User Guide | CG000495 | Rev B

Visium CytAssist Spatial Gene Expression Reagent Kits

for FFPE

For use with:

Visium CytAssist Spatial Gene Expression for FFPE, Human Transcriptome, 6.5 mm, 4 rxns PN-1000520

Visium CytAssist Spatial Gene Expression for FFPE, Mouse Transcriptome, 6.5 mm, 4 rxns PN-1000521

Visium CytAssist Spatial Gene Expression for FFPE, Human Transcriptome, 11 mm, 2 rxns PN-1000522

Visium CytAssist Spatial Gene Expression for FFPE, Mouse Transcriptome, 11 mm, 2 rxns PN-1000523

Visium CytAssist Reagent Accessory Kit, PN-1000499

Visium CytAssist Tissue Slide Cassette, 4 pk, 6.5 mm PN-1000471

Visium CytAssist Tissue Slide Cassette, 4 pk, 11 mm PN-1000472

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

Notices

Document Number

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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
CG000495

Title
Visium CytAssist Spatial Gene Expression Reagent Kits User Guide

Revision
Rev B

Revision Date
November 30, 2022

Specific Changes
- Added guidance on excess silicone removal for Visium CytAssist Tissue Slide Cassettes.
- Added Analytik Jena Biometra as a recommended thermal cycler.
- Updated Probe Release Mix preparation.
- Added Troubleshooting guidance on overloaded traces.

General Changes
Updated for general minor consistency of language and terms throughout.

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Introduction

Reagent Kits

Visium Spatial Gene Expression for FFPE Reagent Kits

Refer to SDS for handling and disposal information.

Reagent Kits	Part Number	Components	Component Part Number
Visium CytAssist Spatial Gene Expression for FFPE, Mouse Transcriptome, 6.5 mm, 4 rxns*	1000521	Visium CytAssist Slide and Cassettes, 6.5 mm, 2 rxns	PN-1000519
		Visium FFPE Reagent Kit v2 - Small	PN-1000436
		Visium Mouse Transcriptome Probe Kit – Small	PN-1000365
Visium CytAssist Spatial Gene Expression for FFPE, Human Transcriptome, 6.5 mm, 4 rxns*	1000520	Visium CytAssist Slide and Cassettes, 6.5 mm, 2 rxns	PN-1000519
		Visium FFPE Reagent Kit v2 - Small	PN-1000436
		Visium Human Transcriptome Probe Kit v2 – Small	PN-1000466
Visium CytAssist Spatial Gene Expression for FFPE, Mouse Transcriptome, 11 mm, 2 rxns**	1000523	Visium CytAssist Slide and Cassettes, 11 mm, 2 rxns	PN-1000518
		Visium FFPE Reagent Kit v2 - Small	PN-1000436
		Visium Mouse Transcriptome Probe Kit – Small	PN-1000365
Visium CytAssist Spatial Gene Expression for FFPE, Human Transcriptome, 11 mm, 2 rxns**	1000522	Visium CytAssist Slide and Cassettes, 11 mm, 2 rxns	PN-1000518
		Visium FFPE Reagent Kit v2 - Small	PN-1000436
		Visium Human Transcriptome Probe Kit v2 – Small	PN-1000466

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

**Also available in a pack of 4 as an 8 rxn kit.

Visium CytAssist Slide and Cassettes, 6.5 mm, 2 rxns PN-1000519

Visium CytAssist Slide and Cassettes, 6.5mm
2 rxns PN-1000519
(store at ambient temperature)

	#	PN
Visium Cassette, 8 port	1	3000811
Visium CytAssist Sliding Gasket, Small (pre-assembled with translator)	2	3000814
Visium CytAssist Cassette Movable Translator (pre-assembled with gasket)	2	3000816
Visium CytAssist Cassette Movable Frame	2	3000813
Visium Slide Seals, 40-pack	1	2000284
Visium CytAssist Spatial Gene Expression Slide v2, 6.5 mm	1	2000550

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Visium CytAssist Slide and Cassettes, 11 mm, 2 rxns PN-1000518

Visium CytAssist Slide and Cassettes, 11mm
2 rxns PN-1000518
(store at ambient temperature)

	#	PN
Visium Cassette, 2 port	1	3000812
Visium CytAssist Sliding Gasket, Large	2	3000815
Visium CytAssist Cassette Movable Frame	2	3000813
Visium Slide Seals, 40-pack	1	2000284
Visium CytAssist Spatial Gene Expression Slide v2, 11 mm	1	2000550

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Visium CytAssist Tissue Slide Cassette, 6.5 mm, 4 Cassettes PN-1000471

Visium CytAssist Tissue Slide Cassette, 6.5mm
4 Cassettes PN-1000471
(store at ambient temperature)

	#	PN
Visium CytAssist Sliding Gasket, Small (pre-assembled with translator)	4	3000814
Visium CytAssist Cassette Movable Translator (pre-assembled with gasket)	4	3000816
Visium CytAssist Cassette Movable Frame	4	3000813

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Visium CytAssist Tissue Slide Cassette, 11 mm, 4 Cassettes PN-1000472

Visium CytAssist Tissue Slide Cassette, 11mm
4 Cassettes PN-1000472
(store at ambient temperature)

	#	PN
Visium CytAssist Sliding Gasket, Large	4	3000815
Visium CytAssist Cassette Movable Frame	4	3000813

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Visium FFPE Reagent Kit v2– Small, PN-1000436

Visium FFPE Reagent Kit - Small
PN-1000436
(store at −20°C)

		#	PN
○	Amp Mix B	1	2000567
●	Extension Enzyme	1	2000389
●	Extension Buffer	1	2000409
●	RNase Enzyme	1	3000593
●	RNase Buffer B	1	2000551
●	Tissue Removal Enzyme	1	3000387
●	Tissue Removal Buffer B*	1	2000543
●	Tissue Removal Buffer Enhancer*	1	2000557
●	Decrosslinking Buffer	1	2000566
●	TS Primer Mix B	1	2000537
●	Block and Stain Buffer	1	2000554

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*Not used in this assay.

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

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

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

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Visium Mouse Transcriptome Probe Kit - Small, PN-1000365

Visium Mouse Transcriptome Probe Kit - Small
PN-1000363
(store at −20°C)

		#	PN
○	FFPE Hyb Buffer	1	2000423
○	FFPE Post-Hyb Wash Buffer	1	2000424
●	Mouse WT Probes – RHS	1	2000455
●	Mouse WT Probes – LHS	1	2000456
○	Probe Ligation Enzyme	1	2000425
○	2X Probe Ligation Buffer	1	2000445
	Post Ligation Wash Buffer	1	2000419

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

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

	#	PN
Dual Index Plate TS Set A	1	3000511

10x Genomics Accessories

Product	Kit and Part Number	Part Number (Item)
10x Magnetic Separator	Visium CytAssist Reagent Accessory Kit 1000499	120250
Low Profile Thermocycler Adapter		3000823

Recommended Thermal Cyclers

Supplier	Description	Part Number
Bio-Rad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module	1851197
Eppendorf	MasterCycler Pro (discontinued)	*North America* 950030010 *International* 6321 000.019
	MasterCycler X50s	*North America* 6311000010
Thermo Fisher Scientific	Veriti 96-Well Thermal Cycler (discontinued)	4375786
Analytik Jena	Biometra TAdvanced 96 SG with 96-well block (silver, 0.2 mL) and gradient function	846-x-070-241

If using thermal cyclers other than BioRad C1000, ramp rates should be adjusted for all the steps as described below

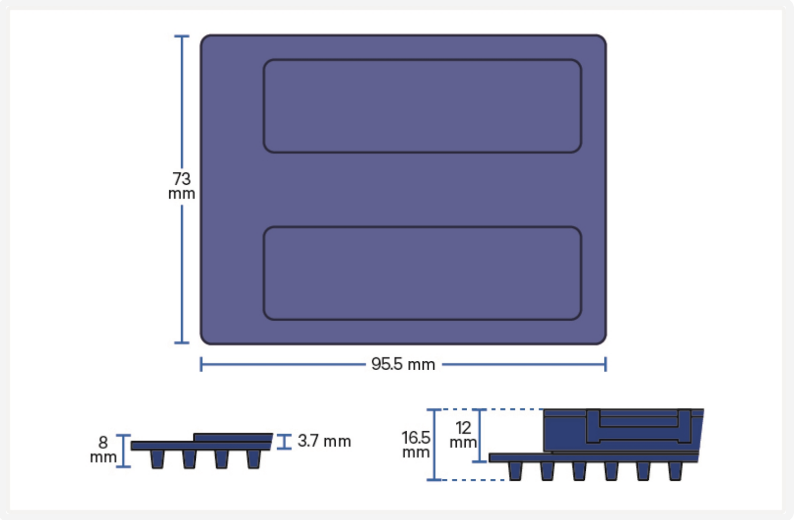
- Eppendorf MasterCycler X50s: 3°C/sec heating and 2°C/sec cooling
- Analytik Jena Biometra TAdvanced: 2°C/sec heating and cooling

Thermal cycler requirements if reactions are performed on a tube:

- Uniform heating of 100 ul volumes
- Temperature-controlled lid
- 96 deep-well block or 0.2 ml block configuration

Thermal cycler requirements if reactions are performed on a slide:

- The thermal cycler must be able to accommodate the low profile plate insert (also referred to as the Low Profile Thermocycler Adapter):
  - Well depth: 4.5 mm
  - Distance between block and heated lid: 12 mm
  - Reaction block dimensions: 95.5 x 73 mm
- The Low Profile Thermocycler Adapter may be difficult to remove from some thermal cyclers. If this is the case, turn off the thermal cycler and place a -20°C cooling pad on top of the Low Profile Thermocycler Adapter for a few minutes prior to attempting removal. Do not handle the Low Profile Thermocycler Adapter while it is hot.



Recommended Real Time qPCR Systems

Supplier	Description	Part Number
Applied Biosystems	QuantStudio 12K Flex system	4471087
Bio-Rad	CFX96 Real-time System (discontinued)	1855096

Imaging System Recommendations

The imaging systems listed below were used by 10x Genomics. Any equivalent system with the listed features may be used for imaging. Consult the Visium CytAssist Gene Expression for FFPE Imaging Guidelines Technical Note (CG000521) for more information.

Imaging Systems & Specifications		
Microscopes (Any equivalent system with the listed features may be used for imaging)		
Supplier	**Model**	**Configuration**
Thermo Fisher Scientific	EVOS M7000	Inverted
Leica	Aperio Versa 8	Upright
	Leica DMi8	Inverted
MetaSystems	Metafer	Upright
Nikon	Nikon Eclipse Ti2	Inverted
BioTek	Cytation 7	Inverted or Upright
Keyence	Keyence BZX800	Inverted
Olympus	VS200	Upright
Zeiss	Imager.Z2	Upright
Microscope Features		
Objectives	• 10X (NA 0.45) • 20X (NA 0.75) • 40X (NA 0.95)	
Brightfield Features (for H&E staining)	• Color camera (3 x 8 bit, 2,424 x 2,424 pixel resolution) • White balancing functionality • Minimum Capture Resolution 2.18 µm/pixel • Exposure times 2-10 milli sec	
Fluorescence Features (for IF Staining)	• Light source (or equivalent) with a wavelength range of 380-680 nm • Monochrome camera (14 bit, 2,424 x 2,424 pixel resolution)	

Imaging Systems & Specifications

- DAPI filter cube (Excitation 392/23, Emission 447/60)
- FITC filter cube (Excitation 466/40, emission 515/30)
- Cy5 filter cube (Excitation 618/50, Emission 698/70)
- TRITC filter cube (Excitation 542/20, Emission 620/52)
- Minimum Capture Resolution 2.18 µm/pixel
- Exposure times 100 milli sec-2 sec

Additional Specifications	
Image Format	Save image as a tiff (preferred) or jpeg
Computer	Computer with sufficient power to handle large images (0.5-5 GB)
Software	Image stitching software (microscope's imaging software or equivalent, like ImageJ)

Additional Kits, Reagents & Equipment

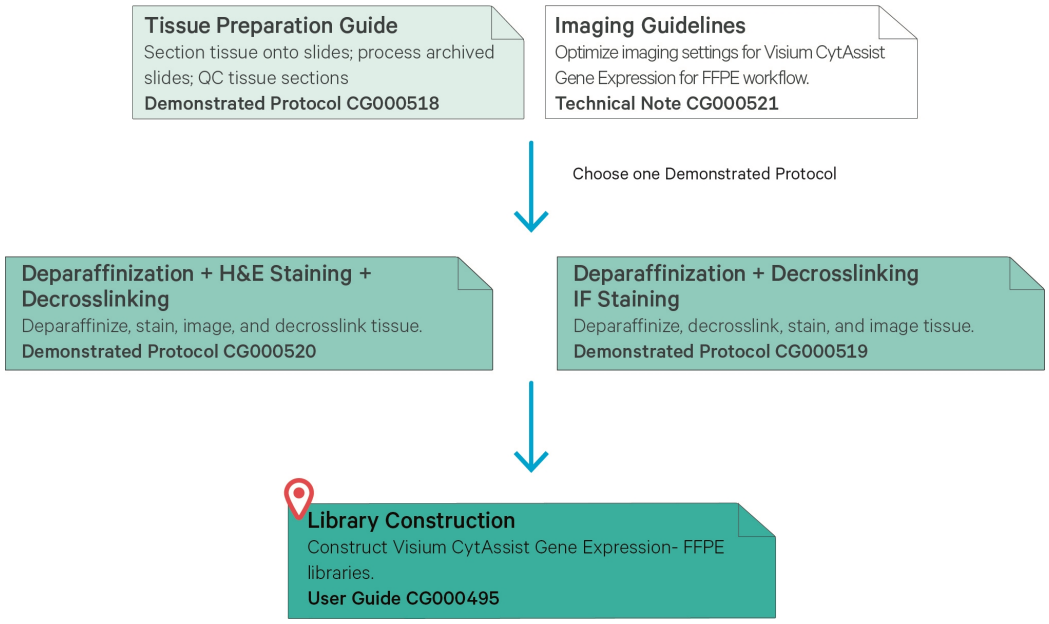
The items in the table below have been validated by 10x Genomics and are highly recommended for the Visium Spatial Reagent Kits protocols. **Substituting materials may adversely affect system performance.** This list does not include standard laboratory equipment such as water baths, centrifuges, vortex mixers, pH meters, freezers etc.

Item	Description	Supplier	Part Number (US)
Plastics			
1.5 ml tubes	DNA LoBind Tubes, 1.5 ml	Eppendorf	022431021
	Low DNA Binding Tubes, 1.5 ml	Sarstedt	72.706.700
2.0 ml tubes (when processing more than 2 slides)	DNA LoBind Tubes, 2.0 ml	Eppendorf	022431048
	Low DNA Binding Tubes, 2.0 ml	Sarstedt	72.695.700
15 ml tubes	15 ml PP Centrifuge Tubes	Corning	430791
50 ml tubes	Self-Standing Polypropylene Centrifuge Tubes (50 ml), sterile	Corning	430921
0.2 ml PCR 8-tube strips *Choose either Eppendorf, USA Scientific, or Thermo Fisher Scientific PCR 8-tube strips*	PCR Tubes 0.2 ml 8-tube strips	Eppendorf	951010022
	TempAssure PCR 8-tube strip	USA Scientific	1402-4700
	MicroAmp 8-Tube Strip, 0.2 ml (see Tips & Best Practices for more information)	Thermo Fisher Scientific	N8010580
	MicroAmp 8 -Cap Strip, clear (see Tips & Best Practices for more information)	Thermo Fisher Scientific	N8010535
Slide mailer/tube	Simport Scientific LockMailer Tamper Evidence Slide Mailer (alternatively, use a 50-ml centrifuge tube)	Thermo Fisher Scientific	22-038-399
	Self-Standing Polypropylene Centrifuge Tubes (50 ml), sterile	Corning	430921
PCR plates and sealing film	Hard-shell PCR Plates 96-well, thin wall (pkg of 50) (Or any compatible PCR Plate)	Bio-Rad	HSP9665
	Microseal 'B' PCR Plate Sealing Film, adhesive (Or any compatible PCR Plate sealing adhesive)	Bio-Rad	MSB1001
Pipette tips	Tips LTS 200UL Filter RT-L200FLR	Rainin	30389240

Item	Description	Supplier	Part Number (US)
	Tips LTS 1ML Filter RT-L1000FLR	Rainin	30389213
	Tips LTS 20UL Filter RT-L20FLR	Rainin	30389226
Wide Bore Pipette tips	Tips RT LTS 200UL FLW	Rainin	30389241
	Tips RT LTS 1000UL FLW	Rainin	30389218
Reagent Reservoirs	Divided Polystyrene Reservoirs	VWR	41428-958
Kits & Reagents			
Nuclease-free Water (not DEPC treated)	Nuclease-free Water	Thermo Fisher Scientific	AM9937
Tris 1M	Tris 1M, pH 8.0, RNase-free	Thermo Fisher Scientific	AM9855G
	Tris 1M, pH 8.0	TEKONOVA	T5080
	UltraPure 1M Tris-HCl, pH 8.0	Invitrogen	15568025
10X PBS	PBS - Phosphate Buffered Saline (10X) pH 7.4, RNase-free	Thermo Fisher Scientific	AM9624
Tween 20	Tween 20 Surfact-Amps Detergent Solution (10% solution)	Thermo Fisher Scientific	28320
qPCR Mix	KAPA SYBR FAST qPCR Master Mix (2X)	KAPA Biosystems (US Only)	KK4600
		Millipore Sigma (Europe, Asia, & Canada)	
SPRIselect Reagent	SPRIselect Reagent Kit	Beckman Coulter	B23318
Ethanol	Ethyl alcohol, Pure (200 Proof, anhydrous)	Millipore Sigma	E7023-500ML
8M KOH	Potassium Hydroxide Solution, 8M	Millipore Sigma	P4494-50ML
20X SSC Buffer	SSC Buffer 20X Concentrate	Millipore Sigma	S66391L
Buffer EB	Qiagen Buffer EB	Qiagen	19086
Eosin	Eosin Y solution, alcoholic	Millipore Sigma	HT110116
	Eosin Y Solution (Modified Alcoholic)	Abcam	ab246824
	Eosin Y with Phloxine 1% alcholic solution	VWR	10143
Ultrapure Water	Ultrapure/Milli-Q water (from Milli-Q Integral Ultrapure Water System or equivalent)		
Equipment			
Pipettes	Pipet-Lite Multi Pipette L8-200XLS+	Rainin	17013805
	Pipet-Lite LTS Pipette L-2XLS+	Rainin	17014393

Item	Description	Supplier	Part Number (US)
	Pipet-Lite LTS Pipette L-10XLS+	Rainin	17014388
	Pipet-Lite LTS Pipette L-20XLS+	Rainin	17014392
	Pipet-Lite LTS Pipette L-100XLS+	Rainin	17014384
	Pipet-Lite LTS Pipette L-200XLS+	Rainin	17014391
	Pipet-Lite LTS Pipette L-1000XLS+	Rainin	17014382
Mini Centrifuge	VWR Mini Centrifuge (or any equivalent mini centrifuge)	VWR	76269-064
Swab	Alpha Polyester Knit TX714A Large Cleanroom Swab	Texwipe	TX714A
Quantification & Quality Control			
Choose Bioanalyzer, TapeStation, or LabChip based on availability & preference.			
Bioanalyzer & associated reagents	2100 Bioanalyzer Laptop Bundle (discontinued)	Agilent	G2943CA
	Replacement 2100 Bioanalyzer Instrument/2100 Expert Laptop Bundle	Agilent	G2939BA/G2953CA
	High Sensitivity DNA Kit	Agilent	5067-4626
TapeStation & associated reagents	4200 TapeStation	Agilent	G2991AA
	High Sensitivity D1000 ScreenTape/Reagents	Agilent	5067-5584/ 5067-5585
	High Sensitivity D5000 ScreenTape/Reagents	Agilent	5067-5592/ 5067-5593
LabChip & associated reagents	LabChip GX Touch HT Nucleic Acid Analyzer	PerkinElmer	CLS137031
	DNA High Sensitivity Reagent Kit	PerkinElmer	CLS760672
Qubit & associated reagents (for determining dilution factor for TapeStation or Bioanalyzer)	Qubit Fluorometer	ThermoFisher Scientific	Q33238
	Qubit Assay Tubes	ThermoFisher Scientific	Q32856
	Qubit dsDNA HS and BR Assay Kits	ThermoFisher Scientific	Q32854
Library Quantification Kit	KAPA Library Quantification Kit for Illumina Platforms	KAPA Biosystems	KK4824

Workflow Overview



Protocol Steps & Timing

Steps	Timing	Stop & Store
Day 1		
Step 1: Probe Hybridization (page 58)		
1.1 Probe Hybridization (page 60)	Overnight	
Day 2		
Step 2: Probe Ligation (page 64)		
2.1 Post-Hybridization Wash (page 66)	20 min	
2.2 Probe Ligation (page 68)	65 min	
2.3 Post-Ligation Wash (page 70)	15 min	STOP 4°C ≤24 h
Step 3: Probe Release & Extension (page 72)		
1.1 CytAssist Enabled RNA Digestion & Probe Release	40 min	
3.2 Probe Extension (page 83)	20 min	STOP 4°C ≤24 h
3.3 Probe Elution (page 84)	15 min	
Step 4: Pre-Amplification and SPRIselect (page 86)		
4.1 Pre-Amplification (page 88)	40 min	
4.2 Pre-Amplification Cleanup - SPRIselect (page 90)	30 min	STOP 4°C ≤72 h or -20°C ≤4 weeks
Step 5: Visium Spatial Gene Expression – FFPE Library Construction (page 91)		
5.1 Cycle Number Determination – qPCR (page 93)	45 min	
5.2 GEX Sample Index PCR (page 95)	40 min	
5.3 GEX Post-Sample Index PCR Cleanup – SPRIselect (page 97)	30 min	STOP -20°C long-term
5.4 GEX Post-Library Construction QC (page 98)	50 min	

Stepwise Objectives

The Visium CytAssist Spatial Gene for FFPE assay is designed to analyze mRNA in tissue sections derived from formalin fixed & paraffin embedded (FFPE) tissue samples. It uses probes targeting the whole transcriptome. Each Visium CytAssist Spatial Gene Expression Slide contains Capture Areas with barcoded spots that include oligonucleotides required to capture gene expression probes. Prior to the assay, tissue sections are deparaffinized, stained, and decrosslinked, and imaged as described in Deparaffinization & Staining Demonstrated Protocols - CG000519 or CG000520.

