CG000239 Rev C

USER GUIDE

Visium Spatial Gene Expression Reagent Kits

FOR USE WITH

Visium Spatial Gene Expression Slide & Reagent Kit, 16 rxns PN-1000184
Visium Spatial Gene Expression Slide & Reagent Kit, 4 rxns PN-1000187
Visium Accessory Kit, PN-1000194
Dual Index Kit TT Set A, 96 rxns PN-1000215

10x GENOMICS

Notices

Document Number

CG000239 • 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	CG000239
Title	Visium Spatial Gene Expression Reagent Kits - User Guide
Revision	Rev C
Revision Date	June 2020

Specific Changes:

- The Tissue Fixation & Staining protocol steps have been removed from the document and are now available in the listed Demonstrated Protocols on the 10x Genomics Support Website:

  Methanol Fixation, H&E Staining & Imaging for Visium Spatial Protocols (Demonstrated Protocol CG000160)

  Methanol Fixation, Immunofluorescence Staining & Imaging for Visium Spatial Protocols (Demonstrated Protocol CG000312)

- Practice glass slide information provided in Additional Kits, Reagents & Equipment table (Shandon ColorFrost Plus Slides 25 x 75 x1 mm).
- Updated sequencing parameter: 90 Read 2 cycles for sequencing Visium Spatial Gene Exression Library.

General Changes:

- Updated step numbers throughout due to removal of fixation and staining steps from this document.
- Updated for general minor consistency of language and terms throughout.

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Visium Spatial Gene Expression Reagent Kits

Visium Spatial Gene Expression Slide & Reagent Kit, 16 rxns PN-1000184

Visium Spatial Gene Expression Reagent Kit, 16 rxns PN-1000186 (store at –20°C)

Visium
Spatial Gene Expression Reagent Kit

	#	PN
Permeabilization Enzyme	1	2000214
RT Reagent	1	2000086
RT Enzyme D	1	2000216
Template Switch Oligo	2	3000228
Reducing Agent B	1	2000087
Second Strand Primer	1	2000217
Second Strand Enzyme	1	2000218
Second Strand Reagent	1	2000219
cDNA Primers	1	2000089
Amp Mix	1	2000047

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Library Construction Kit, 16 rxns PN-1000190 (store at –20°C)

Library Construction Kit

	#	PN
Fragmentation Buffer	1	2000091
Fragmentation Enzyme	1	2000090
Ligation Buffer	1	2000092
DNA Ligase	1	220110
Adaptor Oligos	1	2000094
Amp Mix	1	2000047

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

Visium
Spatial Gene Expression Slide Kit

	#	PN
Visium Spatial Gene Expression Slide	4	2000233
Slide Seal	20	3000279
Slide Cassette	4	3000406
Slide Gasket	4	3000426

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Visium Spatial Gene Expression Reagent Kits

Visium Spatial Gene Expression Slide & Reagent Kit, 4 rxns PN-1000187

Visium Spatial Gene Expression Reagent Kit, 4 rxns PN-1000189 (store at –20°C)

Visium
Spatial Gene Expression Reagent Kit

	#	PN
Permeabilization Enzyme	1	2000214
RT Reagent	1	2000086
RT Enzyme D	1	2000227
Template Switch Oligo	1	3000228
Reducing Agent B	1	2000087
Second Strand Primer	1	2000217
Second Strand Enzyme	1	2000183
Second Strand Reagent	1	2000219
cDNA Primers	1	2000089
Amp Mix	1	2000103

10xGenomics.com

Library Construction Kit, 4 rxns PN-1000196 (store at –20°C)

Library Construction Kit

	#	PN
Fragmentation Buffer	1	2000091
Fragmentation Enzyme	1	2000104
Ligation Buffer	1	2000092
DNA Ligase	1	220131
Adaptor Oligos	1	2000094

10xGenomics.com

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

Visium
Spatial Gene Expression Slide Kit

	#	PN
Visium Spatial Gene Expression Slide	1	2000233
Slide Seals	5	3000279
Slide Cassette	1	3000406
Slide Gasket	1	3000426

10xGenomics.com

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

Dual Index Kit TT Set A

	#	PN
Dual Index Plate TT Set A	1	3000431

Visium 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

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. Hardware compatibility may be tested by using the Visium Spatial Imaging Test Slide. Consult the Visium Spatial Gene Expression Imaging Guidelines Technical Note (CG000241) for more information.

Imaging Systems & Specifications	
Microscopes (Any equivalent system with the listed features may be used for imaging)	
Nikon	Nikon Eclipse Ti2 with brightfield and fluorescence capacity (TRITC)
Molecular Devices	ImageXpress Nano Automated Slide Imaging System
Hamamatsu	NanoZoomer S60
Keyence	Keyence BZX800
BioTek	Cytation 7
Thermo Fisher Scientific	EVOS M7000
Leica	Leica DMi8 Versa 8
Microscope Features	
Objectives	• 4X (Plan APO λ; NA 0.20) • 10X (Plan APO λ; NA 0.45) • 20X (Plan APO λ; NA 0.75)
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 only)	• 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*	• 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) • Cy5 filter cube (Excitation 618/50, Emission 698/70) • TRITC filter cube (Excitation 542/20, Emission 620/52) (required for Immunofluorescence Staining & Tissue Optimization protocols only) • Minimum Capture Resolution 2.18 µm/pixel • Exposure times 100 milli sec-2 sec

* Only required for Visium Spatial Tissue Optimization protocol & Visium Imaging Test Slide verification and if performing Immunofluorescence Staining prior to Tissue Optimization and Gene Expression protocols.

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 mm 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 artifact.

Additional Kits, Reagents & Equipment

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

Supplier	Description	Part Number (US)
Plastics		
Eppendorf	PCR Tubes 0.2 ml 8-tube strips DNA LoBind Tubes, 1.5 ml DNA LoBind Tubes, 2.0 ml (when processing more than 2 slides)	951010022 022431021 022431048
USA Scientific	TempAssure PCR 8-tube strip	1402-4700
Thermo Fisher Scientific	MicroAmp 8-Tube Strip, 0.2 ml MicroAmp 8 -Cap Strip, clear Simport Scientific LockMailer Tamper Evidence Slide Mailer (alternatively, use a 50-ml centrifuge tube)	N8010580 N8010535 22-038-399
Corning	Self-Standing Polypropylene Centrifuge Tubes (50 ml), sterile	430921
Bio-Rad	Hard-shell PCR Plates 96-well, thin wall (pkg of 50) (alternatively, use any compatible PCR Plate) Microseal 'B' PCR Plate Sealing Film, adhesive (alternatively, use any PCR Plate sealing adhesive)	HSP9665 MSB1001
Rainin	Tips LTS 200UL Filter RT-L200FLR Tips LTS 1ML Filter RT-L1000FLR Tips LTS 20UL Filter RT-L10FLR	30389240 30389213 30389226
VWR	Divided Polystyrene Reservoirs	41428-958
Kits & Reagents		
Thermo Fisher Scientific	Nuclease-free Water Low TE Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) Tris 1M, pH 7.0, RNase-free Universal Mouse Reference RNA* (Optional. Alternatively, use any bulk Total RNA. 1µg/µl, RIN ≥ 7) Shandon ColorFrost Plus Slides 25 x 75 x1 mm (Optional)	AM9937 12090-015 AM9850G QS0640 6776214
Fisher Chemical	Hydrochloric Acid Solution, 0.1N	SA54-1
KAPA Biosystems	KAPA SYBR FAST qPCR Master Mix (2X)	KK4600
Beckman Coulter	SPRIselect Reagent Kit	B23318

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

Additional Kits, Reagents & Equipment

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Supplier	Description	Part Number (US)
Kits & Reagents		
Millipore Sigma	Ethanol, Pure (200 Proof, anhydrous) Potassium Hydroxide Solution, 8M SSC Buffer 20X Concentrate Sodium dodecyl sulfate (SDS) solution, 10% in water*	E7023-500ML P4494-50ML S66391L 71736
Qiagen	Qiagen Buffer EB	19086
-	Ultrapure/Milli-Q water (from Milli-Q Integral Ultrapure Water System or equivalent)	
Equipment		
Rainin	Pipet-Lite Multi Pipette L8-200XLS+ Pipet-Lite LTS Pipette L-2XLS+ Pipet-Lite LTS Pipette L-10XLS+ Pipet-Lite LTS Pipette L-20XLS+ Pipet-Lite LTS Pipette L-100XLS+ Pipet-Lite LTS Pipette L-200XLS+ Pipet-Lite LTS Pipette L-1000XLS+	17013805 17014393 17014388 17014392 17014384 17014391 17014382
VWR	VWR Mini Centrifuge (alternatively, use any equivalent mini centrifuge)	76269-064
Quantification & Quality Control		
Agilent	2100 Bioanalyzer Laptop Bundle High Sensitivity DNA Kit 4200 TapeStation High Sensitivity D1000 ScreenTape/Reagents High Sensitivity D5000 ScreenTape/Reagents	G2943CA 5067-4626 G2991AA 5067-5584/ 5067-5585 5067-5592/ 5067-5593
PerkinElmer	LabChip GX Touch HT Nucleic Acid Analyzer DNA High Sensitivity Reagent Kit	CLS137031 CLS760672
KAPA Biosystems	KAPA Library Quantification Kit for Illumina Platforms	KK4824

Choose Bioanalyzer, TapeStation or Lab Chip based on availability & preference.

