10x GENOMICS

User Guide | CG000407 | Rev C

Visium Spatial Gene Expression Reagent Kits for FFPE

For use with:

Visium Spatial Gene Expression Slide Kit, *16 rxns PN-1000185 | 4 rxns PN-1000188*

Visium Tissue Section Test Slides, *4 Pack, PN-1000347*

Visium FFPE Reagent Kit, *Large PN-1000362 | Small PN-1000361*

Visium Human Transcriptome Probe Kit, *Large PN-1000364 | Small PN-1000363*

Visium Mouse Transcriptome Probe Kit, *Large PN-1000366 | Small PN-1000365*

Visium Accessory Kit, *PN-1000194*

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

Notices

Document Number

CG000407 | Rev C

Legal Notices

Licensed Software Updates Warranties

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

Support

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Document Revision Summary

Document Number

CG000407 | Rev C

Title

Visium Spatial Gene Expression Reagent Kits for FFPE - User Guide

Revision

Rev C

Revision Date

November 2021

Specific Changes

- Updated page 7 to indicate that the Visium Tissue Section Test Slides 4 pack (PN-1000347) is not included in the Visium Spatial for FFPE Gene Expression Starter Kits (PN-1000334 & PN-1000335).

General Changes

Updated for general minor consistency of language and terms throughout

Table of Contents

Introduction

Visium Spatial Gene Expression for FFPE Reagent Kits

Visium Accessories

Recommended Thermal Cyclers

Recommended Real Time qPCR Systems

Recommended Imaging Systems

Additional Kits, Reagents & Equipment

Protocol Steps & Timing

Stepwise Objectives

Reagent Kits

Visium Spatial Gene Expression for FFPE Reagent Kits

Refer to SDS for handling and disposal information

Reagent Kits	Part Number	Components (Part Number)
Visium Spatial for FFPE Gene Expression Starter Kit, Human Transcriptome	1000334	Visium Spatial Gene Expression Slide Kit, 16 rxns (PN-1000185)
		Visium FFPE Reagent Kit – Large (PN-1000362)
		Visium Human Transcriptome Probe Kit – Large (PN-1000364)
		Visium Accessory Kit (PN-1000194)
		Dual Index Plate TS Set A (PN-1000251)
Visium Spatial for FFPE Gene Expression Starter Kit, Mouse Transcriptome	1000335	Visium Spatial Gene Expression Slide Kit, 16 rxns (PN-1000185)
		Visium FFPE Reagent Kit – Large (PN-1000362)
		Visium Mouse Transcriptome Probe Kit – Large (PN-1000366)
		Visium Accessory Kit (PN-1000194)
		Dual Index Plate TS Set A (PN-1000251)
Visium Spatial for FFPE Gene Expression Kit, Human Transcriptome, 16 rxns	1000336	Visium Spatial Gene Expression Slide Kit, 16 rxns (PN-1000185)
		Visium FFPE Reagent Kit – Large (PN-1000362)
		Visium Human Transcriptome Probe Kit – Large (PN-1000364)
Visium Spatial for FFPE Gene Expression Kit, Mouse Transcriptome, 16 rxns	1000337	Visium Spatial Gene Expression Slide Kit, 16 rxns (PN-1000185)
		Visium FFPE Reagent Kit, Large (PN-1000362)
		Visium Mouse Transcriptome Probe Kit – Large (PN-1000366)
Visium Spatial for FFPE Gene Expression Kit, Human Transcriptome, 4 rxns	1000338	Visium Spatial Gene Expression Slide Kit, 4 rxns (PN-1000188)
		FFPE Reagent Kit – Small (PN-1000361)
		Visium Human Transcriptome Probe Kit – Small (PN-1000363)
Visium Spatial for FFPE Gene Expression Kit, Mouse Transcriptome, 4 rxns	1000339	Visium Spatial Gene Expression Slide Kit, 4 rxns (PN-1000188)
		Visium FFPE Reagent Kit – Small (PN-1000361)
		Visium Mouse Transcriptome Probe Kit – Small (PN-1000365)

Visium Spatial Gene Expression Slide Kit, 16 rxns PN-1000185

Visium
Spatial Gene Expression Slide Kit
16 rxns, PN-1000185
store at ambient temperature

	#	PN
Visium Spatial Gene Expression Slide	4	2000233
*Visium Slide Seals, 40-pack	1	2000284
Visium Cassette & Gasket Assembly, 4-pack	1	2000282

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Visium Spatial Gene Expression Slide Kit, 4 rxns PN-1000188

Visium
Spatial Gene Expression Slide Kit
4 rxns, PN-1000188
store at ambient temperature

	#	PN
Visium Spatial Gene Expression Slide	1	2000233
*Visium Slide Seals, 12-pack	1	2000283
Visium Cassette & Gasket Assembly, 1-pack	1	2000281

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**Visium Slide Seals may come in varying dimensions and quantities in different lots. Check the number of slide seals in the kit. Additional seals may be required. Refer to page 30 (Visium Slide Seal Application & Removal) of this User Guide for instructions on how to resize seals or cut additional seals.*

Visium Tissue Section Test Slides, 4 Pack PN-1000347

Visium
Tissue Section Test Slides
4 Pack, PN-1000347
store at ambient temperature

	#	PN
Visium Tissue Section Test Slide	4	2000460

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Visium FFPE Reagent Kit – Large PN-1000362

Visium FFPE Reagent Kit – Large
PN-1000362
Store at -20°C

	#	PN
Amp Mix	1	2000047
Extension Enzyme	1	2000427
Extension Buffer	1	2000409
RNase Enzyme	1	3000605
2X RNase Buffer	1	2000411
Perm Enzyme B	1	3000602
Perm Buffer B	1	2000413
TS Primer Mix A	1	2000447

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Visium FFPE Reagent Kit – Small PN-1000361

Visium FFPE Reagent Kit – Small
PN-1000361
Store at -20°C

	#	PN
Amp Mix	1	2000131
Extension Enzyme	1	2000389
Extension Buffer	1	2000408
RNase Enzyme	1	3000593
2X RNase Buffer	1	2000410
Perm Enzyme B	1	3000553
Perm Buffer B	1	2000412
TS Primer Mix A	1	2000447

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Visium Human Transcriptome Probe Kit – Large PN-1000364

Visium
Human Transcriptome Probe Kit – Large
PN-1000364
Store at -20°C

		#	PN
	FFPE Hyb Buffer	1	2000423
	FFPE Post-Hyb Wash Buffer	3	2000424
●	Human WT Probes – RHS	1	2000453
●	Human WT Probes – LHS	1	2000454
○	Probe Ligation Enzyme	1	2000426
○	2X Probe Ligation Buffer	1	2000446
	Post Ligation Wash Buffer	1	2000420

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Visium Human Transcriptome Probe Kit – Small PN-1000363

Visium
Human Transcriptome Probe Kit – Small
PN-1000363
Store at -20°C

		#	PN
	FFPE Hyb Buffer	1	2000423
	FFPE Post-Hyb Wash Buffer	1	2000424
●	Human WT Probes – RHS	1	2000449
●	Human WT Probes – LHS	1	2000450
○	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 – Large PN-1000366

Visium
Mouse Transcriptome Probe Kit – Large
PN-1000366
Store at -20°C

	#	PN
FFPE Hyb Buffer	1	2000423
FFPE Post-Hyb Wash Buffer	3	2000424
● Mouse WT Probes – RHS	1	2000457
● Mouse WT Probes – LHS	1	2000458
○ Probe Ligation Enzyme	1	2000426
○ 2X Probe Ligation Buffer	1	2000446
Post Ligation Wash Buffer	1	2000420

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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
Store at -20°C

	#	PN
Dual Index Plate TS Set A	1	3000511

10x Genomics Accessories

Product	Part Number (Kit)	Part Number (Item)
Thermocycler Adaptor	1000194	3000380
Visium Spatial Imaging Test Slide		2000235
10x Magnetic Separator		230003
Slide Alignment Tool		3000433

Recommended Thermal Cyclers

Supplier	Description	Part Number
Bio-Rad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module	1851197
Eppendorf	MasterCycler Pro *(discontinued)*	North America 950030010 International 6321 000.019
Thermo Fisher Scientific	Veriti 96-Well Thermal Cycler	4375786

Recommended Real Time qPCR Systems

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

Recommended Imaging Systems

The imaging systems listed below were used by 10x Genomics. Any equivalent system with the listed features may be used for imaging. Hardware compatibility may be tested by using the Visium Spatial Imaging Test Slide (included in Visium Accessories Kit). Consult the Visium Spatial Gene Expression for FFPE Imaging Guidelines Technical Note (CG000436) 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
Microscope Features		
Objectives	10X, NA 0.45 20X, NA 0.75 40X, NA 0.95	
Automated Scanning Stage	Microscope tile scanning functionality is required for imaging tissue sections placed on a Capture Area of a Visium Spatial slide.	
Brightfield Features (for H&E staining)	Color camera (3 x 8 bit, 2,424 x 2,424 pixel resolution) White balancing functionality Minimum Capture Resolution 2.18 µm/pixel Exposure times 2-10 milli sec	
Fluorescence Features (for IF staining)	Light source (or equivalent) with a wavelength range of 380-680 nm Monochrome camera (14 bit, 2,424 x 2,424 pixel resolution) DAPI filter cube (Excitation 392/23, Emission 447/60) FITC filter cube (Excitation 480/40, Emission 535/50) TRITC filter cube (Excitation 542/20, Emission 620/52) Cy5 filter cube (Excitation 618/50, Emission 698/70) Minimum Capture Resolution 2.18 µm/pixel Exposure times 100 milli sec-2 sec	

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

Image Capture Guidelines:

The 8 x 8 mm area that includes the fiducial frame and the Capture Area with the tissue section should be represented by ≥2,000 x 2,000 pixel portion of the image.

When setting the microscope for imaging individual Capture Area, the imaging area should be ~1-2 mm beyond the fiducial frame for optimal imaging alignment. Minimize imaging of any adjacent Capture Area/s when taking images of a specific Capture Area with a tissue section. For lossy compression, such as jpeg, the quality level should be kept high enough to represent the fiducial frame crisply and without artifacts.

Additional Kits, Reagents & Equipment

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

Item	Description	Supplier	Part Number
Plastics			
1.5 ml tubes	DNA LoBind Tubes, 1.5 ml	Eppendorf	022431021
2.0 ml tubes	DNA LoBind Tubes, 2.0 ml *when processing more than two slides*	Eppendorf	022431048
0.2 ml 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	Thermo Fisher Scientific	N8010580
	MicroAmp 8-Cap Strip, clear	Thermo Fisher Scientific	N8010535
Slide mailer/tube	Simport Scientific LockMailer Tamper Evidence Slide Mailer	Thermo Fisher Scientific	22-038-399
	Self-Standing Polypropylene Centrifuge Tubes (50 ml), sterile *Alternative to slide mailer*	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	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-L10FLR	Rainin	30389226
Reagent reservoirs	Divided Polystyrene Reservoirs	VWR	41428-958
Kits & Reagents			
Nuclease-free water	Nuclease-free Water (not DEPC-Treated)	Thermo Fisher Scientific	AM9937
Tris 1 M	Tris 1 M, pH 7.0, RNase-free	Thermo Fisher Scientific	AM9850G
Plain glass slides	Fisherbrand Premier Plain Glass Microscope Slides, *Optional*	Thermo Fisher Scientific	12-544-4
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	KK4600
SPRIselect reagent	SPRIselect Reagent Kit	Beckman Coulter	B23318

Choose either Eppendorf, USA Scientific or Thermo Fisher Scientific PCR 8-tube strips.

Additional Kits, Reagents & Equipment

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

Item	Description	Supplier	Part Number
Kits & Reagents			
Ethanol	Ethyl Alcohol, Pure (200 Proof, anhydrous)	Millipore Sigma	E7023-500ML
8 M KOH	Potassium Hydroxide Solution, 8M	Millipore Sigma	P4494-50ML
20X SSC buffer	SSC Buffer 20X Concentrate	Millipore Sigma	S6639-1L
Buffer EB	Qiagen Buffer EB	Qiagen	19086
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
Quantification & Quality Control			
Bioanalyzer & associated reagents	2100 Bioanalyzer Laptop Bundle (discontinued) (Replacement 2100 Bioanalyzer Instrument/ 2100 Expert Laptop Bundle)	Agilent	G2943CA G2939BA/ G2953CA
	High Sensitivity DNA Kit	Agilent	5067-4626
TapeStation & associated reagents	4200 TapeStation	Agilent	G2991AA
	High Sensitivity D1000: ScreenTape/ Reagents	Agilent	5067-5592/ 5067-5593
	High Sensitivity D5000: ScreenTape/ Reagents	Agilent	5067-5584/ 5067-5585
LabChip & associated reagents	LabChip GX Touch HT Nucleic Acid Analyzer	PerkinElmer	CLS137031
	DNA High Sensitivity Reagent Kit	PerkinElmer	CLS760672
Library quantification kit	KAPA Library Quantification Kit for Illumina Platforms	KAPA Biosystems	KK4824

Choose Bioanalyzer, TapeStation or LabChip based on availability & preference.

