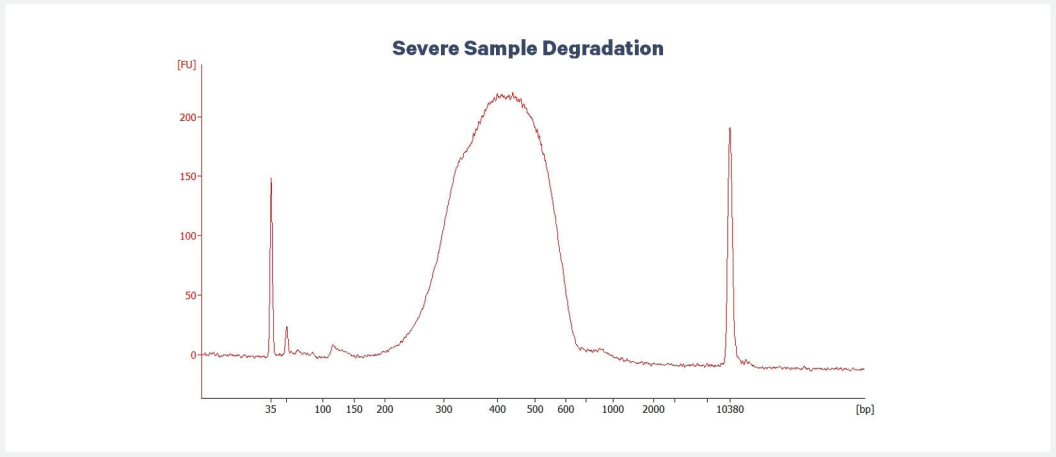
- Poor sample quality
- Issues with pipetting small volumes
- Incorrect preparation of Permeabilization Mix
- Mistake in Permeabilization Mix addition timing
- Failure to neutralize KOH with Tris-HCl 1M, pH 7.0
- Failure to prepare KOH fresh and at the appropriate concentration
- Failure to use fresh reagents during SPRI or using reagents at the wrong concentration
- Failure to add sample to the instrument used for QC
- Leakage from the cassette during workflow

4. Abnormal cDNA and Final Library Traces

Abnormal cDNA Traces

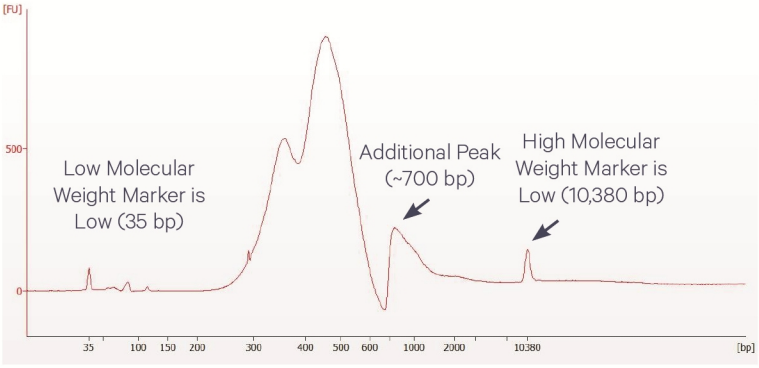
Below are some examples of abnormal cDNA traces. If any of the following are observed, contact support@10xgenomics.com for assistance. See Tips & Best Practices section of this User Guide and Visium HD 3' Fresh Frozen Tissue Preparation Handbook (CG000804) for information on establishing an RNase-free environment.