* Only required for Visium Spatial Tissue Optimization protocol

Protocol Steps & Timing

1-1.5 days

Steps		Timing	Stop & Store
Step 1 – cDNA Synthesis			
1.1	Tissue Permeabilization	Variable	
1.2	Reverse Transcription	65 min	
Step 2 – Second Strand Synthesis & Denaturation			
2.1	Second Strand Synthesis	25 min	
2.2	cDNA Denaturation	15 min	
Step 3 – cDNA Amplification & QC			
3.1	Cycle Number Determination – qPCR	45 min	
3.2	cDNA Amplification	45-60 min	STOP 4°C ≤72 h or –20°C ≤1 week
3.3	cDNA Cleanup – SPRIselect	20 min	STOP 4°C ≤72 h –20°C ≤4 weeks
3.4	cDNA QC & Quantification	50 min	
Step 4 – Visium Spatial Gene Expression Library Construction			
4.1	Fragmentation, End Repair & A-tailing	50 min	
4.2	Post Fragmentation, End Repair & A-tailing Double Sided Size Selection – SPRIselect	30 min	
4.3	Adaptor Ligation	25 min	
4.4	Post Ligation Cleanup- SPRIselect	20 min	
4.5	Sample Index PCR	40 min	STOP 4°C ≤72 h
4.6	Post Sample Index PCR Double Sided Size Selection- SPRIselect	30 min	STOP 4°C ≤72 h or -20°C long term
4.7	Post Library Construction QC	50 min	

Stepwise Objectives

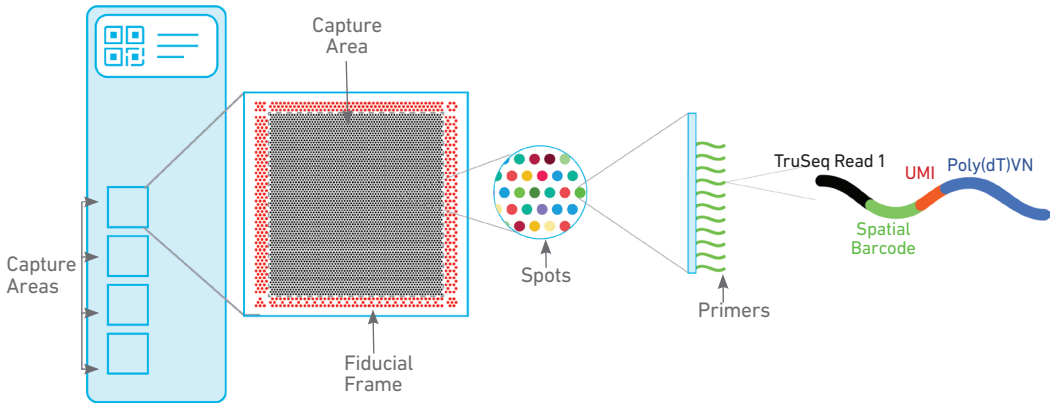
The Visium Spatial Gene Expression Solution measures total mRNA in intact tissue sections and maps the location(s) where gene activity is occurring. Each Visium Spatial Gene Expression Slide contains Capture Areas with gene expression spots that include primers required for capture and priming of poly-adenylated mRNA. Tissue sections placed on these Capture Areas are fixed and stained, as described in Tissue Fixation & Staining Demonstrated Protocols – CG000160 or CG000312, permeabilized, and cellular mRNA is captured by the primers on the gene expression spots. All the cDNA generated from mRNA captured by primers on a specific spot share a common Spatial Barcode. Libraries are generated from the cDNA and sequenced and the Spatial Barcodes are used to associate the reads back to the tissue section images for spatial gene expression mapping.

This document outlines the protocol for generating Visium Spatial Single Cell 3' Gene Expression libraries from tissue sections placed on the Capture Areas of a Visium Spatial Gene Expression Slide.

Visium Spatial Gene Expression Slide

The Visium Spatial Gene Expression Slide includes 4 Capture Areas (6.5 x 6.5 mm), each 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:

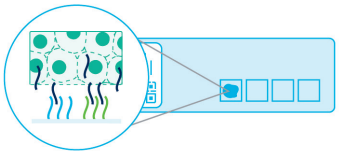
- Illumina TruSeq Read 1 (partial read 1 sequencing primer)
- 16 nt Spatial Barcode (all primers in a specific spot share the same Spatial Barcode)
- 12 nt unique molecular identifier (UMI)
- 30 nt poly(dT) sequence (captures poly-adenylated mRNA for cDNA synthesis).



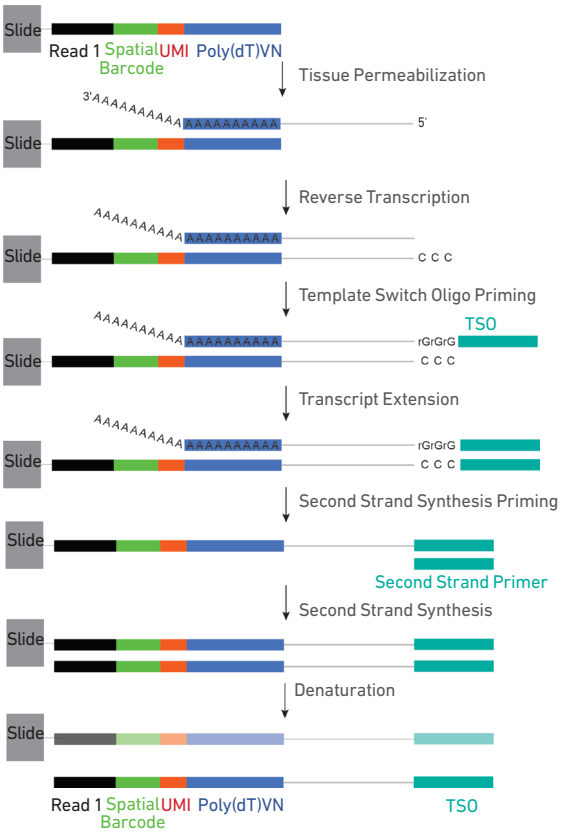
Step 1
Permeabilization & Reverse Transcription

A Permeabilization Enzyme is used to permeabilize the fixed and stained tissue sections on the slide. The poly-adenylated mRNA released from the overlying cells is captured by the primers on the spots. RT Master Mix containing reverse transcription reagents is added to the permeabilized tissue sections. Incubation with the reagents produces spatially barcoded, full-length cDNA from poly-adenylated mRNA on the slide.

Permeabilization



Reactions on slide Capture Areas



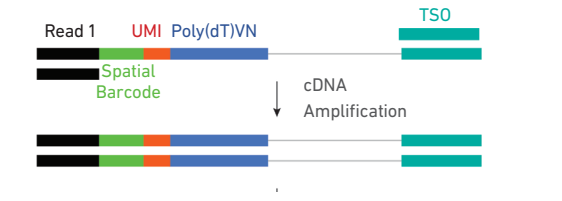
Step 2
Second Strand Synthesis & Denaturation

Second Strand Mix is added to the tissue sections on the slide to initiate second strand synthesis. This is followed by denaturation and transfer of the cDNA from each Capture Area to a corresponding tube for amplification and library construction.

Step 3
cDNA Amplification & QC

After transfer of cDNA from the slide, spatially barcoded, full-length cDNA is amplified via PCR to generate sufficient mass for library construction.

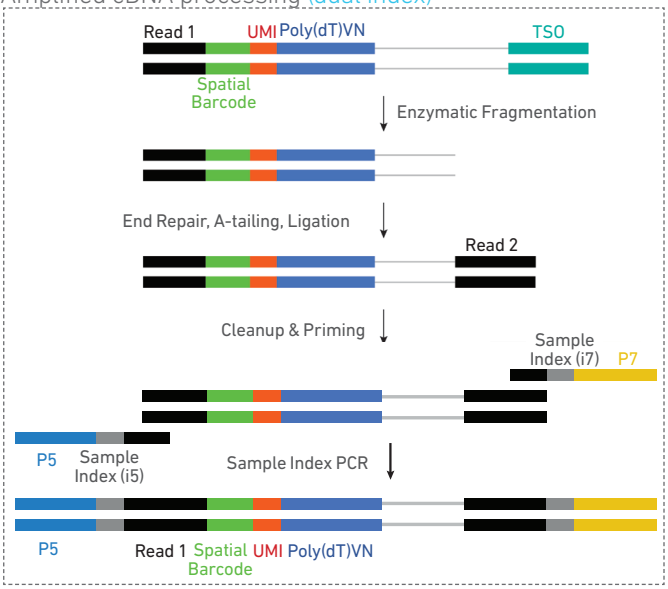
cDNA amplification



Step 4 Visium Spatial Gene Expression Library Construction

Enzymatic fragmentation and size selection are used to optimize the cDNA amplicon size. P5, P7, i7 and i5 sample indexes, and TruSeq Read 2 (read 2 primer sequence) are added via End Repair, A-tailing, Adaptor Ligation, and PCR. The final libraries contain the P5 and P7 primers used in Illumina amplification.

Amplified cDNA processing (dual index)

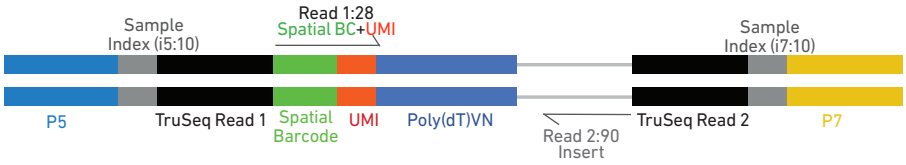


Step 5 Sequencing

A Visium Spatial Gene Expression 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 Read 2 is used to sequence the cDNA fragment. i7 and i5 sample index sequences are incorporated. TruSeq Read 1 and TruSeq Read 2 are standard Illumina sequencing primer sites used in paired-end sequencing.

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

Visium Spatial Gene Expression Library



See Appendix for Oligonucleotide Sequences

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.
- Keep all enzymes and Master Mixes on ice during setup and use. Promptly move reagents back to the recommended storage.
- Use a pH meter to adjust pH as necessary during buffer preparation.

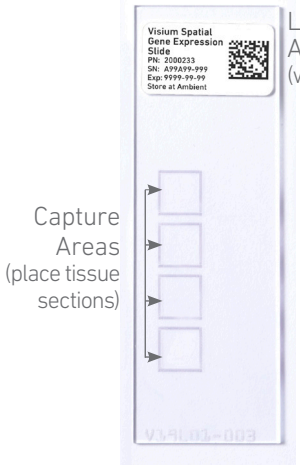
Pipette Calibration

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

Visium Spatial Gene Expression Slide

- Includes 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 Protocols – Tissue Preparation Guide (Demonstrated Protocol CG000240).

Visium Spatial Gene Expression Slide

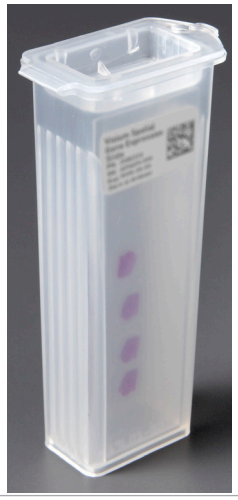


Note the serial number on the slide label; will be required for downstream analysis.

Slide Storage

- Always store slides in a cool, dry environment.
- Store unused slides in original packaging and keep sealed. DO NOT remove dessicant. If necessary, place the sealed container in a secondary container, such as a resealable bag.
- After tissue placement, store the slides at **–80°C** in a sealed container.

Slide Storage



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.
- When immersing slides in water, ensure that the tissue sections are completely submerged.
- 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.

