10x GENOMICS

User Guide | CG000495 | Rev G

Visium CytAssist Spatial Gene Expression Reagent Kits

for Formalin Fixed & Paraffin Embedded (FFPE)
for Fresh Frozen (FF)
for Fixed Frozen (FxF)

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

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

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 G

Revision Date

April 16, 2025

Description of Changes

- Updated tissue slide loading guidance in Instrument Loading Guidelines on page 49
- Updated probe preparation guidance in 1.0 Get Started on page 61.
- Updated tissue slide wiping guidance before instrument loading in 3.1 CytAssist-Enabled RNA Digestion & Tissue Removal on page 78
- Corrected TS Primer Mix B kit part number in 5.0 Get Started on page 95.
- Added information on color balance to Sample Indices on page 105.
- Added loading concentration guidance for NovaSeq X Plus in Library Loading on page 105.

Updated for general minor consistency of language and terms throughout.

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Introduction

Reagent Kits

Visium CytAssist Spatial Gene Expression for FFPE Reagent Kits

Reagent Kits	Part Number	Components	Component Part Number	Quantity
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	2
		Visium FFPE Reagent Kit v2 - Small	PN-1000436	1
		Visium Mouse Transcriptome Probe Kit – Small	PN-1000365	1
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	2
		Visium FFPE Reagent Kit v2 - Small	PN-1000436	1
		Visium Human Transcriptome Probe Kit v2 – Small	PN-1000466	1
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	1
		Visium FFPE Reagent Kit v2 - Small	PN-1000436	1
		Visium Mouse Transcriptome Probe Kit – Small	PN-1000365	1
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	1
		Visium FFPE Reagent Kit v2 - Small	PN-1000436	1
		Visium Human Transcriptome Probe Kit v2 – Small	PN-1000466	1

*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 Tissue Slide Cassette		
Visium CytAssist Sliding Gasket, Small (preassembled with translator)	2	3000814
Visium CytAssist Cassette Movable Translator (preassembled with gasket)	2	3000816
Visium CytAssist Cassette Movable Frame	2	3000813
Visium Slide Seals, 20-pack	1	2000284
Visium CytAssist Spatial Gene Expression Slide v2, 6.5 mm	1	2000549

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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 Tissue Slide Cassette		
Visium CytAssist Sliding Gasket, Large	2	3000815
Visium CytAssist Cassette Movable Frame	2	3000813
Visium Slide Seals, 20-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 (preassembled with translator)	4	3000814
Visium CytAssist Cassette Movable Translator (preassembled 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	2	2000554

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*These tubes may not be included in the kit. They are 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-1000365
(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

Visium CytAssist Reagent Accessory Kit PN-1000499

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

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

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Recommended Thermal Cyclers

Supplier	Description	Part Number
Bio-Rad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (discontinued)	1851197
	PTC Tempo Deepwell Thermal Cycler*	12015392
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
	VeritiPro Thermal Cycler, 96-well**	A48141
Analytik Jena	Biometra TAdvanced 96 SG with 96-well block (silver, 0.2 mL) and gradient function	846-x-070-241 *(where x=2 for 230 V; 4 for 115 V; 5 for 100 V, 50-60 Hz)*

*If used with a single cassette, place the cassette on the side of the adapter furthest away from the thermal cycler hinge to prevent lid lock errors.

**Requires firmware 1.2.0 or later. Open lid slowly to avoid disturbing the cassette. After incubation, if the cassette becomes stuck to the lid, remove carefully. When working with tubes, VeritiPro requires blue MicroAmp 96-Well Tray/Retainer Set for VeritiSystems (PN-4381850), with top piece removed.

Ramp rates should be adjusted for all the steps as described below for the following thermal cyclers:

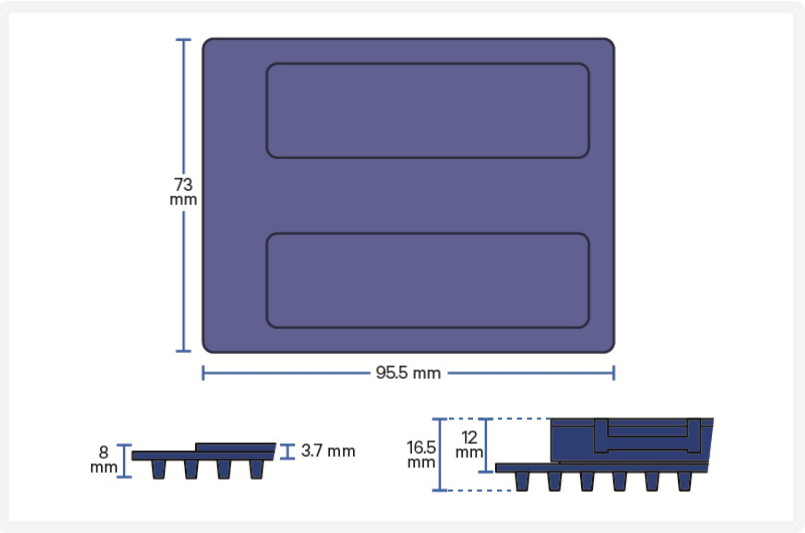
- Eppendorf MasterCycler X50s: 3°C/sec heating and 2°C/sec cooling
- Analytik Jena Biometra TAdvanced: 2°C/sec heating and cooling

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 before 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 Spatial Applications 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 of FFPE tissues)	• Light source (or equivalent) with a wavelength range of 380–680 nm • Monochrome camera (14 bit, 2,424 x 2,424 pixel resolution) • 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	

Imaging Systems & Specifications	
• 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 are 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. For some items, a number of options are listed. Choose item based on availability and preference.

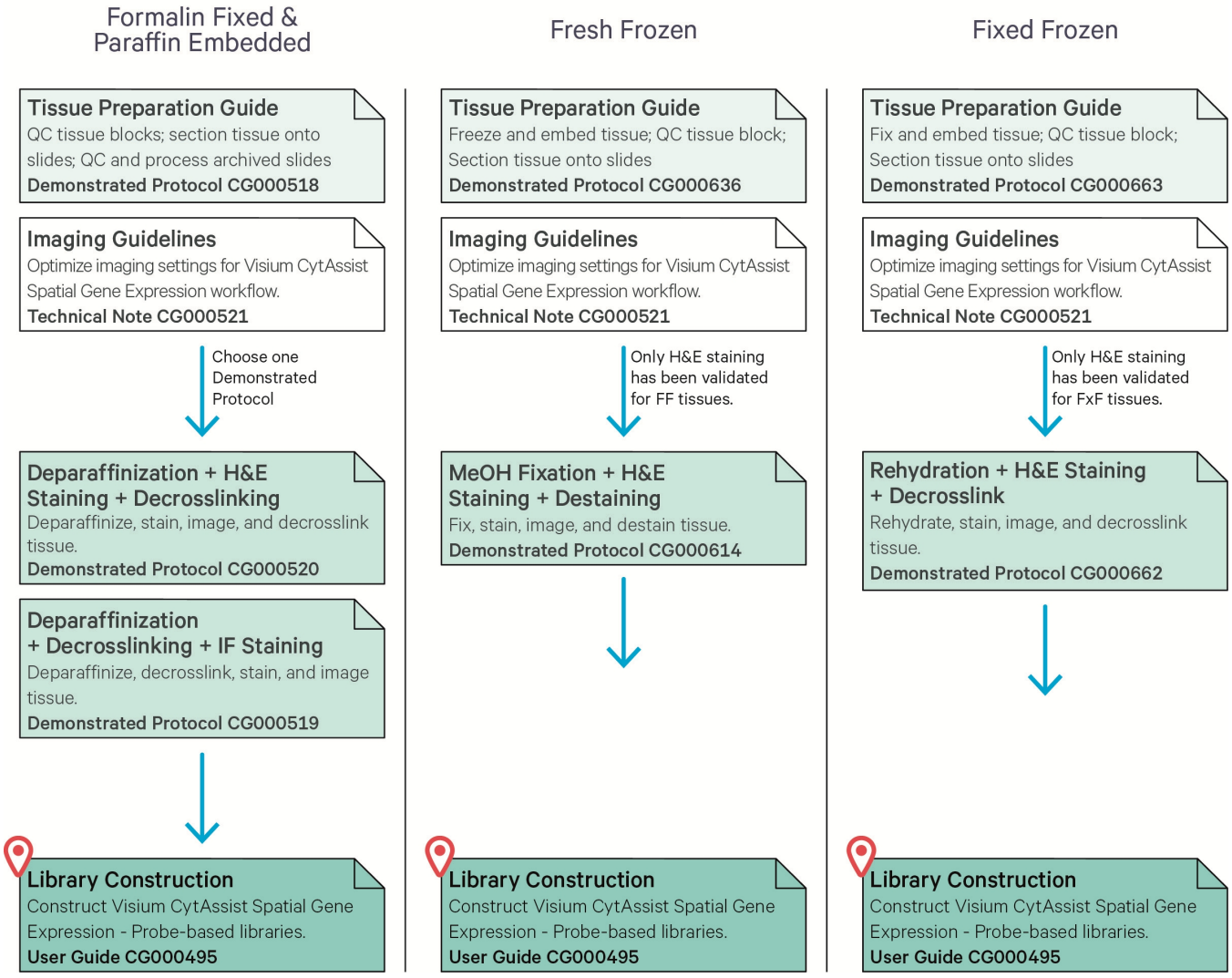
For information on tested glass slides, refer to product-specific documentation.

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
	Tips LTS 1ML Filter RT-L1000FLR	Rainin	30389213
	Tips LTS 20UL Filter RT-L20FLR	Rainin	30389226

Item	Description	Supplier	Part Number (US)
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 Solution, Alcoholic, with Phloxine	Millipore Sigma	HT110332
	Eosin Y with Phloxine 1% alcoholic 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
	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

Item	Description	Supplier	Part Number (US)
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 60)		
1.1 Probe Hybridization (page 62)	Overnight	
Day 2		
Step 2: Probe Ligation (page 66)		
2.1 Post-Hybridization Wash (page 68)	20 min	
2.2 Probe Ligation (page 71)	65 min	
2.3 Post-Ligation Wash (page 73)	15 min	STOP 4°C ≤24 h
Step 3: CytAssist Enabled Probe Release & Extension (page 75)		
3.1 RNA Digestion & Tissue Removal (page 78)	40 min	
3.2 Probe Extension (page 86)	20 min	STOP 4°C ≤24 h
3.3 Probe Elution (page 87)	15 min	
Step 4: Pre-Amplification and SPRIselect (page 89)		
4.1 Pre-Amplification (page 91)	40 min	
4.2 Pre-Amplification Cleanup - SPRIselect (page 93)	30 min	STOP 4°C ≤72 h or -20°C ≤4 weeks
Step 5: Visium CytAssist Spatial Gene Expression – Probe-based Library Construction (page 94)		
5.1 Cycle Number Determination – qPCR (page 96)	45 min	
5.2 Sample Index PCR (page 98)	40 min	
5.3 Post-Sample Index PCR Cleanup – SPRIselect (page 100)	30 min	STOP -20°C long-term
5.4 Post-Library Construction QC (page 101)	50 min	

Stepwise Objectives

The Visium CytAssist Spatial Gene Expression assay is designed to analyze mRNA in tissue sections derived from formalin fixed & paraffin embedded (FFPE), fresh frozen (FF), or fixed frozen (FxF) tissue samples. It uses probes targeting the whole transcriptome. Each Visium CytAssist Spatial Gene Expression Slide v2 contains Capture Areas with barcoded spots that include oligonucleotides required to capture gene expression probes. Before the assay, tissue sections are processed as described in their relevant Demonstrated Protocol. See Workflow Overview on page 19 for documentation references.

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 & Tissue Removal, enabling capture by the spatially barcoded oligonucleotides present on the Visium 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.

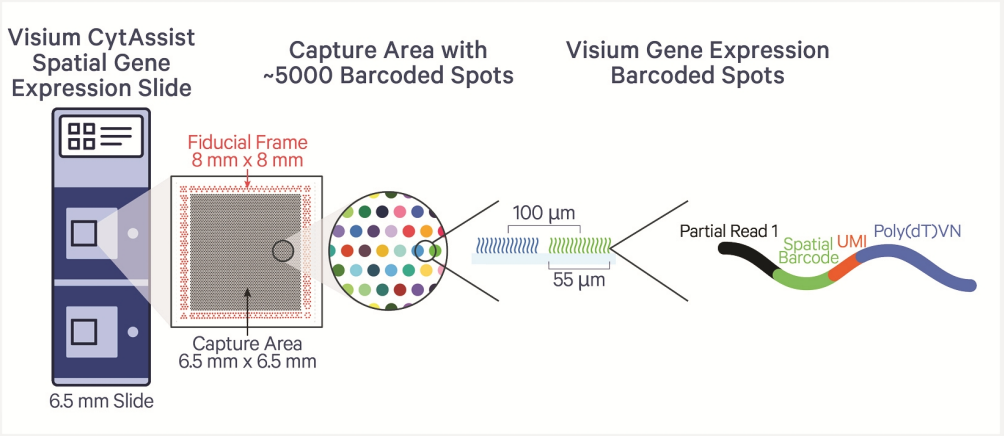
This document outlines the protocol for generating Visium CytAssist Spatial Gene Expression - Probe-based libraries. While this User Guide provides guidance on using both human and mouse probes, only mouse probes have been validated with the FxF assay.

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:

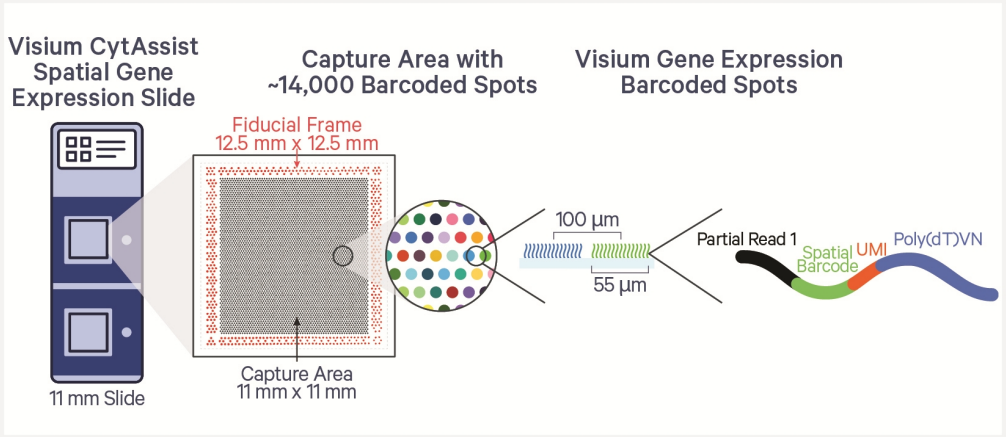
- Gene Expression: Illumina TruSeq partial read 1 sequencing primer, 16 nucleotide (nt) Spatial Barcode, 12 nt unique molecular identifier (UMI), 30 nt poly(dT) sequence (captures ligation product).
- Each Capture Area on the CytAssist Spatial Gene Expression Slide is surrounded by a spacer. This spacer 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

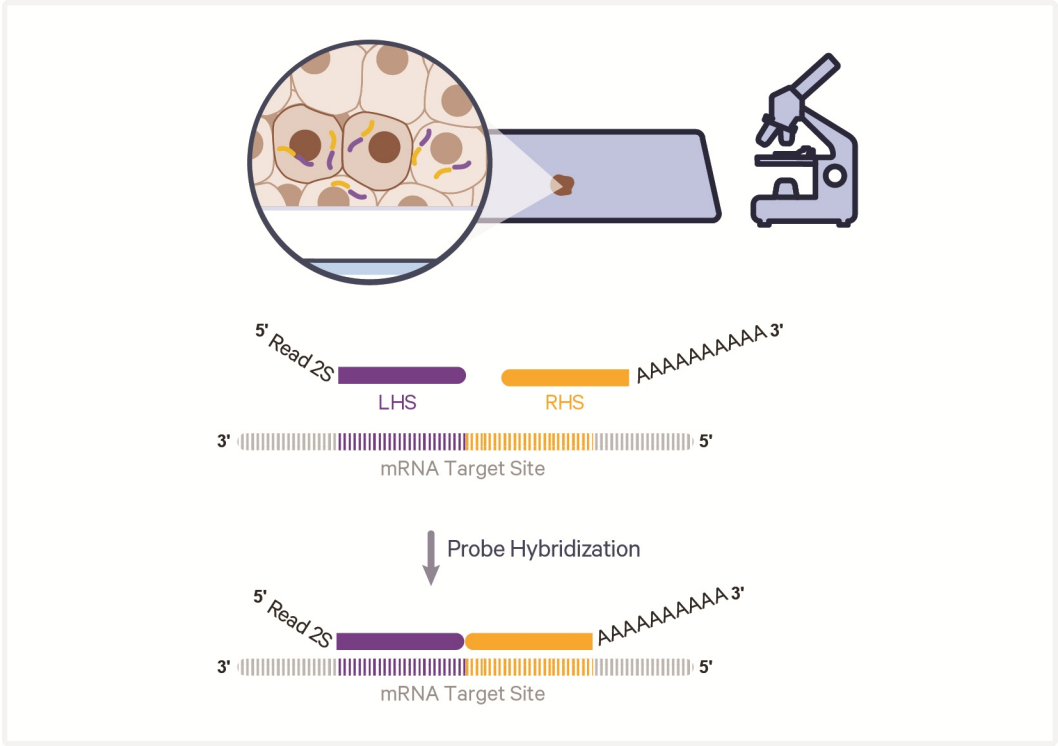
The CytAssist Spatial Gene Expression Slide v2, 11 mm has 2 Capture Areas. Each Capture Area is 11 x 11 mm and defined by a fiducial frame (fiducial frame + Capture Area is 12.5 x 12.5 mm). The Capture Area has ~14,000 barcoded spots. Each spot has the following oligos:

- 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.



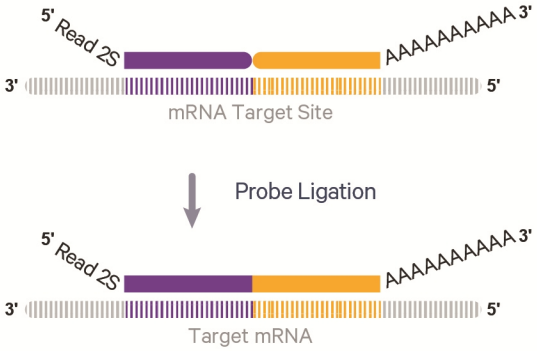
Step 1: Probe Hybridization

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, destained, and decrosslinked tissues. Together, probe pairs hybridize to their complimentary target RNA.



