Visium CytAssist Spatial Gene and Protein Expression

– Custom Add-on Antibody Optimization

Introduction

The Visium CytAssist Spatial Gene and Protein Expression assay is designed to analyze mRNA and protein expression in tissue sections derived from formalin fixed & paraffin embedded (FFPE) samples. 10x Genomics provides a pre-designed antibody panel to analyze protein expression in tissues. To add additional oligo-conjugated antibodies to the panel, an antibody titration is necessary to determine the appropriate antibody concentration for use in the assay. This protocol is used to determine this concentration and should be performed even if the manufacturer provides a recommended dilution for the purchased antibody.

This protocol optimizes the concentration of a single, oligo-conjugated antibody. Antibodies can be purchased from BioLegend or generated through custom conjugation approaches. 10x Genomics does not support the process of independent conjugation of antibodies for Visium applications. Repeat this protocol when optimizing multiple antibodies. This protocol also provides guidance on pooling optimized antibodies. For a discussion on using immunofluorescence vs. sequencing to analyze protein data, refer to Antibody Optimization - Key Considerations.

Contents

Additional Guidance

For information on purchasing BioLegend TotalSeq-Bn antibodies, visit https://www.biolegend.com/en-us/contact.

For information on custom conjugation, visit the Q&A section of the Visium Spatial Gene and Protein Expression page on the 10x Genomics support website.

Consult the Visium CytAssist Spatial Gene and Protein Expression for FFPE - Tissue Preparation Guide (Document CG000660) for information on sectioning FFPE tissue blocks and section placement.

Reagent Kits

Visium Spatial Gene and Protein Expression for FFPE Reagent Kits

Refer to SDS for handling and disposal information.

Visium 6.5 mm Slide Cassette*, 4 pk, PN-1000469

Visium 6.5 mm Slide Cassette, 4 pk
PN-1000469
Store at ambient temperature

	#	PN
Visium Cassette, 8 port	4	3000811

*Additional Visium Cassettes must be purchased for antibody optimization.

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

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

	#	PN
Visium Cassette, 8 port	1	3000811
Visium CytAssist moveable gasket small (preassembled with translator)	2	3000814
Visium CytAssist moveable translator (preassembled with gasket)	2	3000816
Visium CytAssist moveable Cassette, frame	2	3000813
*Visium CytAssist Slide Seals, 40 pack	1	2000284
Visium CytAssist Spatial Gene Expression Slide v2, 6.5 mm	1	2000549

*Only item used for this protocol.

Reagent Kits

Visium Spatial Gene and Protein Expression for FFPE Reagent Kits

Refer to SDS for handling and disposal information.

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

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

	#	PN
Visium Cassette, 2 port	1	3000812
Visium CytAssist moveable gasket large	2	3000815
Visium CytAssist moveable Cassette, frame	2	3000813
*Visium CytAssist Slide Seals, 40 pack	1	2000284
Visium CytAssist Spatial Gene Expression Slide v2, 11 mm	1	2000701

**Only item used for this protocol.*

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

**Only reagent used in this protocol. The amount of Block and Stain Buffer provided in the kit is enough for 3-4 antibody optimizations, while still leaving enough reagent for the main assay. Refer to Antibody Optimization - Key Considerations for more information.*

***May not be present in this kit.*

10x Genomics Accessories

Product	#	Kit & Part Number	Part Number (Item)
Low Profile Plate Insert*	2	Visium CytAssist Reagent Accessory Kit: 1000499	3000823
10x Magnetic Separator	1		120250

**Only item used in this protocol.*

Recommended Thermal Cyclers

Supplier	Description	Part Number
Bio-Rad	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module *(discontinued)*	1851197
	PTC Tempo Deepwell Thermal Cycler*	12015392
Eppendorf	MasterCycler Pro *(discontinued)*	North America 950030010 International 6321 000.019
	MasterCycler X50s	North America 6311000010
Thermo Fisher Scientific	Veriti 96-Well Thermal Cycler *(discontinued)*	4375786
Analytik Jena	Biometra TAdvanced 96 SG with 96-well block (silver, 0.2 mL) and gradient function	846-x-070-241

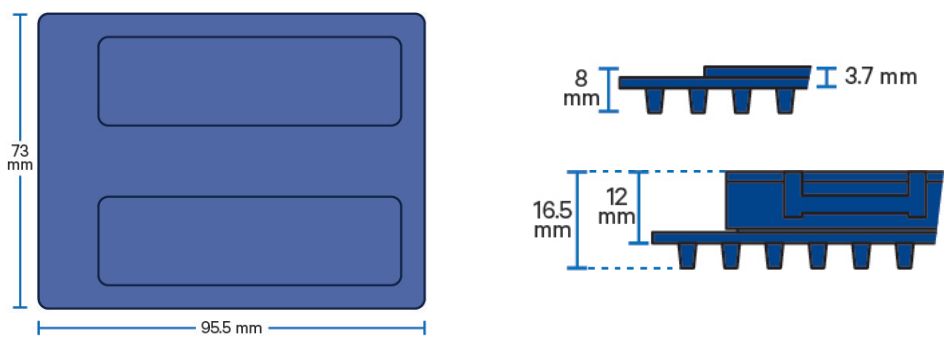
*If used with a single cassette, place the cassette on the side of the adapter furthest away from the thermal cycler hinge to prevent lid lock errors.

For the following thermal cyclers, ramp rates should be adjusted for all steps as described below:

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

Thermal cycler 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



Recommended Real Time qPCR Systems

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

Workflow Overview

Tissue Preparation

Before starting, refer to these documents.

Tissue Preparation Guide
Section tissue onto slides.
Demonstrated Protocol CG000660

Imaging Guidelines
Optimize imaging settings.
Technical Note CG000521

(Optional)

Add-on Antibody Optimization
Determine optimal add-on antibody concentration. (Optional)
Demonstrated Protocol CG000664

Deparaffinization, Staining, Decrosslinking

Choose one demonstrated protocol

Deparaff. + Decross. + IF Stain
Deparaffinize, decrosslink, stain, and image tissue.
Demonstrated Protocol CG000659

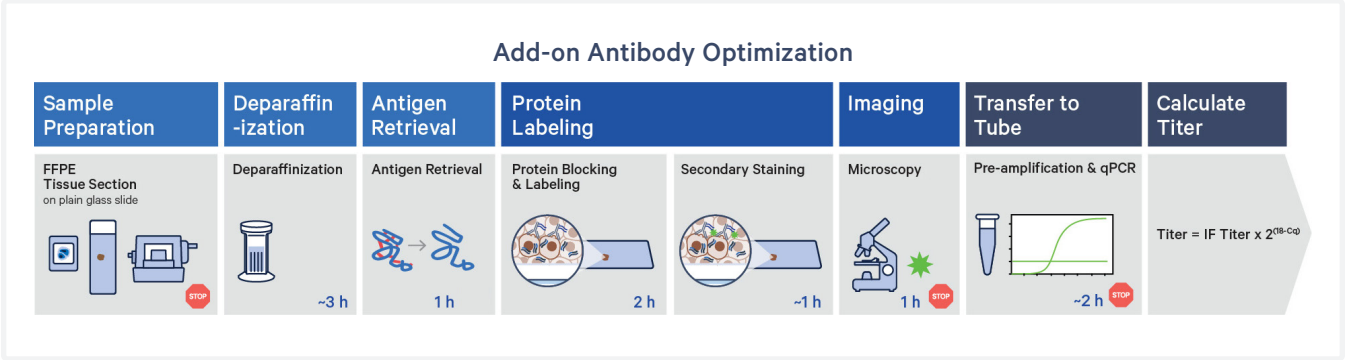
OR

Deparaff. + H&E Stain + Decross.
Deparaffinize, stain, image, and decrosslink tissue.
Demonstrated Protocol CG000658

Library Construction

Library Construction
Construct Visium CytAssist Spatial Gene Expression - Probe-based and Protein Expression libraries.
User Guide CG000494

Visit the 10x Genomics Support website for the most up-to-date documentation.



Specific Reagents & Consumables

For each item, a number of vendor options are listed. Choose item based on availability and preference. **Substituting materials may adversely affect system performance.**

Item	Alternatives/Options	Vendor	Part Number
Amp Mix	AllTaq PCR Core Kit	Qiagen	203123
Xylene	Xylene, Reagent Grade	Millipore Sigma	214736
	Xylene, Histological Grade	Millipore Sigma	534056
Ethanol	Ethyl Alcohol, 200 Proof	Millipore Sigma	E7023
	Ethanol absolute ≥99.5%, TechniSolv, pure (Europe Only)	VWR	83813.360DP
Citrate Buffer	Citrate Buffer, pH 6.0, 10x, Antigen Retriever	Millipore Sigma	C9999-1000ML
Tris 1 M (Tris-HCl)	Tris 1 M, pH 8.0, RNase-free	Thermo Fisher Scientific	AM9855G
	Tris 1 M, pH 8.0	Teknova	T5080
	UltraPure 1M Tris-HCl, pH 8.0	Invitrogen	15568025
PBS	PBS - Phosphate Buffer Saline (10X) pH 7.4, RNase-free	Thermo Fisher Scientific	AM9624
10% 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)	
Nuclease-free water	Nuclease-free Water (not DEPC-Treated)	Thermo Fisher Scientific	AM9937
DAPI	DAPI Solution (1 mg/ml)	Thermo Fisher Scientific	62248
8M KOH	Potassium Hydroxide Solution, 8M	Millipore Sigma	P4494-50ML
20X SSC Buffer	SSC Buffer 20X Concentration	Millipore Sigma	S66391L
Oligo-conjugated Antibodies	TotalSeq-Bn Antibodies	BioLegend	-
Antibody Oligo Primers*	Forward Primer (5'-3'): GTGACTGGAGTTCAGACGTG Reverse Primer (3'-5'): GCTAGGACCGGCCTTAAAGC	-	-
Secondary Antibodies**	Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific	A10037
	Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific	A10042
Autofluorescence Quencher	TrueBlack Lipofuscin Autofluorescence Quencher *Optional*	Biotium	23007
Mounting medium	SlowFade Diamond Antifade Mountant	Thermo Fisher Scientific	S36967
Glycerol	Glycerol	Acros Organics	327255000
	Glycerol Solution	Millipore Sigma	49781
Buffer EB	Qiagen Buffer EB	Qiagen	19086

** Order primers at 100 micromole scale and choose standard desalting purification.*

***Select secondary antibodies based on primary antibody host. Avoid secondary antibodies with fluorescent dyes with emission maxima of 520 ± 20 nm such as FITC, Alexa Fluor 488, and ATTO 488 due to background fluorescence.*

Specific Reagents & Consumables

For each item, a number of vendor options are listed. Choose item based on availability and preference. **Substituting materials may adversely affect system performance.**

Coplin jar/ staining dishes	Coplin Jar	VWR	100500-232
	Staining Dishes	VWR	25608-906
Section dryer oven	Epredia High Capacity Section Dryer *Or any equivalent product. Thermal cycler may also be used for section drying.*	Fisher Scientific	A84600051
Forceps	Excelta 3C-S-SE Tweezers Very Fine Point Straight SS	Excelta	3C-S-SE
1.5 ml tubes	DNA LoBind Tubes, 1.5 ml	Eppendorf	022431021
	Low DNA Binding Tubes, 1.5 ml	Sarstedt	72.706.700
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
PCR Plates and Sealing Film	Hard-shell PCR Plates 96-well, thin wall (pkg of 50) (or any compatible PCR plate)	Bio-Rad	HSP9665
	MIcroseal 'B' PCR Plate Sealing Film adhesive (Or any compatible PCR Plate sealing adhesive)	Bio-Rad	MSB1001
Pipette Tips	Tips LTS 200UL Filter RT-L200 FLR	Rainin	30389240
	Tips LTS 1ML Filter RT-L1000 FLR	Rainin	30389213
	Tips LTS 20UL Filter RT-L20 FLR	Rainin	30389226
Wide Bore Pipette Tips	Tips RT LTS 200UL FLW	Rainin	30389241
Additional Materials			
Razor Blades		-	-
Ultrapure/Milli-Q Water, *from Milli-Q Integral Ultrapure Water System or equivalent*		-	-

Antibody Optimization - Key Considerations

This Demonstrated Protocol enables the addition of oligo-conjugated antibodies to 10x Genomics pre-designed panels in the main assay, resulting in protein expression data for each added marker. Given the cost and labor associated with optimization, it should be performed in situations where visualizing additional proteins of interest via immunofluorescence staining is not ideal.