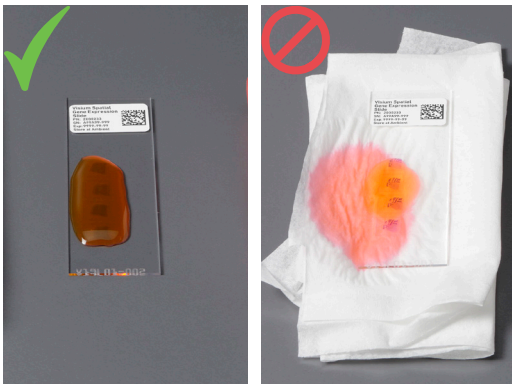
Active Surface with Tissue Sections

Immersing Slide

Correct Incorrect

Reagent on Slide

Correct Incorrect

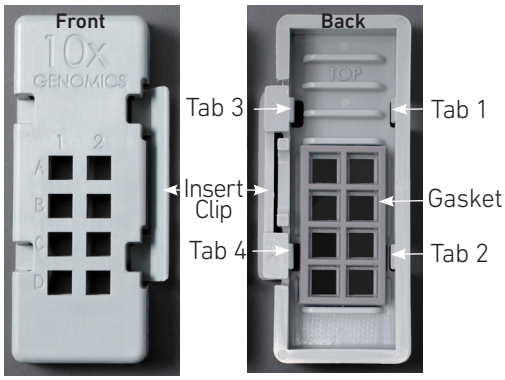


Slides in image are representative.

Slide Cassette

- The Slide Cassette encases the slide and creates leakproof wells for adding reagents.
- Place the slides in the Slide Cassette only when specified.
- The Slide Cassette includes a removable gasket.
- An Insert Clip and four tabs at the back of the Slide Cassette are used for holding the slide in the cassette, as shown.
- The removable gasket corresponds to the Capture Areas on the slides.
- The Slide Cassette may be assembled using the Slide Alignment Tool or manually. Instructions for both are provided in the following section.
- See Slide Cassette Assembly & Removal instructions for details.
- Ensure that the back of the Slide 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.

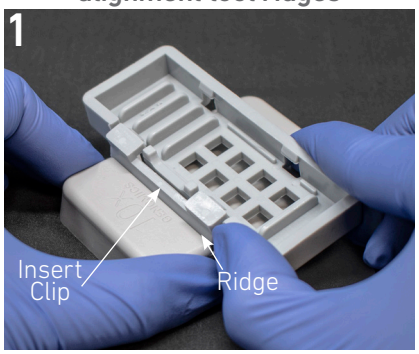
Slide Cassette



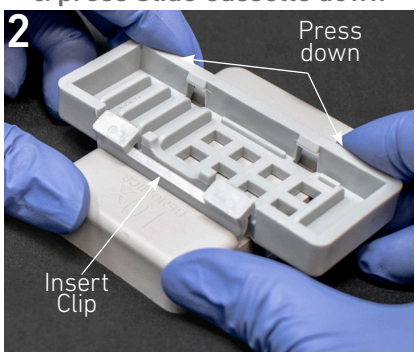
Slide Alignment Tool

Slide Cassette Assembly

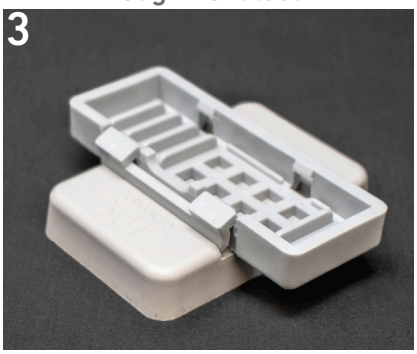
Position Slide Cassette along alignment tool ridges



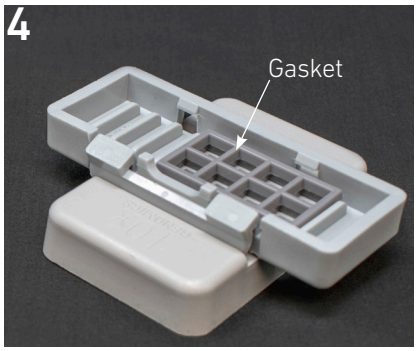
Push Insert Clip along the ridge & press Slide Cassette down



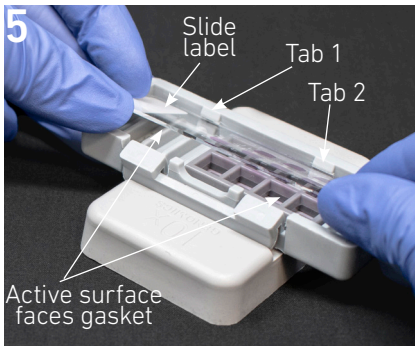
Slide Cassette secured on alignment tool



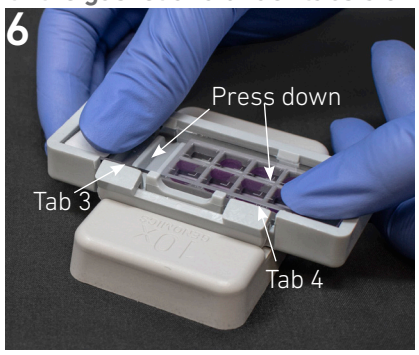
Position Gasket to align with Slide Cassette cutouts



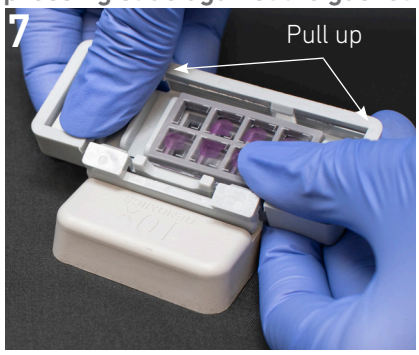
Insert long edge of slide under tabs 1 & 2; ensure slide is flush



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



Remove Slide Cassette while pressing slide against the gasket



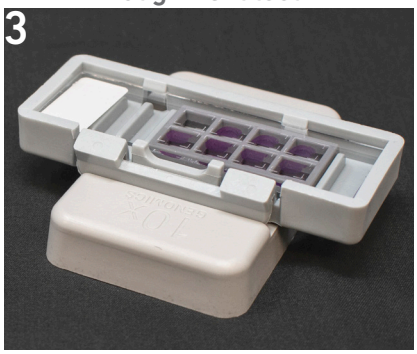
Slide insertion may push gasket out of alignment with slide cutouts. Adjust if necessary.

Slide Cassette Removal*

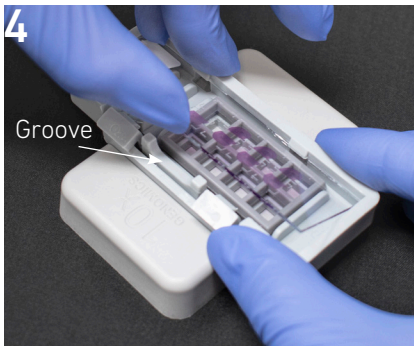
Position Slide Cassette along alignment tool ridges

Push Insert Clip along the ridge & press down

Slide Cassette Sits securely on alignment tool



Lift slide at Slide Cassette groove



*Slide removal not needed for the Visium Spatial Gene Expression protocol.

Manual Slide Cassette Assembly & Removal

Assembly

i. Remove the gasket from the Slide Cassette and re-insert the gasket, ensuring that the gasket and Slide Cassette cutouts are aligned.

ii. Align the label on top of the slide to the top of the Slide Cassette, as shown.

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

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

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

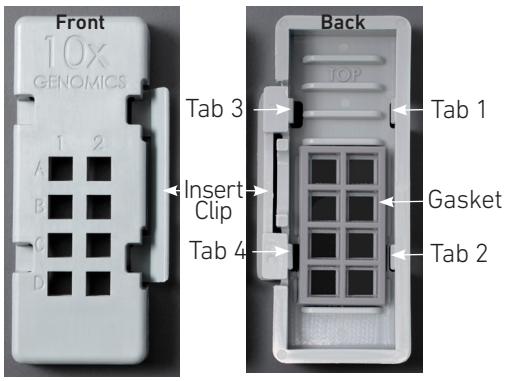
Removal*

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

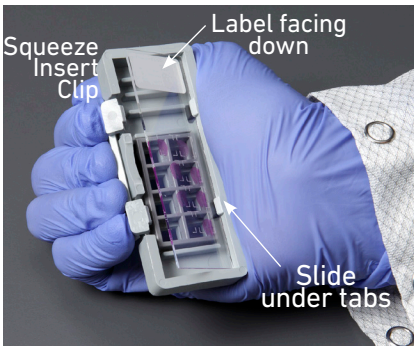
ii. Lift slide at Slide Cassette groove between tabs 3 and 4 until the slide can be removed.

*Slide removal not needed for the Visium Spatial Gene Expression protocol.

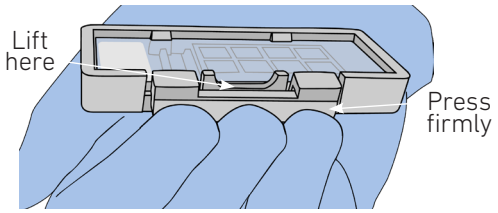
Slide Cassette



Slide Cassette Assembly



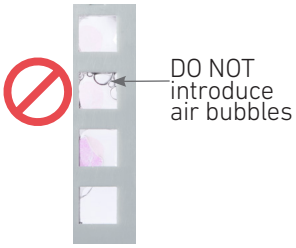
Insert Clip - Press Firmly



Reagent Addition & Removal from Wells

- Place the assembled slide in the Slide Cassette flat on a clean work surface.
- Dispense and remove 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



Slide Seal Application & Removal

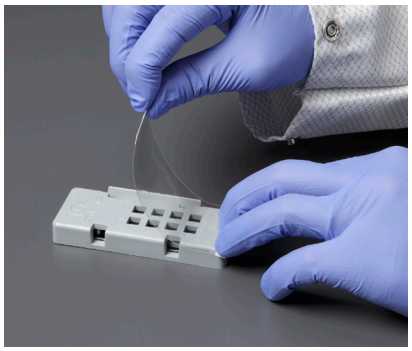
Application

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

Removal

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

Slide Seal Application



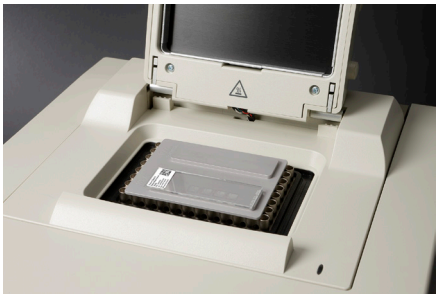
Slide Incubation Guidance

Incubation at a specified temperature

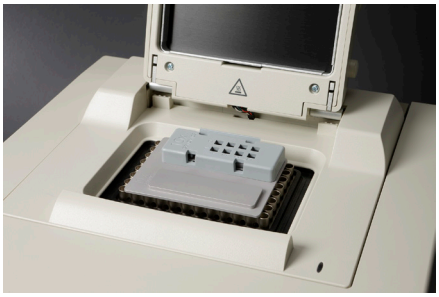
- Position a Thermocycler Adaptor on a thermal cycler that is set at the incubation temperature.
- Ensure that the Thermocycler Adaptor is in contact with the thermal cycler surface uniformly.
- When incubating a slide, position the slide on the Thermocycler Adaptor with the active surface facing up.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

Place Thermocycler Adaptor

Incubate Slide



Incubate Assembled Slide Cassette

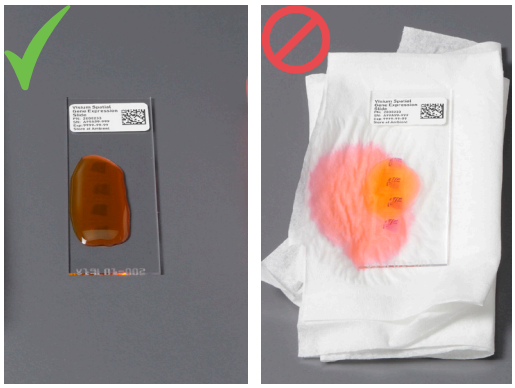


Incubation at room temperature

- Place the slide/Slide 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.

