User Guide | CG000494 | Rev E

# Visium CytAssist Spatial Gene and Protein Expression Reagent Kits

For use with:

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

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

Visium CytAssist Protein Core Reagents PN-1000603

Visium Human Immune Cell Profiling Panel, Small PN-1000607

Visium CytAssist Reagent Accessory Kit, PN-1000499

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

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

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

Dual Index Kit NT Set A, 96 rxns PN-1000242

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

# Notices

### Document Number

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# Document Revision Summary

### Document Number

CG000494

### Title

Visium CytAssist Spatial Gene and Protein Expression Reagent Kits User Guide

### Revision

Rev E

### Revision Date

April 16, 2025

### Description of Changes

- Updated tissue slide loading guidance in Instrument Loading Guidelines on page 52
- Updated probe preparation guidance in 2.0 Get Started on page 67.
- Updated tissue slide wiping guidance before instrument loading in 5.1 CytAssist-Enabled RNA Digestion & Tissue Removal on page 91
- Added information on color balance to Sample Indices on page 127.
- Added loading concentration guidance for NovaSeq X Plus in Library Loading on page 128.

Updated for general minor consistency of language and terms throughout.

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# Introduction


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# Reagent Kits

## Visium CytAssist Spatial Gene Expression for FFPE Reagent Kits

| Reagent Kits | Part Number | Components | Component Part Number | Quantity |
|---|---|---|---|---|
| Visium CytAssist Spatial Gene Expression for FFPE, Human Transcriptome, 6.5 mm, 4 rxns* | 1000520 | Visium CytAssist Slide and Cassettes, 6.5 mm, 2 rxns | PN-1000519 | 2 |
| | | Visium FFPE Reagent Kit v2 - Small | PN-1000436 | 1 |
| | | Visium Human Transcriptome Probe Kit v2 – Small | PN-1000466 | 1 |
| Visium CytAssist Spatial Gene Expression for FFPE, Human Transcriptome, 11 mm, 2 rxns** | 1000522 | Visium CytAssist Slide and Cassettes, 11 mm, 2 rxns | PN-1000518 | 1 |
| | | Visium FFPE Reagent Kit v2 - Small | PN-1000436 | 1 |
| | | Visium Human Transcriptome Probe Kit v2 – Small | PN-1000466 | 1 |

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

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

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

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

| | # | PN |
|---|---|---|
| Visium Cassette, 8 port | 1 | 3000811 |
| Visium Tissue Slide Cassette | | |
| Visium CytAssist Sliding Gasket, Small (preassembled with translator) | 2 | 3000814 |
| Visium CytAssist Cassette Movable Translator (preassembled with gasket) | 2 | 3000816 |
| Visium CytAssist Cassette Movable Frame | 2 | 3000813 |
| Visium Slide Seals, 20-pack | 1 | 2000284 |
| Visium CytAssist Spatial Gene Expression Slide v2, 6.5 mm | 1 | 2000549 |

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

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

| | # | PN |
|---|---|---|
| Visium Cassette, 2 port | 1 | 3000812 |
| Visium Tissue Slide Cassette | | |
| Visium CytAssist Sliding Gasket, Large | 2 | 3000815 |
| Visium CytAssist Cassette Movable Frame | 2 | 3000813 |
| Visium Slide Seals, 20-pack | 1 | 2000284 |
| Visium CytAssist Spatial Gene Expression Slide v2, 11 mm | 1 | 2000550 |

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

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

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

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

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

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

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## Visium Human Immune Cell Profiling Panel* – Small, PN-1000607

**Visium Human Immune Cell Profiling Panel - Small**
**PN-1000607**
(store at -20°C)

| | # | PN |
|---|---|---|
| Human FFPE Immune Profiling Panel, Small | 2 | 2000699 |

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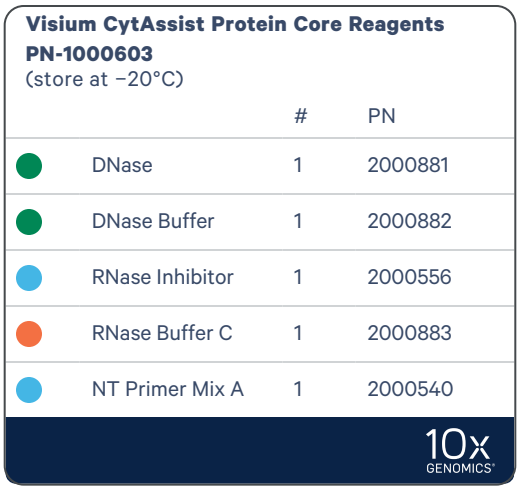
*Each Visium Humane Immune Cell Profiling Panel, Small provides enough reagent for four 6.5 mm reactions or two 11 mm reactions.

## Visium CytAssist Protein Core Reagents*, PN-1000603



**Visium CytAssist Protein Core Reagents**
**PN-1000603**
(store at –20°C)

| | # | PN |
|---|---|---|
| DNase | 1 | 2000881 |
| DNase Buffer | 1 | 2000882 |
| RNase Inhibitor | 1 | 2000556 |
| RNase Buffer C | 1 | 2000883 |
| NT Primer Mix A | 1 | 2000540 |

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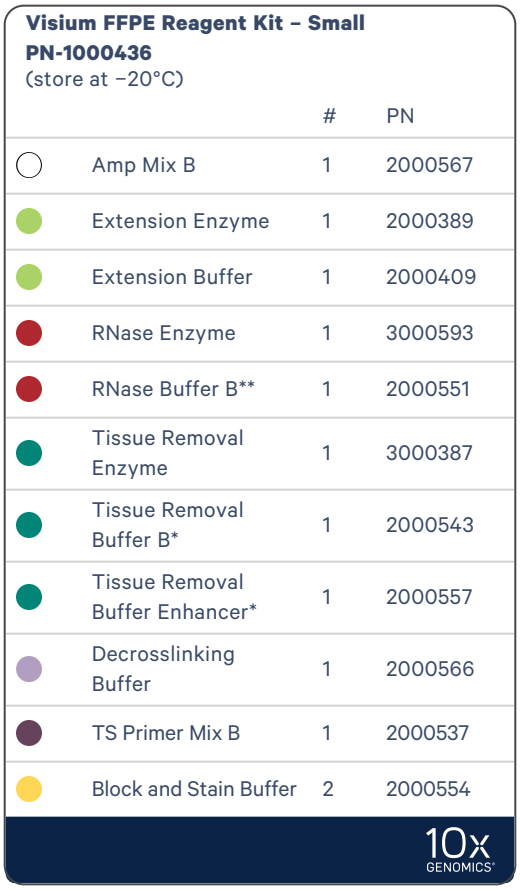
*Each Visium CytAssist Protein Core Reagents Kit provides enough reagent for four 6.5 mm reactions or two 11 mm reactions.

## Visium FFPE Reagent Kit v2 – Small, PN-1000436



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

| | # | PN |
|---|---|---|
| Amp Mix B | 1 | 2000567 |
| Extension Enzyme | 1 | 2000389 |
| Extension Buffer | 1 | 2000409 |
| RNase Enzyme | 1 | 3000593 |
| RNase Buffer B** | 1 | 2000551 |
| Tissue Removal Enzyme | 1 | 3000387 |
| Tissue Removal Buffer B* | 1 | 2000543 |
| Tissue Removal Buffer Enhancer* | 1 | 2000557 |
| Decrosslinking Buffer | 1 | 2000566 |
| TS Primer Mix B | 1 | 2000537 |
| Block and Stain Buffer | 2 | 2000554 |

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*These tubes may not be included in the kit. They are not used in this assay.

**This tube is not used in this assay.

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

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

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

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

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

| | # | PN |
|---|---|---|
| Dual Index Plate TS Set A | 1 | 3000511 |

# Dual Index Kit NT Set A, 96 rxns PN-1000242

**Dual Index Kit NT Set A**
**96 rxns PN-1000242**
(store at –20°C)

| | # | PN |
|---|---|---|
| Dual Index Plate NT Set A | 1 | 3000483 |

# 10x Genomics Accessories

## Visium CytAssist Reagent Accessory Kit PN-1000499

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

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

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# Recommended Thermal Cyclers

| Supplier | Description | Part Number |
|---|---|---|
| Bio-Rad | C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module (discontinued) | 1851197 |
| | PTC Tempo Deepwell Thermal Cycler* | 12015392 |
| Eppendorf | MasterCycler Pro (discontinued) | *North America*<br>950030010<br>*International*<br>6321 000.019 |
| | MasterCycler X50s | *North America*<br>6311000010 |
| Thermo Fisher Scientific | Veriti 96-Well Thermal Cycler (discontinued) | 4375786 |
| | VeritiPro Thermal Cycler, 96-well** | A48141 |
| Analytik Jena | Biometra TAdvanced 96 SG with 96-well block (silver, 0.2 mL) and gradient function | 846-x-070-241<br>*(where x=2 for 230 V; 4 for 115 V; 5 for 100 V, 50-60 Hz)* |

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

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

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

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

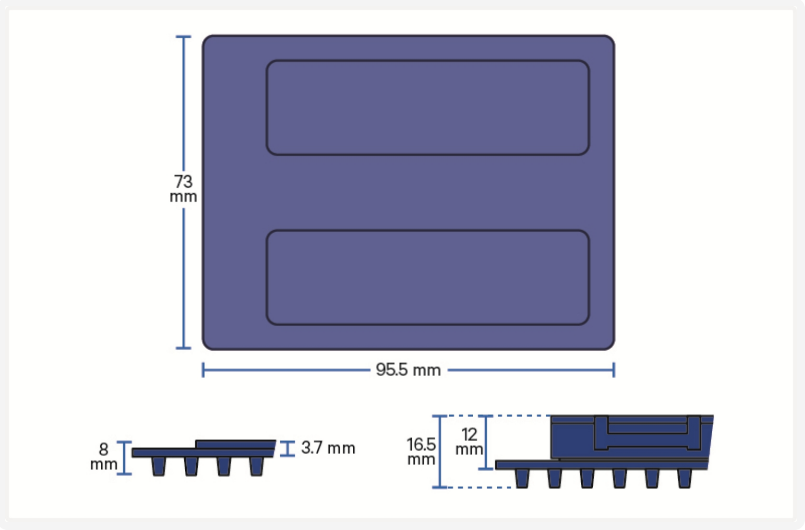
Thermal cycler requirements if reactions are performed on a tube:

- Uniform heating of 100 ul volumes
- Temperature-controlled lid

- 96 deep-well block or 0.2 ml block configuration

Thermal cycler requirements if reactions are performed on a slide:

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




## Recommended Real Time qPCR Systems

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

# Imaging System Recommendations

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

| **Imaging Systems & Specifications** | | |
|---|---|---|
| **Microscopes (Any equivalent system with the listed features may be used for imaging)** | | |
| **Supplier** | **Model** | **Configuration** |
| Thermo Fisher Scientific | EVOS M7000 | Inverted |
| Leica | Aperio Versa 8 | Upright |
| | Leica DMi8 | Inverted |
| MetaSystems | Metafer | Upright |
| Nikon | Nikon Eclipse Ti2 | Inverted |
| BioTek | Cytation 7 | Inverted or Upright |
| Keyence | Keyence BZX800 | Inverted |
| Olympus | VS200 | Upright |
| Zeiss | Imager.Z2 | Upright |
| **Microscope Features** | | |
| Objectives | • 10X (NA 0.45)<br>• 20X (NA 0.75)<br>• 40X (NA 0.95) | |
| Brightfield Features (for H&E staining) | • Color camera (3 x 8 bit, 2,424 x 2,424 pixel resolution)<br>• White balancing functionality<br>• Minimum Capture Resolution 2.18 µm/pixel<br>• Exposure times 2-10 milli sec | |
| Fluorescence Features (for IF Staining) | • Light source (or equivalent) with a wavelength range of 380–680 nm<br>• Monochrome camera (14 bit, 2,424 x 2,424 pixel resolution)<br>• DAPI filter cube (Excitation 392/23, Emission 447/60)<br>• FITC filter cube (Excitation 466/40, emission 515/30)<br>• Cy5 filter cube (Excitation 618/50, Emission 698/70)<br>• TRITC filter cube (Excitation 542/20, Emission 620/52)<br>• Minimum Capture Resolution 2.18 µm/pixel<br>• 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<br>(microscope's imaging software or equivalent, like ImageJ) | |

# Additional Kits, Reagents & Equipment

The items in the table below are validated by 10x Genomics and are highly recommended for the Visium Spatial Reagent Kits protocols. **Substituting materials may adversely affect system performance.** This list does not include standard laboratory equipment such as water baths, centrifuges, vortex mixers, pH meters, freezers etc. For some items, a number of options are listed. Choose item based on availability and preference.

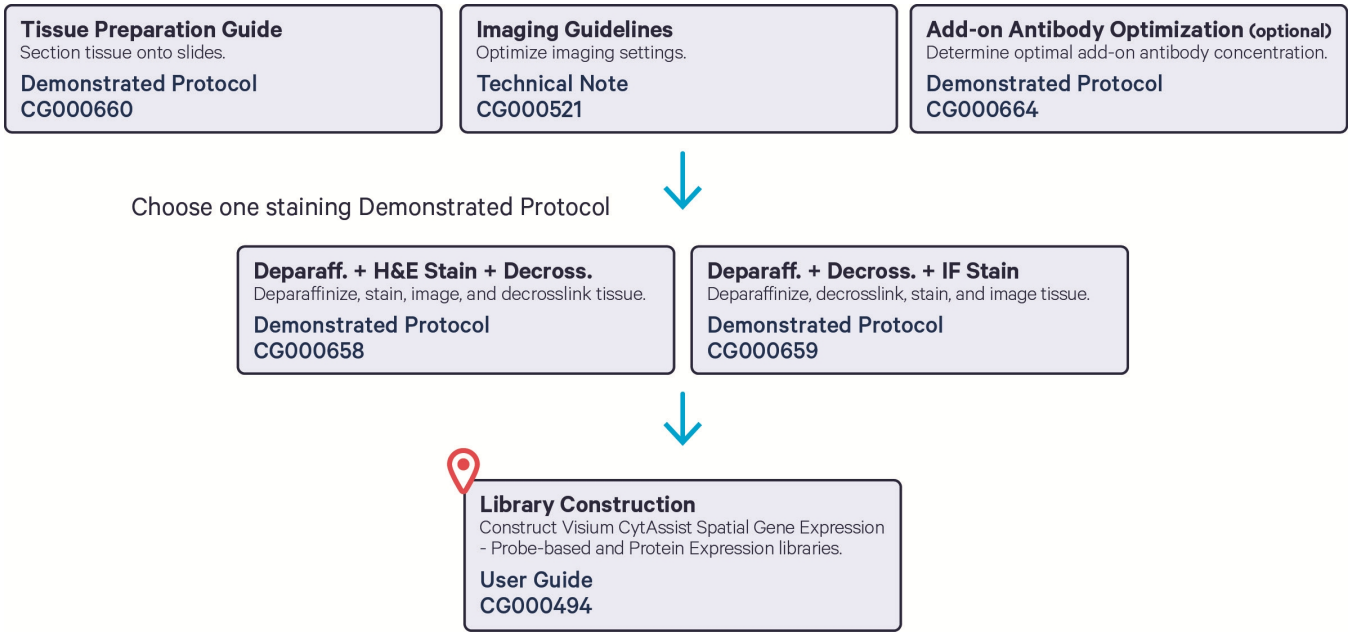
For information on tested glass slides, refer to product-specific documentation.

| Item | Description | Supplier | Part Number (US) |
|---|---|---|---|
| **Plastics** | | | |
| 1.5 ml tubes | DNA LoBind Tubes, 1.5 ml | Eppendorf | 022431021 |
| | Low DNA Binding Tubes, 1.5 ml | Sarstedt | 72.706.700 |
| 2.0 ml tubes (when processing more than 2 slides) | DNA LoBind Tubes, 2.0 ml | Eppendorf | 022431048 |
| | Low DNA Binding Tubes, 2.0 ml | Sarstedt | 72.695.700 |
| 15 ml tubes | 15 ml PP Centrifuge Tubes | Corning | 430791 |
| 50 ml tubes | Self-Standing Polypropylene Centrifuge Tubes (50 ml), sterile | Corning | 430921 |
| 0.2 ml PCR 8-tube strips<br><br>*Choose either Eppendorf, USA Scientific, or Thermo Fisher Scientific PCR 8-tube strips* | PCR Tubes 0.2 ml 8-tube strips | Eppendorf | 951010022 |
| | TempAssure PCR 8-tube strip | USA Scientific | 1402-4700 |
| | MicroAmp 8-Tube Strip, 0.2 ml (see Tips & Best Practices for more information) | Thermo Fisher Scientific | N8010580 |
| | MicroAmp 8 -Cap Strip, clear (see Tips & Best Practices for more information) | Thermo Fisher Scientific | N8010535 |
| Slide mailer/tube | Simport Scientific LockMailer Tamper Evidence Slide Mailer (alternatively, use a 50-ml centrifuge tube) | Thermo Fisher Scientific | 22-038-399 |
| | Self-Standing Polypropylene Centrifuge Tubes (50 ml), sterile | Corning | 430921 |
| PCR plates and sealing film | Hard-shell PCR Plates 96-well, thin wall (pkg of 50)<br>(Or any compatible PCR Plate) | Bio-Rad | HSP9665 |
| | Microseal 'B' PCR Plate Sealing Film, adhesive<br>(Or any compatible PCR Plate sealing adhesive) | Bio-Rad | MSB1001 |
| Pipette tips | Tips LTS 200UL Filter RT-L200FLR | Rainin | 30389240 |
| | Tips LTS 1ML Filter RT-L1000FLR | Rainin | 30389213 |
| | Tips LTS 20UL Filter RT-L20FLR | Rainin | 30389226 |

| Item | Description | Supplier | Part Number (US) |
|---|---|---|---|
| Wide Bore Pipette tips | Tips RT LTS 200UL FLW | Rainin | 30389241 |
| | Tips RT LTS 1000UL FLW | Rainin | 30389218 |
| Reagent Reservoirs | Divided Polystyrene Reservoirs | VWR | 41428-958 |
| **Kits & Reagents** | | | |
| Nuclease-free Water (not DEPC treated) | Nuclease-free Water | Thermo Fisher Scientific | AM9937 |
| Tris 1M | Tris 1M, pH 8.0, RNase-free | Thermo Fisher Scientific | AM9855G |
| | Tris 1M, pH 8.0 | TEKONOVA | T5080 |
| | UltraPure 1M Tris-HCl, pH 8.0 | Invitrogen | 15568025 |
| 10X PBS | PBS - Phosphate Buffered Saline (10X) pH 7.4, RNase-free | Thermo Fisher Scientific | AM9624 |
| Tween 20 | Tween 20 Surfact-Amps Detergent Solution (10% solution) | Thermo Fisher Scientific | 28320 |
| qPCR Mix | KAPA SYBR FAST qPCR Master Mix (2X) | KAPA Biosystems (US Only) | KK4600 |
| | | Millipore Sigma (Europe, Asia, & Canada) | |
| SPRIselect Reagent | SPRIselect Reagent Kit | Beckman Coulter | B23318 |
| Ethanol | Ethyl alcohol, Pure (200 Proof, anhydrous) | Millipore Sigma | E7023-500ML |
| 8M KOH | Potassium Hydroxide Solution, 8M | Millipore Sigma | P4494-50ML |
| 20X SSC Buffer | SSC Buffer 20X Concentrate | Millipore Sigma | S66391L |
| Buffer EB | Qiagen Buffer EB | Qiagen | 19086 |
| Eosin | Eosin Y solution, alcoholic | Millipore Sigma | HT110116 |
| | Eosin Y Solution (Modified Alcoholic) | Abcam | ab246824 |
| | Eosin Y Solution, Alcoholic, with Phloxine | Millipore Sigma | HT110332 |
| | Eosin Y with Phloxine 1% alcoholic solution | VWR | 10143 |
| Ultrapure Water | Ultrapure/Milli-Q water (from Milli-Q Integral Ultrapure Water System or equivalent) | | |
| **Equipment** | | | |
| Pipettes | Pipet-Lite Multi Pipette L8-200XLS+ | Rainin | 17013805 |
| | Pipet-Lite LTS Pipette L-2XLS+ | Rainin | 17014393 |
| | Pipet-Lite LTS Pipette L-10XLS+ | Rainin | 17014388 |
| | Pipet-Lite LTS Pipette L-20XLS+ | Rainin | 17014392 |
| | Pipet-Lite LTS Pipette L-100XLS+ | Rainin | 17014384 |
| | Pipet-Lite LTS Pipette L-200XLS+ | Rainin | 17014391 |
| | Pipet-Lite LTS Pipette L-1000XLS+ | Rainin | 17014382 |
| Mini Centrifuge | VWR Mini Centrifuge (or any equivalent mini centrifuge) | VWR | 76269-064 |

| Item | Description | Supplier | Part Number (US) |
|---|---|---|---|
| **Quantification & Quality Control** | | | |
| *Choose Bioanalyzer, TapeStation, or LabChip based on availability & preference.* | | | |
| Bioanalyzer & associated reagents | 2100 Bioanalyzer Laptop Bundle (discontinued) | Agilent | G2943CA |
| | Replacement 2100 Bioanalyzer Instrument/2100 Expert Laptop Bundle | Agilent | G2939BA/G2953CA |
| | High Sensitivity DNA Kit | Agilent | 5067-4626 |
| TapeStation & associated reagents | 4200 TapeStation | Agilent | G2991AA |
| | High Sensitivity D1000 ScreenTape/Reagents | Agilent | 5067-5584/ 5067-5585 |
| | High Sensitivity D5000 ScreenTape/Reagents | Agilent | 5067-5592/ 5067-5593 |
| LabChip & associated reagents | LabChip GX Touch HT Nucleic Acid Analyzer | PerkinElmer | CLS137031 |
| | DNA High Sensitivity Reagent Kit | PerkinElmer | CLS760672 |
| Qubit & associated reagents (for determining dilution factor for TapeStation or Bioanalyzer) | Qubit Fluorometer | ThermoFisher Scientific | Q33238 |
| | Qubit Assay Tubes | ThermoFisher Scientific | Q32856 |
| | Qubit dsDNA HS and BR Assay Kits | ThermoFisher Scientific | Q32854 |
| Library Quantification Kit | KAPA Library Quantification Kit for Illumina Platforms | KAPA Biosystems | KK4824 |

# Workflow Overview



# Protocol Steps & Timing

| Steps | Timing | Stop & Store |
|---|---|---|
| **Day 1** | | |
| **Step 1: DNase Treatment (page 62)** | | |
| 1.1 DNase Treatment (page 64) | 45 min | |
| **Step 2: Probe Hybridization (page 66)** | | |
| 2.1 Probe Hybridization (page 68) | Overnight | |
| **Day 2** | | |
| **Step 3: Probe Ligation (page 72)** | | |
| 3.1 Post-Hybridization Wash (page 74) | 18 min | |
| 3.2 Probe Ligation (page 77) | 60 min | |
| 3.3 Post-Ligation Wash (page 79) | 12 min | STOP 4°C ≤24 h |
| **Step 4: Protein Labeling (page 81)** | | |
| 4.1 Tissue Blocking (page 83) | 60 min | |
| 4.2 Antibody Incubation (page 84) | Overnight | |
| **Day 3** | | |
| 4.3 Post-Antibody Incubation Wash (page 86) | 25 min | |
| **Step 5: CytAssist Enabled Probe Release & Extension (page 88)** | | |
| 5.1 CytAssist-Enabled RNA Digestion & Tissue Removal (page 91) | 40 min | |
| 5.2 Probe & Antibody Tag Extension (page 100) | 20 min | STOP 4°C ≤2 h |
| 5.3 Probe & Antibody Tag Elution (page 101) | 15 min | |
| **Step 6: Pre-Amplification and SPRIselect (page 103)** | | |
| 6.1 Pre-Amplification (page 105) | 40 min | |
| 6.2 Pre-Amplification Cleanup - SPRIselect (page 107) | 30 min | STOP 4°C ≤72 h or -20°C ≤4 weeks |
| 6.3 Cycle Number Determination – qPCR (page 108) | 45 min | |
| **Step 7: Visium CytAssist Spatial Gene Expression – Probe-based Library Construction (page 111)** | | |
| 7.1 GEX Sample Index PCR (page 113) | 40 min | |
| 7.2 GEX Post-Sample Index PCR Cleanup – SPRIselect (page 115) | 30 min | STOP -20°C long-term |

| Steps | Timing | Stop & Store |
|---|---|---|
| 7.3 GEX Post-Library Construction QC (page 116) | 50 min | |
| **Step 8: Visium CytAssist Spatial Protein Expression Library Construction (page 117)** | | |
| 8.1 Protein Sample Index PCR (page 119) | 40 min | |
| 8.2 Protein Post-Sample Index PCR Cleanup – SPRIselect (page 121) | 30 min |  -20°C long-term |
| 8.3 Protein Post-Library Construction QC (page 122) | 50 min | |

# Stepwise Objectives

The Visium CytAssist Spatial Gene and Protein Expression assay is designed to analyze mRNA and protein in human tissue sections derived from formalin fixed & paraffin embedded (FFPE)tissue samples. It uses a combination of oligo-tagged antibodies and probes targeting the whole transcriptome. Each Visium CytAssist Spatial Gene Expression Slide v2 contains Capture Areas with barcoded spots that include oligonucleotides required to capture both gene expression probes and antibody tags. Before the assay, tissue sections are processed as described in their relevant Demonstrated Protocol. See Workflow Overview on page 20 for documentation references.