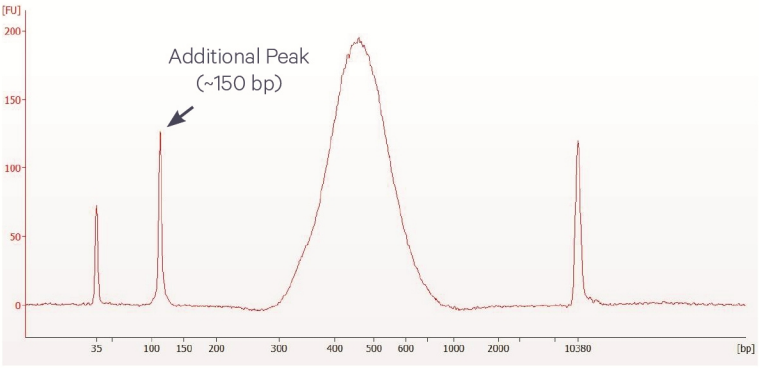
Abnormal Final Library Traces

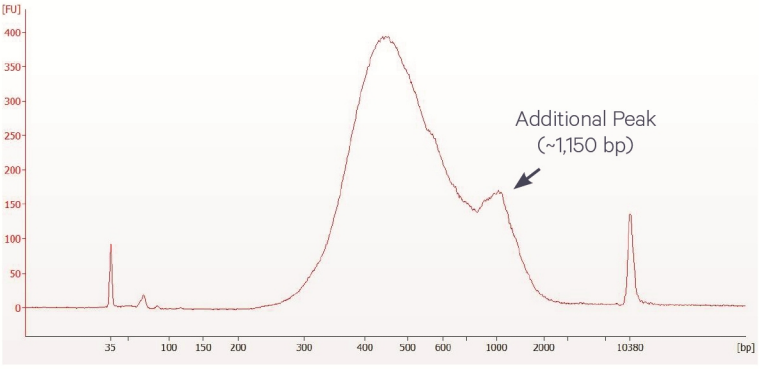
The image below is an example of an overloaded trace. Note the double peak at around 700 bp. The low and high molecular weight markers are in low abundance compared to the sample. Ensure the library was diluted prior to loading per protocol recommendations. If the library was diluted, ensure the library concentration is within the specification range of the automated electrophoresis kit being used. The library can be quantified using a Invitrogen Qubit dsDNA HS Assay Kit and diluted further if appropriate.



Peaks due to Poor SPRI

The additional peaks in the traces below are due to poor SPRI. Ensure that all SPRI cleanup steps are performed accurately.





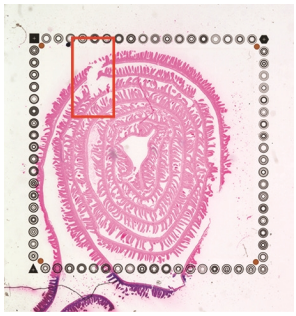
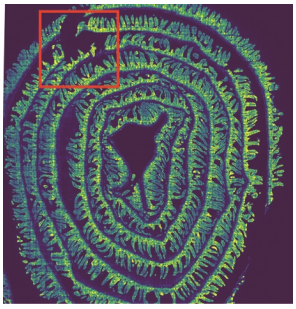
Issues Impacting Tissue Analysis

1. Tissue Detachment and Folding

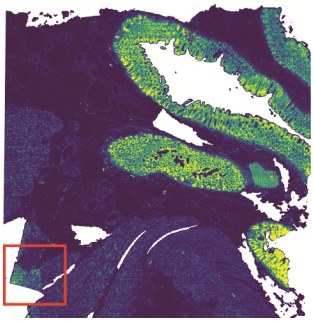
Tissue detachment may result in a lack of Fraction Reads Usable in the region where detachment occurred. If the tissue has folded on itself, this may also cause elevated UMI counts in the overlapping areas or a loss of spatiality due to ineffective permeabilization. Tissue folding may also cause lower UMI capture. Inspect images carefully to identify these areas. For more information on folding that is unrelated to tissue detachment, see 5. Folded Tissue on Tissue Slide on page 108.

Ensure that slides tested by 10x Genomics were used for tissue placement. For a list of tested slides, refer to the Visium HD 3' Spatial Gene Expression Protocol Planner (CG000803) For more information, consult the sample preparation documentation described in Workflow Overview on page 13.