Human or mouse whole transcriptome probe panels, consisting of ~1 (mouse) or ~3 pairs (human) of specific probes for each targeted gene, are added to the tissue, enabling hybridization and ligation of each probe pair. Tissue Slides and Visium CytAssist Spatial Gene Expression v2 Slides are loaded into the Visium CytAssist instrument, where they are brought into proximity with one another. Gene expression probes are released from the tissue upon CytAssist Enabled RNA Digestion & Probe Release, enabling capture by the spatially barcoded oligonucleotides present on the slide surface. The Visium CytAssist Spatial Gene Expression v2 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.

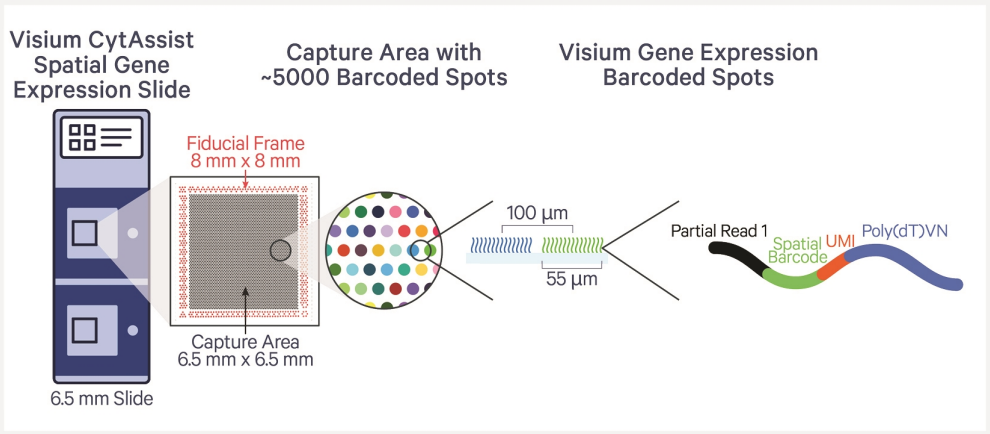
This document outlines the protocol for generating Visium Spatial Gene Expression - FFPE libraries from FFPE tissue sections.

Visium CytAssist Spatial Gene Expression Slides

Visium CytAssist Spatial Gene Expression Slide v2, 6.5 mm

The CytAssist Spatial Gene Expression Slide v2, 6.5 mm has 2 Capture Areas. Each Capture Area is 6.5 x 6.5 mm and defined by a fiducial frame (fiducial frame + Capture Area is 8 x 8 mm). The Capture Area has ~5,000 barcoded spots. Each spot has the following oligos:

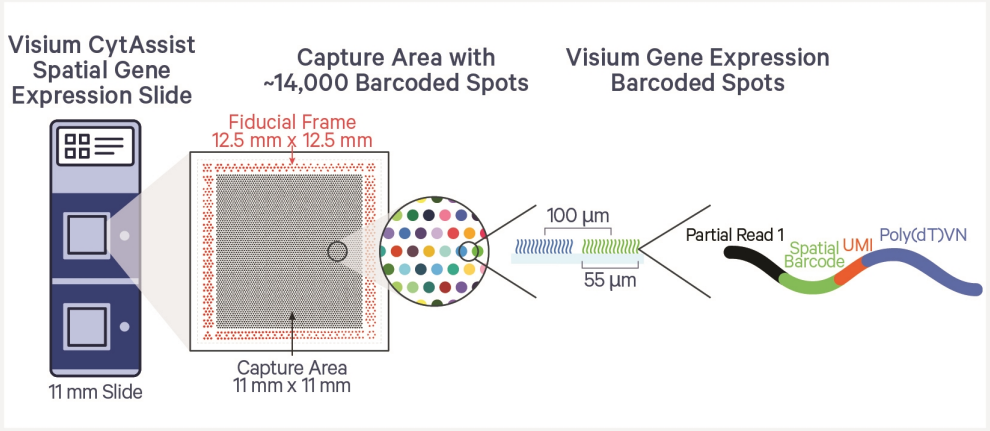
- Gene Expression: Illumina TruSeq partial read 1 sequencing primer, 16 nucleotide (nt) Spatial Barcode, 12 nt unique molecular identifier (UMI), 30 nt poly(dT) sequence (captures ligation product).
- Each Capture Area on the CytAssist Spatial Gene Expression Slide is surrounded by a spacer. This spacer creates a reaction chamber that facilitates proper reagent addition and creates a seal between the CytAssist Spatial Gene Expression Slide and the Tissue Slide.
- The active surface of the slide is defined by a readable label that includes the serial number.



Visium CytAssist Spatial Gene Expression Slide v2, 11 mm

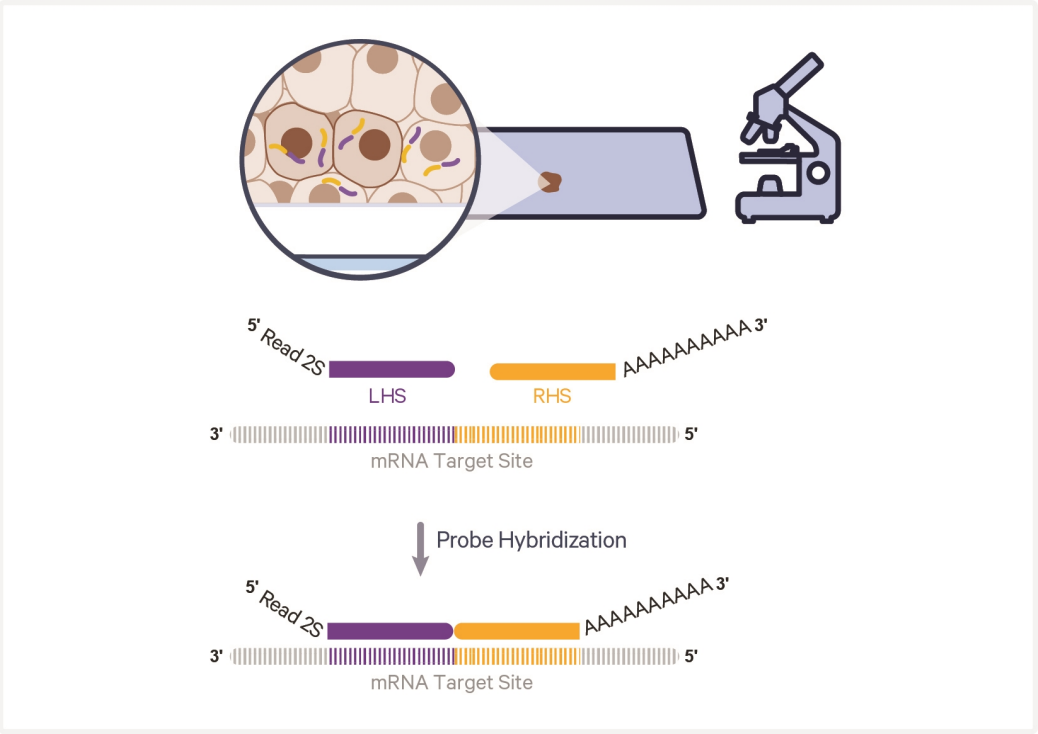
The CytAssist Spatial Gene Expression Slide v2, 11 mm has 2 Capture Areas. Each Capture Area is 11 x 11 mm and defined by a fiducial frame (fiducial frame + Capture Area is 12.5 x 12.5 mm). The Capture Area has ~14,000 barcoded spots. Each spot has the following oligos:

- Gene Expression: Illumina TruSeq partial read 1 sequencing primer, 16 nt Spatial Barcode, 12 nt unique molecular identifier (UMI), 30 nt poly(dT) sequence (captures ligation product)
- Each Capture Area on the CytAssist Spatial Gene Expression Slide is surrounded by a spacer. This spacer creates a reaction chamber that facilitates proper reagent addition and creates a seal between the CytAssist Spatial Gene Expression Slide and the Tissue Slide.
- The active surface of the slide is defined by a readable label that includes the serial number.



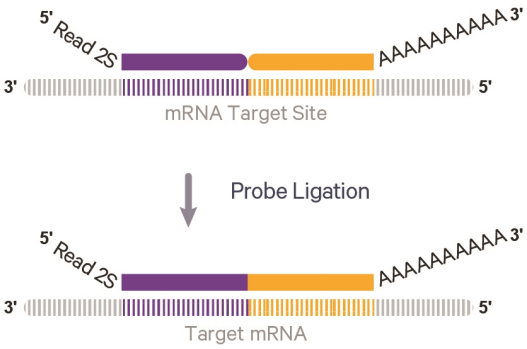
Step 1: Probe Hybridization

The human or mouse whole transcriptome probe panel, consisting of ~3 or ~1 specific probes for each targeted gene respectively, is added to the deparaffinized, stained, and decrosslinked tissues. Together, probe pairs hybridize to their complimentary target RNA.



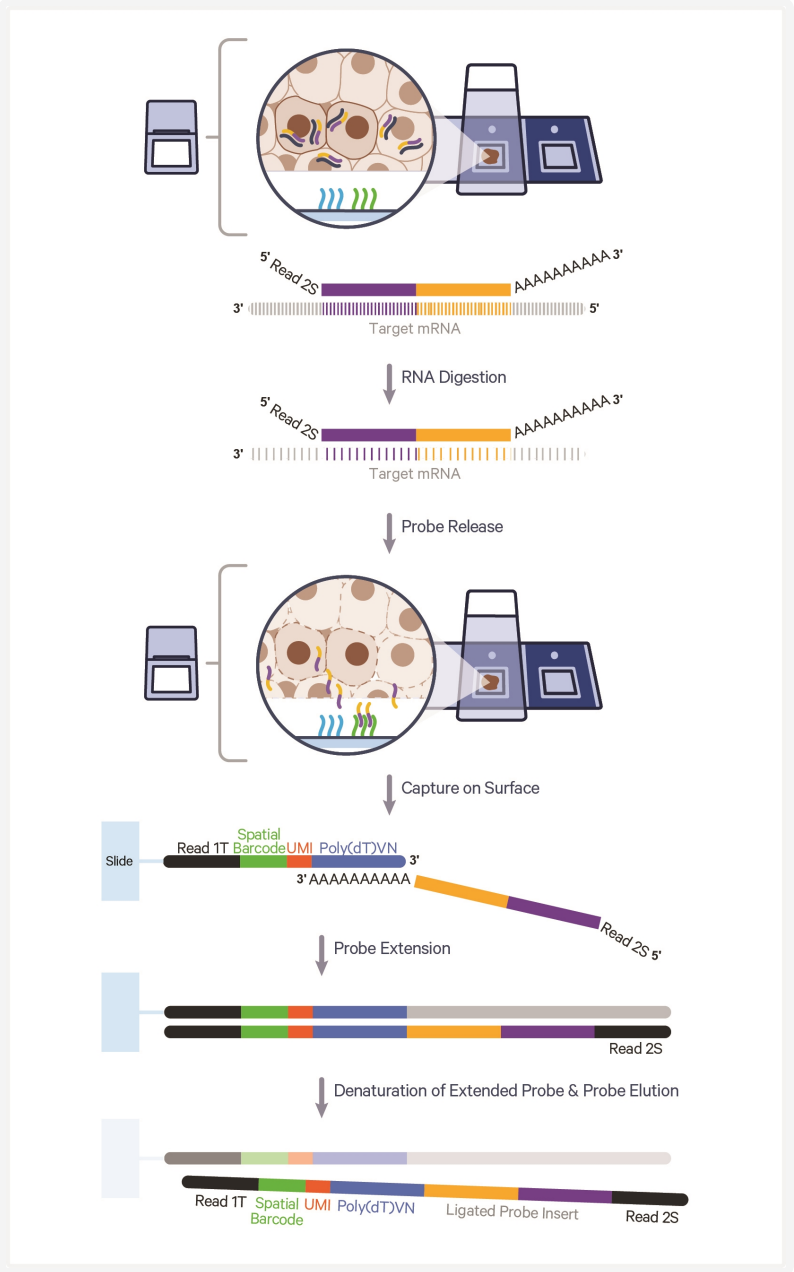
Step 2: Probe Ligation

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



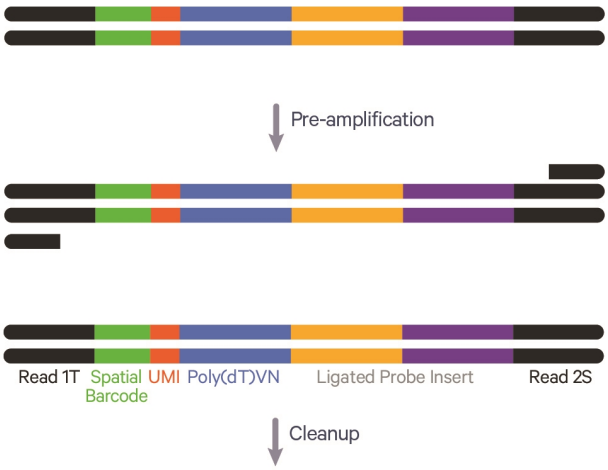
Step 3: CytAssist Enabled RNA Digestion & Oligo Capture

This step occurs on the Visium CytAssist instrument. The single stranded ligation products are released from the tissue upon RNase treatment and tissue removal, and then captured on the Visium slides. Once ligation products are captured, the slides can be removed from the instrument. Probes are extended by incorporating addition of UMI, Spatial Barcode, and partial Read 1. This generates spatially barcoded, ligated probe products, which can then be carried forward for library preparation.



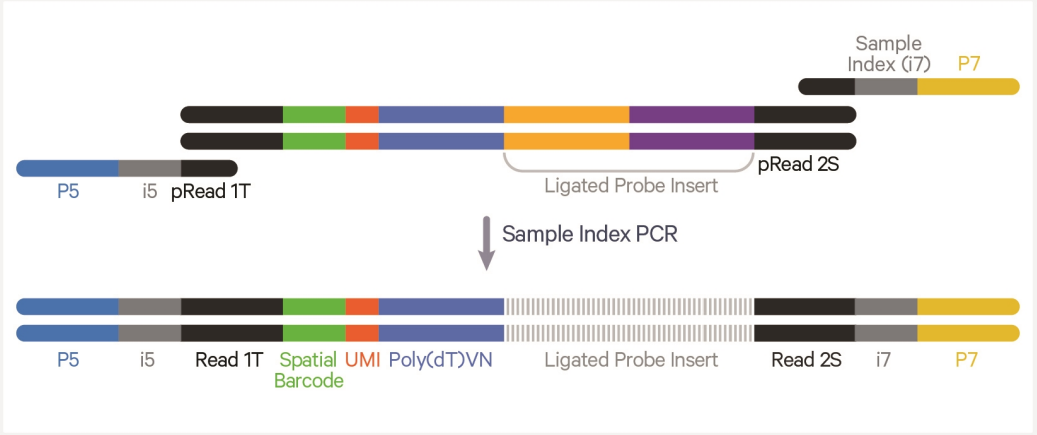
Step 4: Pre-amplification and SPRIselect

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



Step 5: Visium Spatial Gene Expression - FFPE Library Construction

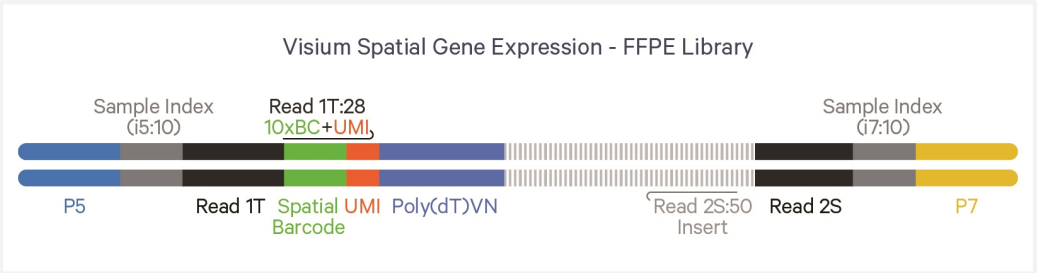
Pre-amplification material is collected for qPCR to determine Sample Index PCR cycle number for gene expression libraries. The amplified material then undergoes indexing via Sample Index PCR generating final library molecules. The final libraries are cleaned up by SPRIselect, assessed on a bioanalyzer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



Sequencing

A Visium Spatial Gene Expression - FFPE library comprises standard Illumina paired-end constructs which begin and end with P5 and P7 adaptors. The 16 bp Spatial Barcode and 12 bp UMI are encoded in TruSeq Read 1, while Small RNA Read 2 (Read 2S) is used to sequence the ligated probe insert.

Illumina sequencer compatibility, sample indices, library loading and pooling for sequencing are summarized in step 8.



See Oligonucleotide Sequences on page 120

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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 version specific update in volume, temperature, instruction, etc.

General Reagent Handling

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

Pipette Calibration

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

Visium Slide Storage

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

Visium Spatial Slide Handling

- 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.
- Minimize exposure of the slides to sources of particles and fibers.
- When pipetting reagent onto a slide, avoid generating bubbles.
- Time between adding Probe Release Mix onto spacers on the Visium CytAssist Spatial Gene Expression v2 Slide on the Visium CytAssist instrument and starting a run should not exceed **5 min**.