Human whole transcriptome probe panels, consisting of ~3 pairs of specific probes for each targeted gene are added to the tissue, enabling hybridization and ligation of each probe pair. Next, the tissue is incubated with an oligo-tagged antibody panel. Tissue slides and Visium CytAssist Spatial Gene Expression v2 Slides are loaded into the Visium CytAssist instrument, where they are brought into proximity with one another. Both gene expression probes and antibody tags are released from the tissue upon CytAssist Enabled RNA Digestion & Tissue Removal, enabling capture by the spatially barcoded oligonucleotides present on the Visium slide surface. The Visium CytAssist Spatial Gene Expression v2 Slide is removed from the Visium CytAssist for downstream library preparation. Paired gene expression and protein libraries are generated from each tissue section and sequenced. Spatial Barcodes are used to associate the reads back to the tissue section images for spatial mapping of gene and protein expression.

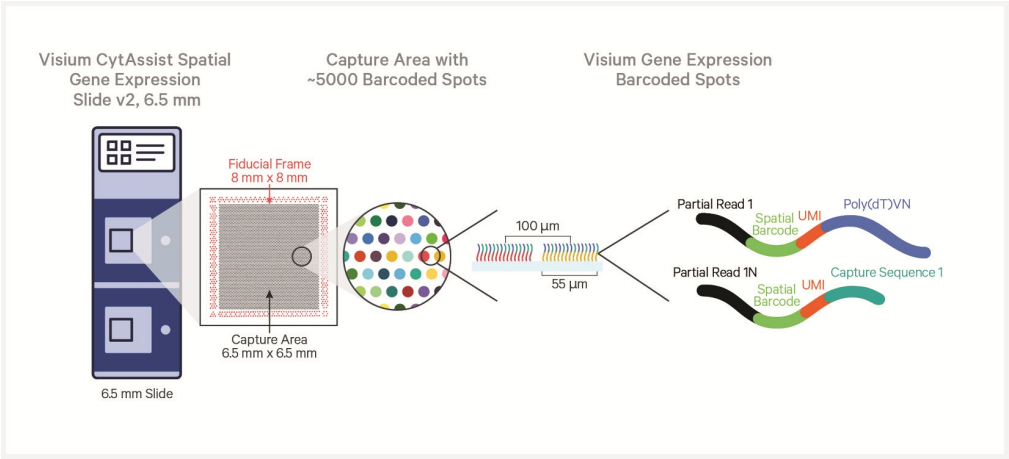
This document outlines the protocol for generating Visium CytAssist Spatial Gene Expression - Probe-based libraries and Visium CytAssist Spatial Protein Expression libraries. This workflow is only supported for human tissues.

# Visium CytAssist Spatial Gene Expression Slides

## Visium CytAssist Spatial Gene Expression Slide v2, 6.5 mm

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

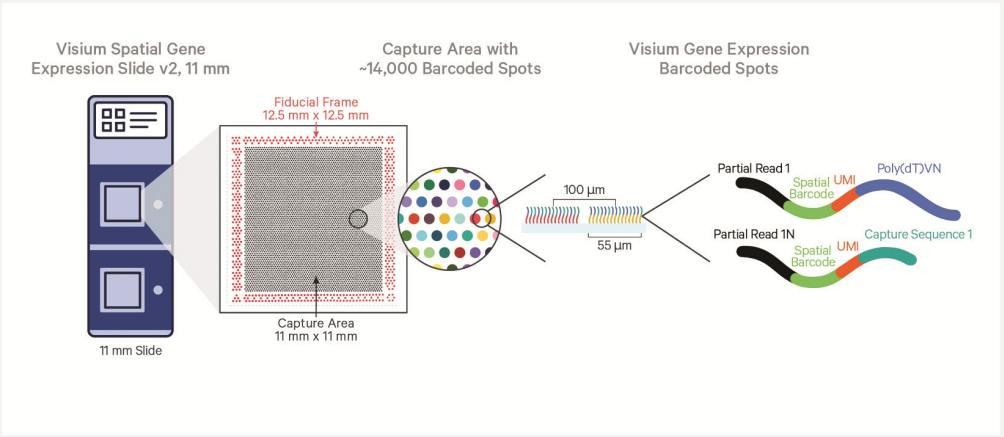
- Gene Expression: Illumina TruSeq partial read 1 sequencing primer, 16 nucleotide (nt) Spatial Barcode, 12 nt unique molecular identifier (UMI), 30 nt poly(dT) sequence (captures ligation product).
- Protein Detection: Illumina Nextera Read 1 (partial read 1 sequencing primer), 16 nt Spatial Barcode, 12 nt Unique molecular identifier (UMI), 16 nt partial capture sequence (captures antibody tags).
- Both gene expression and protein detection oligos within the same spot share the same Spatial Barcode.
- Each Capture Area on the CytAssist Spatial Gene Expression Slide is surrounded by a spacer. This spacer creates a reaction chamber that facilitates proper reagent addition and creates a seal between the CytAssist Spatial Gene Expression Slide and the tissue slide.
- The active surface of the slide is defined by a readable label that includes the serial number.



## Visium CytAssist Spatial Gene Expression Slide v2, 11 mm

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

- Gene Expression: Illumina TruSeq partial read 1 sequencing primer, 16 nt Spatial Barcode, 12 nt unique molecular identifier (UMI), 30 nt poly(dT) sequence (captures ligation product).
- Protein Detection: Illumina Nextera Read 1 (partial read 1 sequencing primer), 16 nt Spatial Barcode, 12 nt unique molecular identifier (UMI), 16 nt partial capture sequence (captures antibody tags).
- Both gene expression and protein detection oligos within the same spot share the same Spatial Barcode.
- Each Capture Area on the CytAssist Spatial Gene Expression Slide is surrounded by a spacer. This spacer creates a reaction chamber that facilitates proper reagent addition and creates a seal between the CytAssist Spatial Gene Expression Slide and the tissue slide.
- The active surface of the slide is defined by a readable label that includes the serial number.

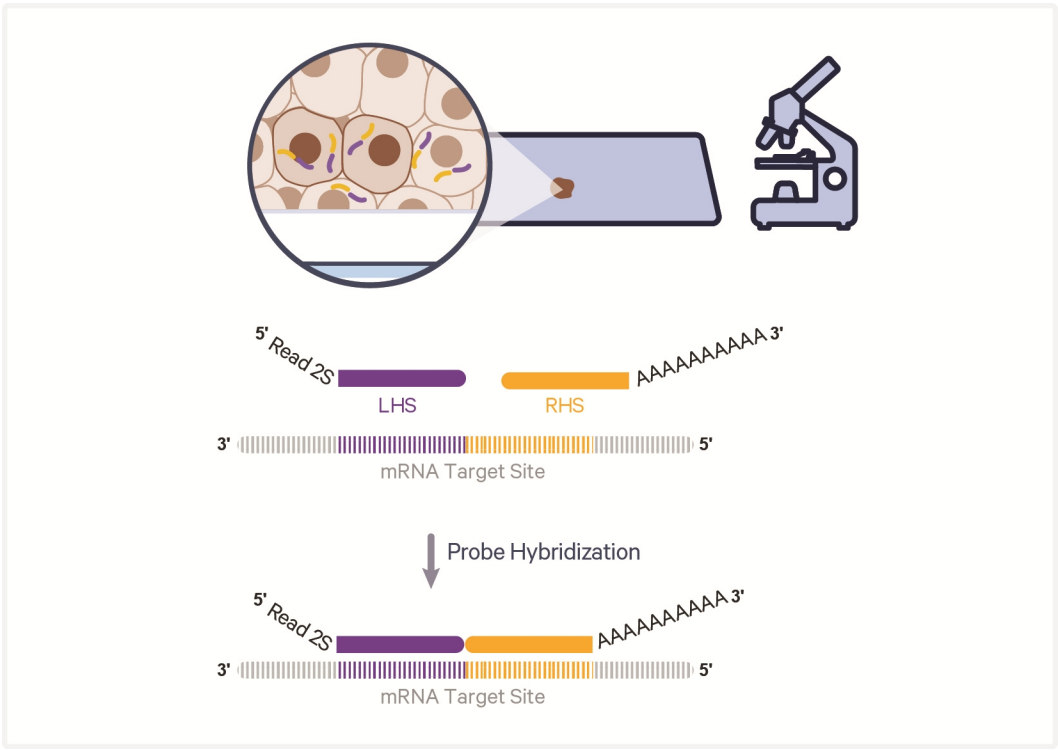


## Step 1: DNase Treatment

Deparaffinized, stained, and decrosslinked tissues in a Visium CytAssist Tissue Slide Cassette are treated with DNase to reduce the likelihood of signal from genomic DNA.

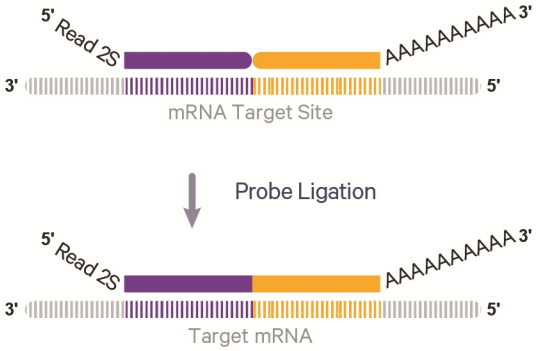
## Step 2: Probe Hybridization

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



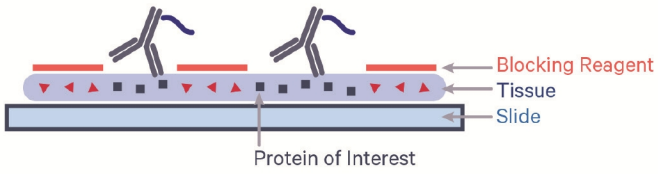
## Step 3: Probe Ligation

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




## Step 4: Protein Labeling

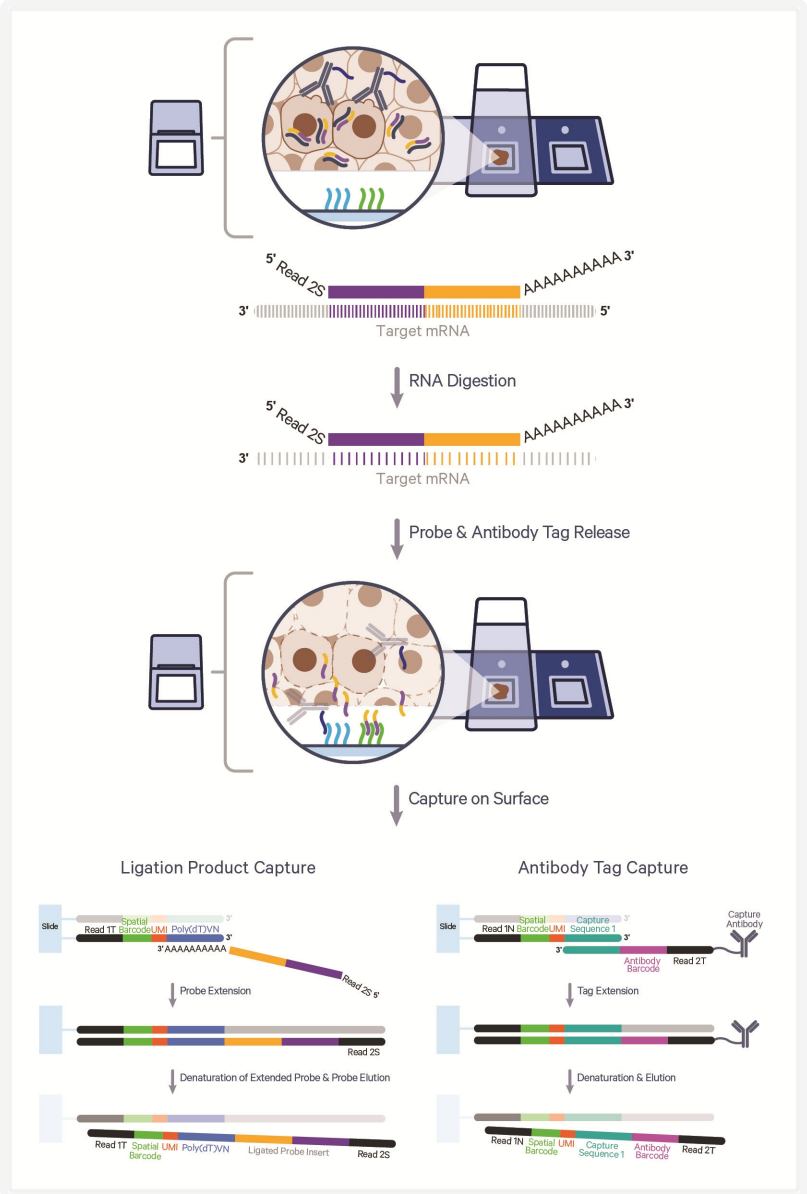
After ligation, the tissue is blocked with Blocking Reagent to minimize nonspecific binding of the antibodies to regions with no target protein expression. After blocking, the tissue is incubated with a mixture of antibodies.



# Step 5: Probe and Antibody Tag Release & Extension

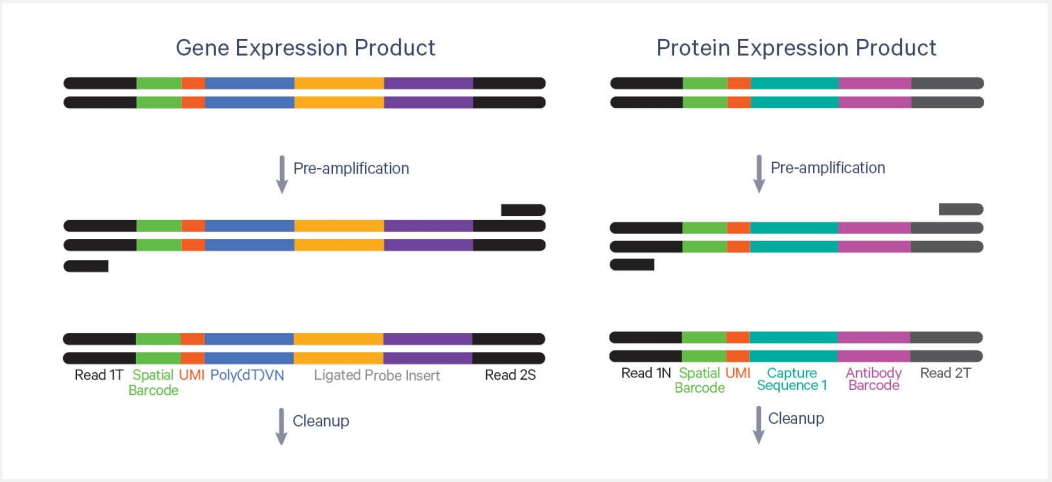
Probe release and capture occurs in the Visium CytAssist instrument. The single stranded ligation products and antibody tags are released from the tissue upon RNase treatment and tissue removal, and then captured on the Visium slide. Once ligation products and antibody tags are captured, the slides can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

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



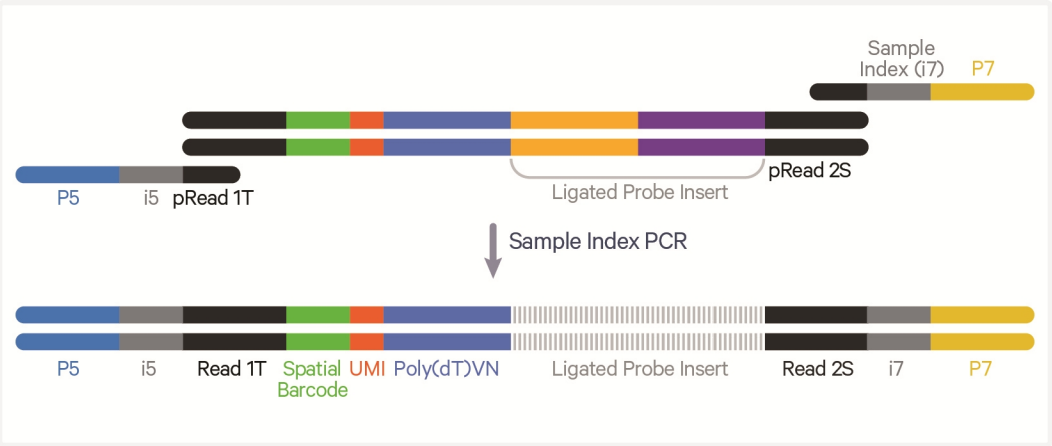
## Step 6: Pre-amplification and SPRIselect

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



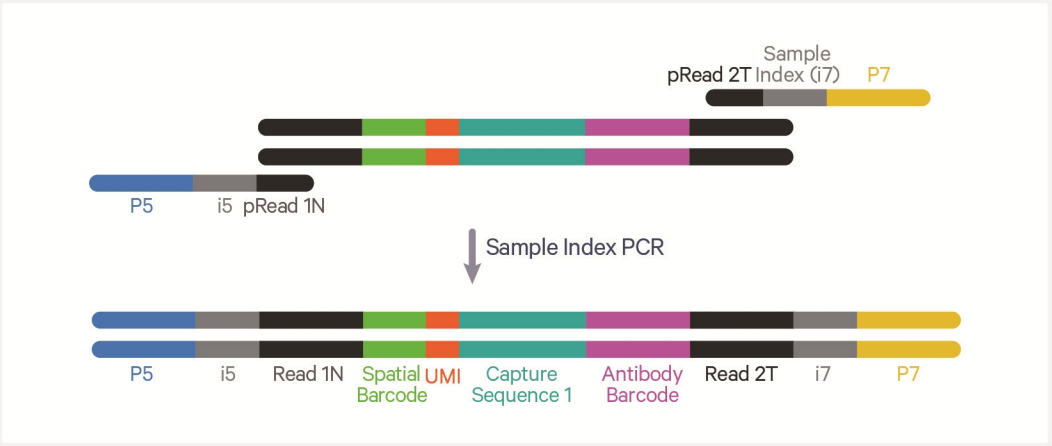

## Step 7: Visium CytAssist Spatial Gene Expression - Probe-based Library Construction

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



# Step 8: Visium CytAssist Spatial Protein Expression Library Construction

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

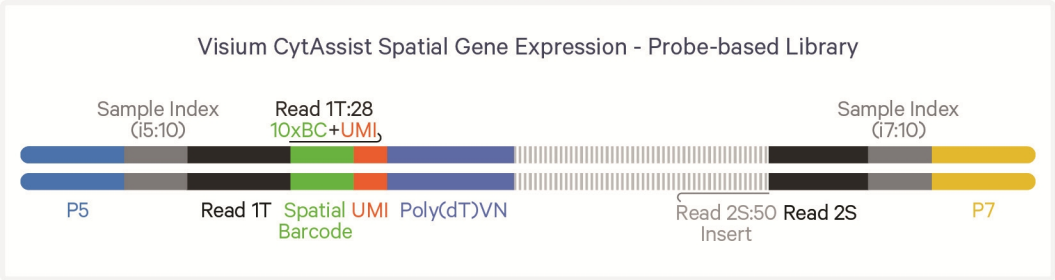


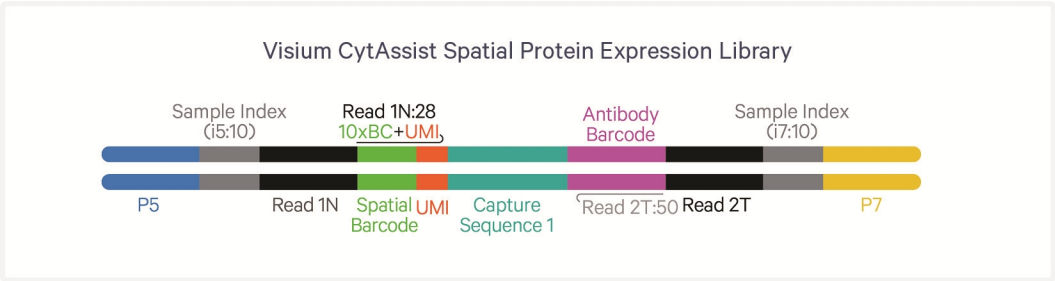
# Sequencing

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

A Visium CytAssist Spatial Protein 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 Nextera Read 1, while TruSeq RNA Read 2 (Read 2) is used to sequence the antibody tag.

Illumina sequencer compatibility, sample indices, and library loading information are summarized in the Sequencing section.




See Oligonucleotide Sequences on page 151

10x GENOMICS

# Tips & Best Practices

## Icons


Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

## General Reagent Handling

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

## Visium Slide Storage

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

## Pipette Calibration

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

## Visium Spatial Slide Handling

- Always wear gloves when handling slides.
- Ensure that the active surface of a slide faces up and is never touched. The active surface is defined by a readable label.
- Minimize exposure of the slides to sources of particles and fibers.
- When pipetting reagent onto a slide, avoid generating bubbles.

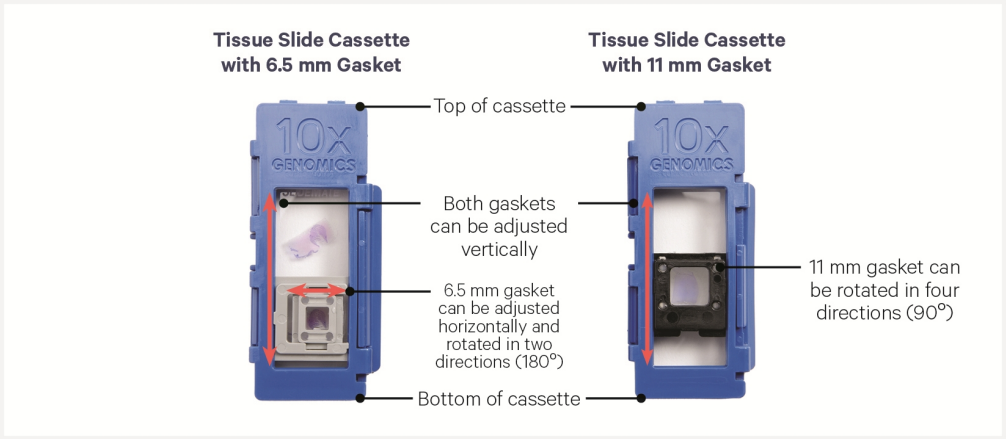
- Time between adding the final reagent to the Probe and Antibody Tag Release Mix and loading the complete Probe and Antibody Tag Release Mix onto spacers on the Visium CytAssist Spatial Gene Expression v2 Slide on the Visium CytAssist instrument and starting a run should not exceed **5 min**. Start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

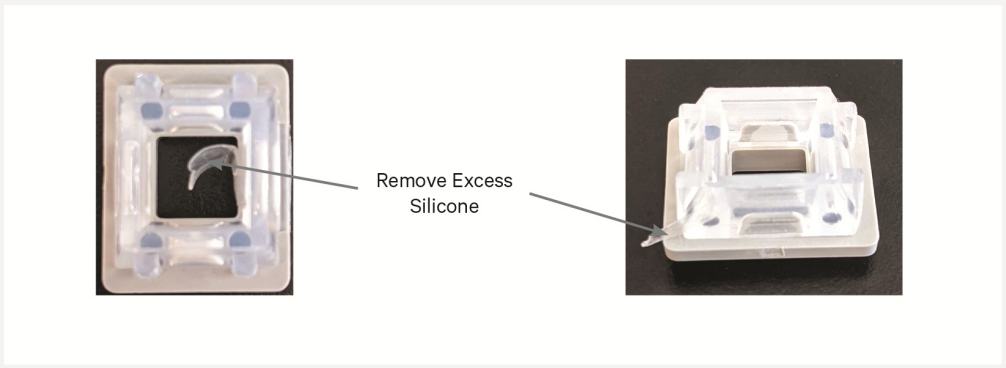
## Tissue Slide Handling

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

## Visium CytAssist Tissue Slide Cassette

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


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


Remove Excess Silicone

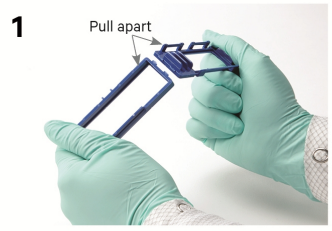
# Visium CytAssist Tissue Slide Cassette Assembly

Assembly instructions apply to both Tissue Slide Cassette sizes.

Wear fresh gloves while assembling Tissue Slide Cassette

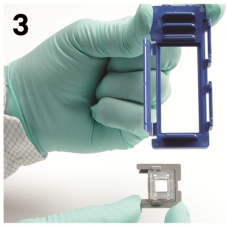
Exercise caution when handling slide edges to prevent injury.

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



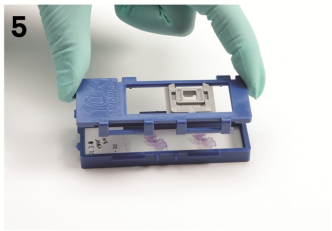

**Place tissue slide into lower half of cassette with tissue facing up**


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

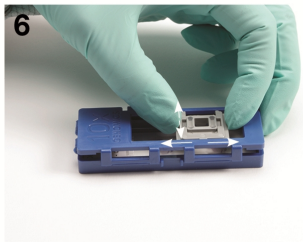



**Securely combine gasket with top half of cassette until the gasket snaps into place.**

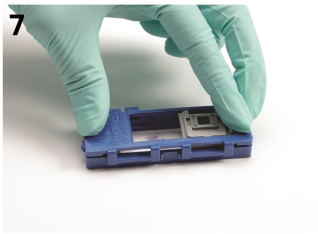

**Gently place top half of cassette over bottom half. DO NOT assemble together until Step 6.**



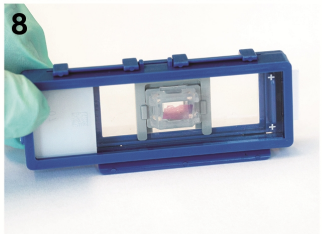

**Adjust gasket such that gasket is over the tissue region of interest. The 6.5 mm gasket can be adjusted horizontally as well as vertically.**




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



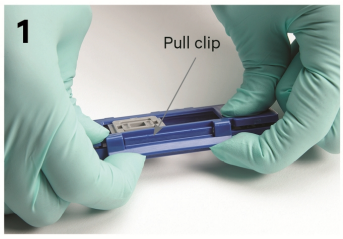

**Turn cassette over and verify tissue region of interest is within gasket. DO NOT move gasket once cassette is closed. If necessary, open cassette and recenter gasket.**



# Visium CytAssist Tissue Slide Cassette Removal

Removal instructions apply to both Tissue Slide Cassette sizes.

