Visium CytAssist Spatial Gene Expression for FFPE – Deparaffinization, Decrosslinking, Immunofluorescence Staining & Imaging

Introduction

The Visium CytAssist Spatial Gene Expression for FFPE assay is designed to analyze mRNA in tissue sections derived from formalin fixed & paraffin embedded (FFPE) tissue samples. The Visium CytAssist instrument requires a glass slide with intact tissue sections as input. This protocol outlines deparaffinization, decrosslinking, immunofluorescence (IF) staining, and imaging of tissue for use with 10x Genomics Visium CytAssist Spatial Gene Expression for FFPE assay. Deparaffinized, decrosslinked, and stained tissue sections are inputs for the downstream Visium Spatial Gene Expression for FFPE workflow.

Additional Guidance

Consult the Visium CytAssist Spatial Gene Expression for FFPE - Tissue Preparation Guide (Document CG000518) for complete information on sectioning FFPE tissue blocks, placing sections on CytAssist compatible slides, and working with archived tissue slides. Consult the Visium CytAssist Spatial Applications Imaging Guidelines Technical Note (Document CG000521) to verify imaging settings prior to starting this Demonstrated Protocol. After completing this Demonstrated Protocol (CG000519), proceed with the Visium CytAssist Spatial Gene Expression User Guide (CG000495).

Contents

2 Reagent Kits

5 Recommended Thermal Cyclers

6 Workflow Overview

7 Specific Reagents & Consumables

9 Tips & Best Practices

25 1. Deparaffinization, Decrosslinking & IF Staining

26 1.1 Preparation - Buffers

29 Protocol Overview

33 1.2 Deparaffinization

35 1.3 Decrosslinking

36 1.4 Immunofluorescence Staining

43 1.5 Coverslipping

44 2. Tissue Imaging

44 2.1 Imaging System Recommendations

45 2.2 Imaging

45 2.3 Coverslip Removal

46 Troubleshooting

Reagent Kits

Visium CytAssist Spatial Gene Expression for FFPE Reagent Kits

Refer to SDS for handling and disposal information

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, 20 pack	1	2000284
Visium CytAssist Spatial Gene Expression Slide v2, 6.5 mm	1	2000549

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, 20 pack	1	2000284
Visium CytAssist Spatial Gene Expression Slide v2, 11 mm	1	2000701

Reagent Kits

Visium CytAssist Spatial Gene Expression for FFPE Reagent Kits

Refer to SDS for handling and disposal information

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

Enough reagent is provided for processing two 6.5 mm slides or one 11 mm slide. Information on how much reagent is used for antibody optimization is provided on page 22. Enough reagent is provided for at least 13 antibody optimizations if using 6.5 mm slides and 6 optimizations if using 11 mm slides.

RNase Inhibitor 40X PN-1000887

RNase Inhibitor 40X
PN-1000887
Store at -20°C

	#	PN
RNase Inhibitor 40X	1	2001488

Reagent Kits

Visium CytAssist Spatial Gene Expression for FFPE Reagent Kits

- *Refer to SDS for handling and disposal information.*
- *Visium and Tissue Slide Cassettes are single use.*
- *Additional Visium Slide Cassettes (PN-1000469) should be purchased for antibody optimization.*
- *Additional Tissue Slide Cassettes (PN-1000471, PN-1000472) must be purchased for each processed section.*

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

10x GENOMICS

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

Visium CytAssist Tissue Slide Cassette, 6.5 mm 4 pk
PN-1000471
Store at ambient temperature

	#	PN
Visium CytAssist moveable gasket small (preassembled with translator)	4	3000814
Visium CytAssist moveable translator (preassembled with gasket)	4	3000816
Visium CytAssist moveable Cassette, frame	4	3000813

10x GENOMICS

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

Visium CytAssist Tissue Slide Cassette, 11 mm 4 pk
PN-1000472
Store at ambient temperature

	#	PN
Visium CytAssist moveable gasket large	4	3000815
Visium CytAssist moveable Cassette, frame	4	3000813

10x GENOMICS

10x Genomics Accessories

Product	#	Part Number (Kit)	Part Number (Item)
Low Profile Plate Insert	2	1000499 (Visium CytAssist Reagent Accessory Kit)	3000823
10x Magnetic Separator	1		230003 or 2000431

Recommended Thermal Cyclers

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

*For single cassette processing, place cassette on 10x Low Profile Thermocycler Adapater in the position farthest from the lid hinge to prevent lid locking 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 Veriti Systems (PN-4381850), with top piece removed.



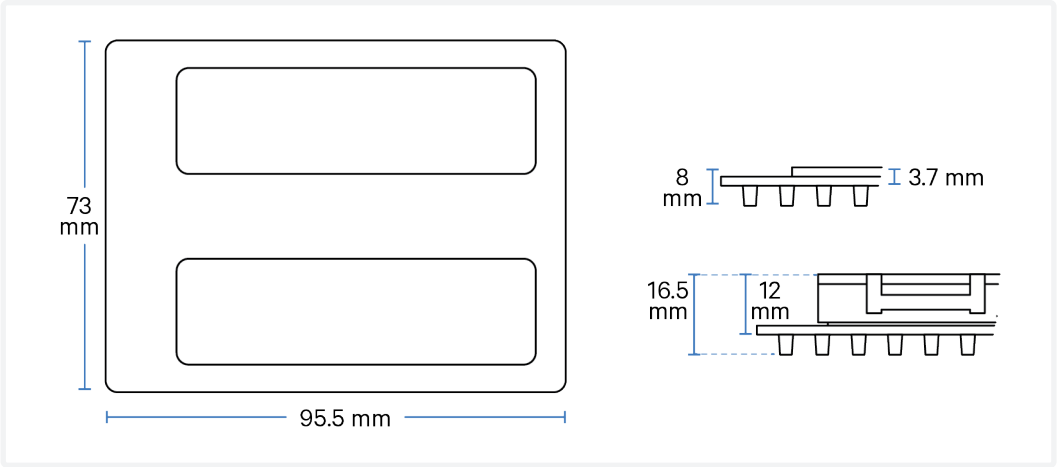
The following thermal cyclers should have their ramp rates adjusted for all steps:

- 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

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 prior to attempting removal. Do not handle the Low Profile Thermocycler Adapter while it is hot.



Workflow Overview

1 Experimental Planning

Before starting, refer to these documents.

Tissue Preparation Guide
Section tissue onto slides; process archived slides; QC tissue sections.
Demonstrated Protocol CG000518

Imaging Guidelines
Optimize imaging settings.
Technical Note CG000521

2 Tissue & Sample Preparation

Detailed guidance for sample preparation.

Deparaffinization + Decrosslinking + IF Staining & Imaging
Deparaffinize, decrosslink, IF stain and image tissue
Demonstrated Protocol CG000519

3 Assay Workflow

Processed prepared tissue slides with the Visium CytAssist instrument.

Library Construction
Construct Visium CytAssist Spatial Gene Expression - Probe-based libraries.
User Guide CG000495

Visit the 10x Genomics Support website for the most updated 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
Xylene	Xylene, Reagent Grade	Millipore Sigma	214736
	Xylene, Histological Grade	Millipore Sigma	534056
Glycerol	Glycerol	Acros Organics	327255000
	Glycerol Solution	Millipore Sigma	49781
Ethanol	Ethyl Alcohol, 200 Proof	Millipore Sigma	E7023
	Ethanol absolute ≥99.5%, TechniSolv, pure (Europe Only)	VWR	83813.360DP
PBS	PBS - Phosphate Buffered Saline (10X) pH 7.4, RNase-free	Thermo Fisher Scientific	AM9624
SSC	SSC Buffer 20x Concentrate	Millipore Sigma	S66391L
10% Tween-20	Tween 20 Surfact-Amps Detergent Solution (10% solution)	Thermo Fisher Scientific	28320
Nuclease-free water	Nuclease-free Water (not DEPC-Treated)	Thermo Fisher Scientific	AM9937
DAPI	DAPI Solution (1 mg/ml)	Thermo Fisher Scientific	62248
Antibodies	-	-	-
RNase inhibitor	Protector RNase Inhibitor *(Alternative to 10x Genomics product)*	Millipore Sigma	3335399001
Mounting medium	SlowFade Diamond Antifade Mountant	Thermo Fisher Scientific	S36967
	SlowFade Gold Antifade Mountant	Thermo Fisher Scientific	S36966
Section dryer oven	Epredia High Capacity Section Dryer	Fisher Scientific	A84600051
Slide holders	Slide Holders, 24-place	VWR	25608-868
Coverslips	Fisherbrand Cover Glasses: Rectangles	Fisher Scientific	12-544-EP
	Cover Glasses, Rectangular	VWR	16004-322
Staining jar/ dishes	Coplin Jar	VWR	100500-232
	Staining Dishes	VWR	25608-906
Green Marker, *Optional, if annotating slide*	Sharpie Argyle Green Permanent Marker *Or any equivalent hue*	Sharpie	1785396
Autofluorescence Quencher *Optional*	TrueBlack Lipofuscin Autofluorescence Quencher	Biotium	23007