Step 2: Probe Ligation

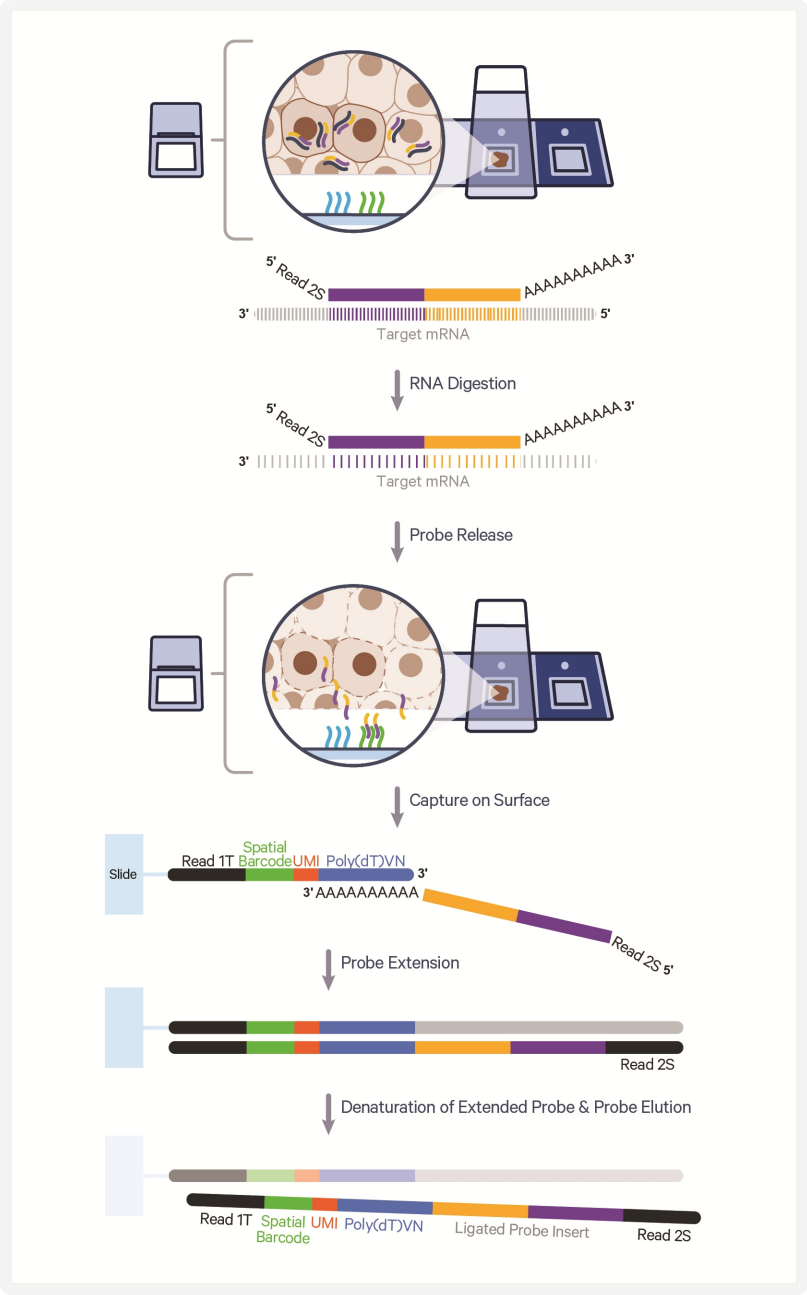
After hybridization, a ligase is added to bridge the junction between the probe pairs that have hybridized to RNA, forming a ligation product.



Step 3: Probe Release & Extension

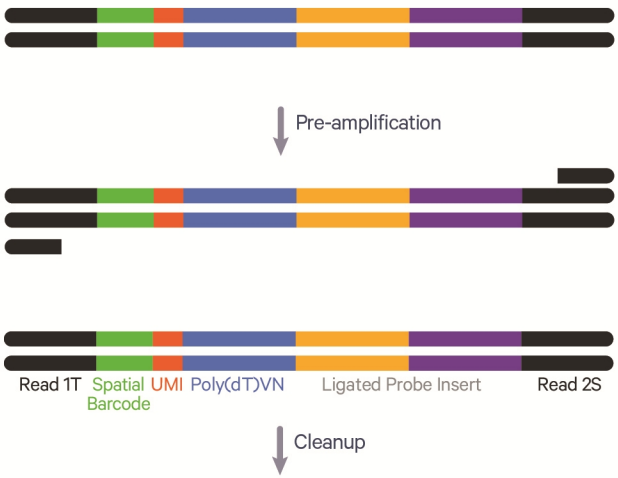
Probe release and capture occurs in the Visium CytAssist instrument. The single stranded ligation products are released from the tissue upon RNase treatment and tissue removal, and then captured on the Visium slide. Once ligation products are captured, the slides can be removed from the instrument.

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



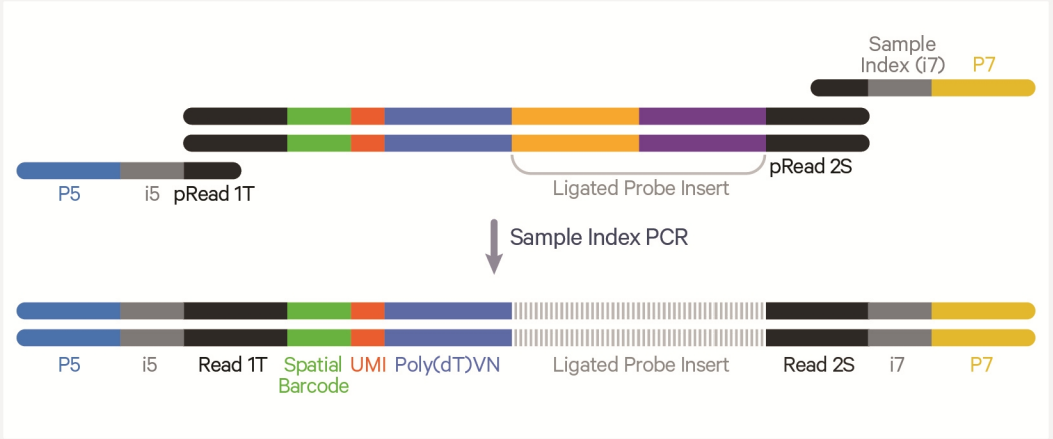
Step 4: Pre-amplification and SPRIselect

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



Step 5: Visium CytAssist Spatial Gene Expression - Probe-based Library Construction

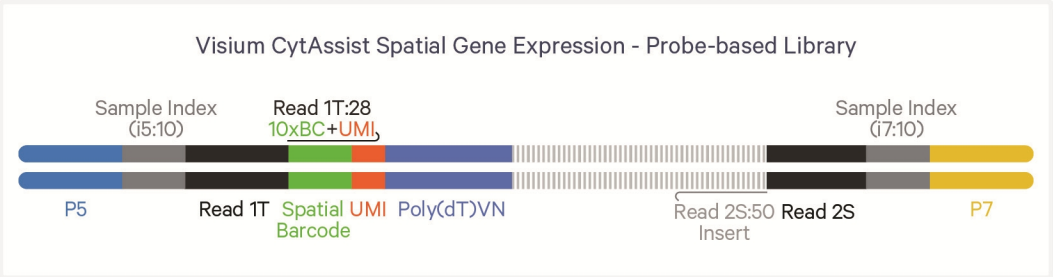
Pre-amplification material is collected for qPCR to determine sample index PCR cycle number for gene expression libraries. The amplified material then undergoes indexing via sample index PCR to generate final library molecules. The final libraries are cleaned up by SPRIselect, assessed on a Bioanalyzer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



Sequencing

A Visium CytAssist Spatial Gene Expression - Probe-based 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, and library loading information are summarized in the Sequencing section.



See Oligonucleotide Sequences on page 131

10x GENOMICS

Tips & Best Practices

Icons

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

General Reagent Handling

- Fully thaw and thoroughly mix reagents before use.
- When pipette mixing, unless otherwise specified, set pipette to 75% of total volume.
- Change gloves frequently to avoid contamination of samples or reagents with DNase.

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.

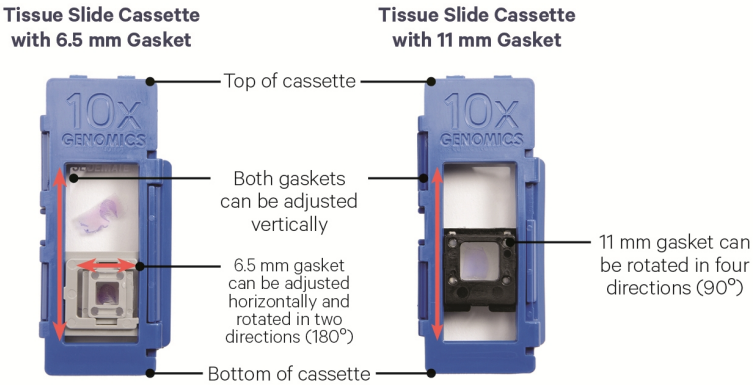
- Time between adding the final reagent to the Probe Release Mix and loading the complete Probe Release Mix onto spacers on the Visium CytAssist Spatial Gene Expression v2 Slide on the Visium CytAssist instrument and starting a run should not exceed **5 min**. Start the run immediately after reagent addition.

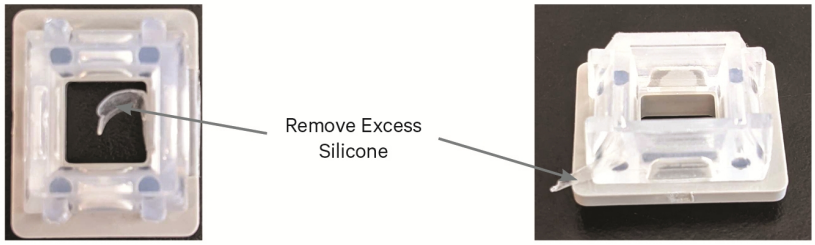
Tissue Slide Handling

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

Visium CytAssist Tissue Slide Cassette

- The Visium CytAssist Tissue Slide Cassette is used to create wells on tissue slides 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 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.
- Before assembly, inspect the moveable gasket to ensure that the gasket perimeter and corners are free of excess silicone. Excess silicone should be safely removed with forceps or a pipette tip.
- Assemble against a white background for easy tissue visualization during alignment.


Tissue Slide Cassette with 6.5 mm Gasket
Tissue Slide Cassette with 11 mm Gasket
Top of cassette
Both gaskets can be adjusted vertically
6.5 mm gasket can be adjusted horizontally and rotated in two directions (180°)
11 mm gasket can be rotated in four directions (90°)
Bottom of cassette


Remove Excess Silicone

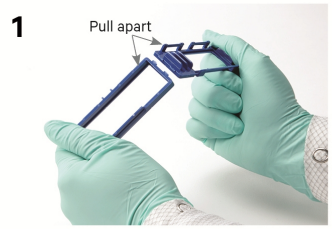
Visium CytAssist Tissue Slide Cassette Assembly

Assembly instructions apply to both Tissue Slide Cassette sizes.

Wear fresh gloves while assembling Tissue Slide Cassette

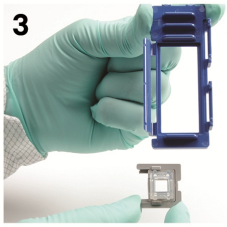
Exercise caution when handling slide edges to prevent injury.

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



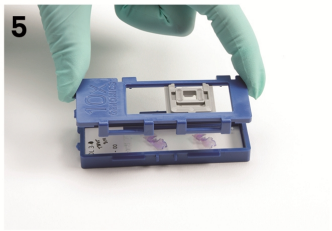
Place tissue slide into lower half of cassette with tissue facing up

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.

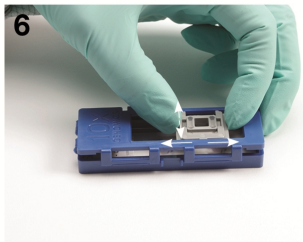


Securely combine gasket with top half of cassette until the gasket snaps into place.

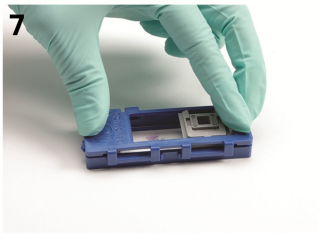
Gently place top half of cassette over bottom half. DO NOT assemble together until Step 6.



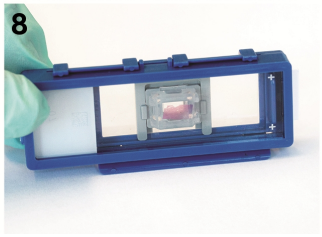
Adjust gasket such that gasket is over the tissue region of interest. The 6.5 mm gasket can be adjusted horizontally as well as vertically.



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



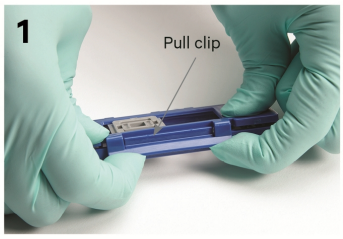
Turn cassette over and verify tissue region of interest is within gasket. DO NOT move gasket once cassette is closed. If necessary, open cassette and recenter gasket.



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

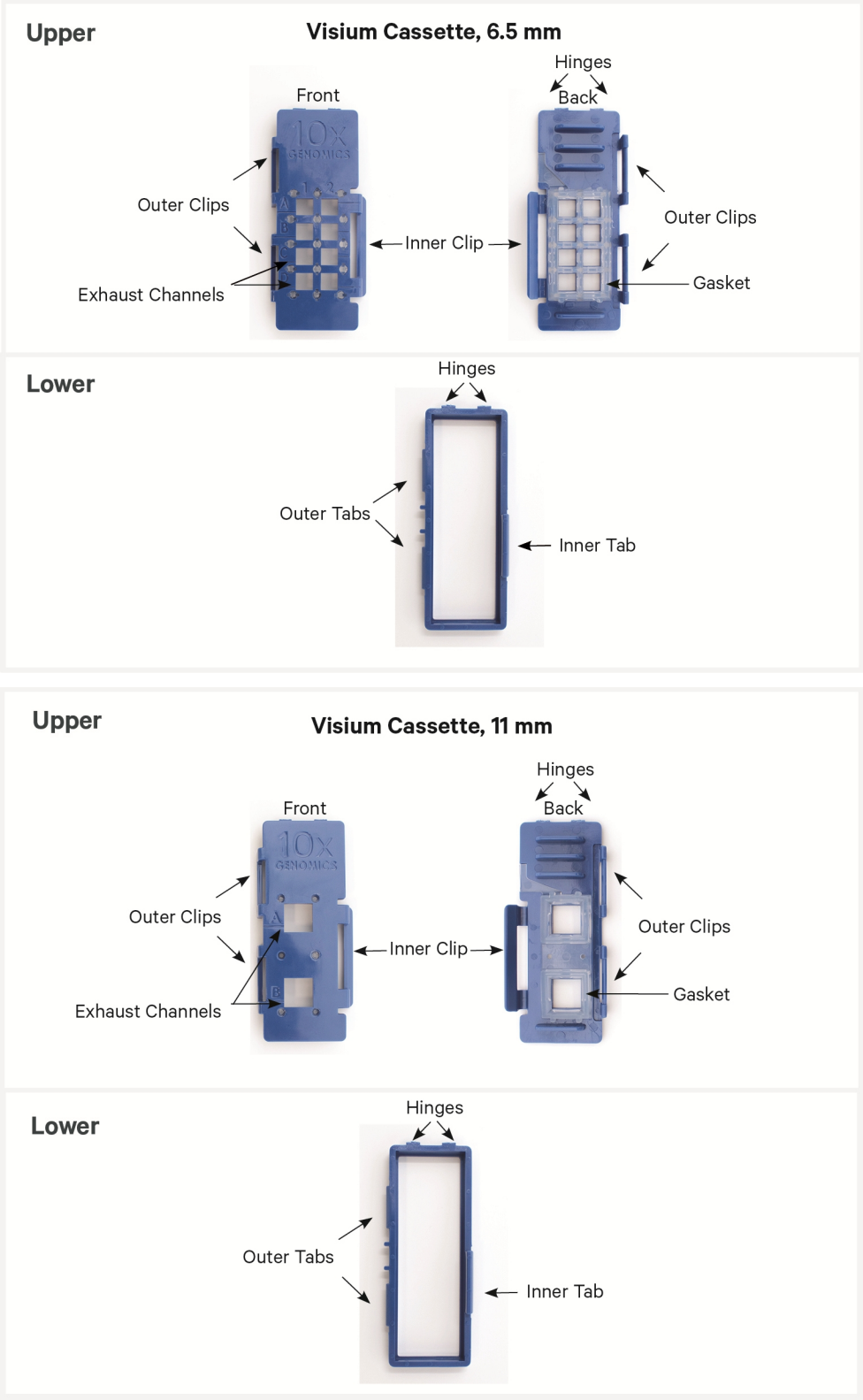


Open cassette by continuing to lift clip upward. If slide sticks to gasket, continue to apply even upward pressure to separate slide from gasket

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

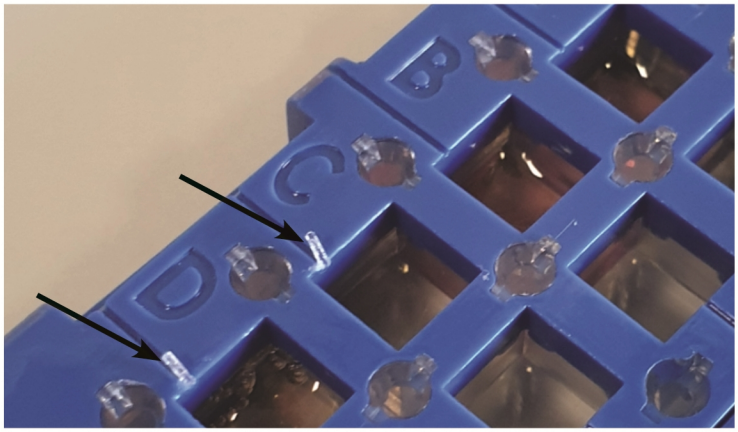
Slides in images are representative.

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 before 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



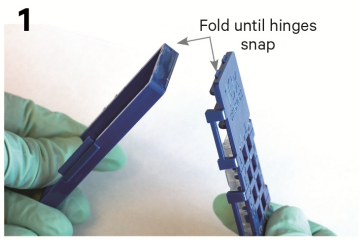
- See Visium Cassette Assembly & Removal instructions for details on assembly and removal.
- Practice assembly with a blank slide (75 x 25 x 1 mm).
- Place the slides in the Visium Cassette only when specified.
- Applying excessive force to the slide may cause the slide to break.

Visium Cassette Assembly

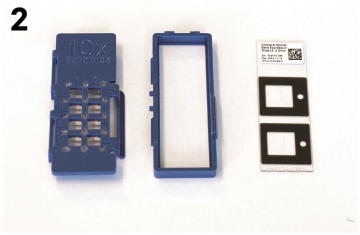
Ensure that the surface of the cassette is dry. Cassette may also be assembled in the hand for comfort.

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

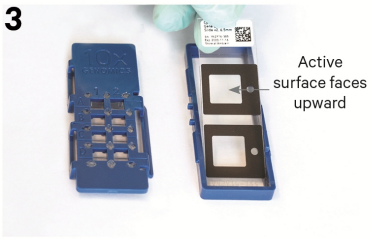
Break cassette into two halves by bending each half at the hinge until they snap apart



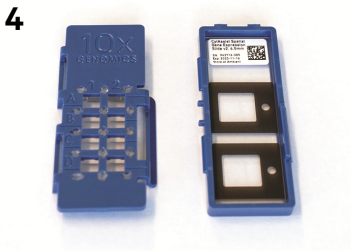
Place upper and lower halves of cassette, and Visium slide, directed upward on bench



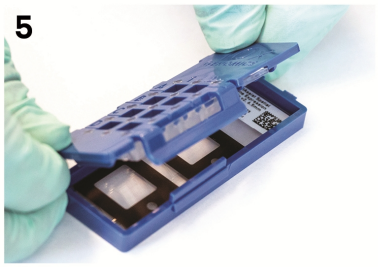
Place Visium slide with active surface facing upwards into lower half of cassette; ensure label is toward hinges



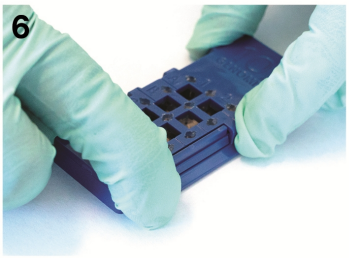
Press slide down into grooves of the bottom half of the cassette until it sits firmly in place



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



Press firmly on top of cassette until it clicks shut



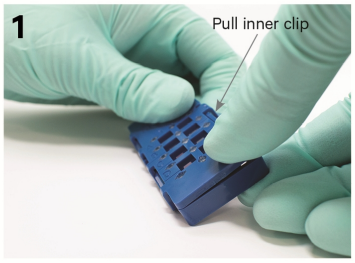
Exercise caution when handling slide edges to prevent injury.