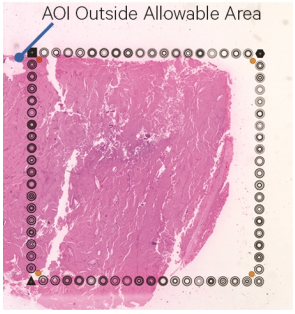
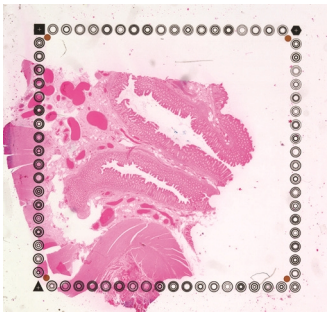
Tissue Detachment May Cause a Loss of Gene Expression

CytAssist Image	UMI Count
	

Tissue Folding May Cause Elevated UMI Counts

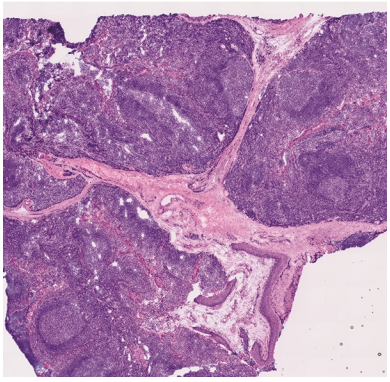
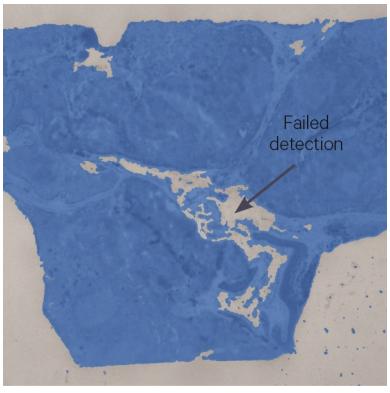
CytAssist Image	UMI Count
	

2. Area of Interest Not Within Allowable Area

Area of Interest Outside of Allowable Area is Not Analyzed	
Tissue Larger than Capture Area	**Tissue Not Properly Aligned**
	

Areas of interest 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. Tissue outside of the allowable area is fine if the tissue outside the allowable area is not an area of interest. Use the Visium CytAssist Alignment Aid to assist in alignment on the instrument. Consult the Visium HD 3' Spatial Gene Expression Protocol Planner (CG000803) for information on tested glass slides.

3. Tissue Segmentation Failure due to High Connective Tissue

Examples of Scenarios that Lead to Tissue Segmentation Failure	
Tissue Composition/Morphology May Result in Failed Tissue Detection	**Tissue Composition/Morphology May Result in Failed Tissue Detection**
	

Space Ranger may fail to detect tissue for a variety of reasons, which may necessitate manual fiducial alignment and tissue detection via Loupe Browser. If the contrast between background and tissue is poor (due to inadequate staining, technical error, or tissue composition) tissue segmentation failure may occur. Consider processing H&E images through Space Ranger's segmentation pipeline (spaceranger segment) prior to proceeding with the

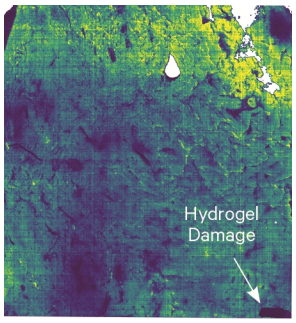
assay to gauge expected performance. For troubleshooting tips related to image quality, refer to the Visium HD Spatial Applications Imaging Guidelines Technical Note (Document CG000688).