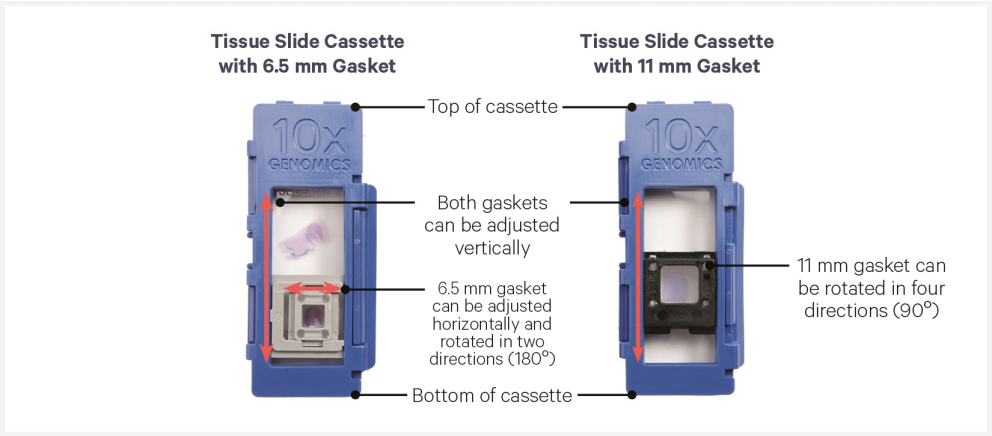
Tissue Slide Handling

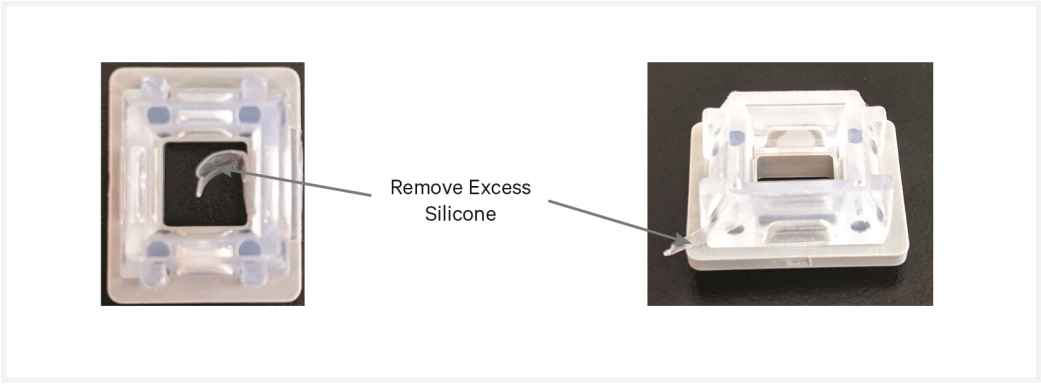
- To ensure compatibility with the Visium CytAssist, tissue sections must be placed in specific areas on a blank slide. Validated slides, as well as appropriate tissue placement areas, are listed in the CytAssist Validated Slides section.

Visium CytAssist Tissue Slide Cassette

- The Visium CytAssist Tissue Slide Cassette is used to create wells on plain glass slides with tissue for reagent addition and removal. It is distinct from the Visium Cassette, which is used to encase Visium CytAssist Spatial Gene Expression Slides.
- The Visium CytAssist Tissue Slide Cassette is a single use item.
- Gaskets are used to create a sealed well around tissue. Gaskets are combined with the complimentary Visium CytAssist Tissue Slide Cassette components to create a complete Visium CytAssist Cassette.
- The appropriately sized Visium CytAssist Tissue Slide Cassette and Gasket will be provided with the Visium CytAssist Spatial Gene Expression Slide kits.
- Reagent mixes for 6.5 mm cassettes and 11 mm cassettes will be differentiated by representative icons next to each table.
- Reagent volumes for 6.5 mm cassettes and 11 mm cassettes will be differentiated by ■ and ▲ symbols respectively.
- Before assembling the Visium CytAssist Tissue Slide Cassette, determine the correct slide orientation with the Tissue Slide Loading Guide (CG000548)
- To ensure that the gasket surrounds the tissue area of interest, gaskets can be adjusted from top to bottom (see image below). 6.5 mm gaskets may also be adjusted from side to side.
- Tissue or area of interest should be centered within the gasket.
- Prior to use, inspect the moveable gasket to ensure that the gasket perimeter and corners are free of excess silicone prior to assembly.
- Excess silicone should be safely removed with forceps or a pipette tip prior to assembly.
- Assembly should occur against a white background for easy tissue

visualization during alignment.





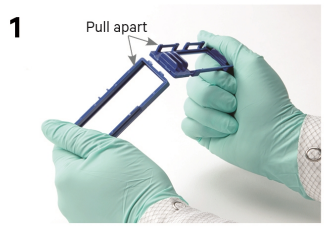
Visium CytAssist Tissue Slide Cassette Assembly

Assembly instructions apply to both Tissue Slide Cassette sizes.

Wear fresh gloves while assembling Tissue Slide Cassette

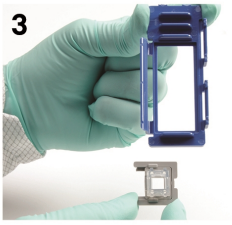
Exercise caution when handling slide edges to prevent injury.

Break cassette into two halves by bending each half at the hinge until they snap apart

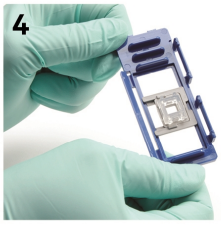


Place tissue slide into lower half of cassette with tissue facing up

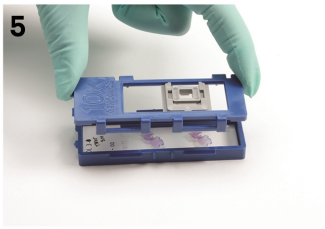
The 6.5 mm gasket can be adjusted horizontally and rotated in two directions (180°) while 11 mm gasket can be rotated in four directions (90°). Determine the appropriate configuration that allows the gasket to encompass the tissue region of interest.



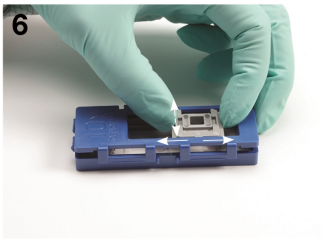
Securely combine gasket with top half of cassette until the gasket snaps into place.



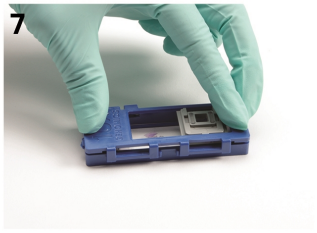
Gently place top half of cassette over bottom half. DO NOT assemble together until Step 6.



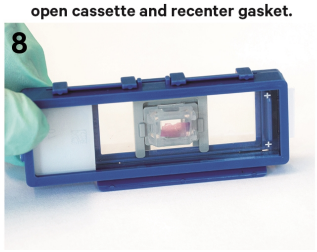
Adjust gasket such that gasket is over the tissue region of interest. The 6.5 mm gasket can be adjusted horizontally as well as vertically.



Apply even pressure on top of cassette until it clicks shut. Verify that clip is completely secured over hinges.



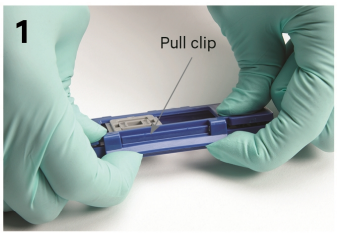
Turn cassette over and verify tissue region of interest is within gasket. DO NOT move gasket once cassette is closed. If necessary, open cassette and recenter gasket.



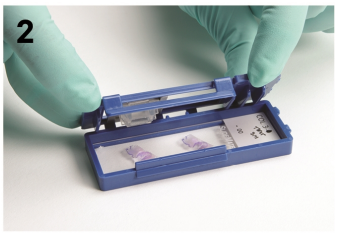
Visium CytAssist Tissue Slide Cassette Removal

Removal instructions apply to both Tissue Slide Cassette sizes.

Pull clip up to detach upper and lower halves of cassette



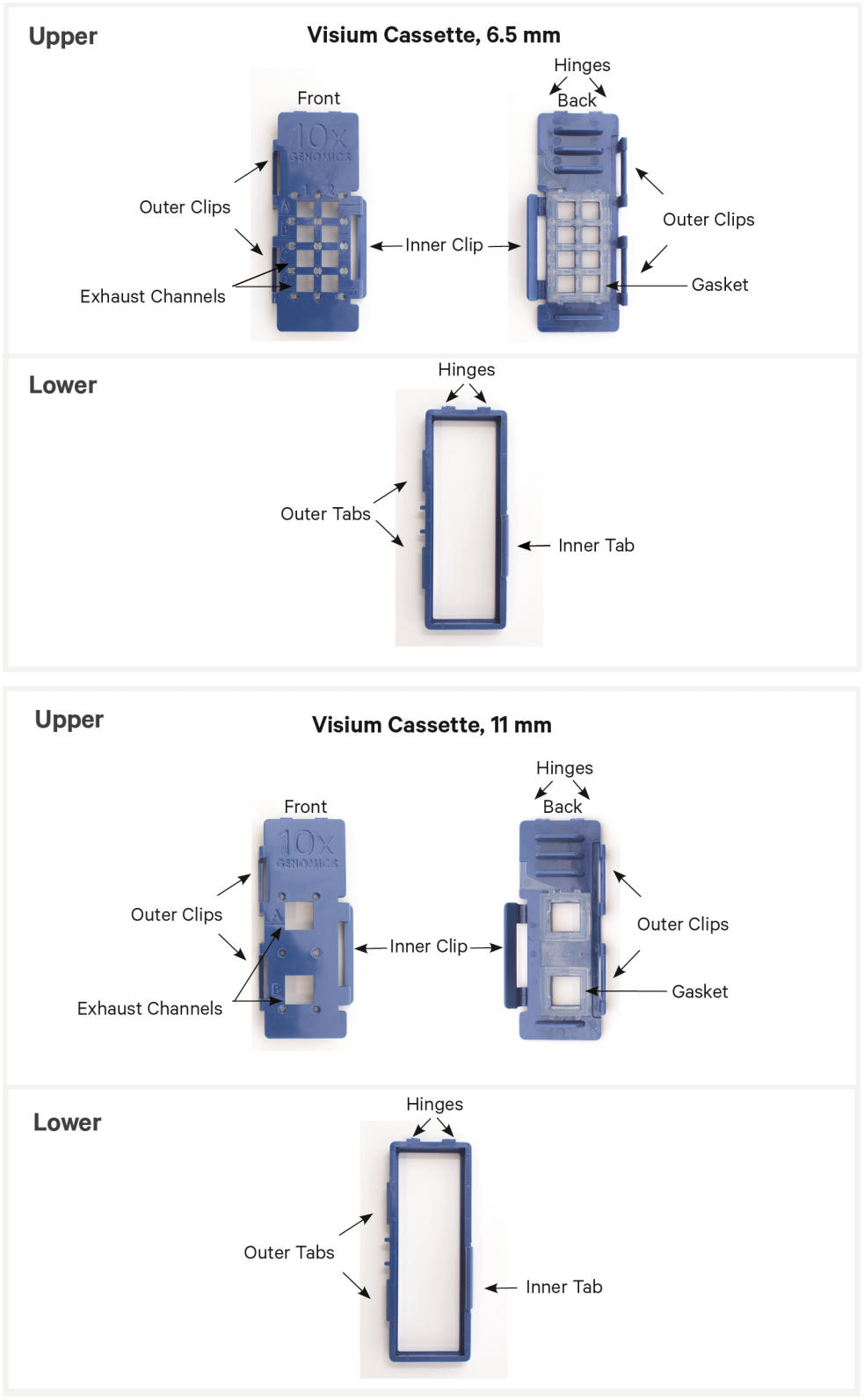
Open cassette by continuing to lift clip upward. If slide sticks to gasket, continue to apply even upward pressure to separate slide from gasket



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

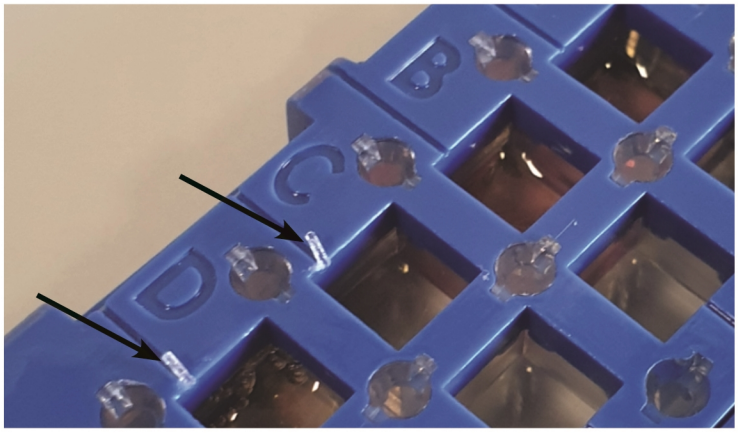
Slides in images are representative.

Visium Cassette



- The Visium Cassette encases the slide and creates leakproof wells for reagent addition.
- The Visium Cassette is a single use item.
- If using a Visium CytAssist 6.5 mm Slide, only wells A1 and D1 of the Visium Cassette are used. If necessary, circle A and D on the cassette with a permanent marker to serve as a reminder.
- Ensure that the Visium Cassette and gasket are free of debris prior to assembly. If placing the top half of the cassette on a surface, ensure the gasket faces away from the surface so it does not collect debris.
- If the exhaust channels have raised pieces of silicone, these pieces are considered excess and must be removed. Run a 10 µl pipette tip through the exhaust channels to ensure they are clear. If excess silicone remains, remove with tweezers or a pipette tip.
- Failure to remove excess silicone may prevent adequate venting of the cassette during heated incubation steps.
- Visually inspect the gasket to ensure it is seated properly.
- If the gasket appears warped, the Visium Cassette is still safe to use as long as the cassette can fully close.

Remove Excess Silicone



- See Visium Cassette Assembly & Removal instructions for details on assembly and removal.
- Practice assembly with a blank slide (75 x 25 x 1 mm).
- Place the slides in the Visium Cassette only when specified.
- Applying excessive force to the slide may cause the slide to break.

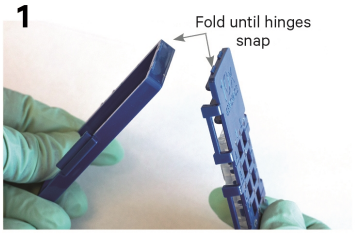
Visium Cassette Assembly

Ensure that the surface of the cassette is dry. Cassette may also be assembled in the hand for comfort.

DO NOT fold over upper and lower halves of cassette before detaching hinges.

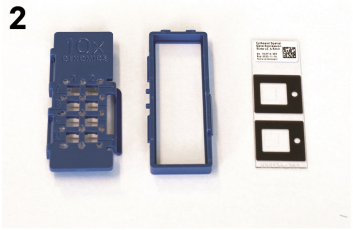
Break cassette into two halves by bending each half at the hinge until they snap apart

1



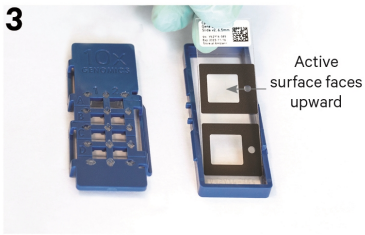
Place upper and lower halves of cassette, and Visium slide, directed upward on bench

2



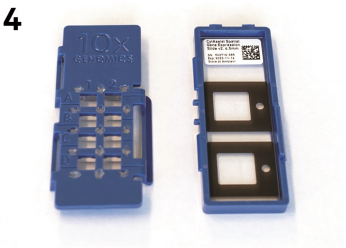
Place Visium slide with active surface facing upwards into lower half of cassette; ensure label is toward hinges

3



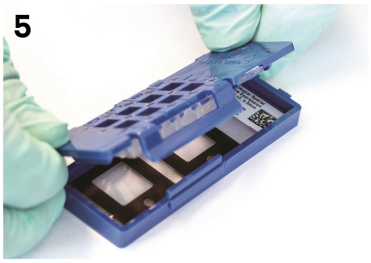
Press slide down into grooves of the bottom half of the cassette until it sits firmly in place

4



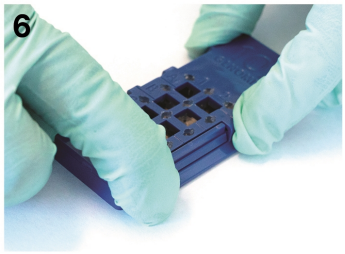
Secure outer clips of top half with outer tab of bottom half

5



Press firmly on top of cassette until it clicks shut

6



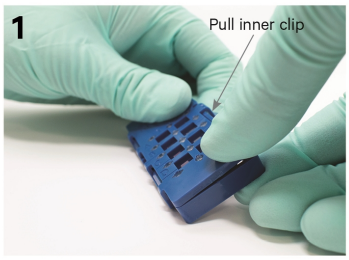
Exercise caution when handling slide edges to prevent injury.

Slides in images are representative.

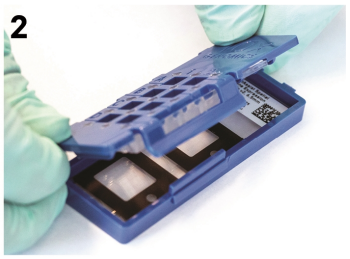
Visium Cassette Removal

Assembly and removal steps apply to both 6.5 and 11 mm Cassettes.

Pull inner clip up from inner tab to detach upper and lower halves of cassette



Open cassette by continuing to lift upper half upward



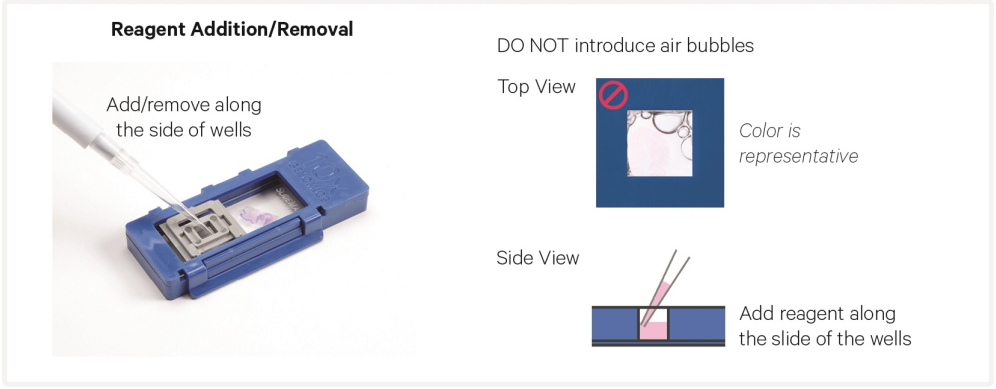
Lift slide out from lower half

Slides in images are representative.

Reagent Addition to Wells

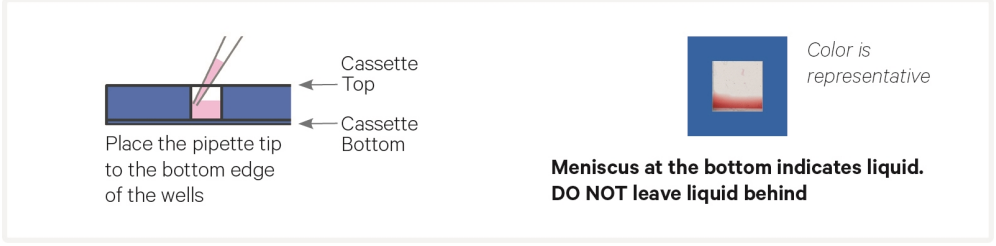
- Assemble slide into the cassette flat on a clean work surface.
- Dispense and remove reagents along the side of the wells without touching the slide surface, tissue sections (when applicable), and without introducing bubbles.
- Always cover the Capture Area or tissue completely when adding reagents to the well. A gentle tap may help spread the reagent more evenly.