Slides in images are representative.

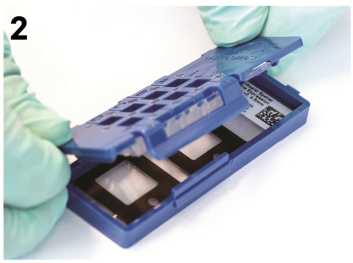
Visium Cassette Removal

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



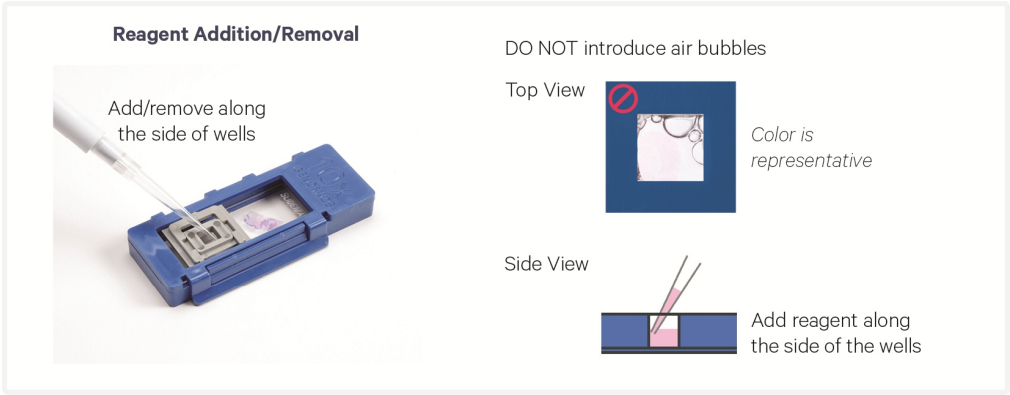
Lift slide out from lower half

Slides in images are representative.

Reagent Addition to & Removal from Wells

Reagent Addition

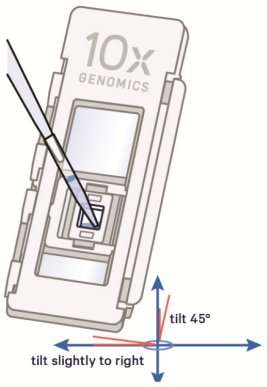
- Assemble slide into the cassette flat on a clean work surface.
- Dispense and remove reagents along the side of the wells without touching the slide surface, tissue sections (when applicable), and without introducing bubbles.
- If applicable, perform washes next to the thermal cycler to avoid significant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



Reagent Removal from Wells

- Slightly tilt the cassette while removing the reagent. DO NOT completely tilt the cassette at a 90 degree angle to avoid liquid from entering other grooves within the gasket. See image below for proper technique.
- Place the pipette tip on the bottom edge of the wells.
- Remove reagents along the side of the wells without touching the tissue sections (when applicable) and without introducing bubbles.
- Remove all liquid from the wells in each step. To ensure complete removal, check the bottom of the well by tilting the cassette slightly. A meniscus at the bottom of the well will indicate the presence of liquid in the well.

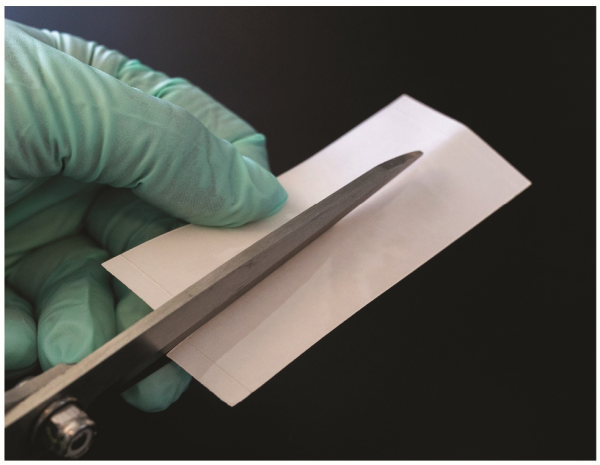
Repeat removal steps until no reagent remains.



Visium Slide Seal Application & Removal

Application

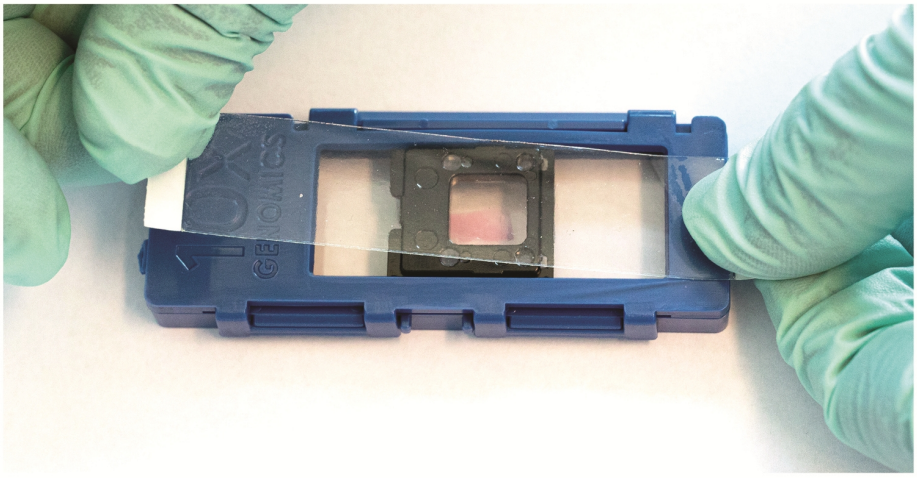
- If applying a Visium Slide Seal to a Tissue Slide Cassette, the seal must be cut in half lengthwise. Cut the seal as shown in the image below. Three pre-cut seals are necessary for this assay.



- 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.

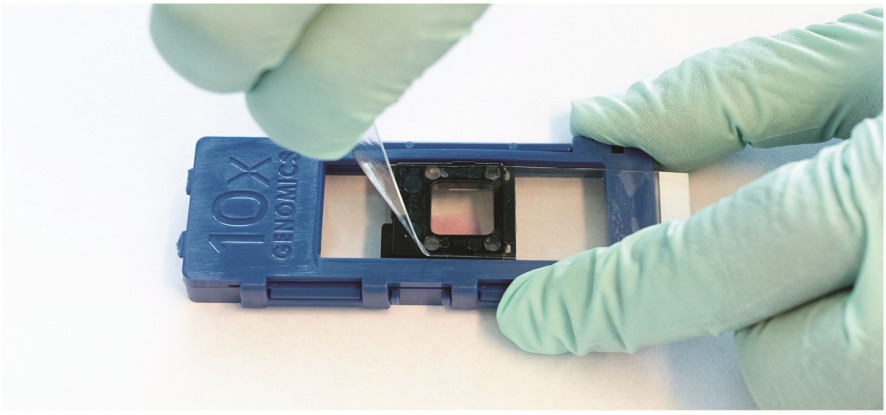
- Use a fresh Visium Slide Seal when prompted during the protocol. Steps that do not require a new slide seal will specify that the slide seal should be pulled back and reapplied instead.

Apply Visium Slide Seal



Removal

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



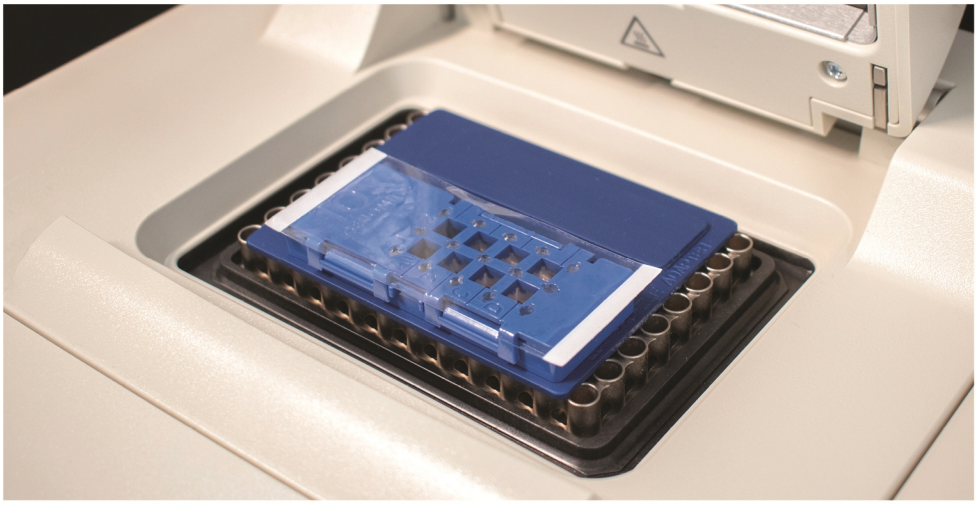
Slide Incubation Guidance

Incubation at a Specified Temperature

- Position a Low Profile Thermocycler Adapter on a thermal cycler that is set at the incubation temperature.
- Ensure that the Low Profile Thermocycler Adapter is in contact with the thermal cycler block surface uniformly. Pre-equilibrate adapters at the HOLD temperature described in the protocol steps for at least five minutes.
- 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. The cassette should snap into place when seated properly 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 overtightening. Image below is for demonstration purposes - thermal cycler lid should be closed when incubating the cassette.



Incubation at Room Temperature

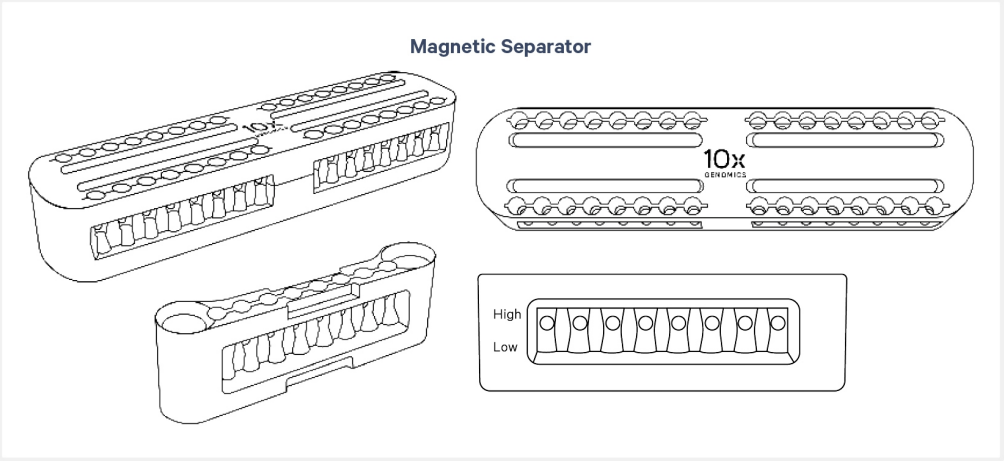
- Place the slide/cassette on a flat, clean, nonabsorbent work surface.

Tissue Detachment on Tissue Slides

- Monitor section adhesion on tissue slides throughout the workflow.
- Ensure that tissue sections are on Superfrost Plus or positively charged slides. A list of tested slides can be found in Visium CytAssist Tested Slides on page 48.
- Tissue detachment before the completion of Probe Release during the workflow can negatively impact performance. If observed, contact support@10xgenomics.com.
- For more information, see Troubleshooting on page 108.

10x Magnetic Separator

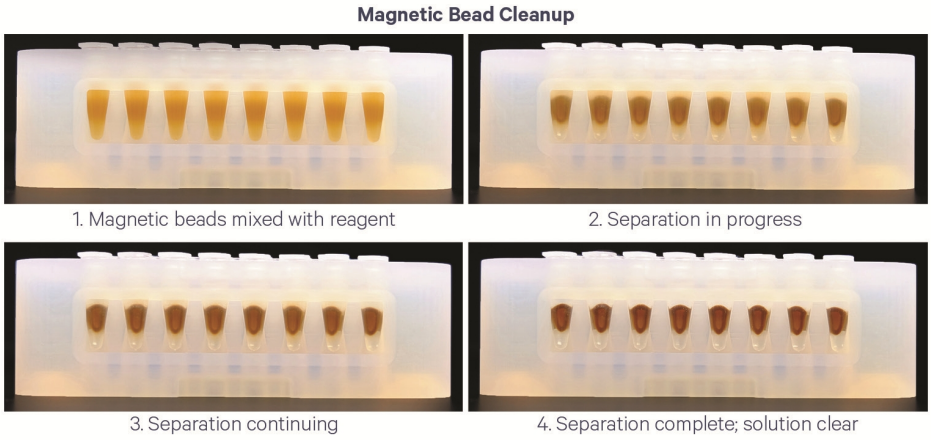
- Images below are illustrative - actual appearance of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.



- Offers two positions of the magnets (high and low) relative to a tube, depending on its orientation.
- Flip the magnetic separator over to switch between high (magnet•**High**) or low (magnet•**Low**) positions.
- If using MicroAmp 8-Tube Strips, use the high position (magnet•**High**) only throughout the protocol.

Magnetic Bead Cleanup Steps

- During magnetic bead-based cleanup steps that specify waiting “until the solution clears”, visually confirm clearing of solution before proceeding to the next step. See panel below for an example. If solution is not clear, leave on magnet until separation is complete.
- The time needed for the solution to clear may vary based on specific step, reagents, volume of reagents, etc.
- Images below are illustrative - actual appearance of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.



SPRIselect Cleanup & Size Selection

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

Sample Indices (i5/i7) in Sample Index PCR

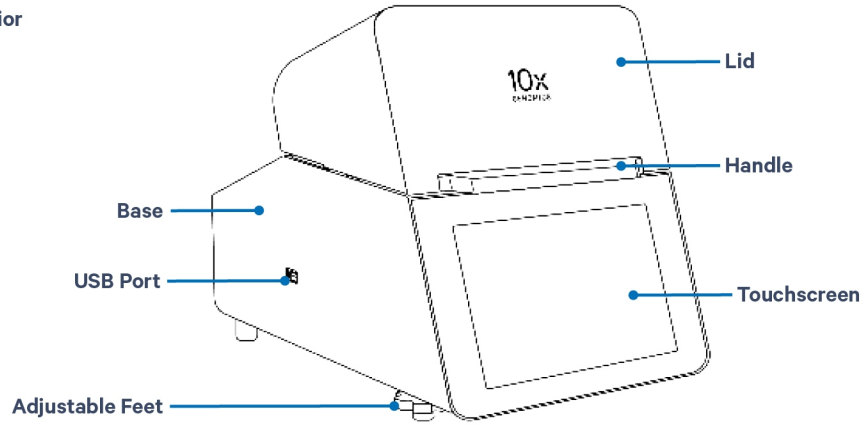
- Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run.
- Each well in the Dual Index Plate TS Set A contains a unique i7 and a unique i5 oligonucleotide.

- To avoid the risk of cross-contamination, use each plate well only once.
- Record the index plate well location for each sample.

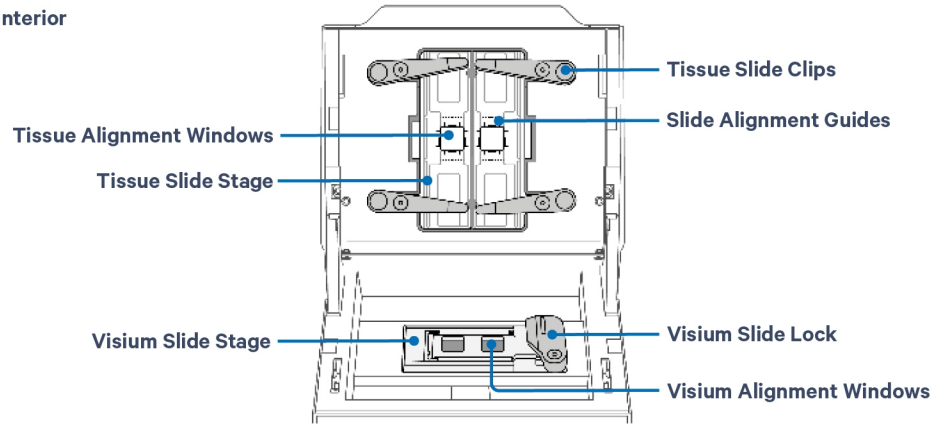
Visium CytAssist

Instrument Orientation

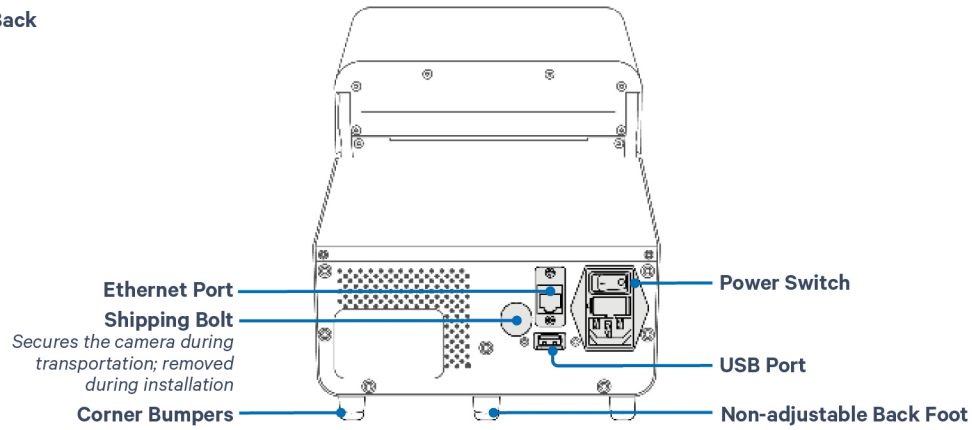
Exterior



Interior



Back



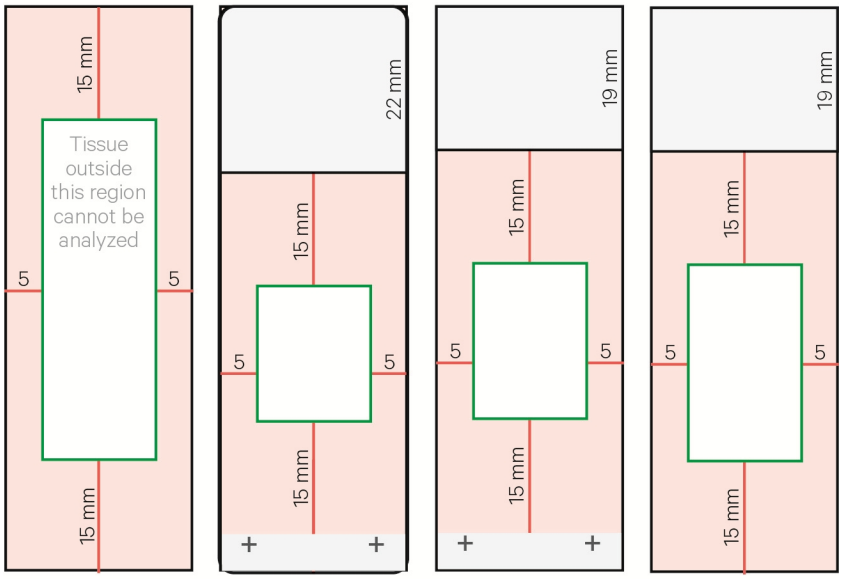
Visium CytAssist Tested Slides

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

Item	Length (mm)	Width (mm)	Thickness (mm)	Ground Corners
Epredia Shandon ColorFrost Plus	75.0	25.0	1.0	No
Fisherbrand SuperFrost Plus	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 ColorFrost Plus

VWR SuperFrost Plus Micro Slide, Premium

Fisherbrand SuperFrost Plus

Sigma-Aldrich Poly Prep

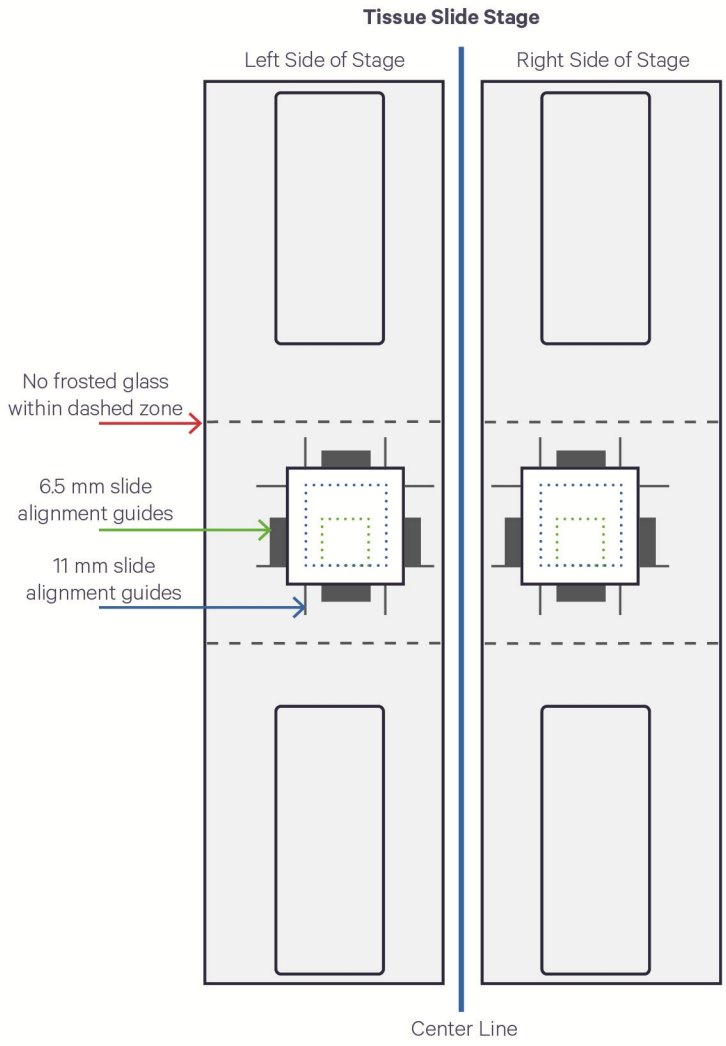
Instrument Loading Guidelines

- Ensure tissue section placement is compatible with Visium CytAssist by using the Visium CytAssist Accessory Kit Quick Reference Card (CG000548). A list of tested slide types can be found in the CytAssist Tested Slides section.
- Consult the Visium CytAssist Training Kit UG (CG000549) for information on practicing tissue slide loading.
- Each tissue slide may be used for one Capture Area on a Visium CytAssist Gene Expression v2 Slide.