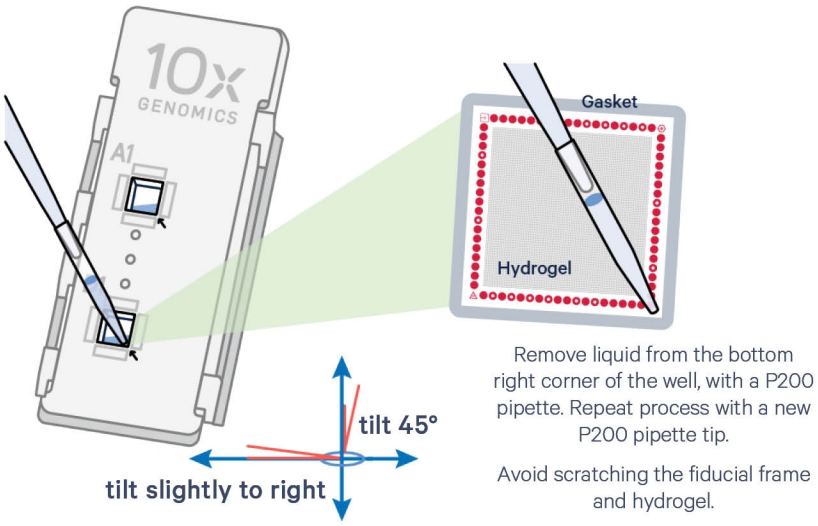
4. Data Loss due to Hydrogel Damage

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 & Removal from Wells on page 26. 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





Appendix

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)	

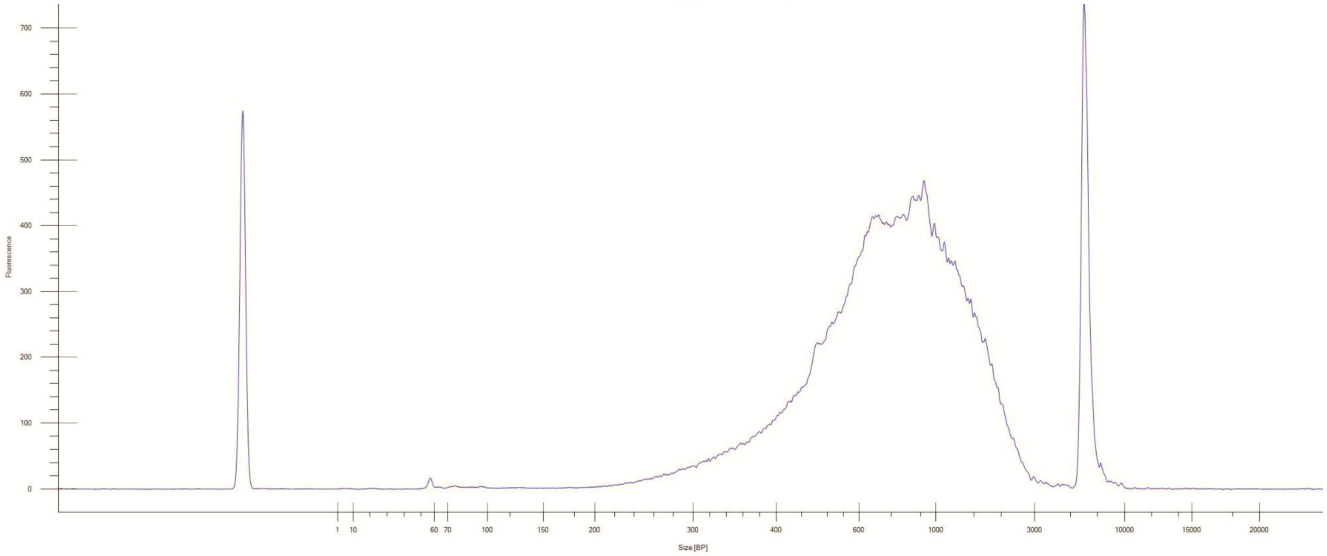
g. Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on insert size derived from the Bioanalyzer/TapeStation trace.