**Pull clip up to detach upper and lower halves of cassette**

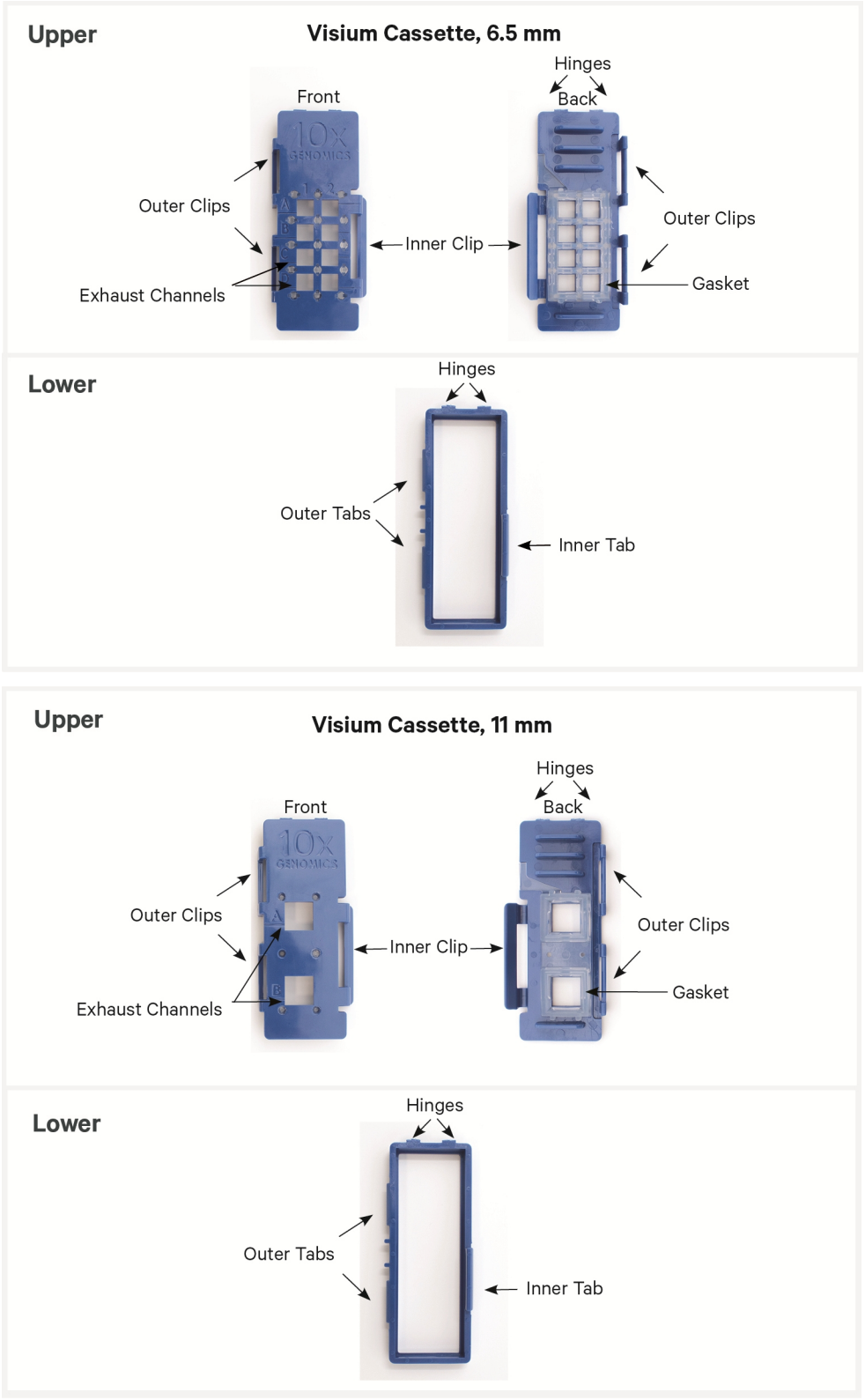



**Open cassette by continuing to lift clip upward. If slide sticks to gasket, continue to apply even upward pressure to separate slide from gasket**


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

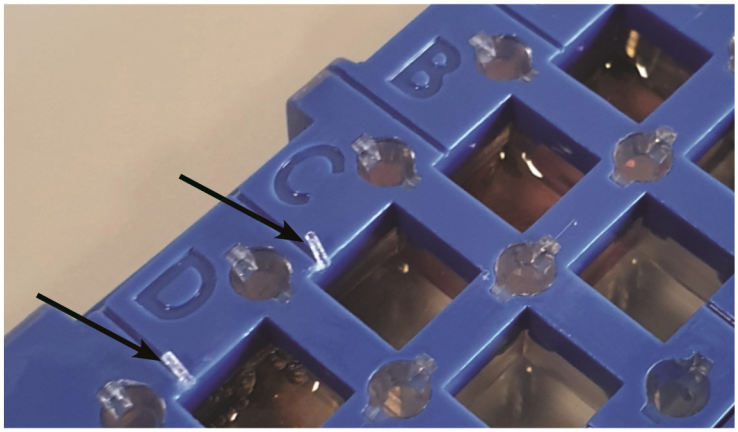

Slides in images are representative.

# Visium Cassette



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

**Remove Excess Silicone**



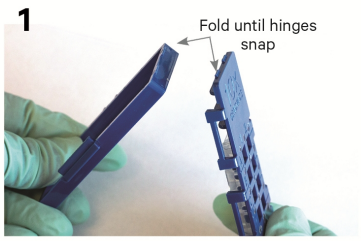

- See Visium Cassette Assembly & Removal instructions for details on assembly and removal.
- Practice assembly with a blank slide (75 x 25 x 1 mm).
- Place the slides in the Visium Cassette only when specified.
- Applying excessive force to the slide may cause the slide to break.

# Visium Cassette Assembly

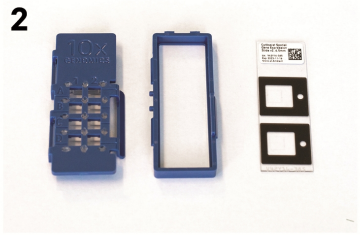
Ensure that the surface of the cassette is dry. Cassette may also be assembled in the hand for comfort.

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

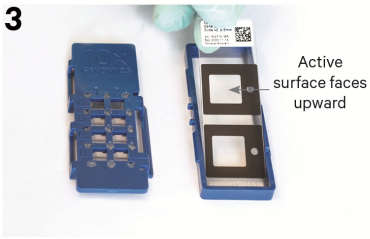
**Break cassette into two halves by bending each half at the hinge until they snap apart**



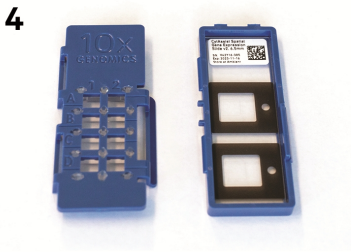

**Place upper and lower halves of cassette, and Visium slide, directed upward on bench**



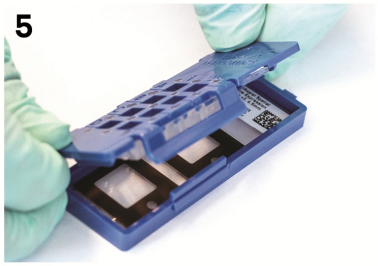

**Place Visium slide with active surface facing upwards into lower half of cassette; ensure label is toward hinges**



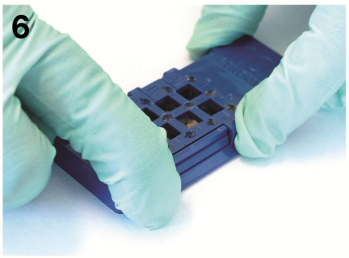

**Press slide down into grooves of the bottom half of the cassette until it sits firmly in place**




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




**Press firmly on top of cassette until it clicks shut**



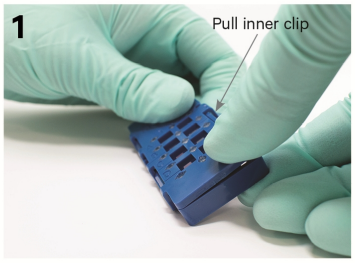

Exercise caution when handling slide edges to prevent injury.

Slides in images are representative.

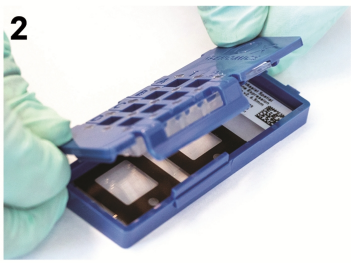
# Visium Cassette Removal

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

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




**Open cassette by continuing to lift upper half upward**



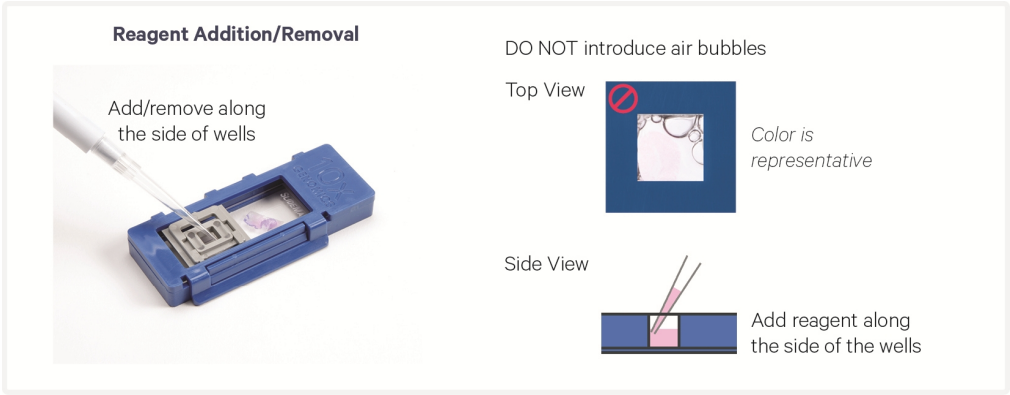

**Lift slide out from lower half**


Slides in images are representative.

# Reagent Addition to & Removal from Wells

## Reagent Addition

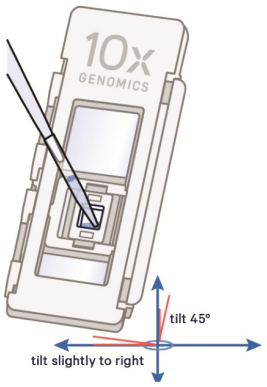
- Assemble slide into the cassette flat on a clean work surface.
- Dispense and remove reagents along the side of the wells without touching the slide surface, tissue sections (when applicable), and without introducing bubbles.
- If applicable, perform washes next to the thermal cycler to avoid significant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




## Reagent Removal from Wells

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

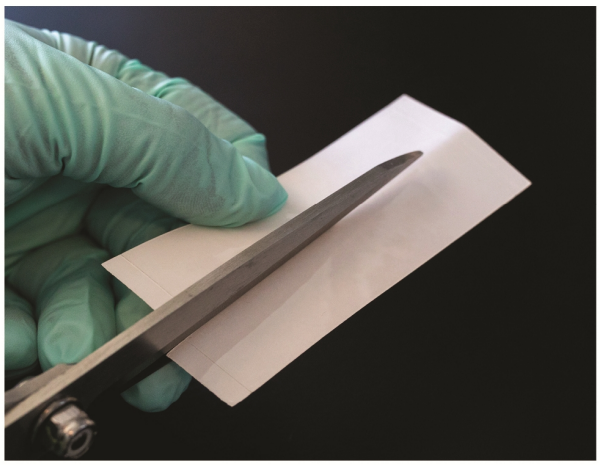
Repeat removal steps until no reagent remains.




# Visium Slide Seal Application & Removal

## Application

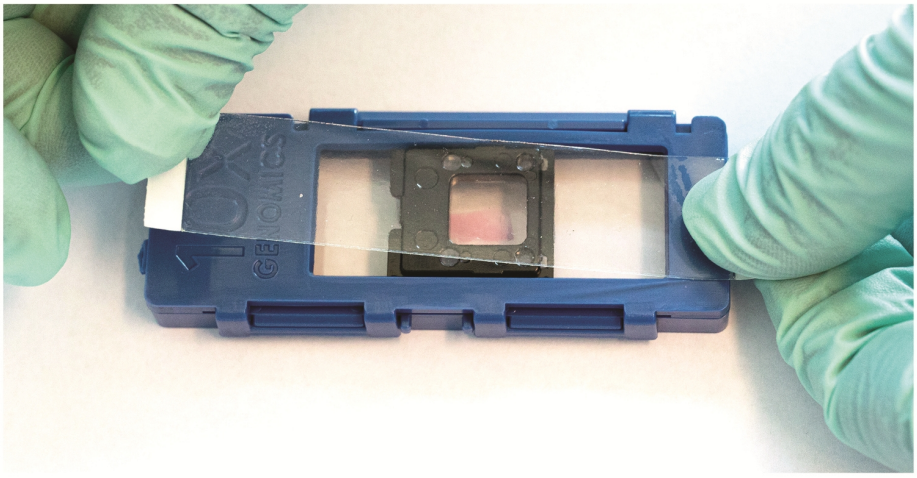
- If applying a Visium Slide Seal to a Tissue Slide Cassette, the seal must be cut in half lengthwise. Cut the seal as shown in the image below. Three pre-cut seals are necessary for this assay.



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

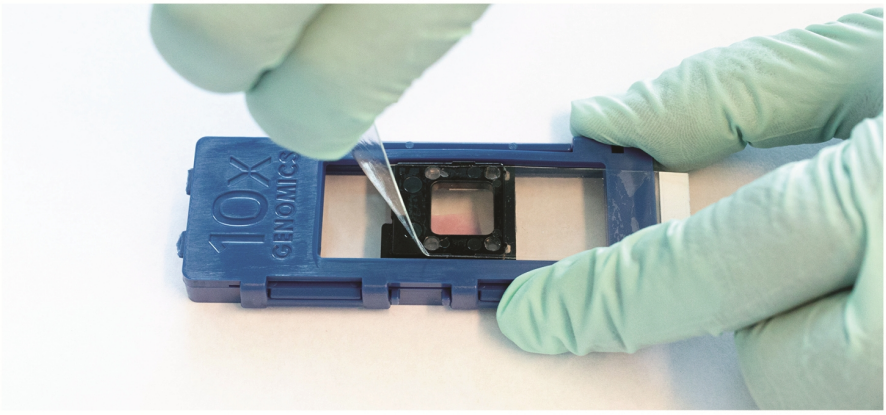
- Use a fresh Visium Slide Seal when prompted during the protocol. Steps that do not require a new slide seal will specify that the slide seal should be pulled back and reapplied instead.

**Apply Visium Slide Seal**




## Removal

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



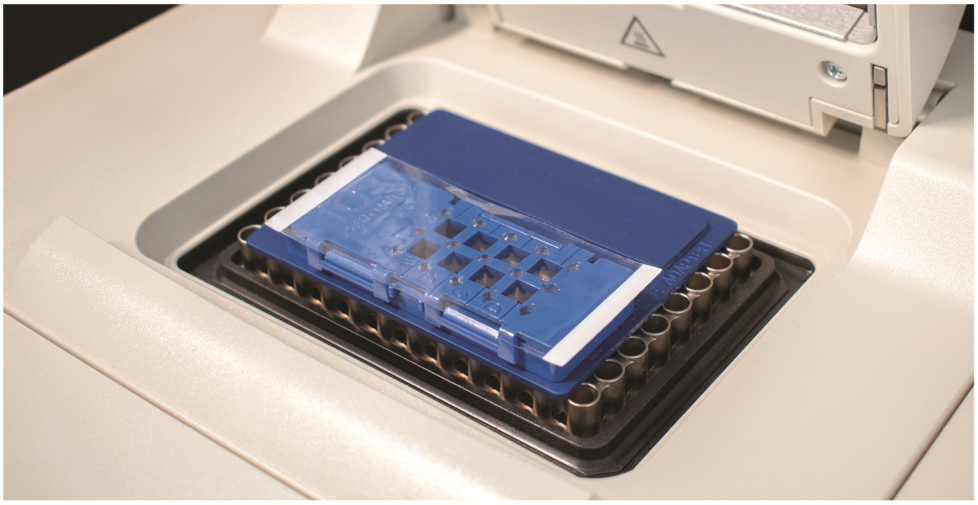
# Slide Incubation Guidance

## Incubation at a Specified Temperature

- Position a Low Profile Thermocycler Adapter on a thermal cycler that is set at the incubation temperature.
- Ensure that the Low Profile Thermocycler Adapter is in contact with the thermal cycler block surface uniformly. Pre-equilibrate adapters at the HOLD temperature described in the protocol steps for at least five minutes.
- When incubating a slide encased in a cassette, place the assembled unit on the Low Profile Thermocycler Adapter with the wells facing up. Ensure the cassette is in complete contact with the adapter. The cassette should always be sealed when on the Low Profile Thermocycler Adapter. The cassette should snap into place when seated properly on the Low Profile Thermocycler Adapter.

### Incubate Assembled Visium Cassette

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



Cassettes with PN-3000811/3000812 are only compatible with the Low Profile Thermocycler Adapter. Use of the wrong Thermocycler Adapter may cause inconsistent heating and slide breakage.

### Incubation at Room Temperature

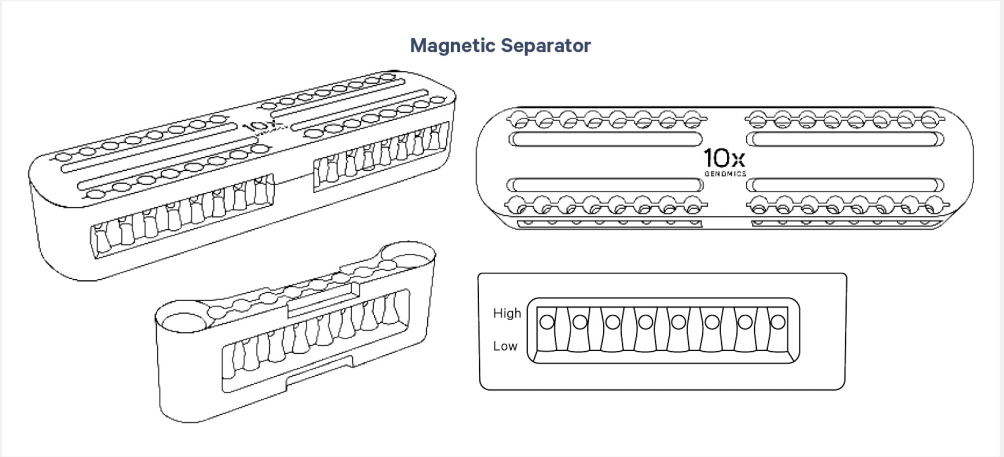
- Place the slide/cassette on a flat, clean, nonabsorbent work surface.

## Tissue Detachment on Tissue Slides

- Monitor section adhesion on tissue slides throughout the workflow.
- Ensure that tissue sections are on Superfrost Plus or positively charged slides. A list of tested slides can be found in Visium CytAssist Tested Slides on page 51.
- Tissue detachment before the completion of Probe and Antibody Tag Release during the workflow can negatively impact performance. If observed, contact support@10xgenomics.com.
- For more information, see Troubleshooting on page 134.

## 10x Magnetic Separator

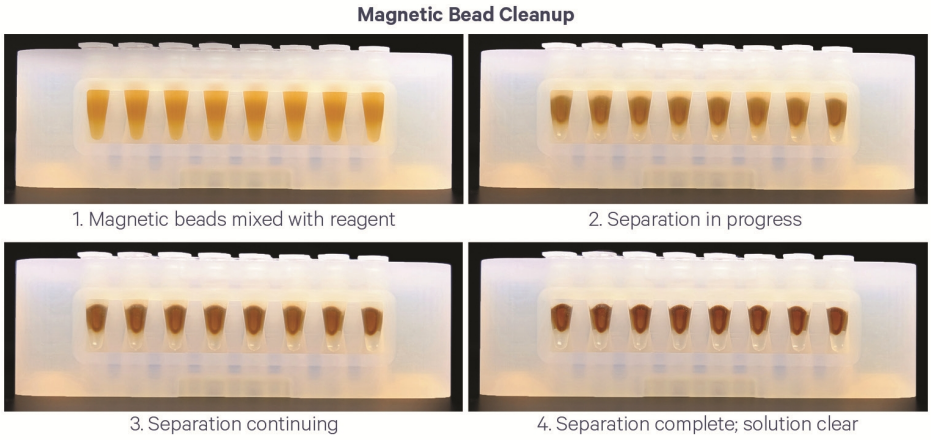
- Images below are illustrative - actual appearance of magnetic separator may vary. Guidance applies to all 10x Magnetic Separators.




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

## Magnetic Bead Cleanup Steps

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




## SPRIselect Cleanup & Size Selection

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

## Sample Indices (i5/i7) in Sample Index PCR

- Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run.
- Each well in the Dual Index Plate TS Set A and NT Set A contains a unique i7 and a unique i5 oligonucleotide. These indices can be pooled to enable simultaneous sequencing of Gene Expression and Protein Expression

libraries.