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.**

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
Wide Bore Pipette Tips	Tips RT LTS 200UL FLW	Rainin	30389241
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
Additional Materials			
Beakers		-	-
Ultrapure/Milli-Q Water, *from Milli-Q Integral Ultrapure Water System or equivalent*		-	-

Tips & Best Practices



Tips & Best Practices section includes additional guidance



Signifies critical step requiring accurate execution



Troubleshooting section includes additional guidance

General Reagent Handling

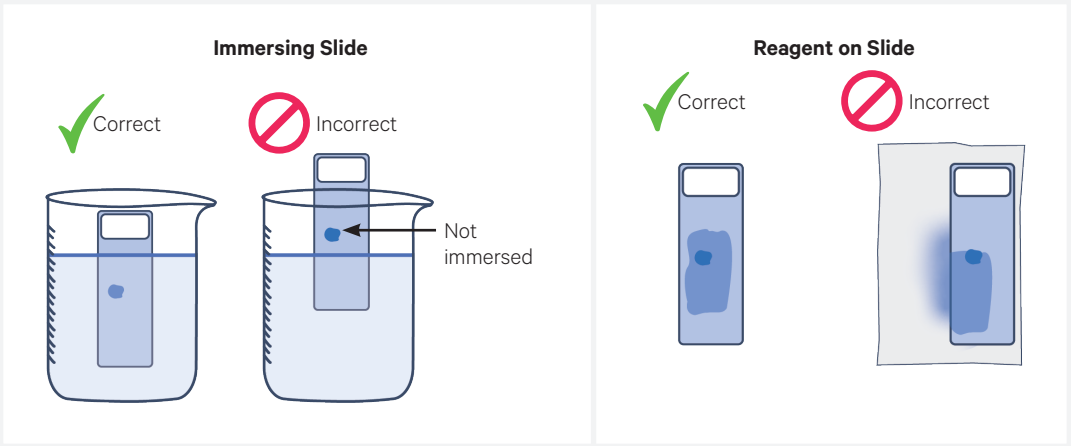
- Thoroughly mix reagents before use.

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 and water, ensure that the tissue sections are completely submerged. DO NOT submerge slide label.
- Keep the slide flat on the bench when adding reagents to the active surface.
- Ensure that no absorbent surface is in contact with the reagents on the slide during incubation.
- DO NOT use a hydrophobic barrier (such as one applied by a PAP pen) on slides, as these barriers can potentially affect assay performance.
- Consider tracing the Tissue Slide Cassette gasket onto the back of the tissue slide to assist in alignment on the CytAssist instrument.
    - Markings may cause automatic tissue registration to fail, resulting in a need for manual registration.
    - If leaving a mark is desired, a green colored marker (Sharpie, PN-1785396) has the least impact on the automatic tissue registration process.

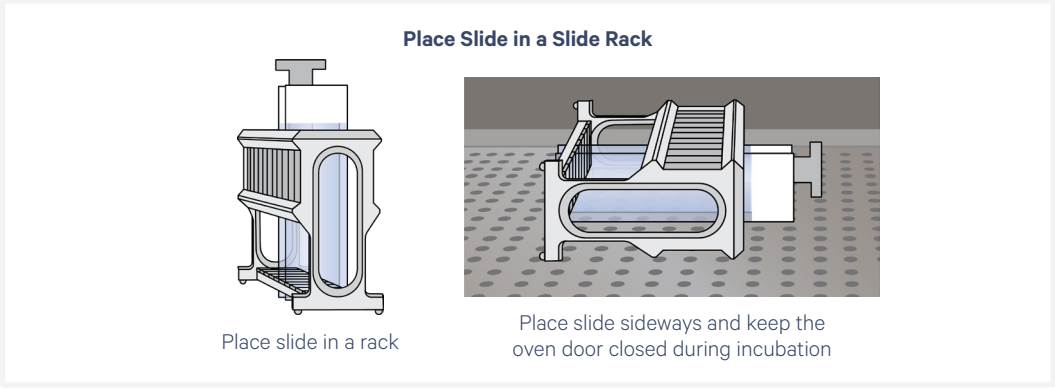


Slide Incubation Guidance

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.
- Close door when incubating the slide in the oven.

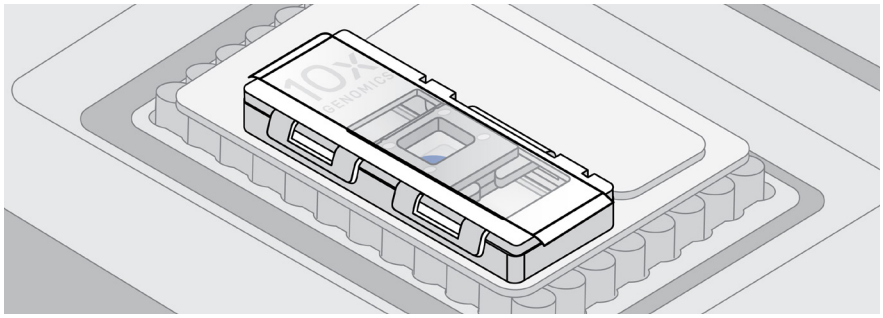


Incubation using a Thermal Cycler:

- 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 without overtightening.
- 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 Cassette in Thermal Cycler



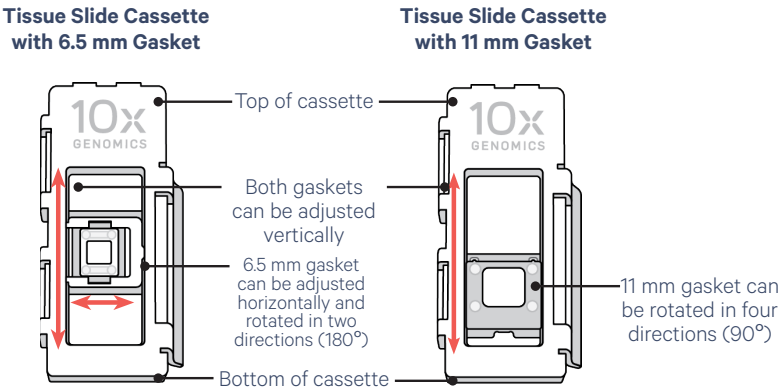
Slide Incubation Guidance

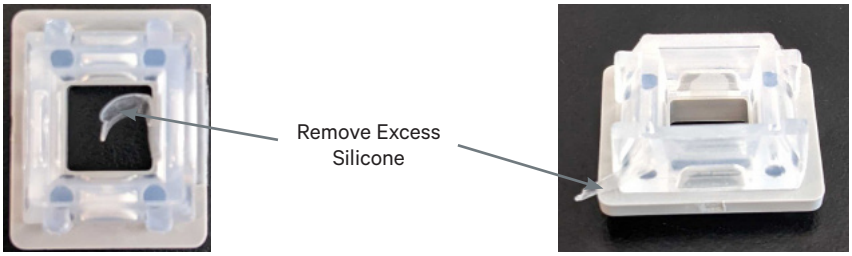
Incubation at room temperature

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

Visium CytAssist Tissue Slide Cassette

- The Visium CytAssist Tissue Slide Cassette encases the slide and creates leak-proof wells for adding reagents on blank slides with tissue.
- The cassette is a single-use item.
- Gaskets are adjustable to ensure that the tissue section or area of interest is encased in a well.
- See Visium CytAssist Tissue Slide Cassette Assembly & Removal instructions for details.
- Before use, inspect the moveable gasket to ensure that the gasket perimeter and corners are free of excess silicone before assembly.
- Excess silicone should be safely removed with forceps or a pipette tip before assembly.
- Assembly should occur against a white background for easy tissue visualization during alignment.
- Practice assembly with a blank slide (75 x 25 x 1 mm).
- Place the slides in the cassette only when specified.





Visium CytAssist Tissue Slide Cassette Assembly

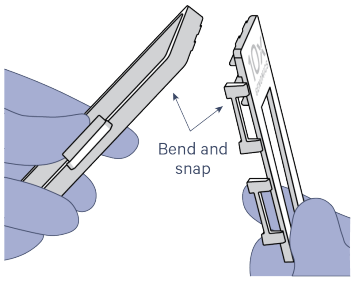
Wear fresh gloves while assembling Tissue Slide Cassette

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

Exercise caution when handling slide edges to prevent injury.

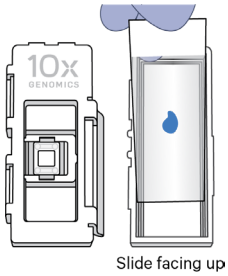
If applicable, break cassette into two halves by bending each half at the hinge until they snap apart

1



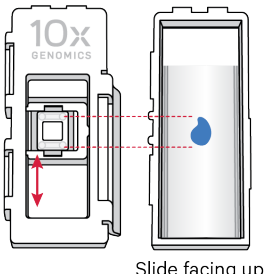
Place tissue slide into lower half of cassette with tissue facing up

2



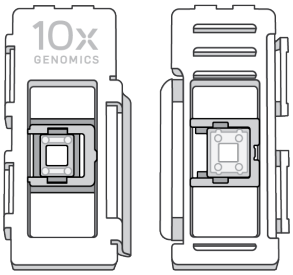
The 6.5 mm gasket can be adjusted horizontally and rotated in two directions (180°) while 11 mm gasket can be rotated in four directions (90°). Determine the appropriate configuration that allows the gasket to encompass the tissue area of interest.