Perkin Elmer LabChip Traces

Protocol Step 5.3 - cDNA QC & Quantification

Representative Trace

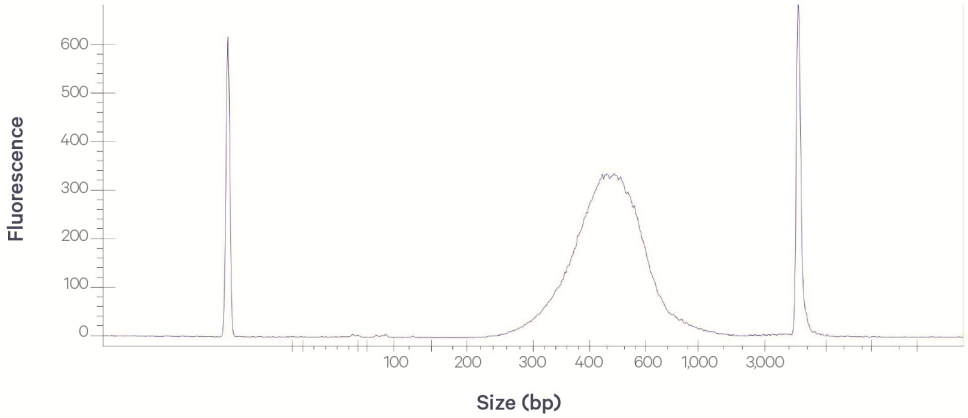
Run manufacturer's recommended volume of diluted sample (1:5 dilution)



Protocol Step 6.7 - 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.

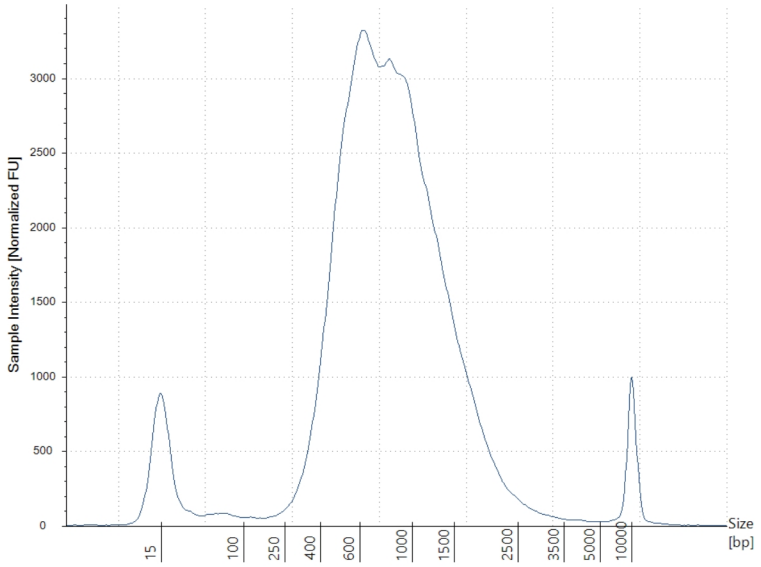
Agilent TapeStation Traces

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

Protocol Step 5.3 - cDNA QC & Quantification

Representative Trace

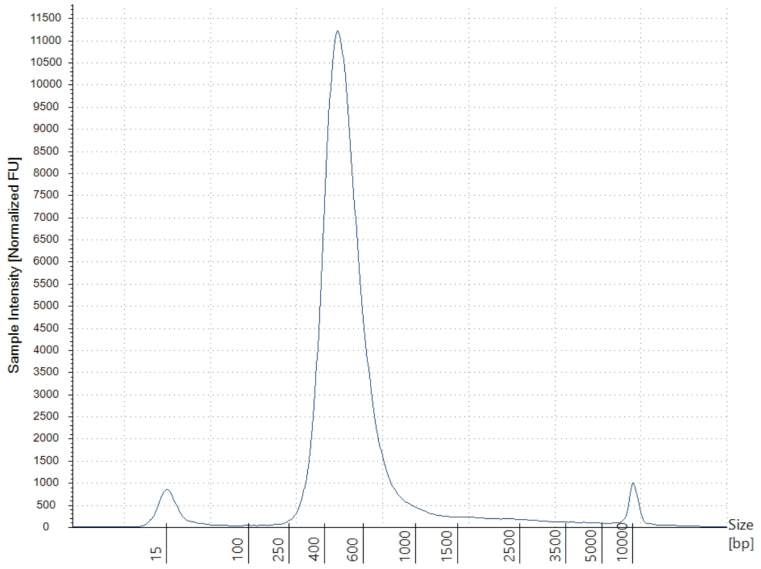
Run manufacturer's recommended volume of diluted sample (1:20 dilution)