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

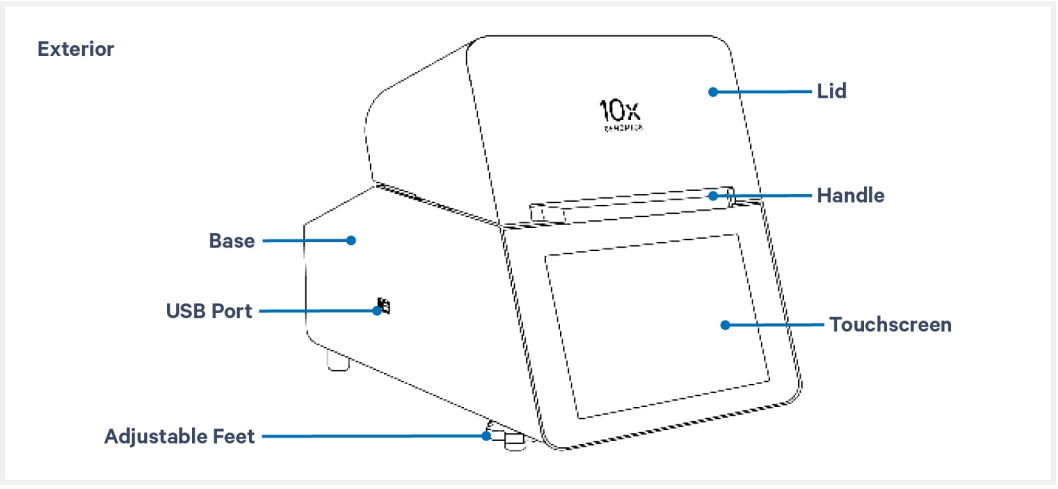
# Visium CytAssist

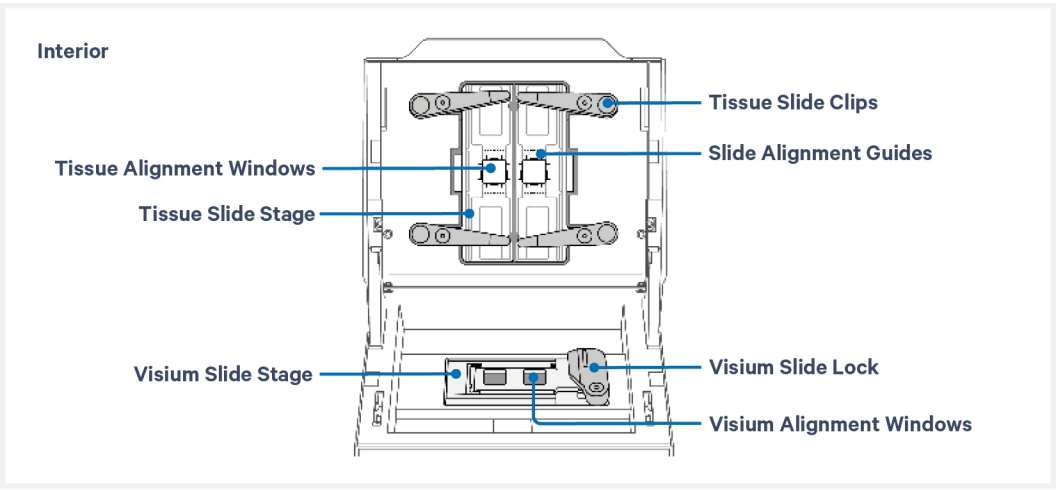

Instrument Orientation 50

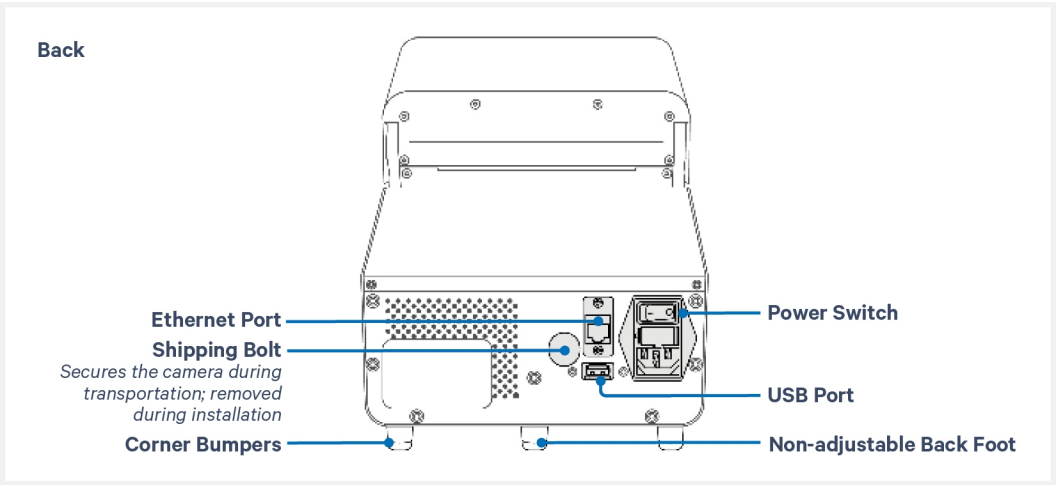
Visium CytAssist Tested Slides 51

Instrument Loading Guidelines 52

# Instrument Orientation



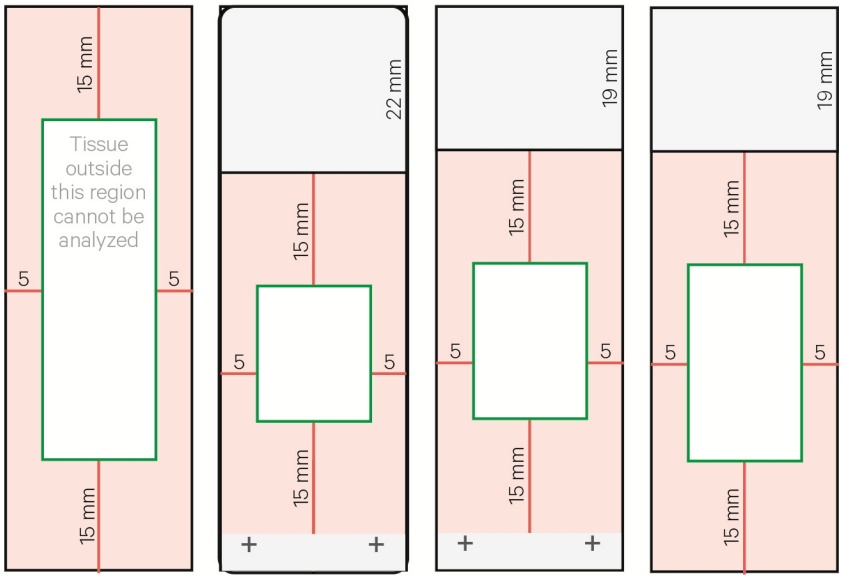
# Visium CytAssist Tested Slides

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

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

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

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




Blank

Epredia Shandon ColorFrost Plus

VWR SuperFrost Plus Micro Slide, Premium

Fisherbrand SuperFrost Plus

Sigma-Aldrich Poly Prep

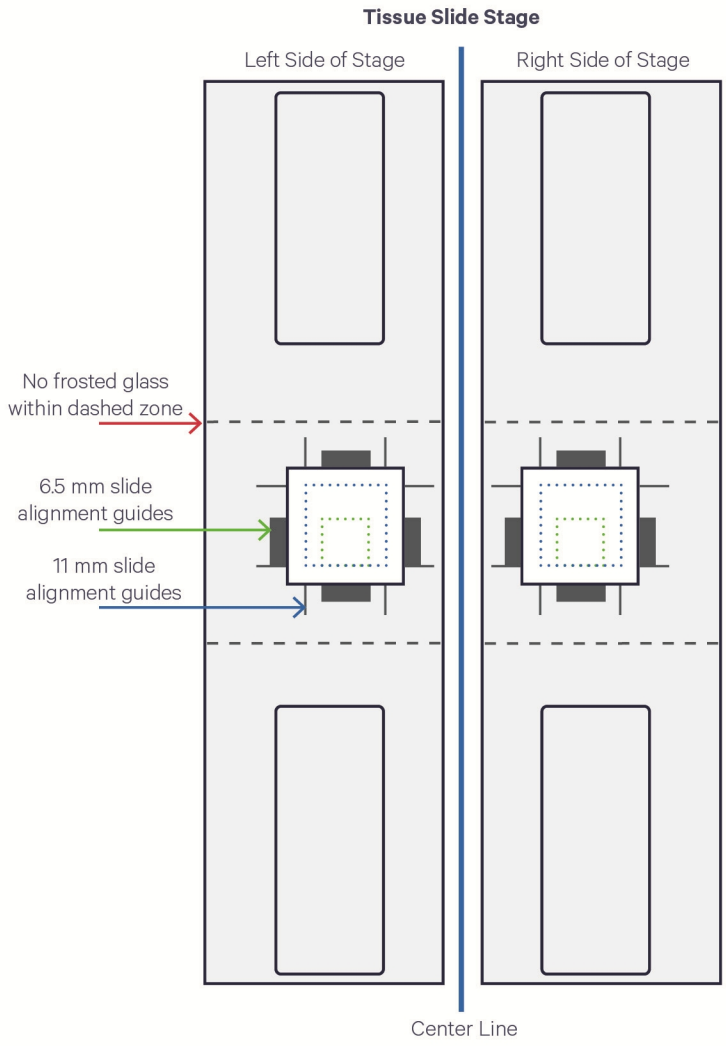
# Instrument Loading Guidelines

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

## Determine Slide Placement

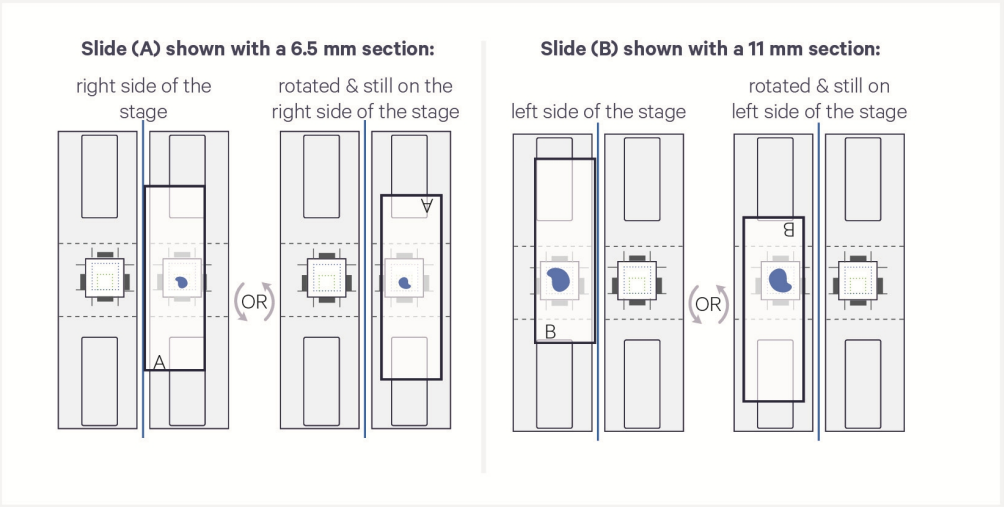
**a.** On the diagram below, align tissue to the center of the alignment guides for 6.5 mm (rectangles) or 11 mm (lines) Capture Areas on either the left or right side of the stage. Align the center of the tissue region of interest to the center of the Capture Area, instead of aligning the edge of the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

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




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

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

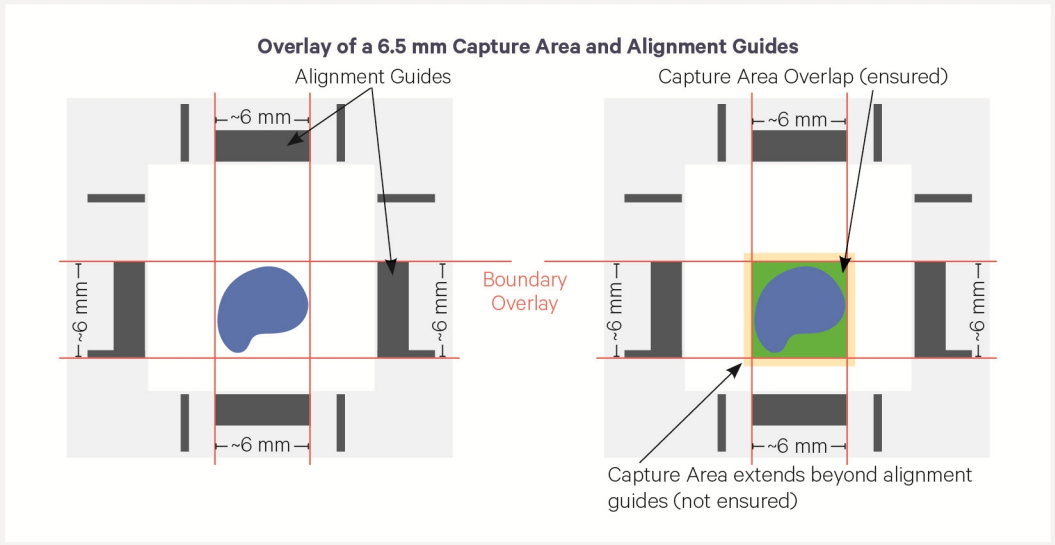


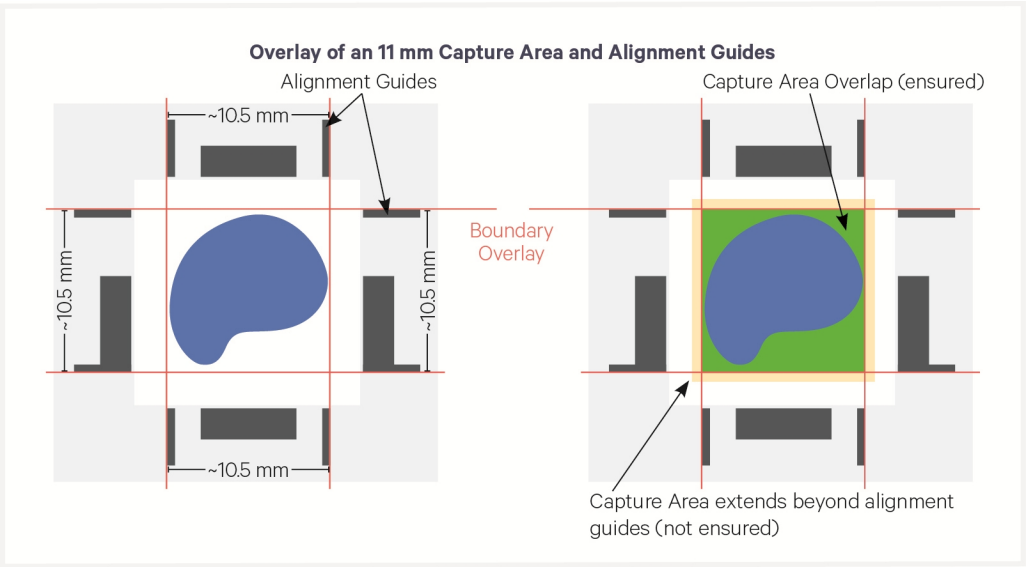

## Fine Adjustments

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

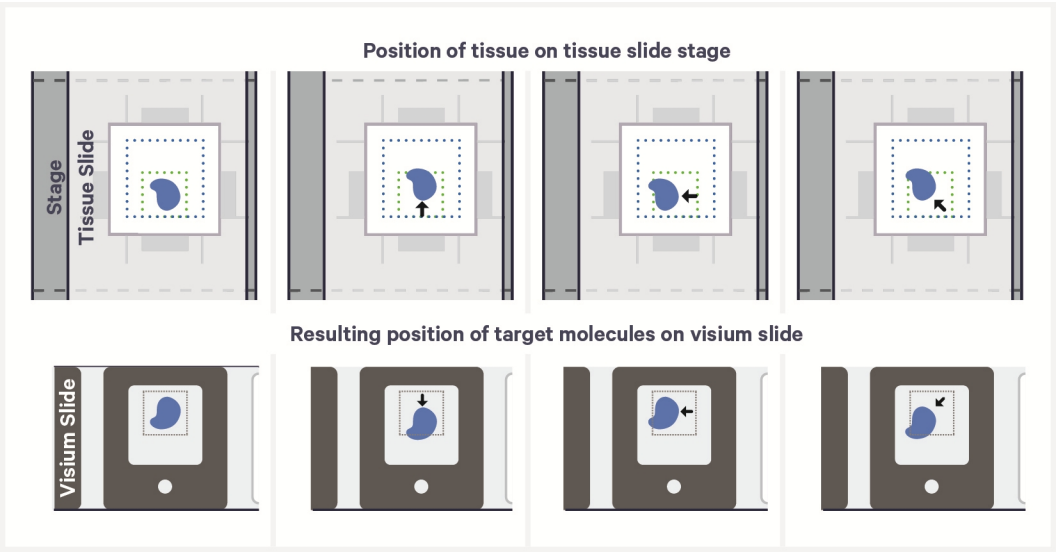
When placing a tissue slide on the Tissue Slide Stage, center the tissue or area of interest within the area inside the outer edges of the alignment guides (area in green in the images below).

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




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

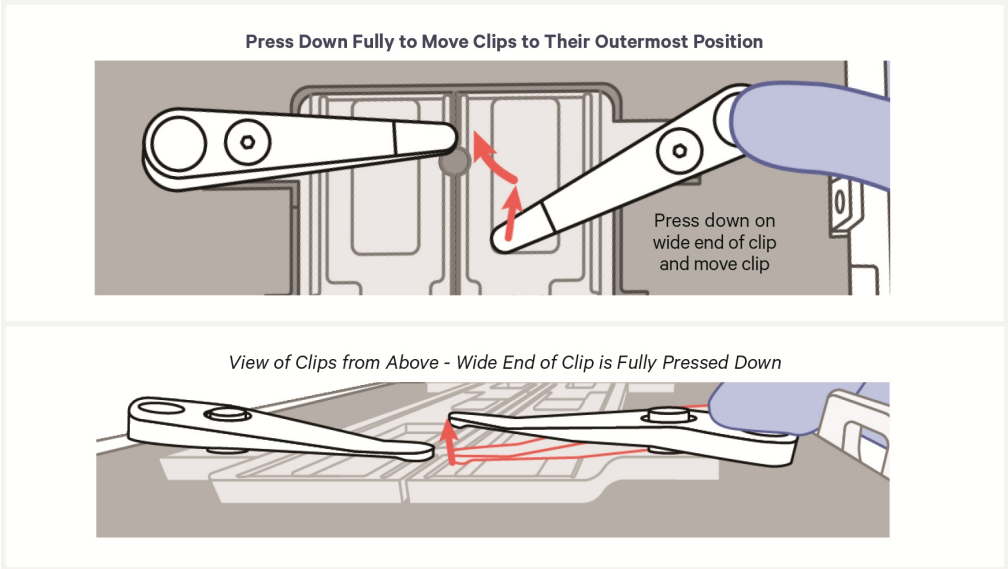



## Tissue Slide Loading

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

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

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




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

*DO NOT touch the tissue section.*

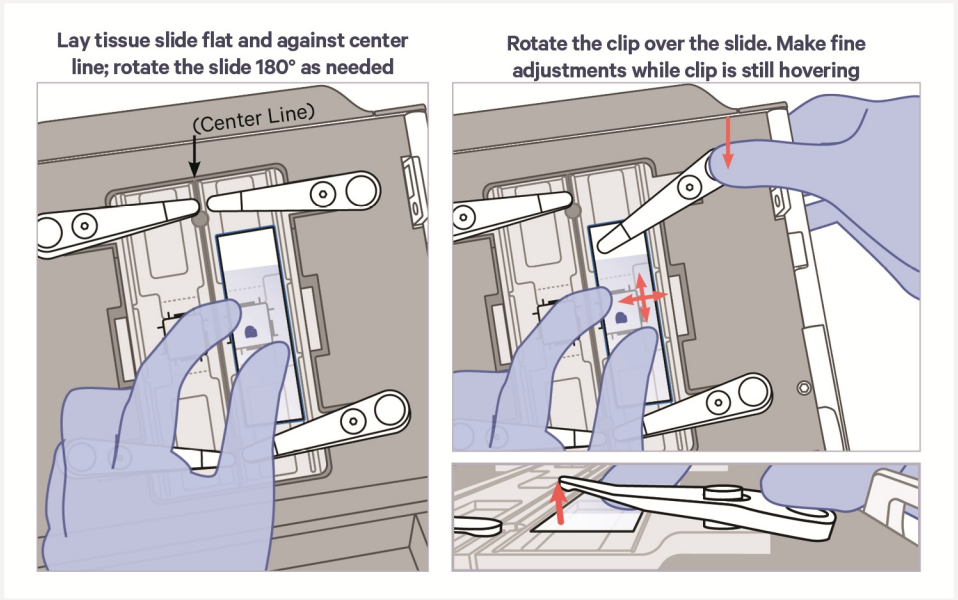
**c.** Use one hand to hold slide in place with two fingers, one on either side of the tissue. Use the other to both cradle the instrument lid and fully press down on the first clip. Rotate the clip over the slide.

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

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

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

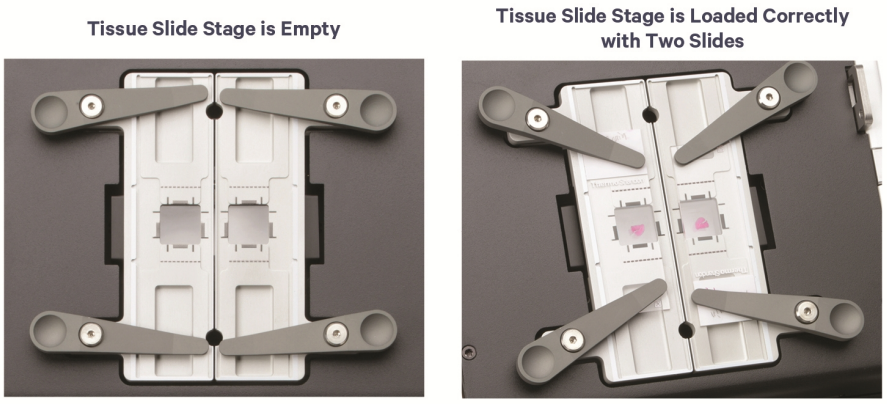
*Reference images below for steps b-d.*

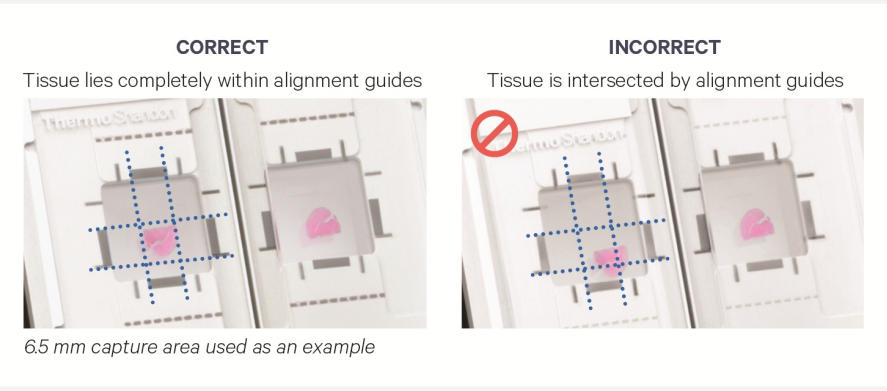



**e.** Continue to hold the slide in place while pressing down fully on the wide end of the second clip. Rotate the second clip onto the slide and gently release.

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

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




*6.5 mm capture area used as an example*

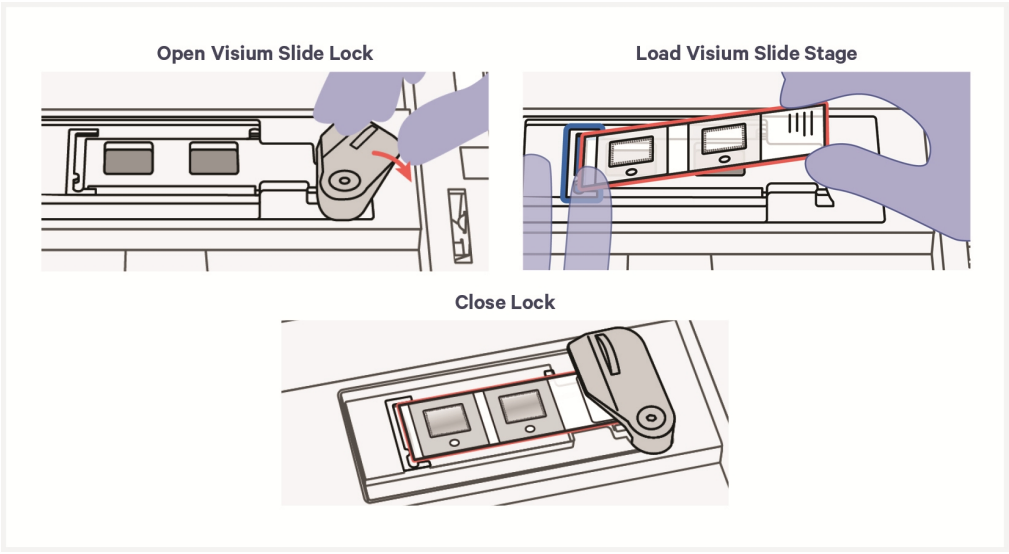
## Visium CytAssist Spatial Slide Loading

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

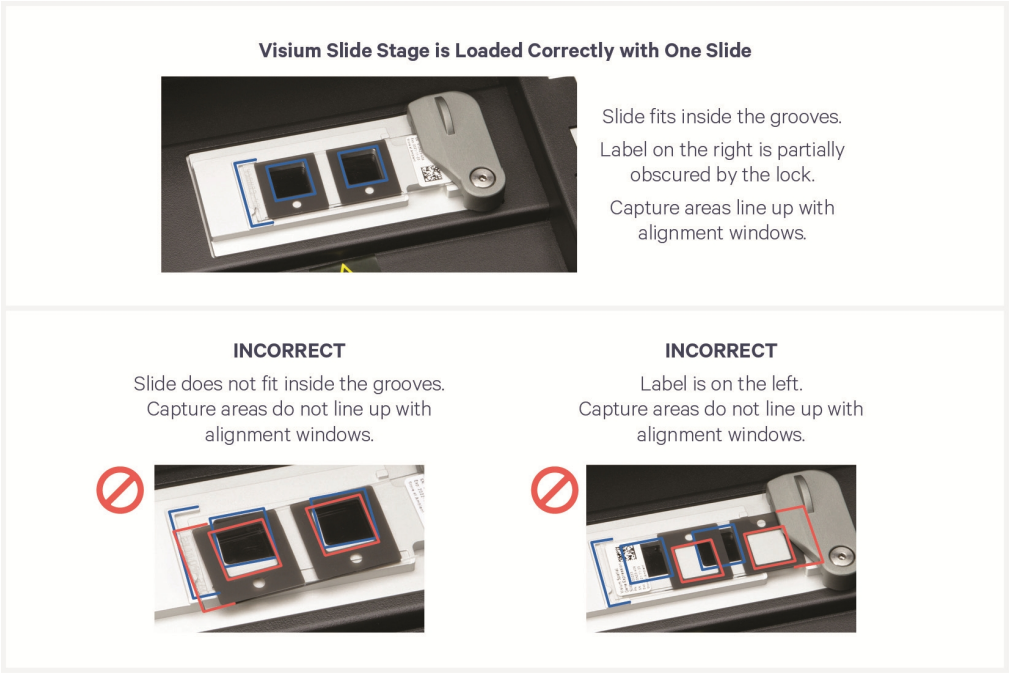
**a.** Open the Visium Slide Lock by using one finger to pivot.

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

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




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



10x GENOMICS

# Sample Preparation & Staining Guidelines

# Sample Preparation

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

Before performing the protocol steps in this User Guide, ensure that all samples have followed the appropriate documentation as described in the Workflow Overview on page 20. Additionally, if using an add-on antibody, optimize the antibody concentration before this User Guide according to the Visium CytAssist Spatial Gene and Protein Expression Add-on Antibody Optimization Protocol (CG000664). Listed below are key considerations described in the tissue preparation documentation.

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

# Step 1:

## DNase Treatment

1.0 Get Started 63

1.1 DNase Treatment 64

# 1.0 Get Started

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

| Items | | 10x PN | Preparation & Handling | Storage |
|---|---|---|---|---|
| **Equilibrate to room temperature** | | | | |
| ☐ | DNase Buffer | Tube: 2000882<br>Kit: 1000603 | Maintain at room temperature. | –20°C |
| **Place on ice** | | | | |
| ☐ | DNase | Tube: 2000881<br>Kit: 1000603 | Thaw on ice. | –20°C |
| **Obtain** | | | | |
| ☐ | Nuclease-free Water | - | - | Ambient |

# 1.1 DNase Treatment

Before starting this protocol, ensure that tissue sections have been stained according to the appropriate protocol. These staining protocols include Decrosslinking, an important step that must be performed before starting the assay. Refer to Workflow Overview on page 20 for more information.