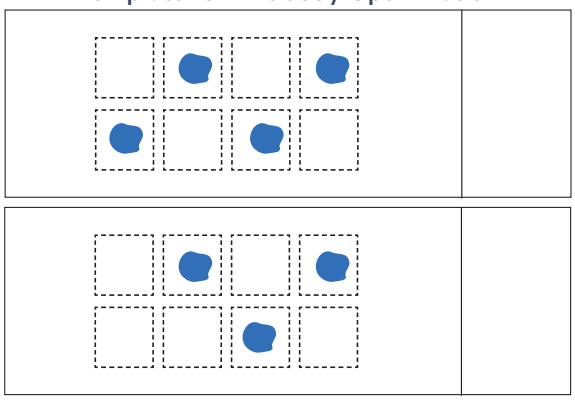
For example, gathering data on 7 additional proteins via immunofluorescence staining is likely not possible due to microscope limitations. Thus, optimizing antibodies for these markers via this protocol and adding them to the main assay for analysis via sequencing is an optimal alternative.

This Demonstrated Protocol provides a suggested titration scheme that requires two tissue slides, two Visium Cassettes, one Low Profile Thermocycler Adapter, and one thermal cycler. The titration scheme can be scaled or modified as needed.

Antibody optimization should be performed on the same experimental block that will be used during the main CytAssist assay, or an equivalent block with similar tissue morphology as the sample of interest. Section multiple tissue sections onto a blank slide in an arrangement compatible with the Visium Slide Cassette (PN-3000811).

The template below can be drawn on the back of a blank slide to assist in section placement. Staggering tissues, as shown below, may aid in tissue placement. The template is to scale. Template is to scale if scaling settings are not modified (select "actual size" or 100%" to print to scale).

Template for Antibody Optimization



If tissue sections are large, score the tissue block such that the area of interest for each tissue section can fit in a square in the template above. For more information on scoring, refer to the Appendix. For information on storing slides with tissue, refer to the Visium CytAssist Spatial Gene and Protein Expression for FFPE - Tissue Preparation Guide (CG000660).

The same region of interest within serial tissue sections should be used for antibody optimization. Staining different regions of tissue between antibody concentrations will yield inconclusive results.

The Block and Stain Buffer used in add-on antibody optimization is also used in the main Visium CytAssist assay. The table below describes the number of antibody optimizations that can be performed while leaving enough reagent for the main assay. The table assumes that IF staining is performed before the main assay. It does not include IF antibody optimization, which will use variable amounts of Block and Stain buffer.

Reagent	PN	Number of Antibody Optimizations
If using 6.5 mm Visium Slides in Main Assay		
Block and Stain Buffer	2000554	4
If using 11 mm Visium Slides in Main Assay		
Block and Stain Buffer	2000554	3

Tips & Best Practices

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

General Reagent Handling

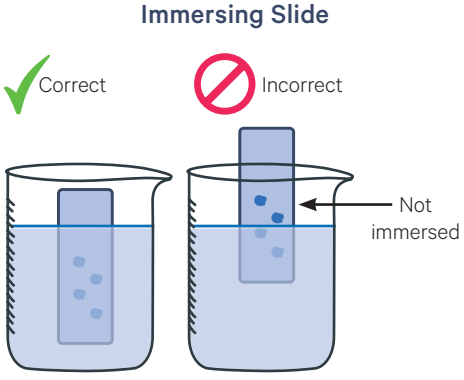
- Thoroughly mix reagents before use.
- Use a pH meter to adjust pH as necessary during buffer preparation.

Pipette Calibration

- Follow manufacturer's calibration and maintenance schedules.

Slide Handling

- Always wear gloves when handling slides.
- DO NOT touch the tissue sections on the slide.
- Minimize exposure of the slides to sources of particles and fibers.
- When immersing slides in deparaffinization solutions, ensure that the tissue sections are completely submerged.
- Keep the slide flat on the bench when adding reagents to the tissue.
- Ensure that no absorbent surface is in contact with the reagents on the slide during incubation.



Slides in images are representative.

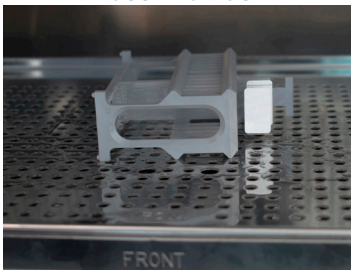
Slide Incubation Guidance

Incubation at a specified temperature

Incubation using a Section Dryer Oven:

- Place the slides in a slide drying rack sideways to minimize paraffin wax entering neighboring tissue sections.
- Close the lid when incubating the slide in the oven.

Place in a Rack

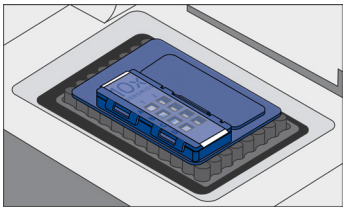


Slides in images are representative.

Incubation using a Thermal Cycler:

- Position a Low Profile Plate Insert (also referred to as Low Profile Thermocycler Adapter) on a thermal cycler that is set at the incubation temperature.
- Move Low Profile Thermocycler Adapter back and forth to ensure that it is seated properly.
- Ensure that the Low Profile Thermocycler Adapter is in contact with the thermal cycler surface uniformly.
- When incubating a slide encased in a cassette, place the assembled unit on the Low Profile Thermocycler Adapter with the wells facing up.
- The cassette should sit flat on the Low Profile Thermocycler Adapter.
- Cassettes should always be sealed when in the Low Profile Thermocycler Adapter.
- Tighten the thermal cycler lid, but do not overtighten.
- After removing the cassette from the thermal cycler, ensure that volume removed from the well is as expected. Volumes lower than what are expected may indicate an improper seal, resulting in evaporation.
- Allow Low Profile Thermocycler Adapter to cool before removing it from the thermal cycler.

Incubate Assembled Cassette

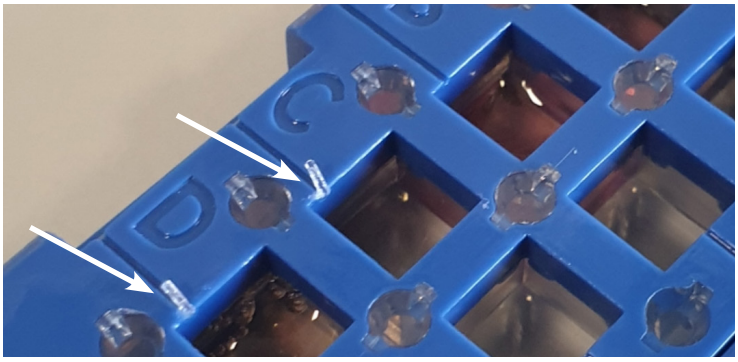


Close lid

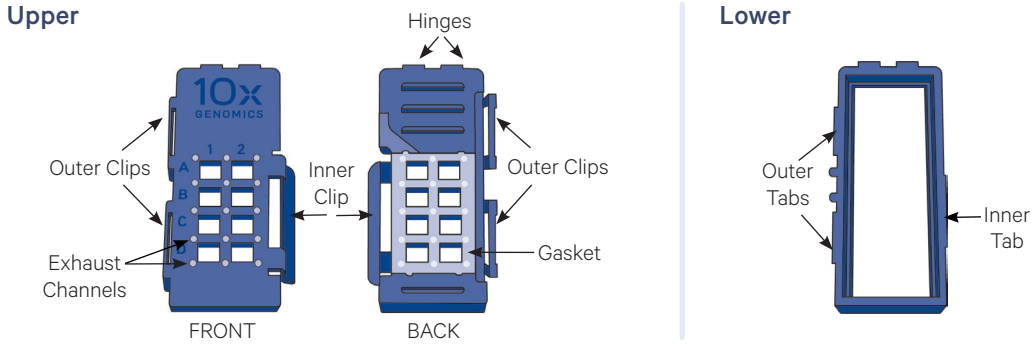
Visium Cassette

- The Visium Cassette encases the slide and creates leakproof wells for reagent addition.
- The Visium Cassette is a single use item.
- Place the slides in the Visium Cassette only when specified.
- See Visium Cassette Assembly & Removal instructions for details on assembly and removal.
- Ensure that the Visium Cassette and slide facing gasket are free of debris before assembly.
- Excess silicone in exhaust channels or within wells, as shown in the image below, should be safely removed with forceps or a pipette tip before cassette assembly.
- Failure to remove excess silicone may prevent adequate venting of the cassette during heated incubation steps.
- If the gasket appears warped, the Visium Cassette is still safe to use as long as the cassette can fully close.
- Visually inspect the gasket to ensure it is seated properly.
- Practice assembly with a blank slide (75 x 25 x 1 mm).
- Applying excessive force to the slide and/or cassette may cause the slide to break.

Remove Excess Silicone



Visium Slide Cassette, 6.5 mm

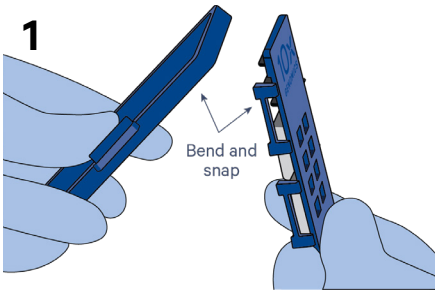


Visium Cassette Assembly

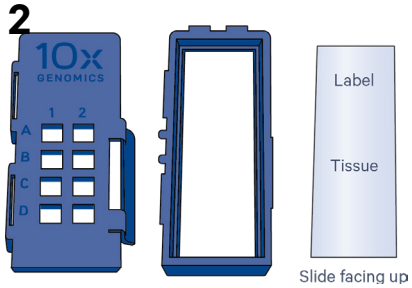
- Ensure that the surface of the cassette is dry.
- Cassette may also be assembled in the hand for comfort.

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

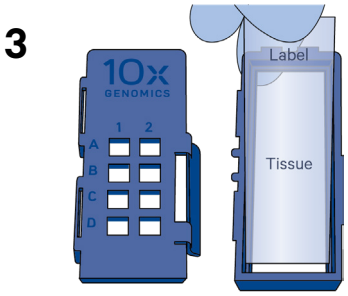
DO NOT close upper and lower halves of cassette before detaching hinges.



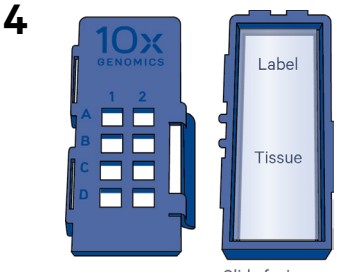
Place upper and lower halves of cassette, and blank, directed upward on bench



Place the slide facing upwards into lower half of cassette; ensure label is toward hinges

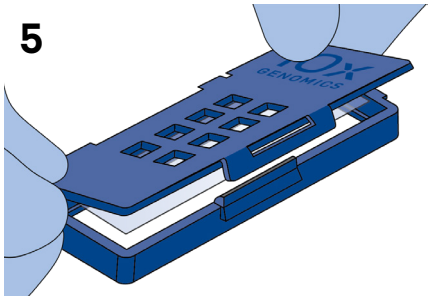


Press slide down into grooves of the bottom half of the cassette until it sits firmly in place

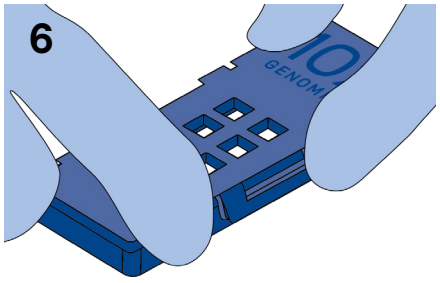


Secure outer clips of top half with outer tab of bottom half

Exercise caution when handling slide edges to prevent injury.