Protocol Steps & Timing

2 days

Steps		Timing	Stop & Store
Step 1 – Probe Hybridization			
1.1	Probe Hybridization	Overnight	
Step 2 – Probe Ligation			
2.1	Post Hybridization Wash	15 min	
2.2	Probe Ligation	65 min	
2.3	Post Ligation Wash	15 min	STOP 4°C ≤24 h
Step 3 – Probe Release & Extension			
3.1	RNA Digestion & Probe Release	75 min	
3.2	Probe Extension	20 min	STOP 4°C ≤72 h
3.3	Probe Elution	15 min	STOP –20°C ≤72 h
Step 4 – Visium Spatial Gene Expression – FFPE Library Construction			
4.1	Cycle Number Determination - qPCR	45 min	
4.2	Sample Index PCR	40 min	STOP 4°C ≤24h
4.3	Post Sample Index PCR - Cleanup	30 min	STOP –20°C long-term
4.4	Post Library Construction QC	50 min	

Stepwise Objectives

Visium Spatial Gene Expression for FFPE assays RNA levels by using probes against the whole transcriptome in intact formalin fixed paraffin embedded (FFPE) tissue sections and maps the location(s) where gene activity is occurring. Each Visium Spatial Gene Expression Slide contains Capture Areas with gene expression spots that include primers required to capture the probes. Tissue sections placed on these Capture Areas are deparaffinized, stained, and decrosslinked, as described in Deparaffinization & Staining Demonstrated Protocols – CG000409 or CG000410.

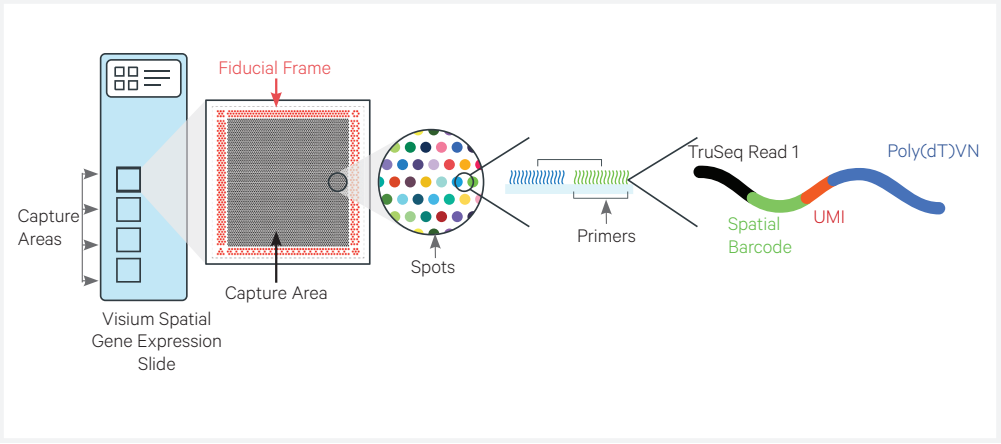
Human or mouse whole transcriptome probe panels, consisting of a pair of specific probes for each targeted gene, are then added to the tissue. These probe pairs hybridize to their gene target and are then ligated to one another. The ligation products are released from the tissue upon RNase treatment and permeabilization. The ligated probe pairs bind with spatially barcoded oligonucleotides present on the Capture Area. All the probes captured by primers on a specific spot share a common Spatial Barcode. Libraries are generated from the probes and sequenced and the Spatial Barcodes are used to associate the reads back to the tissue section images for spatial mapping of gene expression.

This document outlines the protocol for generating Visium Spatial Gene Expression – FFPE libraries from FFPE tissue sections placed on the Capture Areas of a Visium Spatial Gene Expression Slide.

Visium Slide

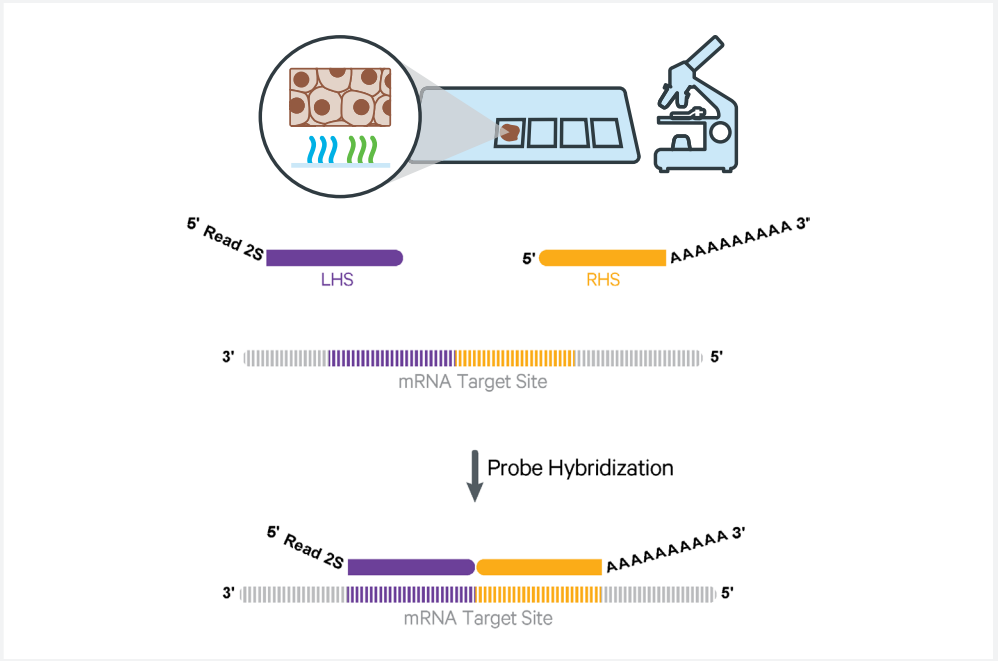
The Visium Spatial Gene Expression Slide has 4 Capture Areas. Each Capture Area is 6.5 x 6.5 mm and defined by a fiducial frame (fiducial frame + Capture Area is 8 x 8 mm). The Capture Area has ~5,000 gene expression spots, each spot with primers that include:

- Illumina TruSeq Read 1 (partial read 1 sequencing primer)
- 16 nt Spatial Barcode (all primers in a specific spot share the same Spatial Barcode)
- 12 nt unique molecular identifier (UMI)
- 30 nt poly(dT) sequence (captures ligation product)