3



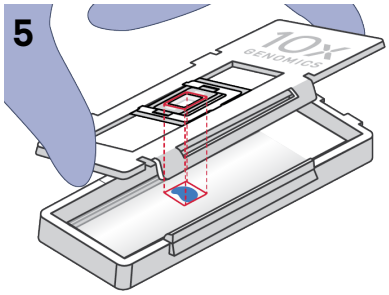
Securely combine gasket with top half of cassette until the gasket snaps into place.

4



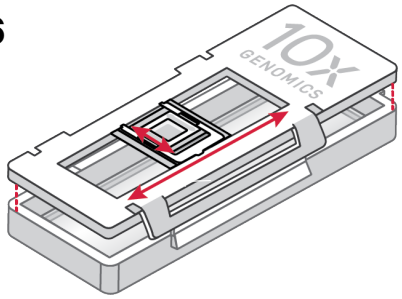
Gently place top half of cassette over bottom half. DO NOT assemble together until Step 7.

5



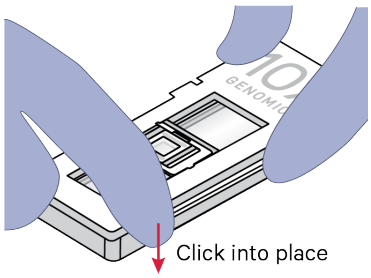
Adjust gasket such that gasket is over the tissue area of interest. The 6.5 mm gasket can be adjusted horizontally as well as vertically.

6



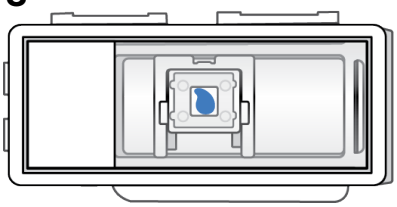
Apply even pressure on top of cassette until it clicks shut. Verify that clip is secured over hinges.

7



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

8

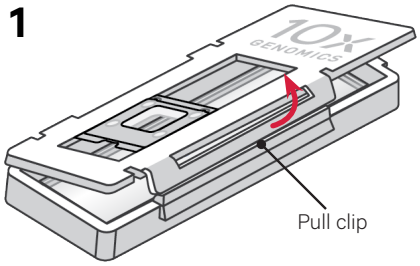


Visium CytAssist Tissue Slide Cassette Removal

Removal instructions are the same for both 6.5 and 11 mm cassettes.

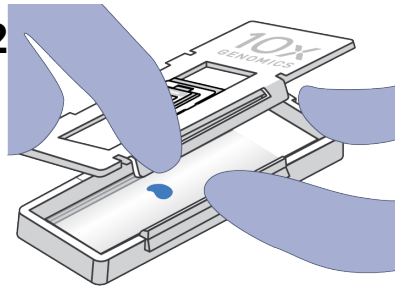
Pull clip up to detach upper and lower halves of cassette

1



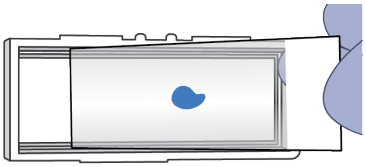
Open cassette by continuing to lift clip upward. If slide sticks to gasket, continue to apply even upward pressure to separate slide from gasket

2



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

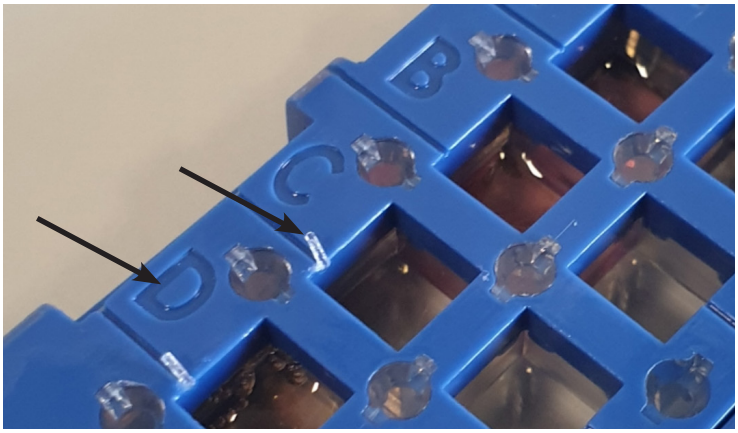
3



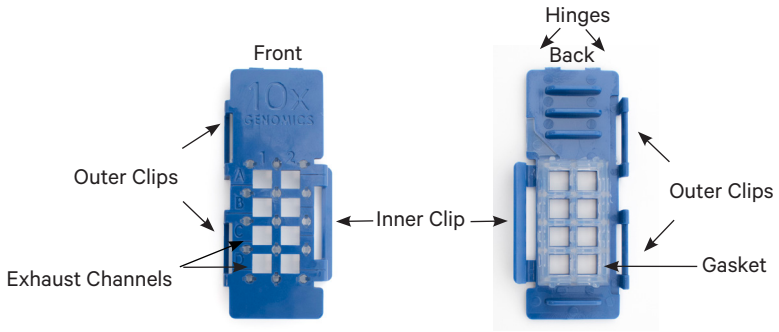
Visium Cassette

- 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 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



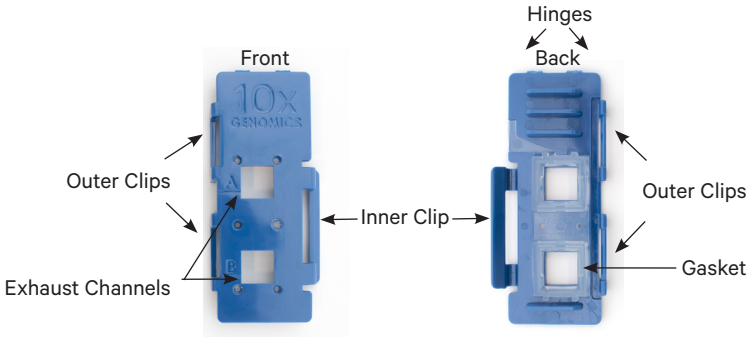
Upper **Visium Slide Cassette, 6.5 mm**



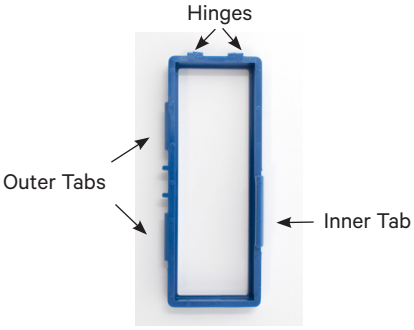
Lower



Upper **Visium Slide Cassette, 11 mm**



Lower

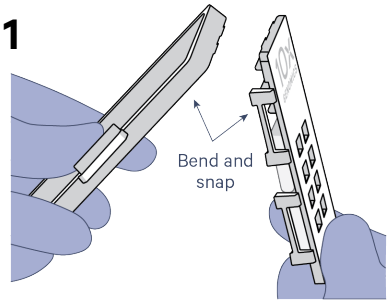


Visium Cassette Assembly

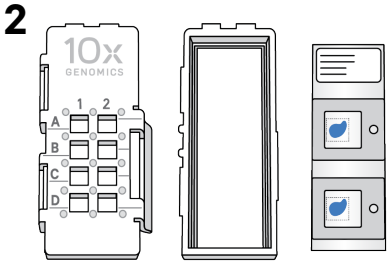
- Ensure that the surface of the cassette is dry.
- Cassette may also be assembled in the hand for comfort.

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

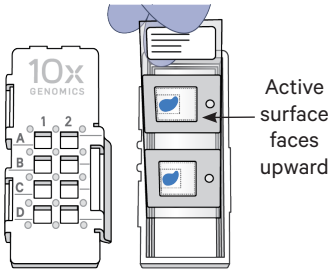
Break cassette into two halves by bending each half at the hinge until they snap apart



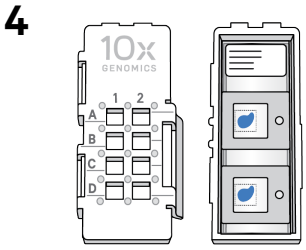
Place upper and lower halves of cassette, and Visium slide, directed upward on bench



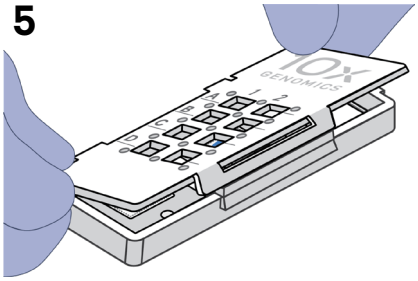
Place Visium slide with active surface facing upwards into lower half of cassette; ensure label is toward hinges



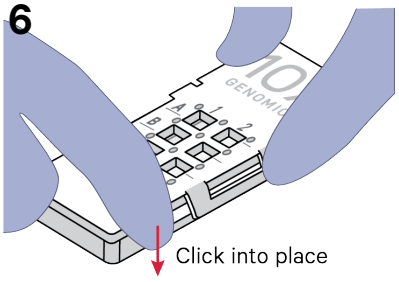
Press slide down into grooves of the bottom half of the cassette until it sits firmly in place



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



Press firmly on top of cassette until it clicks shut



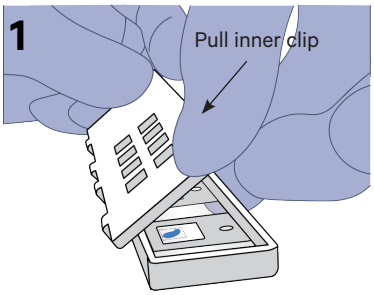
Exercise caution when handling slide edges to prevent injury.