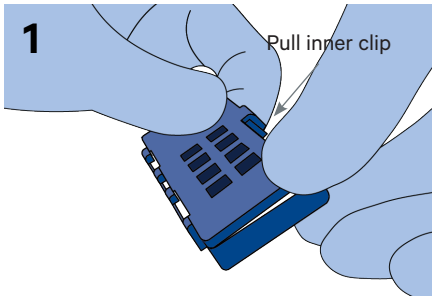


Press firmly on top of cassette until it clicks shut

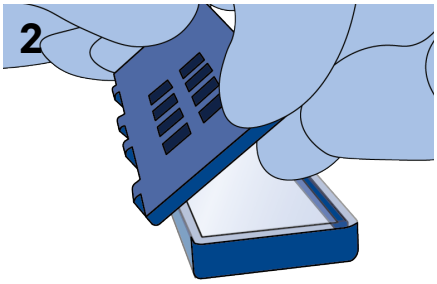


Visium Cassette Removal

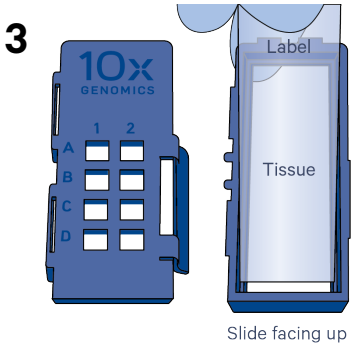
Pull inner clip up from inner tab to detach upper and lower halves of cassette



Open cassette by continuing to lift inner clip upward



Lift slide out from lower half



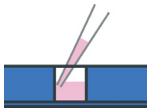
Slide facing up

Reagent Addition to Wells

- Place the assembled slide in the cassette flat on a clean work surface.
- Dispense reagents along the side of the wells without touching the tissue sections and without introducing bubbles.
- Always cover the tissue section completely when adding reagents to the well. A gentle tap may help spread the reagent more evenly.

Reagent Addition/Removal

Side View



Add reagent along the slide of the wells

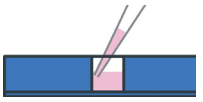
Top View

DO NOT introduce air bubbles

Cassette color is representative

Reagent Removal from Wells

- Slightly tilt the cassette while removing the reagent.
- Place the pipette tip to the bottom edge of the wells.
- Remove reagents along the side of the wells. Do not touch the tissue sections or introduce 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.



Cassette Top

Cassette Bottom

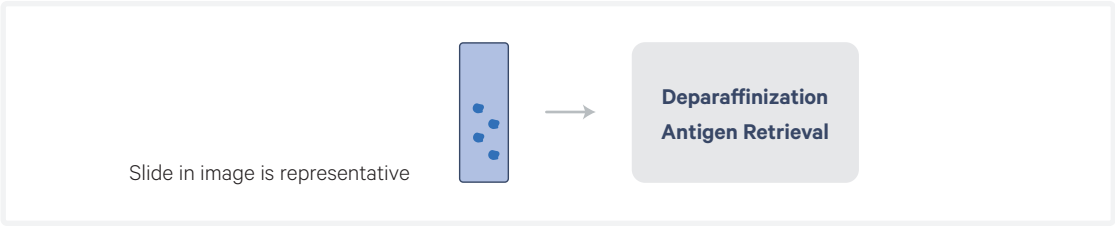
Place the pipette tip to the bottom edge of the wells

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

1. Deparaffinization and Antigen Retrieval

1.0 Overview

This chapter provides guidance on deparaffinization and antigen retrieval of FFPE tissue slides dried overnight in a desiccator according to the Visium CytAssist Spatial Gene and Protein Expression for FFPE - Tissue Preparation Guide (CG000660). A suggested tissue placement template is provided on page 7.



1.1 Preparation - Buffers

For Deparaffinization	
Process two slides per jar. Alternatively, use a slide staining dish. Adjust volumes of deparaffinization solutions and water accordingly and ensure volume fully covers tissue.	
Items	**Preparation & Handling**
☐ Xylene	Label two coplin jars as Xylene Jar 1 and 2. Dispense 30 ml xylene in each.
☐ 100% Ethanol	Label two coplin jars as 100% Ethanol Jar 1 and 2. Dispense 30 ml 100% ethanol in each. *Alternatively, use a 50-ml centrifuge tube or a beaker.*
☐ 96% Ethanol	Label two coplin jars as 96% Ethanol Jar 1 and 2. Dispense 30 ml 96% ethanol in each. *Alternatively, use a 50-ml centrifuge tube or a beaker.*
☐ 70% Ethanol	Label one coplin jar as 70% Ethanol Jar. Dispense 30 ml 70% ethanol. *Alternatively, use a 50-ml centrifuge tube or a beaker.*

Use xylene-resistant dishes for immersion in xylene.

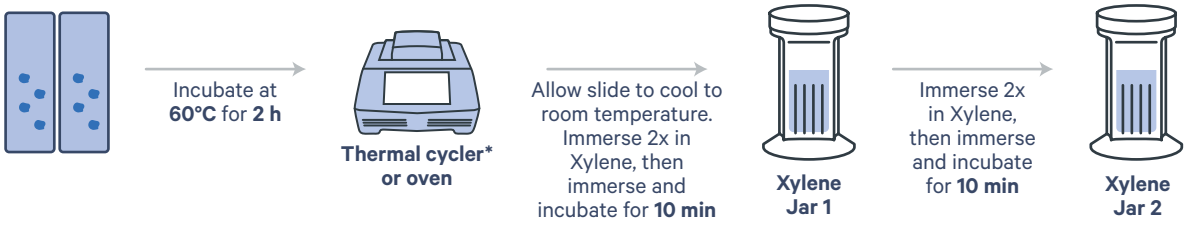
Use xylene-resistant gloves or forceps for deparaffinization.

For Antigen Retrieval	
Items	**Preparation & Handling**
☐ Citrate Buffer, pH 6.0	Store stock buffer at 4°C. Vortex and centrifuge briefly after preparing Citrate Buffer and keep at room temperature. Discard excess Citrate Buffer and store excess stock buffer at 4°C.

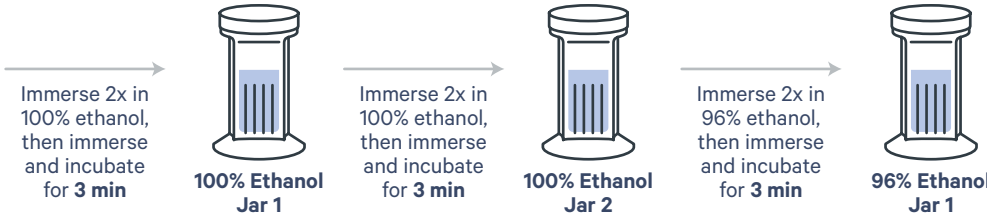
Citrate Buffer	Stock	Final	1X (µl)	7X+ 10% (µl)
Citrate Buffer	10X	1X	25.0	192.5
Nuclease-free Water	-		225.0	1,732.5
Total	-	-	**250.0**	**1,925.0**

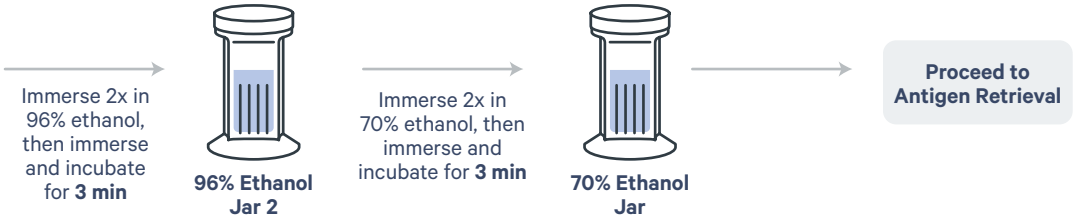
Protocol Overview

Deparaffinization ~3 h



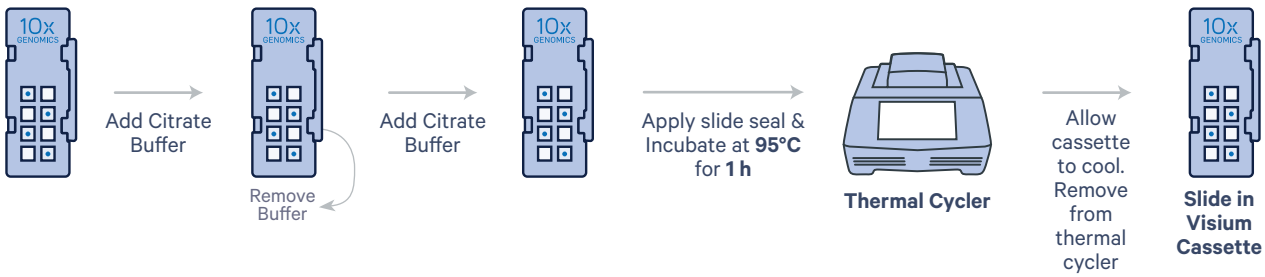
**Keep the thermal cycler lid open during incubation*





Slide in image is representative

Antigen Retrieval ~1 h



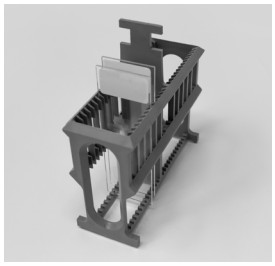
1.2 Deparaffinization

Deparaffinization steps should be performed in a fume hood due to the hazardous nature of xylene.

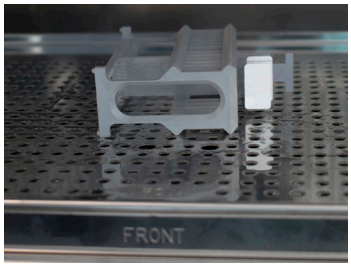
a. Retrieve slides with tissue sections from desiccator after overnight drying.

b. Place slides in a rack in a Section Dryer Oven and incubate uncovered and sideways at **60°C for 2 h**. Keep the oven lid closed during incubation.

Incubation in a Section Dryer Oven



Place slide in a rack



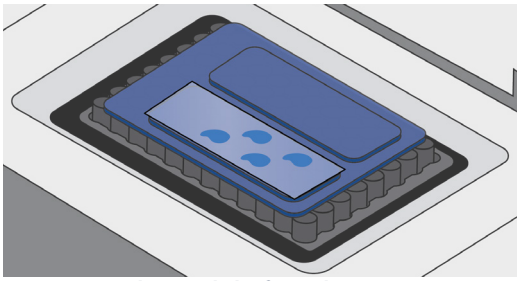
Place slide sideways and keep the oven door closed during incubation

Alternatively, place a Low Profile Thermocycler Adapter on a thermal cycler set at **60°C**. Place slide on the Low Profile Thermocycler Adapter with the tissue side facing up and incubate **2 h at 60°C**.

DO NOT close the thermal cycler lid.

Incubation in a Thermal Cycler

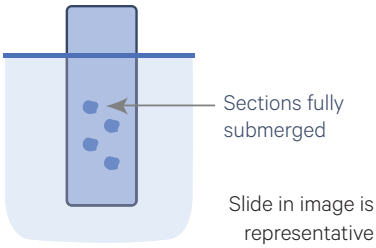
DO NOT close the lid



Incubate Slide for 2 h at 60°C

c. Remove slides from the oven or thermal cycler and allow to cool down to room temperature for **5 min**.