TIPS

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

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

**a.** Prepare a thermal cycler with the following two incubation protocols. Start the DNase Treatment program.

| DNase Treatment | | |
|---|---|---|
| **Lid Temperature** | **Reaction Volume** | **Run Time** |
| 37°C (lid may be turned off if the instrument does not enable 37°C) | 100 µl | 30 Min |
| **Step** | **Temperature** | **Time** *hh:mm:ss* |
| Pre-equilibrate | 37°C | Hold |
| DNase Treatment | 37°C | 00:30:00 |

| DNase Inactivation | | |
|---|---|---|
| **Lid Temperature** | **Reaction Volume** | **Run Time** |
| 75°C | 100 µl | 5 Min |
| **Step** | **Temperature** | **Time** *hh:mm:ss* |
| Heat Inactivation | 75°C | 00:05:00 |
| Hold | 22°C | Hold |

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

| | 6.5 mm Gaskets<br>DNase Mix | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|---|
| ● | DNase Buffer | 2000882 | 35.0 | 77.0 | 154.0 |
| | Nuclease-free Water | - | 28.0 | 61.6 | 123.2 |
| ● | DNase | 2000881 | 7.0 | 15.4 | 30.8 |
| | **Total** | **-** | **70.0** | **154.0** | **308.0** |

| | 11 mm Gaskets<br>DNase Mix | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|---|
| ● | DNase Buffer | 2000882 | 70.0 | 154.0 | 308.0 |
| | Nuclease-free Water | - | 56.0 | 123.2 | 246.4 |
| ● | DNase | 2000881 | 14.0 | 30.8 | 61.6 |
| | **Total** | **-** | **140.0** | **308.0** | **616.0** |

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

**c.** Using a pipette, remove all buffer from each well at the well corners. For H&E stained slides, remove all Decrosslinking buffer. For IF stained slides, remove all PBS.

**d.** Add **■70 µl** or **▲ 140 µl** DNase Mix along the side of each well to uniformly cover the tissue sections, without introducing bubbles.

**e.** Re-apply Visium Slide Seal on each Tissue Slide Cassette and place on the Low Profile Thermocycler Adapter on the thermal cycler. Close the thermal cycler lid.

**f.** Skip Pre-equilibrate step to initiate DNase Treatment.

**g.** After DNase Treatment, **immediately** start the DNase Inactivation program on the thermal cycler.

**h.** Discard gloves and clean workstation before Probe Hybridization.

# Step 2:

## Probe Hybridization

2.0 Get Started 67

2.1 Probe Hybridization 68

# 2.0 Get Started

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

| Items | | | 10x PN | Preparation & Handling | Storage |
|---|---|---|---|---|---|
| **Equilibrate to room temperature** | | | | | |
| ☐ | ○ | FFPE Hyb Buffer | Tube: 2000423<br>Kit: 1000466 | Thaw at room temperature. If precipitate persists, heat at 37°C until dissolved. Avoid vortexing to prevent bubble formation. Pipette mix 10x. Keep the buffer at room temperature after thawing and while performing the workflow. | –20°C |
| **Place on ice** | | | | | |
| ☐ | ● | Human WT Probes v2 - RHS | Tube: 2000657<br>Kit: 1000466 | Thaw at room temperature. Vortex and centrifuge briefly. Maintain on ice. | –20°C |
| ☐ | ● | Human WT Probes v2 - LHS | Tube: 2000658<br>Kit: 1000466 | Thaw at room temperature. Vortex and centrifuge briefly. Maintain on ice. | –20°C |
| **Obtain** | | | | | |
| ☐ | | Nuclease-free Water | - | - | Ambient |
| ☐ | | 10X PBS, pH 7.4 | - | - | Ambient |
| ☐ | | Visium Slide Seals | Component: 2000284<br>Kit: 1000519/1000518 | See Tips & Best Practices. 3 pre-cut slide seals are necessary for the complete workflow. | Ambient |
| ☐ | | 10% Tween-20 | - | - | Ambient |

# 2.1 Probe Hybridization

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

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

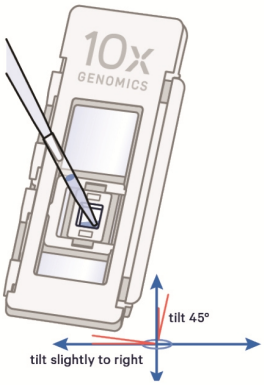
| 6.5 mm Gaskets<br>Pre-Hybridization Mix | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|
| Nuclease-free Water | - | 402.8 | 886.0 | 1,772.1 |
| 10X PBS, pH 7.4 | - | 45.0 | 99.0 | 198.0 |
| 10% Tween-20 | - | 2.3 | 5.0 | 9.9 |
| **Total** | **-** | **450.1** | **990.0** | **1,980.0** |

| 11 mm Gaskets<br>Pre-Hybridization Mix | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|
| Nuclease-free Water | - | 805.5 | 1,772.1 | 3,544.2 |
| 10X PBS, pH 7.4 | - | 90.0 | 198.0 | 396.0 |
| 10% Tween-20 | - | 4.5 | 9.9 | 19.8 |
| **Total** | **-** | **900.0** | **1,980.0** | **3,960.0** |

**b.** Retrieve Tissue Slide Cassettes containing DNase-treated sample from thermal cycler.

**c.** Remove Visium Slide Seals.

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




e. Add ■**150 µl** or ▲**300 µl** Pre-Hybridization Mix along the side of each well to uniformly cover tissue sections, without introducing bubbles.

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


g. Using a pipette, remove all buffer from each well at well corners.

h. Repeat steps e-g twice more for a total of three washes.

TIPS

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

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

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

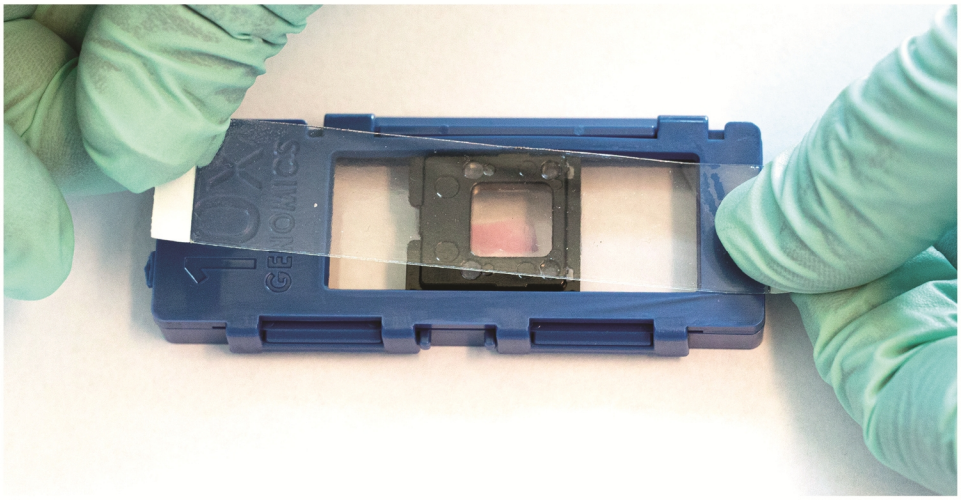
| | 6.5 mm Gaskets<br>Probe Hybridization Mix | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|---|
| ○ | FFPE Hyb Buffer | 2000423 | 70.0 | 154.0 | 308.0 |
| | Nuclease-free Water | - | 10.0 | 22.0 | 44.0 |
| ● | Human WT Probes v2 - RHS | 2000657 | 10.0 | 22.0 | 44.0 |
| ● |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

| | 11 mm Gaskets<br>Probe Hybridization Mix | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|---|
| ○ | FFPE Hyb Buffer | 2000423 | 140.1 | 308.2 | 616.4 |
| | Nuclease-free Water | - | 19.9 | 43.8 | 87.6 |
| ● | Human WT Probes v2 - RHS | 2000657 | 20.0 | 44.0 | 88.0 |
| ● | Human WT Probes v2 - LHS | 2000658 | 20.0 | 44.0 | 88.0 |
| | **Total** | **-** | **200.0** | **440.0** | **880.0** |

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

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

**Apply Visium Slide Seal**



**m.** Skip Pre-equilibrate step to initiate Hybridization.

# Step 3:

## Probe Ligation


3.0 Get Started 73

3.1 Post-Hybridization Wash 74

3.2 Probe Ligation 77

3.3 Post-Ligation Wash 79

# 3.0 Get Started

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

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

# 3.1 Post-Hybridization Wash

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

**a.** Aliquot FFPE Post-Hyb Wash Buffer (**495 µl/per 6.5 mm sample, 990 µl/per 11 mm sample**) and pre-heat to **50°C** in a water bath or thermomixer. Maintain pre-heated wash buffer at **50°C** throughout washes.

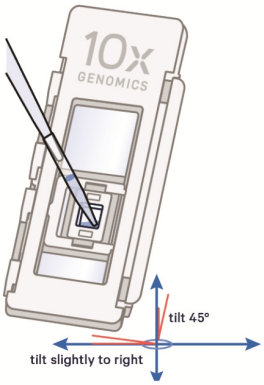
**b.** Prepare 2X SSC Buffer according to the appropriate table. Add reagents in the order listed. Maintain at **room temperature**. DO NOT discard excess buffer, as it will be used for subsequent washes in the protocol.

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

| 11 mm Gaskets<br>SSC Buffer | Stock | Final | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|---|
| SSC | 20X | 2X | 410 | 902 | 1,804 |
| Nuclease-free Water | - | - | 3,690 | 8,118 | 16,236 |
| **Total** | **-** | | **4,100** | **9,020** | **18,040** |

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

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




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

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

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

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

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

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

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

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

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

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

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

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

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

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

# 3.2 Probe Ligation

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

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

| Lid Temperature | Reaction Volume | Run Time |
|---|---|---|
| 37°C (lid may be turned off if the instrument does not enable 37°C) | 100 µl | 1 h |

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

**b.** Prepare Probe Ligation Mix according to the appropriate table shortly before use. Add reagents in the order listed. Pipette mix 10x and centrifuge briefly. DO NOT vortex. Maintain on ice.

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

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

**c.** Remove Visium Slide Seals from each Tissue Slide Cassette and remove all 2X SSC Buffer from each well.

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

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

**f.** Skip Pre-equilibrate step to initiate Ligation.

## 3.3 Post-Ligation Wash

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

Use room temperature Post-Ligation Wash Buffer at first wash step (step 3.3e). Use pre-heated Post-Ligation Wash Buffer at second wash step (step 3.3j).

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

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

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

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

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

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

TIPS

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

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

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

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

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

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

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

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

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

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

**o.** Add ■ **150 µl** or ▲ **300 µl** 2X SSC Buffer prepared in 3.1 Post-Hybridization Wash on page 74 to each well.

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

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

**r.** Re-apply Visium Slide Seals on each Tissue Slide Cassette.


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

10x GENOMICS

# Step 4:

## Protein Labeling


4.0 Get Started 82

4.1 Tissue Blocking 83

4.2 Antibody Incubation 84

4.3 Post-Antibody Incubation Wash 86

# 4.0 Get Started

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

| Items | | 10x PN | Preparation & Handling | Storage |
|---|---|---|---|---|
| **Place on ice** | | | | |
| ☐ | RNase Inhibitor | Tube: 2000556<br>Kit: 1000603 | Pipette mix, centrifuge briefly. Maintain on ice until ready to use. | –20°C |
| **Equilibrate at room temperature** | | | | |
| ☐ | Human Immune Cell Panel | Tube: 2000699<br>Kit: 1000607 | Centrifuge briefly. Maintain at room temperature until ready to use. | –20°C |
| ☐ | Block and Stain Buffer | Tube: 2000554<br>Kit: 1000436 | Thaw at room temperature. Precipitate may be present after thawing. Pipette mix 10-15x with pipette set to 750 µl until solution is homogenous. Maintain at room temperature until ready to use. | –20°C |
| **Obtain** | | | | |
| ☐ | Centrifuge | - | Equilibrate centrifuge to 4°C. | Ambient |
| ☐ | Nuclease-free Water | - | - | Ambient |
| ☐ | 2X SSC | - | Prepared in 3.1 Post-Hybridization Wash on page 74. | Ambient |
| ☐ | 10% Tween-20 | - | - | Ambient |
| ☐ | 10X PBS Buffer | - | - | Ambient |
| ☐ | Visium Slide Seals | Component: 2000284<br>Kit: 1000519/1000518 | See Tips & Best Practices. | Ambient |

# 4.1 Tissue Blocking

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

**a.** Prepare Blocking Mix according to the appropriate table, add reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain at **room temperature**.

| 6.5 mm Gaskets<br>Blocking Mix | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|
| Nuclease-free Water | - | 72.5 | 159.5 | 319.0 |
| Block and Stain Buffer | 2000554 | 25.0 | 55.0 | 110.0 |
| RNase Inhibitor | 2000556 | 2.5 | 5.5 | 11.0 |
| Total | - | 100.0 | 220.0 | 440.0 |

| 11 mm Gaskets<br>Blocking Mix | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|
| Nuclease-free Water | - | 145.0 | 319.0 | 638.0 |
| Block and Stain Buffer | 2000554 | 50.0 | 110.0 | 220.0 |
| RNase Inhibitor | 2000556 | 5.0 | 11.0 | 22.0 |
| Total | - | 200.0 | 440.0 | 880.0 |

**b.** Remove Visium Slide Seals from each Tissue Slide Cassette and remove all 2X SSC Buffer from each well.

**c.** Add ■ **100 µl** or ▲ **200 µl** Blocking Mix to each well.

**d.** Apply a new pre-cut Visium Slide Seal to each Tissue Slide Cassette and incubate at **room temperature** for **1 h**.

**e.** **Immediately** proceed to next step during incubation and begin preparing staining mixes.

## 4.2 Antibody Incubation

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

**a.** Prepare Staining Mix according to the table below. Add reagents in the order listed. The Staining Mix described below is sufficient for one 10x Genomics antibody panel tube. Pipette mix 15x with pipette set to 200 µl. Briefly centrifuge.

One tube is enough for two 6.5 mm samples. If working with two 11 mm samples, two Human Immune Cell Profiling Panel tubes will be required.

| | Staining Mix | 10x PN | Volume (µl) |
|---|---|---|---|
| | Nuclease-free Water | - | 147* |
| ● | Block and Stain Buffer | 2000554 | 52.5 |
| ● | RNase Inhibitor | 2000556 | 10.5 |
| | Total | - | 210.0* |

*If add-on antibodies will be used, add them to this staining mix and reduce the amount of nuclease-free water such that the total volume remains at 210 µl after add-on antibody addition. If adding multiple add-on antibodies, they should be pooled together according to the Visium CytAssist Spatial Gene and Protein Expression Add-on Antibody Optimization Demonstrated Protocol (CG000664).

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

| Lid Temperature | Reaction Volume | Run Time |
|---|---|---|
| 30°C (lid may be turned off if the instrument does not enable 30°C) | 100 µl | Overnight (16 - 24 h) |
| **Step** | **Temperature** | **Time** *hh:mm:ss* |
| Pre-equilibrate | 22°C | Hold |
| Antibody Incubation | 22°C | Overnight (16 - 24 h) |
| Hold | 22°C | Hold |

**c.** Briefly centrifuge Human Immune Cell Profiling Panel.

**d.** Add **210 µl** Staining Mix to each tube of Human Immune Cell Profiling Panel (PN-2000699). One antibody panel tube is enough for two 6.5 mm

samples. Two 11 mm samples will require two tubes.

**e.** Pipette mix 15x with pipette set to 200 µl. Briefly centrifuge.

**f.** If using multiple Human Immune Cell Profiling Panel tubes, briefly centrifuge each panel tube after adding Staining Mix. Combine panels into a single 1.5 ml tube and proceed.

**g.** Incubate resuspended antibody panel for **30 min** at **4°C**.

**h.** During the last **10 min** of Tissue Blocking, centrifuge resuspended antibody panel at **14,000 rcf** for **10 min** at **4°C**.

**i.** Peel back Visium Slide Seals on Tissue Slide Cassettes.

**j.** Remove all Blocking Mix from each well.

**k.** Add **■100 µl** or **▲200 µl** centrifuged, resuspended antibody panel supernatant to each well. Avoid touching the bottom of the tube.

**l.** Re-apply Visium Slide Seals on each Tissue Slide Cassette and place on the Low Profile Thermocycler Adapter on the thermal cycler. Close the thermal cycler lid.

**m.** Skip Pre-equilibrate step to initiate overnight Antibody Incubation.

# 4.3 Post-Antibody Incubation Wash

**a.** Prepare Washing Buffer according to the appropriate table. Add reagents in the order listed. Slowly invert tube 10x and centrifuge briefly.

| 6.5 mm Gaskets<br>Washing Buffer | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|
| Nuclease-free Water | - | 516.0 | 1,135.2 | 2,270.4 |
| 10X PBS Buffer, pH 7.4 | - | 60.0 | 132.0 | 264.0 |
| Tween-20, 10% | - | 24.0 | 52.8 | 105.6 |
| Total | - | 600.0 | 1,320.0 | 2,640.0 |

| 11 mm Gaskets<br>Washing Buffer | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|
| Nuclease-free Water | - | 1,032.0 | 2,270.4 | 4,540.8 |
| 10X PBS Buffer, pH 7.4 | - | 120.0 | 264.0 | 528.0 |
| Tween-20, 10% | - | 48.0 | 105.6 | 211.2 |
| Total | - | 1,200.0 | 2,640.0 | 5,280.0 |

**b.** Prepare 1X PBS according to the appropriate table. Add reagents in the order listed. 1X PBS will also be used during step 5.

| 6.5 mm Gaskets<br>1X PBS | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|
| Nuclease-free Water | - | 2,956.5 | 6,504.3 | 13,008.6 |
| 10X PBS Buffer, pH 7.4 | - | 328.5 | 722.7 | 1,445.4 |
| Total | - | 3,285.0 | 7,227.0 | 14,454.0 |

| 11 mm Gaskets<br>1X PBS | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|
| Nuclease-free Water | - | 3,213.0 | 7,068.6 | 14,137.2 |
| 10X PBS Buffer, pH 7.4 | - | 357.0 | 785.4 | 1,570.8 |
| Total | - | 3,570.0 | 7,854.0 | 15,708.0 |

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

**d.** Remove Visium Slide Seals and remove all Staining Mix from each well.

**e.** Add ■**150 µl** or ▲ **300 µl** Washing Buffer to each well, adding directly onto the tissue without touching the pipette tip to the tissue.

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

**g.** Remove all Washing Buffer from each well.

**h.** **Repeat** steps e-g three times for a total four washes.

**i.** Add ■**150 µl** or ▲ **300 µl** 1X PBS to each well.

# Step 5:

## Probe and Antibody Tag Release & Extension


5.0 Get Started 89

5.1 CytAssist-Enabled RNA Digestion & Tissue Removal 91

5.2 Probe & Antibody Tag Extension 100

5.3 Probe & Antibody Tag Elution 101

# 5.0 Get Started


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

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

| Items | | 10x PN | Preparation & Handling | Storage |
|---|---|---|---|---|
| **Equilibrate to room temperature** | | | | |
| ☐ | RNase Buffer C | Tube: 2000883<br>Kit: 1000603 | Thaw at room temperature. Pipette mix slowly and thoroughly with a wide-bore pipette. DO NOT vortex. | –20°C |
| ☐ | Extension Buffer | Tube: 2000409<br>Kit: 1000436 | Thaw at room temperature, vortex, centrifuge briefly. | –20°C |
| ☐ | Tissue Removal Enzyme | Tube: 3000387<br>Kit: 1000436 | Pipette mix, centrifuge briefly. | –20°C |
| **Place on ice** | | | | |
| ☐ | RNase Enzyme | Tube: 3000593<br>Kit: 1000436 | Pipette mix, centrifuge briefly. Maintain on ice until ready to use. RNase Enzyme is added to the Probe and Antibody Tag Release Mix immediately before running the CytAssist instrument. | –20°C |
| ☐ | Extension Enzyme | Tube: 2000389<br>Kit: 1000436 | Pipette mix, centrifuge briefly. Maintain on ice until ready to use. | –20°C |
| **Obtain** | | | | |
| ☐ | Nuclease-free Water | - | - | Ambient |
| ☐ | Wide-bore Pipette Tips | - | - | Ambient |
| ☐ | Tris 1 M, pH 8.0 (Tris-HCl) | - | Manufacturer's recommendations. | Ambient |
| ☐ | Alcoholic Eosin | - | Manufacturer's recommendations. | Ambient |
| ☐ | PBS | - | Prepared in 4.3 Post-Antibody Incubation Wash on page 86 | Ambient |

| Items | | 10x PN | Preparation & Handling | Storage |
|---|---|---|---|---|
| ☐ | 20X SSC Buffer | - | - | Ambient |
| ☐ | 2X SSC Buffer | - | Prepared in 3.1 Post-Hybridization Wash on page 74. | Ambient |
| ☐ | 8 M KOH Solution | - | Manufacturer's recommendations. | Ambient |
| ☐ | Visium Cassette | Component: 3000811/ 3000812 Kit: 1000519/1000518 | See Tips & Best Practices. | Ambient |
| ☐ | Visium Slide Seals | Component: 2000284 Kit: 1000519/1000518 | See Tips & Best Practices. | Ambient |

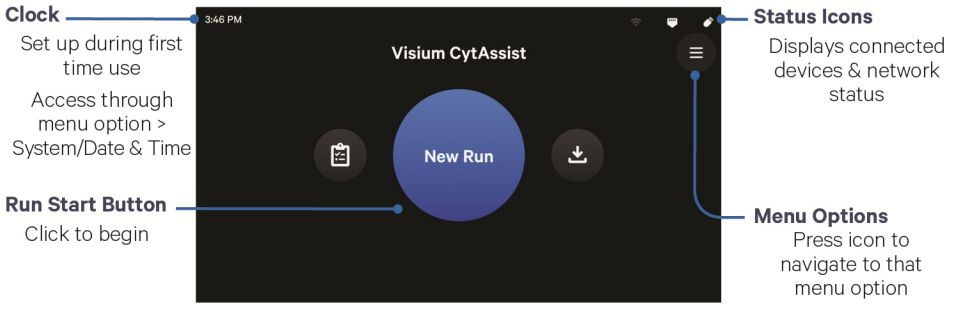
# 5.1 CytAssist-Enabled RNA Digestion & Tissue Removal

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

Keep tissue slides at **4°C** with 1X PBS until ready for CytAssist run.

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

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




**b.** Prepare Probe and Antibody Tag Release Mix shortly before use. Probe and Antibody Tag Release Mix is viscous. Pipette mix thoroughly until solution is homogenous. Avoid generating bubbles. DO NOT vortex. Maintain at room temperature.

| | Probe and Antibody Tag Release Mix | 10x PN | 2 Tissue Slides (µl) (includes overage) |
|---|---|---|---|
| | Nuclease-Free Water | - | 11.3 |
| ● | RNase Buffer C | 2000883 | 50.0 |
| ● | Tissue Removal Enzyme | 3000387 | 4.7 |
| | **Total** | **-** | **66.0** |

**c.** Prepare 1X PBS. Maintain at room temperature.

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

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

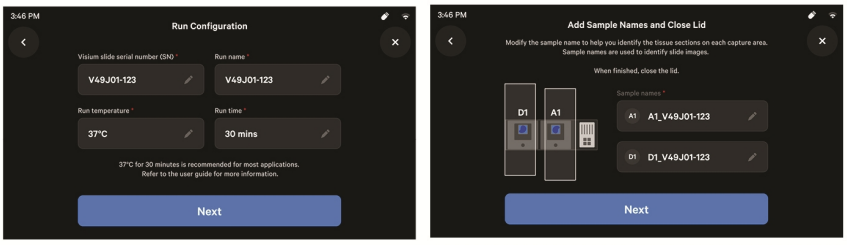
| 6.5 mm Slides<br>10% Eosin | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|
| Alcoholic Eosin | - | 15 | 33 | 66 |
| 1X PBS | - | 135 | 297 | 594 |
| **Total** | **-** | **150** | **330** | **660** |

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

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

**f.** Enter new run information, including:

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




**g.** Using a pipette, remove all 1X PBS Buffer from each well of the Tissue Slide Cassettes.

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

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

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

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

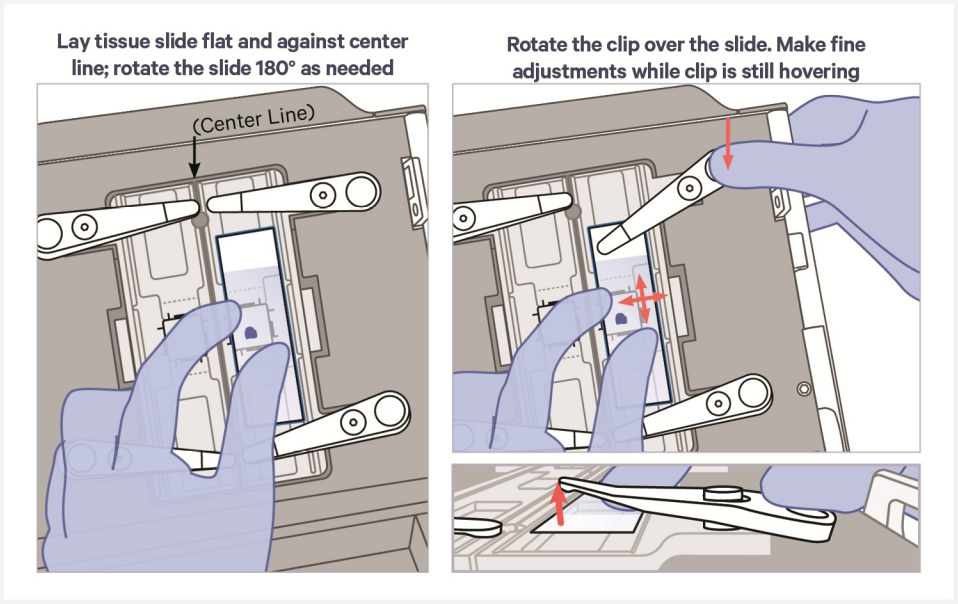
**l.** While holding the slide over liquid waste container, rinse with **1 ml** 1X PBS. DO NOT pipette directly onto tissue.

**DO NOT pipette directly onto tissue.**

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

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

**o.** Wipe back of tissue slides with a laboratory wipe and load into Visium CytAssist. Ensure tissue section is completely dry prior to instrument run. If necessary, repeat slide wafting. See Instrument Loading Guidelines on page 52 for more information.