Slides in images are representative.

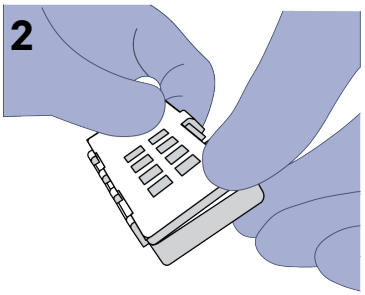
Visium Cassette Removal

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

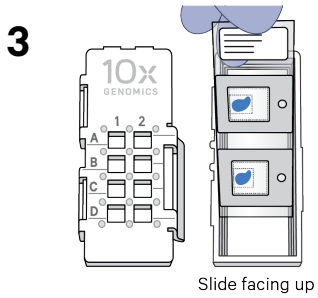
Pull inner clip up from inner tab to detach upper and lower halves of cassette



Open cassette by continuing to lift inner clip upward



Lift slide out from lower half



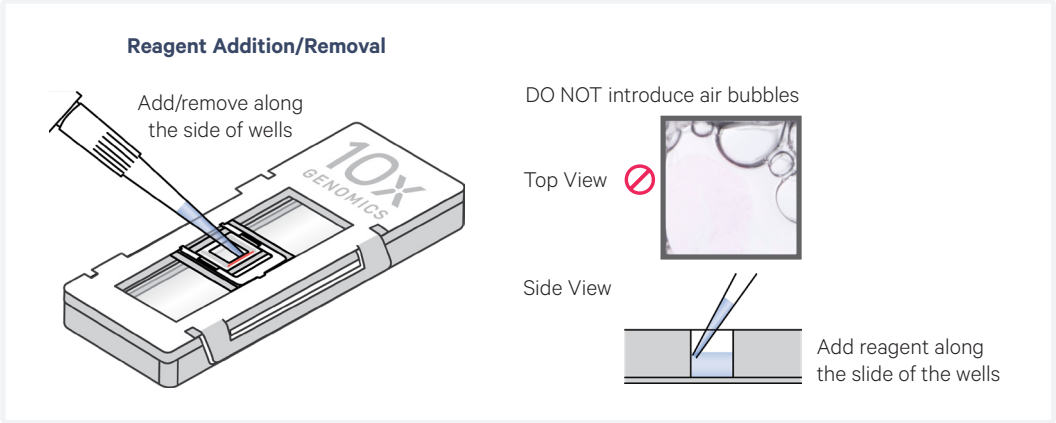
Slide facing up

Slides in images are representative.

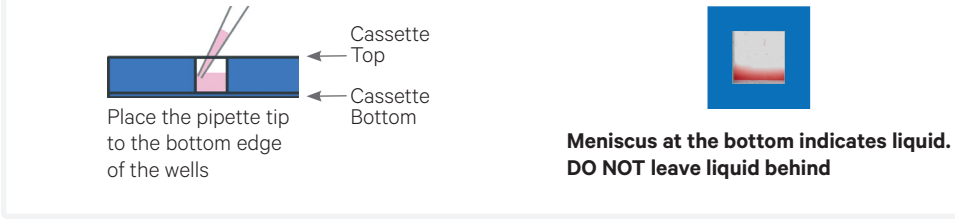
Reagent Addition to Wells

- 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.
- Ensure that no bubbles are introduced in the process.

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 without touching the tissue sections and without introducing bubbles.
- Ensure that no bubbles are introduced in the process.
- Remove all liquid from the wells in each step. To ensure complete removal, check the bottom of the well by tilting the cassette slightly. A meniscus at the bottom of the well will indicate the presence of liquid in the well. Repeat removal steps until no reagent remains.



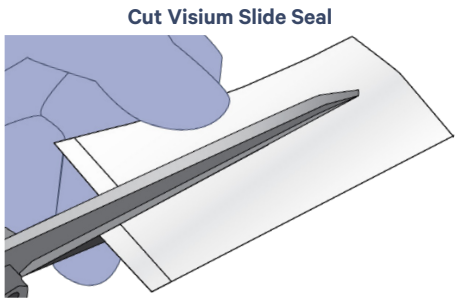
Visium CytAssist Slide Seal Application & Removal

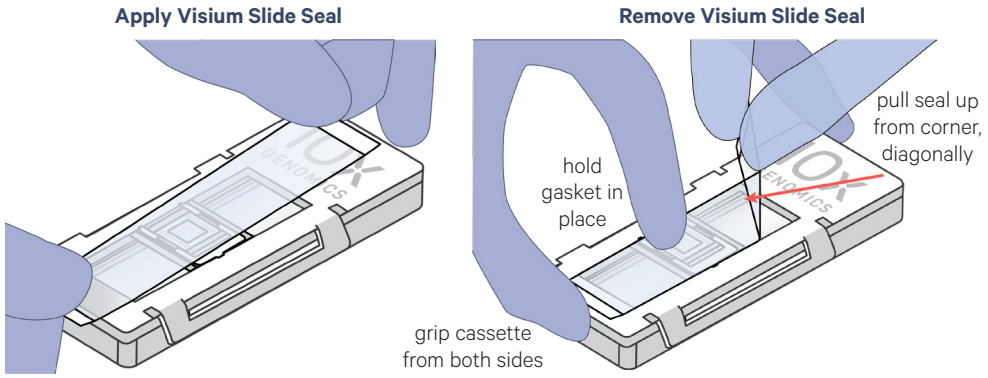
Application

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

Removal

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





Visium CytAssist Tested Slides

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

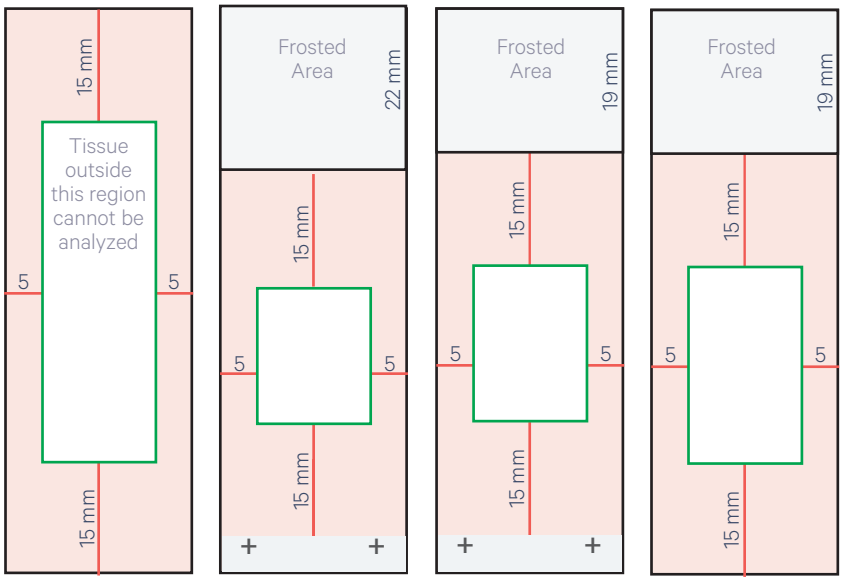
Item	Length (mm)	Width (mm)	Thickness (mm)	Ground Corners
Epredia Shandon ColorFrost Plus	75.0	25.0	1.0	No
Fisherbrand SuperFrost Plus	75.0	25.0	1.0	Available as either
Sigma-Aldrich Poly Prep Slides	75.0	25.0	1.0	No
VWR SuperFrost Plus Micro Slide, Premium	75.0	25.0	1.0	No

If unsure of slide part number, refer to the "generic" slide diagram below for general guidance (images not to scale). 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). The diagrams demonstrate allowable areas that are far enough away from frosted sections to not interfere with gasket closure during the CytAssist assay. Frosted sections include the opaque area of the slide as well as any etching or writing on the slide.

While slides are specified as being 25 mm x 75 mm, manufacturing tolerances may lead to dimensions that are too small or large to be compatible with 10x Genomics products. Tissue slide dimensions must be within 24.8 mm - 25.3 mm in width and 74.4 mm - 76.2 mm in 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



Antibody Optimization

Prior testing of the antibodies is recommended on the same tissue block before performing immunofluorescence staining in combination with the Visium CytAssist Spatial Gene Expression for FFPE workflow. Determination of the optimal antibody concentration is crucial for executing this protocol.



An additional Visium Slide Cassette, 6.5 mm (PN-1000469) must be purchased for antibody optimization.

The amount of Block and Stain Buffer and Decrosslinking Buffer provided in the kit is enough to perform antibody optimization, while still leaving enough reagent for the main assay.