TIPS *When immersing slides in xylene, ensure that the tissue sections are completely submerged.*



d. Gently immerse slides 2x in Xylene Jar 1, then immerse and incubate for **10 min**. Secure jar cap to prevent xylene loss.

e. Gently immerse slides 2x in Xylene Jar 2, then immerse and incubate for **10 min**. Secure jar cap to prevent xylene loss.

f. Gently immerse slides 2x in 100% Ethanol Jar 1, then immerse and incubate for **3 min**.

g. Gently immerse slides 2x in 100% Ethanol Jar 2, then immerse and incubate for **3 min**.

h. Gently immerse slides 2x in 96% Ethanol Jar 1, then immerse and incubate for **3 min**.

i. Gently immerse slides 2x in 96% Ethanol Jar 2, then immerse and incubate for **3 min**.

j. Gently immerse slides 2x in 70% Ethanol Jar, then immerse and incubate for **3 min**.

k. Wipe the back of the slide with a laboratory wipe. Place on a flat, clean, nonabsorbent work surface and air dry.

l. Place slides in Visium Slide Cassettes, 6.5 mm.

TIPS *See Tips & Best Practices for assembly instructions. Practice assembly with a blank slide.*

m. Proceed **immediately** to Antigen Retrieval.

1.3 Antigen Retrieval

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

Lid Temperature	Reaction Volume	Run Time
95°C	100 µl	60 min
Step	**Temperature**	**Time**
Pre-equilibrate	95°C	Hold
Antigen Retrieval	95°C	00:60:00
Cooling	22°C	00:10:00
Hold	22°C	Hold

b. Add **150 µl** Citrate Buffer along the side of the wells containing tissue. Tap cassette gently to ensure uniform coverage.

c. Remove Citrate Buffer from the wells.

d. Add **100 µl** Citrate Buffer along the side of the wells containing tissue. Tap cassette gently to ensure uniform coverage.

e. Apply slide seals on cassettes and place on Low Profile Thermocycler Adapters at **95°C**.

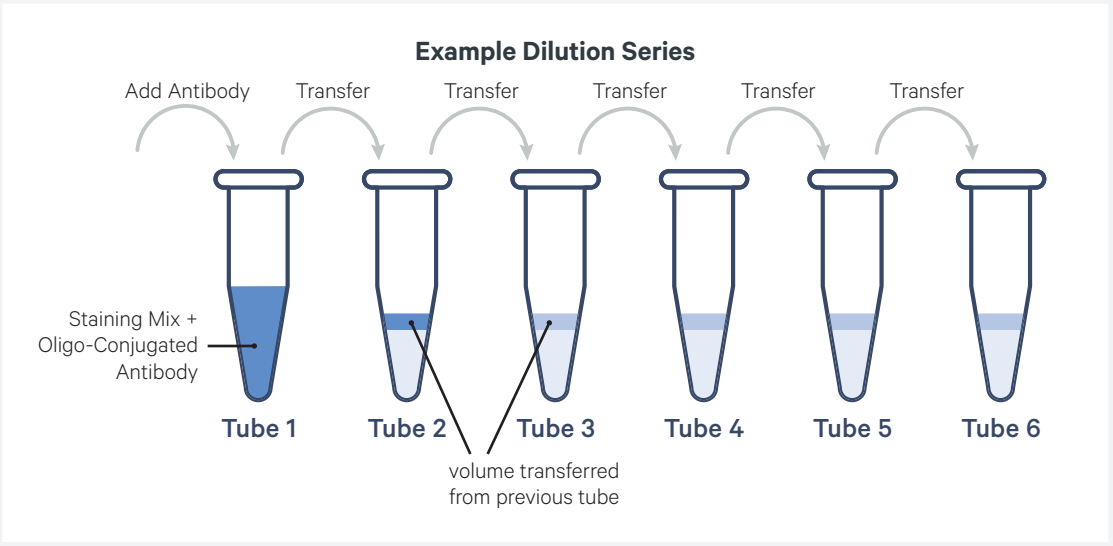
f. Close thermal cycler lid. Skip Pre-equilibrate step and initiate Antigen Retrieval.

2. Blocking and Immunofluorescence Staining

2.0 Overview

This chapter provides guidance on blocking tissue sections and incubating them with a prepared serial dilution of the oligo-conjugated add-on antibody. After incubation with secondary antibodies, the slide is imaged to determine the optimal tissue section for downstream qPCR. Only one antibody should be optimized per optimization experiment. The suggested dilutions provided in this document may be modified to better suit the desired antibody and/or tissue type.

Use secondary antibodies at the manufacturer recommended concentration. Avoid secondary antibodies with fluorescent dyes with emission maxima of 520 ± 20 nm such as FITC, Alexa Fluor 488, and ATTO 488 due to background fluorescence.



2.1 Preparation - Buffers

For Blocking	
Items	**Preparation & Handling**
☐ 1X PBS	Prepare 50 ml of 1X PBS in a 50-ml centrifuge tube by diluting stock PBS in nuclease-free water. Two slides can be processed per tube. Maintain at **room temperature**.
☐ Blocking Mix	Prepare Blocking Mix according to the table. Add reagents in the order listed. Pipette mix 10x with pipette set to 70% of total volume and centrifuge briefly. Maintain at **room temperature**.

Blocking Mix	10x PN	1X (µl)	7X+ 10% (µl)
Nuclease-free Water	-	75.0	577.5
Block and Stain Buffer	2000554	25.0	192.5
Total		**100.0**	**770.0**

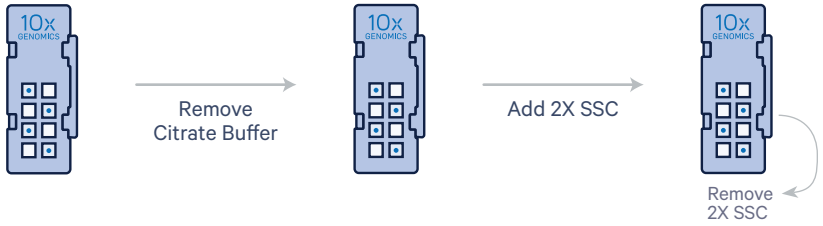
☐ 2X SSC	Prepare 2X SSC according to the table. Add reagents in the order listed. Vortex and centrifuge briefly. Maintain at **room temperature**.

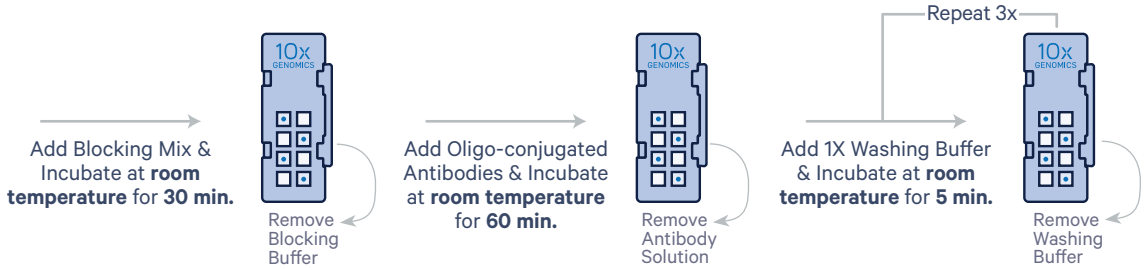
2X SSC	Stock	Final	1X(µl)	7X+ 10% (µl)
SSC	20X	2X	15.0	115.5
Nuclease-free Water	-	-	135.0	1,039.5
Total			**150.0**	**1,155.0**

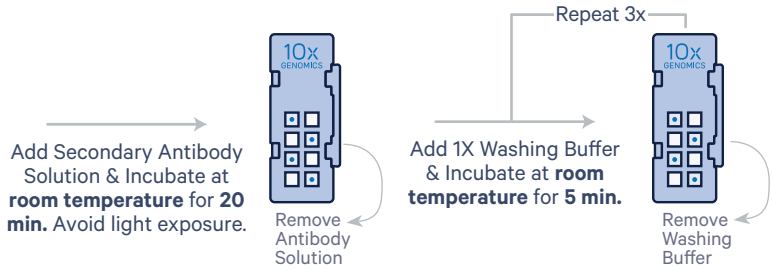
For Coverslipping	
Items	**Preparation & Handling**
☐ Mounting Medium	Invert to mix. Briefly centrifuge to remove bubbles.

Mounting Medium	Stock	Final	1X(µl)	2X+ 15% (µl)
Glycerol	100%	80%	80	184
SlowFade Diamond	100%v	20%	20	46
Total	-	-	**100**	**230**

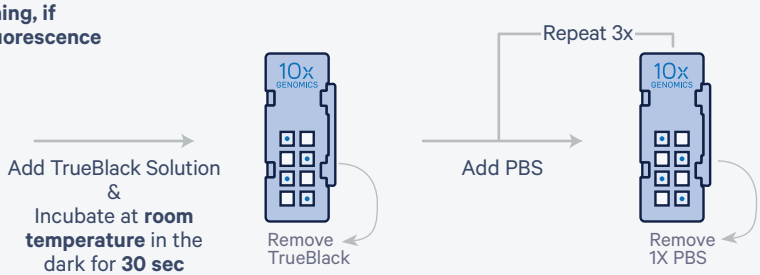
Immunofluorescence Staining – Primary and Secondary Antibodies ~2.5 h

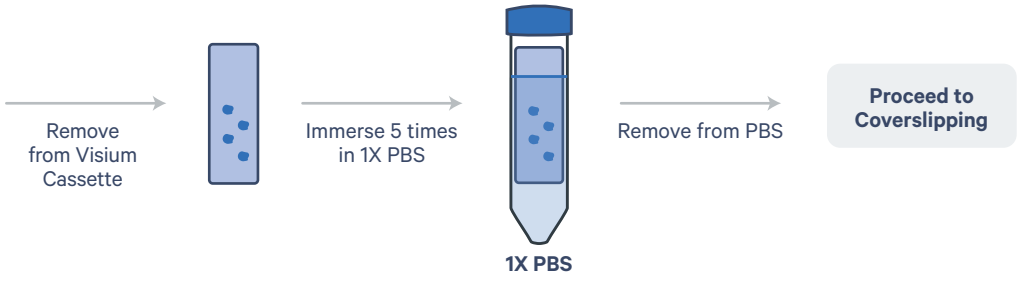






Optional TrueBlack Staining, if tissues have high autofluorescence





2.2 Blocking

a. Remove cassette from Low Profile Thermocycler Adapter and place on a flat, clean work surface.

b. Peel back slide seals.

c. Remove Citrate Buffer from the wells.

d. Add **150 µl** 2X SSC along the side of the wells to uniformly cover the tissue sections, without introducing bubbles. Tap cassette gently to ensure uniform coverage.

e. Remove 2X SSC from the wells.

f. Add **100 µl** Blocking Mix along the side of the wells to uniformly cover the tissue sections, without introducing bubbles. Tap cassette gently to ensure uniform coverage.

g. Re-apply slide seals on cassettes, place on a flat, clean work surface and incubate at **room temperature for 30 min**. Immediately proceed to next step during incubation and begin preparing staining mixes.

2.3 Add-on Antibody Incubation

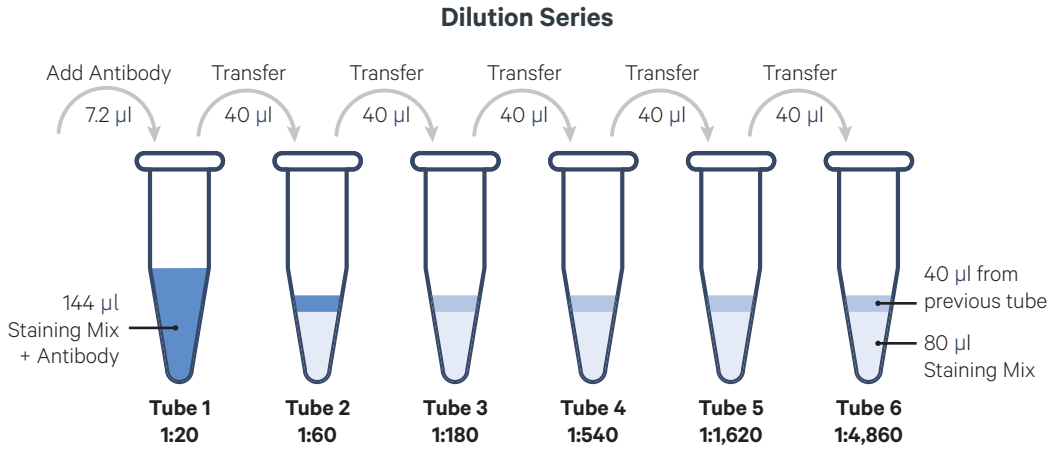
a. Prepare Staining Mix according to the table below, adding reagents in the order listed. 35% overage is to account for required overages during serial dilution.