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


**Visium Slide Stage is Loaded Correctly with One Slide**

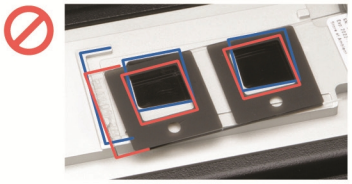
Slide fits inside the grooves.

Label on the right is partially obscured by the lock.

Capture areas line up with alignment windows.

**INCORRECT**

Slide does not fit inside the grooves. Capture areas do not line up with alignment windows.

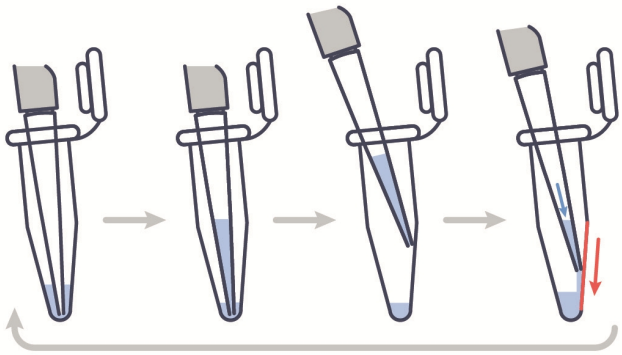


**INCORRECT**

Label is on the left. Capture areas do not line up with alignment windows.

**q.** Add **9 µl** of RNase Enzyme to **66 µl** of Probe and Antibody Tag Release Mix (prepared at step 5.1b). Pipette mix 15x with pipette set to 50 µl. Centrifuge for **10 sec**.

**Pipette Alongside Tube Wall To Avoid Generating Bubbles**

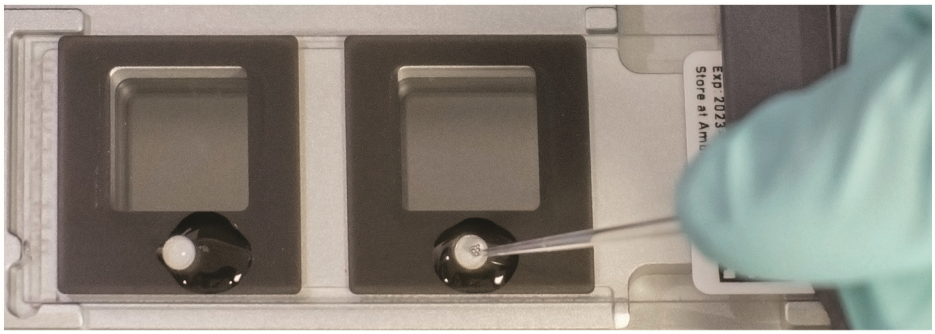


The time between the addition of RNase Enzyme to the Probe and Antibody Tag Release Mix and starting the Visium CytAssist experiment run should be less than **5 min**.

**r.** Slowly dispense **25 µl** of Probe and Antibody Tag Release Mix into the center of each spacer well on the Visium CytAssist Spatial Gene Expression Slide, using a fresh pipette tip for each dispense. Do not push plunger beyond first stop to avoid generating bubbles. If bubbles are generated, pop them with a clean pipette tip.

**Dispense Reagent**

**Pop bubbles with a Clean Pipette Tip**

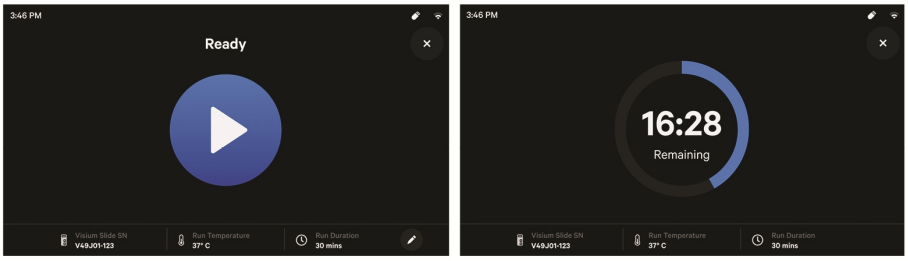


**s.** Close lid and press Next.

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

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

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




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

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

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

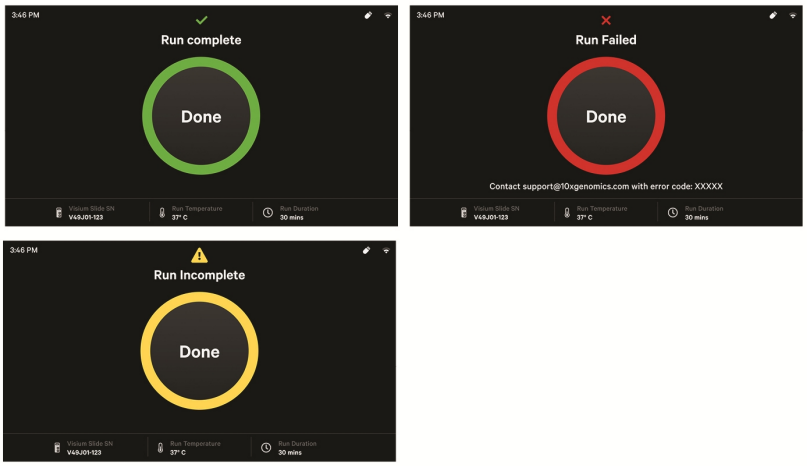
**v.** Prepare Probe and Antibody Tag Extension Mix. Pipette mix. Centrifuge briefly. Maintain on ice.

| | 6.5 mm Slides<br>Probe and Antibody Tag Extension Mix | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|---|
| | Extension Buffer | 2000409 | 73.5 | 161.7 | 323.4 |
| | Extension Enzyme | 2000389 | 1.5 | 3.3 | 6.6 |
| | **Total** | **-** | **75.0** | **165.0** | **330.0** |

| | 11 mm Slides<br>Probe and Antibody Tag Extension Mix | 10x PN | 1X (µl) | 2X +10% (µl) | 4X +10% (µl) |
|---|---|---|---|---|---|
| | Extension Buffer | 2000409 | 196.0 | 431.2 | 862.4 |
| | Extension Enzyme | 2000389 | 4.0 | 8.8 | 17.6 |
| | **Total** | **-** | **200.0** | **440.0** | **880.0** |

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

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



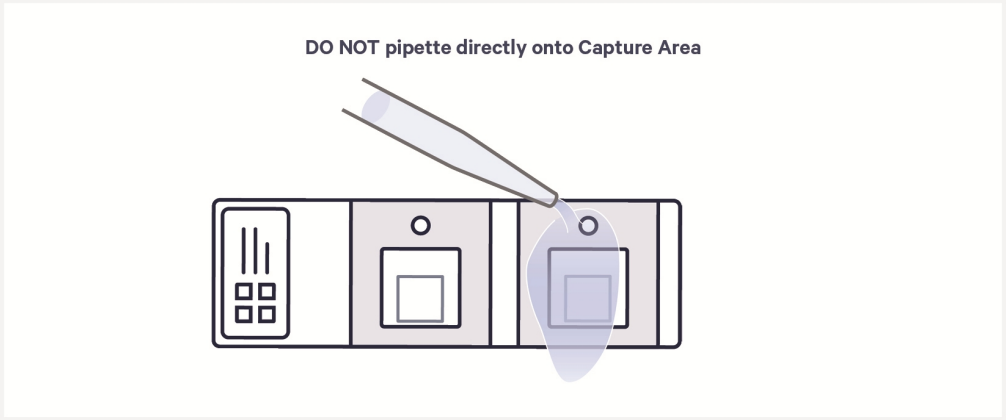
**x.** Click "Done" button and open lid. DO NOT power off the instrument at this time, as it needs to process support data. It is normal for the Visium slide to move after opening instrument.

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

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

**z.** While holding Visium CytAssist Spatial Gene Expression Slide over liquid waste container, rinse each Capture Area with **1 ml** 2X SSC as shown below. DO NOT pipette directly onto Capture Areas, which are surrounded by the fiducial frames.

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




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

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

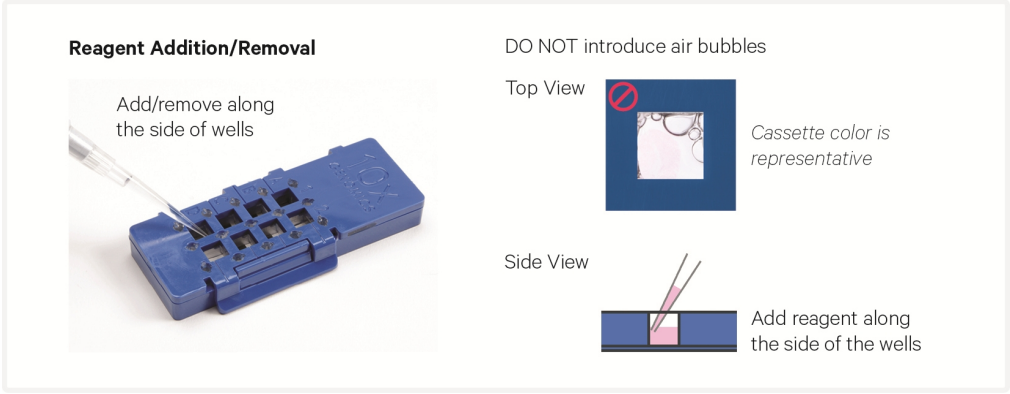
See Visium Cassette section for more information. Some moisture remaining on the Visium CytAssist Spatial Gene Expression slide is normal.

**ac.** Proceed immediately to Probe & Antibody Tag Extension. CytAssist instrument should only be cleaned and data transferred at a safe stopping point.

# 5.2 Probe & Antibody Tag Extension

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

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




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

**c.** Skip Pre-equilibrate step to initiate Probe & Antibody Tag Extension .

**d.** Sample may remain at **4°C** in the thermal cycler for up to **2 h**. This is a safe stopping point to unload and clean the CytAssist instrument. Consult Visium CytAssist Instrument User Guide (CG000542) for more information.

## 5.3 Probe & Antibody Tag Elution

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

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

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

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

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

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

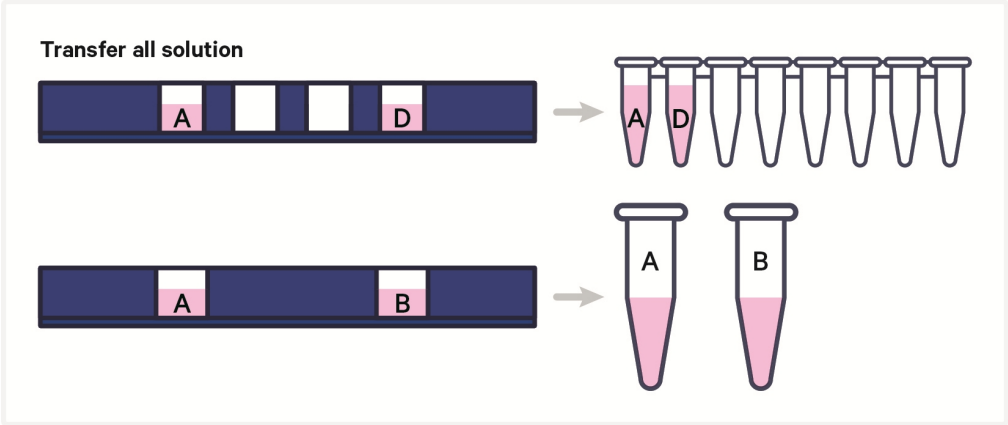
**d.** Add ■ **100 µl** or ▲ **200 µl** 2X SSC Buffer prepared in 3.1 Post-Hybridization Wash on page 74 to each well (A1 and D1 if using a 6.5 mm cassette).

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

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

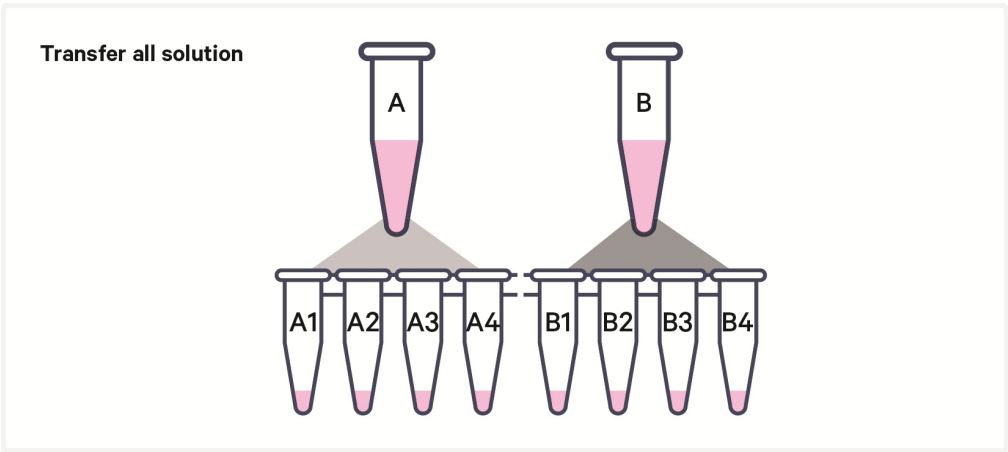
**g.** Incubate at **room temperature** for **10 min**.

**h.** Transfer all solution for each sample containing the probes and antibody tags to a tube in an 8-tube strip if using a 6.5 mm cassette, or 1.5 ml microcentrifuge tube if using an 11 mm cassette. DO NOT leave behind any solution in the wells. *See Tips & Best Practices for reagent removal instructions.*




i. Add ■ **3 µl** or ▲ **12 µl** 1 M Tris-HCl pH 8.0 to each sample. Vortex, centrifuge briefly, and place on ice. Failure to neutralize may result in a loss of signal and lower library complexity.

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



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# Step 6:

## Pre-Amplification and SPRIselect


6.0 Get Started 104

6.1 Pre-Amplification 105

6.2 Pre-Amplification Cleanup - SPRIselect 107

6.3 Cycle Number Determination – qPCR 108

# 6.0 Get Started

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

| Item | | 10x PN | Preparation & Handling | Storage |
|---|---|---|---|---|
| **Equilibrate to room temperature** | | | | |
| ☐ | TS Primer Mix B | Tube: 2000537<br>Kit: 1000436 | Thaw at room temperature, vortex, and centrifuge briefly. | –20°C |
| ☐ | NT Primer Mix A | Tube: 2000540<br>Kit: 1000603 | Thaw at room temperature, vortex, and centrifuge briefly. | –20°C |
| ☐ | Beckman Coulter SPRIselect Reagent | - | Manufacturer's recommendations. | - |
| **Place on ice** | | | | |
| ☐ | Amp Mix B | Tube: 2000567<br>Kit: 1000436 | Vortex, centrifuge briefly. | –20°C |
| **Obtain** | | | | |
| ☐ | Qiagen Buffer EB | - | | Ambient |
| ☐ | 10x Magnetic Separator | Component: 2001212<br>Kit: 1000499 | See Tips & Best Practices. | Ambient |
| ☐ | 80% Ethanol | - | Prepare fresh. Prepare 1 ml per reaction. Store at room temperature. | |

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

# 6.1 Pre-Amplification

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

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

| | 6.5 mm Slides<br>Pre-Amplification Mix | PN | 1X (µl) | 2X + 10% (µl) | 4X + 10% (µl) |
|---|---|---|---|---|---|
| | Nuclease-free Water | | 17.0 | 37.4 | 74.8 |
| ○ | Amp Mix B | 2000567 | 25.0 | 55.0 | 110.0 |
| ● | TS Primer Mix B | 2000537 | 2.5 | 5.5 | 11.0 |
| ● | NT Primer Mix A | 2000540 | 2.5 | 5.5 | 11.0 |
| | **Total** | **-** | **47.0** | **103.4** | **206.8** |

| | 11 mm Slides<br>Pre-Amplification Mix | PN | 1X (µl) | 2X + 10% (µl) | 4X + 10% (µl) |
|---|---|---|---|---|---|
| | Nuclease-free Water | | 68.0 | 149.6 | 299.2 |
| ○ | Amp Mix B | 2000567 | 100.0 | 220.0 | 440.0 |
| ● | TS Primer Mix B | 2000537 | 10.0 | 22.0 | 44.0 |
| ● | NT Primer Mix A | 2000540 | 10.0 | 22.0 | 44.0 |
| | **Total** | **-** | **188.0** | **413.6** | **827.2** |

**b.** Add **47 µl** Pre-Amplification Mix to each tube from 5.3 Probe & Antibody Tag Elution on page 101 (regardless of slide type). Pipette mix and centrifuge briefly.

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

| Lid Temperature | Reaction Volume | Run Time |
|---|---|---|
| 105°C | 100 µl | ~25 min |
| **Step** | **Temperature** | **Time** *hh:mm:ss* |
| 1 | 98°C | 00:03:00 |
| 2 | 98°C | 00:00:15 |
| 3 | 63°C | 00:00:20 |
| 4 | 72°C | 00:00:30 |
| 5 | Go to Step 2, repeat 9X for a total of 10 cycles | |
| 6 | 72°C | 00:01:00 |
| 7 | 4°C | Hold |

## 6.2 Pre-Amplification Cleanup - SPRIselect

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

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

**a.** Vortex to resuspend SPRIselect reagent. Add **120 µl** SPRIselect reagent (1.2X) to each pre-amplification reaction in an 8-tube strip (100 µl) and pipette mix 15x (pipette set to 130 µl).

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

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

**d.** Remove supernatant.

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

**f.** Remove ethanol.

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

**h.** Remove ethanol.

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

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

**k.** Remove from magnet. Add ■ **105 µl** or ▲ **27.5 µl** Buffer EB. Pipette mix 15x (pipette set to 100 µl for samples from 6.5 mm slides or 25 µl for samples from 11 mm slides).

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

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

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

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


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

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

## 6.3 Cycle Number Determination – qPCR

**a.** Dilute TS Primer Mix B and NT Primer Mix A 1:10 in nuclease-free water. The amount of diluted primer mix required to generate the qPCR mixes is described in the table below.

**b.** Prepare **separate** qPCR Mixes on ice according to the tables below. Add reagents in the order listed. Vortex and centrifuge briefly. Maintain on ice.

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

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

*Includes one negative control

| Protein Expression qPCR Mix | Stock | Final | 1X (µl) | 3X* + 10% (µl) | 5X* + 10% (µl) |
|---|---|---|---|---|---|
| **KAPA SYBR FAST qPCR Master Mix** Minimize light exposure | 2X | 1X | 5.0 | 16.5 | 27.5 |
| **Diluted NT Primer Mix A** | - | - | 1.0 | 3.3 | 5.5 |
| **Nuclease-free Water** | - | - | 3.0 | 9.9 | 16.5 |
| **Total** | | | **9.0** | **29.7** | **49.5** |

*Includes 1 negative control

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

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

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

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

**g.** Apply seal and centrifuge briefly. Record which sample is in which well (as well as whether the well contained Gene Expression or Protein Expression qPCR mix) of the qPCR plate.

Only **25 µl** of pre-amplification material is used to generate gene expression libraries, while **25 µl** of pre-amplification material is used to generate protein expression libraries. The remaining **50 µl** (50%) can be stored at **4°C** for up to **72 h** or at **-20°C** for up to **4 weeks** for generating additional libraries.

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

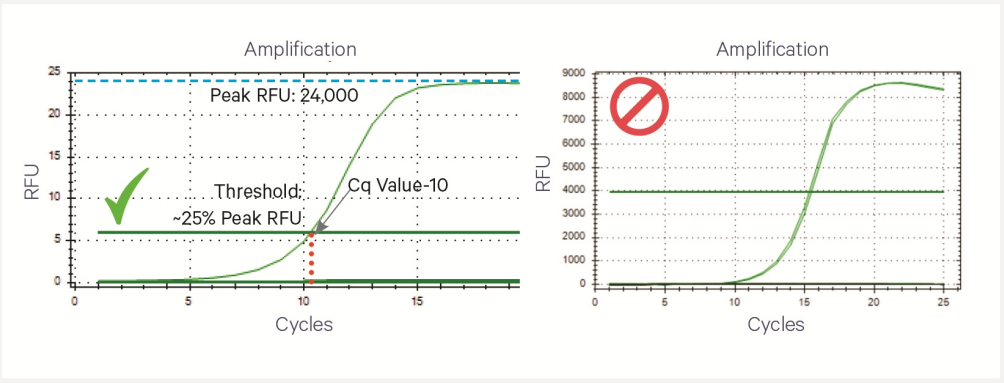
| Lid Temperature | Reaction Volume | Run Time |
|---|---|---|
| 105°C | 10 µl | 35 min |
| **Step** | **Temperature** | **Time** *hh:mm:ss* |
| 1 | 98°C | 00:03:00 |
| 2 | 98°C | 00:00:05 |
| 3 | 63°C | 00:00:30 |
| | Read signal | |
| 4 | Go to step 2, 29x (total of 30 cycles) | - |

**i.** Record the Cq value for each sample.

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

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

### Representative qPCR Amplification Plots




In this User Guide, Visium CytAssist Spatial Gene Expression - Probe-based libraries are constructed before Visium CytAssist Spatial Protein Expression

libraries. If constructing both library types simultaneously, consider that each library preparation may require a separate thermal cycler for sample index PCR due to differences in the required number of cycles between the libraries, as determined by the current step. Separate dual index plates are also required.

If constructing Visium CytAssist Spatial Protein Expression libraries later, store unused pre-amplification material until ready to perform protein expression library construction.

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# Step 7:

## Visium CytAssist Spatial Gene Expression – Probe-based Library Construction


7.0 Get Started 112

7.1 GEX Sample Index PCR 113

7.2 GEX Post-Sample Index PCR Cleanup – SPRIselect 115

7.3 GEX Post-Library Construction QC 116

# 7.0 Get Started

| Item | | 10x PN | Preparation & Handling | Storage |
|---|---|---|---|---|
| **Equilibrate to room temperature** | | | | |
| ☐ | Dual Index Plate TS Set A | Tube: 3000511<br>Kit: 1000251 | Thaw at room temperature, vortex, and centrifuge briefly. | −20°C |
| ☐ | Beckman Coulter SPRIselect Reagent | - | Manufacturer's recommendations. | - |
| ☐ | Agilent TapeStation Screen Tape and Reagents<br>If used for QC | | Manufacturer's recommendations. | - |
| ☐ | Agilent Bioanalyzer High Sensitivity DNA kit<br>If used for QC | - | Manufacturer's recommendations. | - |
| **Place on ice** | | | | |
| ☐ ◯ | Amp Mix B | Tube: 2000567<br>Kit: 1000436 | Vortex, centrifuge briefly. | −20°C |
| ☐ | KAPA SYBR Fast qPCR Master Mix | - | Manufacturer's recommendations. | - |
| **Obtain** | | | | |
| ☐ | Nuclease-free Water | - | - | Ambient |
| ☐ | Qiagen Buffer EB | - | | Ambient |
| ☐ | 10x Magnetic Separator | Component: 2001212<br>Kit: 1000499 | See Tips & Best Practices. | Ambient |
| ☐ | 80% Ethanol | - | Prepare fresh. Prepare 400 µl per reaction. | Ambient |

# 7.1 GEX Sample Index PCR

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

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

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

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

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

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

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

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

Round Cq values up to the nearest whole number and add two cycles (examples below). Samples within ±1 cycle numbers can be combined in a single SI-PCR run at the higher cycle number.

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

**Example Cycle Numbers**

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

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

## 7.2 GEX Post-Sample Index PCR Cleanup – SPRIselect

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

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

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

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

**d.** Remove supernatant.

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

**f.** Remove ethanol.

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

**h.** Remove ethanol.

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

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

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

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

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

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

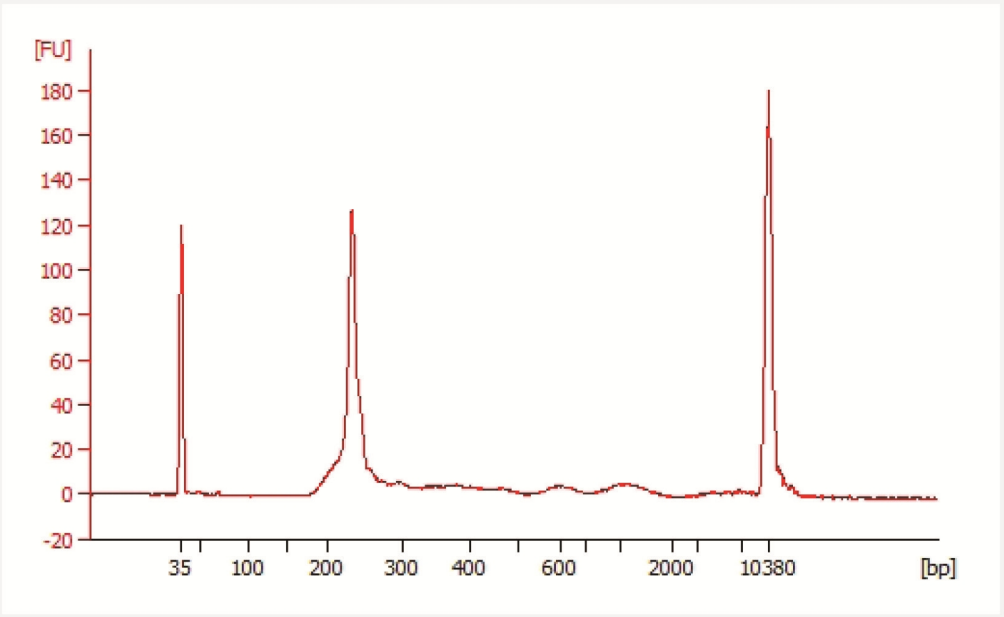

**o.** Store at **-20°C** for **long-term** storage.

## 7.3 GEX Post-Library Construction QC

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

**b.** Run **1 µl** of diluted sample on an Agilent Bioanalyzer High Sensitivity chip. If peak is too small or flat, retry with a lower dilution. See Troubleshooting on page 134 for more information.