Protocol Step 6.7 - GEX Post Library Construction QC

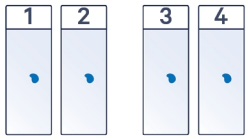
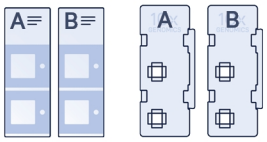
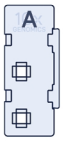
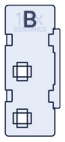
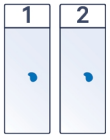
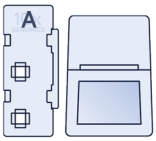
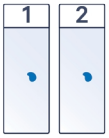
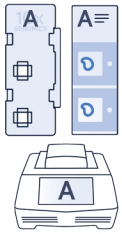
Representative Trace

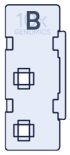
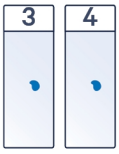
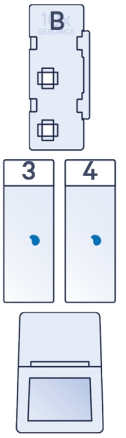
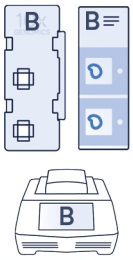
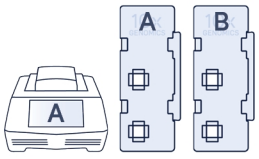
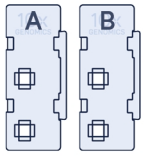
Run manufacturer's recommended volume of diluted sample (1:20 dilution)



HD 3' Workflow Overview for Processing 4 Tissue Slides with 1 Visium CytAssist Instrument

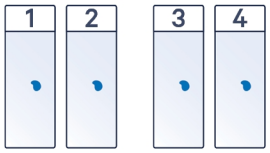
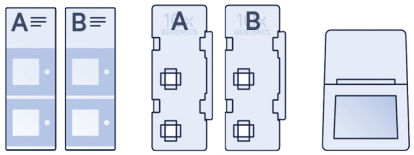
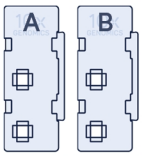
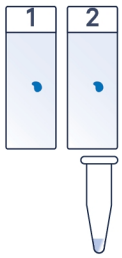
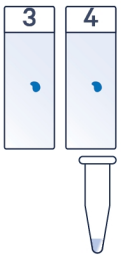
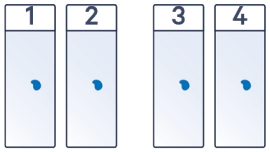
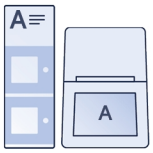
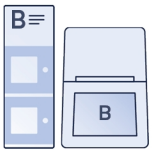
Two thermal cyclers are required. Scale volume calculations appropriately. If using multiple beakers, ensure they are cleaned according 2.2 Destaining on page 59. This table is only a reference. Consult the detailed steps in this user guide for execution.