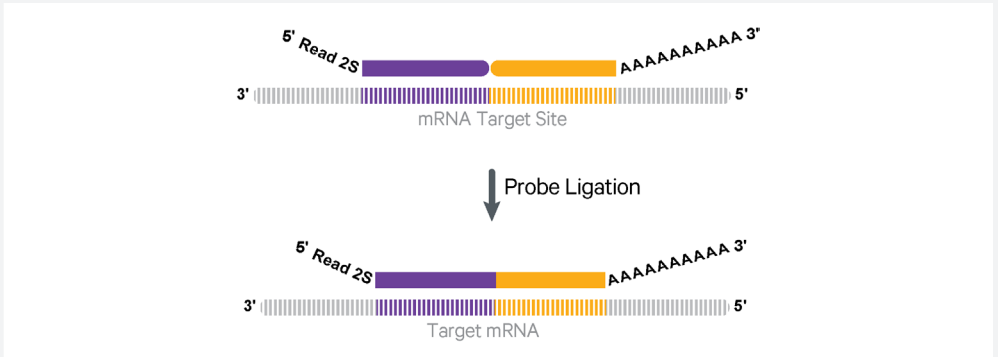
Step 1
Probe Hybridization

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



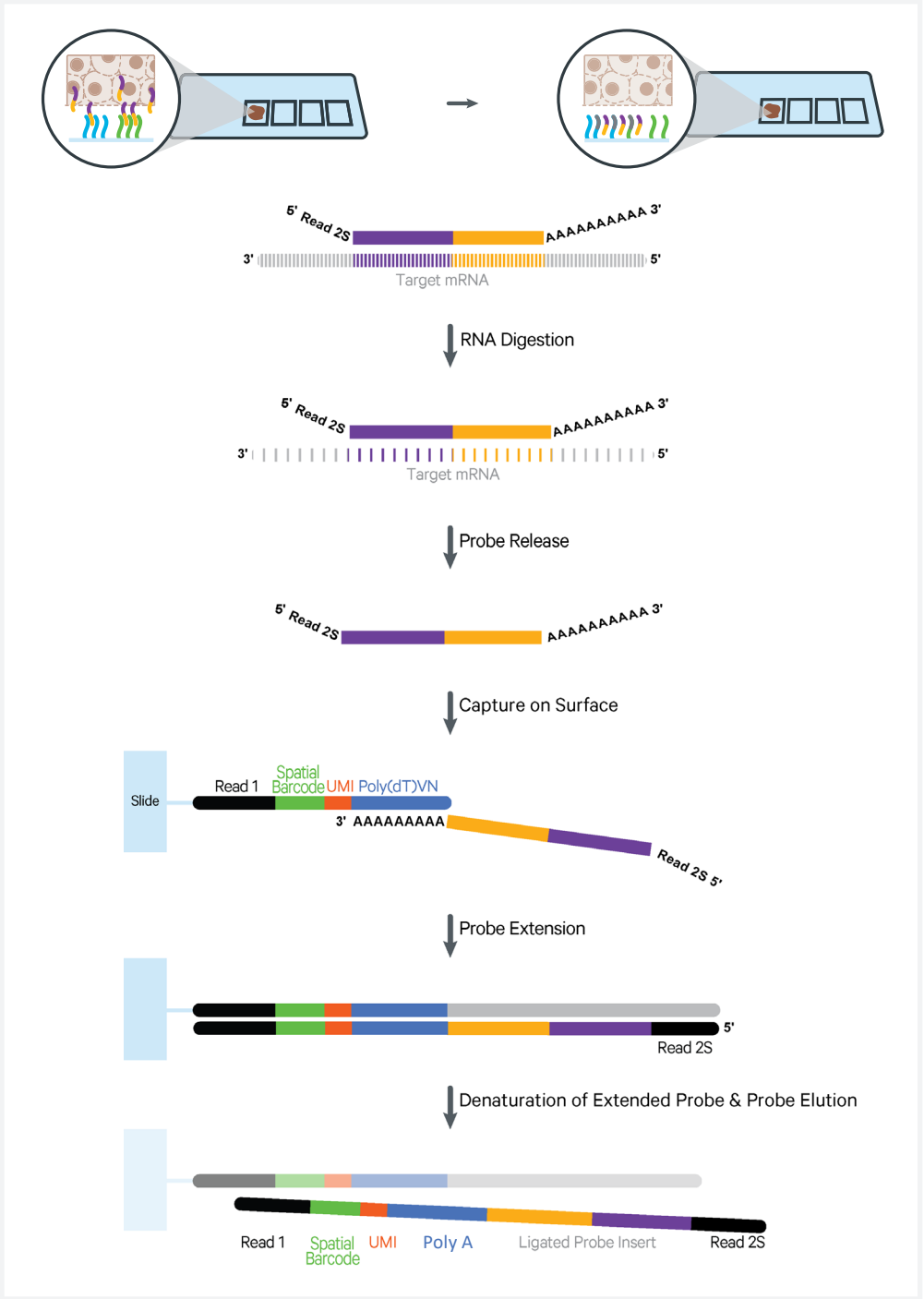
Step 2
Probe Ligation

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



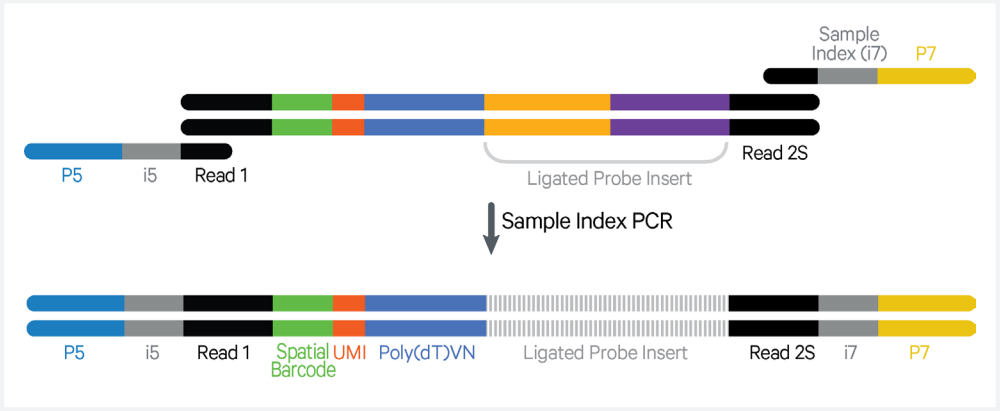
Step 3 Probe Release & Extension

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



Step 4 Visium Spatial Gene Expression - FFPE Library Construction

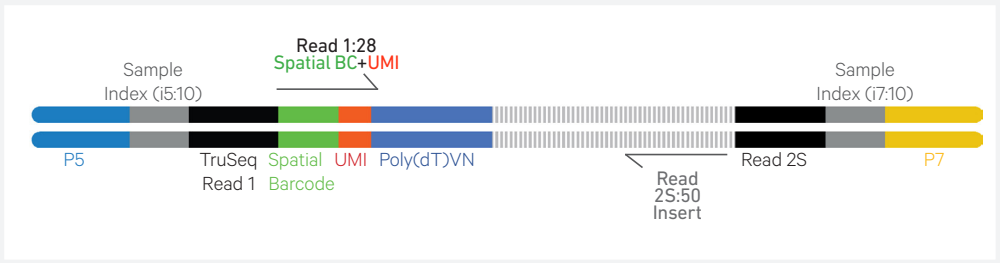
The spatially barcoded, ligated probe products are released from the slide and harvested for qPCR to determine Sample Index PCR cycle number. The products then undergo indexing via Sample Index PCR. This, in turn, generates final library molecules that are cleaned up by SPRIselect, assessed on a bioanalyzer or a similar instrument, quantified, and then sequenced.



Step 5 Sequencing

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

Illumina sequencer compatibility, sample indices, library loading and pooling for sequencing are summarized in step 5.



See Appendix for Assay Scheme and Library Sequence

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Tips & Best Practices

Icons

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

General Reagent Handling

- Fully thaw and thoroughly mix reagents before use.
- Use a pH meter to adjust pH as necessary during buffer preparation.

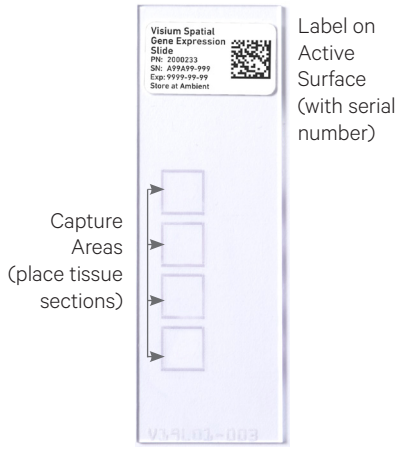
Pipette Calibration

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

Visium Slides

- Visium slides include 4 Capture Areas (6.5 x 6.5 mm), each with ~5,000 unique gene expression spots.
- Each gene expression spot includes primers with a unique Spatial Barcode (see Stepwise Objectives for additional information).
- The active surface of the slide is defined by a readable label that includes the serial number.
- The tissue sections are always placed on the active surface of the Capture Areas. For more information, consult the Visium Spatial Gene Expression for FFPE – Tissue Preparation Guide (Demonstrated Protocol CG000408).

Visium Spatial Gene Expression Slide



Note the serial number on the slide label; required for downstream analysis.

Slide Storage

- Always store slides in a cool, dry environment.
- Store unused slides in original packaging and keep sealed. DO NOT remove desiccant. If necessary, place the sealed container in a secondary container, such as a resealable bag.
- After tissue placement, store the slides at **room temperature** in a low moisture environment such as a desiccator.

Slide Handling

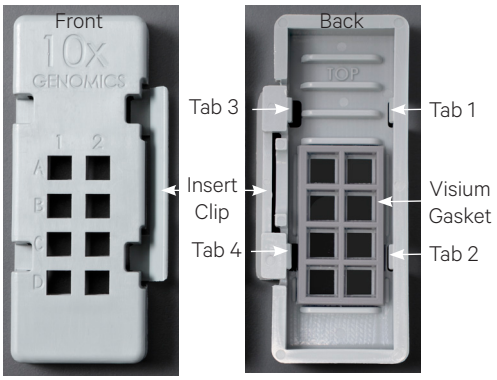
- Always wear gloves when handling slides.
- Ensure that the active surface of a slide faces up and is never touched. The orientation of the label on the slide defines the active surface.
- The tissue sections should always be on the active surface of the slide. DO NOT touch the tissue sections on the slide.
- Minimize exposure of the slides to sources of particles and fibers.
- Keep the slide flat on the bench when adding reagents to the active surface.
- Ensure that no absorbent surface is in contact with the reagents on the slide during incubation.

Active Surface with Tissue Sections

Visium Cassette

- The Visium Cassette encases the slide and creates leakproof wells for adding reagents.
- Place the slides in the Visium Cassette only when specified.
- The Visium Cassette includes a removable Visium Gasket.
- An Insert Clip and four tabs at the back of the Visium Cassette are used for holding the slide in the cassette, as shown.
- The removable Visium Gasket corresponds to the Capture Areas on the slides.
- The Visium Cassette may be assembled using the Slide Alignment Tool or manually. Instructions for both are provided in the following section.
- See Visium Cassette Assembly & Removal instructions for details.
- Ensure that the back of the Visium Cassette is facing the user prior to assembly. The active surface of the slide with tissue sections will face down such that the slide label is no longer readable.
- Practice assembly with a plain glass slide (75 x 25 x 1 mm).
- Applying excessive force to the slide may cause the slide to break.

Visium Cassette



Slide Alignment Tool

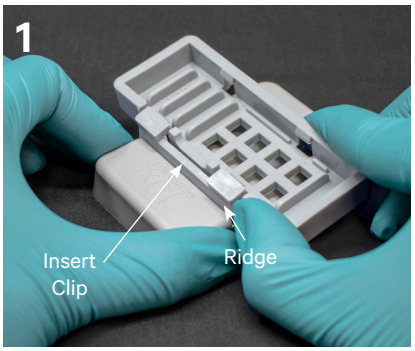
Visium Cassette Assembly

Exercise caution when handling slide edges to prevent injury.

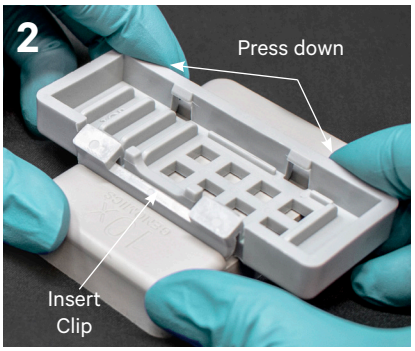
Slide insertion may push Visium Gasket out of alignment with slide cutouts. Adjust if necessary.

Slides in images are representative.

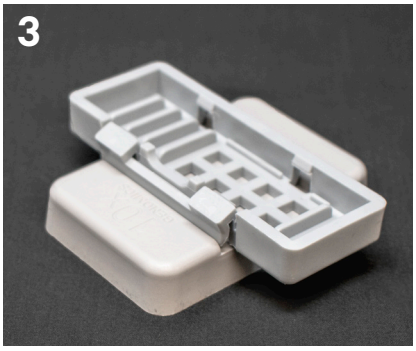
Position Visium Cassette along alignment tool ridges



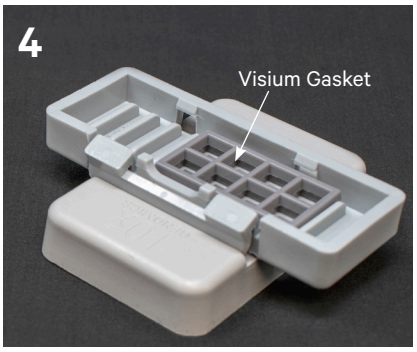
Push Insert Clip along the ridge & press Visium Cassette down



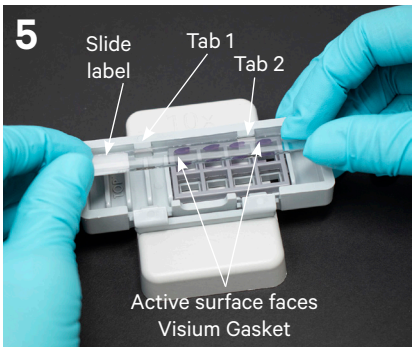
Visium Cassette secured on alignment tool



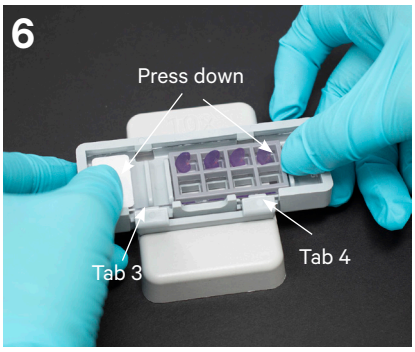
Position Visium Gasket to align with Visium Cassette cutouts



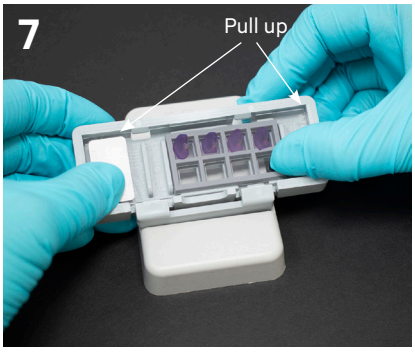
Insert long edge of slide under tabs 1 & 2; ensure slide is flush



Press slide down until it is flush with the Visium Gasket and under tabs 3 & 4

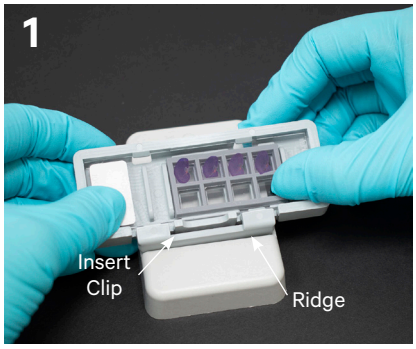


Remove Visium Cassette while pressing slide against the Visium Gasket

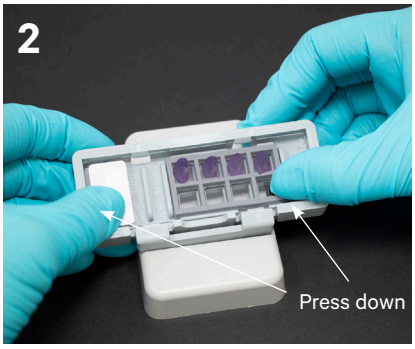


Visium Cassette Removal

Position Visium Cassette along alignment tool ridges

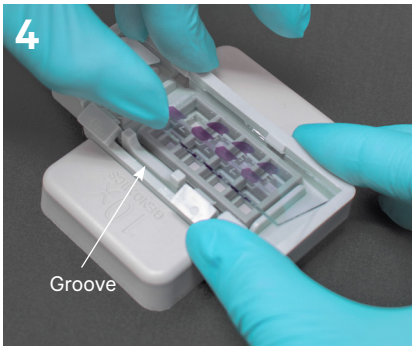


Push Insert Clip along the ridge & press down



Visium Cassette sits securely on alignment tool

Lift slide at Visium Cassette groove



Slides in images are representative.

Manual Visium Cassette Assembly & Removal

Assembly

a. Remove the Visium Gasket from the Visium Cassette and re-insert the Visium Gasket, ensuring that the Visium Gasket and Visium Cassette cutouts are aligned.

b. Align the label on top of the slide to the top of the Visium Cassette, as shown.

c. Insert the slide under tabs 1 and 2. Ensure that the long edge of the slide is flush with the side of the Visium Cassette.