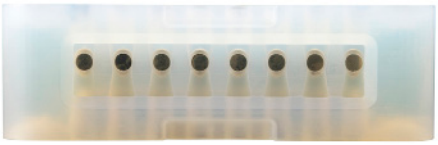
Slide Incubation

Correct Incorrect



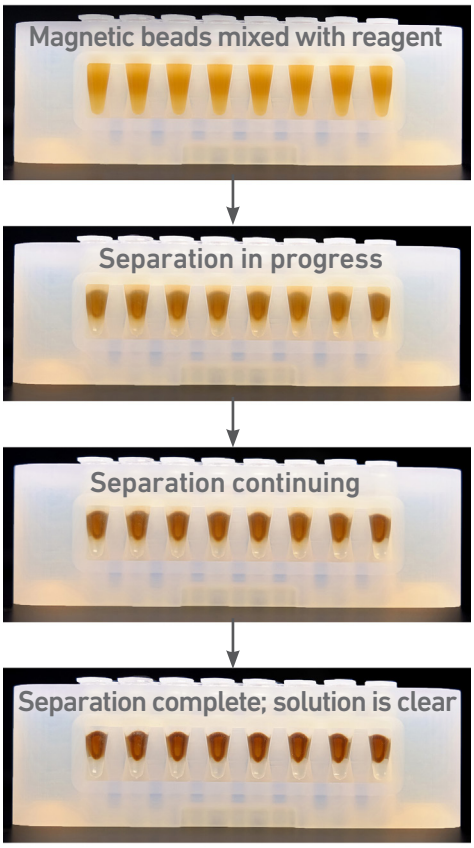
10x Magnetic Separator

- 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

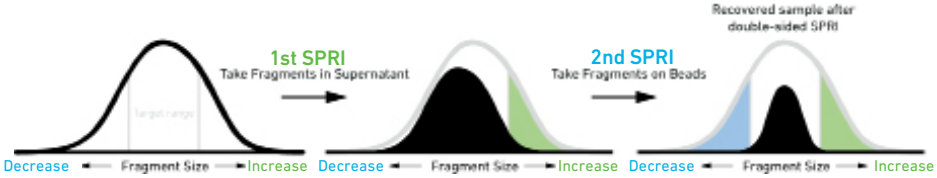
- After aspirating the desired volume of SPRIselect reagent, examine the pipette tips before dispensing to ensure the correct volume is transferred.
- Pipette mix thoroughly as insufficient mixing of sample and SPRIselect reagent will lead to inconsistent results.
- Use fresh preparations of 80% Ethanol.

Tutorial — SPRIselect Reagent:DNA Sample Ratios

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

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

Schematic of Double Sided Size Selection



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

Tutorial — Double Sided Size Selection

Step a – First SPRIselect: Add **50 µl** SPRIselect reagent to **100 µl** sample (**0.5X**).

Ratio = $\frac{\text{Volume of SPRIselect reagent added to the sample}}{\text{Volume of DNA sample}} = \frac{50\ \mu l}{100\ \mu l} = \mathbf{0.5X}$

Step b – Second SPRIselect: Add **30 µl** SPRIselect reagent to supernatant from step a (**0.8X**).

Ratio = $\frac{\text{Total Volume of SPRIselect reagent added to the sample (step a + b)}}{\text{Original Volume of DNA sample}} = \frac{50\ \mu l + 30\ \mu l}{100\ \mu l} = \mathbf{0.8X}$

Enzymatic Fragmentation

- Ensure enzymatic fragmentation reactions are prepared on ice and then loaded into a thermal cycler pre-cooled to **4°C** prior to initiating the Fragmentation, End Repair, and A-tailing incubation steps.

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 TT Set A contains a unique i7 and a unique i5 oligonucleotide.

Sample Preparation, Fixation & Staining Guidelines

Sample Preparation Guidelines

Proper tissue handling and preparation techniques are critical in preserving the morphological quality of the tissue sections and subsequent transcript profiling using Visium Spatial protocols.

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

Consult the Visium Spatial Protocols – Tissue Preparation Guide for complete information (Demonstrated Protocol CG000240).

Key Considerations
Slide Handling (before sectioning)
☐ Equilibrate Visium slides to cryostat temperature before cryosectioning.
☐ 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.
Freezing and Embedding
☐ Snap freeze samples in a bath of isopentane and liquid nitrogen.
☐ Store frozen samples at **-80°C** in a sealed container for **long-term** storage prior to embedding.
Cryosectioning
☐ Equilibrate OCT tissue block to the cryostat chamber temperature for **30 min**.
☐ Place tissue sections on the Capture Area within the fiducial frame on the slide.

Slide Handling (after sectioning)
☐ Maintain slides containing sections in a low moisture environment.
☐ Keep slides cold and transport slides on dry ice.
☐ DO NOT leave slides at room temperature.
Sample Storage
☐ Store slides individually in a sealed container at **-80°C** for up to **4 weeks** to avoid multiple freeze thaw cycles. If necessary, place the sealed container in a secondary container, such as a resealable bag.

Fixation & Staining Guidelines

Proper tissue fixation and staining should be performed before executing the Permeabilization & cDNA Synthesis steps.

Consult Demonstrated Protocols (available on the 10x Genomics Support website) for fixing and staining tissue sections:

Methanol Fixation, H&E Staining & Imaging for Visium Spatial Protocols (Demonstrated Protocol CG000160)

OR

Methanol Fixation, Immunofluorescence Staining & Imaging for Visium Spatial Protocols (Demonstrated Protocol CG000312)

DO NOT proceed with Permeabilization & Reverse Transcription without performing appropriate fixation, staining, and imaging (if applicable) for the tissue sections on the appropriate Visium slide.

Tissue Optimization Guidelines

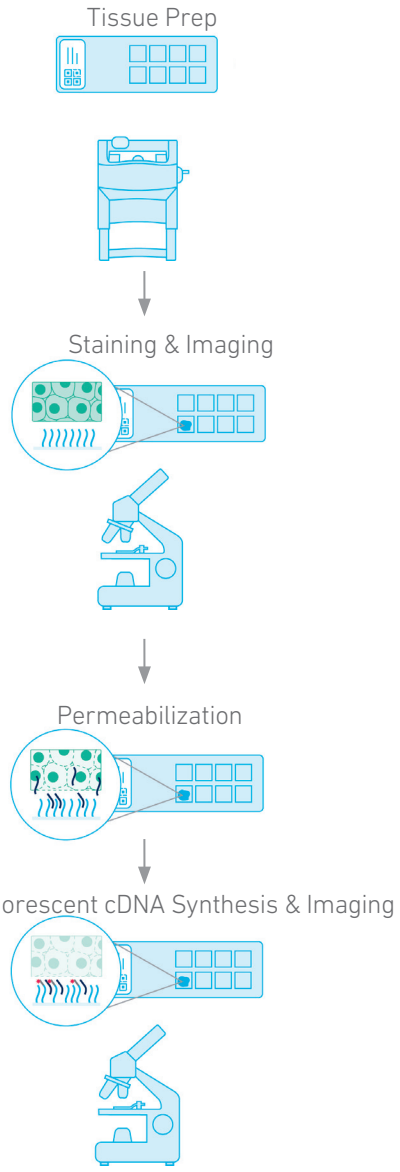
Tissue Optimization Guidelines

Prior to using a new tissue type for generating Visium Spatial Gene Expression libraries, the permeabilization time should be optimized. Failure to optimize the permeabilization time can diminish the efficiency of the assay significantly.

Refer to the Visium Spatial Gene Expression Reagent Kits – Tissue Optimization User Guide (CG000238) for the complete protocol for optimizing permeabilization time for any tissue of interest.

Briefly, previously fixed and stained tissue sections on 7 Capture Areas on a Visium Tissue Optimization slide are permeabilized for different times. mRNA released during permeabilization binds to oligonucleotides on the Capture Areas. Fluorescent cDNA is synthesized on the slide and imaged. The permeabilization time that results in maximum fluorescence signal with the lowest signal diffusion is optimal. If the signal is the same at two time points, the longer permeabilization time is considered optimal.

Tissue Optimization Workflow



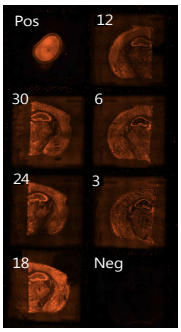
Choose the permeabilization time that results in maximum fluorescence signal with the lowest signal diffusion. If the signal is the same at two time points, choose the longer permeabilization time.

Example: Tissue Permeabilization Time Course

Mouse brain sections were imaged after permeabilization and fluorescent cDNA synthesis, using a Nikon Eclipse Ti2 microscope.

- Positive control: Strong fluorescent signal.
- Negative Control: No fluorescent signal.
- Optimal signal: **18 min**.
  Use for Visium Spatial Gene Expression protocol.