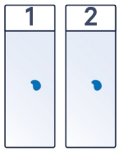
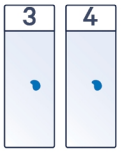
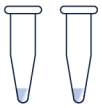
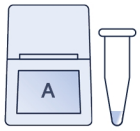
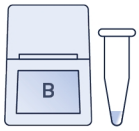
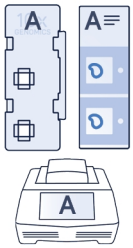
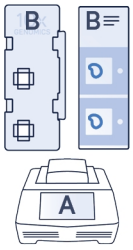
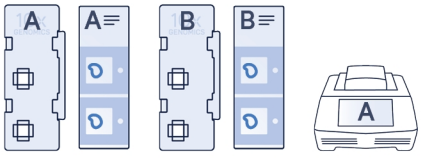
HD 3' Workflow: 4 Tissue Slides, 1 CytAssist		
Step	**Run A**	**Run B**
Sample Prep		
Tissue slides 1 - 4 *prep only 2 slides at a time*		• Prepare tissue slides via sectioning, staining and imaging • Store coverslipped at 4°C for up to 72 h
Step 1: Visium HD Slide & CytAssist Preparation		
1.1 Visium HD Slide Wash Visium HD Slides A & B *at the same time*		• Thaw & wash Visium HD Slides • Place slides in Visium cassettes with 0.1X SSC
Equilibration for Visium slide A	 • Remove SSC and replace with Pre-Equilib Mix • Prep CytAssist instrument	 • Store Visium HD Slide B in final 0.1X SSC wash at room temperature for <2 h
A - Step 2: Coverslip Removal & Destaining		
2.1 Coverslip Removal **2.2 Destaining** Tissue slides 1 & 2 *at the same time*	 • Decoverslip • Destain • Prep Perm Mix (amount for 2 tissue slides)	*Continue to store* • *Tissue slides 3 & 4 at 4°C* • *Visium HD Slide B at room temperature*
A - Step 3: CytAssist-Enabled mRNA Capture & Reverse Transcription 1		
3.1 CytAssist-Enabled mRNA Capture Start CytAssist (Run A) with tissue slides 1 & 2, Visium HD Slide A	  • Remove Pre-Equilib Mix • Load Visium HD Slide on CytAssist to dry completely (10 min) • Load tissue slides 1 & 2 into CytAssist instrument • Add Perm Enzyme B and Reducing Agent B to Perm Mix, dispense onto Visium slide spacer wells • Close lid and start run • Prep thermal cycler A • Prep RT Master Mix 1	
CytAssist (Run A) complete **3.2 Reverse Transcription** Reverse Transcription 1 for Visium HD Slide A	 • Open CytAssist • Wash Visium HD Slide A • Place in Visium Cassette and add RT Master Mix 1 • Apply seal and place on thermal cycler A	

HD 3' Workflow: 4 Tissue Slides, 1 CytAssist		
Step	**Run A**	**Run B**
B - Step 2: Coverslip Removal & Destaining		
Equilibration for Visium HD Slide B	 *Hold in thermal cycler*	 • Remove SSC and replace with Pre-Equilib Mix • Prep CytAssist instrument
2.1 Coverslip Removal **2.2 Destaining** Tissue slides 3 & 4 *at the same time*		 • Decoverslip • Destain • Prep Perm Mix
B - Step 3: CytAssist-Enabled mRNA Capture & Reverse Transcription 1		
3.1 CytAssist-Enabled mRNA Capture *Ensure 20 min cool-down period after completion of Run A* Start CytAssist (Run B) with tissue slides 3 & 4, Visium HD Slide B		 • Remove Pre-Equilib Mix • Load Visium HD Slide on CytAssist to dry completely (10 min) • Load tissue slides 3 & 4 into CytAssist instrument • Add Perm Enzyme B and Reducing Agent B to Perm Mix, dispense onto Visium slide spacer wells • Close lid and start run • Prep thermal cycler B • Prep RT Master Mix 1
CytAssist (Run B) complete **3.2 Reverse Transcription** Reverse Transcription 1 for Visium HD Slide B		 • Open CytAssist • Wash Visium HD Slide B • Place in Visium Cassette and add RT Master Mix 1 • Apply seal and place on thermal cycler B
Step 3: Reverse Transcription 2 & Denaturation		
Reverse Transcription 2 for Visium HD Slides A & B		• Prep thermal cycler A • Remove seal from Visium cassettes and remove RT Master Mix • Add then remove SSC buffer • Add RT Master Mix 2 • Apply seal and place on thermal cycler A
3.3 Denaturation		*Proceed with Visium HD Slides A & B through the rest of the user guide, accounting for higher volumes*

HD 3' Workflow Overview for Processing 4 Tissue Slides with 2 Visium CytAssist Instruments

Two thermal cyclers are required. A slide rack capable of holding four slides is needed for processing four tissue slides at a time. Scale volume calculations appropriately. If using multiple beakers, ensure they are cleaned according to 2.2 Destaining on page 59. This table is only a reference. Consult the detailed steps in this user guide for execution.