**Representative Trace**




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

**Alternate QC Methods:**

- Agilent TapeStation
- LabChip

See Appendix on page 147 for representative traces

See Post Library Construction Quantification on page 148

10x GENOMICS

# Step 8:

## Visium CytAssist Spatial Protein Expression Library Construction


8.0 Get Started 118

8.1 Protein Sample Index PCR 119

8.2 Protein Post-Sample Index PCR Cleanup – SPRIselect 121

8.3 Protein Post-Library Construction QC 122

# 8.0 Get Started

| Item | | 10x PN | Preparation & Handling | Storage |
|---|---|---|---|---|
| **Equilibrate to room temperature** | | | | |
| ☐ | Dual Index Plate NT Set A | Component: 3000483<br>Kit: 1000242 | Thaw at room temperature, vortex, and centrifuge briefly. | −20°C |
| ☐ | Beckman Coulter SPRIselect Reagent | - | Manufacturer's recommendations. | - |
| ☐ | Agilent TapeStation Screen Tape and Reagents<br>If used for QC | | Manufacturer's recommendations. | - |
| ☐ | Agilent Bioanalyzer High Sensitivity DNA kit<br>If used for QC | - | Manufacturer's recommendations. | - |
| **Place on ice** | | | | |
| ☐ ◯ | Amp Mix B | Tube: 2000567<br>Kit: 1000436 | Vortex, centrifuge briefly. | −20°C |
| **Obtain** | | | | |
| ☐ | Nuclease-free Water | - | - | Ambient |
| ☐ | Qiagen Buffer EB | - | | Ambient |
| ☐ | 10x Magnetic Separator | Component: 230003 or 2000431<br>Kit: 1000499 | See Tips & Best Practices. | Ambient |
| ☐ | 80% Ethanol | - | Prepare fresh. | Ambient |

# 8.1 Protein 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-1000242 Dual Index Plate NT Set A) used.

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

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

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

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

**e.** Add **5 µl** of an individual Dual Index Plate NT Set A (PN-3000483) to each tube and record the well ID used. Pipette mix and centrifuge briefly.

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

| Lid Temperature | Reaction Volume | Run Time |
|---|---|---|
| 105°C | 100 µl | ~25-40 min |
| **Step** | **Temperature** | **Time** *hh:mm:ss* |
| 1 | 98°C | 00:03:00 |
| 2 | 98°C | 00:00:15 |
| 3 | 63°C | 00:00:20 |
| 4 | 72°C | 00:00:30 |
| 5 | Go to step 2, use the Cq Value +2 as the total # of cycles. See table below for total # of cycle examples. | |
| 6 | 72°C | 00:01:00 |
| 7 | 4°C | Hold |

Round Cq values to the nearest whole number and add two cycles, as shown in the examples below.

### Example Cycle Numbers

| Cq Value from qPCR | +2 | Total Cycles |
|---|---|---|
| 12.2 | +2 | 15 |
| 13.5 | +2 | 16 |
| 19.7 | +2 | 22 |

## 8.2 Protein Post-Sample Index PCR Cleanup – SPRIselect

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

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

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

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

**d.** Remove the supernatant.

**e.** 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 two washes.

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

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

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

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

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

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


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

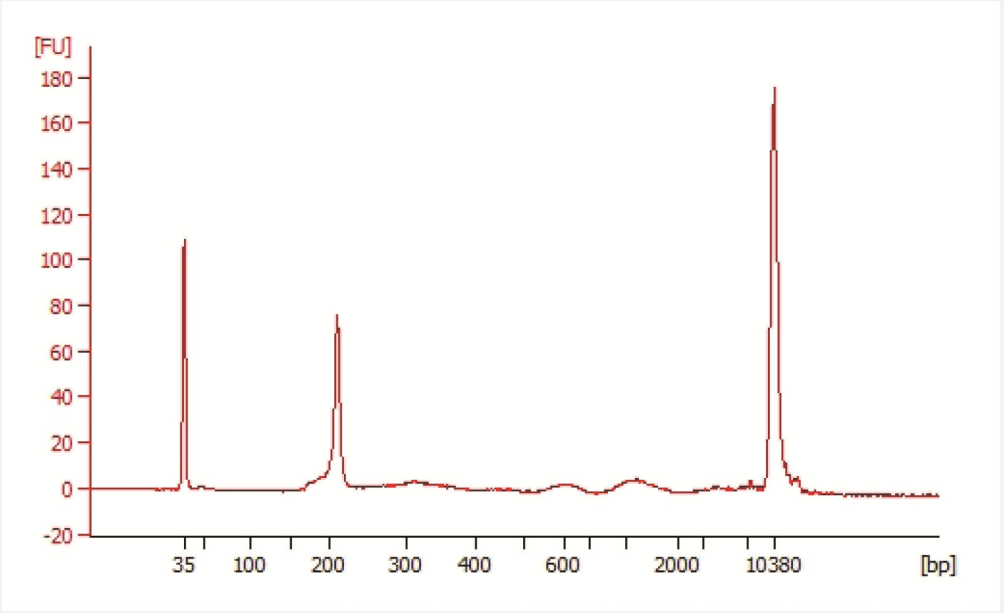
# 8.3 Protein Post-Library Construction QC

**a.** Quantify library using a Nanodrop or Qubit Fluorometer.

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

**c.** Run **1 µl** of sample on an Agilent Bioanalyzer High Sensitivity chip.

**Representative Trace**




Determine the average fragment size from the Bioanalyzer trace. The expected average fragment size is 210 bp. This will be used as the insert size for library quantification.

**Alternate QC Method:**

- Agilent TapeStation
- LabChip

See Appendix on page 147 for representative traces

See Post Library Construction Quantification on page 148

10x GENOMICS

# Sequencing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Sequencing Type & Run Parameters 127

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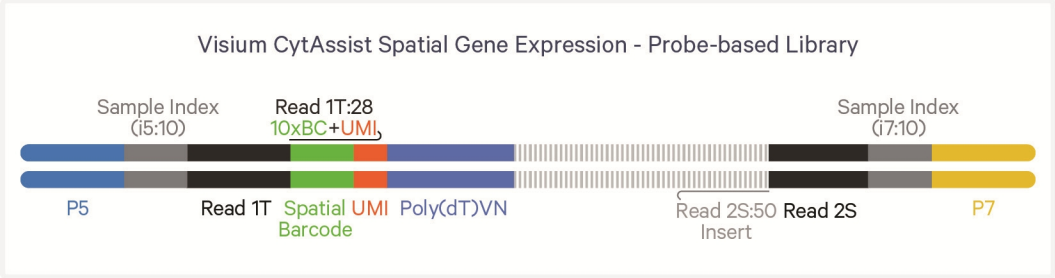
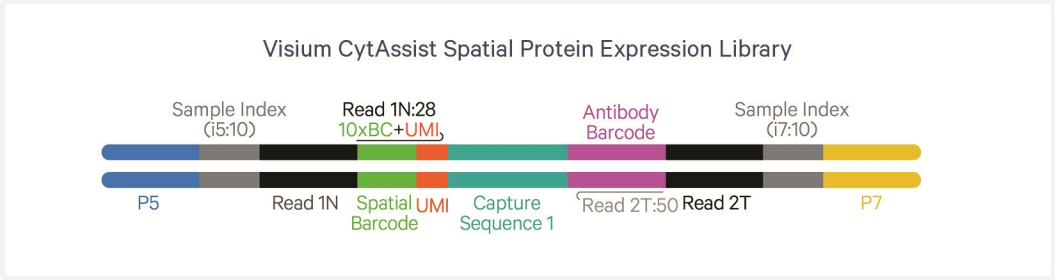
# Sequencing Libraries

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



# Sequencing Depth

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

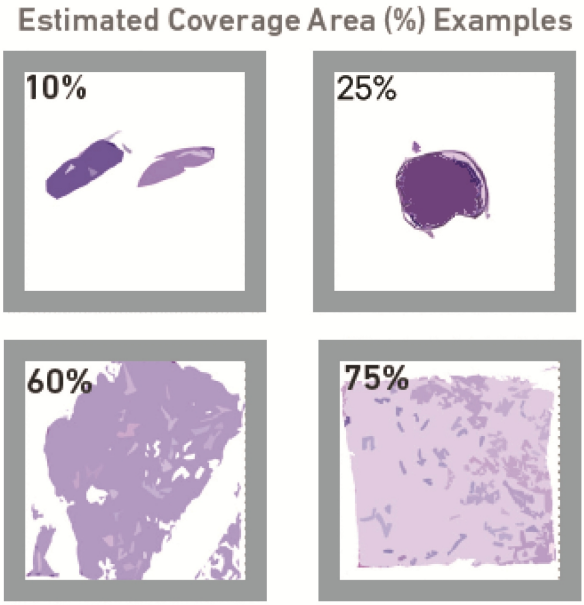
The examples below are for 6.5 mm slides. The total number of spots/Capture Area should be adjusted based on the Capture Area size (the number of spots for 6.5 mm slides is 4,992 and the number of 11 mm slides is 14,336).

Sequencing Depth for Visium CytAssist Spatial Gene Expression – Probe-based libraries:

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

### Example: Sequencing Depth for a Sample

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

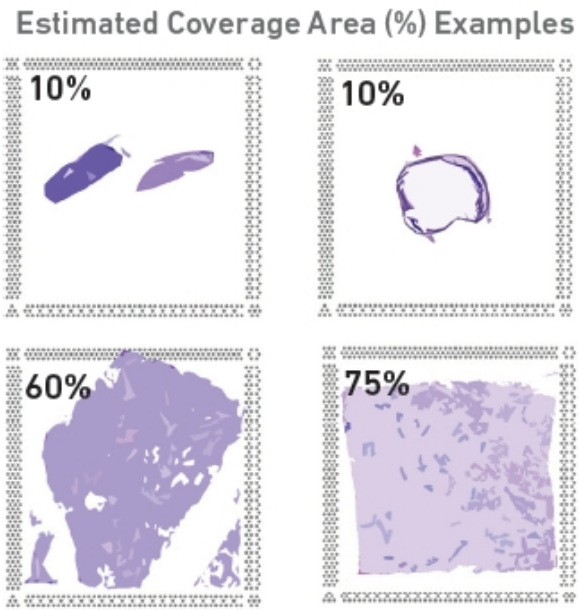


## Sequencing Depth for Visium CytAssist Spatial Protein Expression libraries:

| | |
|---|---|
| **Sequencing Depth/spot** | Minimum 5,000 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 5,000 read pairs/spot
- **Example calculation for 60% coverage:** (0.60 x 5,000 total spots) x 5,000 read pairs/spot= 15 million total read pairs for that sample



# Sequencing Type & Run Parameters

Use the sequencing run type and parameters indicated.

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

| Visium CytAssist Spatial Protein Expression Library |
| --- |
| Paired-end, dual indexed sequencing |
| Read 1: 28 cycles<br>i7 Index: 10 cycles<br>i5 Index: 10 cycles<br>Read 2S: 50 cycles |

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

# Illumina Sequencer Compatibility

Libraries generated from this User Guide are compatible with the following sequencers. Some variation in assay performance is expected based on sequencer choice. Data from tested sequencers are shown in Sequencing Metrics on page 130.

- MiSeq
- NextSeq 500/550
- NextSeq 1000/2000
- NovaSeq 6000
- NovaSeq X Plus
- iSeq

# Sample Indices

Each well of the Dual Index Kit TS and NT Set A (PN-1000251 and PN-1000242) contains a mix of one unique i7 and one unique i5 sample index. If multiple samples are pooled in a sequence lane, the sample index name (i.e. the Dual

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

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

## Library Loading

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

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

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

## Library Pooling

Visium CytAssist Spatial Gene Expression – Probe-based libraries and Protein Expression libraries should be pooled for sequencing, taking into account differences in tissue covered spot on a Capture Area and per-spot read depth requirements between each library. Samples using 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 Pooling Example

| Libraries | Sequencing Depth (read pairs per tissue-covered spot) | Library Pooling Ratio |
|---|---|---|
| Visium CytAssist Spatial Gene Expression – Probe-based | 25,000 | 5 |
| Visium CytAssist Spatial Protein Expression | 5,000 | 1 |

# Sequencing Metrics

To compare sequencing metrics across Illumina platforms, Visium CytAssist Spatial Gene Expression - Probe-based and Visium CytAssist Spatial Protein Expression libraries were generated from two Hematoxylin & Eosin (H&E) stained human tonsil tissue samples.FFPE samples were processed according to documentation listed in the Workflow Overview on page 20. Libraries were pooled and sequenced at the indicated run parameters to generate the % Base and % ≥Q30 plots shown in the following figures. These same libraries were used to illustrate sequence compatibility. All libraries were quantified with the KAPA DNA Quantification Kit and sequenced with 1% PhiX. Though only FFPE libraries are shown in this section, libraries from fresh frozen (FF) and fixed frozen (FxF) tissue sections are expected to perform similarly. All libraries followed the following sequencing configuration and run parameters:

- Targeted sequencing depth: 25,000 read pairs per tissue-covered spot (Probe-based libraries) or 5,000 read pairs per tissue-covered spot (Protein Expression libraries).
- Paired-end, dual indexing: Read 1: 28 cycles; i7 Index: 10 cycles; i5 Index: 10 cycles; Read 2: 50 cycles

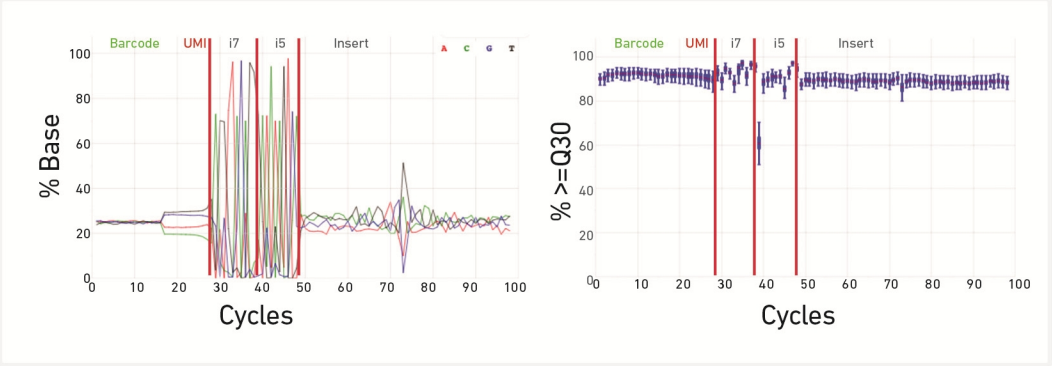
% Base by cycle and % ≥Q30 Quality Score distribution showed highly consistent profiles for all sequencing platforms tested. The data serve as guidelines for assessing the quality of library sequencing. Additional factors that may contribute to overall success of a sequencing run and impact downstream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

- Starting with a high-quality tissue block.
- Generating sequencing data from sections that remain adhered to the slide.
- Final libraries with fragment lengths within the expected size range for each library type.
- Reliable and accurate library quantification using the KAPA qPCR DNA Quantification Kit for Illumina Platforms (Roche, KK4824) and the average insert size determined by Agilent Bioanalyzer, Perkin Elmer LabChip, or Agilent TapeStation QC. Alternative methods to KAPA qPCR, such as Qubit, for final library quantification may result in underquantification and result in overloading flow cells.
- Sequencing platform loading concentrations follow recommendations described in Library Loading on page 128, which are based on KAPA qPCR quantification. Overloading/over-clustering may result in poor run performance, decrease sequencing quality, and lower total data output as compared to optimally loaded runs. The loading recommendations for an

individual sequencer are listed as general guidance and additional optimization may be required.

### Probe-based Libraries Pooled with Protein Expression Libraries

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

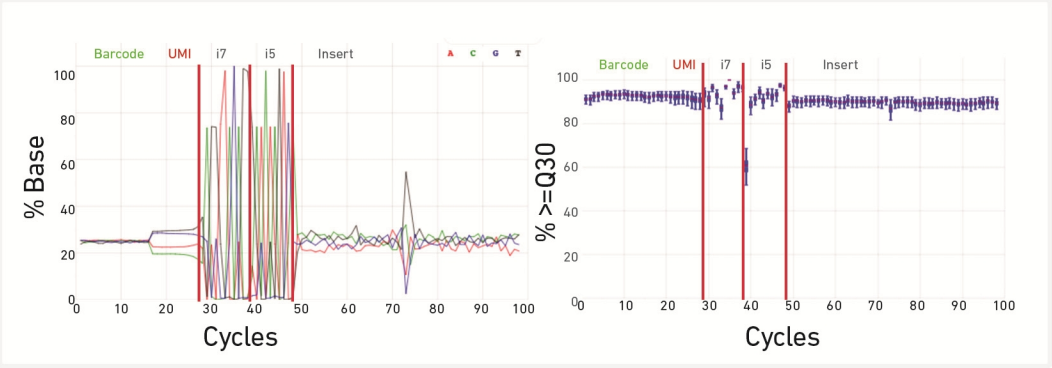



Libraries were sequenced on the indicated Illumina sequencers. "Data by Cycle" plots from the Illumina SAV software displaying the percentage of base calls and Q30 quality scores are shown. Mapped Reads (%) refers to Reads Mapped Confidently to Probe Set for Probe-based (GEX) libraries. Mapped Reads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

| Instr. | Load Conc. (pM) | Cluster Density (K/mm$^2$) | %PF | R1 | R2 | R1 | i7 | i5 | R2 | GEX | PEX |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Yield per Lane (Gb) | | % ≥Q30 | | | | Mapped Reads (%) | |
| NextSeq 500 | 1.6 | 119 | 96.62 | 2.01 | 3.66 | 98.50 | 97.34 | 98.31 | 96.98 | 97.0 | 96.4 |
| NextSeq 2000 | 650 | 4,974 | 78.10 | 14.06 | 25.51 | 96.47 | 95.16 | 94.95 | 96.29 | 97.2 | 96.4 |
| NovaSeq 6000 | 300 | 2,961 | 82.72 | 13.29 | 24.10 | 91.60 | 92.98 | 87.45 | 88.81 | 96.2 | 96.3 |

### Probe-based Libraries Alone

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

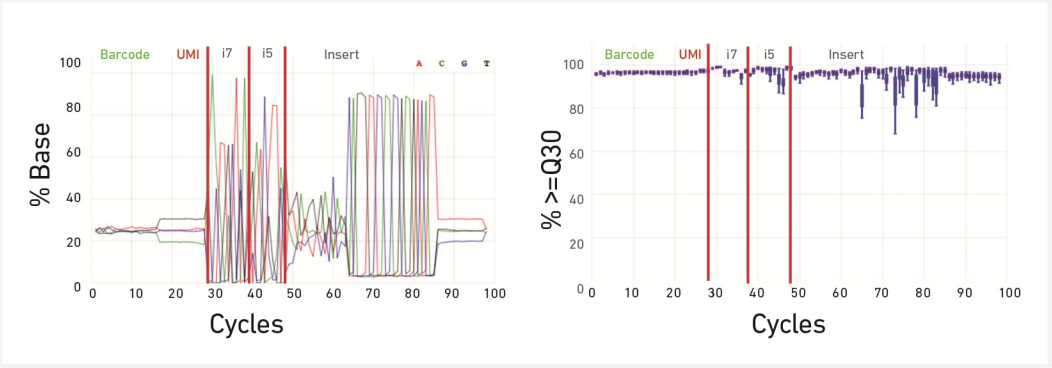



Libraries were sequenced on the indicated Illumina sequencers. "Data by Cycle" plots from the Illumina SAV software displaying the percentage of base calls and Q30 quality scores are shown. Mapped Reads (%) refers to Reads Mapped Confidently to Probe Set for Probe-based (GEX).

| Instr. | Load Conc. (pM) | Cluster Density (K/mm$^2$) | %PF | R1 | R2 | R1 | i7 | i5 | R2 | GEX |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Yield per Lane (Gb) | | % ≥Q30 | | | | Mapped Reads (%) |
| NextSeq 500 | 1.6 | 120.75 | 97.00 | 2.05 | 3.72 | 98.49 | 97.53 | 94.66 | 96.98 | 96.9 |
| NovaSeq 6000 | 300 | 2,961 | 83.42 | 13.37 | 24.25 | 92.27 | 94.19 | 88.84 | 89.48 | 96.4 |

### Protein Expression Libraries Alone

Representative % Base and % ≥Q30 plots are from a pool of two Protein Expression libraries sequenced on a NovaSeq SP flow cell.




Libraries were sequenced on the indicated Illumina sequencers. "Data by Cycle" plots from the Illumina SAV software displaying the percentage of base calls and Q30 quality scores are shown. Mapped Reads (%) refers to Fraction Antibody Reads for Protein Expression (PEX) libraries.

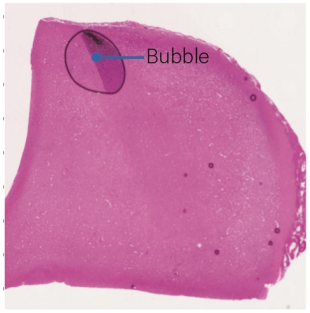
| Instr. | Load Conc. (pM) | Cluster Density (K/mm$^2$) | %PF | R1 | R2 | R1 | i7 | i5 | R2 | PEX |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Yield per Lane (Gb) | | % ≥Q30 | | | | Mapped Reads (%) |
| NextSeq 2000 | 650 | 4,974 | 80 | 3.46 | 6.29 | 96.36 | 97.22 | 96.52 | 94.69 | 96.4 |

# Troubleshooting

# Before CytAssist Instrument Run

## 1. Bubbles during Coverslipping

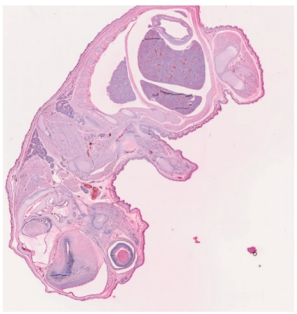
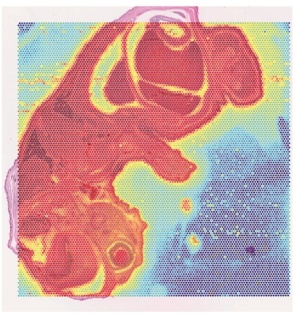
A bubble could be generated during coverslipping.

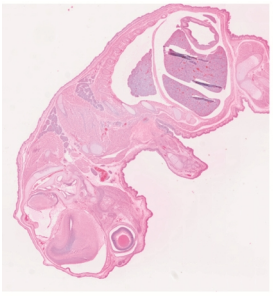
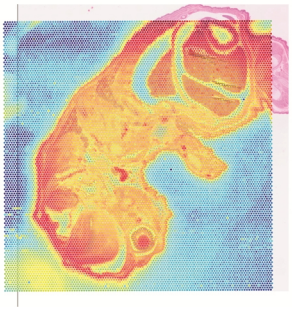
| Bubbles may cause blackening of tissue |
| --- |
| **H&E Stain** |
|   |

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

## 2. Tissue Thickness Outside of Specifications

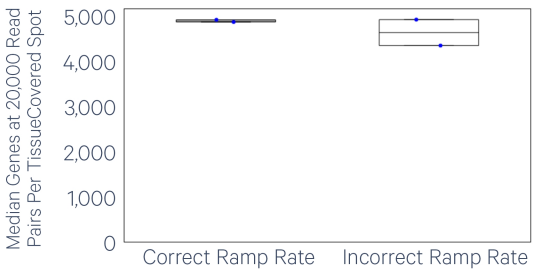
FFPE tissue sections should be between 3 and 10 µm. Sections outside these specifications may result in reduced performance. Mouse sections shown below are for demonstration purposes only. Mouse tissues are not supported for this application.

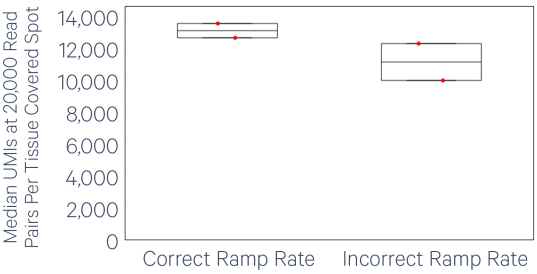
| Thin Sections May Lead to Decreased Sensitivity | |
| --- | --- |
| **H&E Stained Tissue at Recommended Thickness** | **Mean Panel UMI Count at 20,000 Raw Reads per Spot: 14,645** |
|  |  |

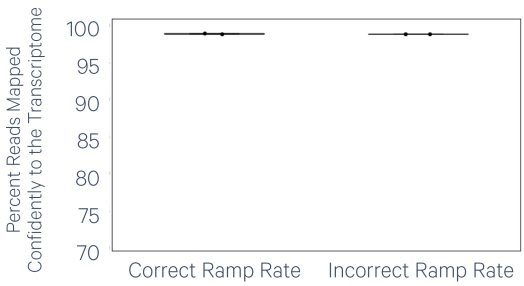
| Thin Sections May Lead to Decreased Sensitivity | |
|---|---|
| **H&E Stained Tissue Below Recommended Thickness** | **Mean Panel UMI Count at 20,000 Raw Reads per Spot: 8,511** |
|  |  |

## 3. Incorrect Thermal Cycler Ramp Rate

Incorrect thermal cycler ramp rates negatively impact assay sensitivity (mean UMI counts and mean genes per tissue-covered spot), but has no impact on assay quality (reads mapped confidently to the probe set). These data show that using a Biometra TAdvanced thermal cycler at the incorrect ramp rate (8°C/sec heating and cooling) vs. the correct ramp rate (2°C/sec heating and cooling) negatively impacts assay sensitivity. Use the recommended ramp rates as described in Recommended Thermal Cyclers on page 14.