Reagent Addition/Removal



Reagent Removal from Wells

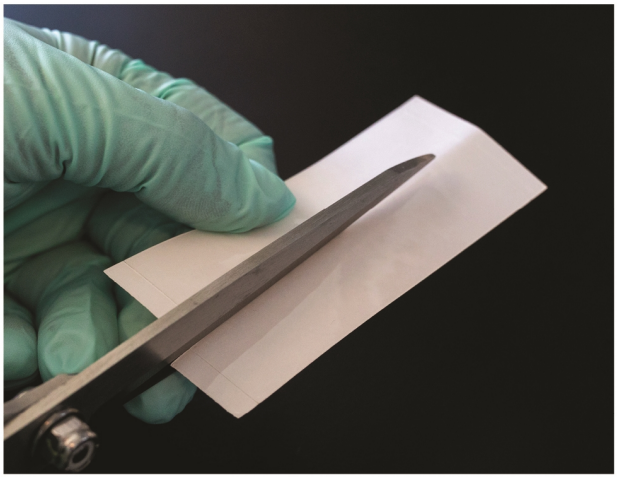
- Assemble slide into the cassette flat on a clean work surface.
- Slightly tilt the cassette while removing the reagent.
- Place the pipette tip on the bottom edge of the wells.
- Remove reagents along the side of the wells without touching the tissue sections (when applicable) and without introducing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



Visium Slide Seal Application & Removal

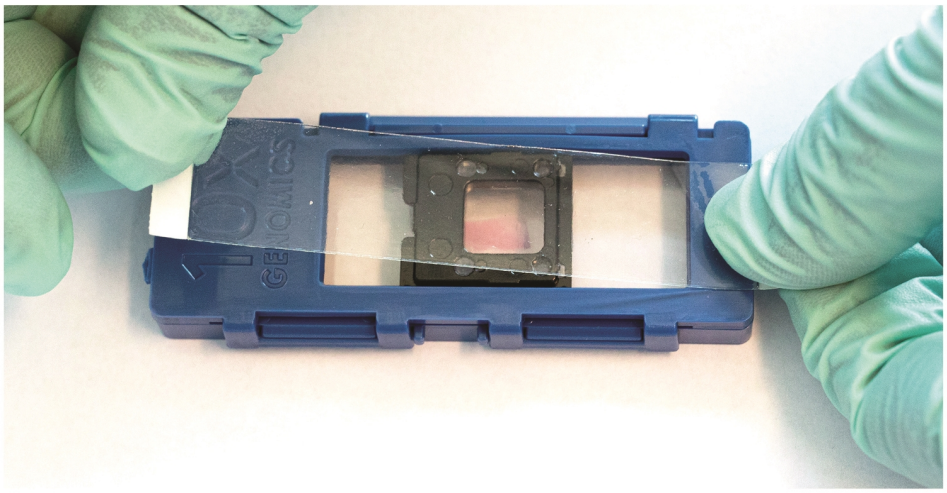
Application

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



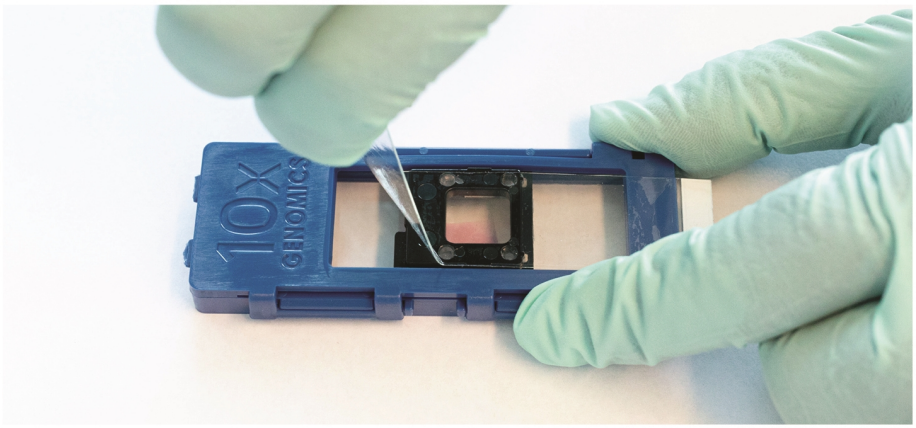
- Place the cassette flat on a clean work surface. Ensure surface of cassette is dry.
- Remove the back of the adhesive Visium Slide Seal.
- Align the Visium Slide Seal with the surface of the cassette and apply while firmly holding the cassette with one hand.
- Press on the Visium Slide Seal to ensure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

Apply Visium Slide Seal



Removal

- Place the cassette flat on a clean work surface.
- Carefully pull on the Visium Slide Seal from the edge while firmly holding the cassette. Ensure that no liquid splashes out of the wells.



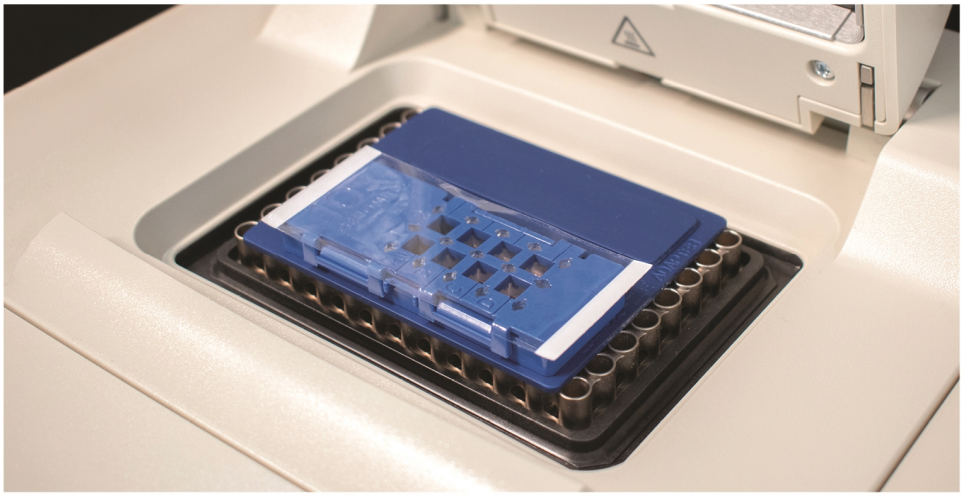
Slide Incubation Guidance

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.
- When incubating a slide encased in a cassette, place the assembled unit on the Low Profile Thermocycler Adapter with the wells facing up. Ensure the cassette is in complete contact with the adapter. The cassette should always be sealed when on the Low Profile Thermocycler Adapter.

Incubate Assembled Visium Cassette

- Open and close the thermal cycler lid gently. If the lid is adjustable, tighten lid only as much as necessary. Avoid over-tightening. Image below is for demonstration purposes - thermal cycler lid should be closed when incubating the cassette.



Incubation at room temperature

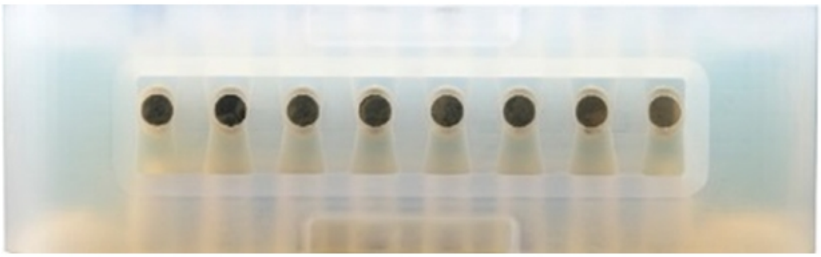
- Place the slide/cassette on a flat, clean, non-absorbent work surface.

Tissue Detachment on Tissue Slides

- Monitor section adhesion on tissue slides throughout the workflow.
- Ensure that tissue sections are on superfrost or positively charged slides. A list of tested slides can be found in Visium CytAssist Tested Slides on page 48.
- Tissue detachment prior to the completion of CytAssist Mediated Probe Release during the workflow can negatively impact performance. If observed, contact support@10xgenomics.com.
- For more information, consult Troubleshooting on page 103.

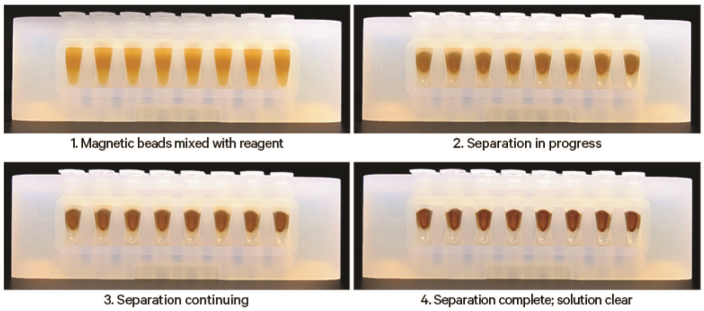
10x Magnetic Separator

- 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.



SPRIselect Cleanup & Size Selection

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

Sample Indices (i5/i7) in Sample Index PCR

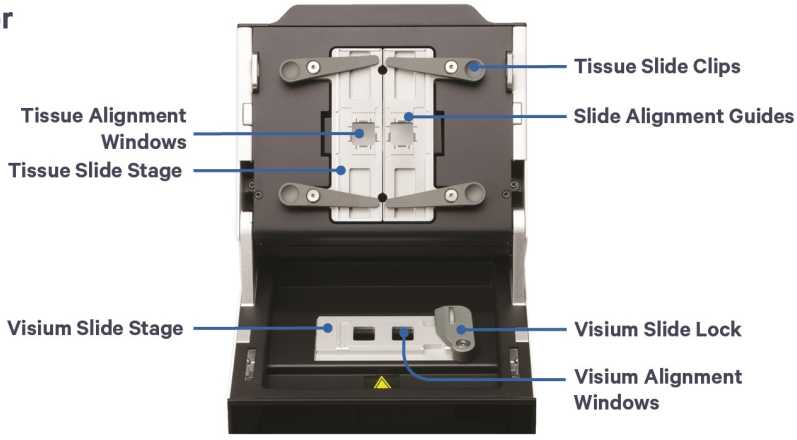
- Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run.
- Each well in the Dual Index Plate TS Set A contains a unique i7 and a unique i5 oligonucleotide.
- To avoid the risk of cross-contamination, use each plate well once.

Visium CytAssist

Instrument Orientation

Exterior

Interior



Back

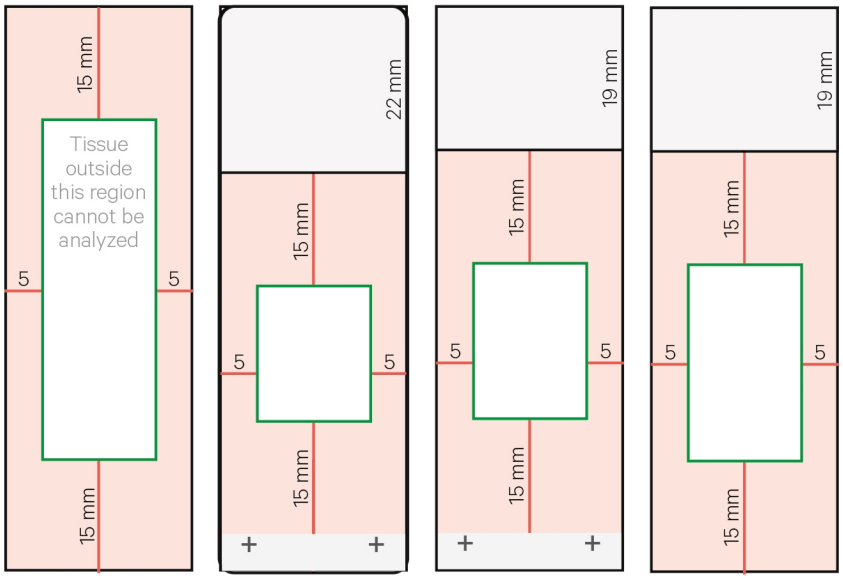
Visium CytAssist Tested Slides

The following slides have been tested for use with the Visium CytAssist instrument.

Item	Length (mm)	Width (mm)	Thickness (mm)	Ground Corners
Epredia Shandon SuperFrost	75.0	25.0	1.0	No
Fisher SuperFrost/ColorFrost	75.0	25.0	1.0	Available as either
Sigma-Aldrich Poly Prep Slides	75.0	25.0	1.0	No
VWR SuperFrost Plus Micro Slide, Premium	75.0	25.0	1.0	No

If unsure of slide part number, refer to the diagram below for guidance. Diagrams for verifying that tissue sections are placed in the allowable area can also be found in the Visium CytAssist Quick Reference Cards - Accessory Kit (Document CG000548).

While slides are specified as being 25 x 75 mm, manufacturing tolerances may lead to dimensions that are incompatible with 10x Genomics products. Slide dimensions must be within 24.8 mm - 25.3 mm (width) and 74.4 mm - 76.2 mm (length) to fit the Visium CytAssist Tissue Slide Cassette. Minimum slide dimensions: 24.8 x 74.4 mm. Maximum slide dimensions: 25.3 x 76.2 mm.



Blank

Epredia Shandon SuperFrost

Fisher SuperFrost/ ColorFrost

VWR SuperFrost Plus Micro Slide, Premium

Sigma-Aldrich Poly Prep

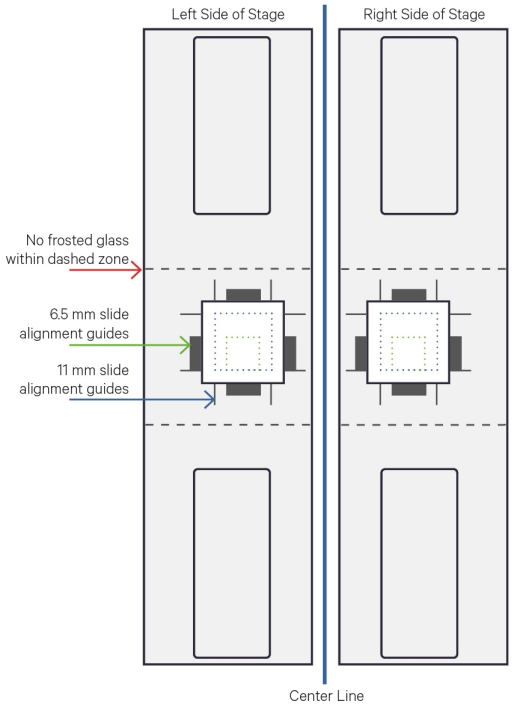
CytAssist Loading Guidelines

- Ensure tissue slide is compatible with Visium CytAssist by using the CytAssist Tissue Area Check Guide (CG0000548). A list of validated slide types and allowable tissue areas can be found in the CytAssist Validated Slides section.
- Each Tissue Slide may be used for one Capture Area on a Visium CytAssist Gene Expression v2 Slide.

Tissue Slide Loading

a. Ensure the tissue on each slide fits within the allowable area of the Tissue Slide Stage.

b. Align tissue within the center of the 6.5 mm slide alignment guides (rectangles) or the 11 mm slide alignment guides (lines) on either the left or right side of the stage.

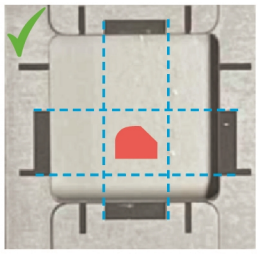


The dotted outlines of the capture areas that are shown in diagram are not present on the instrument's surface.

Slides should not overlap the center line.

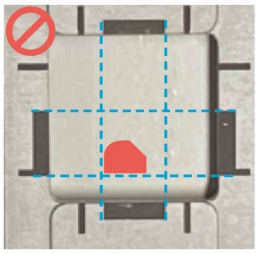
CORRECT

Extend lines from the alignment guides to create an imaginary square. Place the tissue on the center of that square.



INCORRECT

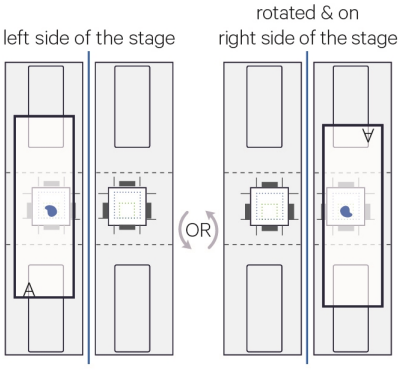
Tissue is within the alignment guides, but should be centered within the imaginary square, not aligned with the top or bottom line.



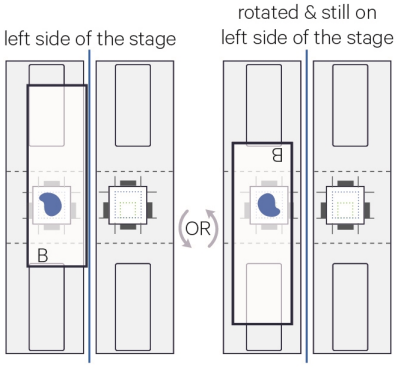
6.5 mm capture area used as an example

c. If necessary, rotate the slide 180° as shown. Place off-center tissues closer to the center line. Slides should not overlap the center line.

Slide (A) shown with a 6.5 mm section:



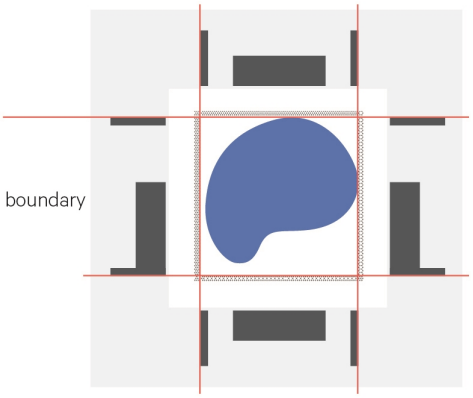
Slide (B) shown with a 11 mm section:



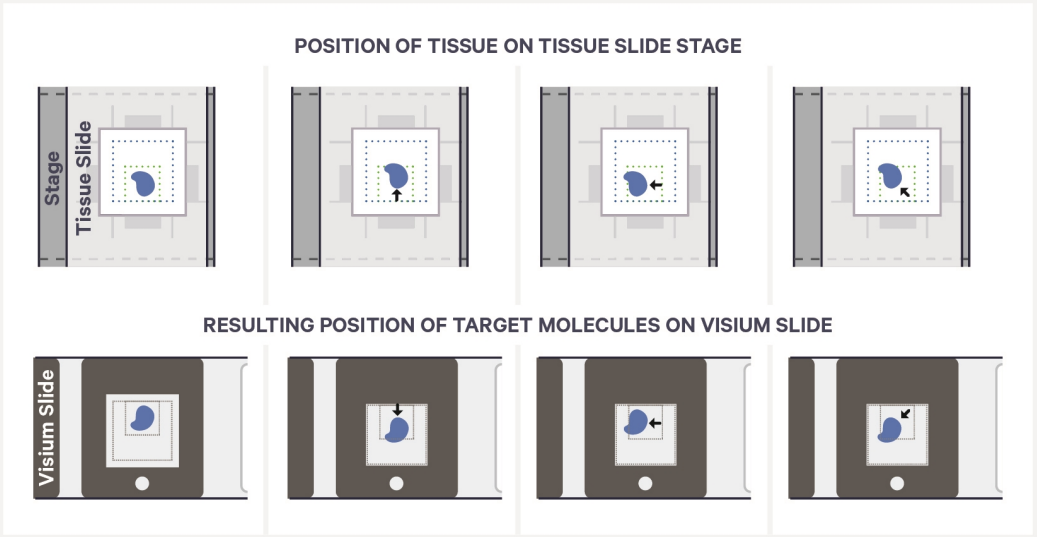
Overlay of a 11 mm fiducial frame and the alignment guides

Note that the **outside** of the alignment guides mark the boundary of the fiducial frame on a Visium CytAssist Slide capture area.

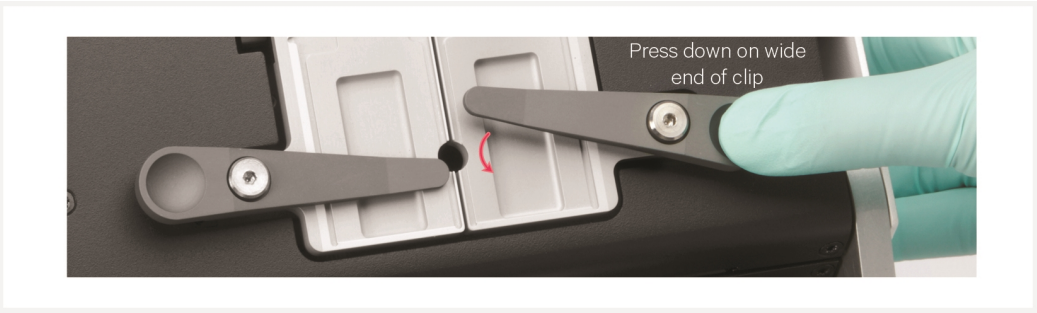
When placing a tissue slide on the Tissue Slide Stage, tissue may come up to the edge of the 11 mm alignment guides.