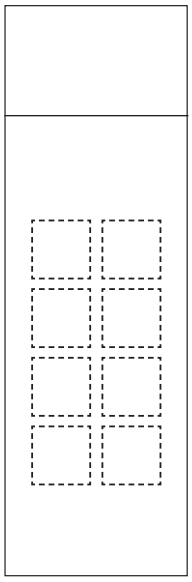
6.5 mm Slides			
Reagent	**PN**	**Staining Method**	**Reactions* Available for Antibody Optimization**
Block and Stain Buffer	2000554	Fluorophore Conjugated Primary Antibodies	50
		Primary and Secondary Antibodies	34
Decrosslinking Buffer	2000566	-	13
11 mm Slides			
Reagent	**PN**	**Staining Method**	**Reactions* Available for Antibody Optimization**
Block and Stain Buffer	2000554	Fluorophore Conjugated Primary Antibodies	25
		Primary and Secondary Antibodies	17
Decrosslinking Buffer	2000566	-	6

*Reactions refers to the number of antibody dilutions that can be prepared, given the volume of each well in the Visium Cassette. For example, the example dilution series on page 24 tests seven antibody optimization reactions.

- Optimal antibody concentrations for this Demonstrated Protocol may differ from other applications. Composition of reagents and buffers may also differ from other immunofluorescence applications.
- Ensure that enough reactions are available for the main assay after running optimization experiments.
- When optimizing the antibody for a single tissue type, ensure that stained slides can be imaged according to the imaging guidelines listed in Visium CytAssist Spatial Applications Imaging Guidelines Technical Note (CG000521).

- Below is a suggested optimization workflow. Antibody optimization may be performed according to preference, as long as tissues are tested using the immunofluorescence staining protocol described in this document.
    - To optimize antibody concentration, draw representative frames on the back of a 75 x 25 x 1 mm blank slide using the example slide layout.

Template for Antibody Optimization



    - Tissue sections must be trimmed to fit within each 6.5 mm area on the template.
    - Place tissue sections in the frames on the front of the slide for compatibility with the Visium Cassette.
    - Execute the Deparaffinization, Decrosslinking & Immunofluorescence Staining protocol using a range of antibody concentrations. A starting concentration of 0.01 µg/µl (0.7 µg/sample) is recommended.
    - To reduce autofluorescence, TrueBlack reagent may be added.
    - Select the antibody concentration that results in the specific staining of desired cells, while minimizing nonspecific background staining.
- Autofluorescence quenchers are added following immunofluorescence staining and may result in the reduction of fluorescence signal. Additional optimization and increase in antibody concentration may be required to properly visualize immunostaining.
- An example dilution layout is provided below. DAPI and merged images are provided to show the presence of breast cancer tissue for each antibody dilution. Dilutions are of recombinant Anti-Vimentin antibody conjugated to Alexa Fluor 594 (BioLegend, PN677804, 0.5 mg/ml). A 1:100 dilution (0.35 µg/sample) was considered optimal in this example.

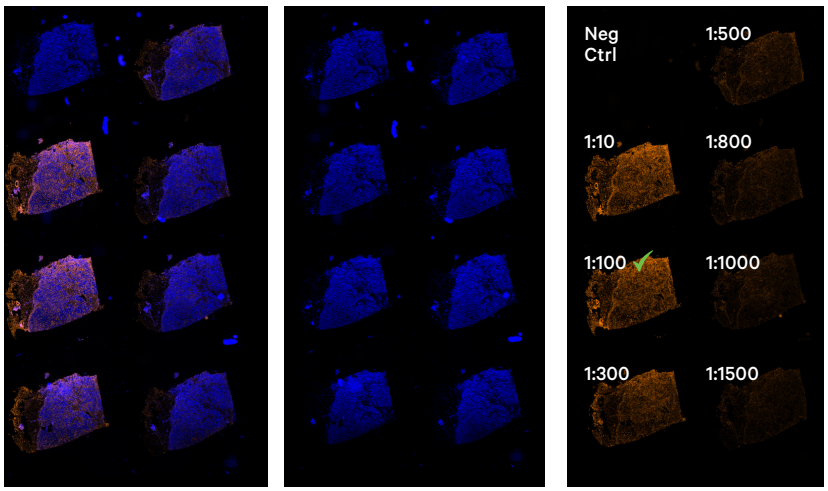
- Example calculations:
  - Stock antibody concentration is 0.5 µg/µl and desired concentration is 0.01 µg/µl (50X dilution).

Reagents	Volume (µl)
Block and Stain Buffer	17.5
RNase Inhibitor	3.5
Antibody	1.4
Nuclease-free Water	47.6
Total	**70.0**

  - Stock antibody concentration is 0.5 µg/µl and desired concentration is 0.0025 µg/µl (200X dilution).
    - This calculation requires pipetting a small volume of antibody. Dilute the stock concentration first (for example, dilute 10X to 0.05 µg/µl) then proceed with the following reagent table:

Reagents	Volume (µl)
Block and Stain Buffer	17.5
RNase Inhibitor	3.5
Antibody	3.5
Nuclease-free Water	45.5
Total	**70.0**

Example Fluorophore Conjugated Primary Antibody Dilution Series



1. Deparaffinization, Decrosslinking & Immunofluorescence (IF) Staining

1.0 Overview

This chapter provides guidance on deparaffinization, decrosslinking, and immunofluorescence (IF) staining of slides containing FFPE tissue sections that are dried overnight in a desiccator.

- Additional Tissue Slide Cassettes (PN-1000471, PN-1000472) must be purchased for each processed section.
- Ensure that microscope settings have been verified and imaging programs have been created prior to starting this program. Consult the Visium CytAssist Spatial Applications Imaging Guidelines Technical Note (CG000521) for more information.
- If staining using fluorophore conjugated primary antibodies, proceed directly to step 1.4A (Immunofluorescence staining- Fluorophore Conjugated Primary Antibodies) after completing step 1.3 (Decrosslinking).
- If staining using primary and secondary antibodies, proceed directly to step 1.4B (Immunofluorescence staining- Primary and Secondary Antibodies) after completing step 1.3 (Decrosslinking).



Slide in image is representative

1.1 Preparation - Buffers

For Deparaffinization Prepare fresh weekly, process two slides per jar	
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 enough 100% ethanol to fully submerge tissue in each.
☐ 96% Ethanol	Label two coplin jars as 96% Ethanol Jar 1 and 2. Dispense enough 96% ethanol to fully submerge tissue in each.
☐ 70% Ethanol	Label one coplin jar as 70% Ethanol Jar. Dispense enough 70% ethanol to fully submerge tissue.
☐ Milli-Q or UltraPure Water	Label one coplin jar as Water Jar. Dispense enough water to fully submerge. Alternatively, use a 50-ml centrifuge tube or a beaker.

Alternatively, a slide staining dish can also be used in place of a coplin jar. Adjust the volumes of deparaffinization solutions and water accordingly. Use xylene-resistant dishes for immersion in xylene. Use xylene-resistant gloves or forceps for deparaffinization. Prepare fresh reagents every week.

For Decrosslinking and Staining	
Items	**Preparation & Handling**
☐ 1X PBS	Prepare 50 ml 1X PBS using nuclease-free water.
☐ 2X SSC	Prepare 1 ml 2X SSC using nuclease-free water.
☐ 70% Ethanol	Optional, if performing TrueBlack Quenching. Prepare 1 ml of 70% Ethanol by diluting stock ethanol with nuclease-free water.
☐ Wash Buffer	Prepare Wash Buffer according to the appropriate table below, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain on ice.

If using a fluorophore conjugated primary antibody:



6.5 mm Gaskets					
Wash Buffer	**Stock**	**Final**	**1X (µl)**	**2X+ 10% (µl)**	**4X+ 10% (µl)**
PBS	10X	1X	60.0	132.0	264.0
Tween-20	10%	0.4%	24.0	52.8	105.6
Nuclease-free Water	-		516.0	1,135.2	2,270.4
Total	**-**	**-**	**600.0**	**1,320.0**	**2,640.0**



11 mm Gaskets					
Wash Buffer	**Stock**	**Final**	**1X (µl)**	**2X+ 10% (µl)**	**4X + 10% (µl)**
PBS	10X	1X	120.0	264.0	528.0
Tween-20	10%	0.4%	48.0	105.6	211.2
Nuclease-free Water	-		1,032.0	2,270.4	4,540.8
Total	**-**	**-**	**1,200.0**	**2,640.0**	**5,280.0**

☐ Wash Buffer

If using a primary and secondary antibody:



6.5 mm Gaskets Wash Buffer	Stock	Final	1X (µl)	2X+ 10% (µl)	4X+ 10% (µl)
PBS	10X	1X	120.0	264.0	528.0
Tween-20	10%	0.4%	48.0	105.6	211.2
Nuclease-free Water	-		1,032.0	2,270.4	4,540.8
Total	**-**	**-**	**1,200.0**	**2,640.0**	**5,280.0**



11 mm Gaskets Wash Buffer	Stock	Final	1X (µl)	2X+ 10% (µl)	4X + 10% (µl)
PBS	10X	1X	240.0	528.0	1,056.0
Tween-20	10%	0.4%	96.0	211.2	422.4
Nuclease-free Water	-		2,064.0	4,540.8	9,081.6
Total	**-**	**-**	**2,400.0**	**5,280.0**	**10,560.0**

☐ 1X Blocking Buffer

Prepare Blocking Buffer on ice according to the appropriate table below, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain on ice.