Permeabilization Time Course (min)



Step 1

Permeabilization & Reverse Transcription

1.0 Permeabilization & Reverse Transcription

CHECKLIST – GET STARTED!			
Items	**10x PN**	**Preparation & Handling**	**Storage**
Prepare & equilibrate to 37°C			
☐ **Permeabilization Enzyme**	2000214	**Immediately** before use, centrifuge briefly and resuspend in 1.2 ml HCl (0.1N), pipette mix, centrifuge briefly, verify no precipitate. Equilibrate to 37°C. Store unused resuspended enzyme at –20°C. DO NOT freeze-thaw more than 3x.	–20°C
Equilibrate to room temperature			
☐ **RT Reagent**	2000086	Thaw, vortex, verify no	–20°C
☐ **Template Switch Oligo**	3000228	Centrifuge briefly, resuspend in 80 µl Low TE Buffer. Vortex 15 sec at maximum speed, centrifuge briefly, leave at room temperature for ≥ 30 min. After resuspension, store at –80°C.	–20°C
☐ **Reducing Agent B**	2000087	Thaw, vortex, verify no	–20°C
Place on ice			
☐ **RT Enzyme D**	2000216/ 2000227	Pipette mix, centrifuge briefly.	–20°C
☐ **Nuclease-free Water**	-	-	Ambient
☐ **20X SSC**	-	-	Ambient
☐ **Slide Cassette**	3000406	See Tips & Best Practices.	Ambient
☐ **Slide Seals**	3000279	See Tip & Best Practices	Ambient
☐ **Low TE Buffer**	-	-	-
☐ **Hydrochloric Acid Solution**	-	-	-
Prepare			

☐ **0.1X SSC** (can be prepared ahead of time)	**0.1X SSC** *Store at room temperature*	**Stock**	**Final**	**50 ml (50 slides)**
	SSC	20X	0.1X	250 µl
	Water (Ultrapure/ Milli-Q)	-	-	49.75 ml

DO NOT proceed with Permeabilization & cDNA Synthesis without performing appropriate fixation, staining, and imaging (if applicable) for the tissue sections on the Visium slide.

1.1 Tissue Permeabilization

Retrieve the Visium Tissue Optimization Slide with **fixed & stained** tissue sections. If a coverslip was mounted on the slide for imaging, remove the coverslip. Consult the Demonstrated Protocol used for tissue staining for coverslip removal instructions. Ensure Permeabilization Enzyme is resuspended and is maintained at **37°C**.

If Methanol Fixation, Immunofluorescence Staining & Imaging was performed (CG000312), the slide will be in the Slide Cassette with wash buffer in the wells. Using a pipette, remove wash buffer from the well corners and proceed immediately to step c.

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 (may be 50°C if instrument does not enable 37°C)		*
Step	**Temperature**	**Time**
Pre-equilibrate	37°C	Hold
Permeabilization	37°C	*Determined by Tissue Optimization protocol.

b. Place the slide in the Slide Cassette*. See Tips & Best Practices for assembly instructions. Practice assembly with a blank slide.

c. Add **70 µl** Permeabilization Enzyme along the side of the wells to uniformly cover the tissue sections, without introducing bubbles.

Tap Slide Cassette gently to ensure uniform coverage.

d. Apply Slide Seal on the Slide Cassette and place the Slide Cassette on the Thermocycler Adaptor at **37°C**.

e. Close the thermal cycler lid and incubate for the pre-determined permeabilization time (tissue type specific).

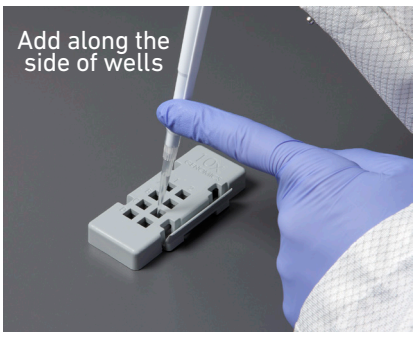
Consult the Visium Spatial Gene Expression Reagent Kits – Tissue Optimization User Guide (CG000238) for the complete protocol for optimizing permeabilization time for any tissue of interest.

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

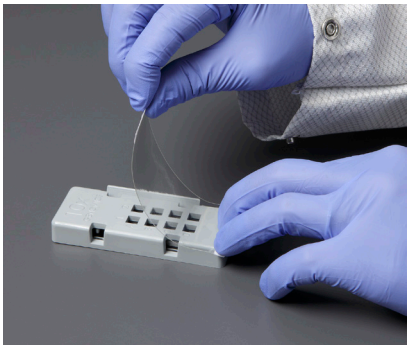
g. Using a pipette, remove Permeabilization Enzyme from the well corners.

h. Add **100 µl** 0.1X SSC to the wells.

Add Reagent



Apply Slide Seal



1.2 Reverse Transcription

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

Lid Temperature	Reaction Volume	Run Time
53°C	-	45 min
Step	**Temperature**	**Time**
Pre-equilibrate	53°C	Hold
Reverse Transcription	53°C	00:45:00
Hold	4°C	Hold

b. Prepare RT Master Mix on ice. Pipette mix 10x and centrifuge briefly.

RT Master Mix *Add reagents in the order listed.*	PN	Volume/slide + 10% (µl)	Volume/2 slides + 10% (µl)
Nuclease-free Water	-	166.3	332.6
● **RT Reagent**	2000086	82.7	165.4
● **Template Switch Oligo**	3000228	22.9	45.8
○ **Reducing Agent B**	2000087	6.6	13.2
● **RT Enzyme D**	2000216/ 2000227	51.5	103.0
Total	-	**330**	**660**

c. Remove 0.1X SSC from the wells.

d. Add **75 µl** RT Master Mix to each well.

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

f. Skip Pre-equilibrate step to initiate Reverse Transcription.

Step 2

Second Strand Synthesis & Denaturation

2.1 Second Strand Synthesis

2.2 Denaturation

2.0 Second Strand Synthesis

CHECKLIST – GET STARTED!			
Items	**10x PN**	**Preparation & Handling**	**Storage**
Equilibrate to room temperature			
☐ **Second Strand Reagent**	2000219	Thaw, vortex, centrifuge briefly.	–20°C
☐ **Second Strand Primer**	2000217	Thaw, vortex, centrifuge briefly.	–20°C
Place on ice			
☐ **Second Strand Enzyme**	2000218/ 2000183	Pipette mix, centrifuge briefly.	–20°C
Obtain			
☐ **Nuclease-free Water**	-		Ambient
☐ **Qiagen Buffer EB**	-	Manufacturer's recommendations.	Ambient
☐ **Tris 1 M, pH 7.0** (Tris-HCl)	-	Manufacturer's recommendations.	Ambient
☐ **Slide Seals**	3000279	See Tip & Best Practices.	Ambient
Prepare			

☐ **0.08 M KOH** (prepare 500 µl/slide)	**0.08 M KOH** *Store at room temperature*	**Stock**	**Final**	**500 µl**
	KOH	8 M	0.08 M	5 µl
	Water (Nuclease-free or ultrapure/Milli-Q)	-	-	495 µl

2.1 Second Strand Synthesis

a. Remove the Slide Cassette from the thermal cycler and place on a flat, clean work surface.

b. Leave the Thermocycler Adaptor on the thermal cycler. Prepare the thermal cycler with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
65°C	-	15 min
Step	**Temperature**	**Time**
Pre-equilibrate	65°C	Hold
Second Strand Synthesis	65°C	00:15:00
Hold	4°C	Hold

c. Remove RT Master Mix from the wells.

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

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

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

g. Add **100 µl** EB to each well.

h. Prepare Second Strand Mix on ice. Vortex and centrifuge briefly.

Second Strand Mix *Add reagents in the order listed*	PN	Volume/slide + 10% (µl)	Volume/2 slides + 10% (µl)
Second Strand Reagent	2000219	305.8	611.6
Second Strand Primer	2000217	17.6	35.2
Second Strand Enzyme	2000218/ 2000183	6.6	13.2
Total	-	**330**	**660**

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

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

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

l. Skip Pre-equilibrate step to initiate Second Strand Synthesis.

2.2 Denaturation

a. At the end of incubation, using a pipette, remove reagents from the wells.

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

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

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

e. Incubate **10 min** at **room temperature**.

f. Add **5 µl** Tris (1 M, pH 7.0) to 4 tubes in an 8-tube strip (4 tubes will be used for each slide).

g. Transfer **35 µl** sample from each well to a corresponding tube containing Tris in the 8-tube strip.
DO NOT discard sample. ~**1-2 µl** volume variation is expected.

h. Vortex, centrifuge briefly, and place on ice.

The Slide Cassette and slide may be discarded.

Step 3

cDNA Amplification & QC

3.1 Cycle Number Determination – qPCR

3.2 cDNA Amplification

3.3 cDNA Cleanup – SPRIselect

3.4 cDNA QC & Quantification

3.0 cDNA Amplification & QC

CHECKLIST – GET STARTED!			
Item	**10x PN**	**Preparation & Handling**	**Storage**
Equilibrate to room temperature			
☐ 🟢 **cDNA Primers**	2000089	Thaw, vortex, 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.	-
☐ **DNA High Sensitivity Reagent Kit** If LabChip used for QC	-	Manufacturer's recommendations.	-
Place on ice			
☐ **KAPA SYBR FAST qPCR Master Mix** Minimize light exposure	-	Vortex, centrifuge briefly.	–20°C
☐ ◯ **Amp Mix**	2000047/ 2000103	Vortex, centrifuge briefly.	–20°C
Obtain			
☐ **Qiagen Buffer EB**	-	-	Ambient
☐ **Nuclease-free Water**	-	-	-
☐ **qPCR Plate**	-	-	-
☐ **10x Magnetic Separator**	230003	See Tips & Best Practices.	Ambient
☐ **Prepare 80% Ethanol** Prepare 15 ml for 4 reactions (1 slide)	-	Prepare fresh.	Ambient
Special Equipment			
☐ **Real Time qPCR System**			

3.1 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*	PN	5X* + 10% (µl) *Includes 1 negative control	9X* + 10% (µl) *Includes 1 negative control
Nuclease-free Water	-	20.4	36.6
KAPA SYBR FAST qPCR Master Mix Minimize light exposure	-	27.5	49.5
cDNA Primers	2000089	1.7	3.0
Total	-	**49.6**	**89.1**

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

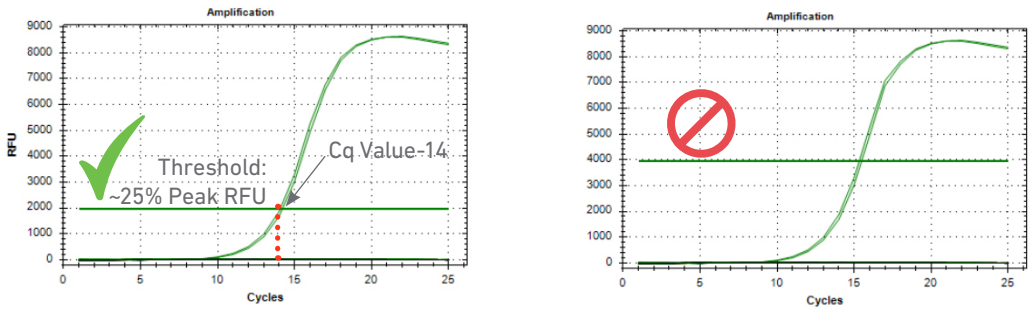
c. Transfer **1 µl** sample from **step 2.2h** to the qPCR plate well containing the qPCR Mix. Pipette mix, centrifuge briefly (if using a negative control, add **1 µl** nuclease-free water to the corresponding well).