Staining Mix	10x PN	1X (µl)	7X + 35% (µl)
Nuclease-free water	-	52.5	496.1
Block and Stain Buffer	2000554	17.5	165.4
Total	**-**	**70.0**	**661.5**

b. Prepare dilution series on ice according to the table below, adding reagents in the order listed. Flick the oligo-conjugated antibody tube 4x to mix before starting the dilution series. Change pipette tips between tubes. Pipette mix each dilution 10x with pipette set to 70% of total volume, then transfer indicated volume to next tube. Maintain on ice.

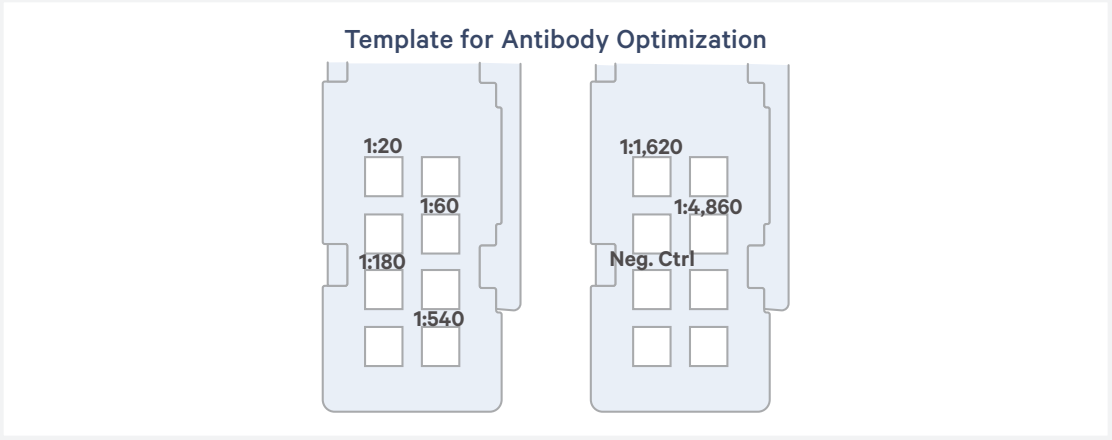
Add-on Antibody Dilution Series	Tube 1 1:20	Tube 2 1:60	Tube 3 1:180	Tube 4 1:540	Tube 5 1:1,620	Tube 6 1:4,860
Oligo-conjugated Antibody	7.2	-	-	-	-	-
Volume from Previous Tube	-	40.0	40.0	40.0	40.0	40.0
Staining Mix	136.8	80.0	80.0	80.0	80.0	80.0
Total*	**144**	**120.0**	**120.0**	**120.0**	**120.0**	**120.0**

**total volume before generating the subsequent dilution*



**volumes in tubes are shown after volume transfer from the previous tube*

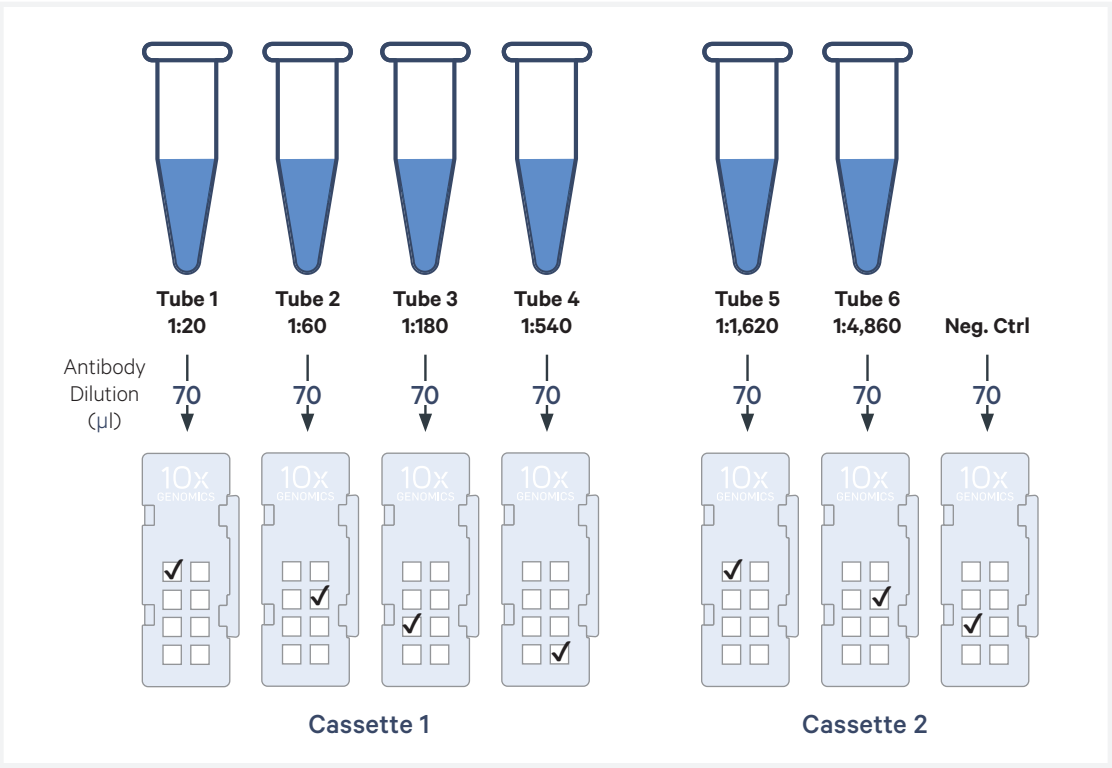
c. Retrieve cassettes and label with the antibody dilutions that will be added to the tissue section.



d. Peel back slide seals and using a pipette, remove all the Blocking Mix from the well corners.

e. Gently tap oligo-conjugated antibody tubes to mix and briefly centrifuge.

f. Add **70 µl from each antibody dilution** along the side of the appropriate well. Tap gently to ensure uniform coverage.



g. Add **70 µl of Staining Mix** along the side of the negative control well.

h. Re-apply slide seal on the cassettes and incubate at **room temperature** for **1 h**.

2.4 Secondary Antibody Incubation

a. Prepare Wash Buffer according to the table below. Add reagents in the order listed. Slowly invert tube 10x and centrifuge briefly. Maintain on ice.

Wash Buffer	Stock	Final	1X (µl)	7X+ 10% (µl)
Nuclease-free Water	-	-	1,032.0	7,946.4
10X PBS Buffer, pH 7.4	10X	1X	120.0	924.0
Tween-20, 10%	10%	0.4%	48.0	369.6
Total			**1,200.0**	**9,240.0**

b. Prepare Secondary Antibody Solution on ice according to the table below, adding reagents in the order listed. Use secondary antibodies at the manufacturer recommended concentration. Pipette mix 10x and centrifuge briefly. Maintain on ice and in the dark.

Secondary Antibody Solution	Stock	Final	1X (µl)	7X + 10% (µl)
Block and Stain Buffer	4X	1X	17.5	134.8
Secondary Antibody	Variable	Variable	Variable	Variable
DAPI Solution	100X	1X	0.7	5.4
Nuclease-free Water	-	-	Variable	Variable
Total	**-**	**-**	**70.0**	**539.0**

c. *Optional* - Prepare 1X TrueBlack Solution according to the table below, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain at room temperature.

1X TrueBlack Solution	Stock	Final	1X (µl)	7X + 10% (µl)
TrueBlack Lipofuscin Quencher	20X	1X	3.5	27.0
70% Ethanol	-	-	66.5	512.0
Total	**-**	**-**	**70.0**	**539.0**

d. After Oligo-conjugated Antibody Incubation, peel back slide seal and using a pipette, remove all the Staining Mix from the well corners.

e. Add **150 µl** Wash Buffer to each well, adding directly onto the tissue.

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

g. Remove all Wash Buffer from the wells.

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

i. Add **70 µl** Secondary Antibody Solution along the side of the wells. Tap gently to ensure uniform coverage.

j. Re-apply slide seal on the cassette.

k. Incubate for **20 min** at **room temperature** in the dark.

l. Peel back slide seal and remove Secondary Antibody Solution.

m. Add **150 µl** Wash Buffer along the side of the wells.

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

o. Remove Wash Buffer from the wells.

p. Repeat m-o three more times for a total of four washes.

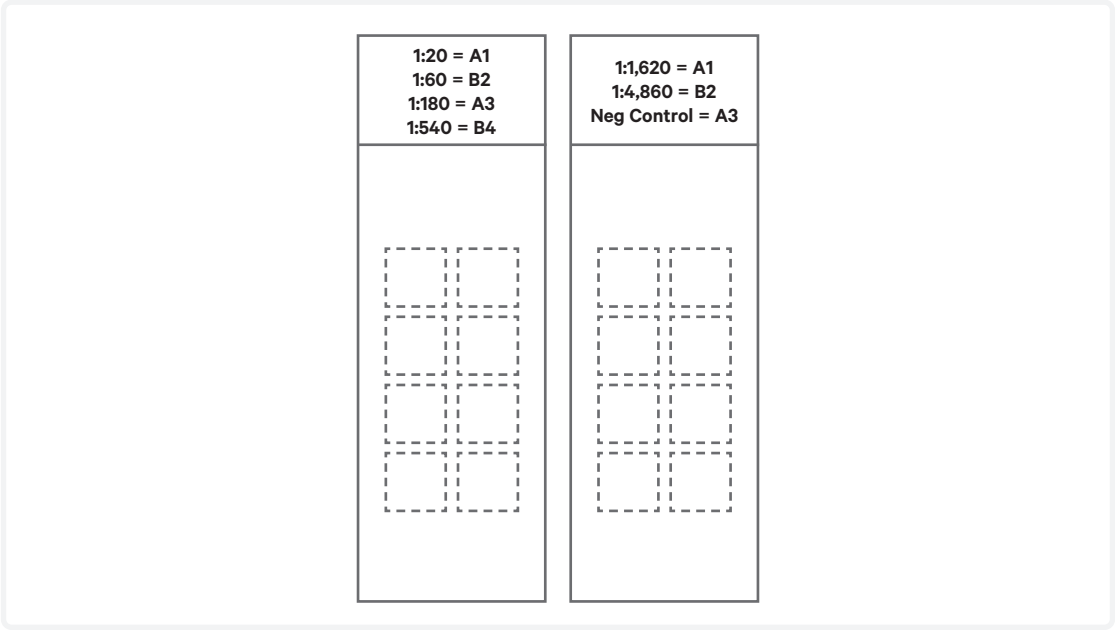
q. *Optional* - TrueBlack Quenching

- Add **70 µl** 1X TrueBlack Solution along the side of the wells.
- Incubate for **30 sec at room temperature**.
- Remove all 1X TrueBlack Solution from the wells.
- Add **150 µl** 1X PBS along the side of the wells.
- Remove all PBS from the wells.
- **Repeat** PBS washes two more times for a total of three washes.

r. Remove slide from cassette.

TIPS *See Tips & Best Practices for removal instructions.*

s. Annotate the top of the slide with the tested antibody dilutions.



t. Gently immerse slides 5x in 1X PBS in 50-ml centrifuge tube.

u. Remove slide from the PBS and proceed immediately to Coverslipping.