6.5 mm Gaskets 1X Blocking Buffer	Stock	Final	1X (µl)	2X+ 10% (µl)	4X+ 10% (µl)
Block and Stain Buffer	4X	1X	25.0	55.0	110.0
RNase Inhibitor	40X	1X	2.5	5.5	11.0
Nuclease-free Water	-	-	72.5	159.5	319.0
Total	**-**	**-**	**100.0**	**220.0**	**440.0**



11 mm Gaskets 1X Blocking Buffer	Stock	Final	1X (µl)	2X+ 10% (µl)	4X + 10% (µl)
Block and Stain Buffer	4X	1X	50.0	110.0	220.0
RNase Inhibitor	40X	1X	5.0	11.0	22.0
Nuclease-free Water	-	-	145.0	319.0	638.0
Total	**-**	**-**	**200.0**	**440.0**	**880.0**

Items	Preparation & Handling
☐ Diluted Decrosslinking Buffer	Thaw Decrosslinking Buffer at room temperature. Vortex and centrifuge briefly after preparing Diluted Decrosslinking Buffer.



6.5 mm Gaskets Diluted Decrosslinking Buffer	Stock	Final	1X (µl)	2X+ 10% (µl)	4X+ 10% (µl)
Decrosslinking Buffer	10X	1X	25	55	110
Nuclease-free Water	-		225	495	990
Total	**-**	**-**	**250**	**550**	**1,100**



11 mm Gaskets Diluted Decrosslinking Buffer	Stock	Final	1X (µl)	2X+ 10% (µl)	4X+ 10% (µl)
Decrosslinking Buffer	10X	1X	50	110	220
Nuclease-free Water	-		450	990	1,980
Total	**-**	**-**	**500**	**1,100**	**2,200**

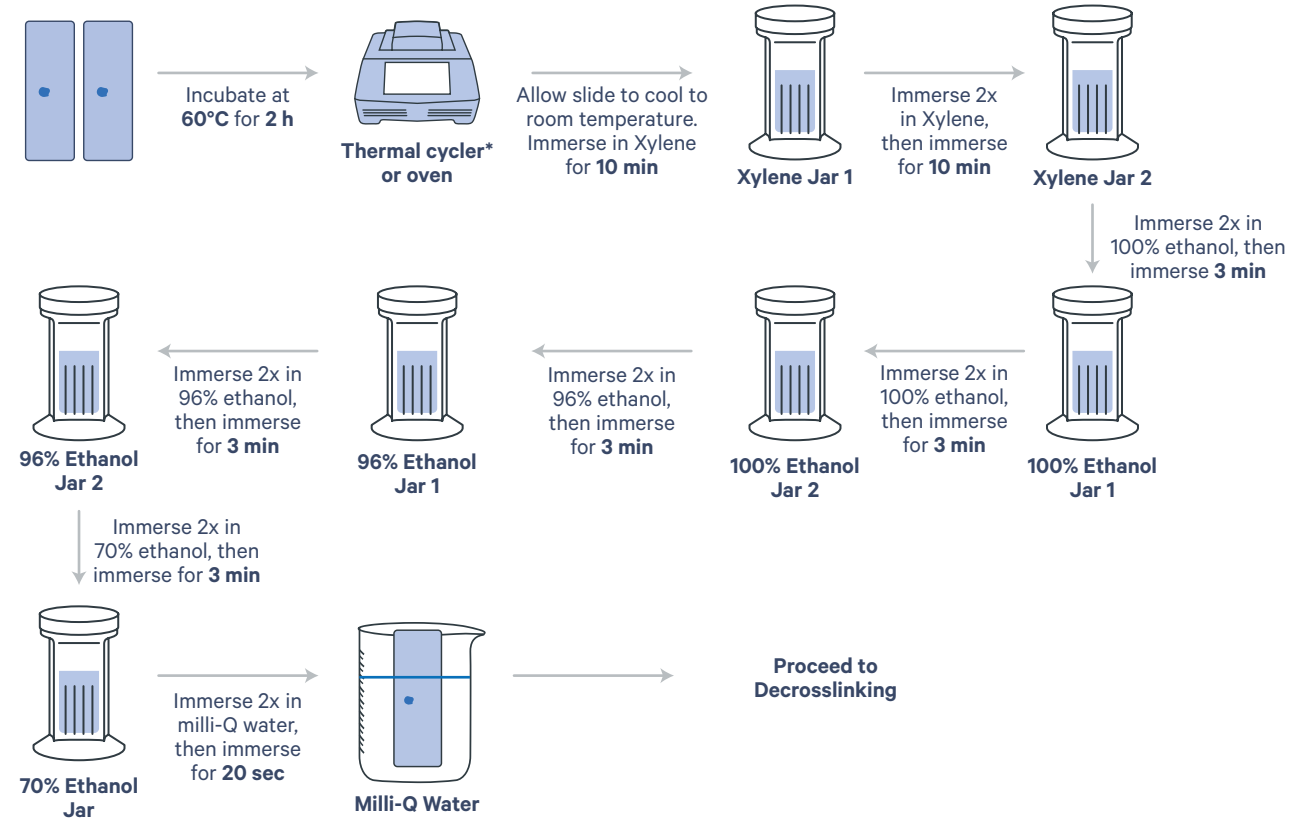
For Coverslipping

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%	20%	20	46
Total	**-**	**-**	**100**	**230**