Determine Slide Placement

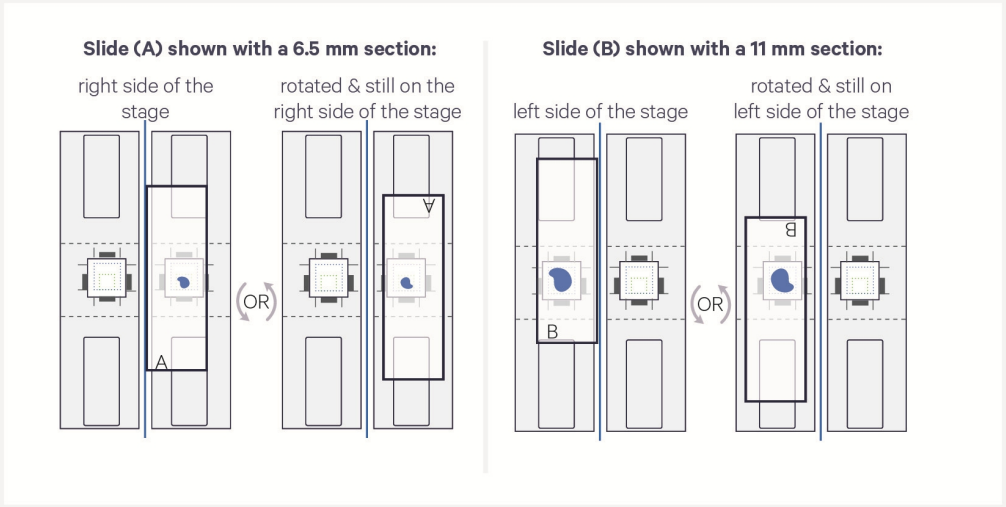
a. On the diagram below, align tissue to the center of the alignment guides for 6.5 mm (rectangles) or 11 mm (lines) Capture Areas on either the left or right side of the stage. Align the center of the tissue region of interest to the center of the Capture Area, instead of aligning the edge of the region to the edge of the Capture Area.

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



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

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

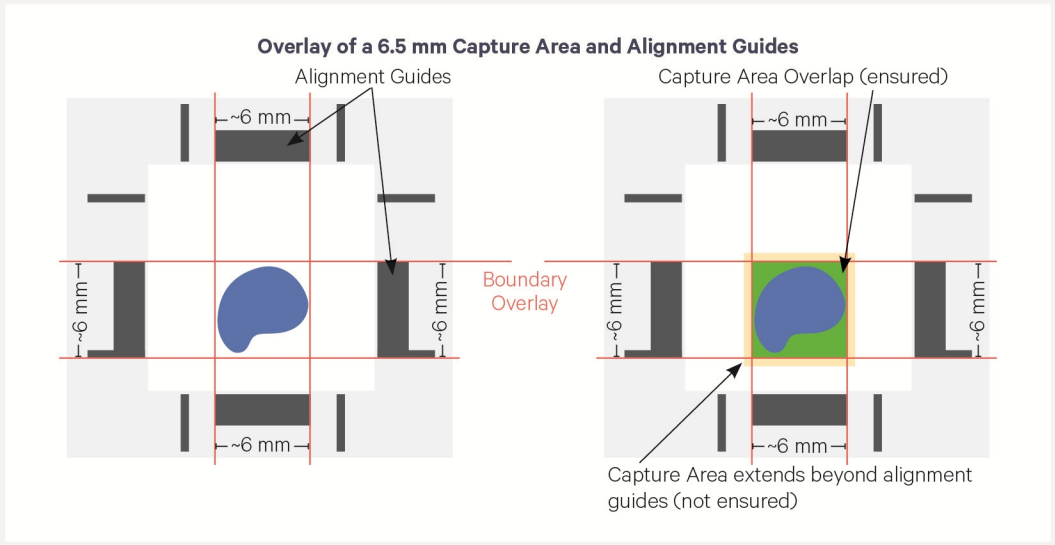


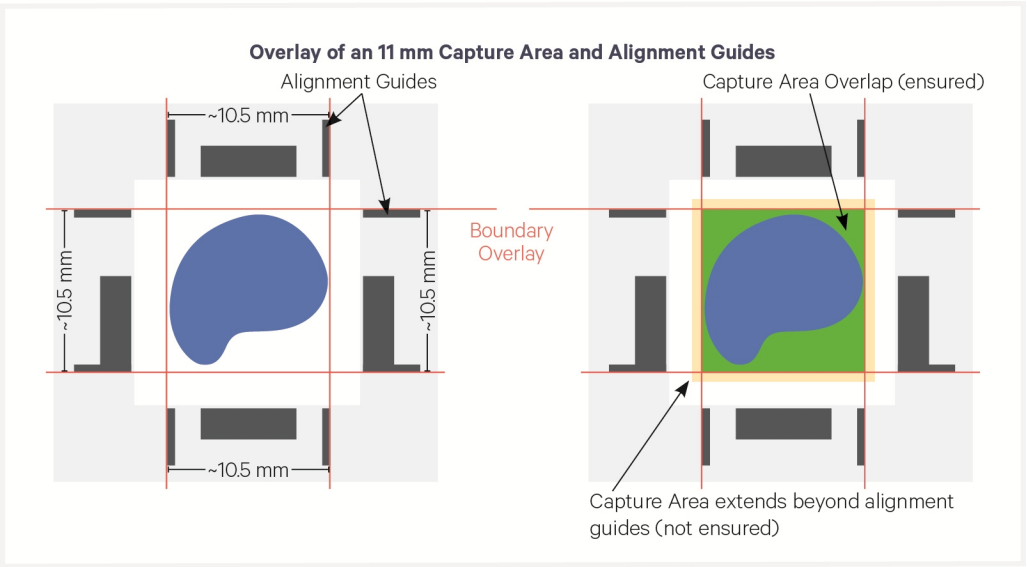
Fine Adjustments

The **outside** of the alignment guides, shown in red, mark the inner boundaries of the fiducial frame on a Capture Area. They mark a distance **approximately** 6 mm (6.5 mm slides) or 10.5 mm (11 mm slides) wide on each side and surround the target region ensured to align within the fiducial frame.

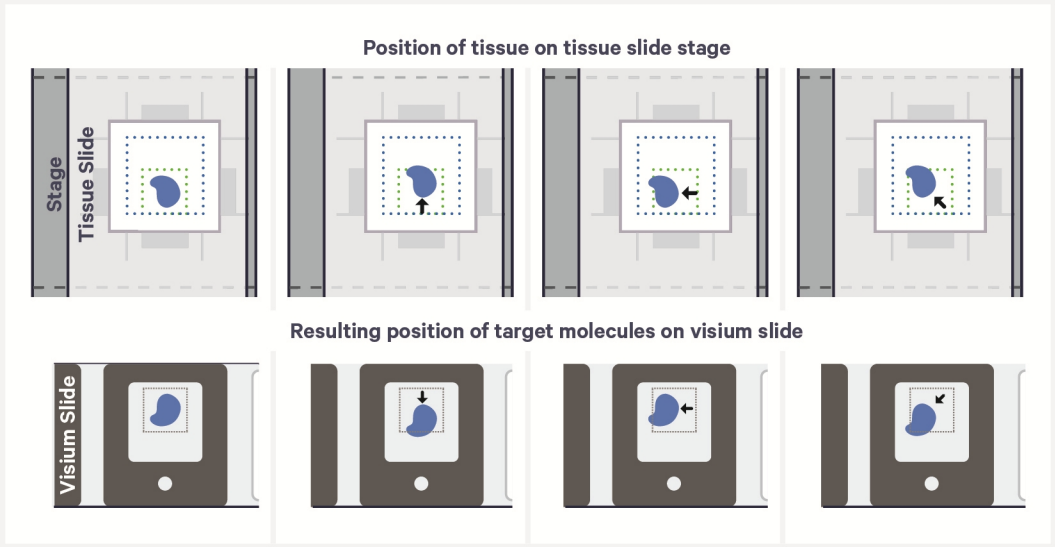
When placing a tissue slide on the Tissue Slide Stage, center the tissue or area of interest within the area inside the outer edges of the alignment guides (area in green in the images below).

Alignment guide dimensions and locations are designed with a high tolerance to minimize data loss. Tissue within the alignment guides is ensured to overlap with the Capture Area during a successful run. Tissue outside of these guides may be captured (area in yellow in the images below), but it is not ensured. 10x Genomics recommends placing high-priority tissue areas within the alignment guides.





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

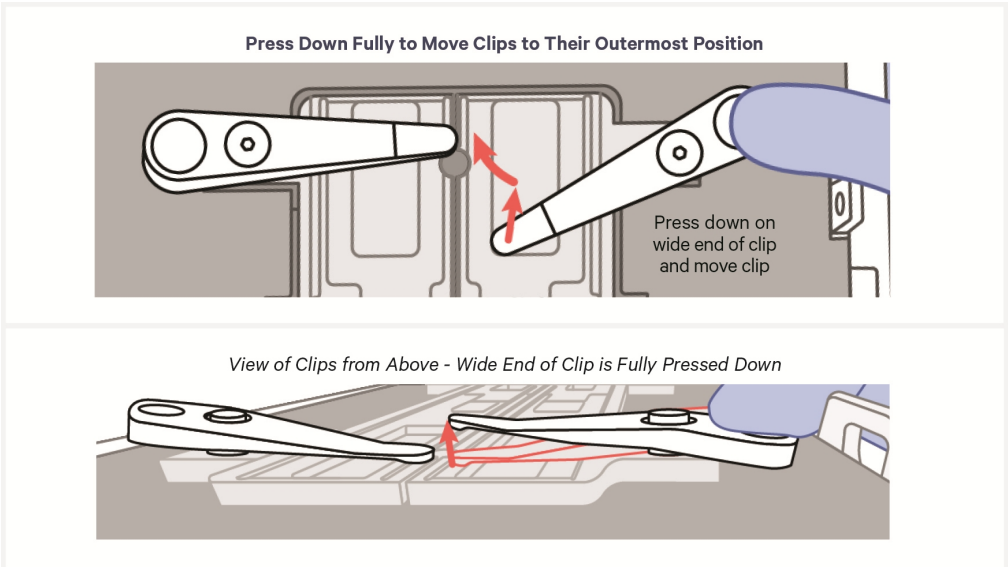


Tissue Slide Loading

Tissue slide loading instructions are updated in this document revision (Rev G)

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

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



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

DO NOT touch the tissue section.

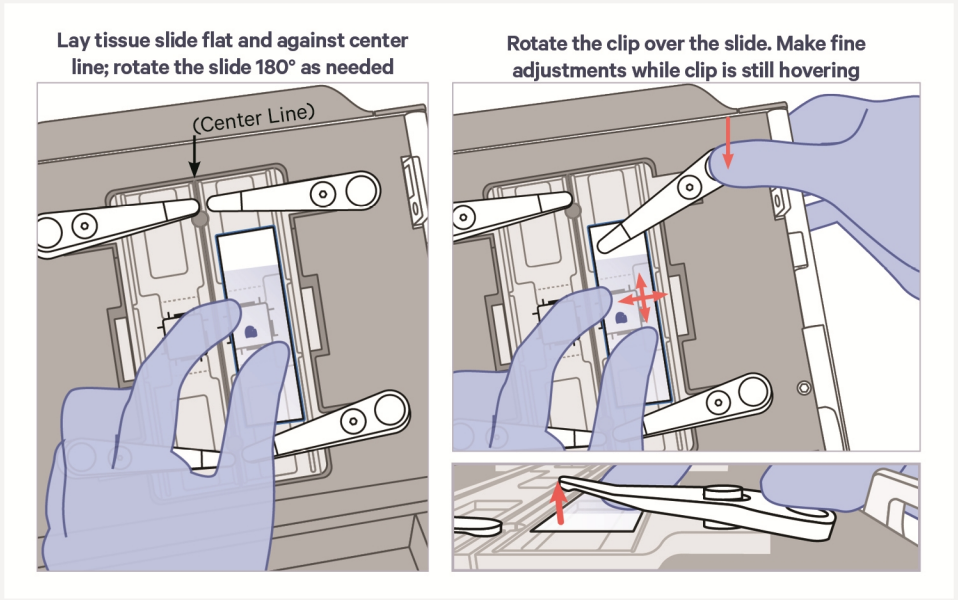
c. Use one hand to hold slide in place with two fingers, one on either side of the tissue. Use the other to both cradle the instrument lid and fully press down on the first clip. Rotate the clip over the slide.

d. While clip is hovering over the slide, make fine adjustments to align the tissue or area of interest to the center of the alignment guides. Gently release when adjustments are complete.

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

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

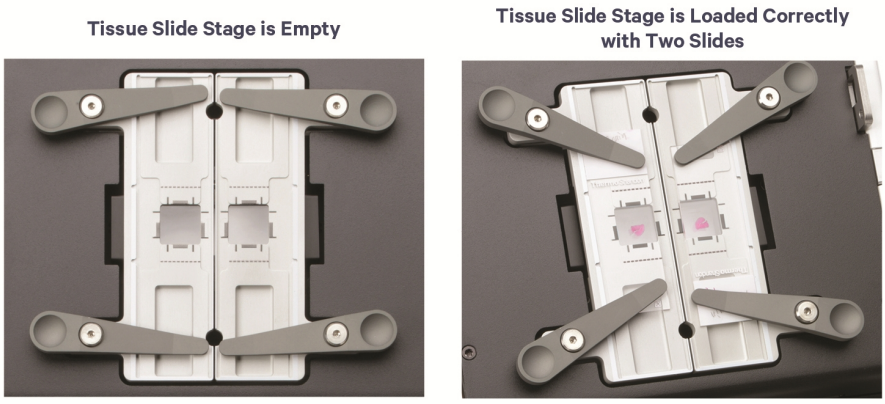
Reference images below for steps b-d.

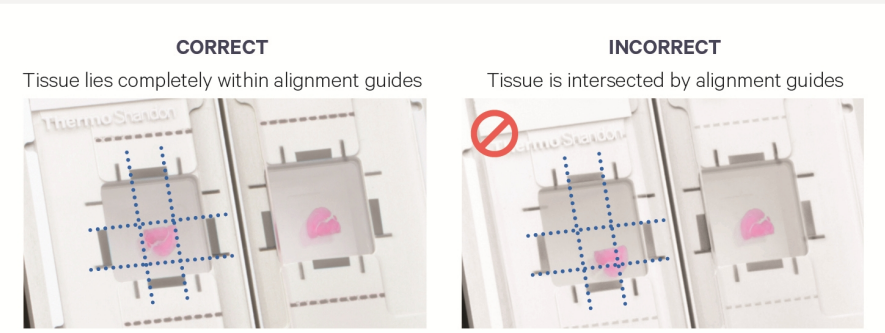


e. Continue to hold the slide in place while pressing down fully on the wide end of the second clip. Rotate the second clip onto the slide and gently release.

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

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





6.5 mm capture area used as an example

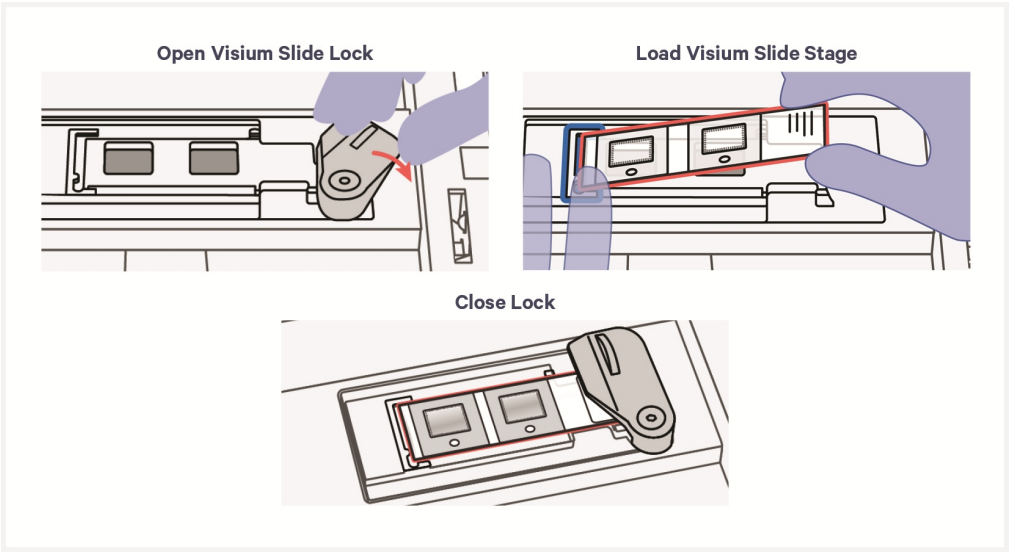
Visium CytAssist Spatial Slide Loading

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

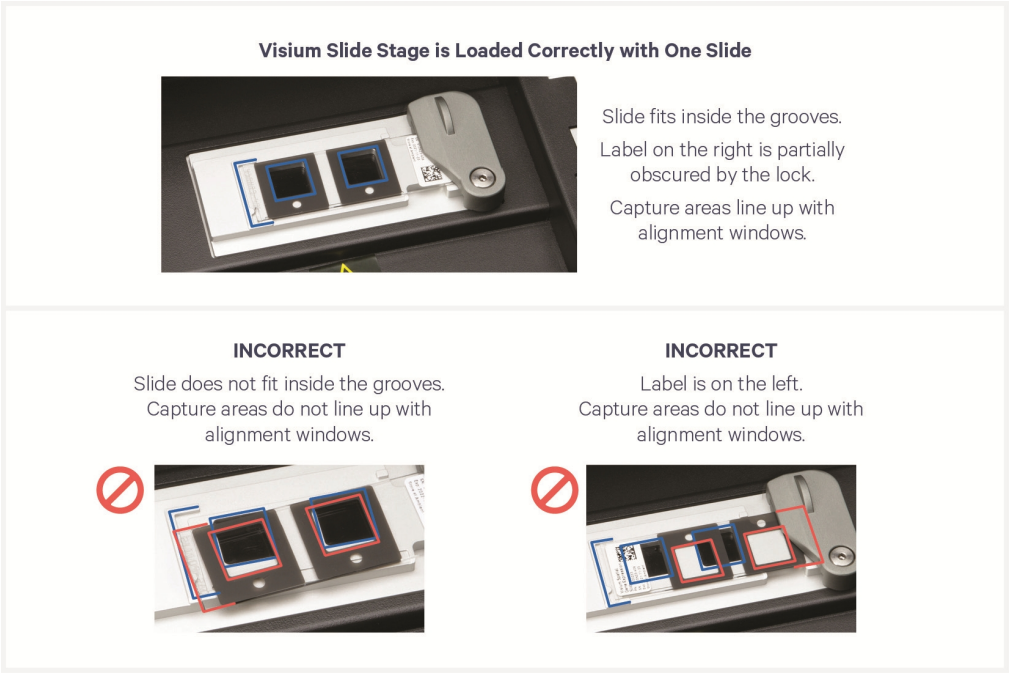
a. Open the Visium Slide Lock by using one finger to pivot.