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

Lid Temperature	Reaction Volume	Run Time
-	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, for a total of 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



3.2 cDNA Amplification

a. Prepare cDNA Amplification Mix on ice. Vortex and centrifuge briefly.

cDNA Amplification Mix *Add reagents in the order listed*	PN	4X + 10% (µl)	8X + 10% (µl)
○ **Amp Mix**	2000047/ 2000103	220	440
● **cDNA Primers**	2000089	66	132
Total	-	**286**	**572**

b. Add **65 µl** cDNA Amplification Mix to remaining **~35 µl** sample from step 2.2h.

c. Pipette mix 15x (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	~45-60 min
Step	**Temperature**	**Time**
1	98°C	00:03:00
2	98°C	00:00:15
3	63°C	00:00:20
4	72°C	00:01:00
5	Go to Step 2, 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

Cycle number examples determined based on rounding the Cq Value.

Cq Value from qPCR	Total Cycles
12.2	12 cycles
13.5	14 cycles
15.7	16 cycles

STOP **e.** Store at **4°C** for up to **72 h** or at **–20°C** for up to **1 week**, or proceed to the next step.

3.3 cDNA Cleanup – SPRIselect

a. Vortex to resuspend the SPRIselect reagent. Add **60 µl** SPRIselect reagent **(0.6X)** to each sample (100 µl) and pipette mix 15x (pipette set to 150 µl).

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

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

d. Remove the supernatant.

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

f. Remove the ethanol.

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

h. Centrifuge briefly and place on the magnet•**Low**.

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

j. Remove from the magnet. Add **40.5 µl** Buffer EB. Pipette mix 15x (pipette set to 40 µl).

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

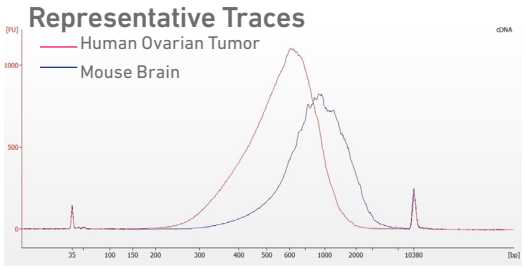
l. Place the tube strip on the magnet•**Low** until the solution clears.

m. Transfer **40 µl** sample to a new tube strip.

n. Store at **4°C** for up to **72 h** or at **–20°C** for up to **4 weeks**, or proceed to the next step.

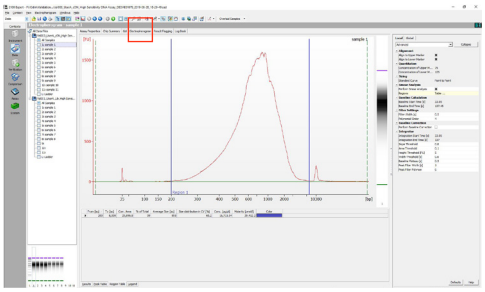
3.4 cDNA QC & Quantification

a. Run 1 µl of sample on an Agilent Bioanalyzer High Sensitivity chip.
cDNA profile may vary depending on tissue type and quality.
Lower molecular weight product (35-150 bp) may be present. This is normal and does not affect sequencing or application performance.

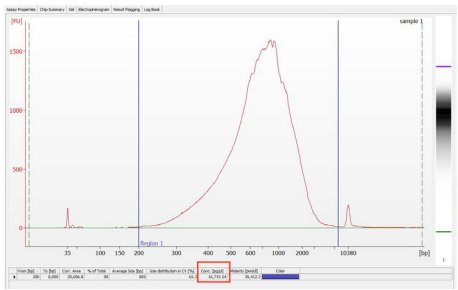


EXAMPLE CALCULATION

i. Select Region
Under the "Electropherogram" view choose the "Region Table". Manually select the region of ~200 – ~9000 bp.



ii. Note Concentration [pg/µl]



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

Example Calculation of cDNA Total Yield

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

Total cDNA Yield

$$= \frac{\text{Conc'n (pg/µl) x Elution Volume (µl)}}{\text{1000 (pg/ng)}}$$

$$= \frac{\text{16,715.54 (pg/µl) x 40 (µl)}}{\text{1000 (pg/ng)}} = 668.6.\ \text{ng}$$

The carry forward cDNA volume is specified in step 4.1.
Refer to step 4.5e for appropriate number of Sample Index PCR cycles based on carry forward cDNA/input mass.

Alternate Quantification Methods:

- Agilent TapeStation
- LabChip

See Appendix for representative traces

Step 4

Spatial Gene Expression Library Construction

4.1 Fragmentation, End Repair & A-tailing

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

4.3 Adaptor Ligation

4.4 Post Ligation Cleanup – SPRIselect

4.5 Sample Index PCR

4.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect

4.7 Post Library Construction QC

4.0 Visium Spatial Gene Expression Library Construction

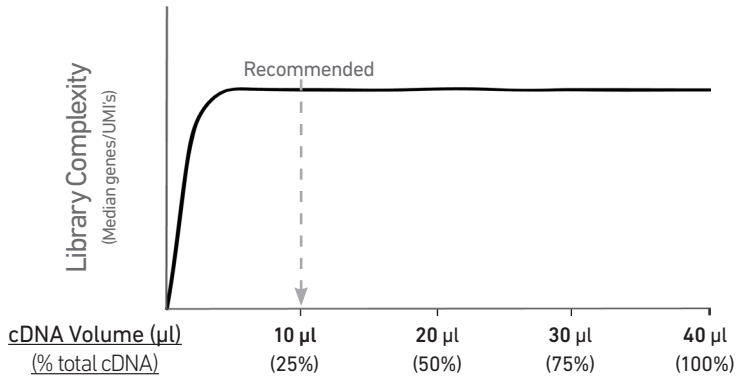
CHECKLIST – GET STARTED!			
Item	**10x PN**	**Preparation & Handling**	**Storage**
Equilibrate to room temperature			
☐ **Fragmentation Buffer**	2000091	Vortex, verify no precipitate, centrifuge briefly.	–20°C
☐ **Adaptor Oligos**	2000094	Vortex, centrifuge briefly.	–20°C
☐ **Ligation Buffer**	2000092	Vortex, verify no precipitate, centrifuge briefly.	–20°C
☐ **Dual Index Plate TT Set A**	3000431	-	–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.	-
☐ **DNA High Sensitivity Reagent Kit** If LabChip used for QC	-	Manufacturer's recommendations.	-
Place on ice			
☐ **Fragmentation Enzyme**	2000090/ 2000104	Pipette mix, centrifuge briefly before using.	–20°C
☐ **DNA Ligase**	220110/ 220131	Pipette mix, centrifuge briefly before using.	–20°C
☐ **Amp Mix**	2000047/ 2000103	Vortex, centrifuge briefly.	–20°C
☐ **KAPA Library Quantification Kit for Illumina Platforms**	-	Manufacturer's recommendations.	-
Obtain			
☐ **Qiagen Buffer EB**	-		Ambient
☐ **10x Magnetic Separator**	230003	See Tips & Best Practices.	Ambient
☐ **Prepare 80% Ethanol** Prepare 20 ml for 8 reactions	-	Prepare fresh.	Ambient

Step Overview (Step 4.1d)

Correlation between input & library complexity

A Visium Spatial Gene Expression library is generated using a fixed proportion (10 µl, 25%) of the total cDNA (40 µl) obtained at step 3.3. The complexity of this library will be comparable to one generated using a higher proportion (>25%) of the cDNA. The remaining proportion (30 µl, 75%) of the cDNA may be stored at **4°C** for up to **72 h** or at **–20°C** for longer-term storage (up to **4 weeks**).

Correlation: cDNA input & Library Complexity



Note that irrespective of the total cDNA yield (ng), which may vary based on tissue type, coverage of Capture Area by tissue section, and tissue thickness, this protocol has been optimized for a broad range of input mass (ng), as shown in the example below. The total number of SI PCR cycles (step 4.5d) should be optimized based on carrying forward a fixed proportion (10 µl, 25%) of the total cDNA yield calculated during Post cDNA Amplification QC & Quantification (step 3.4).

Example: Library Construction Input Mass & SI PCR Cycles

Tissue Type	Tissue Covered Capture Area (%)	Total cDNA Amplification Cycles	Total cDNA Yield (ng)	cDNA Input into Fragmentation		SI PCR Cycle Number
				Volume (µl)	Mass (ng)	
High RNA Content		17	412	10	102	13
		15	928	10	232	10
Low RNA Content		17	128	10	32	14
		15	536	10	134	12

4.1 Fragmentation, End Repair & A-tailing

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

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

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

Fragmentation Mix *Add reagents in the order listed*	PN	4X + 10% (µl)	8X + 10% (µl)
Fragmentation Buffer	2000091	22	44
Fragmentation Enzyme	2000090/ 2000104	44	88
Total	-	**66**	**132**

c. Transfer ONLY **10 µl** purified cDNA sample from cDNA Cleanup (step 3.3m) to a tube strip maintained on ice.
Note that only **10 µl** (25%) cDNA sample is sufficient for generating Visium Spatial Gene Expression library. The remaining **30 µl** (75%) cDNA sample can be stored at **4°C** for up to **72 h** or at **–20°C** for up **to 4 weeks** for generating additional libraries.

d. Add **25 µl** Buffer EB to each sample.

e. Add **15 µl** Fragmentation Mix to each sample.

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

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

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

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

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

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

c. Place on the magnet•**High** until the solution clears. DO NOT discard supernatant.

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

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

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

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

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

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

j. Remove the ethanol.

k. **Repeat** steps i and j for a total of 2 washes.

l. Centrifuge briefly. Place on the magnet•**Low** until the solution clears. Remove remaining ethanol. DO NOT over dry to ensure maximum elution efficiency.

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

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

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

p. Transfer **50 µl** sample to a new tube strip.

4.3 Adaptor Ligation

a. Prepare Adaptor Ligation Mix. Pipette mix and centrifuge briefly.