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

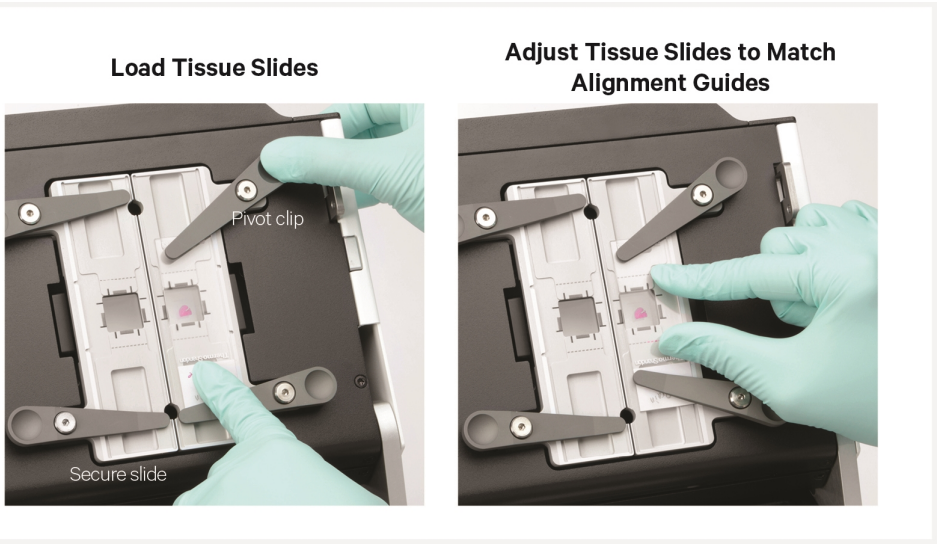


d. Press down on the wide end of clip to lift and pivot the narrow end of the clip. Pivot the clips to their outermost position.

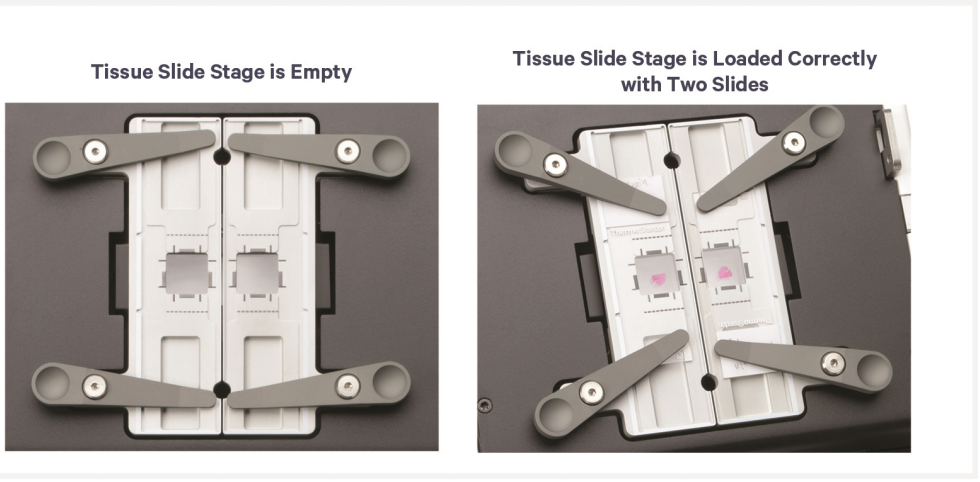


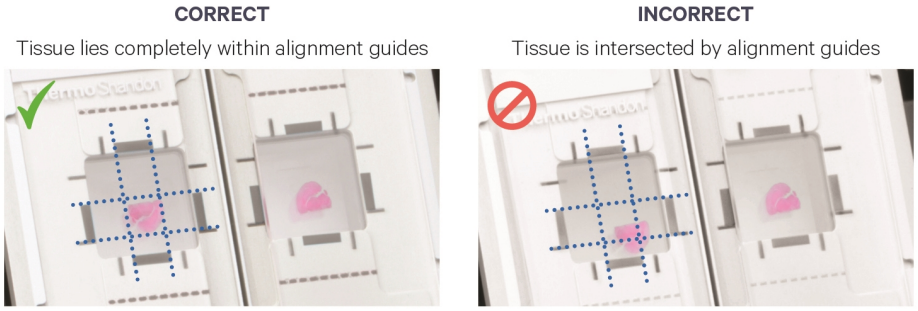
e. Lay the tissue slide flat against the stage surface. Both hands needed: use one hand to hold the slide in place and the other to pivot the clips and overlap the slide. Ensure that at least one clip secures the slide before adjusting the position further.

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



g. If only one tissue on one slide will be analyzed, use a blank slide for the second position on the Tissue Slide Stage.





6.5 mm capture area used as an example

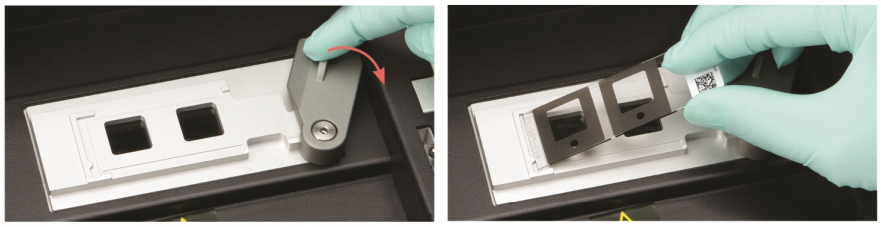
Visium CytAssist Spatial Slide Loading

Prior to loading, record the slide serial number and note which tissue is placed in which Capture Area.

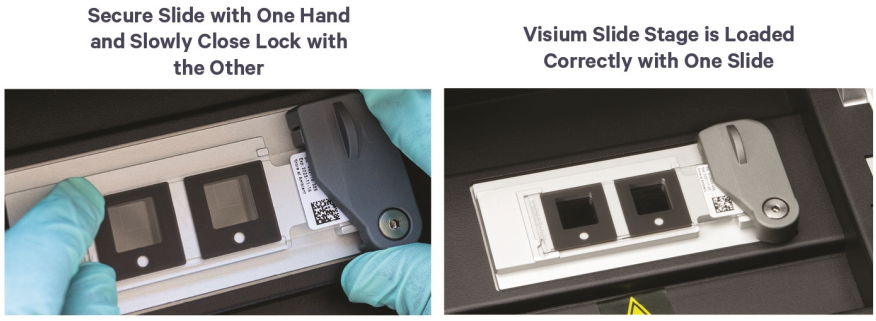
a. Open the Visium Slide Lock by using one finger to pivot.

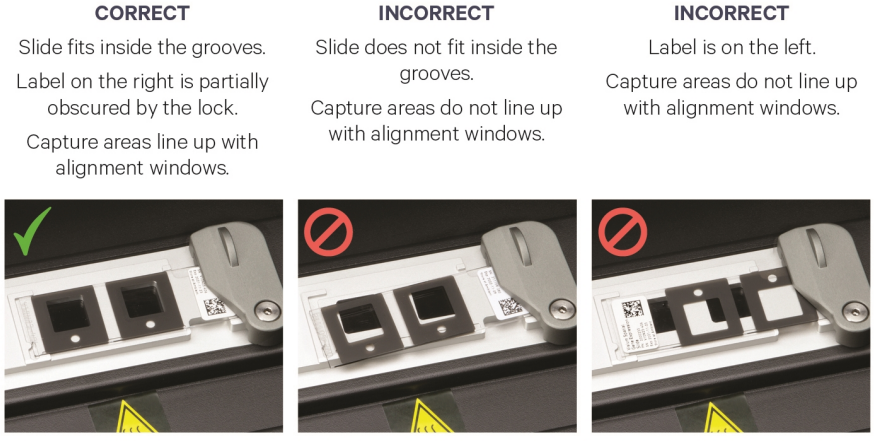
b. Line up the slide with the label face up and oriented to the right.

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



d. Hold the slide in place with one hand while slowing closing the Visium Slide Lock. The lock will partially obscure the slide label when correctly secured.





10x GENOMICS

Sample Preparation & Staining Guidelines

Sample Preparation Guidelines

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

Listed below are some key considerations for preparing samples that are compatible with the Visium Spatial protocols.

Consult the Visium CytAssist Spatial Gene Expression for FFPE - Tissue Preparation Guide (CG000518), which includes Tips & Best Practices for tissue sectioning and section placement.

Key Considerations	
Slide Handling (before tissue placement)	
FFPE Tissue Sectioning & Section Placement	
☐	Assess RNA quality of the FFPE tissue block or from archived sections.
☐	Practice sectioning and section placement with a non-experimental block prior to placing sections on slides.
☐	Section the FFPE tissue block using a microtome and place sections on the compatible glass slides using a water bath.
☐	Place tissue sections on the allowable area on the slide. Allowable area may vary depending on slide choice. Refer to CytAssist Validated Slides for more information.
Slide Handling (after tissue placement)	
☐	Store the slides containing FFPE sections for up to two weeks in a desiccator at room temperature.

Deparaffinization, Staining & Decrosslinking Guidelines

FFPE tissue sections should be processed using one of the following staining Demonstrated Protocols before proceeding with Visium CytAssist Spatial Gene for FFPE. Consult Demonstrated Protocols CG000519 and CG000520 (available on the 10x Genomics support website) for details.

DO NOT proceed with User Guide steps without performing appropriate deparaffinization, staining, imaging, and decrosslinking with the tissue sections.

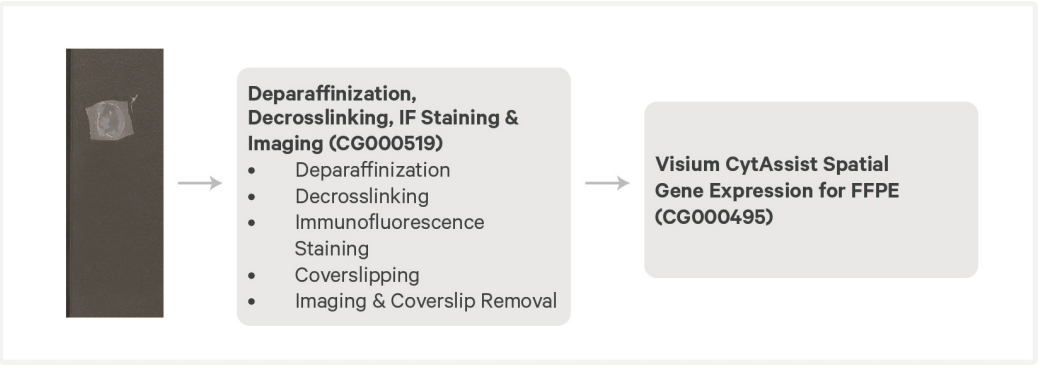
Deparaffinization, H&E Staining, Imaging & Decrosslinking

Consult Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, H&E Staining, Imaging & Decrosslinking (Demonstrated Protocol CG000520). In this protocol, slides containing FFPE tissue sections are first deparaffinized and then stained with Hematoxylin and Eosin (H&E). The stained sections are then coverslipped and imaged. After the coverslip is removed, a decrosslinking step is performed. Once the slides are decrosslinked, proceed directly to the User Guide.

Deparaffinization, H&E Staining, Imaging & Decrosslinking Workflow

Deparaffinization, Decrosslinking, IF Staining & Imaging

Consult Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, Decrosslinking, IF Staining & Imaging (Demonstrated Protocol CG000519). In this protocol, slides containing FFPE tissue sections are first deparaffinized and decrosslinked to make protein target epitopes accessible by immunofluroescence antibodies. The slides are then stained with fluorescently labeled antibodies, coverslipped, and imaged. Once the immunostained tissue sections are imaged and the coverslip is removed, proceed directly to the User Guide.



Step 1:

Probe Hybridization

1

1.0 Get Started

Each 10x Genomics reagent tube is good for two 6.5 mm slides or one 11 mm slide.

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	FFPE Hyb Buffer	2000423	Thaw at room temperature. If precipitate persists, heat at 37°C until dissolved. Avoid vortexing to prevent bubble formation. Pipette mix 10x. Keep the buffer at room temperature after thawing and while performing the workflow, then return to -20°C when finished	–20°C
Place on ice				
☐ ●	Human WT Probes v2 - RHS	2000657	Thaw on ice. Vortex and centrifuge briefly.	–20°C
☐ ●	Human WT Probes v2 - LHS	2000658	Thaw on ice. Vortex and centrifuge briefly.	–20°C
☐ ●	Mouse WT Probes - RHS	2000455	Thaw on ice. Vortex and centrifuge briefly.	–20°C
☐ ●	Mouse WT Probes - LHS	2000456	Thaw on ice. Vortex and centrifuge briefly.	–20°C
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	10X PBS, pH 7.4	-	-	Ambient
☐	Visium CytAssist Tissue Slide Cassette, 6.5 or 11 mm Gasket	1000519/ 1000520 1000471/ 1000472	See Tips & Best Practices.	Ambient
☐	Visium Slide Seals	2000284	See Tips & Best Practices. 3 pre-cut slide seals are necessary for the complete workflow.	Ambient
☐	10% Tween-20	-	-	Ambient

1.1 Probe Hybridization

Before starting this protocol, ensure that tissue sections have been appropriately deparaffinized, stained, imaged, and decrosslinked. Consult Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, H&E Staining, Imaging & Decrosslinking (Demonstrated Protocol CG000520) or Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, Decrosslinking, IF Staining & Imaging (Demonstrated Protocol CG000519).

TIPS

During reagent removal steps, ensure that **ALL the liquid is removed** from the wells. See Tips & Best Practices for guidance on Reagent Removal.

■ *denotes volumes for 6.5 mm gaskets and* ▲ *denotes volumes for 11 mm gaskets.*

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

6.5 mm Gaskets Pre-Hybridization Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
Nuclease-free Water	-	89.5	196.9	393.8
10X PBS, pH 7.4	-	10.0	22.0	44.0
10% Tween-20	-	0.5	1.1	2.2
Total	-	100.0	220.0	440.0

11 mm Gaskets Pre-Hybridization Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
Nuclease-free Water	-	179.0	393.8	787.6
10X PBS, pH 7.4	-	20.0	44.0	88.0
10% Tween-20	-	1.0	2.2	4.4
Total	-	200.0	440.0	880.0

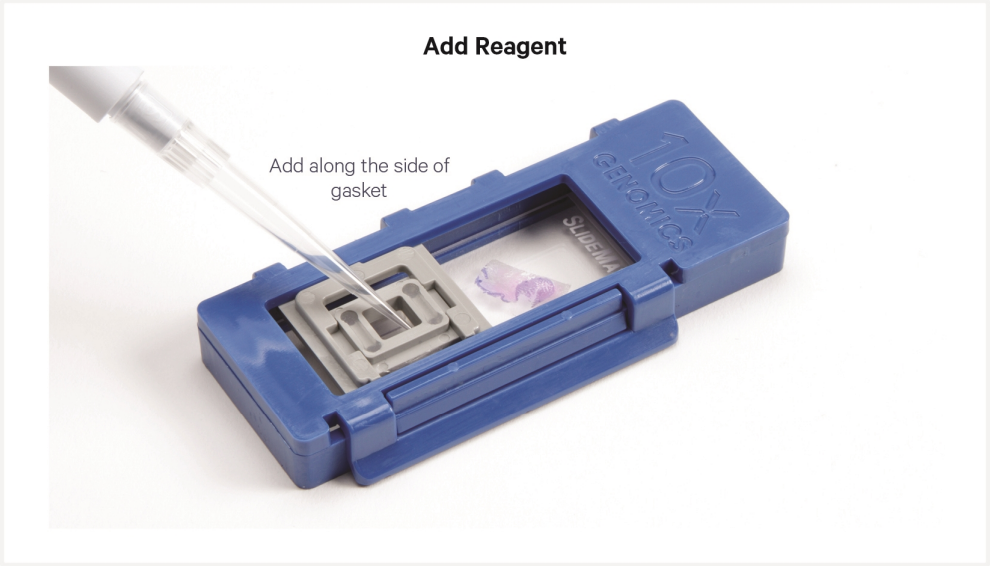
b. Retrieve Tissue Slide Cassette containing H&E stained or IF stained sections and peel back Visium Slide Seal.

c. Using a pipette, remove all buffer from well corners. For H&E stained slide, remove all Decrosslinking buffer.

d. Add ■ **100 µl** or ▲ **200 µl** Pre-Hybridization Mix along the side of the wells to uniformly cover the tissue sections, without introducing bubbles.

e. Re-apply Visium Slide Seal on the Tissue Slide Cassette.

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



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

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

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

	6.5 mm Gaskets Probe Hybridization Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
	Nuclease-free Water	-	10.0	22.0	44.0
	FFPE Hyb Buffer	2000423	70.0	154.0	308.0
●	Human WT Probes v2 - RHS or Mouse WT Probes - RHS	2000657 or 2000455	10.0	22.0	44.0
●	Human WT Probes v2 - LHS or Mouse WT Probes - LHS	2000658 or 2000456	10.0	22.0	44.0
	Total	-	100.0	220.0	440.0

	11 mm Gaskets Probe Hybridization Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
	Nuclease-free Water	-	19.9	43.8	87.6
	FFPE Hyb Buffer	2000423	140.1	308.2	616.4
●	Human WT Probes v2 - RHS or Mouse WT Probes - RHS	2000657 or 2000455	20.0	44.0	88.0
●	Human WT Probes v2 - LHS or Mouse WT Probes - LHS	2000658 or 2000456	20.0	44.0	88.0
	Total	-	200.0	440.0	880.00

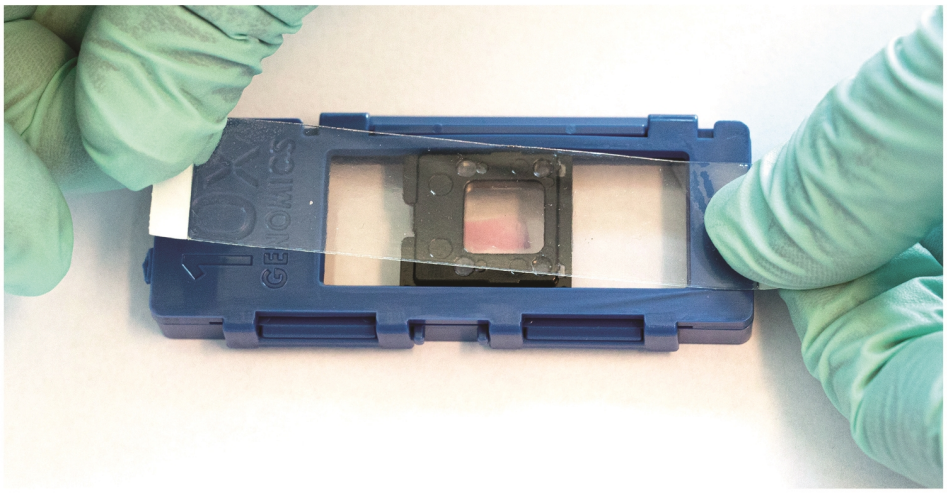
i. Remove Visium Slide Seal from the Tissue Slide Cassette.

j. Remove all Pre-Hybridization Mix from the wells.

k. Add ■ **100 µl** or ▲ **200 µl** room temperature Probe Hybridization Mix to each well.

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

Apply Visium Slide Seal



m. Skip Pre-equilibrate step to initiate Hybridization.

Step 2:

Probe Ligation

2

2.0 Get Started

Each 10x Genomics reagent tube is good for two 6.5 mm slides or one 11 mm slide.

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

2.1 Post-Hybridization Wash

■ *denotes volumes for 6.5 mm gaskets and* ▲ *denotes volumes for 11 mm gaskets.*

a. Aliquot FFPE Post-Hyb Wash Buffer (**495 µl/per 6.5 mm sample, 990 µl/per 11 mm sample**) and pre-heat to **50°C**.

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

6.5 mm Gaskets SSC Buffer	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
SSC	20X	2X	355	781	1,562
Nuclease-free Water	-	-	3,195	7,029	14,058
Total	-		3,550*	7,810*	17,182*

11 mm Gaskets SSC Buffer	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
SSC	20X	2X	410	902	1,804
Nuclease-free Water	-	-	3,690	8,118	16,236
Total	-		4,100*	9,020*	19,844*

*Volume of 2X SSC Buffer is sufficient for washes in all subsequent steps.

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

d. Peel back Visium Slide Seal and using a pipette, remove all Probe Hybridization Mix from the well.

e. **Immediately** add ■ **150 µl** or ▲ **300 µl** pre-heated FFPE Post-Hyb Wash Buffer to each well. Avoid well drying or cooling to room temperature. Removal and addition of buffers should be done quickly.