2.5 Coverslipping

a. Place slide on a flat, clean, non-absorbent work surface. Some residual droplets may remain.

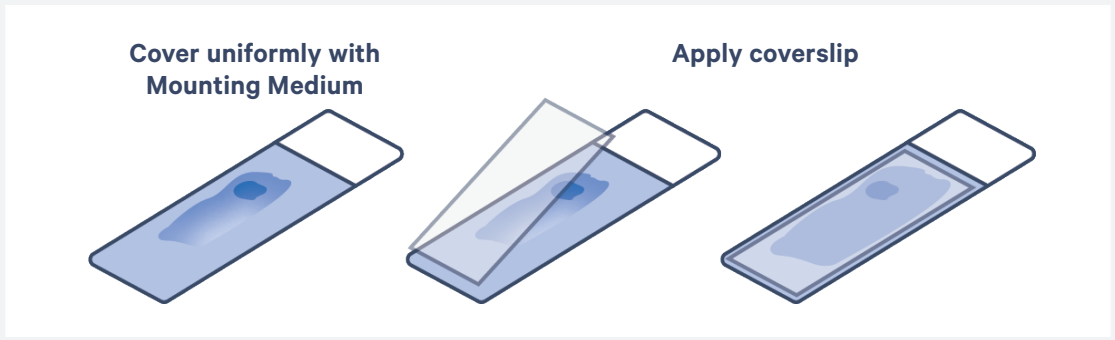
b. Using a **wide-bore** pipette tip, add **100 µl** Mounting Medium to uniformly cover all tissue sections on slides. Avoid generating bubbles.

c. Apply the coverslip at an angle on one end of the slide. Slowly lower the coverslip, without introducing bubbles. Allow the mounting medium to spread and settle.

d. Remove any large excess of mounting media by carefully wicking away from the edge of the coverslip with a laboratory wipe. Do not move the coverslip and disturb the tissue.

e. Once coverslipping is complete, **immediately** proceed with imaging.

DO NOT let the attached coverslip dry.
DO NOT use Cytoseal or nail polish for securing the coverslip. This protocol is designed for use with freshly sectioned tissues.



3. Tissue Imaging

3.0 Overview

This chapter provides guidance on imaging tissue slides.

3.1 Imaging System Recommendations

The following table shows imaging systems used by 10x Genomics in the development of this protocol. Any equivalent imaging setup can be used as an alternative. Adjust illumination power and exposure time to obtain a bright image without saturation.

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

Fluorescence Recommended Configuration
10x Magnification
Light source (or equivalent) with a wavelength range of 380–680 nm
Monochrome camera (14 bit, 2,424 x 2,424 pixel resolution)
DAPI filter cube (Excitation 392/23, Emission 447/60)
FITC filter cube (Excitation 466/40, Emission 515/30)
TRITC filter cube (Excitation 542/20, Emission 620/52)
Cy5 filter cube (Excitation 618/50, Emission 698/70)
2.18 µm/pixel minimum capture resolution

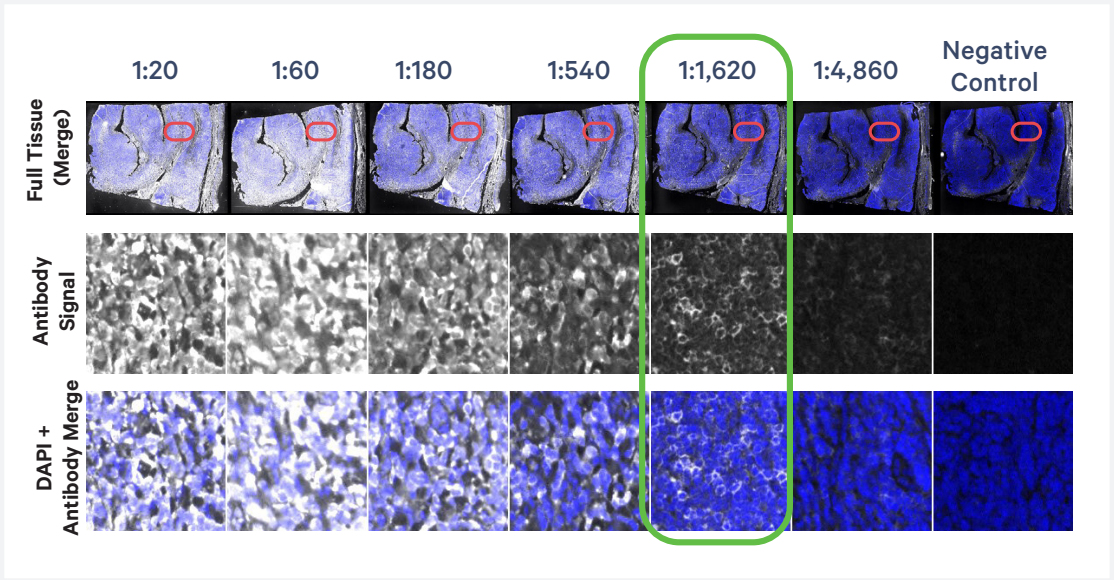
3.2 Imaging

a. Image all tissue sections individually at 10X magnification using fluorescence imaging settings. Save each image. Consult the Visium CytAssist Imaging Guidelines Technical Note (CG000521) for additional information.

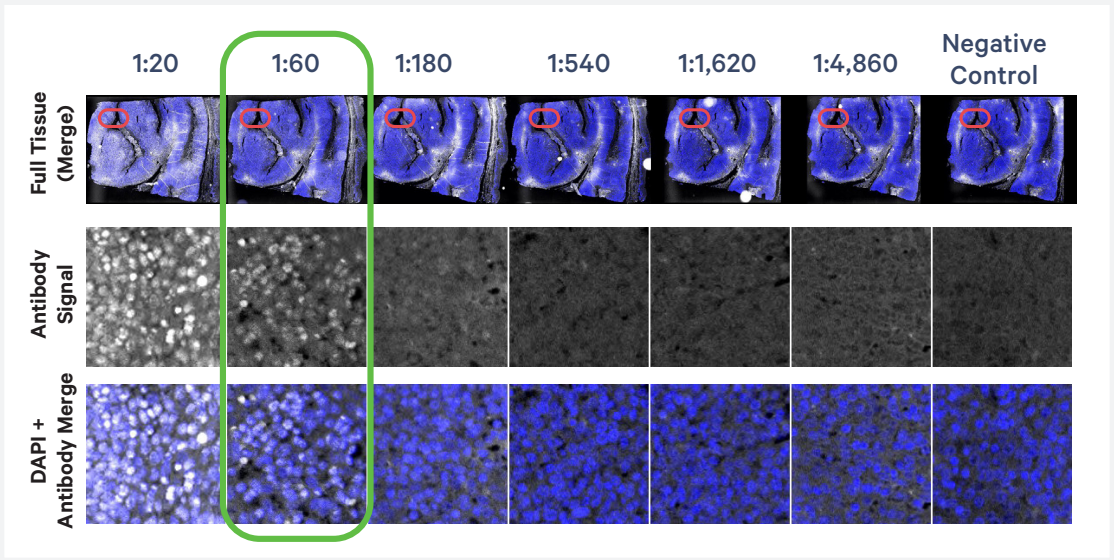
The image exposure can be set such that the highest concentration of antibody (1:20 in the provided example) is not oversaturated when imaged. After determining exposure settings, use the same settings for all tissue sections.

b. Zoom into a section of the tissue and compare the antibody signal within the same area of interest across dilution images. Examine the DAPI + antibody signal merged image to determine if antibody signal is appearing in expected regions of tissue. Tools such as ImageJ may be used to assist in visualization.

c. After imaging, determine the antibody dilution that results in images with the highest signal to noise ratio. Proceed with downstream parts of this workflow only if IF staining reveals expected spatiality of the antibody. If antibody signal for all dilutions appears similar to the negative control, or if the optimal dilution is very high (1:20 in the example below), select a different clone. If antibody signal appears similar across dilutions, but different from the negative control, additional dilutions may be required.



In the example above, 1:1,620 was selected as the optimal dilution, as indicated in green. This dilution resulted in an image with the best signal to noise ratio and expected distribution of the antibody (cytoplasm). Images in the second and third rows are enlarged images from the regions indicated in red.



In the example above, 1:60 was selected as the optimal dilution, as indicated in green. This dilution resulted in an image with the best signal to noise ratio and expected distribution of the antibody (cytoplasm). Images in the second and third rows are enlarged images from the regions indicated in red.

d. Proceed **immediately** to Coverslip Removal or store slides flat in a box or slide book, in the dark, for 16-20 h at 4°C. Only the slide with the area of interest will require coverslip removal.

4. Tissue Scraping and Oligo Elution

4.0 Overview

This chapter provides guidance on coverslip removal, removing stained tissue from tissue slides and eluting antibody oligos.

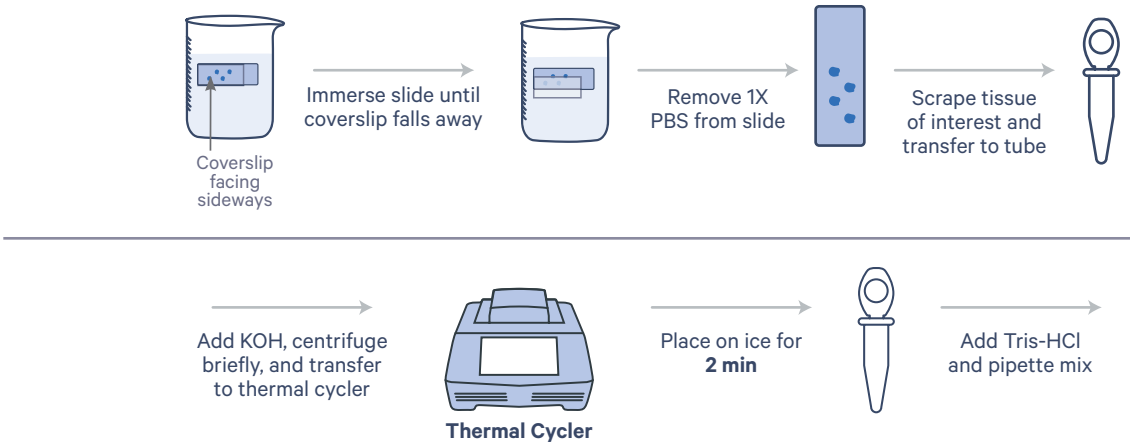
4.1 Preparation - Buffers

For Oligo Elution	
Items	**Preparation & Handling**
☐ KOH Mix	Prepare 0.08 M KOH Mix according to the table shortly before use, adding reagents in the order listed. Vortex and centrifuge briefly. Maintain at **room temperature**. 2X reagent preparation instructions are provided to avoid pipetting small volumes.

KOH Mix	Stock	Final	1X (µl)	2X+ 10% (µl)
Nuclease-free Water	-	-	49.5	108.9
KOH	8M	0.08M	0.5	1.1
Total			**50.0**	**110.0**

Protocol Overview

Coverslip Removal, Tissue Scraping, and Oligo Elution ~40 min



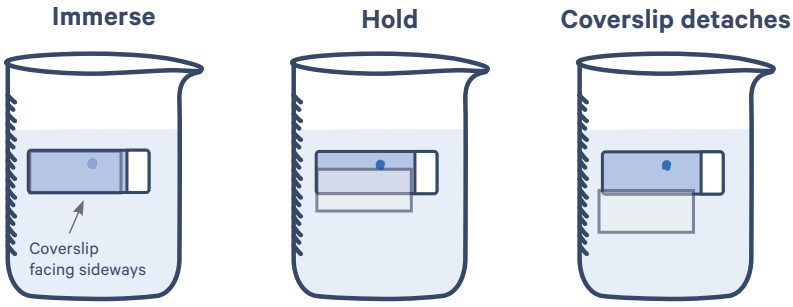
4.2 Coverslip Removal and Tissue Scraping

a. Prepare a 0.2-ml tube strip on ice.

b. Immerse the slide sideways/horizontal in a beaker containing 1X PBS with the coverslipped surface fully sideways to prevent the coverslip from dragging across the tissue.

c. Hold the slide in 1X PBS until the coverslip slowly separates away from the slide.