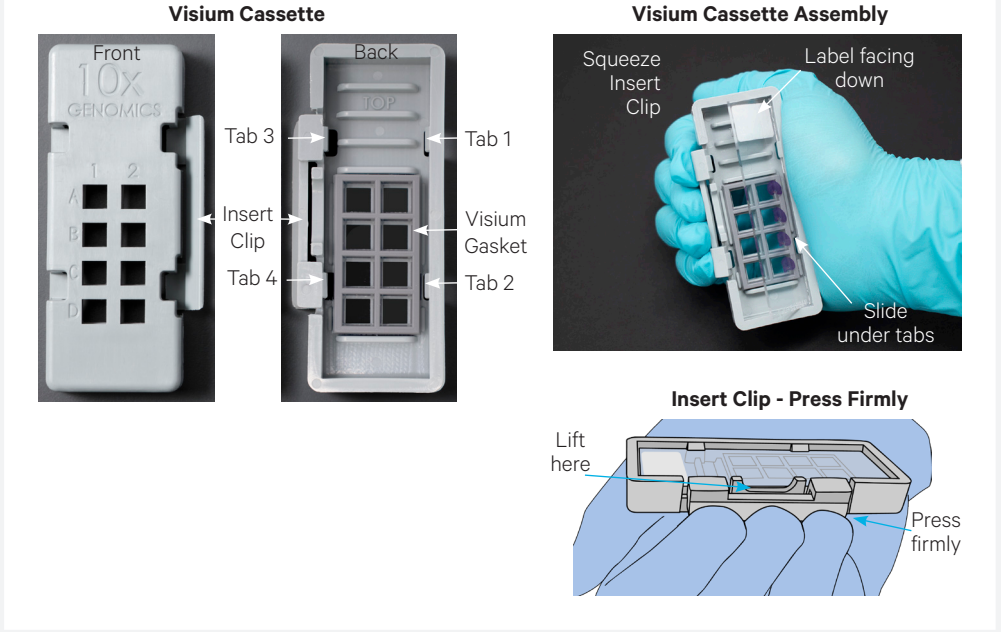
d. Press the insert clip **very firmly** by applying even force on the lower part of the insert clip.

e. Place a finger in between tab 3 and the top of the cassette, and one finger between tab 4 and the bottom of the cassette.
Press down on the slide evenly until the slide is under each tab and release the insert clip.

Removal

a. Press the insert clip very firmly to release the slide from the cassette.

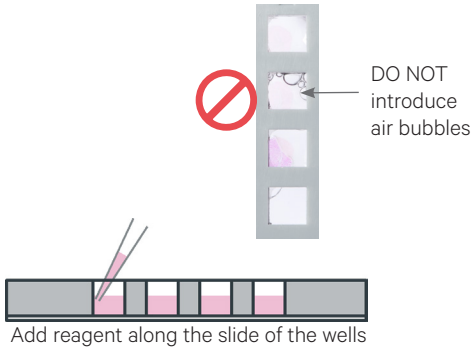
b. Lift slide at Visium Cassette groove between tabs 3 and 4 until the slide can be removed.



Reagent Addition to Wells

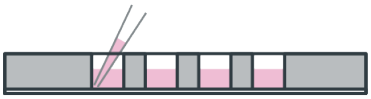
- Place the assembled slide in the Visium Cassette flat on a clean work surface.
- Dispense reagents along the side of the wells without touching the tissue sections and without introducing bubbles.
- Always cover the tissue section completely when adding reagents to the well. A gentle tap may help spread the reagent more evenly.
- Ensure that no bubbles are introduced in the process.

Reagent Addition/Removal

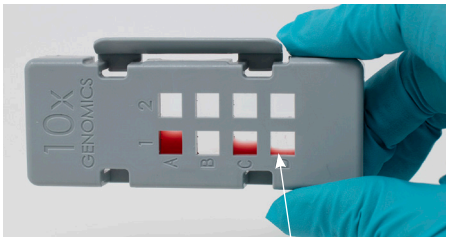


Reagent Removal from Wells

- Place the assembled slide in the Visium Cassette flat on a clean work surface.
- Slightly tilt the Visium Cassette while removing the reagent.
- Place the pipette tip to the bottom edge of the wells.
- Remove reagents along the side of the wells without touching the tissue sections and without introducing bubbles.
- Ensure that no bubbles are introduced in the process.
- 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.



Place the pipette tip to the bottom edge of the wells



Meniscus at the bottom indicates liquid, DO NOT leave liquid behind

Post Hybridization & Post Ligation Washes

- Post hybridization and post ligation washes are critical for assay performance. Failure to perform the correct number of washes can significantly reduce the fraction of targeted reads usable.
- Washing for less than the recommended time and reagent carry over during washes can also reduce the fraction of targeted reads usable.
- Remove all liquid from the well when washing, and refer to appropriate step for correct number of washes and incubation times.

Visium Slide Seal Application & Removal

To generate new or resize Visium Slide Seals, use one of the provided seals (PN-2000283/2000284) as a template to cut additional seals from MicroSeal 'B' PCR Plate Sealing Film (PN-MSB1001; listed in Additional Kits, Reagents & Equipment). Contact support@10xgenomics.com if assistance is required.

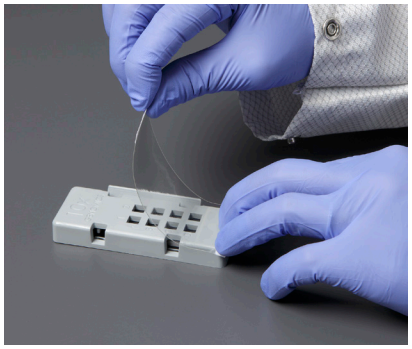
Application

- Place the Visium Cassette flat on a clean work surface.
- Remove the back of the adhesive Visium Slide Seal.
- Align the Visium Slide Seal with the surface of the Visium Cassette and apply while firmly holding the Visium Cassette with one hand.
- Press on the Visium Slide Seal to ensure uniform adhesion.

Removal

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

Visium Slide Seal Application



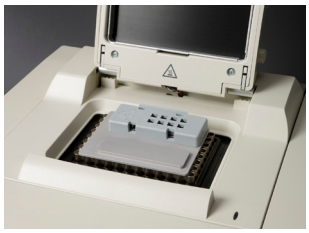
Slide Incubation Guidance

Incubation at a specified temperature

- Position a Thermocycler Adaptor on a thermal cycler that is set at the incubation temperature.
- Ensure that the Thermocycler Adaptor is in contact with the thermal cycler surface uniformly.
- When incubating a slide encased in a Visium Cassette, place the assembled unit on the Thermocycler Adaptor with the wells facing up. The Visium Cassette should always be sealed when on the Thermocycler Adaptor.

Place Thermocycler Adaptor

Incubate Assembled Visium Cassette



Incubation at room temperature

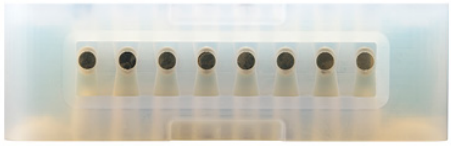
- Place the slide/Visium Cassette on a flat, clean, non-absorbent work surface.
- Ensure that no absorbent surface is in contact with the reagents on the slide during incubation.

Tissue Detachment on Visium Slides

- Monitor section adhesion on the Visium slides throughout the workflow.
- Tissue detachment during the workflow can impact performance.

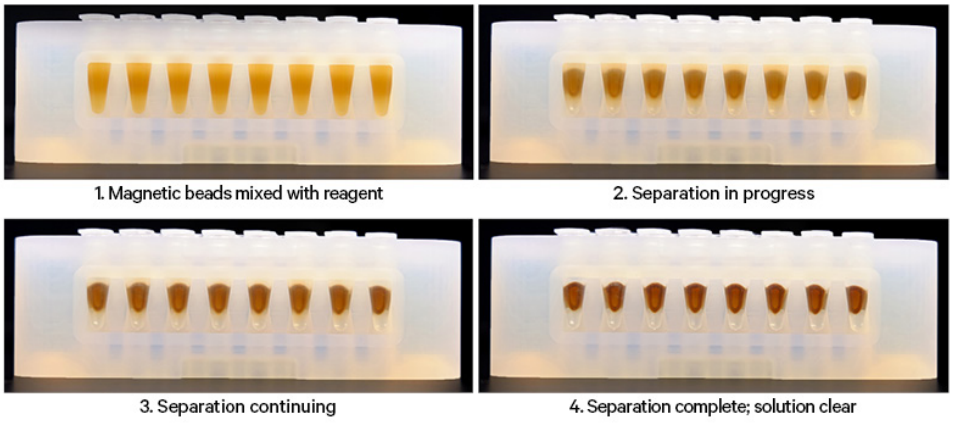
10x Magnetic Separator

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



Magnetic Bead Cleanup Steps

- During magnetic bead based cleanup steps that specify waiting "until the solution clears", visually confirm clearing of solution before proceeding to the next step. See adjacent panel for an example.
- The time needed for the solution to clear may vary based on specific step, reagents, volume of reagents etc.



SPRIselect Cleanup & Size Selection

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

Sample Indices (i5/i7) in Sample Index PCR

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

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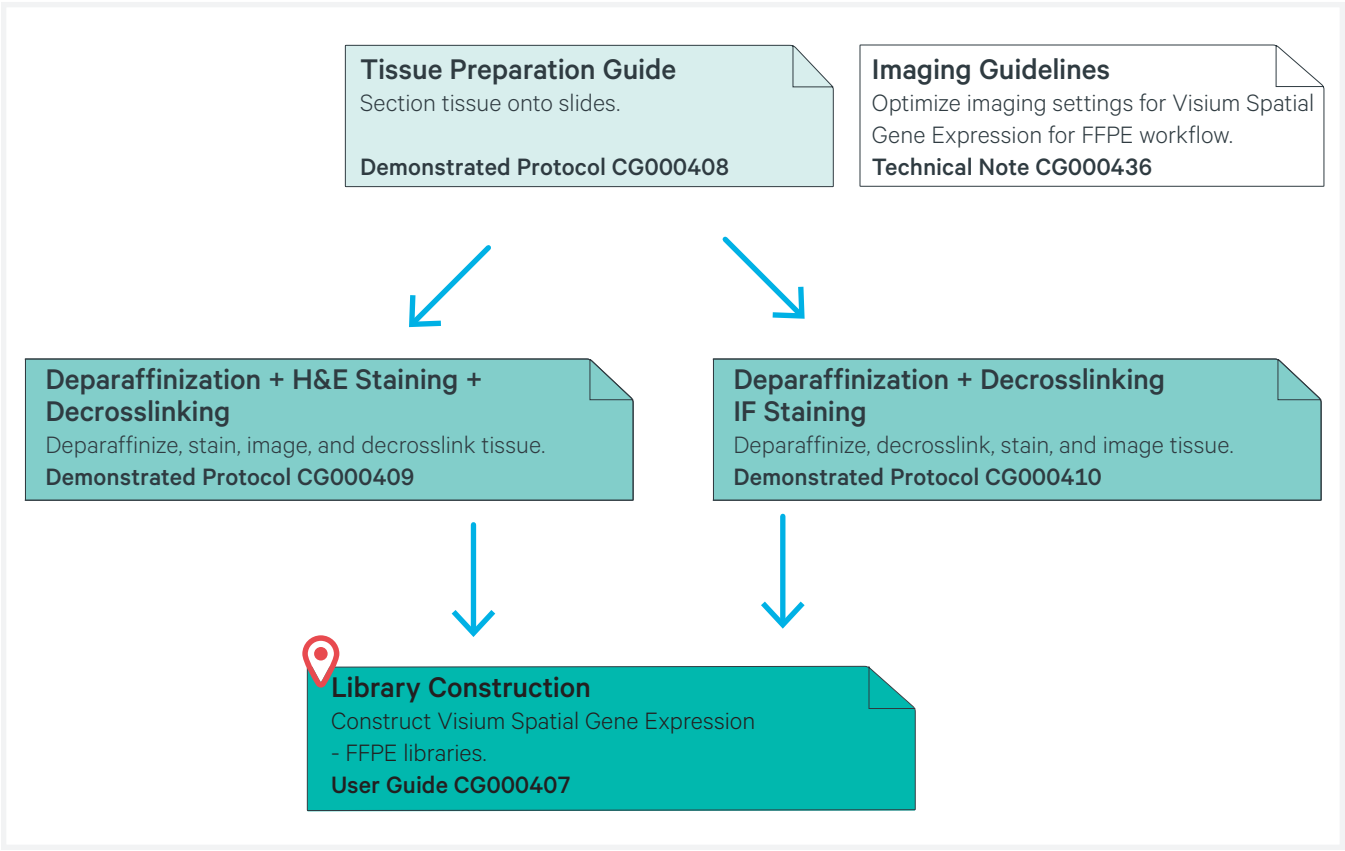
Sample Preparation, Deparaffinization & Staining Guidelines

Workflow Overview

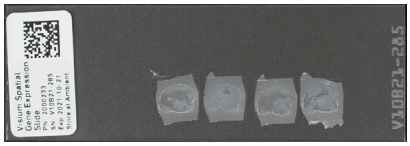


Sample Preparation Guidelines

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

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

Consult the Visium Spatial Gene Expression for FFPE – Tissue Preparation Guide for complete information (Demonstrated Protocol CG000408), including Tips & Best Practices for tissue sectioning and section placement.