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

g. Skip the Hybridization step and initiate Post-Hybridization Wash. Incubate in the thermal cycler at **50°C** for **5 min**.

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

i. Peel back Visium Slide Seal and using a pipette, remove all FFPE Post-Hyb Wash Buffer from the wells.

j. **Immediately** add ■**150 µl** or ▲**300 µl** pre-heated FFPE Post-Hyb Wash Buffer to each well. Avoid well drying or cooling to room temperature. Removal and addition of buffers should be done quickly.

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

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

m. **Repeat** steps h-l one more time for a total of three washes.

n. Remove the Visium Slide Seal and remove FFPE Post-Hyb Wash Buffer.

o. Add ■ **150 µl** or ▲ **300 µl** 2X SSC Buffer to each well and re-apply seal.

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

2.2 Probe Ligation

■ *denotes volumes for 6.5 mm gaskets and* ▲ *denotes volumes for 11 mm gaskets.*

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

Lid Temperature	Reaction Volume	Run Time
37°C (lid may be turned off if the instrument doesn't enable 37°C)	100 µl	1 h

Step	Temperature	Time *hh:mm:ss*
Pre-equilibrate	37°C	Hold
Ligation	37°C	01:00:00
Hold	4°C	Hold

b. Prepare Probe Ligation Mix according to the appropriate table shortly before use. Add reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain on ice.

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

	11 mm Gaskets Probe Ligation Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
	Nuclease-free Water	-	52.0	114.4	228.8
◯	2X Probe Ligation Buffer	2000445	65.0	143.0	286.0
◯	Probe Ligation Enzyme	2000425	13.0	28.6	57.2
	Total	-	130.0	286.0	572.0

c. Remove all 2X SSC Buffer from the wells.

d. Add ■ **60 µl** or ▲ **130 µl** Probe Ligation Mix directly to tissue sections, without introducing bubbles. Tap Tissue Slide Cassette gently to ensure uniform coverage.

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

f. Skip Pre-equilibrate step to initiate Ligation.

2.3 Post-Ligation Wash

■ *denotes volumes for 6.5 mm gaskets and* ▲ *denotes volumes for 11 mm gaskets.*

Use room temperature Post-Ligation Wash Buffer at the first wash step (step 2.3e). Use pre-heated Post-Ligation Wash Buffer at the second wash step (step 2.3j).

a. Pre-heat Post-Ligation Wash Buffer (■ **110 µl/sample** or ▲ **220 µl/sample**) to **57°C**. Only **100 µl** per 6.5 mm sample or **200 µl** per 11 mm sample is needed.

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

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

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

d. Remove the Visium Slide Seal and using a pipette, remove all Probe Ligation Mix from all wells.

e. Immediately add ■**100 µl** or ▲ **200 µl room temperature** Post-Ligation Wash Buffer to each well. Removal and addition of buffers should be done quickly.

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

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

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

i. Peel back the Visium Slide Seal and using a pipette, remove all Post-Ligation Wash Buffer.

j. Add ■ **100 µl** or ▲ **200 µl pre-heated** Post-Ligation Wash Buffer to each well.

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

the thermal cycler lid.

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

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

n. Peel back the Visium Slide Seal and using a pipette, remove all Post Ligation Wash Buffer.

o. Add ■ **150 µl** or ▲ **300 µl** 2X SSC Buffer prepared at step 2.1b to each well.

p. Remove all 2X SSC Buffer

q. Add ■ **150 µl** or ▲ **300 µl** 2X SSC Buffer to each well.

r. Re-apply Visium Slide Seal on the Tissue Slide Cassette.

s. Store at **4°C** for up to **24 h** or allow to come to room temperature for **5 min** and proceed to next step.

Step 3:

Probe Release & Extension

3

3.0 Get Started

Each 10x Genomics reagent tube is good for two 6.5 mm slides or one 11 mm slide.

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	RNase Buffer B	2000551	Thaw at room temperature. Pipette mix slowly and thoroughly with a wide-bore pipette. DO NOT vortex.	–20°C
☐	Extension Buffer	2000409	Thaw at room temperature, vortex, centrifuge briefly.	–20°C
☐	Tissue Removal Enzyme	3000387	Pipette mix, centrifuge briefly. Maintain at room temperature until ready to use.	–20°C
Place on ice				
☐	RNase Enzyme	3000593	Pipette mix, centrifuge briefly. Maintain on ice until ready to use.	–20°C
☐	Extension Enzyme	2000389	Pipette mix, centrifuge briefly. Maintain on ice until ready to use.	–20°C
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	Wide-bore Pipette Tips	-	-	Ambient
☐	Tris 1 M, pH 8.0 (Tris-HCl)	-	Manufacturer's recommendations.	Ambient
☐	Alcoholic Eosin	-	Manufacturer's recommendations.	Ambient
☐	1X PBS	-	Manufacturer's recommendations. Prepare 1X solution from stock with nuclease-free water.	Ambient
☐	2X SSC Buffer	-	Prepared at step 2.1b.	Ambient
☐	8 M KOH Solution	-	Manufacturer's recommendations.	Ambient
☐	Visium Cassette	3000811/ 3000812	See Tips & Best Practices.	Ambient
☐	Visium Slide Seals	2000284	See Tips & Best Practices.	Ambient

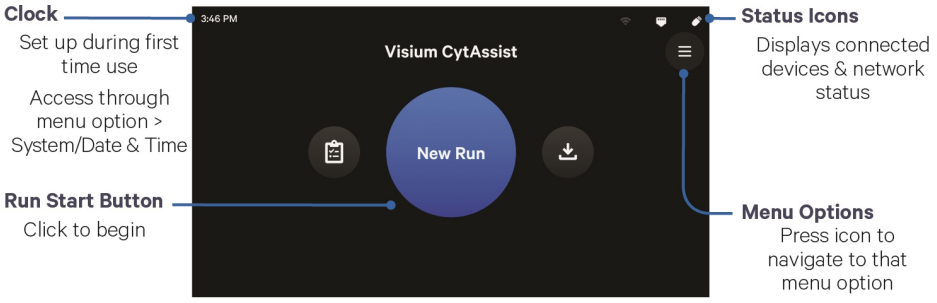
3.1 CytAssist Enabled RNA Digestion & Tissue Removal

■ *denotes volumes for 6.5 mm gaskets and* ▲ *denotes volumes for 11 mm gaskets.*

If processing more than two tissue slides, keep remaining tissue slides at **4°C** with 2X SSC buffer.

a. Ensure that Visium CytAssist is powered on, clean, and ready for an experimental run.

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



b. Prepare Probe Release Mix shortly before use. Probe Release Mix is viscous. Pipette mix thoroughly until solution is homogenous. Maintain on ice.

Probe Release Mix	10x PN	2 Tissue Slides (µl) (includes overage)
Nuclease-free Water	-	15.8
RNase Buffer B	2000551	50.0
RNase Enzyme	3000593	4.5
Total	-	70.3

c. Prepare 10% Eosin shortly before use. Vortex and centrifuge briefly.

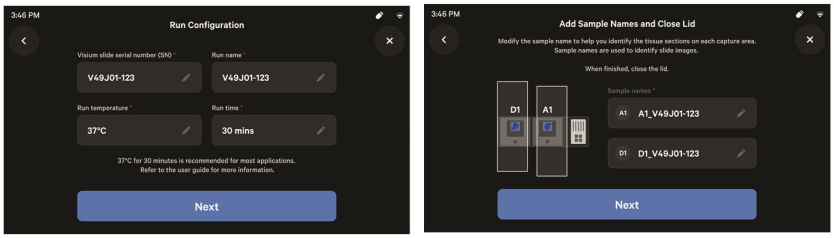
6.5 mm Slides 10% Eosin	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
Alcoholic Eosin	-	15	33	66
1X PBS	-	135	297	594
Total	-	150	330	660

11 mm Slides 10% Eosin	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
Alcoholic Eosin	-	30	66	132
1X PBS	-	270	594	1,188
Total	-	300	660	1,320

d. Press blue Run Start Button on the touchscreen to initiate run.

e. Enter new run information, including:

- Visium Slide serial number. Ensure serial number is accurate.
- Custom run name, temperature, and time (**37°C** for **30 min** is recommended for most applications)
- Sample names



f. Using a pipette, remove all 2X SSC Buffer from the wells of the Tissue Slide.

g. Remove Tissue Slide from Tissue Slide Cassette. See Visium CytAssist Tissue Slide Cassette Removal for instructions.

h. Add ■ **150 µl** or ▲ **300 µl** 10% Eosin to uniformly cover each tissue section per slide.

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

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

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

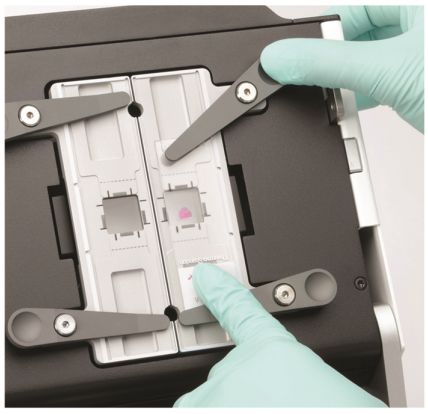
DO NOT pipette directly onto tissue.

l. Repeat k two more times for a total of three washes.

m. Gently flick slide back and forth to remove excess PBS. Remove any excess PBS with a laboratory wipe without damaging the tissue sections.

n. Wipe back of Tissue Slides with a laboratory wipe and load into Visium CytAssist. See Loading Guidelines for more information.

Load Tissue Slides



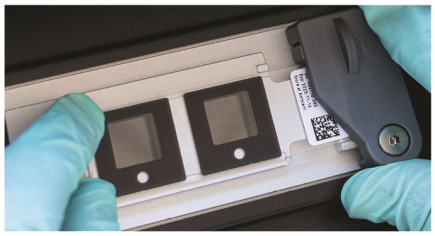
Adjust Tissue Slides to Match Alignment Guides

o. Load Visium CytAssist Spatial Gene Expression Slide onto Visium Slide Stage and close Visium Slide Lock.

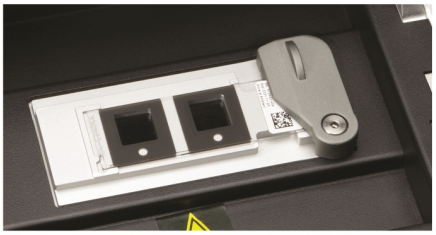
Open Visium Slide Lock

Load Visium Slide

Secure Slide with One Hand and Slowly Close Lock with the Other



Visium Slide Stage is Loaded Correctly with One Slide

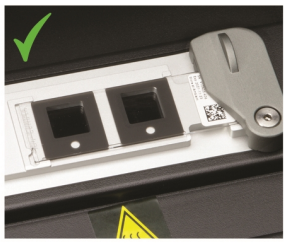


CORRECT

Slide fits inside the grooves.

Label on the right is partially obscured by the lock.

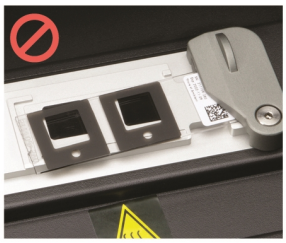
Capture areas line up with alignment windows.



INCORRECT

Slide does not fit inside the grooves.

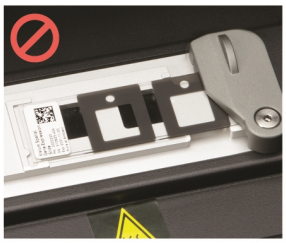
Capture areas do not line up with alignment windows.



INCORRECT

Label is on the left.

Capture areas do not line up with alignment windows.



p. Remove Probe Release Mix from ice.

q. Pipette mix Tissue Removal Enzyme (PN-3000387) and centrifuge briefly. Add **4.7 µl** of Tissue Removal Enzyme to **70.3 µl** of Probe Release Mix (prepared at step 3.1b). Pipette mix 15x with pipette set to 50 µl. Centrifuge for **5 sec**.

The time between the addition of Tissue Removal Enzyme to Probe Release Mix and starting the Visium CytAssist experiment run should be less than **5 min**.

r. Dispense **25 µl** of Probe Release Mix into each spacer well on the Visium CytAssist Spatial Gene Expression Slide.

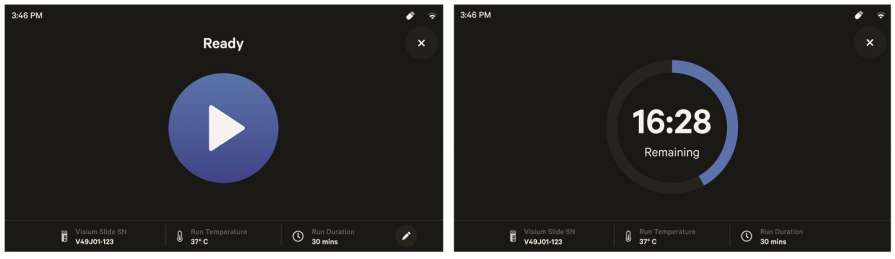
Dispense Reagent

s. Close the lid.

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

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

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



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

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

Lid Temperature	Reaction Volume	Run Time
45°C (lid may be turned off if the instrument doesn't enable 45°C)	100 µl	15 min
Step	**Temperature**	**Time**
Pre-equilibrate	45°C	Hold
Probe Extension	45°C	00:15:00
Hold	4°C	Hold

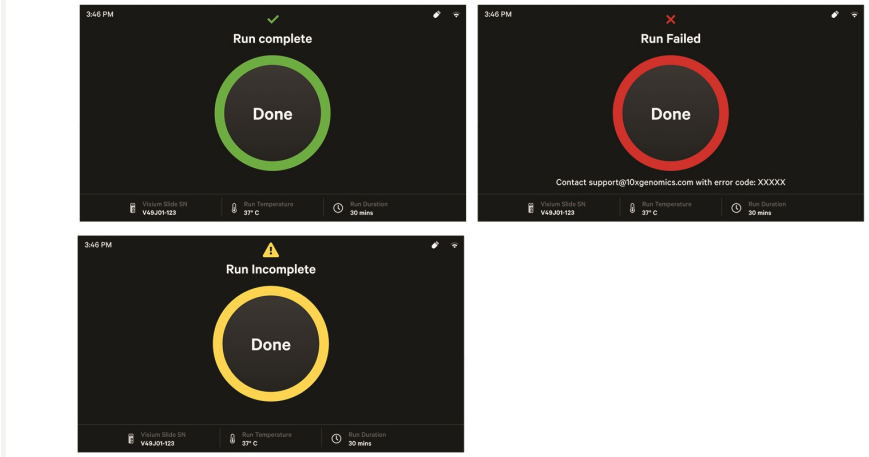
v. Prepare Probe Extension Mix. Pipette mix. Maintain on ice.

	6.5 mm Slides Probe Extension Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
●	Extension Buffer	2000409	73.5	161.7	323.4
●	Extension Enzyme	2000389	1.5	3.3	6.6
	Total	-	75.0	165.0	330.0

	11 mm Slides Probe Extension Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
●	Extension Buffer	2000409	196.0	431.2	862.4
●	Extension Enzyme	2000389	4.0	8.8	17.6
	Total	-	200.0	440.0	880.0

w. 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.



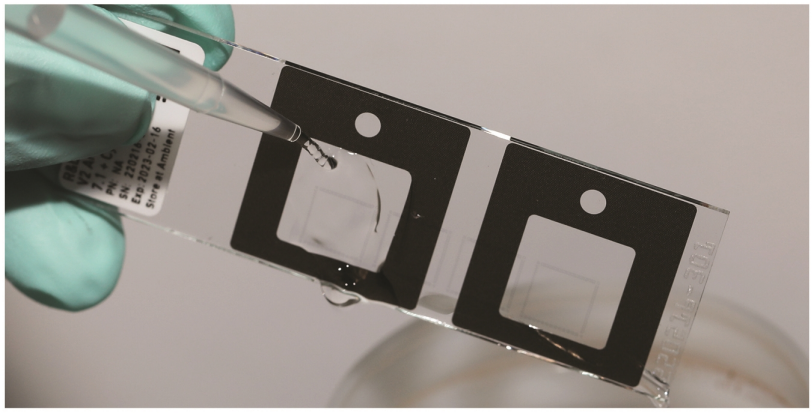
x. Click the "Done" button and open the lid. DO NOT power off the instrument at this time, as it needs to process support data. Slide may move after opening instrument.

y. Remove Visium CytAssist Spatial Gene Expression slide. It is normal if tissue remains on the Tissue Slides after run completion.

z. While holding the Visium CytAssist Spatial Gene Expression slide over the liquid waste container, rinse each Capture Area with **1 ml** 2X SSC.
DO NOT pipette directly onto Capture Areas.

Rinse slide near Visium CytAssist instrument to ensure prompt washing.

DO NOT pipette directly onto Capture Area.



aa. Repeat step z two more times for a total of three washes per Capture Area.

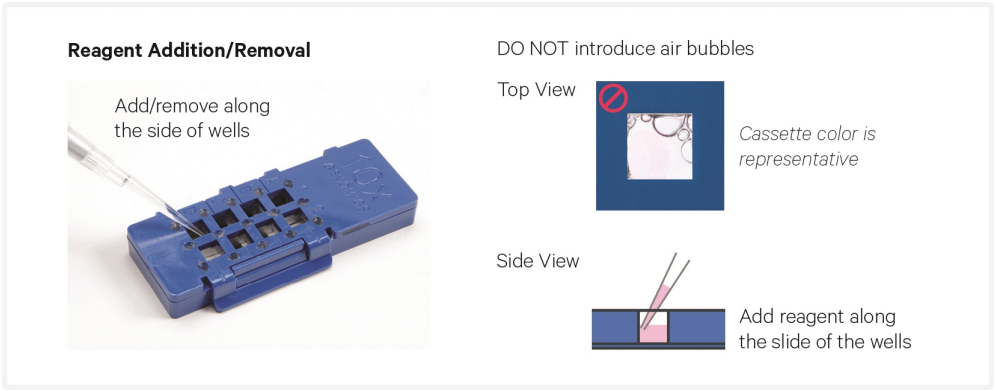
ab. Place Visium CytAssist Spatial Gene Expression slide in a new 6.5 or 11 mm Visium Cassette. See Visium Cassette section for more information. Some moisture remaining on the Visium CytAssist Spatial Gene Expression slide is normal.

3.2 Probe Extension

■ *denotes volumes for 6.5 mm slides and* ▲ *denotes volumes for 11 mm slides.*

Seal for the Visium Cassette should not be cut.

a. Add ■ **75 µl** or ▲ **200 µl** Probe Extension Mix to each well (to A1 and D1 if using a 6.5 mm cassette). Gently tap the Visium Cassette to ensure uniform coverage of the Capture Area.



b. Apply a new uncut 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.

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

STOP

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

3.3 Probe Elution

■ *denotes volumes for 6.5 mm slides and* ▲ *denotes volumes for 11 mm slides.*

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

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

11 mm Slides KOH Mix	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
Nuclease-free Water	-	-	198.0	435.6	871.2
KOH	8 M	0.08 M	2.0	4.4	8.8
Total	-	-	200.0	440.0	880.0

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

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

d. Add ■ **100 µl** or ▲ **200 µl** 2X SSC Buffer prepared at step 2.1 to each well (A1 and D1 if using a 6.5 mm cassette).

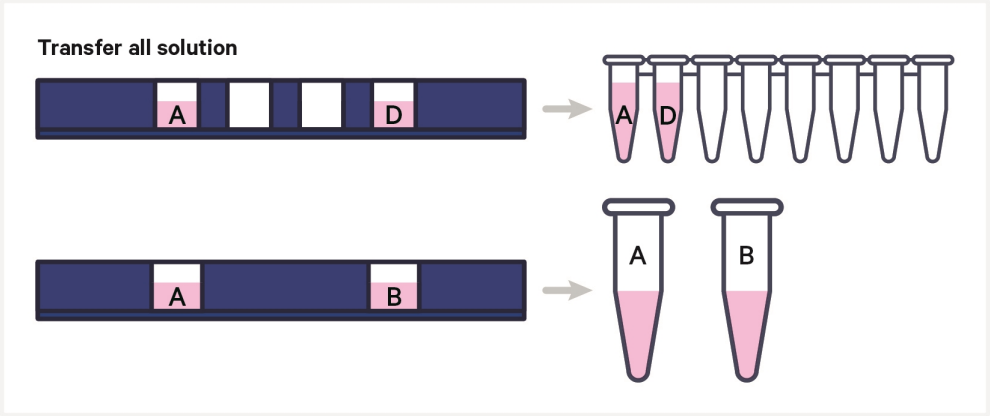
e. Remove all 2X SSC Buffer from the wells.

f. Add ■ **50 µl** or ▲ **200 µl** 0.08 M KOH Mix to each well (A1 and D1 if using a 6.5 mm cassette). Gently tap the Visium Cassette to ensure uniform coverage of the Capture Area.