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

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



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



10x GENOMICS

Sample Preparation & Staining Guidelines

Sample Preparation

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

Before performing the protocol steps in this User Guide, ensure that all samples have followed the appropriate documentation as described in the Workflow Overview on page 19. Listed below are key considerations described in the tissue preparation documentation.

Key Considerations for FFPE Samples	
FFPE Tissue Sectioning & Section Placement	
☐	Assess RNA quality of the FFPE tissue block or from archived sections.
☐	Practice sectioning and section placement with a nonexperimental block before placing sections on slides.
☐	Section the tissue block and place sections on compatible blank slides using a water bath.
☐	Place tissue sections on the allowable area on the slide. Allowable area may vary depending on slide choice. See CytAssist Tested Slides for more information.
Tissue Slide Handling	
☐	Ensure slides are completely dried after tissue placement.
☐	Store the slides containing FFPE sections for up to two weeks in a desiccator at room temperature.

Key Considerations for FF Samples	
Freezing and Embedding	
☐	Snap freeze samples in a bath of isopentane and liquid nitrogen.
☐	Embed frozen samples in OCT.
☐	Store frozen samples in a sealed container at -80°C for long-term storage.
Slide Handling	
☐	Equilibrate slides to cryostat temperature before cryosectioning.
Cryosectioning	
☐	Practice sectioning and section placement with a nonexperimental block before placing sections on slides.
☐	Equilibrate OCT tissue block to the cryostat chamber temperature for 30 min.
☐	Section the tissue block and place sections on compatible blank slides.
☐	Assess RNA quality of the tissue block.
Tissue Slide Handling	
☐	Maintain slides containing sections in a low moisture environment.
☐	Keep slides cold and transport slides on dry ice.
☐	Store tissue slides in a slide mailer at -80°C for up to two months.

Key Considerations for FxF Samples	
Fixation, Embedding, and Freezing	
☐	Fix samples in 4% PFA or formaldehyde
☐	Cryopreserve samples in a sucrose gradient.
☐	Embed fixed samples in OCT and freeze.
Sample Block Storage	
☐	Store frozen samples in a sealed container at -80°C for long-term storage.
Slide Handling	
☐	Equilibrate slides to cryostat temperature before cryosectioning.
Cryosectioning	
☐	Assess RNA quality of the tissue block.
☐	Practice sectioning and section placement with a nonexperimental block before placing sections on slides.
☐	Equilibrate OCT tissue block to the cryostat chamber temperature for 30 min.
☐	Section the tissue block and place sections on compatible blank slides.
Tissue Slide Handling	
☐	Maintain slides containing sections in a low moisture environment.
☐	Keep slides cold and transport slides on dry ice.
☐	Store tissue slides in a slide mailer at -80°C for up to two months.

Step 1:

Probe Hybridization

1.0 Get Started

Each 10x Genomics reagent tube is enough for two 6.5 mm Visium Slides (four Tissue Slides) or one 11 mm Visium slide. Number of reactions in reagent preparation tables refers to the number of Tissue Slides.

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐ ○	FFPE Hyb Buffer	Tube: 2000423 Kit: 1000466	Thaw at room temperature. If precipitate persists, heat at 37°C until dissolved. Avoid vortexing to prevent bubble formation. Pipette mix 10x. Keep the buffer at room temperature after thawing and while performing the workflow.	–20°C
Place on ice				
☐ ●	Human WT Probes v2 - RHS	Tube: 2000657 Kit: 1000466	Thaw at room temperature. Vortex and centrifuge briefly. Maintain on ice.	–20°C
☐ ●	Human WT Probes v2 - LHS	Tube: 2000658 Kit: 1000466	Thaw at room temperature. Vortex and centrifuge briefly. Maintain on ice.	–20°C
☐ ●	Mouse WT Probes - RHS	Tube: 2000455 Kit: 1000365	Thaw at room temperature. Vortex and centrifuge briefly. Maintain on ice.	–20°C
☐ ●	Mouse WT Probes - LHS	Tube: 2000456 Kit: 1000365	Thaw at room temperature. Vortex and centrifuge briefly. Maintain on ice.	–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	Component: 2000284 Kit: 1000519/1000518	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 stained according to the appropriate protocol. See Workflow Overview on page 19 for more information.

TIPS

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

■ *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. Vortex and centrifuge briefly. Maintain at **room temperature**.

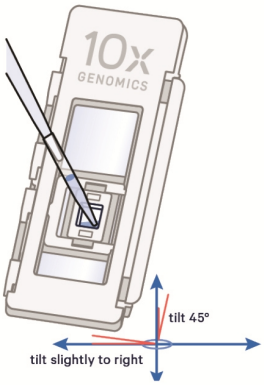
6.5 mm Gaskets Pre-Hybridization Mix	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
Nuclease-free Water	-	89.5	196.9	393.8
10X PBS, pH 7.4	-	10.0	22.0	44.0
10% Tween-20	-	0.5	1.1	2.2
Total	**-**	**100.0**	**220.0**	**440.0**

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 Cassettes containing H&E stained or IF stained sections.

c. Peel back Visium Slide Seals.

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



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

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

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

TIPS

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

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

Lid Temperature	Reaction Volume	Run Time
Hybridization	50°C	Overnight (16 - 24 h)
Post Hybridization Wash	50°C	Hold

i. 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)
	FFPE Hyb Buffer	2000423	70.0	154.0	308.0
	Nuclease-free Water	-	10.0	22.0	44.0
●	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)
	FFPE Hyb Buffer	2000423	140.1	308.2	616.4
	Nuclease-free Water	-	19.9	43.8	87.6
●	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**	**880.0**

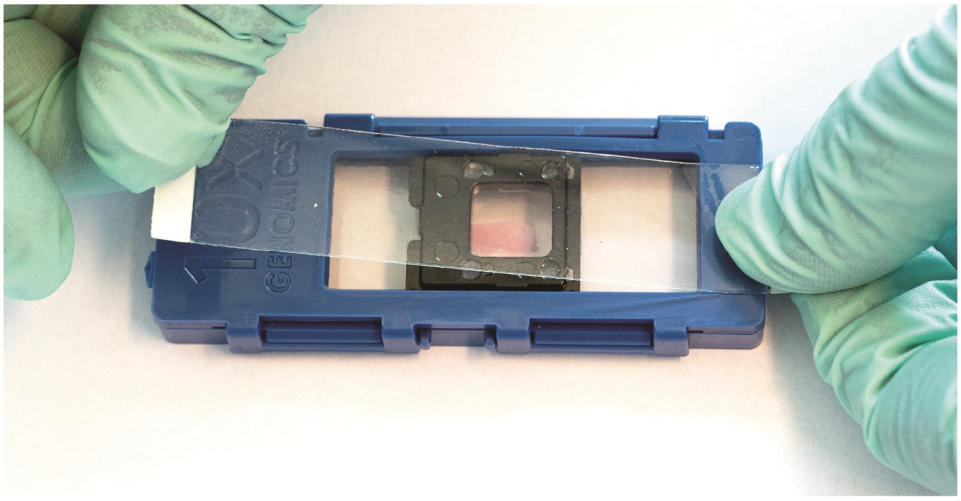
j. Remove Visium Slide Seals from Tissue Slide Cassettes.

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

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

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

Apply Visium Slide Seal



n. Skip Pre-equilibrate step to initiate Hybridization.

Step 2:

Probe Ligation

2.0 Get Started

Each 10x Genomics reagent tube is enough for two 6.5 mm Visium Slides (four Tissue Slides) or one 11 mm Visium slide. Number of reactions in reagent preparation tables refers to the number of Tissue Slides.

Items		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐ ◯	FFPE Post-Hyb Wash Buffer	Tube: 2000424 Kit: 1000466	Thaw at room temperature. If precipitate persists, heat at 37°C until dissolved. Vortex briefly. Flick tube until liquid settles at the bottom of the tube.	–20°C
☐ ◯	2X Probe Ligation Buffer	Tube: 2000445 Kit: 1000466	Thaw at room temperature until no precipitate remains. Vortex and centrifuge briefly.	–20°C
☐ ◯	Post-Ligation Wash Buffer	Tube: 2000419 Kit: 1000466	Thaw at room temperature. If precipitate persists, heat at 37°C until dissolved. Vortex.	–20°C
Place on ice				
☐ ◯	Probe Ligation Enzyme	Tube: 2000425 Kit: 1000466	Centrifuge briefly. Maintain on ice.	–20°C
Obtain				
☐	Nuclease-free Water	-	-	Ambient
☐	20X SSC Buffer	-	-	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** in a water bath or thermomixer. Maintain pre-heated wash buffer at **50°C** throughout washes.

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.

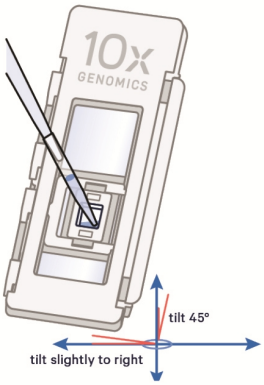
6.5 mm Gaskets SSC Buffer	Stock	Final	1X (µl)	2X +10% (µl)	4X +10% (µl)
SSC	20X	2X	356	781	1,562
Nuclease-free Water	-	-	3,195	7,029	14,058
Total	**-**		**3,550**	**7,810**	**15,620**

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**	**18,040**

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

TIPS

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



e. **Immediately** add ■ **150 µl** 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 each Tissue Slide Cassette and place on Low Profile Thermocycler Adapter on pre-heated thermal cycler. Close thermal cycler lid. See Tips & Best Practices for more information on cassette incubation on a thermal cycler.

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

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

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

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

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

k. **Immediately** add ■**150 µl** 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.

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

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

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

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

p. Add ■ **150 µl** or ▲ **300 µl** 2X SSC Buffer to each well and re-apply Visium Slide Seal on each Tissue Slide Cassette.

q. 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 does not enable 37°C)	100 µl	1 h
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-equilibrate	37°C	Hold
Ligation	37°C	01:00:00
Hold	4°C	Hold

b. Prepare Probe Ligation Mix according to the appropriate table shortly before use. Add reagents in the order listed. Pipette mix 10x and centrifuge briefly. DO NOT vortex. 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 Visium Slide Seals from each Tissue Slide Cassette and remove all 2X SSC Buffer from each well.

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

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

f. Skip Pre-equilibrate step to initiate Ligation.

2.3 Post-Ligation Wash

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

Use room temperature Post-Ligation Wash Buffer at first wash step (step 2.3e). Use pre-heated Post-Ligation Wash Buffer at 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 Cassettes from the Low Profile Thermocycler Adapter and place on a flat, clean work surface.

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

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

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

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.

TIPS

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

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

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

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

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

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

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

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

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

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

o. Add ■ **150 µl** or ▲ **300 µl** 2X SSC Buffer prepared in 2.1 Post-Hybridization Wash on page 68 to each well.

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

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

r. Re-apply Visium Slide Seals on each 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

3.0 Get Started

Each 10x Genomics reagent tube is enough for two 6.5 mm Visium Slides (four tissue slides) or one 11 mm Visium slide. Number of reactions in reagent preparation tables refers to the number of Tissue Slides. After instrument run, number of reactions refers to the number of wells in a Visium Cassette.

Ensure that the Visium CytAssist is powered on, is clean, and ready to perform an experimental run.

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	Tube: 2000409 Kit: 1000436	Thaw at room temperature, vortex, centrifuge briefly.	–20°C
☐	Tissue Removal Enzyme	Tube: 3000387 Kit: 1000436	Pipette mix, centrifuge briefly. Maintain at room temperature until ready to use. Tissue Removal Enzyme is added to the Probe Release Mix immediately before running the CytAssist instrument.	–20°C
Place on ice				
☐	RNase Enzyme	Tube: 3000593 Kit: 1000436	Pipette mix, centrifuge briefly. Maintain on ice until ready to use.	–20°C
☐	Extension Enzyme	Tube: 2000389 Kit: 1000436	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
☐	10X PBS	-	Use 10X PBS stock to prepare 1X PBS.	Ambient

Items		10x PN	Preparation & Handling	Storage
☐	20X SSC Buffer	-	-	Ambient
☐	2X SSC Buffer	-	Prepared in 2.1 Post-Hybridization Wash on page 68.	Ambient
☐	8 M KOH Solution	-	Manufacturer's recommendations.	Ambient
☐	Visium Cassette	Component: 3000811/ 3000812 Kit: 1000519/1000518	See Tips & Best Practices.	Ambient
☐	Visium Slide Seals	Component: 2000284 Kit: 1000519/1000518	See Tips & Best Practices.	Ambient

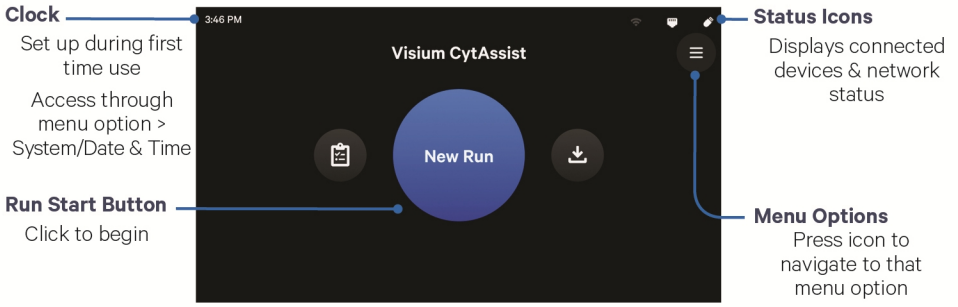
3.1 CytAssist-Enabled RNA Digestion & Tissue Removal

■ *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. Avoid generating bubbles. DO NOT vortex. 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 1X PBS. Maintain at room temperature.

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

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

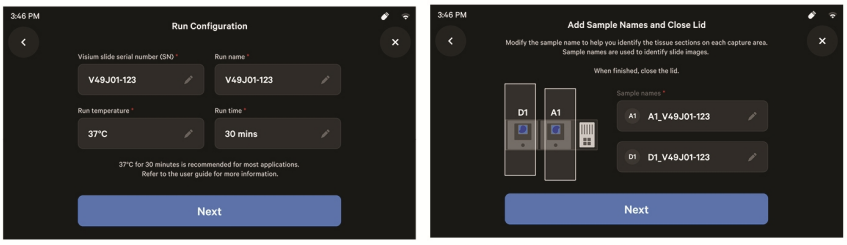
6.5 mm Slides 10% Eosin	10x PN	1X (µl)	2X +10% (µl)	4X +10% (µl)
Alcoholic Eosin	-	15	33	66
1X PBS	-	135	297	594
Total	**-**	**150**	**330**	**660**

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**

e. Press blue New Run Button on touchscreen to initiate run.

f. 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



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

h. Remove tissue slides from Tissue Slide Cassettes. See Visium CytAssist Tissue Slide Cassette Removal for instructions.

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

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

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

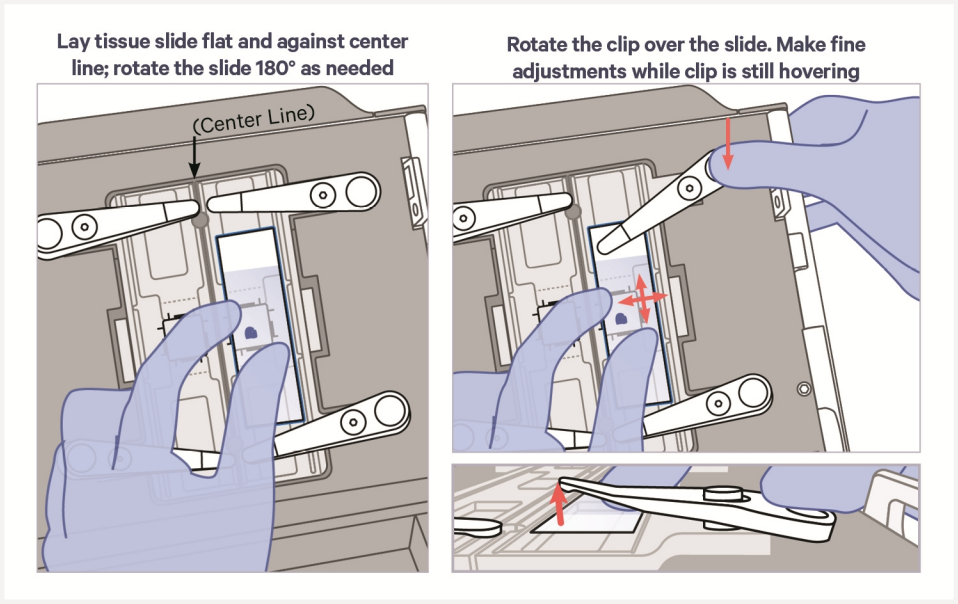
l. While holding the slide over liquid waste container, rinse with **1 ml** 1X PBS. DO NOT pipette directly onto tissue.

DO NOT pipette directly onto tissue.

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

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

o. Wipe back of tissue slides with a laboratory wipe and load into Visium CytAssist. Ensure tissue section is completely dry prior to instrument run. If necessary, repeat slide wafting. See Instrument Loading Guidelines on page 49 for more information.



p. Load Visium CytAssist Spatial Gene Expression Slide against the grooves of Visium Slide Stage and close Visium Slide Lock.

Visium Slide Stage is Loaded Correctly with One Slide

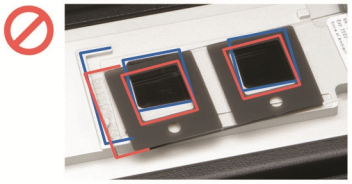
Slide fits inside the grooves.

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.

q. Remove Probe Release Mix from ice.

r. 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. Avoid generating bubbles. Centrifuge for **5 sec**.

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

s. Slowly dispense **25 µl** of Probe Release Mix into the center of each spacer well on the Visium CytAssist Spatial Gene Expression Slide, using a fresh pipette tip for each dispense. Do not push plunger beyond first stop to avoid generating bubbles.