Key Considerations
Slide Handling (before sectioning)
☐ Store unused slides in original packaging and keep sealed. DO NOT remove desiccant. If necessary, store original packaging in a secondary container such as a resealable bag.
FFPE Tissue Sectioning & Section Placement
☐ Assess RNA quality of the FFPE tissue block.
☐ Section the FFPE tissue block using a microtome and place sections on the Visium Spatial slides using a water bath.
☐ Place tissue sections on the Capture Area within the fiducial frame on the slide.

Slide Handling (after sectioning)
☐ Store the slides containing FFPE sections for up to 2 weeks in a desiccator.

Deparaffinization, Staining & Decrosslinking Guidelines

FFPE tissue sections should be deparaffinized, stained, imaged, and decrosslinked before proceeding with Visium Spatial Gene Expression for FFPE. Consult Demonstrated Protocols (available on the 10x Genomics Support website) for details.

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

Deparaffinization, H&E Staining, Imaging, & Decrosslinking

Consult Visium Spatial Gene Expression for FFPE – Deparaffinization, H&E Staining, Imaging & Decrosslinking (Demonstrated Protocol CG000409). In this protocol, Visium slides containing FFPE tissue sections are first deparaffinized and then stained with Hematoxylin and Eosin (H&E). The stained slides are then coverslipped and imaged. After the coverslip is removed, a decrosslinking step is performed. Once the slides are decrosslinked, proceed directly to the User Guide.

Deparaffinization, H&E Staining, Imaging & Decrosslinking Workflow

Deparaffinization, H&E Staining, Imaging & Decrosslinking (CG000409)
- Deparaffinization
- H&E Staining
- Coverslipping
- Imaging & Coverslip Removal
- Decrosslinking

→ **Visium Spatial Gene Expression for FFPE (CG000407)**

Deparaffinization, Decrosslinking, IF Staining & Imaging

Consult Visium Spatial Gene Expression for FFPE – Deparaffinization, Decrosslinking, IF Staining & Imaging (Demonstrated Protocol CG000410). In this protocol, Visium slides containing FFPE tissue sections are first deparaffinized and then decrosslinked. The slides are then stained with fluorescently labeled antibodies, coverslipped, and imaged. Once the immunostained tissue sections are imaged and the coverslip is removed, proceed directly to the User Guide.

Deparaffinization, Decrosslinking, IF Staining & Imaging Workflow

Deparaffinization, Decrosslinking, IF Staining & Imaging (CG000410)
- Deparaffinization
- Decrosslinking
- Immunofluorescence Staining
- Coverslipping
- Imaging & Coverslip Removal

→ **Visium Spatial Gene Expression for FFPE (CG000407)**

Step 1

Probe Hybridization

1.1 Probe Hybridization

1

Get Started

1.0 Probe Hybridization

	Item	10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	**FFPE Hyb Buffer**	2000423	Thaw at room temperature. If precipitate persists, heat at 37°C until dissolved. Avoid vortexing to prevent bubble formation. Keep the buffer at room temperature after thawing.	–20°C
Place on ice				
☐	**Perm Enzyme B**	3000602/ 3000553	Pipette mix, centrifuge briefly. Maintain on ice until ready to use. Dilute 200X by adding 1 µl Perm Enzyme B in 199 µl Buffer EB.	–20°C
Probes for human samples				
☐	**Human WT Probes – RHS**	2000453/ 2000449	Thaw on ice. Vortex and centrifuge briefly.	–20°C
☐	**Human WT Probes – LHS**	2000454/ 2000450	Thaw on ice. Vortex and centrifuge briefly.	–20°C
Probes for mouse samples				
☐	**Mouse WT Probes – RHS**	2000457/ 2000455	Thaw on ice. Vortex and centrifuge briefly.	–20°C
☐	**Mouse WT Probes – LHS**	2000458/ 2000456	Thaw on ice. Vortex and centrifuge briefly.	–20°C
Obtain				
☐	**Nuclease-free Water**	-	-	Ambient
☐	**Visium Cassette & Gasket Assembly**	2000282/ 2000281	See Tips & Best Practices	Ambient
☐	**Visium Slide Seals**	2000284/ 2000283	See Tips & Best Practices	Ambient
☐	**Buffer EB**	-	-	Ambient
☐	**10% Tween-20**	-	-	Ambient

1.1 Probe Hybridization

Before starting this protocol, ensure that slide has been appropriately deparaffinized, stained, imaged, and decrosslinked. Consult Visium Spatial Gene Expression for FFPE – Deparaffinization, H&E Staining, Imaging & Decrosslinking (Demonstrated Protocol CG000409) and Visium Spatial Gene Expression for FFPE – Deparaffinization, Decrosslinking, IF Staining & Imaging (Demonstrated Protocol CG000410).

TIPS

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

a. Prepare Pre-hybridization Mix shortly before using and keep at **room temperature**. Pipette mix 10x and centrifuge briefly.

Pre-hybridization Mix *Add reagents in the order listed. Maintain at room temperature.*	**10x PN**	**1X (µl)**	**4X+ 10% (µl)**	**8X + 10% (µl)**
Nuclease-free Water	-	89.0	391.6	783.2
10X PBS	-	10.0	44	88
Diluted Perm Enzyme B Dilute Perm Enzyme B 200X in Buffer EB before using. DO NOT use concentrated stock directly.	3000602/ 3000553	0.5	2.2	4.4
10% Tween-20	-	0.5	2.2	4.4
Total	**-**	**100.0**	**440.0**	**880.0**

b. Retrieve the Visium Cassette containing H&E stained or IF stained sections and remove the Visium Slide Seal.

c. Using a pipette, remove all buffer from the well corners. For H&E stained slide, remove all TE buffer. For IF stained slide, remove all PBS.

d. Add **100 µl** Pre-hybridization Mix along the side of the wells to uniformly cover the tissue sections, without introducing bubbles.

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

Add Reagent

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

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

g. Prepare Probe Hybridization Mix shortly before using and keep at room temperature. Pipette mix 10x and centrifuge briefly.

Probe Hybridization Mix *Add reagents in the order listed. Maintain at room temperature.*	**10x PN**	**1X (µl)**	**4X + 10% (µl)**	**8X + 10% (µl)**
Nuclease-free Water	-	10.0	44.0	88.0
FFPE Hyb Buffer	2000423	70.0	308.0	616.0
Human WT Probes –RHS **Or** **Mouse WT Probes – RHS**	2000453/ 2000449 Or 2000457/ 2000455	10.0	44.0	88.0
Human WT Probes – LHS **Or** **Mouse WT Probes – LHS**	2000454/ 2000450 Or 2000458/ 2000456	10.0	44.0	88.0
Total	**-**	**100.0**	**440.0**	**880.0**

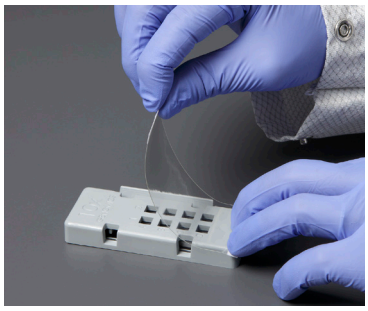
h. Remove all Pre-hybridization Mix from the wells.

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

j. Apply Visium Slide Seal on the cassette and place on the Thermocycler Adaptor on the pre-heated thermal cycler. Close the thermal cycler lid.

k. Skip Pre-equilibrate step to initiate Hybridization.

Apply Visium Slide Seal



10x GENOMICS

Step 2

Probe Ligation

2.1 Post Hybridization Wash

2.2 Probe Ligation

2.3 Post Ligation Wash

2

Get Started

2.0 Probe Ligation

	Item	10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐	**FFPE Post-Hyb Wash Buffer**	2000424	Thaw at room temperature. If precipitate persists, heat at 37°C until dissolved. Vortex and centrifuge briefly.	–20°C
☐ ◯	**2X Probe Ligation Buffer**	2000446/ 2000445	Thaw at room temperature. Vortex and centrifuge briefly.	–20°C
☐	**Post Ligation Wash Buffer**	2000420/ 2000419	Thaw at room temperature. If precipitate persists, heat at 37°C until dissolved. Vortex and centrifuge briefly. The tube is filled to the top. Pipette carefully.	–20°C
Place on ice				
☐ ◯	**Probe Ligation Enzyme**	2000426/ 2000425	Thaw on ice. Centrifuge briefly.	–20°C
Obtain				
☐	**Nuclease-free Water**	-	-	Ambient
☐	**20X SSC Buffer**	-	-	Ambient

2.1 Post Hybridization Wash

a. Pre-heat FFPE Post-Hyb Wash Buffer (**495 µl/per sample)** to **50°C**.

b. Prepare 2X SSC Buffer.

SSC Buffer *Add reagents in the order listed. Maintain at room temperature.*	Stock	Final	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
SSC	20X	2X	120.0	528.0	1056.0
Nuclease-free Water	-	-	1080.0	4752.0	9504.0
Total	-		**1200.0***	**5280.0***	**10560.0***

**This volume of 2X SSC Buffer is sufficient for washes in all the subsequent steps.*

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

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

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

f. Apply Visium Slide Seal on the cassette and place on the Thermocycler Adaptor on the pre-heated thermal cycler. Close the thermal cycler lid.

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

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

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

j. **Immediately** add **150 µl pre-heated** FFPE Post-Hyb Wash Buffer to each well. Avoid well drying or cooling to room temperature.

k. Apply Visium Slide Seal on the cassette and place on the Thermocycler Adaptor on the pre-heated thermal cycler. Close the thermal cycler lid.

l. Initiate Post Hybridization Wash. Incubate in the thermal cycler at **50°C** for **5 min**.

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

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

o. **Repeat** j-n one more time.

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

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

2.2 Probe Ligation

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

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

Step	Temperature	Time
Pre-equilibrate	37°C	Hold
Ligation	37°C	01:00:00
Hold	4°C	Hold

b. Prepare Probe Ligation Mix shortly before using. Pipette mix 10x and centrifuge briefly.

Probe Ligation Mix *Add reagents in the order listed. Maintain on ice.*	10x PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	24.0	105.6	211.2
◯ **2X Probe Ligation Buffer**	2000446/ 2000445	30.0	132.0	264.0
◯ **Probe Ligation Enzyme**	2000426/ 2000425	6.0	26.4	52.8
Total	**-**	**60.0**	**264.0**	**528.0**

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

d. Add **60 µl** Probe Ligation Mix along the side of the wells to uniformly cover the tissue sections, without introducing bubbles.
Tap Visium Cassette gently to ensure uniform coverage.

e. Apply a new Visium Slide Seal on the Visium Cassette and place on the Thermocycler Adaptor on the pre-heated thermal cycler.
Close the thermal cycler lid. Discard old slide seals.

f. Skip Pre-equilibrate step to initiate Ligation.

2.3 Post Ligation Wash

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

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

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

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

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

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

e. Immediately add **100 µl room temperature** Post Ligation Wash Buffer to each well. The Post Ligation Wash Buffer should be at **room temperature**. Avoid well drying.

f. Apply Visium Slide Seal on the Visium Cassette and place on the Thermocycler Adaptor on the pre-heated thermal cycler. Close the thermal cycler lid.

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

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

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

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

k. Apply Visium Slide Seal on the Visium Cassette and place on the Thermocycler Adaptor on the pre-heated thermal cycler. Close the thermal cycler lid.

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

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

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

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

p. Remove all 2X SSC buffer.

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

STOP

r. Let the slides cool to **room temperature** and proceed to next step or apply Visium Slide Seal on the Visium Cassette and store the slides in 2X SSC Buffer at **4°C** for up to **24 h**.