Protocol Overview

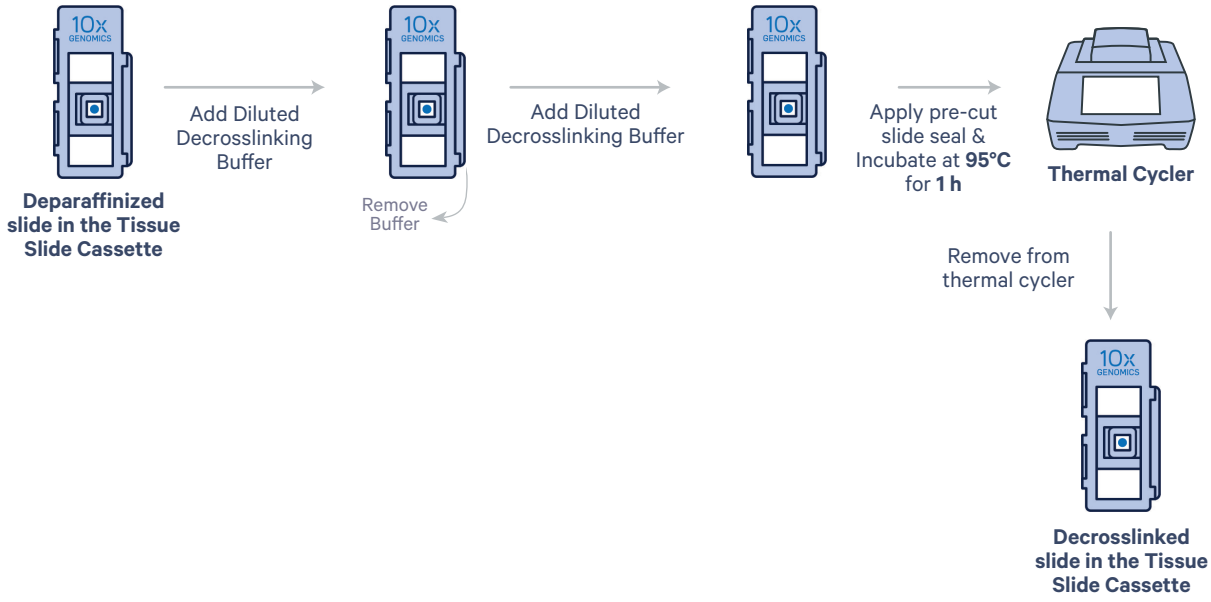
Deparaffinization ~3 h



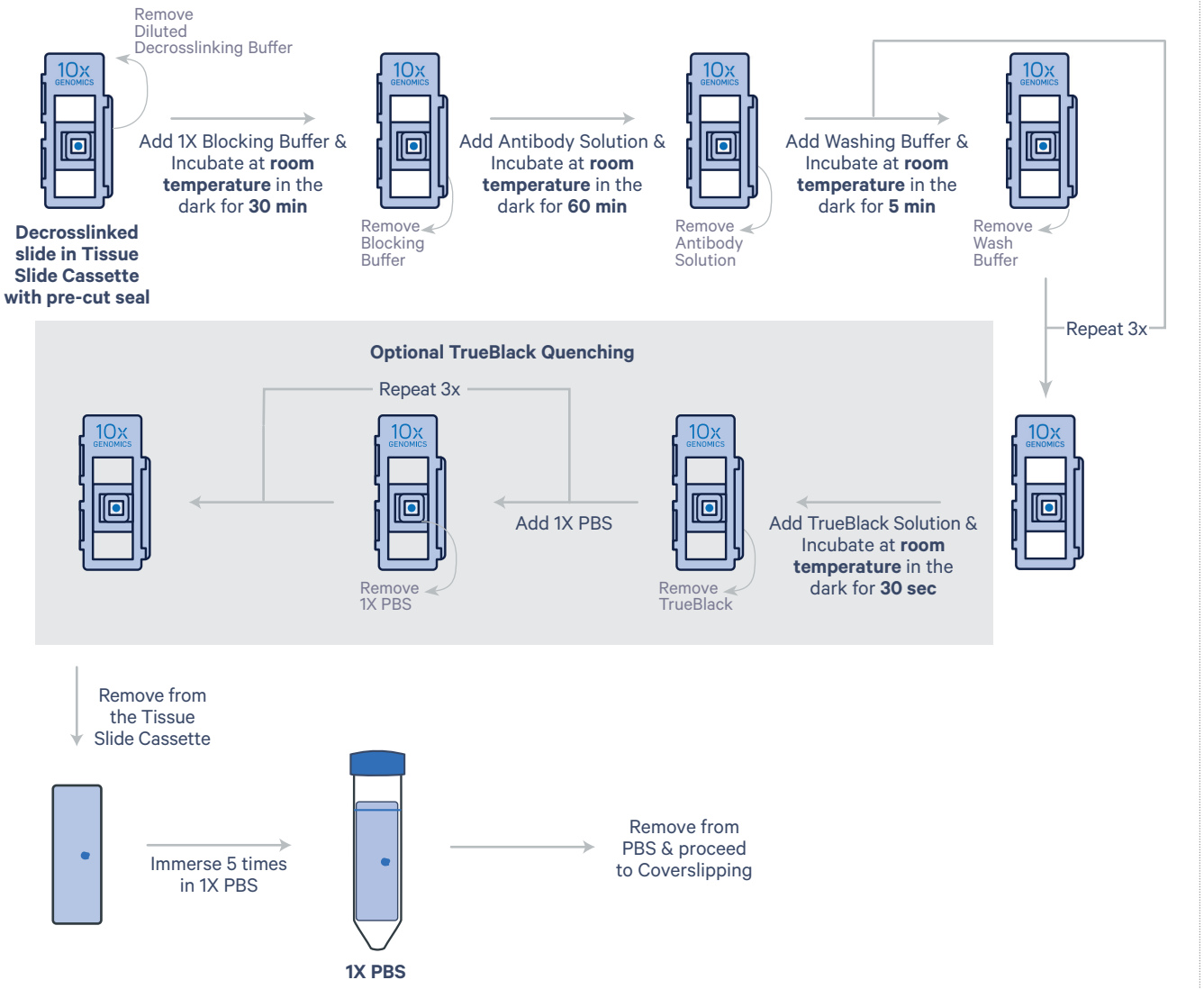
Slide in image is representative

**Keep the thermal cycler lid open during incubation*

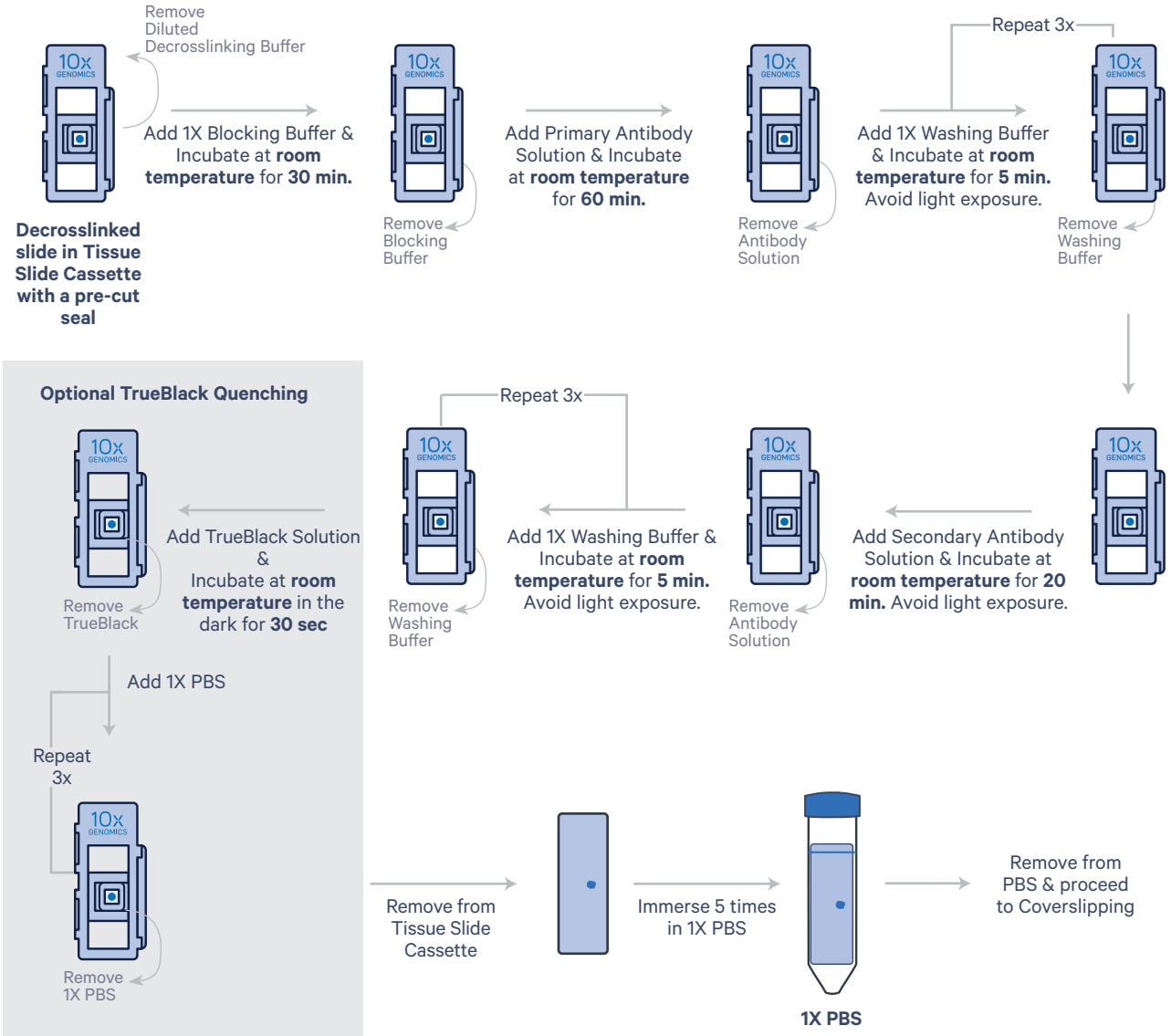
Decrosslinking ~1 h



Immunofluorescence Staining – Fluorophore Conjugated Primary Antibodies ~1.5 h



Immunofluorescence Staining – Primary and Secondary Antibodies ~2.5 h



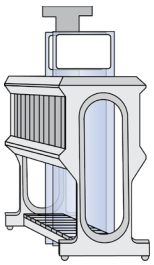
1.2 Deparaffinization

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

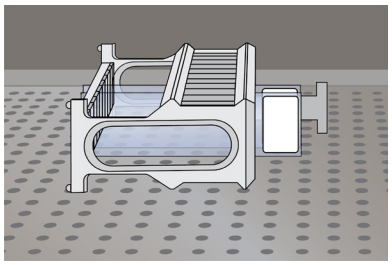
a. Retrieve slides with tissue sections from the desiccator after overnight drying.

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

Incubation in a Section Dryer Oven



Place slides in a rack



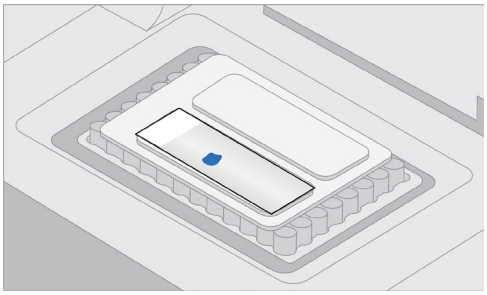
Place slide sideways and keep the oven door closed during incubation

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 on a Thermal Cycler

DO NOT close the lid



Incubate Slide for 2 h at 60°C

Slide in image is representative.

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

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 for **10 min.** Secure jar cap to prevent xylene loss.

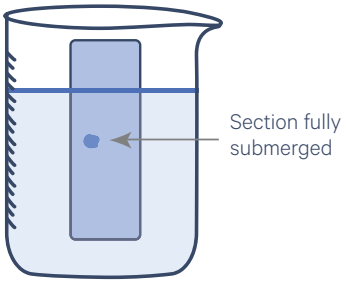
e. Gently immerse slides 2x in Xylene Jar 2, then immerse for **10 min**.

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

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

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

Submerge in Xylene/Ethanol



Slide in image is representative

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

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

k. Gently immerse slides 2x Water Jar, then immerse for **20 sec**.

l. Let the slides air dry and proceed to Decrosslinking.

1.3 Decrosslinking

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

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

Lid Temperature	Reaction Volume	Run Time
95°C	-	1 h
Step	**Temperature**	**Time**
Pre-equilibrate	95°C	Hold
Decrosslinking	95°C	00:60:00
Cooling	22°C	00:10:00
Hold	22°C	Hold

b. Place the slide in the Visium CytAssist Tissue Slide Cassette.