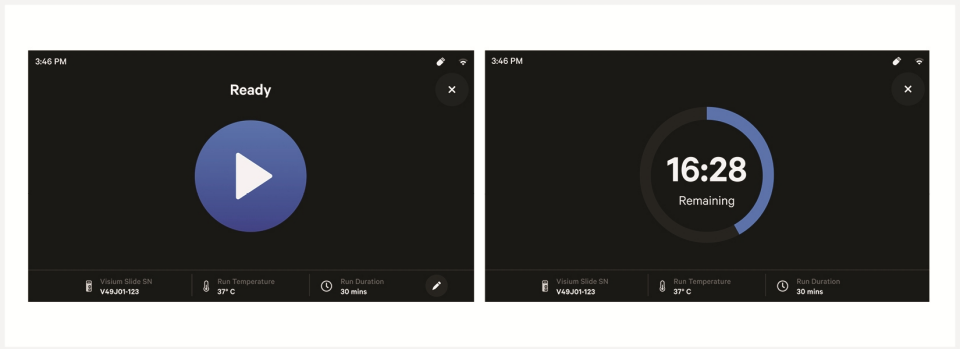
Dispense Reagent

t. Close lid and press Next.

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

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

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



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

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

Lid Temperature	Reaction Volume	Run Time
45°C (lid may be turned off if the instrument does not 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

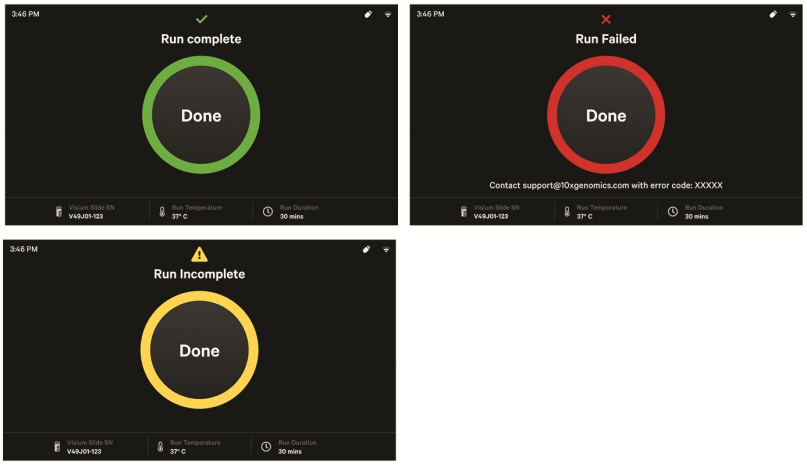
w. Prepare Probe Extension Mix. Pipette mix. Centrifuge briefly. Maintain on ice.

	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**

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

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



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

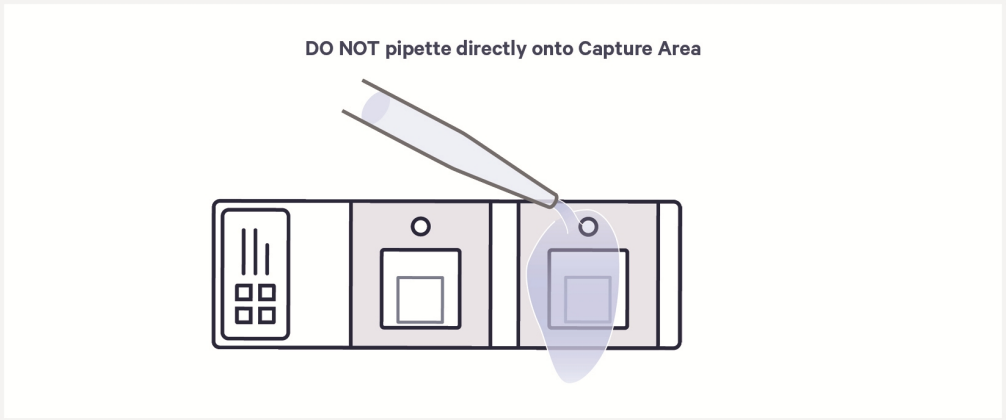
z. Remove Visium CytAssist Spatial Gene Expression Slide. It is normal if tissue remains of tissue slides after run completion.

Leave the CytAssist lid open to facilitate cleaning of the instrument at a safe stopping point.

aa. While holding Visium CytAssist Spatial Gene Expression Slide over liquid waste container, rinse each Capture Area with **1 ml** 2X SSC as shown below.

DO NOT pipette directly onto Capture Areas, which are surrounded by the fiducial frames.

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



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

ac. Place Visium CytAssist Spatial Gene Expression slide in a new 6.5 or 11 mm Visium Cassette.

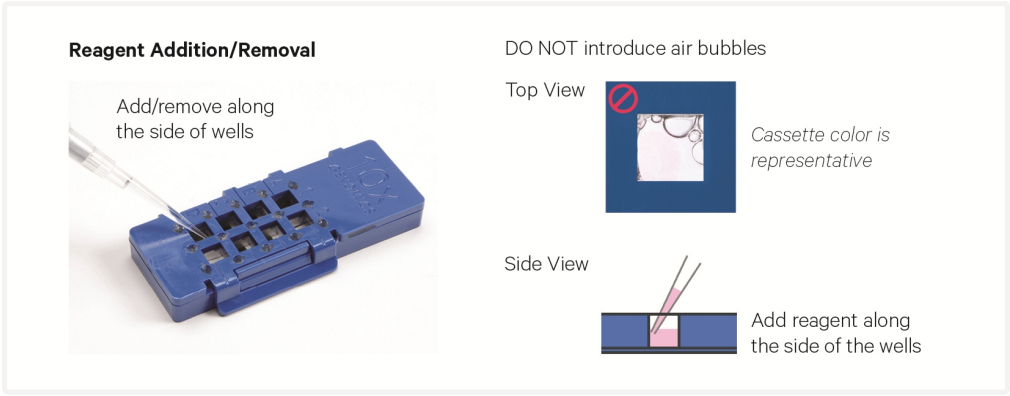
See Visium Cassette section for more information. Some moisture remaining on the Visium CytAssist Spatial Gene Expression slide is normal.

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

3.2 Probe Extension

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

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



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

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

STOP

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

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

3.3 Probe Elution

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

a. Prepare fresh 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**. Discard unused KOH Mix after use.

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

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

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

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

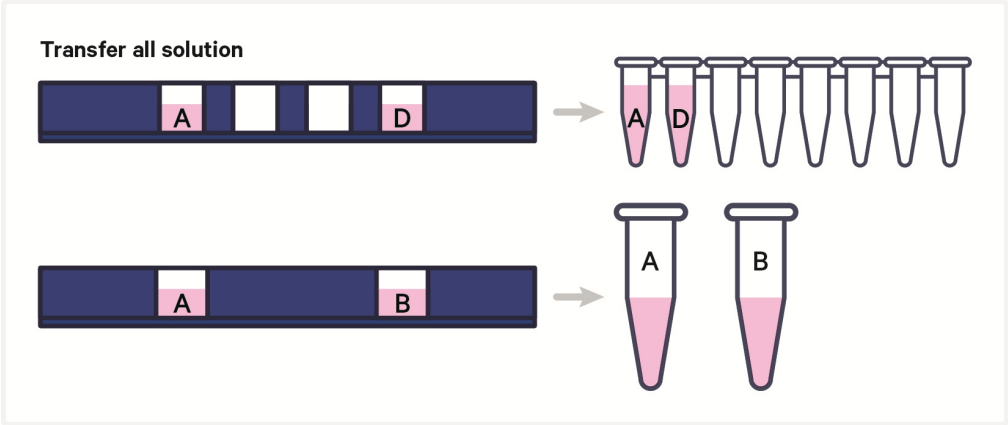
d. Add ■ **100 µl** or ▲ **200 µl** 2X SSC Buffer prepared in 2.1 Post-Hybridization Wash on page 68 to each well (A1 and D1 if using a 6.5 mm cassette).

e. Remove all 2X SSC Buffer from 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.

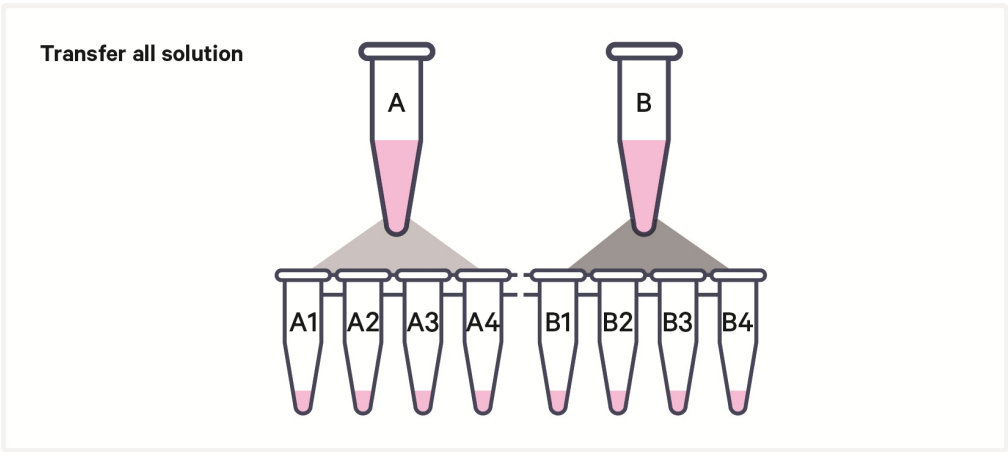
g. Incubate at **room temperature** for **10 min**.

h. Transfer all solution for each sample containing the probes 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. *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. Failure to neutralize may result in a loss of signal and lower library complexity.

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.



10x GENOMICS

Step 4:

Pre-Amplification and SPRIselect

4.0 Get Started

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

Item		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	TS Primer Mix B	Tube: 2000537 Kit: 1000436	Thaw at room temperature, vortex, and centrifuge briefly.	–20°C
☐	Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendations.	-
Place on ice				
☐	Amp Mix B	Tube: 2000567 Kit: 1000436	Vortex, centrifuge briefly.	–20°C
Obtain				
☐	Qiagen Buffer EB	-		Ambient
☐	10x Magnetic Separator	Component: 2001212 Kit: 1000499	See Tips & Best Practices.	Ambient
☐	80% Ethanol	-	Prepare fresh. Prepare 1 ml per reaction. Store at room temperature.	

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

4.1 Pre-Amplification

If working with samples from 11 mm slides, 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)
	Nuclease-free Water		19.5	42.9	85.8
	Amp Mix B	2000567	25.0	55.0	110.0
●	TS Primer Mix B	2000537	2.5	5.5	11.0
	Total	**-**	**47.0**	**103.4**	**206.8**

	11 mm Slides Pre-Amplification Mix	PN	1X (µl)	2X + 10% (µl)	4X + 10% (µl)
	Nuclease-free Water		78.0	171.6	343.2
	Amp Mix B	2000567	100.0	220.0	440.0
●	TS Primer Mix B	2000537	10.0	22.0	44.0
	Total	**-**	**188.0**	**413.6**	**827.2**

b. Add **47 µl** Pre-Amplification Mix to each tube from 3.3 Probe Elution on page 87 (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	~25 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:03:00
2	98°C	00:00:15
3	63°C	00:00:20
4	72°C	00:00:30
5	Go to Step 2, repeat 9X for a total of 10 cycles	
6	72°C	00:01:00
7	4°C	Hold

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 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 130 µl).

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

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

d. Remove supernatant.

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

f. Remove ethanol.

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

h. Remove ethanol.

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

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

k. Remove from magnet. Add ■ **105 µl** 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 tube strip on magnet •**High** for samples from 6.5 mm slides or •**Low** for samples from 11 mm slides until 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.

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.

10x GENOMICS

Step 5:

Visium CytAssist Spatial Gene Expression – Probe-based Library Construction

5.0 Get Started

Item		10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐ ●	TS Primer Mix B	Tube: 2000537 Kit: 1000436	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	Tube: 3000511 Kit: 1000251	Thaw at room temperature, vortex, and centrifuge briefly.	–20°C
☐	Beckman Coulter SPRIselect Reagent	-	Manufacturer's recommendations.	-
☐	Agilent TapeStation Screen Tape and Reagents If used for QC		Manufacturer's recommendations.	-
☐	Agilent Bioanalyzer High Sensitivity DNA kit If used for QC	-	Manufacturer's recommendations.	-
Place on ice				
☐ ○	Amp Mix B	Tube: 2000567 Kit: 1000436	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	Component: 2001212 Kit: 1000499	See Tips & Best Practices.	Ambient
☐	80% Ethanol	-	Prepare fresh. Prepare 400 µl per reaction.	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 one negative control					

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

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

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

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

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.

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

Lid Temperature	Reaction Volume	Run Time
105°C	10 µl	35 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:03:00
2	98°C	00:00:05

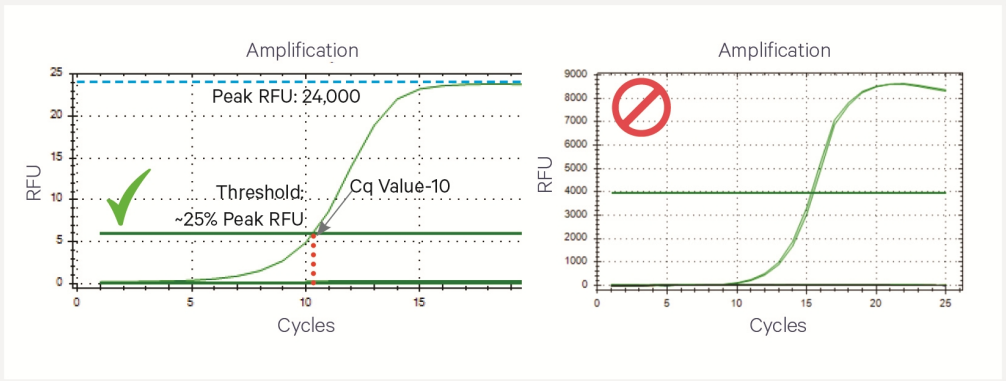
Lid Temperature	Reaction Volume	Run Time
3	63°C	00:00:30
	Read signal	
4	Go to step 2, 29x (total of 30 cycles)	-

g. Record the Cq value for each sample.

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

If the Cq value is > 20 cycles , contact support@10xgenomics.com before proceeding.

Representative qPCR Amplification Plots



5.2 Sample Index PCR

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

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

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

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

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

e. Add **5 µl** of an individual Dual Index Plate 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 (examples below). Samples within ±1 cycle numbers can be combined in a single SI-PCR run at the higher cycle number.

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

Example Cycle Numbers

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

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

5.3 Post-Sample Index PCR Cleanup – SPRIselect

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

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

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

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

d. Remove supernatant.

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

f. Remove ethanol.

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

h. Remove ethanol.

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

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

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

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

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

n. Transfer **25 µl** sample to a new tube strip on ice.

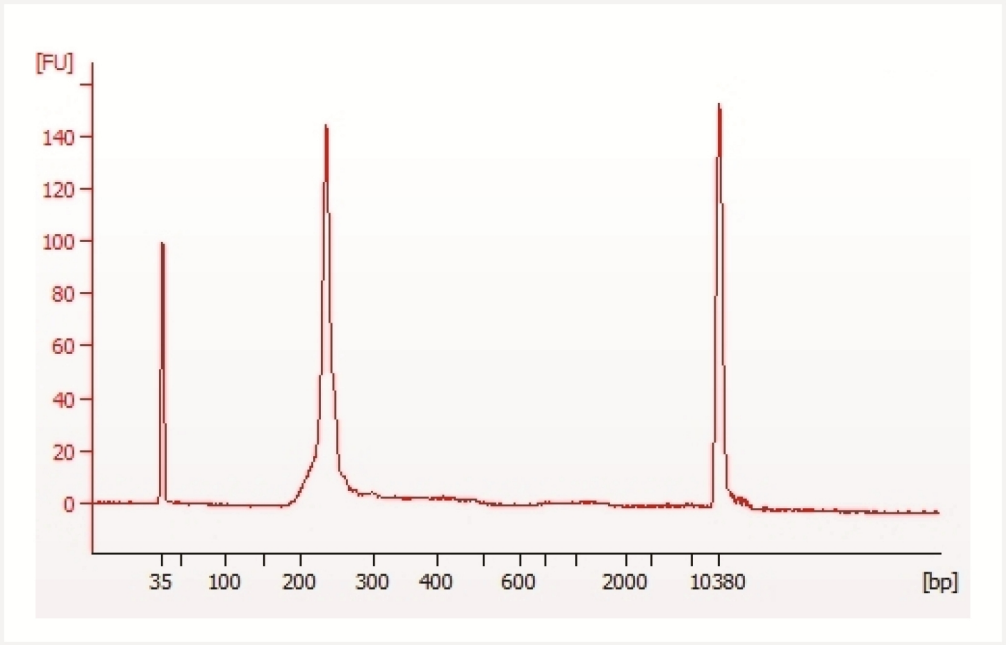
o. Store at **-20°C** for **long-term** storage.

5.4 Post-Library Construction QC

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

b. Run **1 µl** of diluted sample on an Agilent Bioanalyzer High Sensitivity chip. If peak is too small or flat, retry with a lower dilution. See Troubleshooting on page 108 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 Methods:

- Agilent TapeStation
- LabChip

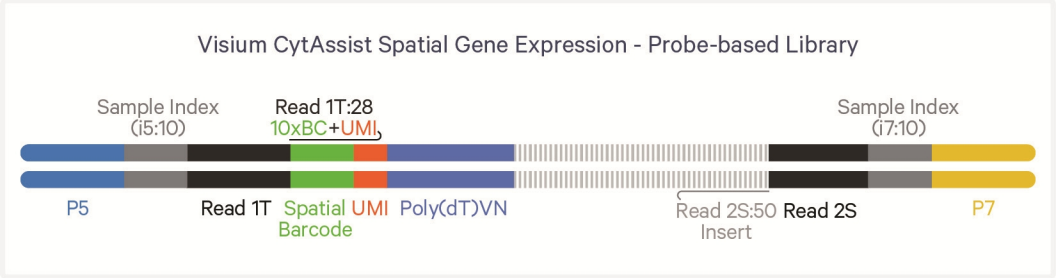
See Appendix on page 123 for representative traces

See Post Library Construction Quantification on page 128

Sequencing

Sequencing Libraries

Libraries contain 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 (Read 1T), while i7 and i5 sample index sequences are incorporated as the index read. Read 1T, and Small RNA Read 2 (Read 2S) are standard Illumina sequencing primer sites used in paired-end sequencing. Read 1T are used to sequence the 16 bp Spatial Barcode and 12 bp UMI. Read 2S is used to sequence the Ligated Probe Insert. Sequencing these libraries produces a standard Illumina BCL data output folder.



Sequencing Depth

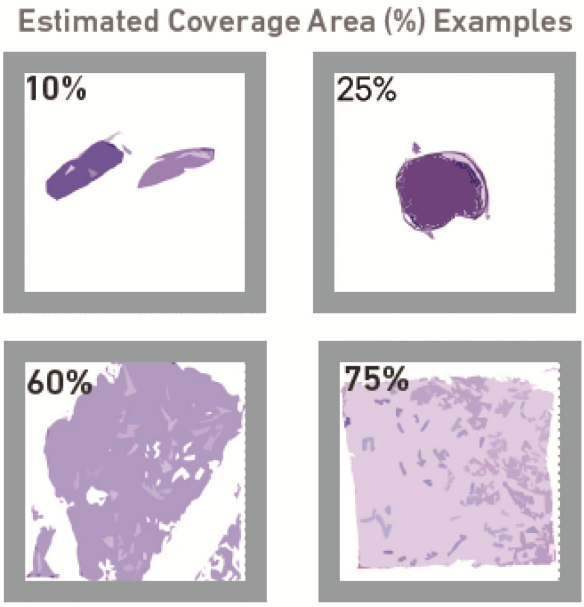
Calculating sequencing depth requires estimating the approximate Capture Area (%) covered by tissue. This may be performed visually or by using the Visium Manual Alignment Wizard in Loupe Browser for a more accurate measurement. See examples below for estimating coverage area visually. If using Loupe 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.