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Step 3

Probe Release & Extension

3.1 RNA Digestion & Probe Release
3.2 Probe Extension
3.3 Probe Elution

3

Get Started

3.0 Probe Release & Extension

	Item	10x PN	Preparation & Handling	Storage
Equilibrate to room temperature				
☐ 🟢	**Extension Buffer**	2000409/ 2000408	Thaw at room temperature, vortex, centrifuge briefly.	–20°C
☐ 🔴	**2X RNase Buffer**	2000411/ 2000410	Thaw at room temperature, vortex, centrifuge briefly.	–20°C
☐ 🔵	**Perm Buffer B**	2000413/ 2000412	Thaw at room temperature. DO NOT vortex.	–20°C
Place on ice				
☐ 🟢	**Extension Enzyme**	2000427/ 2000389	Pipette mix, centrifuge briefly. Maintain on ice until ready to use.	–20°C
☐ 🔵	**Perm Enzyme B**	3000602/ 3000553	Pipette mix, centrifuge briefly. Maintain on ice until ready to use.	–20°C
☐ 🔴	**RNase Enzyme**	3000605/ 3000593	Pipette mix, centrifuge briefly. Maintain on ice until ready to use.	–20°C
Obtain				
☐	**Nuclease-free Water**	-		Ambient
☐	**Tris 1 M, pH 7.0** *(Tris-HCl)*	-	Manufacturer's recommendations.	Ambient
☐	**2X SSC Buffer**	-	Prepared at step 2.1b.	Ambient
☐	**8 M KOH Solution**	-	Manufacturer's recommendations.	Ambient
☐	**Visium Slide Seals**	2000284/ 2000283	See Tips & Best Practices.	Ambient

3.1 RNA Digestion & Probe Release

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

Lid Temperature	Reaction Volume	Run Time
37°C (lid may be turned off if the instrument doesn't enable 37°C)	100 µl	~ 70 min

Step	Temperature	Time
Pre-equilibrate	37°C	Hold
RNA Digestion	37°C	00:30:00
Hold	37°C	Hold
Permeabilization	37°C	00:40:00

b. Prepare RNase Mix shortly before using. Vortex and centrifuge briefly.

RNase Mix *Maintain on ice*	10x PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Nuclease-free Water	-	33.0	145.2	290.4
2X RNase Buffer	2000411/ 2000410	37.5	165.0	330.0
RNase Enzyme	3000605/ 3000593	4.5	19.8	39.6
Total	-	**75.0**	**330.0**	**660.0**

c. Using a pipette, remove all 2X SSC Buffer from the wells. If the slide was stored overnight, remove the Visium Slide Seal before removing the 2X SSC Buffer.

d. Add **75 µl** RNase Mix to each well. Gently tap the cassette to ensure uniform coverage of the Capture Area.

e. Apply a new Visium Slide Seal on the Visium Cassette and place on the Thermocycler Adaptor on the pre-heated thermal cycler. Close the thermal cycler lid. Discard old slide seals.

f. Skip Pre-equilibrate step to initiate RNA Digestion.

g. Prepare Permeabilization Mix shortly before using and pipette mix 10x. DO NOT vortex.

Permeabilization Mix *Maintain at room temperature*	10x PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Perm Buffer B	2000413/ 2000412	70.3	309.4	618.8
Perm Enzyme B	3000602/ 3000553	4.7	20.6	41.2
Total	-	**75.0**	**330.0**	**660.0**

h. After the RNA Digestion is complete, remove the Visium Cassette from the Thermocycler Adaptor and place on a flat, clean work surface.

i. Remove the Visium Slide Seal and using a pipette, remove all RNase Mix from the wells.

j. Add **75 µl** Permeabilization Mix to each well. Gently tap the cassette to ensure uniform coverage of the Capture Area.

k. Apply a new Visium Slide Seal on the Visium Cassette and place on the Thermocycler Adaptor on the pre-heated thermal cycler. Close the thermal cycler lid. Discard old slide seals.

l. Skip Hold step to initiate Permeabilization.

m. After the permeabilization is complete, remove the Visium Cassette from the Thermocycler Adaptor and place on a flat, clean work surface.

n. Remove the Visium Slide Seal and using a pipette, remove all Permeabilization Mix from the wells.
The tissue might disintegrate during Permeabilization. This is normal and does not affect performance.

o. Add **175 µl** 2X SSC Buffer prepared at step 2.1b to the each well.

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

q. **Repeat** o-p one more time.

r. Add **175 µl** 2X SSC Buffer to the each well and proceed **immediately** to Probe Extension.

3.2 Probe Extension

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

Lid Temperature	Reaction Volume	Run Time
45°C (lid may be turned off if the instrument doesn't enable 45°C)	100 µl	15 min

Step	Temperature	Time
Pre-equilibrate	45°C	Hold
Probe Extension	45°C	00:15:00
Hold	4°C	Hold

b. Prepare Probe Extension Mix shortly before using. Vortex and centrifuge briefly.

Probe Extension Mix *Maintain on ice*	10x PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Extension Buffer	2000409/ 2000408	73.5	323.4	646.8
Extension Enzyme	2000427/ 2000389	1.5	6.6	13.2
Total		**75.0**	**330.0**	**660.0**

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

d. Add **75 µl** Probe Extension Mix to each well. Gently tap the cassette to ensure uniform coverage of the Capture Area.

e. Apply a new Visium Slide Seal on the Visium Cassette and place on the Thermocycler Adaptor on the pre-heated thermal cycler. Close the thermal cycler lid. Discard old slide seals.

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

STOP

g. After the Probe Extension is complete, **immediately** proceed to next step or store slides at **4°C** for up to **72 h**. DO NOT remove the Visium Slide Seal during storage.

3.3 Probe Elution

a. Prepare 0.08 M KOH Mix. Vortex and centrifuge briefly.

KOH Mix *Maintain at room temperature*	Stock	Final	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
KOH	8 M	0.08 M	0.4	1.8	3.5
Nuclease-free Water	-	-	39.6	174.2	348.5
Total	-		**40.0**	**176.0**	**352.0**

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

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

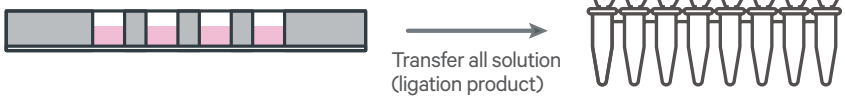
d. Add **100 µl** 2X SSC Buffer prepared at step 2.1b to each well.

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

f. Add **40 µl** 0.08 M KOH Mix to each well. Gently tap the cassette to ensure uniform coverage of the Capture Area.

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

h. Transfer all solution containing the ligation product to an 8-tube strip. DO NOT leave behind any solution in the wells. The solution might contain tissue pieces. Transfer all the solution even if there is tissue.
See Tips & Best Practices for reagent removal instructions.



i. Add **5 µl** 1 M Tris-HCl pH 7.0 to the solution in the 8-tube strip. Vortex, centrifuge briefly, and place on ice.

STOP

j. Proceed to next step or store at **-20°C** for up to **72 h.**

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Step 4

Visium Spatial Gene Expression – FFPE Library Construction

4.1 Cycle Number Determination – qPCR

4.2 Sample Index PCR

4.3 Post Sample Index PCR Cleanup – SPRIselect

4.4 Post Library Construction QC

Get Started

4.0 Visium Spatial Gene Expression – FFPE Library Construction

		Item	10x PN	Preparation & Handling	Storage
		Equilibrate to room temperature			
☐		**Dual Index Plate TS Set A**	3000511	Thaw at room temperature, vortex, and centrifuge briefly.	–20°C
☐	●	**TS Primer Mix A**	2000447	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 kit** *If used for QC*	-	Manufacturer's recommendations.	-
		Place on ice			
☐	◯	**Amp Mix**	2000047/ 2000113	Vortex, centrifuge briefly.	–20°C
☐		**KAPA SYBR FAST qPCR Master Mix**	-	Manufacturer's recommendations.	-
		Obtain			
☐		**Nuclease-free Water**	-	-	Ambient
☐		**Qiagen Buffer EB**	-	Manufacturer's recommendations.	Ambient
☐		**80% Ethanol**	-	Prepare fresh.	Ambient
☐		**10x Magnetic Separator**	230003	See Tips & Best Practices.	Ambient

4.1 Cycle Number Determination – qPCR

a. Prepare qPCR Mix on ice. Vortex and centrifuge briefly.

qPCR Mix *Add reagents in the order listed. Maintain on ice.*	Stock	Final	1X (µl)	5X* + 10% (µl) *Includes 1 negative control	9X* + 10% (µl)
Nuclease-free Water	-	-	4.0	22.0	39.6
KAPA SYBR FAST qPCR Master Mix *Minimize light exposure*	2X	1X	5.0	27.5	49.5
TS Primer Mix A (PN-2000447)	-	-	1.0	5.5	9.9
Total			**10.0**	**55.0**	**99.0**

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

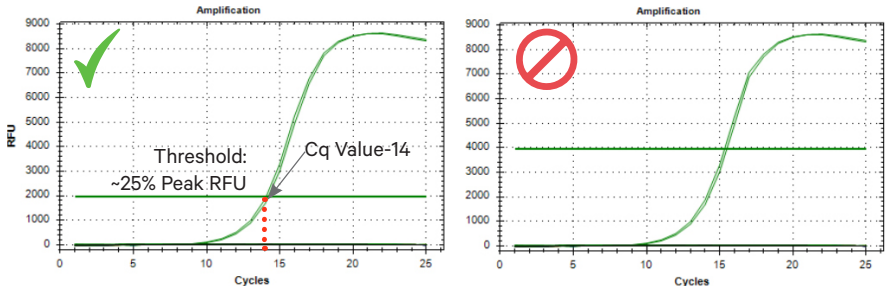
c. Transfer **1 µl** sample to the qPCR plate well containing the qPCR Mix. Pipette mix, centrifuge briefly. If using a negative control, add **1 µl** nuclease-free water to the corresponding well. Briefly centrifuge.

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

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

e. Record the Cq Value for each sample. The threshold for determining the Cq Value should be set along the exponential phase of the amplification plot, at ~25% of the peak fluorescence value.

Representative qPCR Amplification Plots



4.2 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-1000251/PN-3000511 Dual Index Kit/Plate TS Set A well ID) used.

b. Add **50 µl** Amp Mix (PN-2000047 or 2000131) to ~**45 µl** sample.

c. Add **5 µl** of an individual Dual Index TS Set A to each well and record the well ID used. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.

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

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25-40 min
Step	**Temperature**	**Time**
1	98°C	00:01:00
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 as the total # of cycles. See table below for total # of cycle examples	
6	72°C	00:01:00
7	4°C	Hold

Example: Cycle number examples determined based on rounding the Cq Value

Cq Value from qPCR	Total Cycles
12.2	12
13.5	14
19.7	20

STOP

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

4.3 Post Sample Index PCR Cleanup – SPRIselect

a. Vortex to resuspend the SPRIselect reagent. Add **85 µl** SPRIselect Reagent **(0.85X)** to each sample. Pipette mix 15x (pipette set to 180 µl).

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

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

d. Remove the supernatant.

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

f. Remove the ethanol.

g. **Repeat** steps e and f for a total of 2 washes.

h. Centrifuge briefly. Place on the magnet•**Low**. Remove remaining ethanol. Air dry for **2 min**. DO NOT exceed 2 min as this will decrease elution efficiency.

i. Remove from the magnet. Add **25.5 µl** Buffer EB. Pipette mix 15x.

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

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

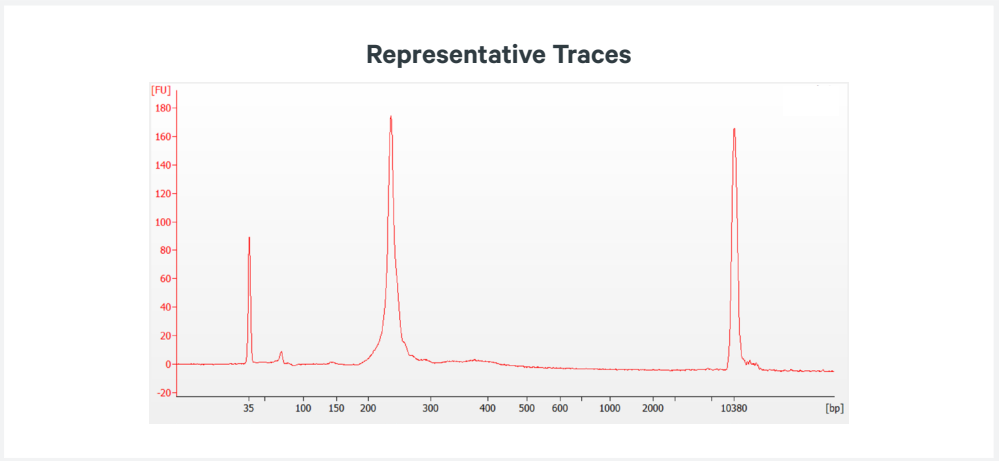
l. Transfer **25 µl** to a new tube strip.