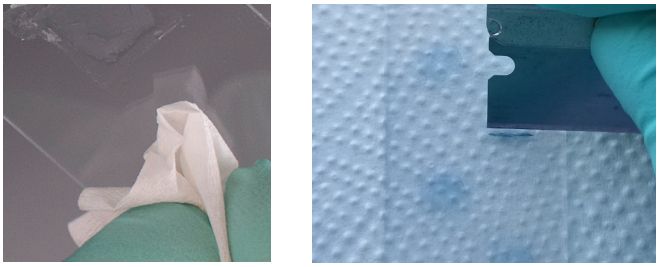
To avoid damaging the tissue sections or causing tissue detachment, DO NOT move the slide up and down, shake forcibly or manually move the coverslip.



d. Gently immerse slide 10x in the 1X PBS to ensure all mounting medium is removed.

e. Remove excess liquid by gently flicking the slide, then removing any remaining liquid with a laboratory wipe. Do not touch tissue.

Tissue Scraping



Wipe excess liquid. Scrape tissue.

f. While the tissue section is wet, identify the tissue section that resulted in the IF image with the highest signal-to-noise ratio. Place the slide flat and carefully scrape entire stained portion of the desired tissue section with a clean blade in a single motion.

g. Transfer tissue section to the bottom of one pre-chilled 0.2-ml tube in a tube strip with a clean forcep, avoiding the walls of the tube. DO NOT vortex or otherwise disturb the tube until Oligo Elution.

4.3 Oligo Elution

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

Lid Temperature	Reaction Volume	Run Time
95°C	100 µl	~30 min
Step	**Temperature**	**Time**
Pre-equilibrate	95°C	Hold
Oligo Elution	95°C	30 min
Cool Down	4°C	2 min

b. Add **50 µl** 0.08 M KOH Mix to the sample. Tissue section should be fully immersed in KOH mix. Centrifuge briefly.

c. Transfer tube strip to pre-heated thermal cycler. Close the thermal cycler lid.

d. Skip Pre-equilibrate step to initiate Oligo Elution.

e. After Cool Down is complete, immediately place tube strip on ice for **2 min.**

f. Add **3 µl** 1 M Tris-HCl pH 8.0 to the sample.

g. Pipette mix 30x with pipette set to 35 µl.

5. Pre-Amplification and qPCR

5.0 Overview

This chapter provides guidance on pre-amplification of antibody oligos and qPCR. The Cq values determined via qPCR are used to select the ideal add-on antibody concentration for use in the full assay.

The Pre-Amplification mix requires purchasing the following primers (choose standard desalting purification). Prepare a 100 µM stock and store at -20°C. Prepare final primer mix as described in the table below.

Forward Primer (5'-3'): GTGACTGGAGTTCAGACGTG
Reverse Primer (5'-3'): GCTAGGACCGGCCTTAAAGC

5.1 Preparation - Reagents and Buffers

For Pre-Amplification

Items	Preparation & Handling
☐ Amp Mix *(from Qiagen)*	Vortex, centrifuge briefly.
☐ 40 µM Primer Mix	Prepare 40 µM Primer Mix according to the table below. Thaw forward and reverse primer stock solutions at room temperature, vortex, and centrifuge briefly before using. Maintain 40 µM Primer Mix at room temperature.

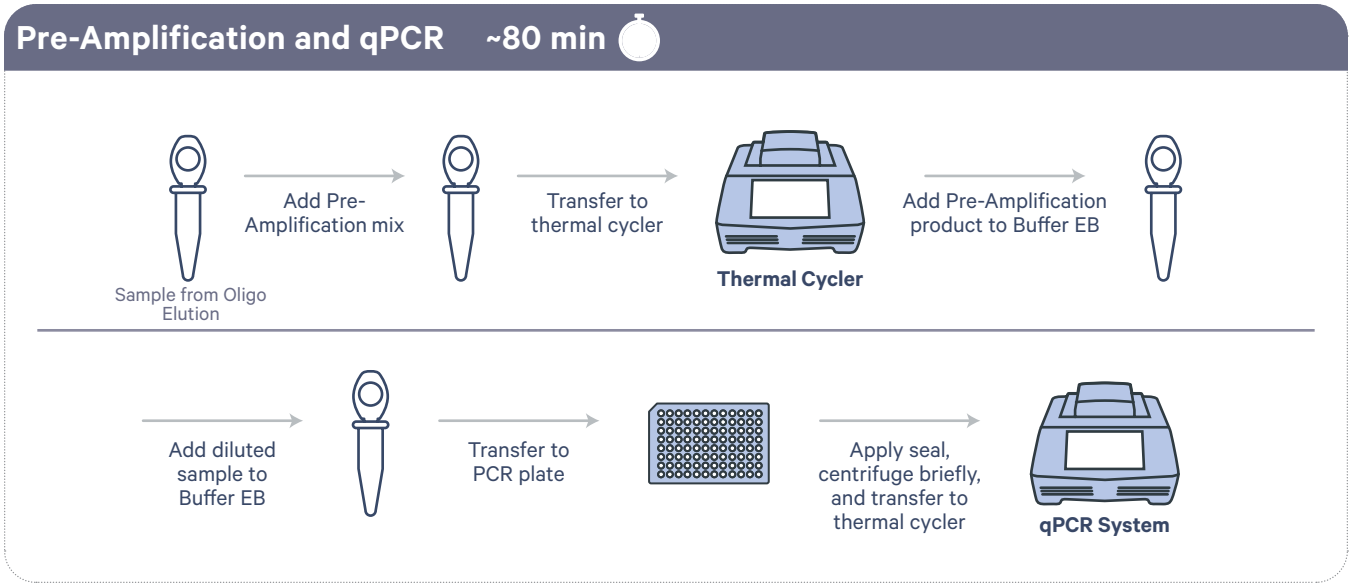
40 uM Primer Mix	Stock	Final	1X (µl)
Forward Primer	100 µM	40 µM	80
Reverse Primer	100 µM	40 µM	80
Nuclease-free water	-	-	40
Total			**200**

☐ Pre-Amplification Mix

Prepare Pre-Amplification Mix on ice according to the table below. All components are contained within the Qiagen AllTaq Core Kit. Add reagents in the order listed. Pipette mix and centrifuge briefly. Maintain on **ice**.

Pre-Amplification Mix	1X + 10% (µl)
AllTaq PCR Buffer	22.0
Nuclease-free Water	11.5
dNTP Mix	2.2
MgCl2	8.8
40 µM Primer Mix	2.8
AllTaq DNA Polymerase	4.4
Total	**51.7**

Protocol Overview



5.2 Pre-Amplification

a. Add **47 µl** Pre-Amplification mix to tube from Oligo Elution. Pipette mix and centrifuge briefly.

b. Incubate in a thermal cycler with the appropriate protocol below.

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	30 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	95°C	00:02:00
2	98°C	00:00:15
3	55°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

5.3 qPCR

a. Prepare qPCR Mix on ice according to the table below. Add reagents in the order listed. Centrifuge briefly. Maintain on ice.

qPCR Mix	Stock	Final	1X (µl)	4X+ 10%* (µl)
Nuclease-free Water	-	-	3.9	17.2
KAPA SYBR FAST qPCR Master Mix	2X	1X	5.0	22.0
40 µM Primer Mix	40 µM	0.4 µM	0.1	0.4
Total			**9.0**	**39.6**

b. Vortex samples from Pre-Amplification for **5 sec**, then centrifuge for **30 sec**.

c. Add **2 µl** Pre-Amplification product to **8 µl** Buffer EB. Pipette mix thoroughly.

d. Add **2 µl** diluted sample from 5.3c to **198 µl** Buffer EB. Pipette mix thoroughly.

e. Add **9 µl** qPCR Mix to one well per sample in a qPCR plate, including a well for a negative control. Sample may be run in triplicate.

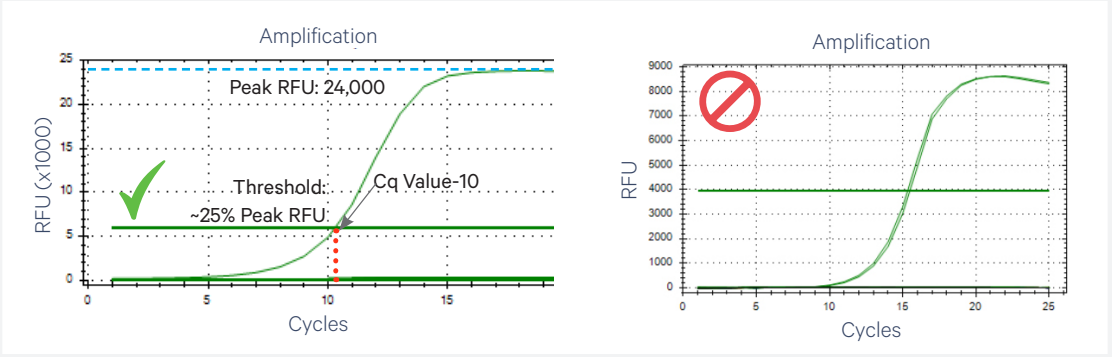
f. Transfer **1 µl** of diluted sample from 5.3d to each qPCR plate well containing qPCR Mix. If using a negative control, add **1 µl** nuclease-free water to the corresponding well.

g. Apply seal, and centrifuge briefly. Record which wells are for sample or for the negative control.

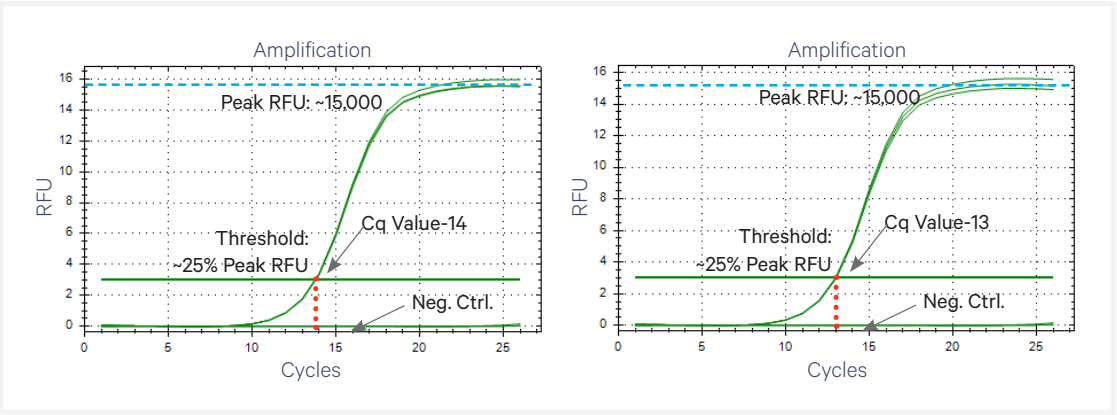
h. Prepare a qPCR system with the following protocol, place the plate in the thermal cycler, and start the program.

Lid Temperature	Reaction Volume	Run Time
105°C	10 µl	30 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:03:00
2	98°C	00:00:15
3	63°C	00:00:30
	Read Signal	
4	Go to step 2, repeat 24X for a total of 25 cycles	

i. 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.



Additional representative examples are shown below. Select the average peak among the three traces.



5.4 Antibody Concentration Selection

Use the Cq value determined in the previous step and the dilution value to calculate the antibody dilution to use in the main assay. Use the following formula:

Antibody Dilution for Main Assay = IF Dilution * $2^{(18-\text{Cq Value})}$

For Cq values above 18, use 18 as the value for the calculation.

Below are example calculations for a sample with an ideal IF dilution of 1:180 and a Cq value of 11.