Adaptor Ligation Mix *Add reagents in the order listed*	PN	4X + 10% (µl)	8X + 10% (µl)
Ligation Buffer	2000092	88	176
DNA Ligase	220110/ 220131	44	88
Adaptor Oligos	2000094	88	176
Total	-	**220**	**440**

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

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

Lid Temperature	Reaction Volume	Run Time
30°C	100 µl	15 min
Step	**Temperature**	**Time**
1	20°C	00:15:00
2	4°C	Hold

4.4 Post Ligation Cleanup – SPRIselect

a. Vortex to resuspend SPRIselect Reagent. Add **80 µl** SPRIselect Reagent **(0.8X)** to each sample. Pipette mix 15x (pipette set to 150 µl).

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

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

d. Remove the supernatant.

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

f. Remove the ethanol.

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

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

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

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

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

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

m. Transfer **30 µl** sample to a new tube strip.

4.5 Sample Index PCR

a. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x Sample Index name (PN-1000215 Dual Index Plate TT Set A well ID) used.

b. Add **50 µl** Amp Mix (PN-2000047 or 2000103) to **30 µl** sample.

c. Add **20 µl** of an individual Dual Index TT 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	00:00:45
2	98°C	00:00:20
3	54°C	00:00:30
4	72°C	00:00:20
5	Go to step 2, see below for # of cycles	
6	72°C	00:01:00
7	4°C	Hold

The total cycles should be optimized based on 25% carry forward cDNA yield/input calculated during Post cDNA Amplification QC & Quantification (step 3.4)

Recommended cycle numbers

cDNA Input	Total Cycles
0.25-25 ng	14-16
25-150 ng	12-14
150-500 ng	10-12
500-1,000 ng	8-10
1,000-1,500 ng	6-8
>1500 ng	5

STOP

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

4.6 Post Sample Index PCR Double Sided Size Selection – SPRIselect

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

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

c. Place on the magnet•**High** until the solution clears. DO NOT discard supernatant.

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

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

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

g. Place the magnet•**High** until the solution clears.

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

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

j. Remove the ethanol.

k. **Repeat** steps i and j for a total of 2 washes.

l. Centrifuge briefly. Place on the magnet•**Low**. Remove remaining ethanol.

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

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

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

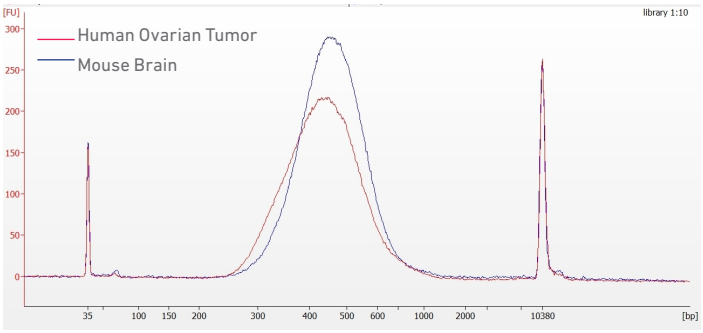
p. Transfer **35 µl** to a new tube strip.

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

4.7 Post Library Construction QC

a. Run **1 µl** of sample (1:10 dilution) on an Agilent Bioanalyzer High Sensitivity chip.

Representative Traces



A smaller peak (~200-600 bp) may be present in some tissue types (e.g. mouse brain).

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

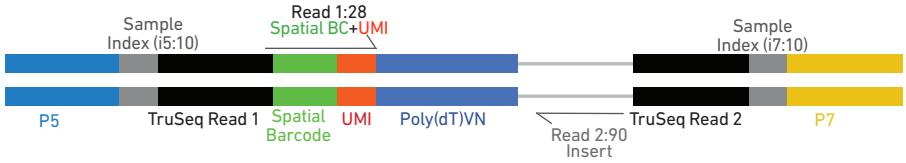
Sequencing

5

Sequencing Libraries

Visium Spatial Gene Expression 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 Read 2 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. Sequencing these libraries produce a standard Illumina BCL data output folder.

Visium Spatial Gene Expression Library



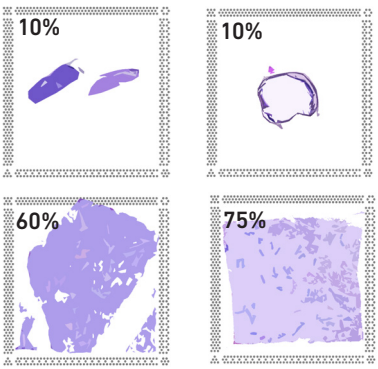
Sequencing Depth

Sequencing Depth/spot	Minimum 50,000 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 50,000 read pairs/spot
- **Example calculation for 60% coverage:** (0.60 x 5,000 total spots) x 50,000 read pairs/spot= 150 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 2: 90 cycles

Illumina Sequencer Compatibility

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

- MiSeq
- NextSeq 500/550
- HiSeq 2500 (Rapid Run)
- HiSeq 3000/4000
- NovaSeq
- iSeq

Sample Indices

Each well of the Dual Index Kit TT Set A (PN-1000215) contains a mix of one unique i7 and one unique i5 sample index. If multiple samples are pooled in a sequence lane, the sample index name (i.e. the Dual Index TT Set A plate well ID, SI-TT-) is needed in the sample sheet used for generating FASTQs with "spaceranger mkfastq". Samples utilizing the same sample index should not be pooled together or run on the same flow cell lane, as this would not enable correct sample demultiplexing.

Library Loading

Once quantified and normalized, the Visium Spatial Gene Expression 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
HiSeq 2500 (RR)	11	1
HiSeq 4000	240	1
NovaSeq	150**/300	1
iSeq	150	1

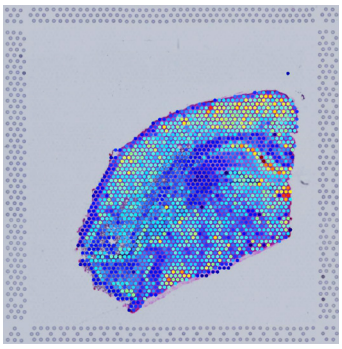
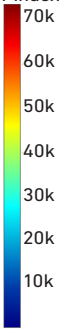
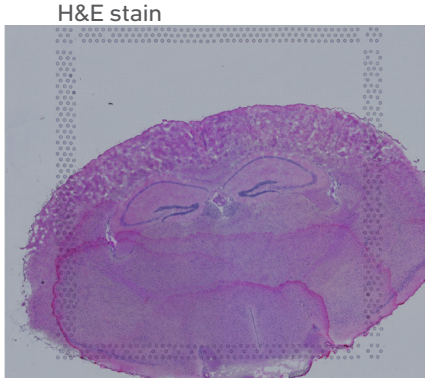
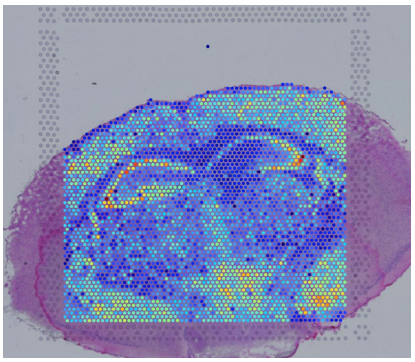
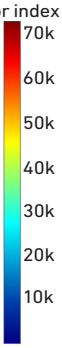
** Use 150 pM loading concentration for Illumina XP workflow.

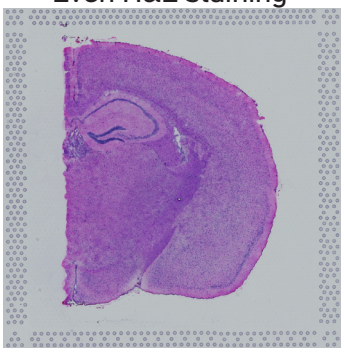
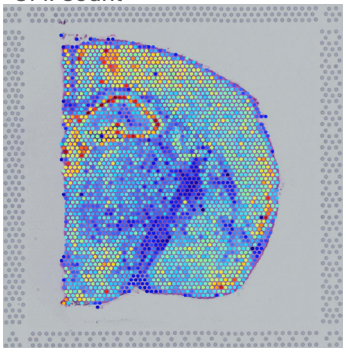
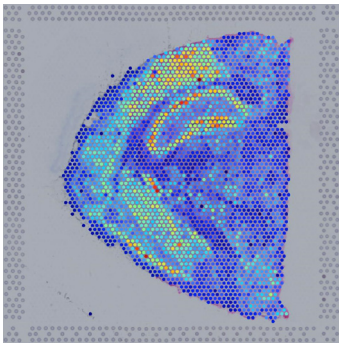
Library Pooling

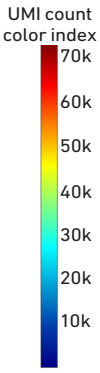
The Visium Spatial Gene Expression libraries may be pooled for sequencing, taking into account the differences in tissue covered spot on a Capture Area and per-spot read depth requirements between each library. Samples utilizing the same sample index should not be pooled together, or run on the same flow cell lane, as this would not enable correct sample demultiplexing.

Troubleshooting

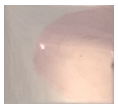
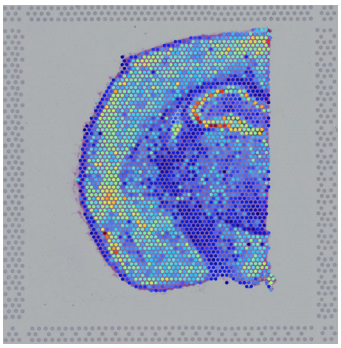
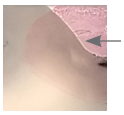
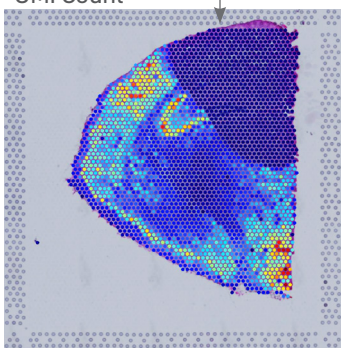
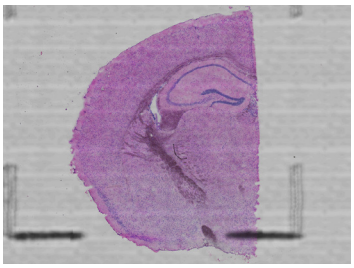
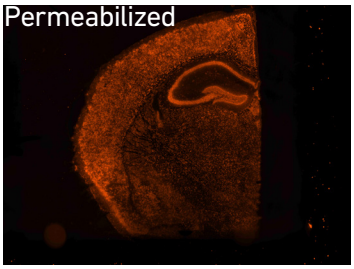
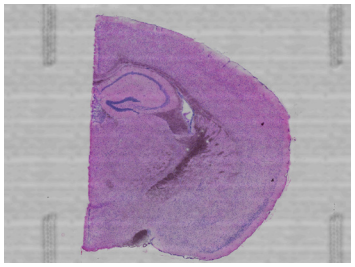
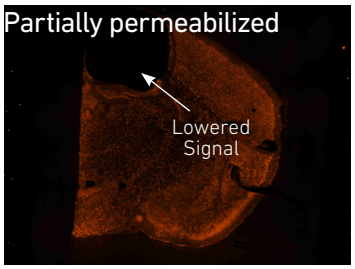
6

STEP	NOTES
Tissue Folding – Impact on UMI Count	Folded tissue H&E stain  UMI count  UMI count color index  Folded tissue section can result in OCT induced tissue damage, impacting permeabilization, and diminishing assay sensitivity. However, the data derived from the rest of the tissue portions (not folded) can be analyzed.
Tissue Placement – Impact on UMI Count	Fiducials are obstructed H&E stain  UMI Count  UMI count color index  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.