STOP

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

4.4 Post Library Construction QC

a. Run **1 µl** of sample (1:5 dilution) on an Agilent Bioanalyzer High Sensitivity chip.



b. Determine the average fragment size from the Bioanalyzer trace. This will be used as the insert size for library quantification.

Alternate QC Method:

- Agilent TapeStation
- LabChip

See Appendix for representative traces

See Appendix for Post Library Construction Quantification

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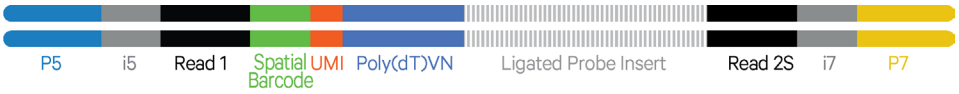
Sequencing

5

Sequencing Libraries

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

Visium Spatial Gene Expression – FFPE Library



Sequencing Depth

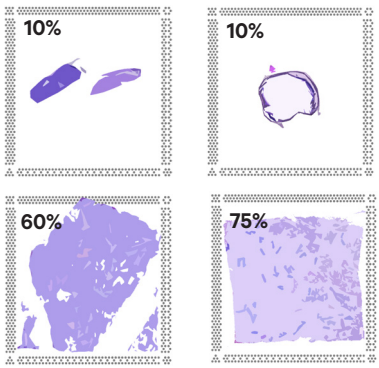
Sequencing Depth/spot	Minimum 25,000 read pairs per tissue covered spot on Capture Area
Sequencing Depth/sample	See example calculation below

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

Estimated Coverage Area (%) Examples



Sequencing Type & Run Parameters

Use the sequencing run type and parameters indicated.

Dual Index Library	
Paired-end, dual indexed sequencing	
Read 1: 28 cycles i7 Index: 10 cycles i5 Index: 10 cycles Read 2S: 50 cycles*	**Visium Spatial Gene Expression – FFPE libraries may be pooled with Visium Spatial Gene Expression libraries generated from fresh frozen samples. In that case, use 90 cycles for Read 2S. If pooling the two different library types, Visium Gene Expression - FFPE libraries should not be more than 40% of the pool.*

Illumina Sequencer Compatibility

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

- MiSeq
- NextSeq 500/550
- NextSeq 2000
- NovaSeq
- iSeq

Sample Indices

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

Library Loading

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

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

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

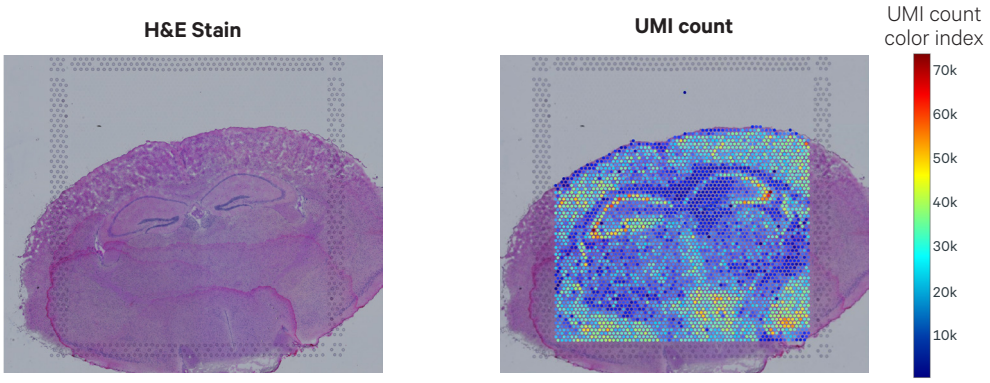
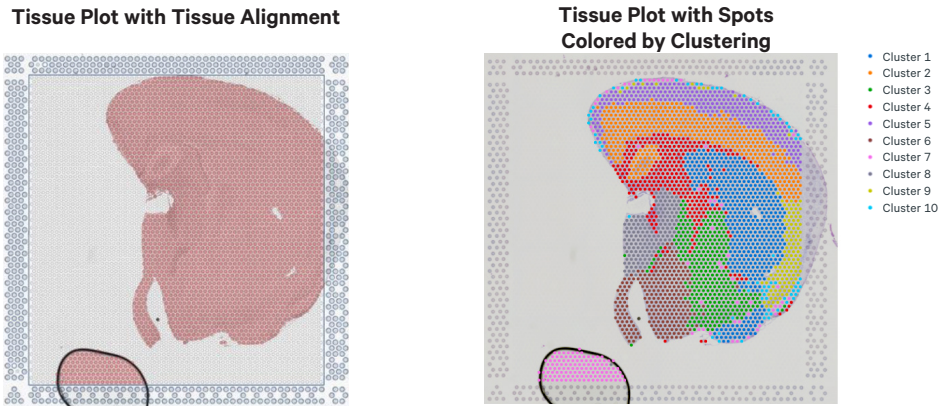
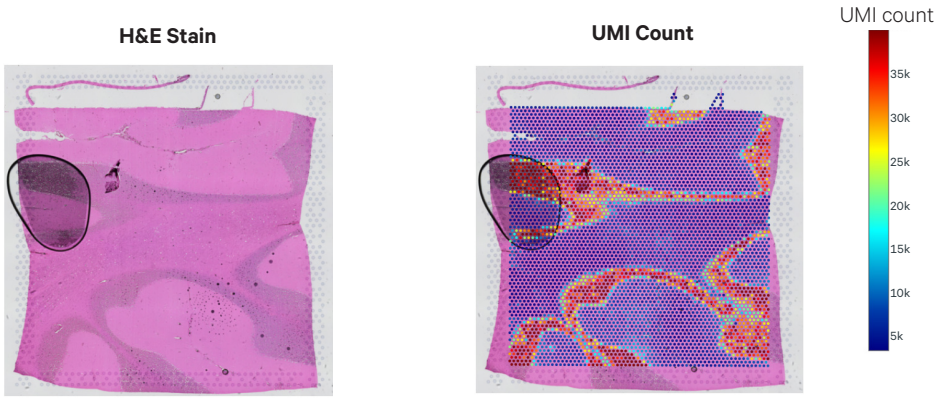
Library Pooling

Pooling Visium Spatial Gene Expression – FFPE Libraries

The Visium Spatial Gene Expression – FFPE 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.

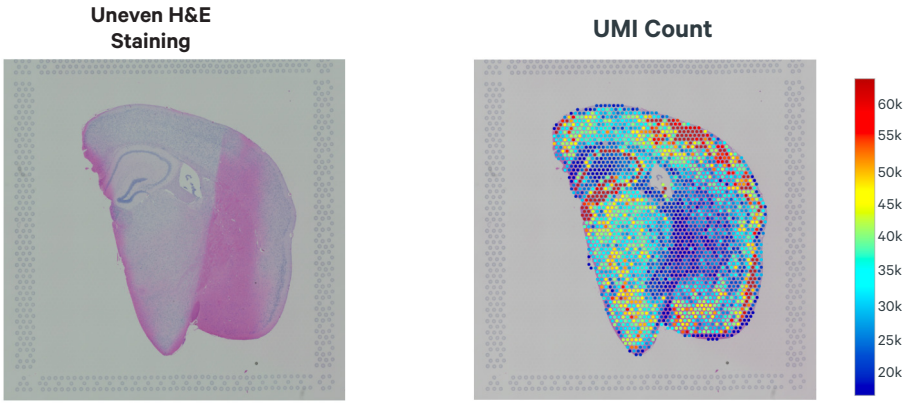
Troubleshooting

6

STEP	NOTES
Tissue Placement – Impact on UMI Count	**Fiducials are obstructed**  Fiducial obstruction may result in image analysis failure. Placement must be correct before the workflow begins. If necessary, software will prompt users to manually align tissue images during analysis.
Bubbles during Coverslipping	 A bubble could be generated during coverslipping. Software may identify it as tissue and a cluster may be associated with it. In that case, perform manual alignment and identification of the tissue.
Bubbles during Coverslipping	 A bubble could be generated during coverslipping. 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 region can still be analyzed.

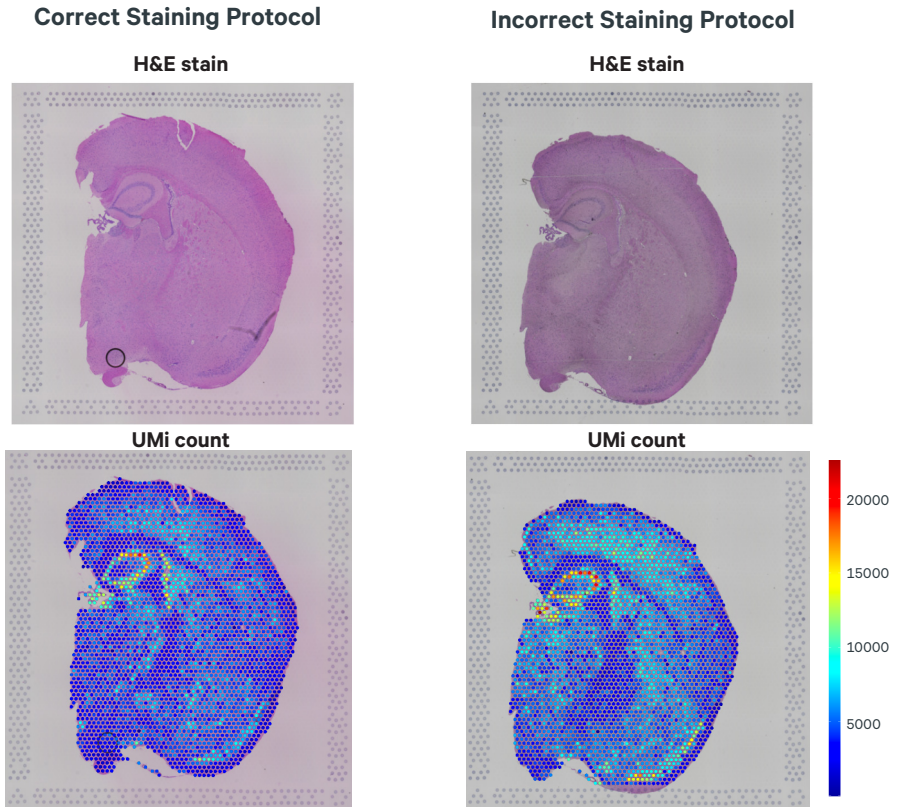
STEP	NOTES

Uneven Tissue Staining – Impact on UMI Count



Ensure that staining reagents are applied to the tissue uniformly and adequate washes are performed. A gentle tap may help spread the reagent uniformly. Uneven staining does not diminish sensitivity and spatial resolution, and the data derived from the unevenly stained tissue portions can still be analyzed.

Incorrect Staining Protocol – Impact on UMI Count

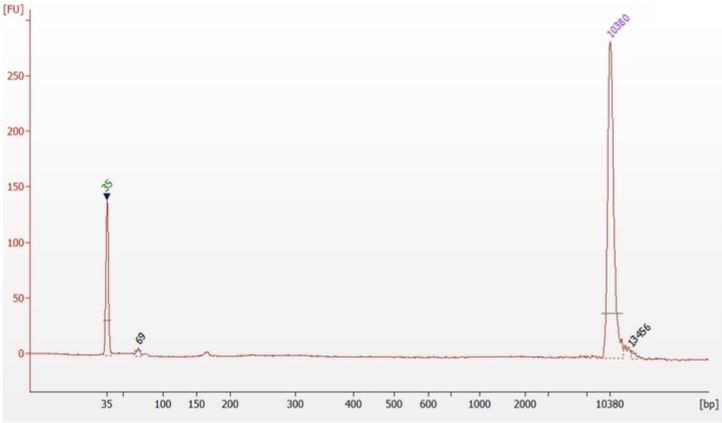


Ensure the correct staining protocol is followed. If the staining protocol recommended in Demonstrated Protocol Methanol Fixation, H&E Staining & Imaging for Visium Spatial Protocols (CG000160) is followed, lower quality images will be obtained. However, incorrect staining does not diminish sensitivity and spatial resolution, and the data derived from the unevenly stained tissue portions can still be analyzed.

STEP	NOTES

Flat Line in BioAnalyzer Library Trace

Failure to neutralize the KOH after probe elution from the slide, will result in a normal qPCR output but no peak will be visible in the BioAnalyzer trace.