Antibody Dilution for Main Assay = IF Dilution * $2^{(18-\text{Cq Value})}$

Antibody Dilution for Main Assay = 180 * $2^{(18-11)}$

Antibody Dilution for Main Assay = 180 * $2^{(7)}$

Antibody Dilution for Main Assay = 180 * 128

Antibody Dilution for Main Assay = 23,040

The antibody should be diluted 1:23,040 during the Protein Labeling step of the Visium Spatial Gene and Protein Expression User Guide (CG000494).

To avoid pipetting extreme volumes, split this dilution into two parts. First, make a 200X stock solution.

$C_1V1 = C_2V_2$
(23,040)(1) = (200)(x)
23,040 = 200x
x = 115.2

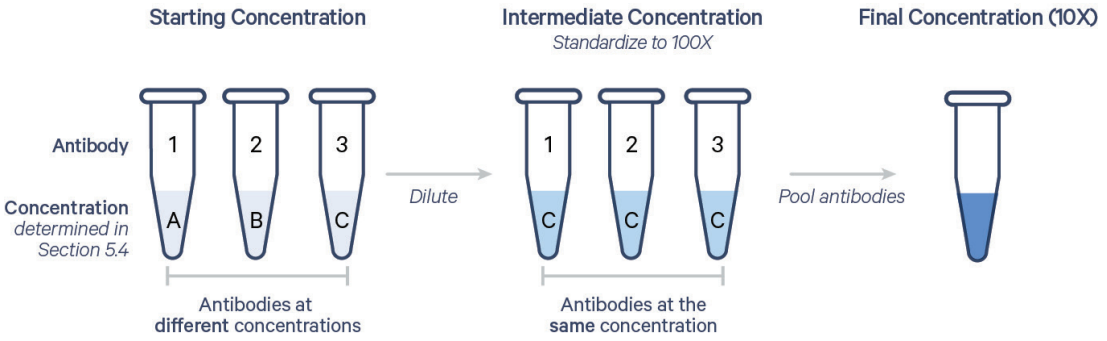
Add 1 µl of stock antibody to 114.2 µl of Block and Stain buffer to generate 115.2 µl of 200X stock solution.

Add 1.1 µl of this 200X solution to the antibody staining mix in the Protein Labeling step of the Visium Spatial Gene and Protein Expression User Guide (CG000494). This additional dilution will bring the add-on antibody to the appropriate dilution of 1:23,040.

5.5 Antibody Pooling

Optimize each antibody separately before pooling. Prepare antibody pool right before use. If an antibody has an optimal dilution of 1:100 or lower (i.e. 1:95), add the antibody directly during the Antibody Incubation step of the CytAssist Spatial Gene and Protein Expression Reagent Kits User Guide (CG000494) instead of pooling.

Prepare a 10X antibody pool that will be diluted 1:10 during the main assay. The maximum volume of antibody pool that can be added during the main assay is 147 µl. To avoid dilutions that require pipetting small volumes, dilute antibodies in two parts.



1. Bring all antibodies to the same intermediate concentration (100X), so that the **same volume** from each antibody stock can be added to the pool.

2. Pool each antibody (at 100X) in PBS such that each antibody is diluted again 1:10. Because all antibodies are now at 100X, the same volume from each antibody can be added to the pool. This creates a pool where each antibody is at 10X concentration.

This antibody pool will be combined with the 10x Genomics pre-designed panel during the Antibody Incubation step of the CytAssist Spatial Gene and Protein Expression Reagent Kits User Guide (CG000494). This step further dilutes the pool 1:10, resulting in antibodies at the appropriate 1X working concentration.

An example antibody pooling calculation is provided on the next page. Additionally, an excel sheet to assist with calculations is available on the 10x Genomics support site as the Visium CytAssist Spatial Gene and Protein Expression Add-on Antibody Pooling Calculator (Document CG000693).

5.5 Antibody Pooling

An example of pooling three antibodies is shown below.

1. Bring each antibody that will be pooled to 100X.

Add-on Antibody	Antibody 1	Antibody 2	Antibody 3
Optimal Dilution Determined in Step 5.4	1:10,000	1:1,000	1:100
Dilution required to Bring Antibody Stock to 100X	1:100	1:10	None

2. Dilute each antibody stock according to the intermediary dilution noted above.

Intermediate Dilution	Antibody 1	Antibody 2	Antibody 3
Antibody Stock (µl)	1.0	1.0	No Dilution Needed
1X PBS (µl)	99.0	9.0	
Total	**100.0**	**10.0**	**-**

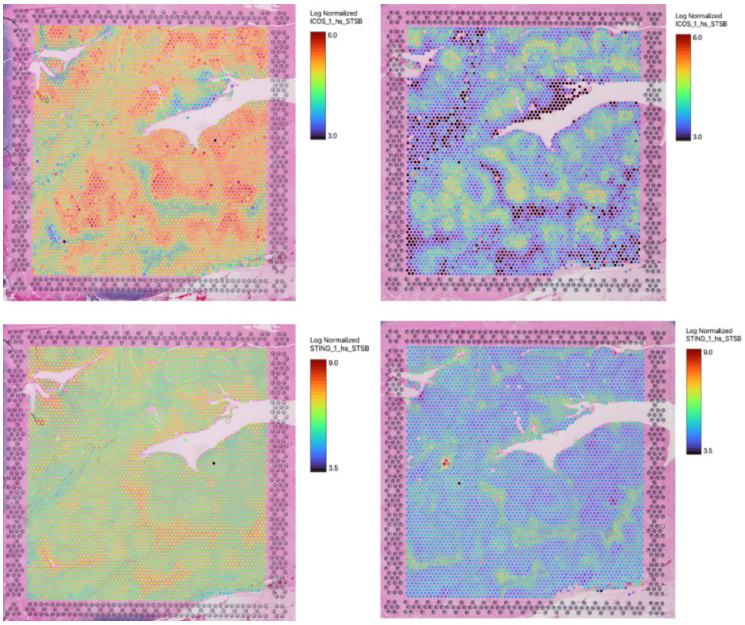
3. Pool antibodies with PBS such that each antibody is diluted 1:10. This results in a pool that has each antibody at 10X concentration. The scheme below may be modified to accommodate additional antibodies. 21 µl of this pool will be carried forward to the main assay.

10X Antibody Pool	Volume from Intermediate Dilution (µl)
Antibody 1	2.5
Antibody 2	2.5
Antibody 3	2.5
PBS	17.5
Total	**25**

5.6 Data Analysis

For information regarding data analysis, refer to the 10x Genomics software support website.

Troubleshooting

	Notes
High UMI Distribution Throughout Tissue	High UMI distribution across the tissue section may indicate that the chosen antibody concentration is too high. A lower concentration of add-on antibody may result in UMI distributions that more closely match the expected spatial distribution of the detected antigen. A high add-on antibody concentration will not affect assay performance, but may be result in high background. **Antibody Concentration Too High** **Optimal Antibody Concentration** 

Appendix

A1: Trimming/Scoring the Block

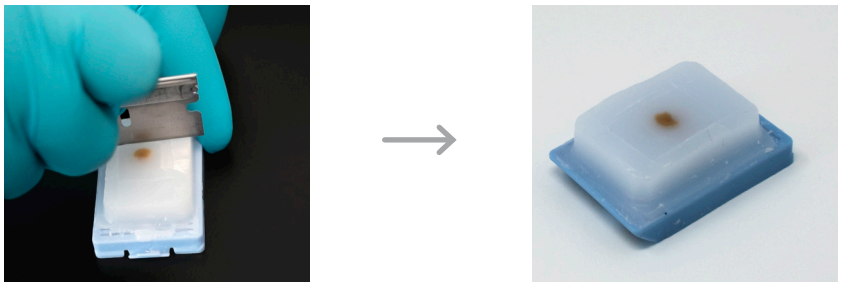
Before starting, wipe down all the surfaces and work areas with RNaseZap RNase decontaminating solution. A tissue section of ≤6.5 x 6.5 mm is compatible with the Visium Cassette.

FFPE Tissue Block >6.5 x 6.5 mm & Tissue ≤6.5 x 6.5 mm

For tissue samples that fit within the Visium Cassette wells and are embedded in a large tissue block, the excess paraffin around the tissue can be trimmed to remove the excess and obtain smaller sections.

- After facing, remove the tissue block from the microtome and start trimming.
- Using a razor blade, remove excess paraffin from around the tissue, so it is not larger than the Fiducial Frame.
- Retain paraffin edges around the tissue samples to enhance tissue attachment on the slide.

Trimming large tissue block containing tissues that fit the Visium Cassette

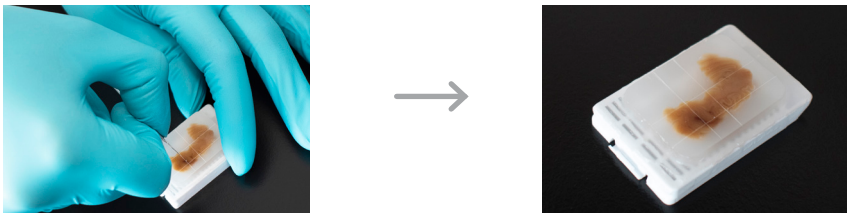


FFPE Tissue Block & Tissue >6.5 x 6.5 mm

Tissue samples larger than wells in the Visium Cassette can be scored using a razor blade to generate smaller sections. See Appendix A2 for alternative Triming/Scoring of FFPE Tissue Block & Tissue >6.5 X 6.5 mm.

- Remove the tissue block from the microtome and start scoring.
- To score, glide a razor blade over the surface of the tissue to introduce a shallow cut. A deep incision may lead to tissue damage and disintegration.

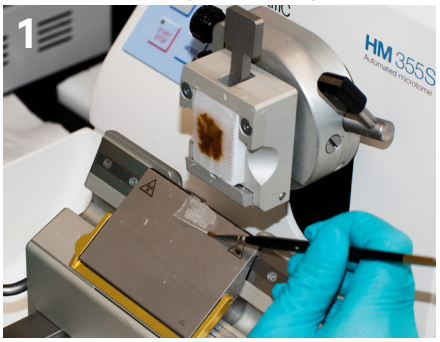
Scoring tissues larger than wells in the Visium Cassette



A2: Alternative Trimming/Scoring of FFPE Block & Tissue >6.5 X 6.5 mm

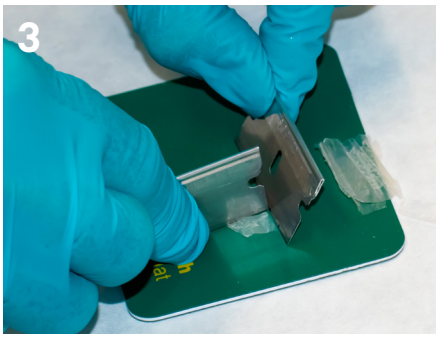
Keep all surface and materials RNase free. Avoid tearing of the FFPE sections during cutting. Keep the 5 µm FFPE sections from curling.

Set the microtome to 5 µm for tissue block and begin sectioning

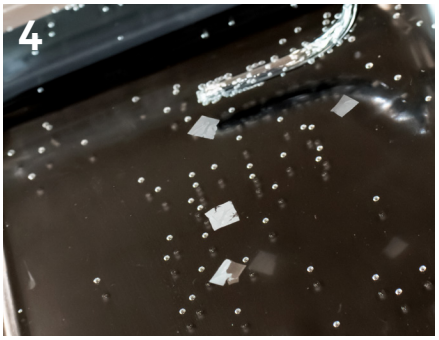


Collect sections with a paintbrush and place onto a cutting mat

Cut tissue sections into 6.5 mm sections to fit in the Capture Areas on the Visium Spatial slide



Place sections in the water bath



Align section edge with desired location on the slide and pull upward

Slides in images are representative.

Document Revision Summary

Document Number	CG000664
Title	Visium CytAssist Spatial Gene and Protein Expression for FFPE – Add-on Antibody Optimization
Revision	Rev A
Revision Date	August 2023

Specific Changes:

N/A

General Changes:

N/A

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