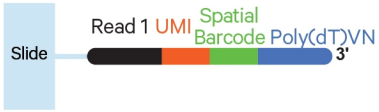
HD 3' Workflow: 4 Tissue Slides, 2 CytAssists		
Step	**Run A, CytAssist A**	**Run B, CytAssist B**
Sample Prep		
Tissue slides 1 - 4 *at the same time*		• Prepare tissue slides via sectioning, staining and imaging • Store coverslipped at 4°C for up to 72 h
Step 1: Visium HD Slide & CytAssist Preparation		
1.1 Visium HD Slide Wash Visium HD Slides A & B *at the same time*		• Thaw & wash Visium HD Slides • Place slides in Visium cassettes with 0.1X SSC
Equilibration for Visium HD Slides A & B		• Remove SSC and replace with Pre-Equilib Mix • Prep CytAssist instrument
Step 2: Coverslip Removal & Destaining		
2.1 Coverslip Removal 2 Tissue slides *at a time*	 • Decoverslip • Prep 2 tubes of Perm Mix (amount for 2 tissue slides each)	 • Decoverslip • Prep 2 tubes of Perm Mix (amount for 2 tissue slides each)
2.2 Destaining Tissue slides 1-4 *at the same time*		• Destain
Step 3: CytAssist-Enabled mRNA Capture, Reverse Transcription, & Denaturation		
Load CytAssists A & B	 • Remove Pre-Equilib Mix • Load Visium HD Slide A on CytAssist to dry completely (10 min)	 • Set timer for 2 min (starts the offset between CytAssist A & B) • After 2 min, remove Pre-Equilib Mix • Load Visium HD Slide B on CytAssist to dry completely (10 min) • Set timer for 2 min (again)

HD 3' Workflow: 4 Tissue Slides, 2 CytAssists		
Step	**Run A, CytAssist A**	**Run B, CytAssist B**
Load CytAssists A & B	 • After 2 min, load tissue slides 1 & 2 into CytAssist instrument	 • Load tissue slides 3 & 4 into CytAssist instrument
Prep Perm Mix		 • Add Perm Enzyme B and Reducing Agent B to Perm Mix tubes
Start CytAssist A, then after 2 min, start CytAssist B	 • Dispense Perm Mix onto Visium slide spacer wells • Close lid and start run	 • Dispense Perm Mix onto Visium slide spacer wells • Close lid and start run
Prep thermal cyclers		 • Prep thermal cycler A • Prep 2x volume of RT Master Mix 1
3.2 Reverse Transcription CytAssist A (Run A) complete *2 min later* CytAssist B (Run B) complete Reverse Transcription 1 for Visium HD Slides A & B	 • Open CytAssist • Wash Visium HD Slide A • Place in Visium Cassette and add RT Master Mix 1 • Apply seal and place on thermal cycler A (don't start yet)	*2 min offset*
		 • Open CytAssist • Wash Visium HD Slide B • Place in Visium Cassette and add RT Master Mix 1 • Apply seal and place on thermal cycler A • Start Reverse Transcription 1 with slides A & B
Reverse Transcription 2 for Visium HD Slides A & B		*Proceed with Visium HD Slides A & B through the rest of the user guide, accounting for higher volumes*

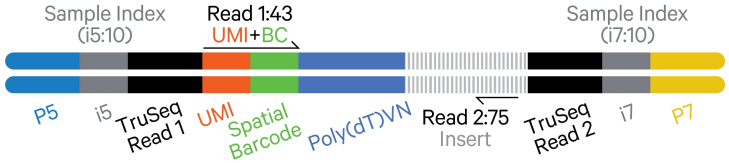
Oligonucleotide Sequences

Visium HD 3' Spatial Gene Expression Slide Primers

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



Visium HD 3' Library



5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-UMI-SpatialBarcode-GVV-T30-VN-cDNA_Insert-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'

3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-UMI-SpatialBarcode-CBB-A30-BN-cDNA_Insert-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'