Visium CytAssist Spatial Gene Expression - Probe-based Library
Paired-end, dual indexed sequencing
Read 1T: 28 cycles i7 Index: 10 cycles i5 Index: 10 cycles Read 2S: 50 cycles*

Visium CytAssist Spatial Gene Expression - Probe-based libraries may be pooled for sequencing, taking into account the differences in tissue-covered spot on a Capture Area and per-spot read depth requirements between each library. 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.

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.

- MiSeq
- NextSeq 500/550

- NextSeq 1000/2000
- NovaSeq 6000
- NovaSeq X Plus
- 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 Plate TS Set A plate well ID, SI-TS-) is needed in the sample sheet used for generating FASTQs with "spaceranger mkfastq". Samples utilizing the same sample index should not be pooled together or run on the same flow cell lane, as this would not enable correct sample demultiplexing.

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

Library Loading

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

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

Instrument	Loading Concentration (pM)	PhiX (%)
MiSeq	11	1
NextSeq 500/550	1.8	1
NextSeq 1000/2000	650	1
NovaSeq 6000 Standard	100-150	1
NovaSeq 6000 XP Workflow	150-200	1
NovaSeq X Plus	300-400	1
iSeq	150	1

Sequencing Metrics

The representative metrics presented below were derived from a pool of eleven H&E-stained human tonsil tissue samples. FFPE samples were processed according to documentation listed in the Workflow Overview on page 19. Libraries were pooled and sequenced at the indicated run parameters to generate the % Base and % ≥Q30 plots shown in the following figures. These same libraries were used to illustrate sequence compatibility. All libraries were quantified with the KAPA DNA Quantification Kit and sequenced with 1% PhiX. Though only FFPE libraries are shown in this section, libraries from fresh frozen (FF) and fixed frozen (FxF) tissue sections are expected to perform similarly. All libraries followed the following sequencing configuration and run parameters:

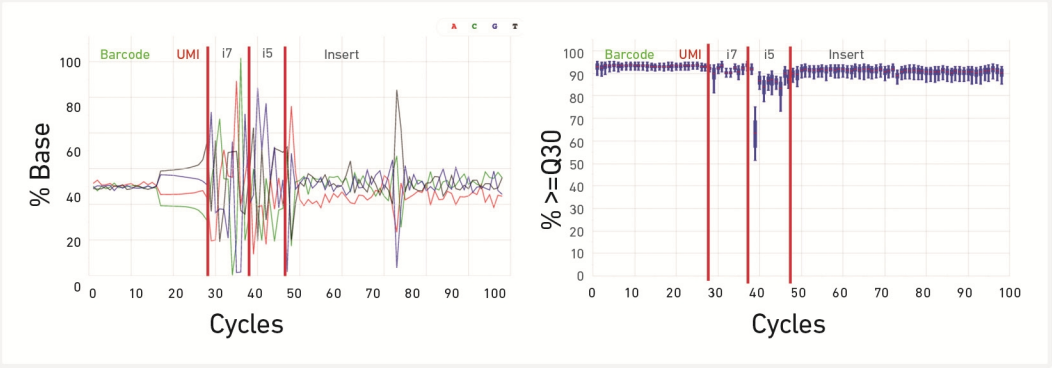
- Targeted sequencing depth: 25,000 read pairs per tissue-covered spot.
- Paired-end, dual indexing: Read 1: 28 cycles; i7 Index: 10 cycles; i5 Index: 10 cycles; Read 2: 50 cycles

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

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

Probe-based Libraries

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



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

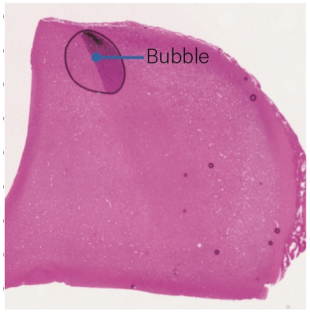
Instr.	Load Conc. (pM)	% Occupancy	%PF	R1	R2	R1	i7	i5	R2	GEX
				Yield per Lane (Gb)		% ≥Q30				Mapped Reads (%)
NovaSeq 6000	300	98	70	24.4	44.3	93.2	91.7	83.7	91.1	97.9

Troubleshooting

Before CytAssist Instrument Run

1. Bubbles during Coverslipping

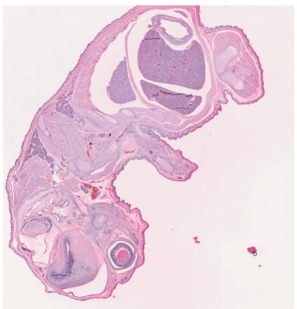
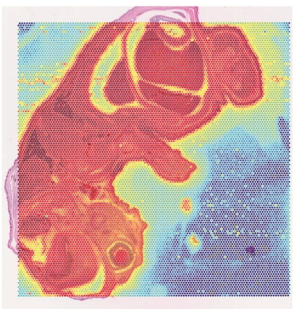
A bubble could be generated during coverslipping.

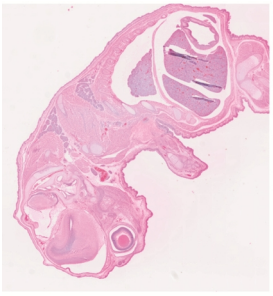
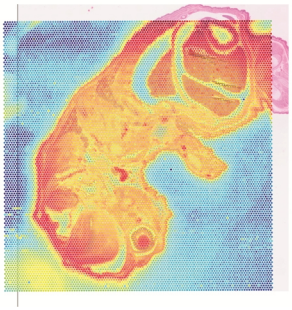
Bubbles may cause blackening of tissue
H&E Stain


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.

2. Tissue Thickness Outside of Specifications

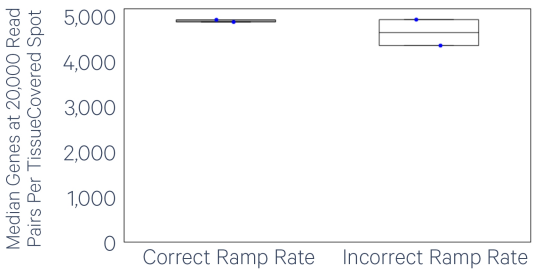
FFPE tissue sections should be between 3 and 10 µm. FF and FxF tissue sections should be between 10 and 20 µm. Sections outside these specifications may result in reduced performance.

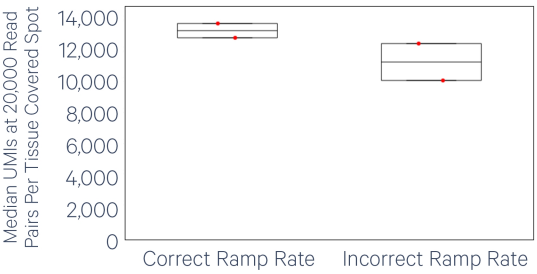
Thin Sections May Lead to Decreased Sensitivity	
H&E Stained Tissue at Recommended Thickness	**Mean Panel UMI Count at 20,000 Raw Reads per Spot: 14,645**
	

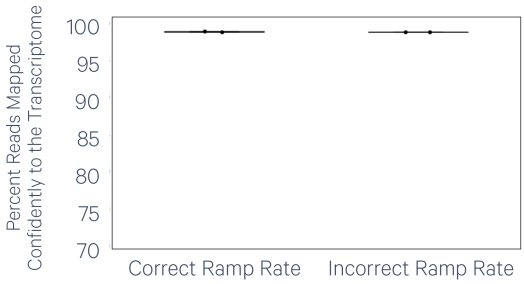
Thin Sections May Lead to Decreased Sensitivity	
H&E Stained Tissue Below Recommended Thickness	**Mean Panel UMI Count at 20,000 Raw Reads per Spot: 8,511**
	

3. Incorrect Thermal Cycler Ramp Rate

Incorrect thermal cycler ramp rates negatively impact assay sensitivity (mean UMI counts and mean genes per tissue-covered spot), but has no impact on assay quality (reads mapped confidently to the probe set). These data show that using a Biometra TAdvanced thermal cycler at the incorrect ramp rate (8°C/sec heating and cooling) vs. the correct ramp rate (2°C/sec heating and cooling) negatively impacts assay sensitivity. Use the recommended ramp rates as described in Recommended Thermal Cyclers on page 12.







4. Number of Washes

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

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

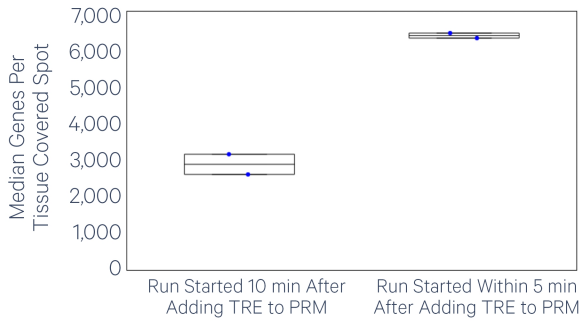
5. Incorrect Visium Slide Loading

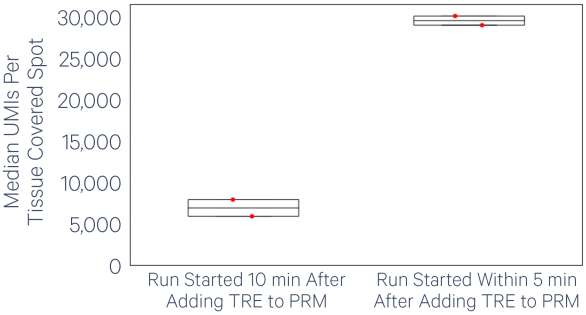
Loading the Visium Slide incorrectly onto the Visium Slide Stage may result in slide breakage. Ensure that the slide sits within the grooves of the Visium Slide Stage, with the label facing toward the right. The images below show the Visium Slide loaded correctly and incorrectly.

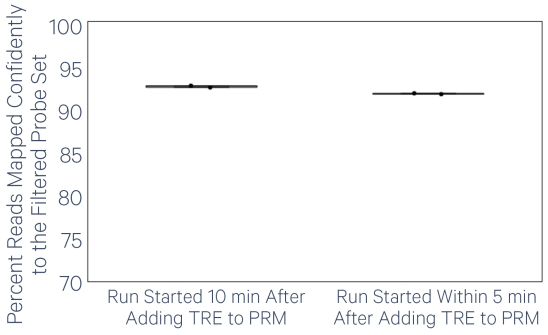
In the event of slide breakage, remove all traces of broken glass to avoid damaging the instrument. Exercise caution when removing glass to prevent injury.

6. Delayed Addition of Tissue Removal Enzyme to Probe Release Mix

While the effect of a delay in adding Tissue Removal Enzyme (TRE) to the Probe Release Mix (PRM) longer than ten minutes is unknown, a short delay (up to ten minutes) is shown to negatively impact assay sensitivity (median UMI counts and median genes per tissue-covered spot), but has no impact on assay quality (percent reads mapped confidently to the probe set). 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**.


Median Genes Per Tissue Covered Spot
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
Run Started 10 min After Adding TRE to PRM
Run Started Within 5 min After Adding TRE to PRM

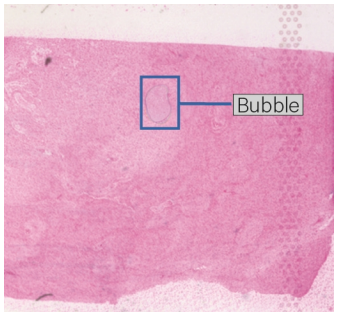
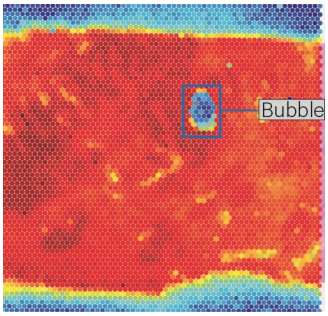

Median UMIs Per Tissue Covered Spot
30,000
25,000
20,000
15,000
10,000
5,000
0
Run Started 10 min After Adding TRE to PRM
Run Started Within 5 min After Adding TRE to PRM


Percent Reads Mapped Confidently to the Filtered Probe Set
100
95
90
85
80
75
70
Run Started 10 min After Adding TRE to PRM
Run Started Within 5 min After Adding TRE to PRM

During CytAssist Instrument Run

1. Bubbles Trapped During Visium CytAssist Run

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

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

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

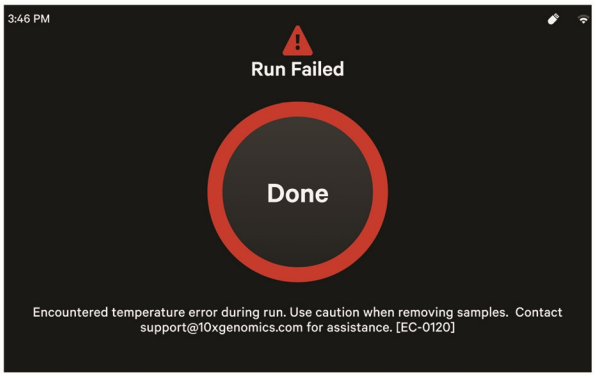
Avoid generating bubbles during reagent dispensing by pipetting slowly and avoiding expelling air from the pipette tip. Additionally, ensure that the frosted area of the tissue slides are outside of the alignment guide lines on the Tissue Slide Stage and that tissue slides are compatible with the CytAssist instrument.

2. Reagent Flow Failure

Inappropriate flow of reagents during a Visium CytAssist experiment run may result in transcript mislocation. If the UMI map appears abnormal (complete loss of UMIs from a significant portion of the tissue), contact support@10xgenomics.com.

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

3. Visium CytAssist Overheating



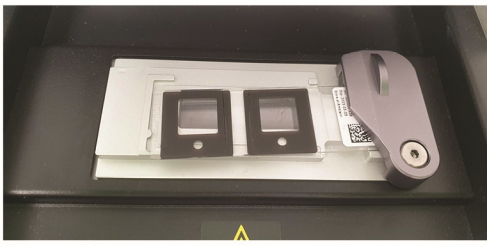
If the Visium CytAssist instrument overheats during an experiment run, the run will fail. A warning message will appear instructing users to carefully remove slides and contact support@10xgenomics.com.

After CytAssist Instrument Run

1. Slide Popped Off of Visium Slide Stage

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

Visium Slide Popped out of the Grooves



2. Visium CytAssist Slide Removal Delayed

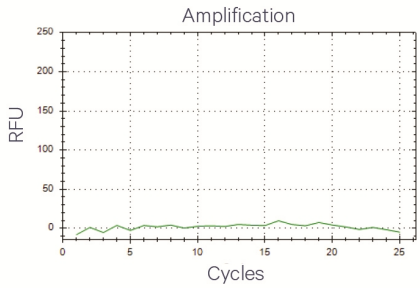
A delay in removing and processing the Visium CytAssist Spatial Gene Expression Slide after run completion may impact data quality. Immediately proceed with slide washes while adjacent to the instrument.

3. No qPCR Amplification

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

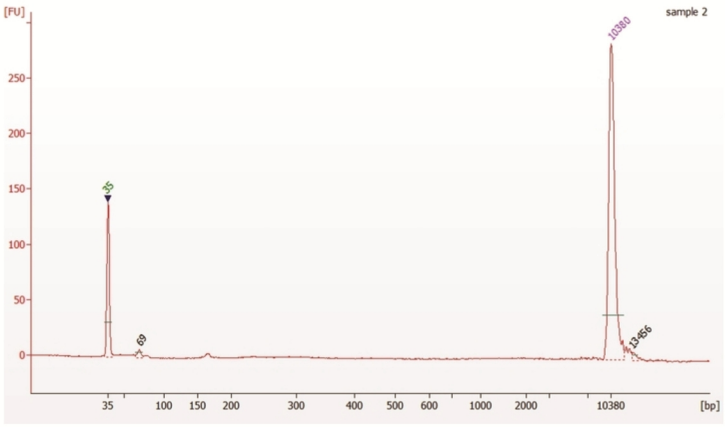
- Incorrect qPCR machine programming (i.e. detecting a fluorophore other than SYBR Green)
- Issues with pipetting small volumes
- Failure to neutralize KOH
- Failure to add primers to pre-amp or qPCR mix
- Unusually low recovery from tissue due to high percentage of connective tissue in Capture Area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

- Leakage from the cassette during workflow



4. Flat Line in Bioanalyzer Library Trace

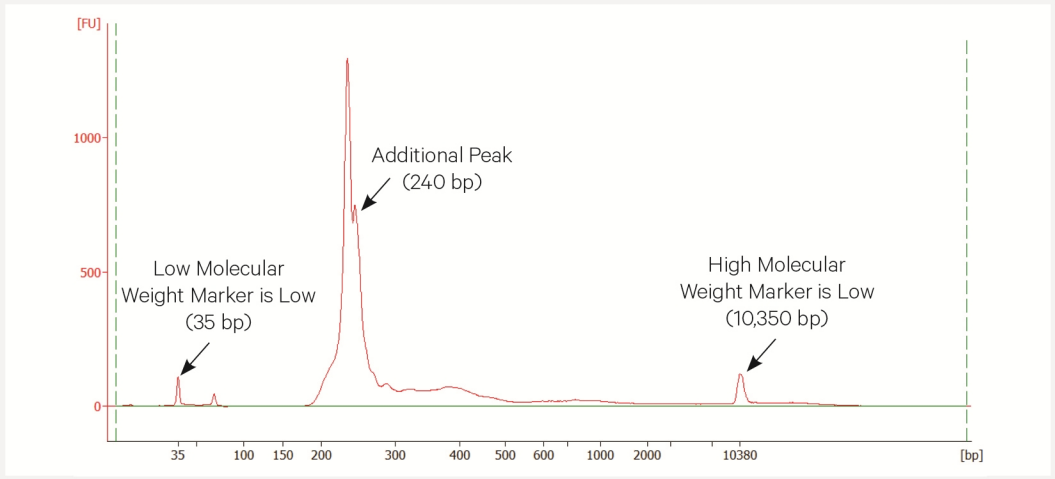
A normal qPCR output but no peak visible in the Bioanalyzer trace may be due to a mistake in the SI-PCR step, errors during SPRIselect cleanup, an overdilution of the final library, Bioanalyzer issue, or thermal cycler failure.



5. Overloaded or Overamplified Trace

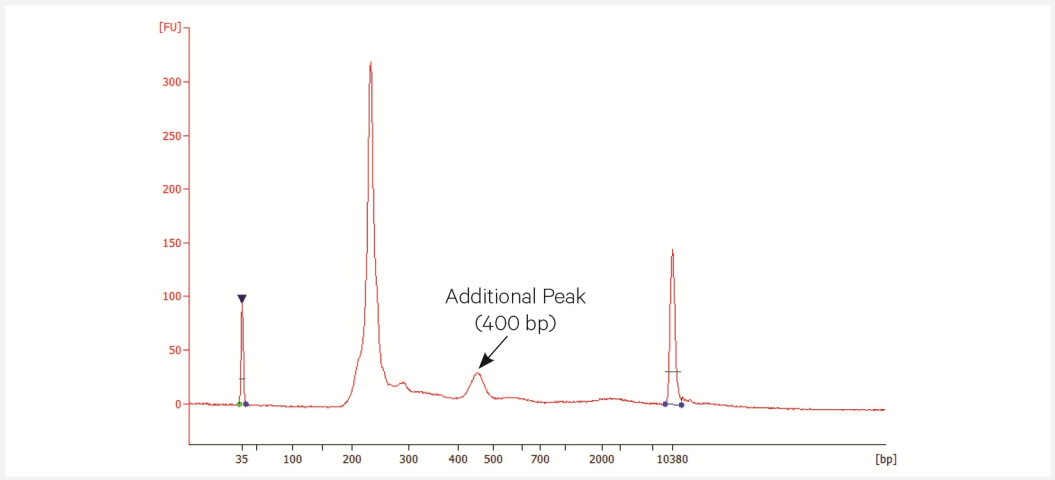
Overloaded Trace

The image below is an example of an overloaded trace. Note the double peak at around 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 in between.