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

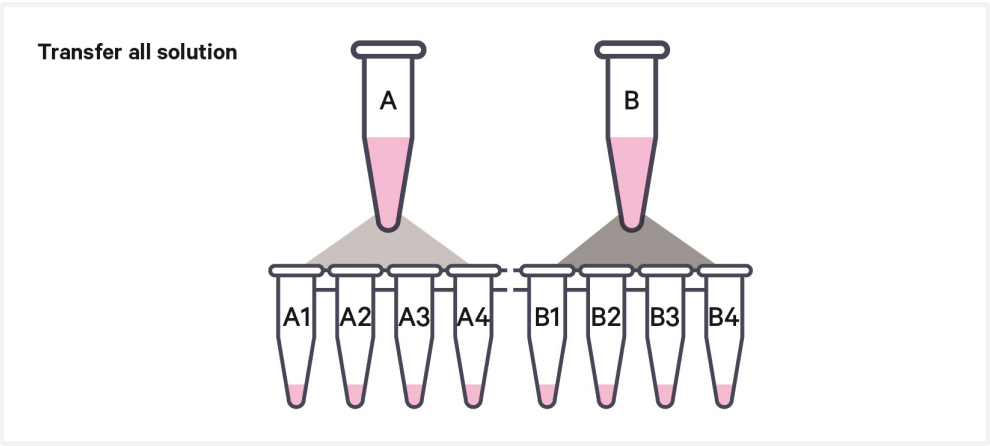
h. Transfer all solution for each sample containing the ligation product to a tube in an 8-tube strip if using a 6.5 mm cassette, or 1.5 ml microcentrifuge tube if using an 11 mm cassette. DO NOT leave behind

any solution in the wells. Failure to neutralize may result in a loss of signal and lower library complexity. *See Tips & Best Practices for reagent removal instructions*.



i. Add ■ **3 µl** or ▲ **12 µl** 1 M Tris-HCl pH 8.0 to each sample. Vortex, centrifuge briefly, and place on ice.

j. If using 11 mm Slides, divide each **212 µl** neutralized sample among four tubes in an 8-tube strip so that each tube contains **53 µl** of the neutralized sample. If necessary, add enough nuclease-free water to arrive at the correct volume.



Step 4:

Pre-Amplification and SPRIselect

4

4.0 Get Started

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

4.1 Pre-Amplification

If working with samples from 11 mm slides, note that during Probe Elution, each 11 mm sample was split into four replicate tubes in an 8-tube strip. Thus, all reactions during pre-amplification will have four times the number tubes as Capture Areas.

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

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

	11 mm Slides Pre-Amplification Mix	PN	1X* (µl)	2X* + 10% (µl)	4X* + 10% (µl)
○	Amp Mix B	2000567	100	220	440
	Nuclease-free Water		78	171.6	343.2
●	TS Primer Mix B	2000537	10	22	44
	Total	-	188	413.6	827.2

*Refers to original number of Capture Areas

b. Add **47 µl** Pre-Amplification Mix to each tube from step 3.3 Probe Elution (regardless of slide type). 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	~30-45 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	00:00:30
5	Go to Step 2, repeat 9X for a total of 10 cycles	
6	72°C	00:01:00
7	4°C	Hold

4.2 Pre-Amplification Cleanup - SPRIselect

■ *denotes volumes for samples from 6.5 mm slides and* ▲ *denotes volumes for samples from 11 mm slides.*

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

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

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

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

d. Remove the supernatant.

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

f. Remove the ethanol.

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

h. Remove the ethanol.

i. Centrifuge briefly and place on the 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 the magnet. Add ■ **105 µl** or ▲ **27.5 µl** Buffer EB. Pipette mix 15x (pipette set to 100 µl for samples from 6.5 mm slides or 25 µl for samples from 11 mm slides).

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

m. Place the tube strip on the magnet •**High** for samples from 6.5 mm slides or •**Low** for samples from 11 mm slides until the solution clears.

n. For samples from 6.5 mm slides, transfer **100 µl** sample to a new tube strip.

For samples from 11 mm slides, pool replicate samples together (Four **25 µl** samples for a total of **100 µl** per capture area), vortex and centrifuge briefly.

STOP

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

After this point in the protocol, all instructions are the same regardless of slide type used.

Step 5:

Visium Spatial Gene Expression – FFPE Library Construction

5

5.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐ ●	TS Primer Mix B	2000537	Thaw at room temperature, vortex, and centrifuge briefly. Dilute amount needed for assay 1:10 in nuclease-free water.	–20°C
☐	Dual Index Plate TS Set A	3000511	Thaw at room temperature, vortex, and centrifuge briefly.	–20°C
☐	Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendations.	-
☐	Agilent TapeStation Screen Tape and Reagents If used for QC		Manufacturer's recommendations.	-
☐	Agilent Bioanalyzer High Sensitivity DNA kit If used for QC	-	Manufacturer's recommendations.	-
Place on ice				
☐ ○	Amp Mix B	2000567	Vortex, centrifuge briefly.	–20°C
☐	KAPA SYBR Fast qPCR Master Mix	-	Manufacturer's recommendations.	-
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	Qiagen Buffer EB	-		Ambient
☐	10x Magnetic Separator	230003	See Tips & Best Practices.	Ambient
☐	80% Ethanol	-	Prepare fresh.	Ambient

5.1 Cycle Number Determination – qPCR

a. Prepare qPCR Mix on ice according to the table below. Add reagents in the order listed. Vortex and centrifuge briefly. Maintain on ice. Refer to Get Started table for dilution instructions.

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

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

b. Add **9 µl** qPCR Mix to each well in a qPCR plate (a well for negative control may be included).

c. Dilute sample (1:5 in nuclease-free water) sample from Pre-Amplification Cleanup - SPRIselect. Pipette mix, centrifuge briefly.

d. Transfer **1 µl** diluted sample from Pre-Amplification Cleanup - SPRIselect to the qPCR plate well containing qPCR Mix. If using a negative control, add **1 µl** nuclease-free water to the corresponding well. Pipette mix, apply seal, and centrifuge briefly. Record which sample is in which well of the qPCR plate.

Note that only **25 µl** of pre-amplification material is used to generate gene expression libraries. The remaining **75 µl** (75%) can be stored at **4°C** for up to **72 h** or at **-20°C** for up to **4 weeks** for generating additional libraries.

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

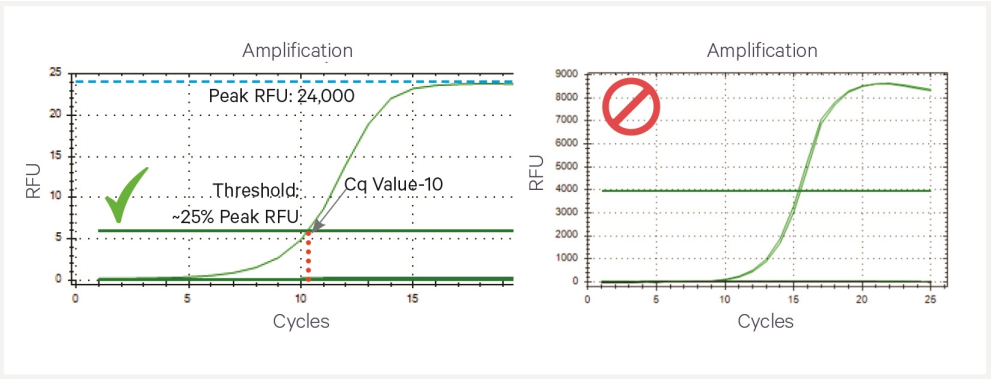
Lid Temperature	Reaction Volume	Run Time
105°C	10 µl	35 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:03:00

Lid Temperature	Reaction Volume	Run Time
2	98°C	00:00:05
3	63°C	00:00:30
	Read signal	
4	Go to step 2, 29x (total of 30 cycles)	-

f. Record the Cq Value for each sample.

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

Representative qPCR Amplification Plots



5.2 GEX Sample Index PCR

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

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

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

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

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

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

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

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

Round Cq values up to the nearest whole number and add two cycles, as shown in the examples below. Samples within ±1 cycle numbers can be combined in a single SI-PCR run at the higher cycle number. Do not combine samples if the cycle number difference is greater than 1 to avoid library over-

amplification.

Example Cycle Numbers

Cq Value from qPCR	+2	Total Cycles
7.2	+2	10
8.5	+2	11
13.7	+2	16

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

5.3 GEX Post-Sample Index PCR Cleanup – SPRIselect

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

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

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

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

d. Remove the supernatant.

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

f. Remove the ethanol.

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

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

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

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

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

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

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

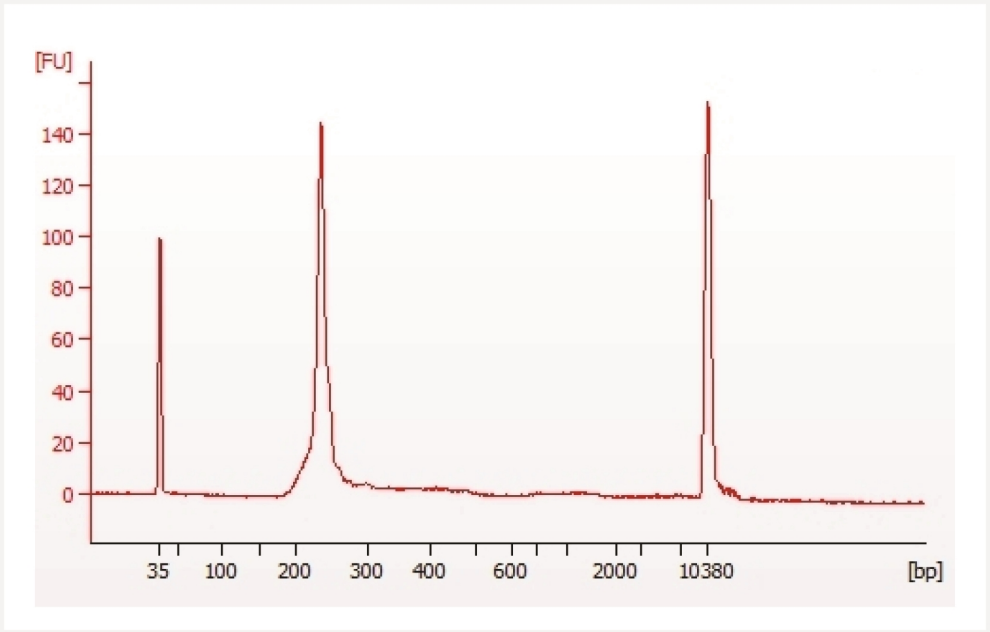
n. Store at **-20°C** for **long-term** storage.

5.4 GEX Post-Library Construction QC

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

b. Run **1 µl** of sample on an Agilent Bioanalyzer High Sensitivity chip. If peak is too small or flat, re-try with a lower dilution. Refer to Troubleshooting for more information.

Representative Trace



Determine the average fragment size from the Bioanalyzer trace. The expected average fragment size is 240 bp. This will be used as the insert size for library quantification.

Alternate QC Method:

- Agilent TapeStation
- LabChip

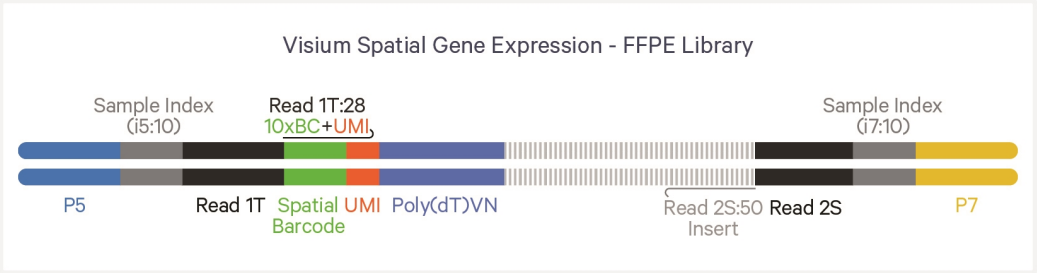
See Appendix on page 116 for representative traces

See Post Library Construction Quantification on page 117

Sequencing

Sequencing Libraries

Visium Spatial Gene Expression - FFPE libraries comprise standard Illumina paired-end constructs which begin with P5 and end with P7. 16 bp Spatial Barcodes are encoded at the start of TruSeq Read 1, while i7 and i5 sample index sequences are incorporated as the index read. TruSeq Read 1 and Small RNA Read 2 (Read 2S) are standard Illumina sequencing primer sites used in paired-end sequencing. TruSeq Read 1 are used to sequence the 16 bp Spatial Barcode and 12 bp UMI. Small RNA Read 2 (Read 2S) is used to sequence the Ligated Probe Insert. Sequencing these libraries produces a standard Illumina BCL data output folder.



Sequencing Depth

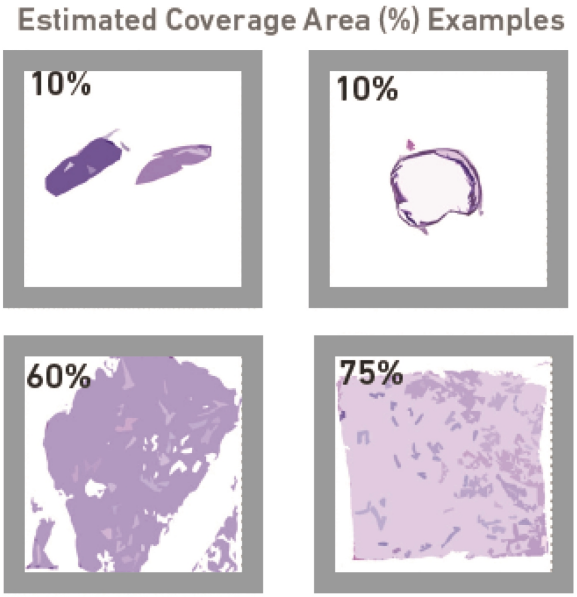
Calculating sequencing depth requires estimating the approximate Capture Area (%) covered by tissue. This may be performed visually or by using the Visium Manual Alignment Wizard in Loupe Browser for a more accurate measurement. See examples below for estimating coverage area visually. If using Loupe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

The examples below are for 6.5 mm slides. The total number of spots/Capture Area should be adjusted based on the Capture Area size (the number of spots for 6.5 mm slides is 4,992 and the number of 11 mm slides is 14,336).

Sequencing Depth/spot	Minimum 25,000 read pairs per tissue covered spot on Capture Area
Sequencing Depth/sample	See example calculation below

Example: Sequencing Depth for a Sample

- **Estimate the approximate Capture Area (%)** covered by the tissue section.
- **Calculate total sequencing depth=** (Coverage Area x total spots on the Capture Area) x 25,000 read pairs/spot
- **Example calculation for 60% coverage:** (0.60 x 5,000 total spots) x 25,000 read pairs/spot= 75 million total read pairs for that sample



Sequencing Type & Run Parameters

Use the sequencing run type and parameters indicated.

Dual Index Library
Paired-end, dual indexed sequencing
Read 1: 28 cycles i7 Index: 10 cycles i5 Index: 10 cycles Read 2S: 50 cycles*

*Visium Spatial Gene Expression – FFPE libraries may be pooled with Visium Spatial Gene Expression libraries generated from fresh frozen samples. If pooling the two different library types, Visium Spatial Gene Expression – FFPE libraries should not occupy more than 40% of the pool.

Illumina Sequencer Compatibility

The compatibility of the listed sequencers has been verified by 10x Genomics. Some variation in assay performance is expected based on sequencer choice. For more information about performance variation, visit the 10x Genomics Support website.

- MiSeq
- NextSeq 500/550

- NextSeq 2000
- NovaSeq
- iSeq

Sample Indices

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

Library Loading

Once quantified and normalized, the Visium Spatial Gene Expression –FFPE libraries should be denatured and diluted as recommended for Illumina sequencing platforms. Refer to Illumina documentation for denaturing and diluting libraries. Refer to the 10x Genomics Support website, for more information.

Instrument	Loading Concentration (pM)	PhiX (%)
MiSeq	11	1
NextSeq 500/550	1.8	1
NextSeq 2000	650	1
NovaSeq	150**/300	1
iSeq	150	1

** Use 150 pM loading concentration for Illumina XP workflow.

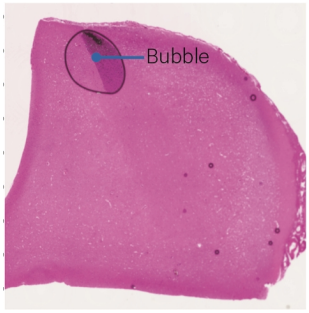
Troubleshooting

Bubbles during Coverslipping

A bubble could be generated during coverslipping.

Bubbles may cause blackening of tissue

H&E Stain

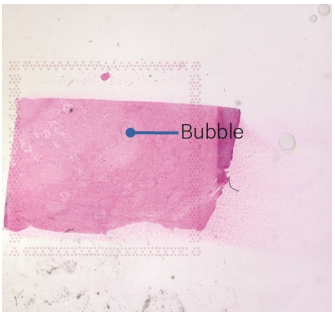


A bubble could be generated during coverslipping. Avoid generating bubbles during mounting medium dispensing by pipetting slowly and avoiding expelling air from the pipette tip. If the bubble is on the tissue, blackening of the tissue could occur. However, this does not diminish sensitivity and spatial resolution, and the data derived from the blackened area can still be analyzed.

Bubbles Trapped During CytAssist Enabled RNA Digestion and Tissue Removal

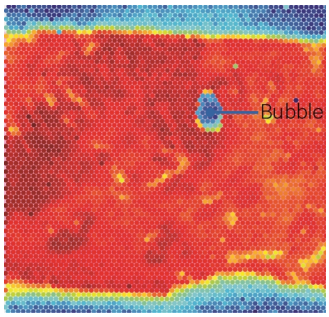
Bubbles may Result in no Usable Sequencing Reads during the CytAssist Assay

Eosin Stained Tissue



Some eosin may be washed off during an instrument run, as shown on the right side of this image. This does not affect performance.

UMI Counts



Bubbles during a Visium CytAssist experiment run are rare, but may result in a lack of useable reads in the tissue area where the bubbled occurred. Avoid

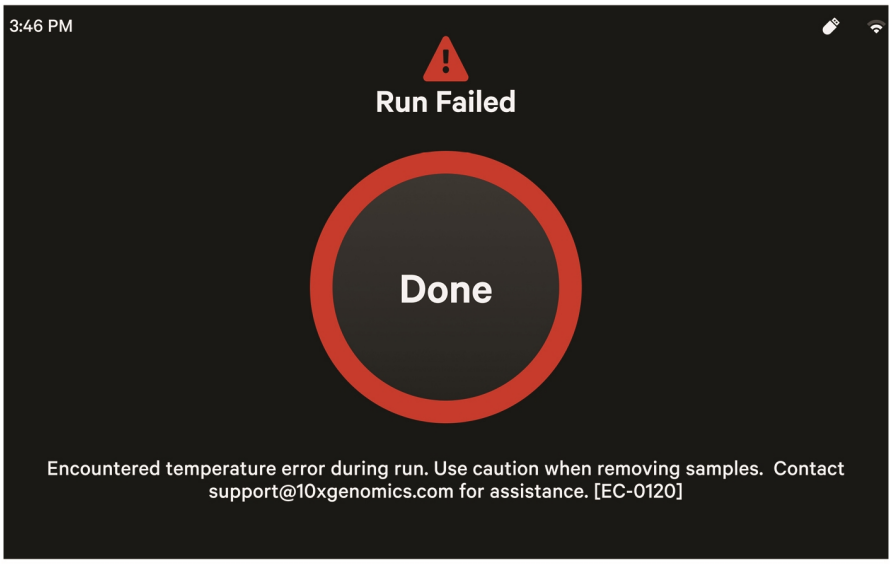
generating bubbles during reagent dispensing by pipetting slowly and avoiding expelling air from the pipette tip.