STEP	CORRECT	INCORRECT
Tissue Staining – Impact on UMI Count	Even H&E staining  UMI Count 	Uneven H&Estaining  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 may diminish sensitivity and spatial resolution. However, the data derived from the evenly stained tissue portions can be analyzed.

STEP	CORRECT	INCORRECT
1.1 Tissue Permeabilization – Reagent Coverage	 Slide Cassette Well Tissue section covered completely UMI Count 	 Slide Cassette Well Tissue section not covered UMI Count 
1.1 Tissue Permeabilization – Time	 	 
2.2 Denaturation – Partial	Cover the tissue section uniformly with 35 µl 0.08 M KOH to prevent partial denaturation.	
3.1 No Cq Value	Ensure that correct KOH dilution (0.08 M) is used at step 2.2d. Also, confirm that the qPCR mix includes KAPA SYBR FAST dye.	
3.4 Flat cDNA Trace (Cq value observed)	Flat cDNA trace, even though Cq value was observed at step 3.1. Failure to properly neutralize KOH by addition of Tris (1 M, pH 7.0) at step 2.2f negatively impacts cDNA amplification efficiency (no impact on qPCR amplification, hence Cq value is observed).	

Ensure that permeabilization reagents are applied to the tissue uniformly. Uneven permeabilization will diminish sensitivity and spatial resolution. However, the data derived from the optimally permeabilized tissue portions can be

Ensure that permeabilization times are optimized for each tissue type using the Visium Spatial Tissue Optimization protocol prior to beginning this workflow. Sub-optimal permeabilization will diminish sensitivity and spatial resolution.

Appendix

Post Library Construction Quantification
Agilent TapeStation Traces
LabChip Traces
Oligonucleotide 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	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.

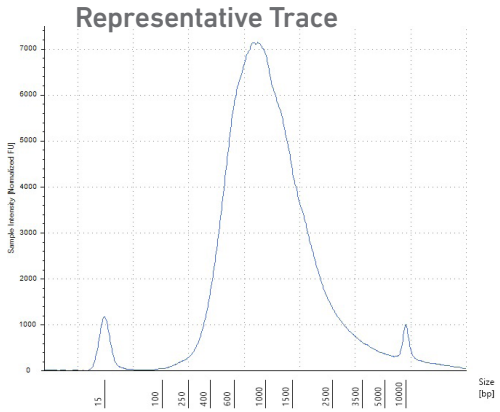
Agilent TapeStation Traces

Agilent TapeStation Traces

Agilent TapeStation High Sensitivity D5000 ScreenTape was used.
Protocol steps correspond to the Visium Spatial Gene Expression Reagent Kits User Guide (CG000239).

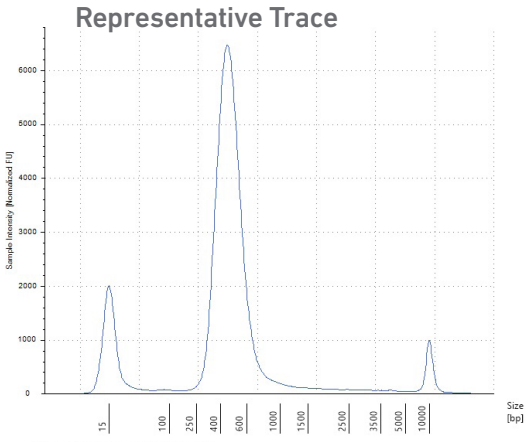
Protocol Step 3.4 – cDNA QC & Quantification

Run 2 µl sample mixed with 2 µl loading buffer. Ensure dilution factor is factored in when calculating cDNA yield/µl (divide by 2).



Protocol Step 4.7 – Post Library Construction QC

Run 2 µl diluted sample (1:10 dilution) mixed with 2 µl loading buffer.



LabChip Traces

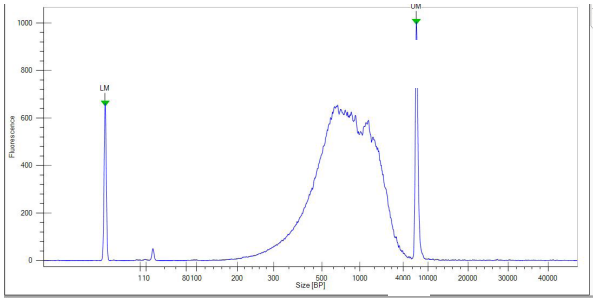
LabChip Traces

DNA High Sensitivity Reagent Kit was used.
Protocol steps correspond to the Visium Spatial Gene Expression Reagent Kits User Guide (CG000239).

Protocol Step 3.4 – cDNA QC & Quantification

Run 10 µl undiluted sample.
cDNA yield calculation is same as Agilent Bioanalyzer traces.

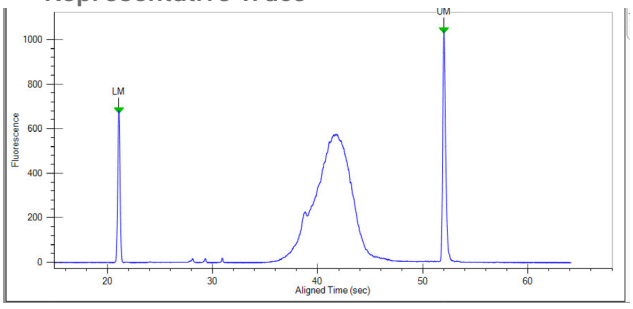
Representative Trace



Protocol Step 4.7 – Post Library Construction QC

Run 10 µl diluted sample (1:10 dilution).

Representative Trace



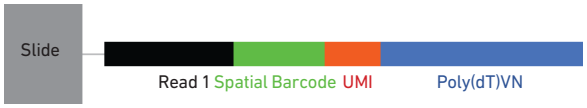
Oligonucleotide Sequences

Protocol steps correspond to the Visium Spatial Gene Expression Reagent Kits User Guide (CG000239)

Protocol Step 1.2 – Reverse Transcription

Slide Primers

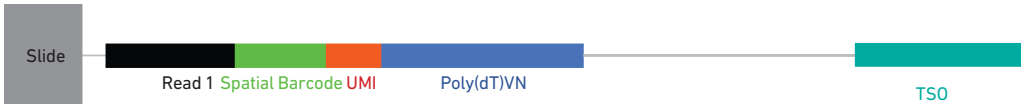
5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'



Template Switch Oligo PN -3000228

5'-AAGCAGTGGTATCAACGCAGAGTACATrGrGrG-3'

cDNA



5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-cDNA_Insert-CCCATGTACTCTGCGTTGATACCACTGCTT-3'

Protocol Step 2.1 – Second Strand Synthesis

Second Strand Primer PN -2000217

5'-AAGCAGTGGTATCAACGCAGAG-3'

Second Strand

3'-GATGTGCTGCGAGAAGGCTAGA-N16-N12-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAABN-cDNA_Insert-GGGTACATGAGACGCAACTATGGTGACGAA-5'

Protocol Step 3.2 – cDNA Amplification

cDNA Primers PN-2000089

Forward Primer: Partial Read 1

5'-CTACACGACGCTCTTCCGATCT-3'

Reverse Primer: Partial TSO

5'-AAGCAGTGGTATCAACGCAGAG-3'

Amplification Products

5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-cDNA_Insert-CCCATGTACTCTGCGTTGATACCACTGCTT-3'

3'-GATGTGCTGCGAGAAGGCTAGA-N16-N12-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAABN-cDNA_Insert-GGGTACATGAGACGCAACTATGGTGACGAA-5'

Oligonucleotide Sequences

Protocol steps correspond to the Visium Spatial Gene Expression Reagent Kits User Guide (CG000239)

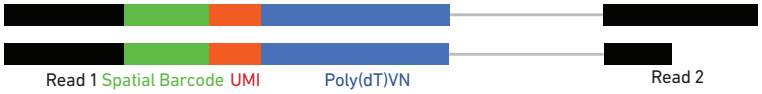
Protocol Step 4.3 – Adaptor Ligation

Adaptor Oligos
PN -2000094

Partial Read 2

5'- GATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'
3'-TCTAGCCTTCTCG-5'

Ligation Product



5'-CTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-cDNA_Insert-AGATCGGAAGAGC ACACGTCTGAACTCCAGTCAC-3'
3'-GATGTGCTGCGAGAAGGCTAGA-N16-N12-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAABN-cDNA_Insert-TCTAGCCTTCTCG-5'

Protocol Step 4.5 – Sample Index PCR

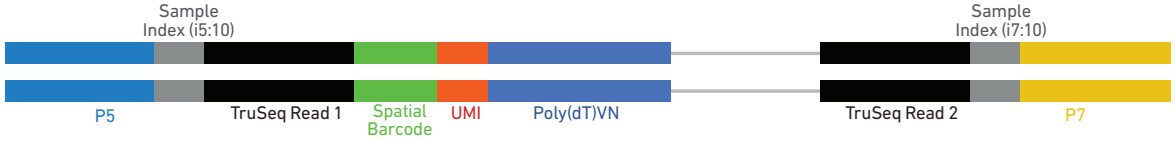
Dual Indexing

Dual Index TT Set A PN-1000215

5'-AATGATACGGCGACCACCGAGATCTACAC-N10-ACACTCTTTCCCTACACGACGCTC-3'

5'-CAAGCAGAAGACGGCATACGAGAT-N10- GTGACTGGAGTTCAGACGTGT-3'

Sample Index PCR Product



5'-AATGATACGGCGACCACCGAGATCTACAC--N10-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-N16-N12-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-cDNA_Insert-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-N10-ATCTCGTATGCCGTCTTCTGCTTG-3'
3'-TTACTATGCCGCTGGTGGCTCTAGATGTG--N10-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-N16-N12-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAABN-cDNA_Insert-TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-N10-TAGAGCATACGGCAGAAGACGAAC-5'