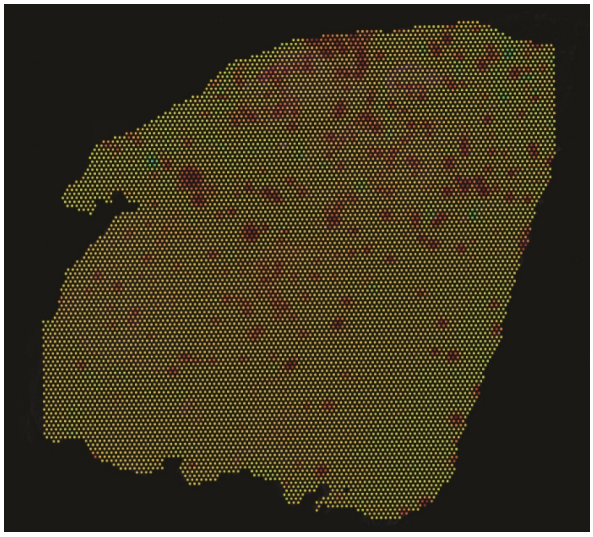

## 4. Number of Washes

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

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

## 5. High Signal Spots in Tissue Section

**Areas of High Signal Intensity in Tissue Section**



Areas of high signal intensity that do not align with tissue morphology, show in the same areas across several antibodies, and are not consistent between replicates are indicative of antibody aggregates. Ensure that the antibody mix is properly centrifuged. Do not touch the bottom of the tube when removing the antibody mix.

## 6. Incorrect Visium Slide Loading

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

**Visium Slide Stage is Loaded Correctly with One Slide**

Slide fits inside the grooves.

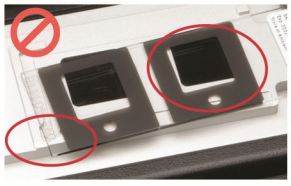
Label on the right is partially obscured by the lock.

Capture areas line up with alignment windows.

**INCORRECT**

Slide does not fit inside the grooves.

Capture areas do not line up with alignment windows.

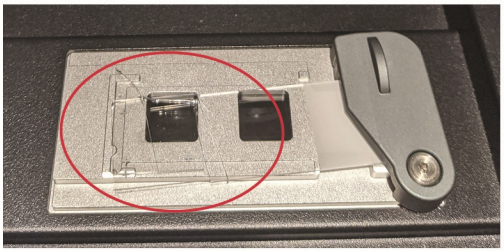


**INCORRECT**

Label is on the left.

Capture areas do not line up with alignment windows.

**Slide is Broken due to Improper Loading**

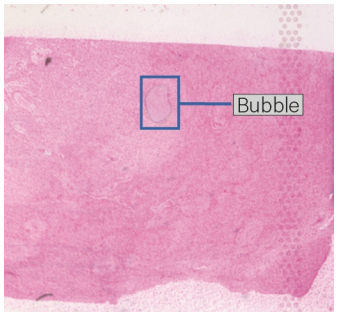
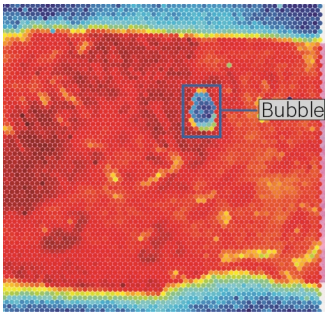


In the event of slide breakage, remove all traces of broken glass to avoid damaging the instrument. Exercise caution when removing glass to prevent injury.

# During CytAssist Instrument Run

## 1. Bubbles Trapped During Visium CytAssist Run

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

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



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

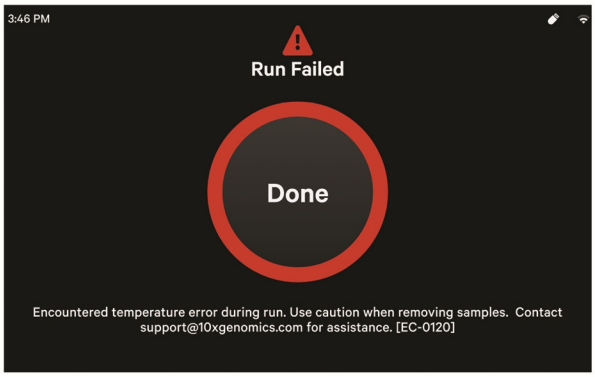
Avoid generating bubbles during reagent dispensing by pipetting slowly and avoiding expelling air from the pipette tip. Additionally, ensure that the frosted area of the tissue slides are outside of the alignment guide lines on the Tissue Slide Stage and that tissue slides are compatible with the CytAssist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

## 2. Reagent Flow Failure

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

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

# 3. Visium CytAssist Overheating



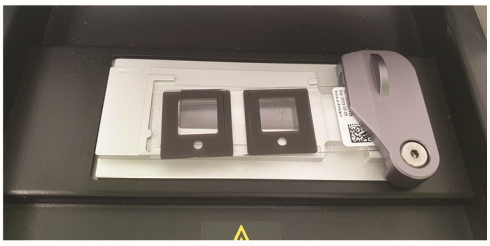

If the Visium CytAssist instrument overheats during an experiment run, the run will fail. A warning message will appear instructing users to carefully remove slides and contact support@10xgenomics.com.

# After CytAssist Instrument Run

## 1. Slide Popped Off of Visium Slide Stage

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

**Visium Slide Popped out of the Grooves**



## 2. Visium CytAssist Slide Removal Delayed

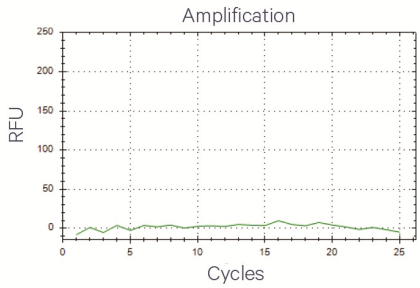
A delay in removing and processing the Visium CytAssist Spatial Gene Expression Slide after run completion may impact data quality. Immediately proceed with slide washes while adjacent to the instrument.

## 3. No qPCR Amplification

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

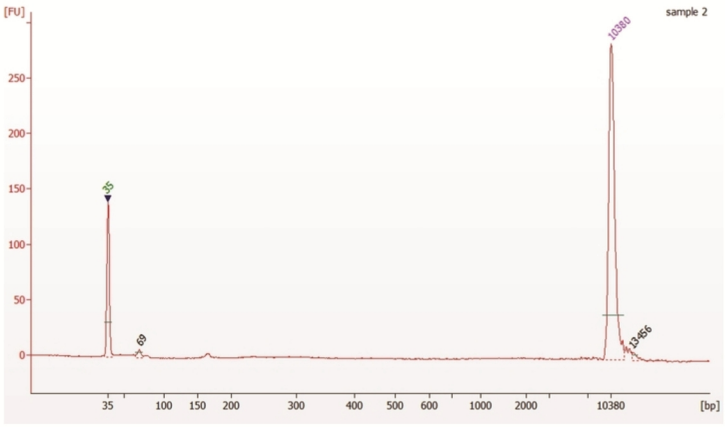
- Incorrect qPCR machine programming (i.e. detecting a fluorophore other than SYBR Green)
- Issues with pipetting small volumes
- Failure to neutralize KOH
- Failure to add primers to pre-amp or qPCR mix
- Unusually low recovery from tissue due to high percentage of connective tissue in Capture Area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

- Leakage from the cassette during workflow




## 4. Flat Line in Bioanalyzer Library Trace

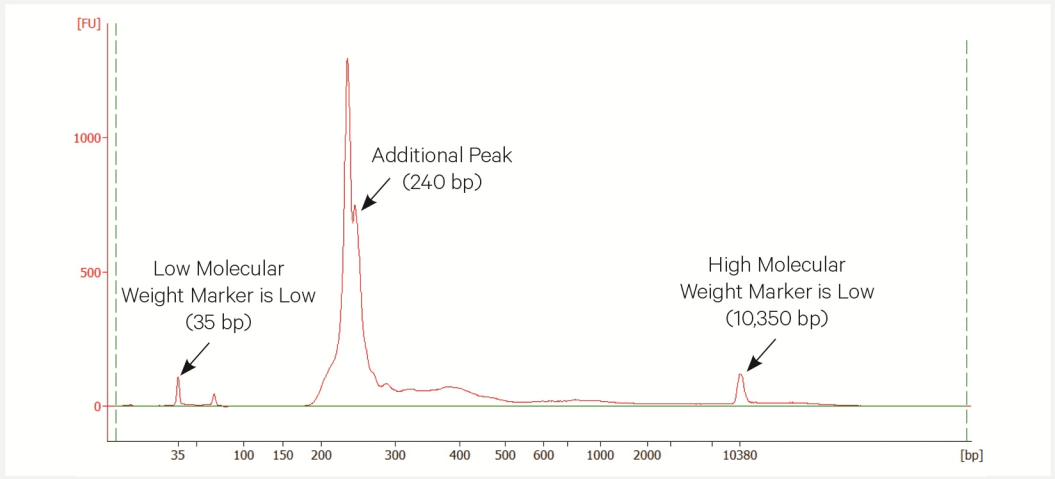
A normal qPCR output but no peak visible in the Bioanalyzer trace may be due to a mistake in the SI-PCR step, errors during SPRIselect cleanup, an overdilution of the final library, Bioanalyzer issue, or thermal cycler failure.




## 5. Overloaded or Overamplified Trace

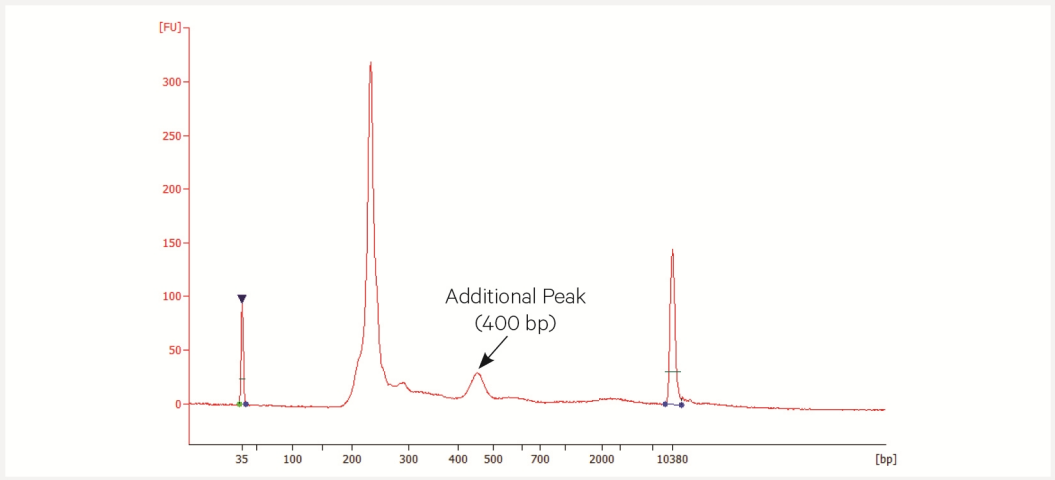
### Overloaded Trace

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




### Overamplified Trace

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




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

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

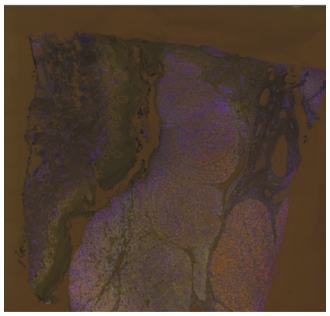
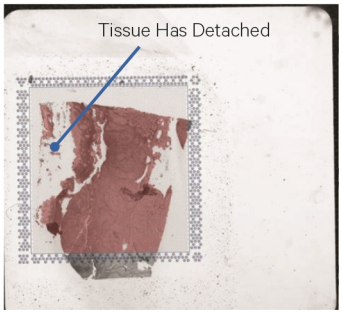
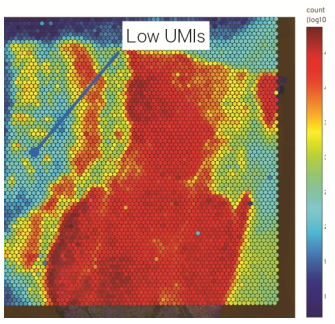
# Issues Impacting Tissue Analysis

## 1. Tissue Detachment and Folding

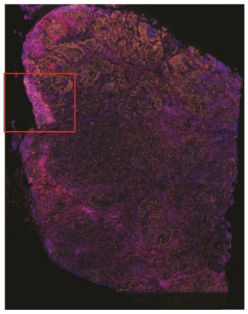
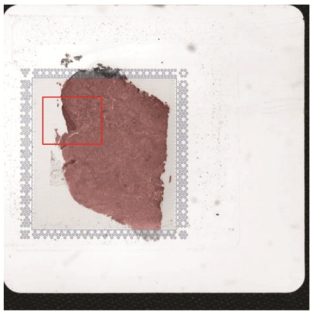
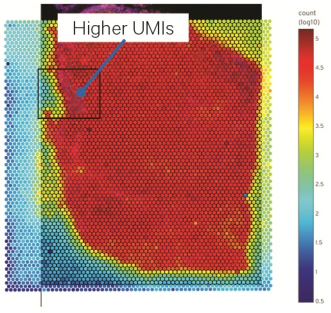
Tissue detachment may result in a lack of Fraction Reads Usable in the region where detachment occurred. If the tissue has folded on itself, this may also cause elevated UMI counts in the overlapping areas or a loss of spatiality due to ineffective permeabilization. Tissue folding may also cause lower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

Ensure that slides tested by 10x Genomics were used for tissue placement. For more information, consult the sample preparation documentation described in Workflow Overview on page 20.If tissue detachment is observed during this workflow, contact support@10xgenomics.com

**Tissue Detachment May Cause a Loss of Gene Expression**

| IF Stained Tissue | CytAssist Image | UMI Count |
|---|---|---|
|  |  |  |

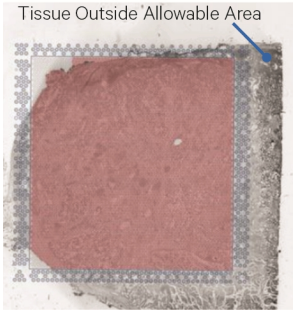
**Tissue Folding May Cause Elevated UMI Counts**

| H&E Stained Tissue | CytAssist Image | UMI Count |
|---|---|---|
|  |  |  |

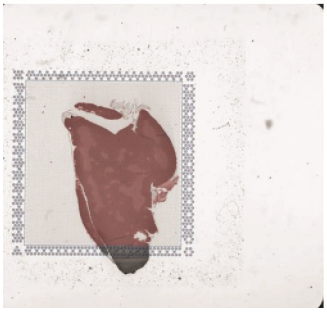
## 2. Area of Interest Not Within Allowable Area

**Area of Interest Outside of Allowable Area is Not Analyzed**

**Tissue Larger than Capture Area**




**Tissue Not Properly Aligned**

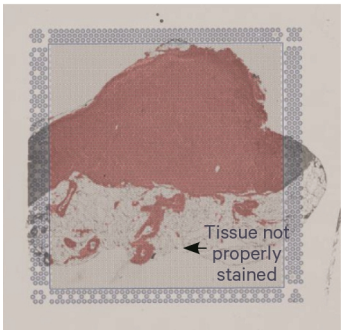


Areas of interest that are not placed within the allowable area on compatible glass slides will not be analyzed. This may occur if the tissue is larger than the Capture Area or if the tissue slide is not properly aligned when loading onto the Tissue Slide Stage. Tissue outside of the allowable area is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

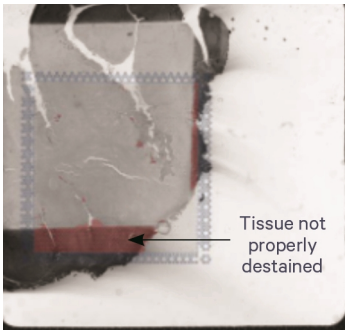
## 3. Tissue Segmentation Failure due to High Connective Tissue

**Examples of Scenarios that Lead to Tissue Segmentation Failure**

**Inadequate Staining Causes Poor Tissue Segmentation**



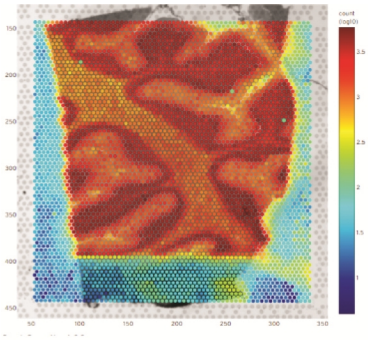

**H&E Stained Tissues Outside of Gasket May Not Undergo Destaining**




Space Ranger may fail to detect tissue for a variety of reasons, which may necessitate manual fiducial alignment and tissue detection via Loupe Browser. If the contrast between background and tissue is poor (due to inadequate staining, technical error, or tissue composition) tissue segmentation failure may occur (left image). If large tissues that exceed the gasket area of the cassette re-enter the capture area, they may cause a tissue segmentation failure in Space Ranger if they re-enter the Capture Area due to the contrast of this tissue against tissue within the Capture Area (right image).

# 4. Tissue within Capture Area Not Analyzed

**Gasket Covers Portion of Tissue within Capture Area**

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

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

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

10x GENOMICS

# Appendix


Post Library Construction Quantification 148

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Oligonucleotide Sequences 151

# Post Library Construction Quantification

**a.** Thaw KAPA Library Quantification Kit for Illumina Platforms.

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

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

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

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

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

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

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

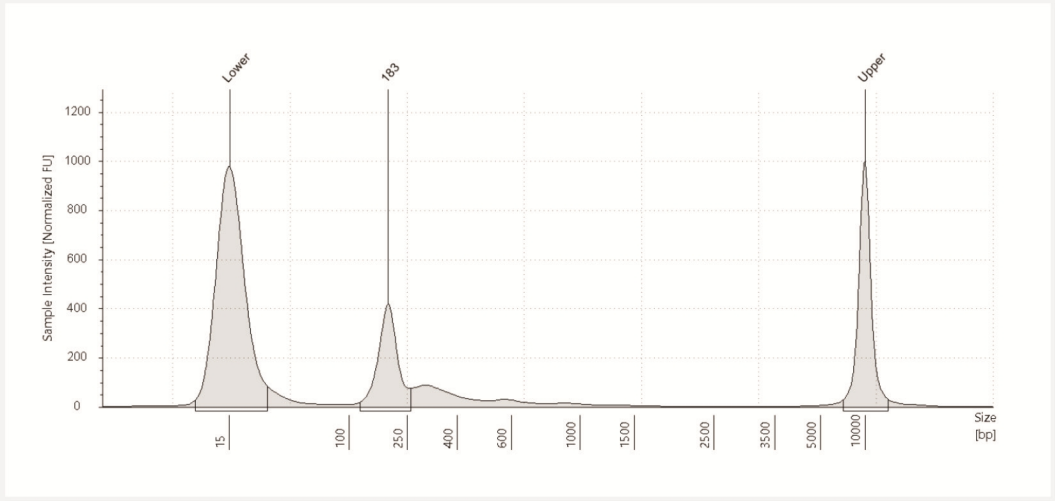
**g.** Follow the manufacturer's recommendations for qPCR-based quantification. For library quantification for sequencer clustering, determine the concentration based on insert size derived from the Bioanalyzer/TapeStation trace.

# Agilent TapeStation Traces

**Protocol Step 7.3 - Protein Post Library Construction QC**

**Representative Trace**

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

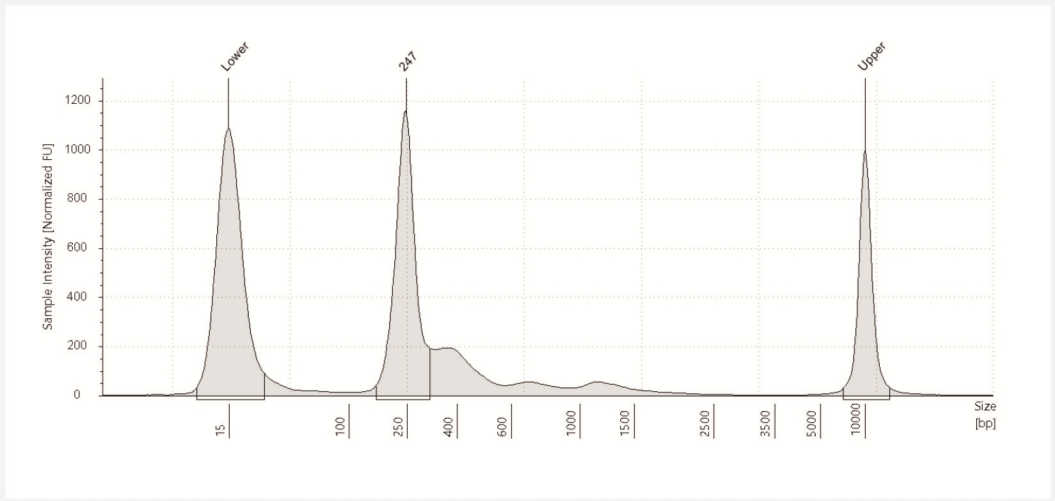



Agilent TapeStation High Sensitivity D5000 ScreenTape was used. Protocol steps correspond to the steps in this User Guide.

**Protocol Step 5.3 - GEX Post Library Construction QC**

**Representative Trace**

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

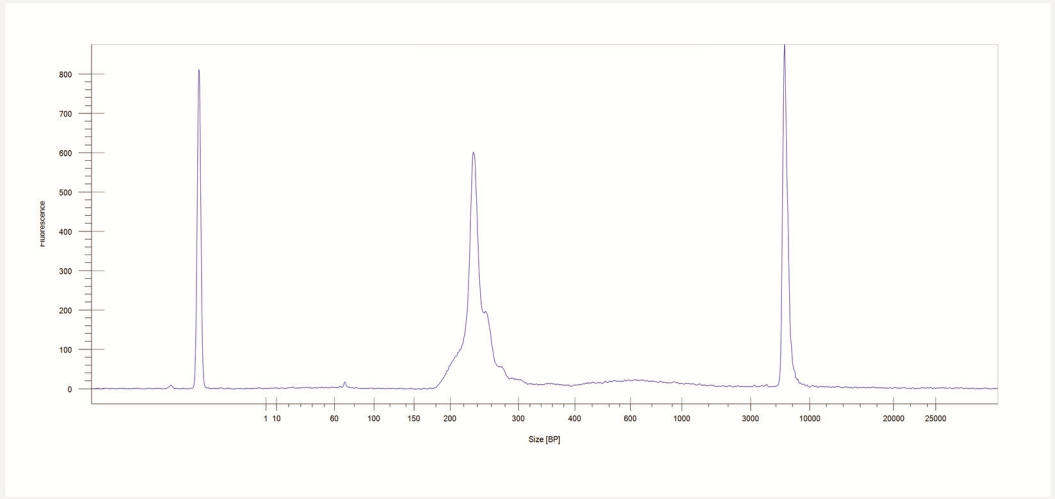


# LabChip Traces

**Protocol Step 5.3 - GEX Post Library Construction QC**

**Representative Trace**

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

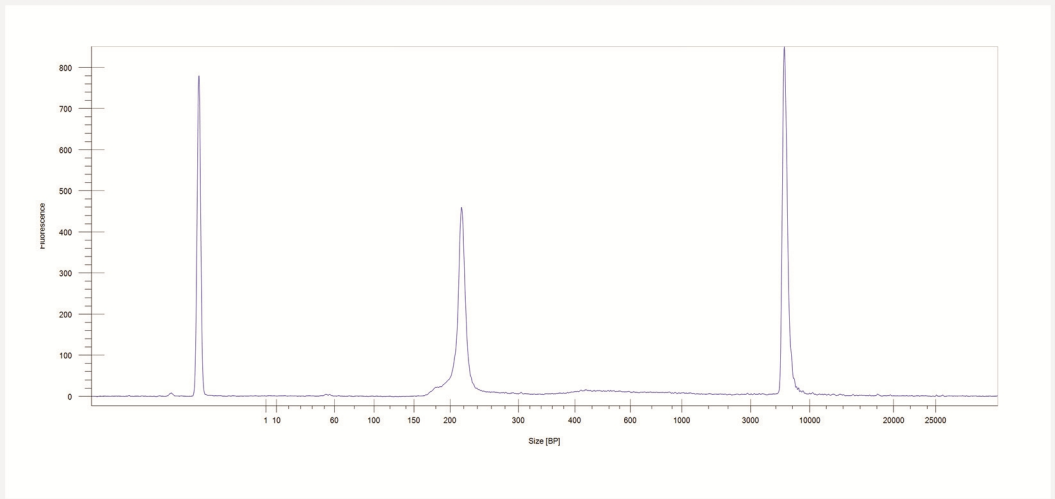



DNA High Sensitivity Reagent Kit was used. Protocol steps correspond to the steps in this User Guide.

**Protocol Step 7.3 - Protein Post Library Construction QC**

**Representative Trace**

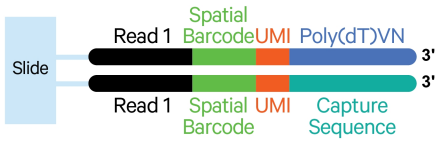
**Run manufacturer's recommended volume of diluted sample (1:5 dilution)**



# Oligonucleotide Sequences

## Slide Primers

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




5'-GTCAGATGTGTATAAGAGACAG-N16-N12-N16-3'

## Visium CytAssist Spatial Gene Expression - Probe-based Library


## Visium CytAssist Spatial Protein Expression Library