Reagent Flow Failure

Inappropriate flow of reagents during a Visium CytAssist experiment run may result in transcript mislocation. If UMI map appears abnormal (>50% of UMIs outside of tissue), contact support@10xgenomics.com.

Ensure that frosted areas of slide are not within the dashed zone on the Visium Tissue Slide Stage. Keep Visium Spatial and tissue slides free from 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 laboratory wipe.

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.

Number of Washes

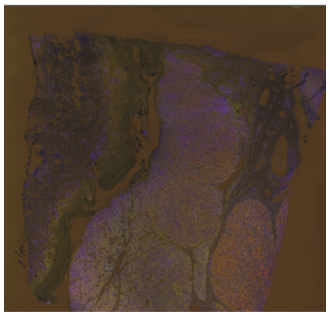
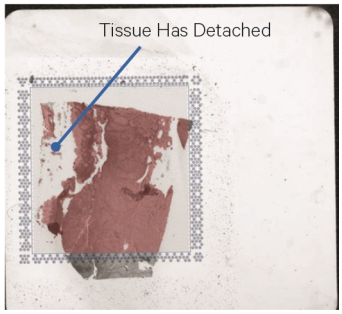
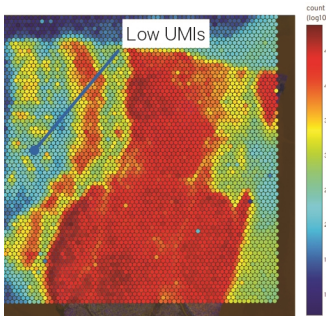
Post hybridization and post ligation washes are critical for assay performance. Failure to perform the correct number of washes can reduce the fraction of targeted reads usable (see table below). A similar effect is observed when washing for less than the recommended 5 min, or when reagent is carried over during the washes. Remove all liquid from the well when washing, and refer to User Guide for correct number of washes and incubation times.

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

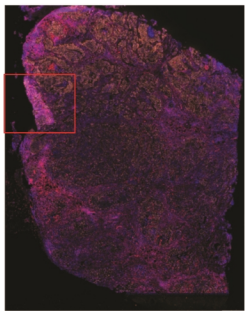
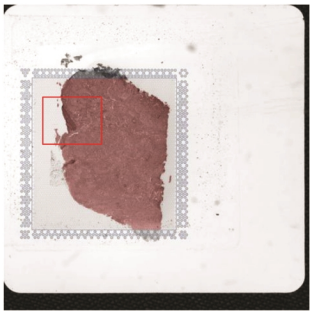
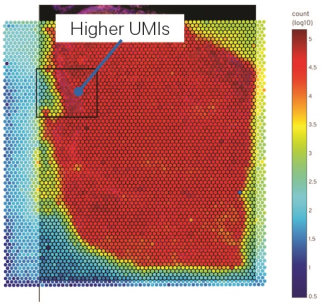
Tissue Detachment and Folding

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

Tissue Detachment May Cause a Loss of Gene Expression

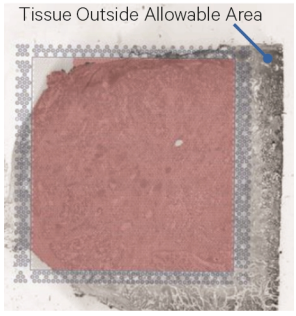
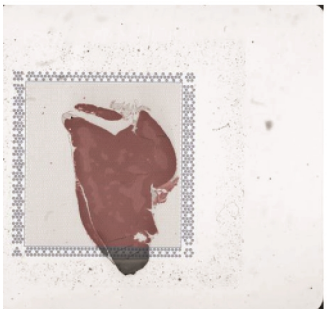
IF Stained Tissue	CytAssist Image	UMI Count
		

Tissue Folding May Cause Elevated UMI Counts

H&E Stained Tissue	CytAssist Image	UMI Count
		

Tissue Not Within Allowable Area

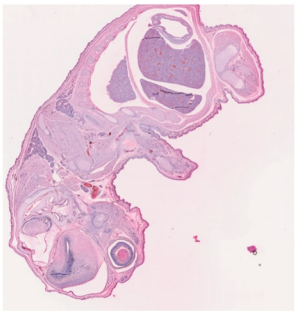
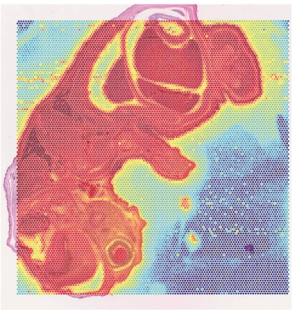
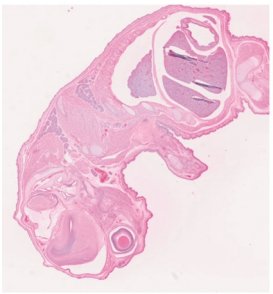
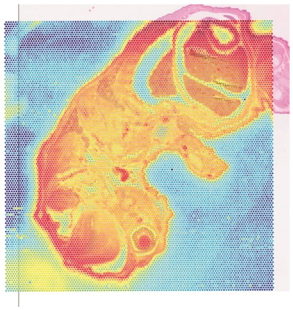
Tissue Outside of Allowable Area is Not Analyzed

Tissue Larger than Capture Area	Tissue Not Properly Aligned
	

Tissues that are not placed within the allowable area on approved 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 into the CytAssist instrument. Refer to CytAssist Validated Slides for guidance on allowable areas.

Tissue Outside of Specifications

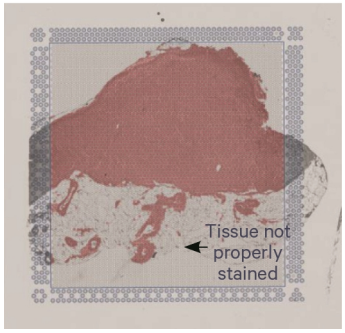
Tissue sections should be between 3 and 10 µm. Sections that are too thin may lead to a decrease in sensitivity.

Thin Sections May Lead to Decreased Sensitivity	
H&E Stained Tissue at Recommended Thickness	**Mean Panel UMI Count at 20,000 Raw Reads per Spot: 14,645**
	
H&E Stained Tissue Below Recommended Thickness	**Mean Panel UMI Count at 20,000 Raw Reads per Spot: 8,511**
	

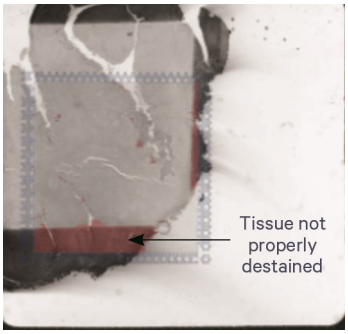
Tissue Segmentation Failure

Examples of Scenarios that Lead to Tissue Segmentation Failure

Inadequate Staining Causes Poor Tissue Segmentation



H&E Stained Tissues Outside of Gasket May Not Undergo Destaining

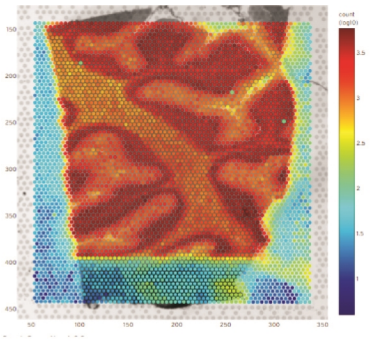


Space Ranger may fail to detect tissue for a variety of reasons which may necessitate manual fiducial alignment and tissue detection via Loupe Browser. Two example scenarios that may lead to tissue segmentation failure are described below:

- If the contrast between background and tissue is poor, due to inadequate staining for example, tissue segmentation failure may occur (left image).
- If large tissues that exceed the gasket area of the cassette re-enter the capture area, they may cause a tissue segmentation failure in Space Ranger if they re-enter the Capture Area due to the contrast of this tissue against tissue within the Capture Area (right image).

Gasket Obscures Capture Area

Gasket Covers Portion of Tissue within Capture Area

H&E Stained Tissue	Tissue Plot with Spots Colored by Clustering
	

If the gasket covers a portion of the tissue within the Capture Area, that area will not be exposed to the appropriate reagents; thus, analytes will not be captured. In Loupe Browser, manual alignment must be performed to exclude the tissue area obscured by the gasket. This issue can be prevented by ensuring that the positioning of the gasket within the Tissue Slide Cassette does not obscure the Capture Area after Tissue Slide Cassette assembly.

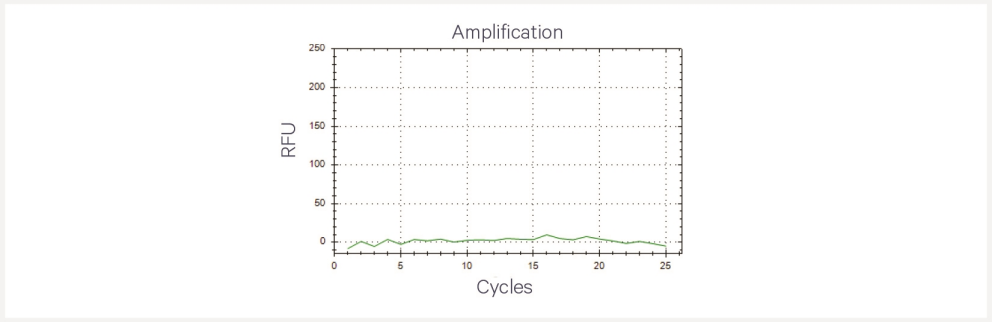
Visium CytAssist Slide Removal Delayed

A delay in removing and processing the Visium CytAssist Spatial Gene Expression Slide after run completion may impact data quality.

No qPCR Amplication

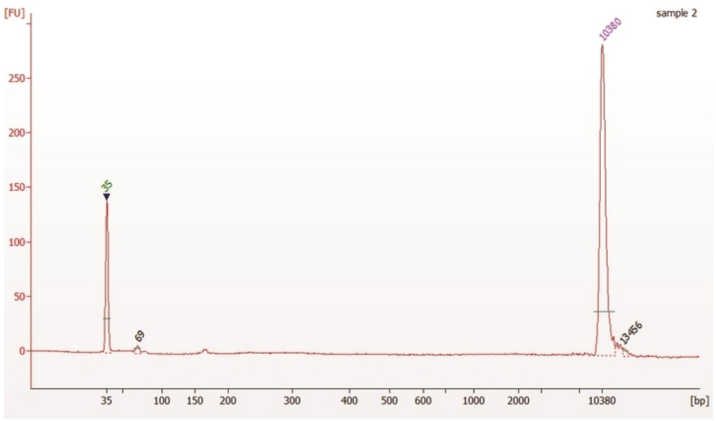
No amplification during Cycle Number Determination may be due to the following:

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



Flat Line in BioAnalyzer Library Trace

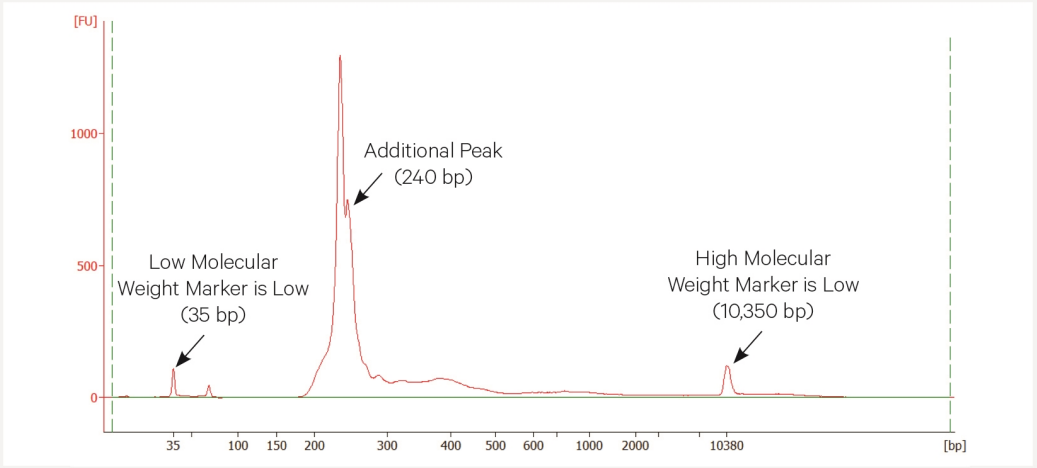
A normal qPCR output but no peak visible in the BioAnalyzer trace may be due to a mistake in the SI-PCR step, an overdilution of the final library, Bioanalyzer issue, or thermal cycler failure.



Overloaded or Overamplified Trace

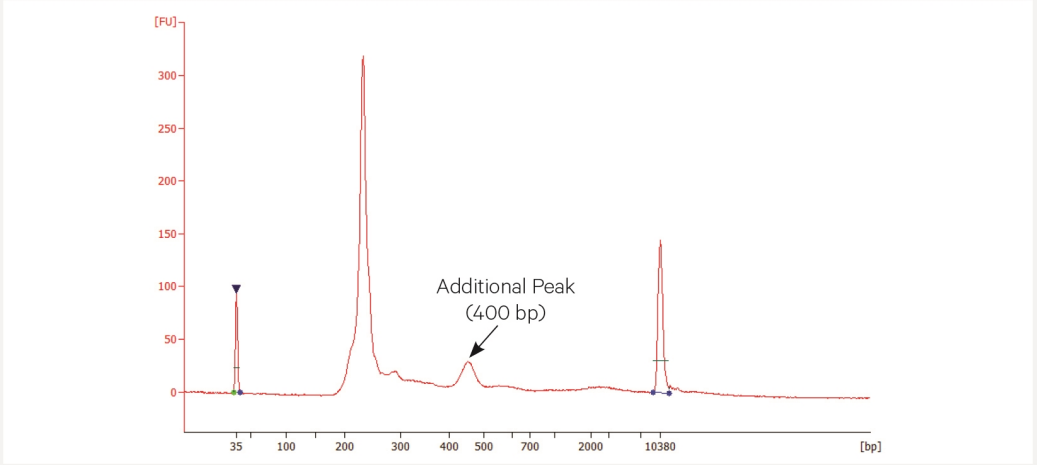
Overloaded Trace

The image below is an example of an overloaded trace. Note the double peak at around 240 bp. The low and high molecular weight markers are in low abundance compared to the sample. Ensure the library was diluted 1:50 prior to loading per protocol recommendations. If the library was diluted, ensure the library concentration is within the specification range of the automated electrophoresis kit being used. The library can be quantified using a Qubit and diluted further if appropriate.



Overamplified Trace

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



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

For example, a sample with C(t) value of 7.5 should go through 10 cycles during SI-PCR (round up to 8 and add 2) and a sample with a Cq value of 9.2 should go through 12 cycles during SI-PCR (round up to 10 and add 2). If running together, samples should undergo 11 cycles during SI-PCR.

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)	

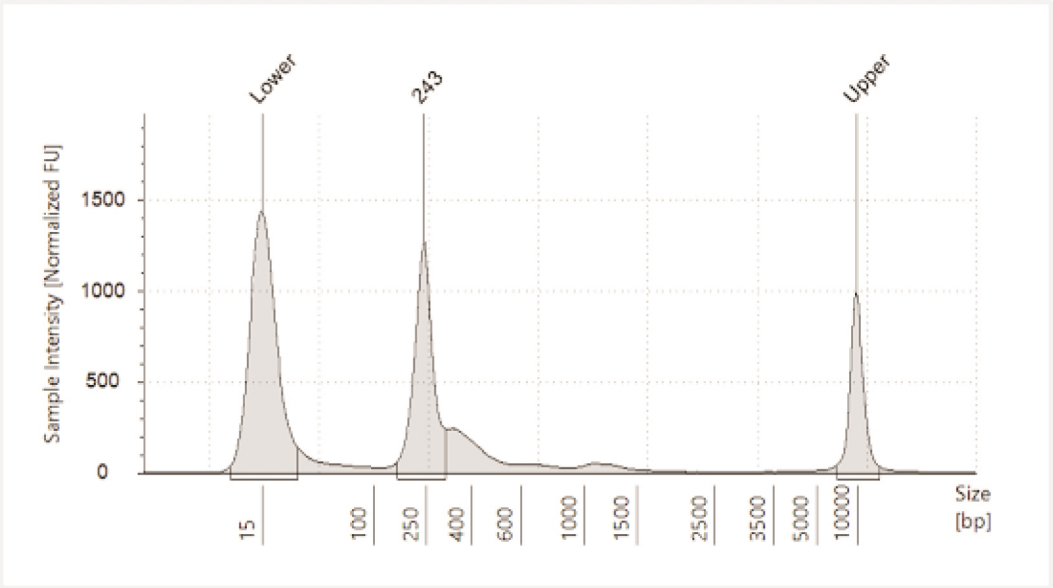
g. Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on insert size derived from the Bioanalyzer/TapeStation trace.

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 - GEX Post Library Construction QC

Representative Trace
Run manufacturer's recommended volume of diluted sample (1:50 dilution)

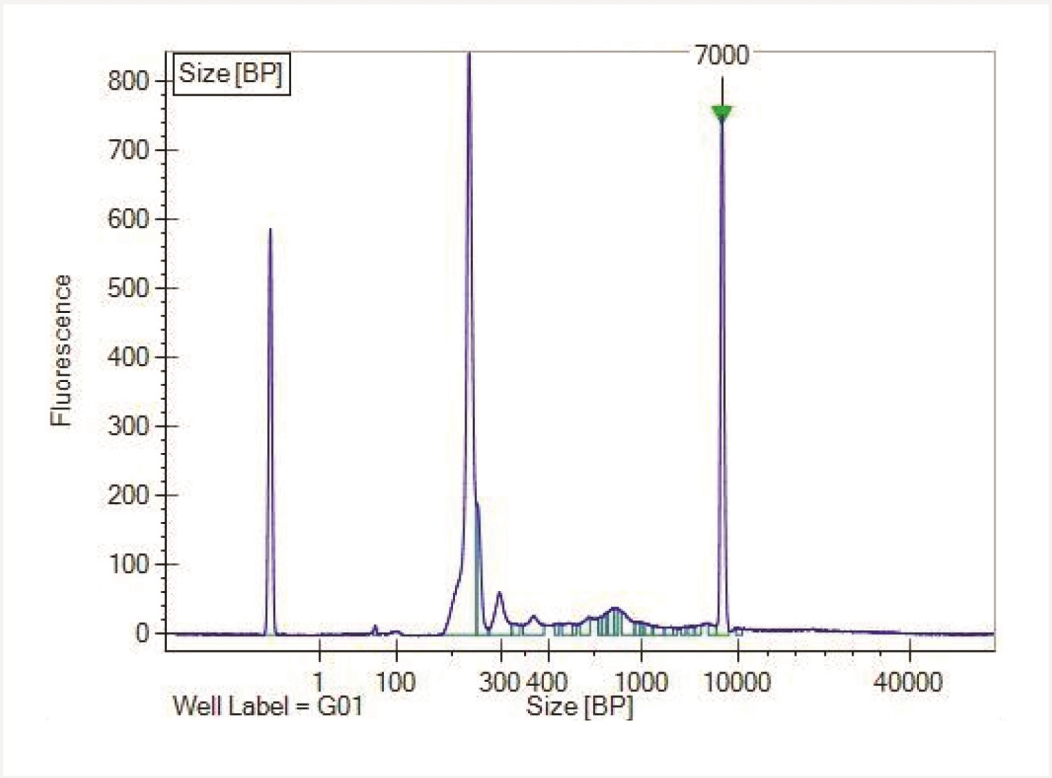


LabChip Traces

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

Protocol Step 5.3 - GEX Post Library Construction QC

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



Oligonucleotide Sequences

Slide Primers

5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'

Visium Spatial Gene Expression - FFPE Gene Expression Library

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-Ligated_Probe_Insert-TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'
3'-TTACTATGCCGCTGGTGGCTCTAGATGTG-N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAABN-Ligated_Probe_Insert-ACCTTAAGAGCCCACGGTTCCTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'