For example, a sample with Cq 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.

5. Drop in Read 2 Q30 Score

A drop in Read 2 Q30 Score may be due to an improper sequencing configuration. The example data below shows the effect of modifying the 10x Genomics recommended sequencing configuration (Read 1: 28 cycles; i7 Index: 10 cycles; i5 Index: 10 cycles; Read 2: 50 cycles) for Visium CytAssist Spatial Gene Expression - Probe-based libraries.

In this example, the deviation is due to reading more than 50 bases for Read 2 (in this case, 90). Q30 can be recovered by trimming the read to 50 bp. Space Ranger only considers the first 50 bp of Read 2 in probe-based assays; thus, even without trimming, analysis with Space Ranger would not be impacted by the extra sequencing. If the deviation had been due to reading fewer than 50 bases, impacts to data analysis would be expected.

10x Genomics Recommended Sequencing Configuration (28, 10, 10, 50)

Instr.	Load Conc. (pM)	% Occupancy	%PF	R1	R2	R1	i7	i5	R2	GEX
				Yield per Lane (Gb)		% ≥Q30				Mapped Reads (%)
NovaSeq 6000	300	98	70	24.4	44.3	93.2	91.7	83.7	91.1	97.9

Modified Sequencing Configuration (28, 10, 10, 90)

Instr.	Load Conc. (pM)	% Occupancy	%PF	R1	R2	R1	i7	i5	R2	GEX
				Yield per Lane (Gb)		% ≥Q30				Mapped Reads (%)
NovaSeq 6000	300	95.4	77.5	79.9	263.6	92.3	90.0	74.6*	81.4	98.2

*This library was sequenced alone, which likely resulted in the lower i5 Q30 score.

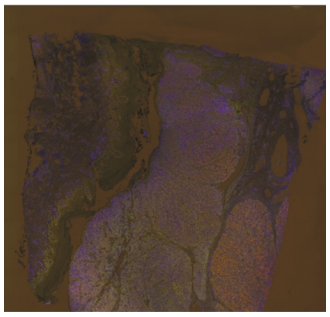
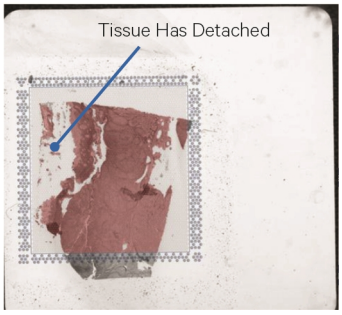
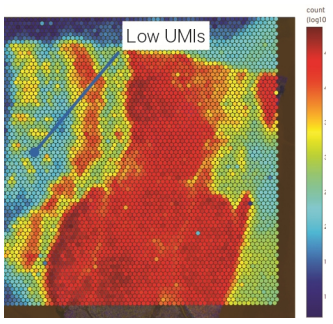
Issues Impacting Tissue Analysis

1. Tissue Detachment and Folding

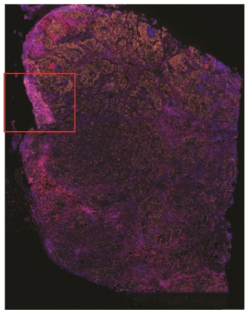
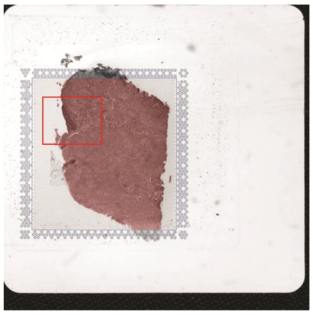
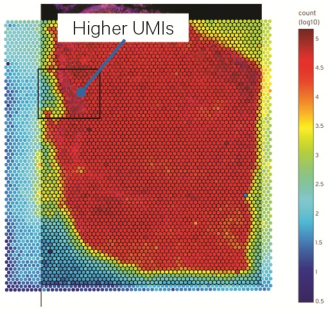
Tissue detachment may result in a lack of Fraction Reads Usable in the region where detachment occurred. If the tissue has folded on itself, this may also cause elevated UMI counts in the overlapping areas or a loss of spatiality due to ineffective permeabilization. Tissue folding may also cause lower UMI capture. Inspect images carefully to identify these areas.

Ensure that slides tested by 10x Genomics were used for tissue placement. For more information, consult the sample preparation documentation described in Workflow Overview on page 19.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
		

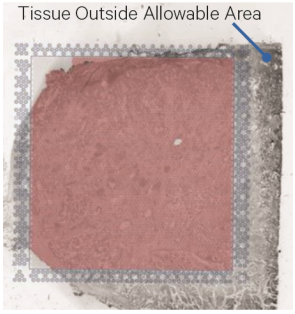
Tissue Folding May Cause Elevated UMI Counts

H&E Stained Tissue	CytAssist Image	UMI Count
		

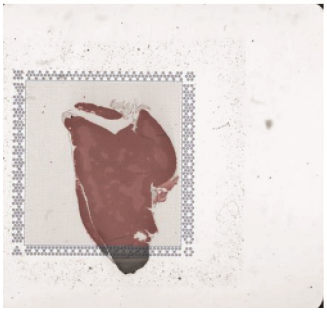
2. Area of Interest Not Within Allowable Area

Area of Interest Outside of Allowable Area is Not Analyzed

Tissue Larger than Capture Area



Tissue Not Properly Aligned

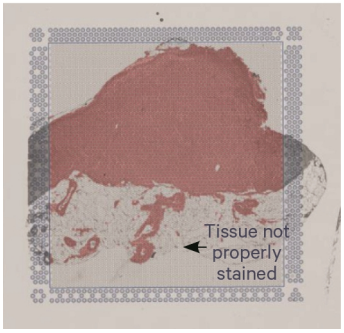


Areas of interest that are not placed within the allowable area on compatible glass slides will not be analyzed. This may occur if the tissue is larger than the Capture Area or if the tissue slide is not properly aligned when loading onto the Tissue Slide Stage. Tissue outside of the allowable area is fine if the tissue outside the allowable area is not an area of interest.

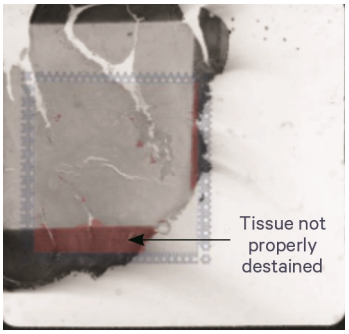
3. Tissue Segmentation Failure due to High Connective Tissue

Examples of Scenarios that Lead to Tissue Segmentation Failure

Inadequate Staining Causes Poor Tissue Segmentation

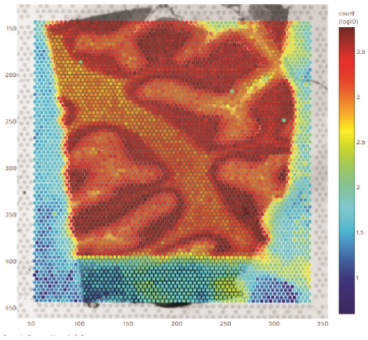


H&E Stained Tissues Outside of Gasket May Not Undergo Destaining



Space Ranger may fail to detect tissue for a variety of reasons, which may necessitate manual fiducial alignment and tissue detection via Loupe Browser. If the contrast between background and tissue is poor (due to inadequate staining, technical error, or tissue composition) 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).

4. Tissue within Capture Area Not Analyzed

Gasket Covers Portion of Tissue within Capture Area	
H&E Stained Tissue	**Tissue Plot with Spots Colored by Clustering**
	

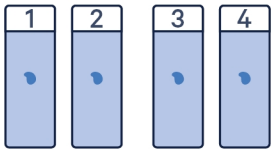
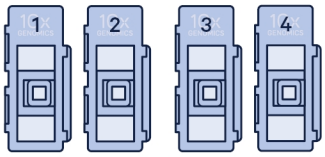
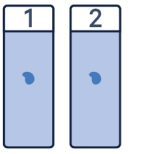
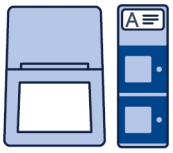
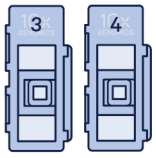
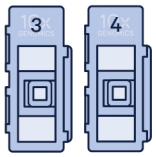
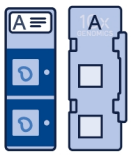
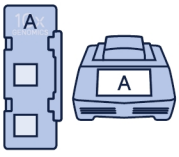
If the gasket covers a portion of the tissue within the Capture Area, that area will not be exposed to the appropriate reagents; thus, probes will not be captured. Gasket obstruction may be caused by improper gasket assembly, resulting in the gasket being placed directly onto areas of interest. In Loupe Browser, manual alignment must be performed to exclude the tissue area obscured by the gasket. Ensure that the area of interest is centered within the gasket.

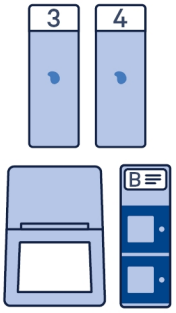
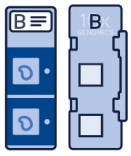
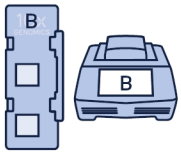
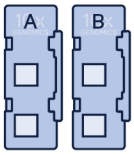
Additionally, tissue placed within the Capture Area may be excluded from the assay workflow due to improper tissue slide alignment when loading the tissue slide into the Visium CytAssist instrument.

Appendix

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

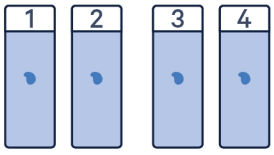
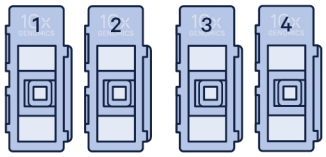
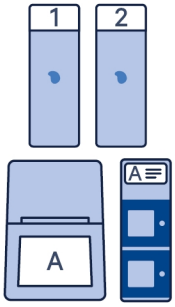
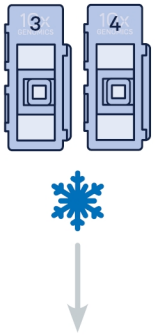
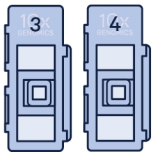
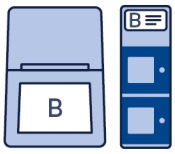
To execute this workflow, two thermal cyclers are required. This table is meant as a reference only. Consult the detailed steps in this user guide for execution.

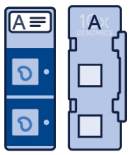
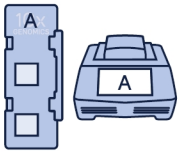
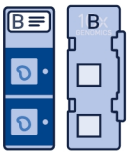
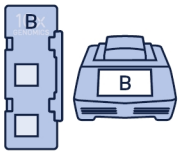
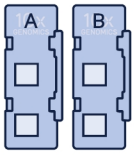
Standard Workflow: 4 Slides, 1 CytAssist		
Step	**Run A**	**Run B**
Sample Prep		
Tissue slides 1 - 4 *at the same time*		• Prepare tissue slides via sectioning, staining and imaging • Destaining and Decrosslinking
Step 1: Probe Hybridization, Step 2: Probe Ligation		
Tissue slides 1 - 4 *at the same time*		• Probe Hybridization • Post-Hybridization Wash • Probe Ligation • Post-Ligation Wash
Step 3: Probe Release & Extension		
3.1 **CytAssist Enabled RNA Digestion & Tissue Removal** Start CytAssist (Run A) with tissue slides 1 & 2	  • Prep CytAssist • Prep reagents (without enzyme) • Stain slides 1 & 2 with Eosin • Load slides on CytAssist • Add enzyme, pipette Probe Release Mix onto Visium slide • Close lid and start run • Prep thermal cycler A • Prep Probe Extension Mix	  • Store slides 3 & 4 at 4°C with 2X SSC buffer in tissue slide cassettes
Equilibration for tissue slides 3 & 4 *during Run A*	 *Once CytAssist has completed the run and is cooling down, begin equilibration for slides 3 & 4.*	 • Remove slides 3 & 4 from storage • Equilibrate for 5 min (room temp.) • Prep reagents (without enzyme)
CytAssist (Run A) complete	 • Open CytAssist • Wash Visium slide A • Place in Visium Cassette *Use 2 port cassette for 11 mm (shown) or 8 port cassette for 6.5 mm*	
3.2 **Probe Extension** for Visium Slide A	 • Add Probe Extension Mix • Apply seal and place on thermal cycler A	

Standard Workflow: 4 Slides, 1 CytAssist		
Step	**Run A**	**Run B**
Start CytAssist (Run B) with tissue slides 3 & 4 *Ensure 20 min cool-down period after completion of Run A*		 • Clean and prep CytAssist • Stain slides 3 & 4 with Eosin • Load slides on CytAssist • Add enzyme, pipette Probe Release Mix onto Visium slide • Close lid and start run • Prep thermal cycler B • Prep Probe Extension Mix
CytAssist (Run B) complete		 • Open CytAssist • Wash Visium slide B • Place in Visium Cassette *Use 2 port cassette for 11 mm (shown) or 8 port cassette for 6.5 mm*
3.2 Probe Extension for Visium Slide B		 • Add Probe Extension Mix • Apply seal and place on thermal cycler B
3.3 Probe Elution		*Proceed with Visium slides A & B*

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

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

Standard Workflow: 4 Slides, 2 CytAssists		
Step	**Run A, CytAssist A**	**Run B, CytAssist B**
Sample Prep		
Tissue slides 1 - 4 *at the same time*	 • Prepare tissue slides via sectioning, staining and imaging • Destaining and Decrosslinking	
Step 1: Probe Hybridization, Step 2: Probe Ligation		
Tissue slides 1 - 4 *at the same time*	 • Probe Hybridization • Post-Hybridization Wash • Probe Ligation • Post-Ligation Wash	
Step 3: Probe Release & Extension		
3.1 CytAssist Enabled RNA Digestion & Tissue Removal Start CytAssist (Run A) with tissue slides 1 & 2	 • Prep CytAssist A • Prep reagents (without enzyme) • Stain slides 1 & 2 with Eosin • Load slides on CytAssist • Add enzyme, pipette Probe Release Mix onto Visium slide • Close lid and start run • Prep thermal cycler A • Prep Probe Extension Mix	 • Store slides 3 & 4 at 4°C with 2X SSC buffer in tissue slide cassettes
Equilibration for tissue slides 3 & 4 *during Run A*		 • Remove slides 3 & 4 from storage • Equilibrate for 5 min (room temp.)
Start CytAssist (Run B) with tissue slides 3 & 4 *~10 min after start of Run A*		 • Prep CytAssist B • Prep reagents (without enzyme) • Stain slides 3 & 4 with Eosin • Load slides 3 & 4 on CytAssist B • Add enzyme, pipette Probe Release Mix onto Visium slide • Close lid and start run • Prepare thermal cycler B

Standard Workflow: 4 Slides, 2 CytAssists		
Step	**Run A, CytAssist A**	**Run B, CytAssist B**
CytAssist (Run A) complete	 • Open CytAssist A • Wash Visium slide A • Place in Visium Cassette *Use 8 port cassette for 6.5 mm (shown) or 2 port cassette for 11 mm*	
3.2 Probe Extension for Visium Slide A	 • Add Probe Extension Mix • Apply seal and place on thermal cycler A	
CytAssist (Run B) complete		 • Open CytAssist B • Wash Visium slide B • Place in Visium Cassette *Use 2 port cassette for 11 mm (shown) or 8 port cassette for 6.5 mm*
3.2 Probe Extension for Visium Slide B		 • Add Probe Extension Mix • Apply seal and place on thermal cycler B
3.3 Probe Elution	 *Proceed with Visium slides A & B*	

Post Library Construction Quantification

a. Thaw KAPA Library Quantification Kit for Illumina Platforms.

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

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

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

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

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

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

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

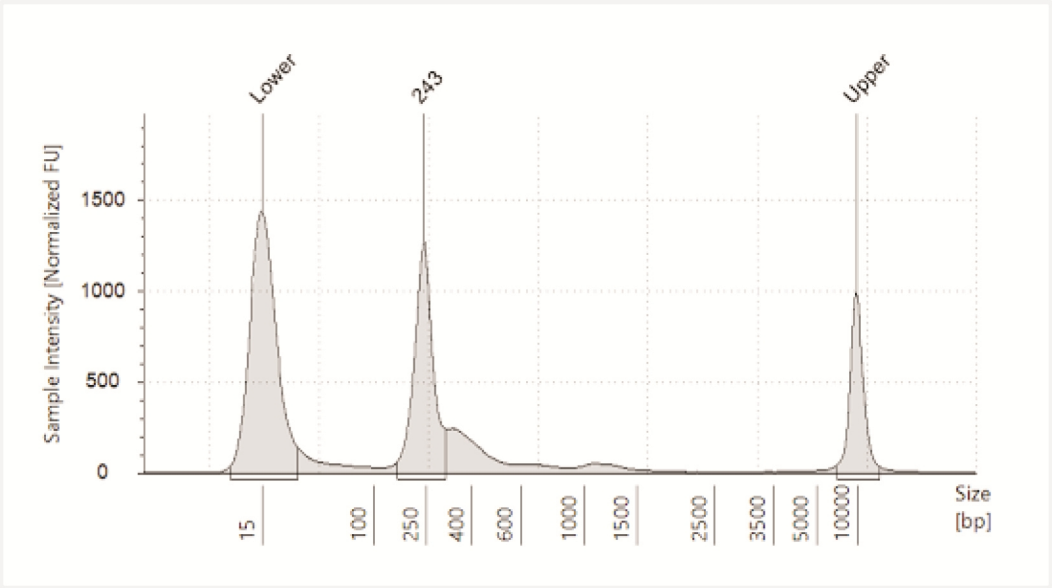
g. Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on insert size derived from the Bioanalyzer/TapeStation trace.

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)

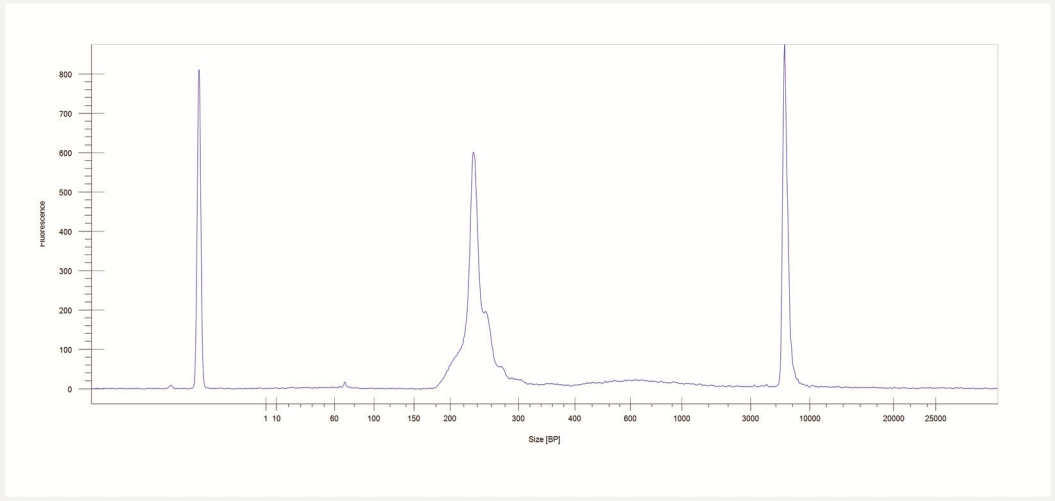


LabChip Traces

Protocol Step 5.3 - GEX Post Library Construction QC

Representative Trace

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



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

Oligonucleotide Sequences

Slide Primers

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

Visium CytAssist Spatial Gene Expression - Probe-based Library