Number of Washes

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

Wash	Number of Washes	Fraction Targeted Reads Usable (Mean)
Post Hybridization Wash	1	0.2905
	2	0.4125
	3	0.7895
Post Ligation Wash	1	0.6925
	2	0.7475

Tissue Detachment

Tissue detachment can result in a loss of gene expression complexity and spatiality in Visium Spatial Gene Expression – FFPE libraries. If tissue detachment is observed during the workflow, contact support@10xgenomics.com.

Appendix

Post Library Construction Quantification

Agilent TapeStation Traces

LabChip Traces

Assay Scheme and Sequences

7

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. Incubate in a thermal cycler with the following protocol.

Step	Temperature	Run 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.

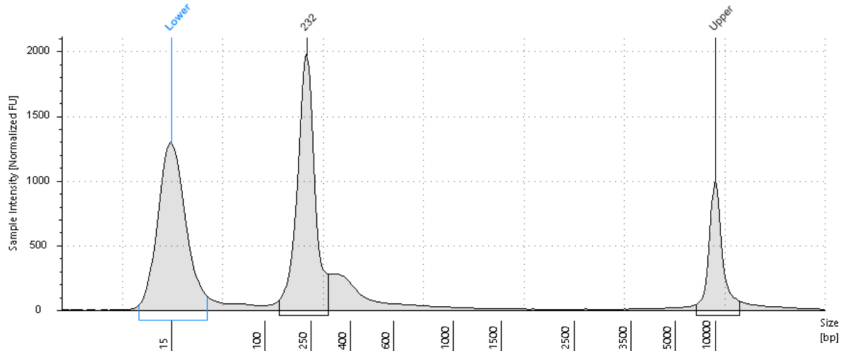
Agilent TapeStation Traces

Agilent TapeStation High Sensitivity D5000 ScreenTape was used. Protocol steps correspond to the Visium Spatial Gene Expression for FFPE User Guide (CG000407).

Protocol Step 4.4 – Post Library Construction QC

Representative Trace

Run 2 µl diluted sample (1:5 dilution) mixed with 2 µl loading buffer.



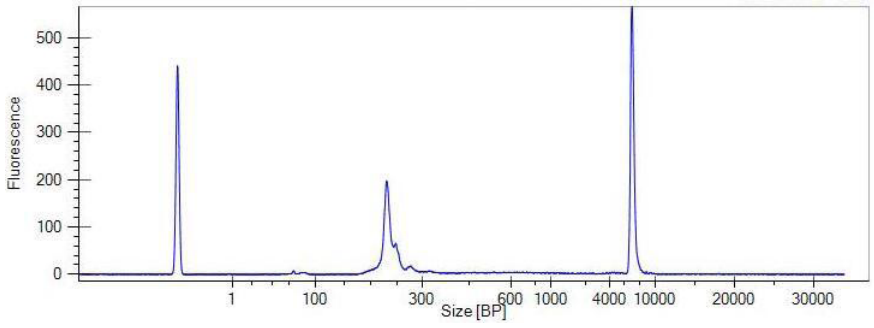
LabChip Traces

DNA High Sensitivity Reagent Kit was used.
Protocol steps correspond to the Visium Spatial Gene Expression for FFPE User Guide (CG000407).

Protocol Step 4.4 – Post Library Construction QC

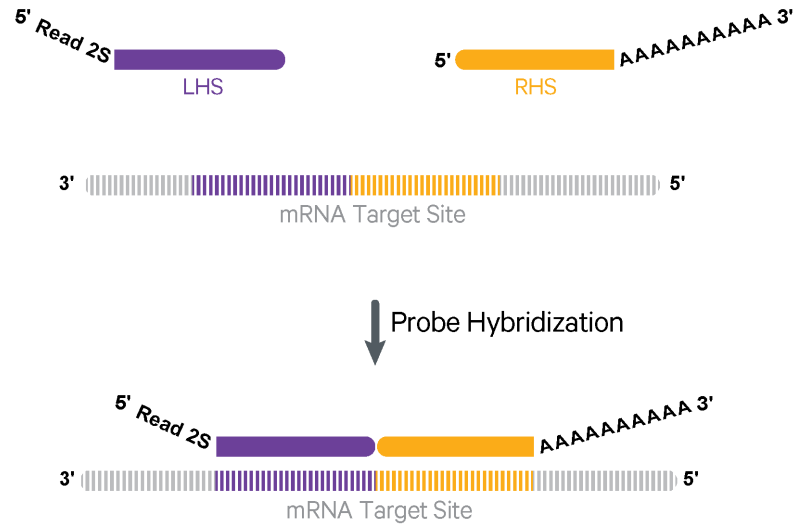
Representative Trace

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

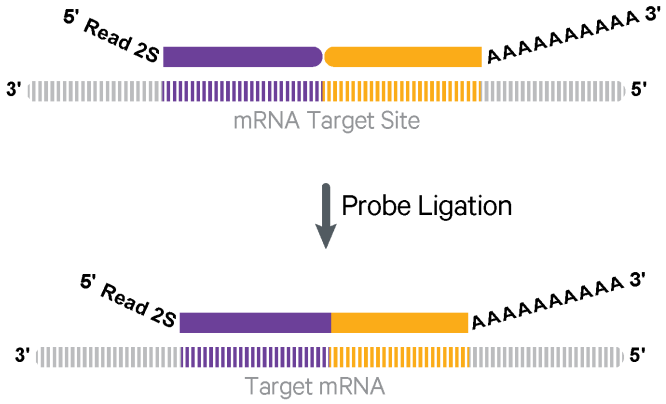


Assay Scheme

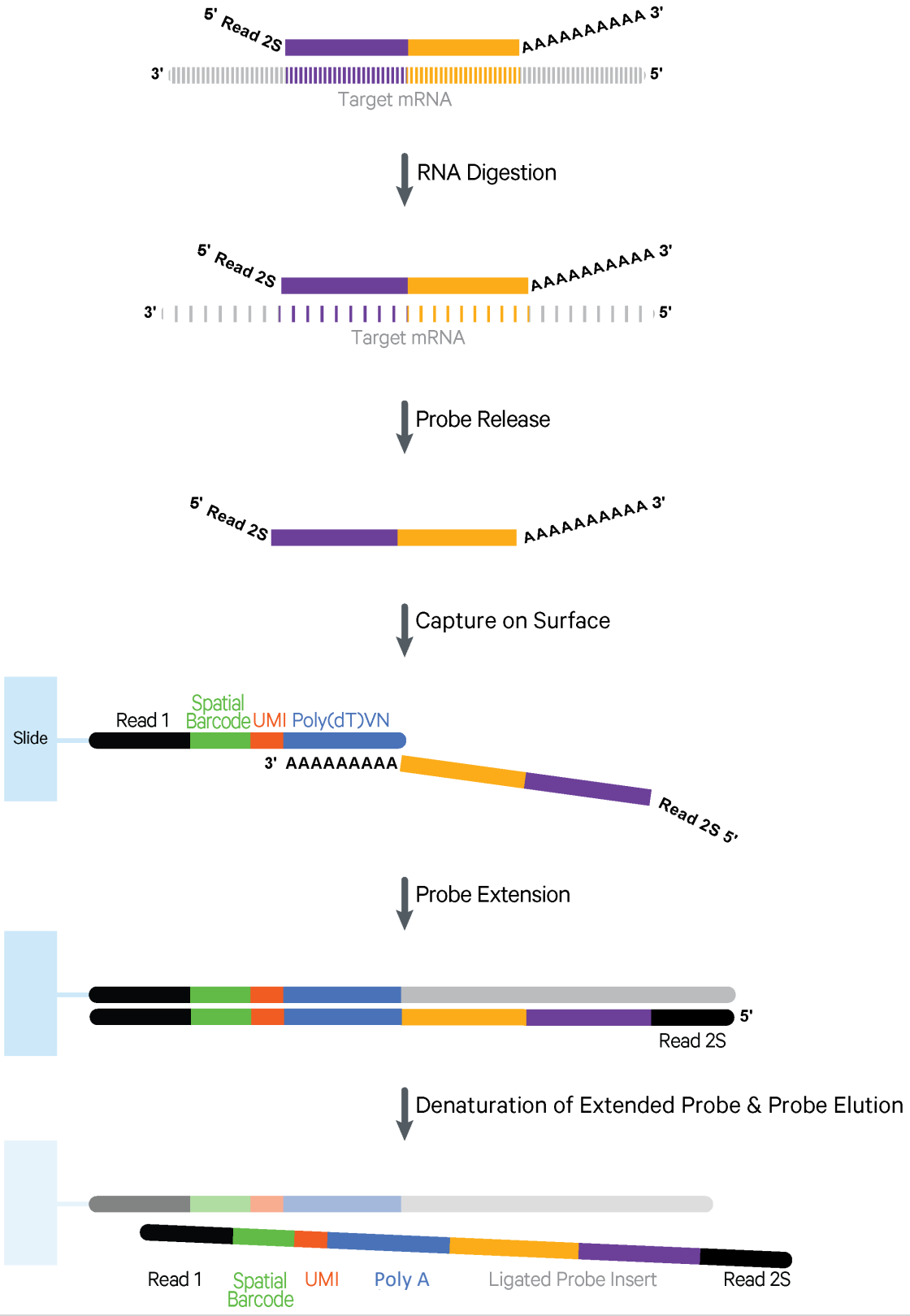
Probe Hybridization



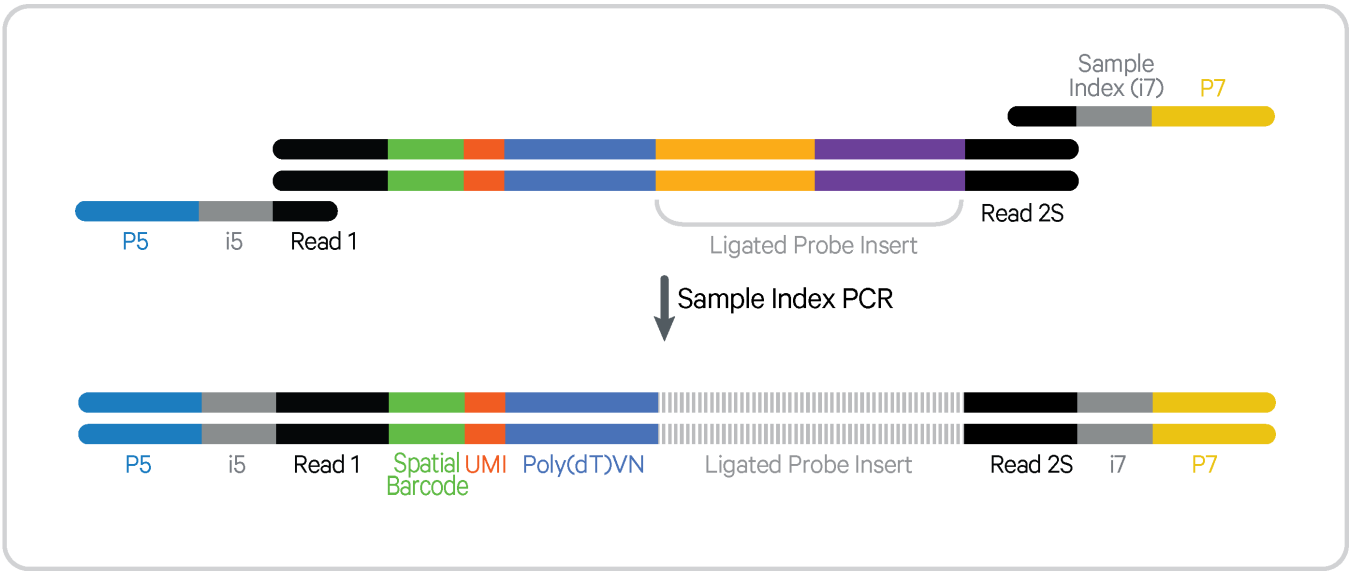
Probe Ligation



Probe Release & Extension



Visium Spatial Gene Expression – FFPE Library Construction

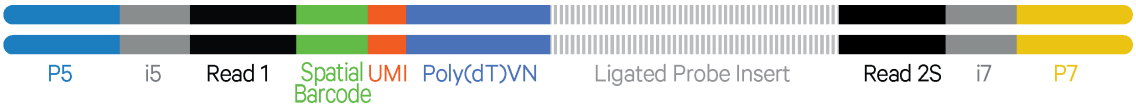


Sequences

Slide Primers

5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'

Visium Spatial Gene Expression – FFPE Library



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