TIPS

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

c. Add **■150 µl or ▲300 µl** Diluted Decrosslinking Buffer along the side of the wells.

d. Remove all Decrosslinking Buffer from the wells.

e. Add **■100 µl or ▲200 µl** Diluted Decrosslinking Buffer along the side of the wells.

f. Apply a pre-cut Visium Slide Seal on the cassette and place the cassette on the Low Profile Thermocycler Adapter at **95°C**.

g. Close the thermal cycler lid. Skip pre-equilibrate step and initiate Decrosslinking.

h. After decrosslinking is complete, remove the cassette from the Low Profile Thermocycler Adapter and place on a flat, clean work surface.

i. Peel back slide seal and using a pipette, remove all Decrosslinking Buffer from the well corners.

j. Add **■150 µl or ▲300 µl** 2X SSC along the side of the wells to uniformly cover the tissue sections, without introducing bubbles.

k. Proceed **immediately** to appropriate Immunofluorescence Staining protocol.

1.4 Immunofluorescence Staining

Choose appropriate staining protocol depending upon the antibodies used.
■ *denotes volumes for 6.5 mm gaskets and* ▲ *denotes volumes for 11 mm gaskets*

1.4A Immunofluorescence Staining – Fluorophore Conjugated Primary Antibodies

a. Prepare Antibody Solution on ice, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain on ice.

If using primary and secondary antibody, proceed directly to 1.4B.



6.5 mm Gaskets Antibody Solution	Stock	Final	1X (µl)	2X+ 10% (µl)	4X+ 10% (µl)
Block and Stain Buffer	4X	1X	17.5	38.5	77.0
RNase Inhibitor	40X	2X	3.5	7.7	15.4
Antibody #1	Variable	Variable	Variable	Variable	Variable
Antibody #2 (Optional)	Variable	Variable	Variable	Variable	Variable
Antibody #3 (Optional)	Variable	Variable	Variable	Variable	Variable
DAPI Solution	100X	1X	0.7	1.5	3.1
Nuclease-free Water	Variable	Variable	Variable	Variable	Variable
Total	-	-	**70.0**	**154.0**	**308.0**



11 mm Gaskets Antibody Solution	Stock	Final	1X (µl)	2X+ 10% (µl)	4X + 10% (µl)
Block and Stain Buffer	4X	1X	35.0	77.0	154.0
RNase Inhibitor	40X	2X	7.0	15.4	30.8
Antibody #1	Variable	Variable	Variable	Variable	Variable
Antibody #2 (Optional)	Variable	Variable	Variable	Variable	Variable
Antibody #3 (Optional)	Variable	Variable	Variable	Variable	Variable
DAPI Solution	100X	1X	1.4	3.1	6.2
Nuclease-free Water	Variable	Variable	Variable	Variable	Variable
Total	-	-	**140.0**	**308.0**	**616.0**

TIPS

Antibody dilution can change depending on the antibody, ranging from 1:50 down to 1:1000. Antibody volumes will depend on concentrations determined during antibody optimization. Add an appropriate volume of nuclease-free water based on the amount of added antibody to achieve the stated total volume.

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

If using primary and secondary antibody, proceed directly to 1.5B.



6.5 mm Slides 1X TrueBlack Solution	Stock	Final	1X (µl)	2X+ 10% (µl)	4X+ 10% (µl)
TrueBlack Lipofuscin Quencher	20X	1X	3.5	7.7	15.4
70% Ethanol	-	-	66.5	146.3	292.6
Total	**-**	**-**	**70.0**	**154.0**	**308.0**



11 mm Slides 1X TrueBlack Solution	Stock	Final	1X (µl)	2X+ 10% (µl)	4X + 10% (µl)
TrueBlack Lipofuscin Quencher	20X	1X	7.0	15.4	30.8
70% Ethanol	-	-	133.0	292.6	585.2
Total	**-**	**-**	**140.0**	**308.0**	**616.0**

c. Remove all 2X SSC from the wells.

d. Add ■**100 µl or** ▲**200 µl** 1X Blocking Buffer along the side of the wells.

e. Re-apply Visium Slide Seal to the Visium CytAssist Tissue Slide Cassette.

f. Incubate for **30 min** at **room temperature**.

g. Peel back slide seal from the cassette and remove all Blocking Buffer from the wells.

h. Add ■**70 µl or** ▲**140 µl** Antibody Solution along the side of the wells. Tap gently to ensure uniform coverage.

i. Re-apply slide seal to the cassette.

j. Incubate for **1 h** at **room temperature** in the dark.

k. Peel back slide seal and remove Antibody Solution.

l. Add ■**150 µl or** ▲**300 µl** Wash Buffer along the side of the wells.

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

n. Remove all Wash Buffer from the wells.

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

p. *Optional* - TrueBlack Quenching

- Add ■**70 µl or ▲140 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 or ▲300 µl** 1X PBS along the side of the wells.
- Remove all PBS from the wells.
- Repeat PBS washes three more times for a total of four washes.

q. Remove slide from cassette.



See Tips & Best Practices for removal instructions.

r. Gently immerse slide 5x in 1X PBS in 50-ml centrifuge tube. If processing multiple slides, use a separate 50-ml centrifuge tube filled with PBS for each slide.

s. Remove slide from the PBS and proceed **immediately** to Coverslipping.



DO NOT let the slide dry.

1.4B Immunofluorescence Staining – Primary and Secondary Antibodies

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

a. Prepare Primary Antibody Solution on ice according to the appropriate table, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain on ice.



6.5 mm Gaskets Primary Antibody Solution	Stock	Final	1X (µl)	2X+ 10% (µl)	4X+ 10% (µl)
Block and Stain Buffer	4X	1X	17.5	38.5	77.0
RNase Inhibitor	40X	2X	3.5	7.7	15.4
Antibody #1	Variable	Variable	Variable	Variable	Variable
Antibody #2 (Optional)	Variable	Variable	Variable	Variable	Variable
Antibody #3 (Optional)	Variable	Variable	Variable	Variable	Variable
Nuclease-free Water	Variable	Variable	Variable	Variable	Variable
Total	-	-	**70.0**	**154.0**	**308.0**



11 mm Gaskets Primary Antibody Solution	Stock	Final	1X (µl)	2X+ 10% (µl)	4X + 10% (µl)
Block and Stain Buffer	4X	1X	35.0	77.0	154.0
RNase Inhibitor	40X	2X	7.0	15.4	30.8
Antibody #1	Variable	Variable	Variable	Variable	Variable
Antibody #2 (Optional)	Variable	Variable	Variable	Variable	Variable
Antibody #3 (Optional)	Variable	Variable	Variable	Variable	Variable
Nuclease-free Water	Variable	Variable	Variable	Variable	Variable
Total	-	-	**140.0**	**308.0**	**616.0**



Antibody dilution can change depending on the antibody, generally ranging from 1:50 up to 1:1000. Antibody volumes will depend on concentrations determined during antibody optimization.

c. Prepare Secondary Antibody Solution on ice according to the appropriate table, adding reagents in the order listed. Pipette mix 10x and centrifuge briefly. Maintain on ice.



6.5 mm Gaskets Secondary Antibody Solution	Stock	Final	1X (µl)	2X+ 10% (µl)	4X+ 10% (µl)
Block and Stain Buffer	4X	1X	17.5	38.5	77.0
RNase Inhibitor	40X	2X	3.5	7.7	15.4
Antibody #1	Variable	Variable	Variable	Variable	Variable
Antibody #2 (Optional)	Variable	Variable	Variable	Variable	Variable
Antibody #3 (Optional)	Variable	Variable	Variable	Variable	Variable
DAPI Solution	100X	1X	0.7	1.5	3.1
Nuclease-free Water	Variable	Variable	Variable	Variable	Variable
Total	-	-	**70.0**	**154.0**	**308.0**



11 mm Gaskets Secondary Antibody Solution	Stock	Final	1X (µl)	2X+ 10% (µl)	4X + 10% (µ)
Block and Stain Buffer	4X	1X	35.0	77.0	154.0
RNase Inhibitor	40X	2X	7.0	15.4	30.8
Antibody #1	Variable	Variable	Variable	Variable	Variable
Antibody #2 (Optional)	Variable	Variable	Variable	Variable	Variable
Antibody #3 (Optional)	Variable	Variable	Variable	Variable	Variable
DAPI Solution	100X	1X	1.4	3.1	6.2
Nuclease-free Water	Variable	Variable	Variable	Variable	Variable
Total	-	-	**140.0**	**308.0**	**616.0**



Antibody dilution can change depending on the antibody, ranging from 1:200 up to 1:1000. Antibody volumes will depend on concentrations determined during antibody optimization.

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



6.5 mm Gaskets 1X TrueBlack Solution	Stock	Final	1X (µl)	2X+ 10% (µl)	4X+ 10% (µl)
TrueBlack Lipofuscin Quencher	-	-	3.5	7.7	15.4
70% Ethanol	-	-	66.5	146.3	292.6
Total	**-**	**-**	**70.0**	**154.0**	**308.0**



11 mm Gaskets 1X TrueBlack Solution	Stock	Final	1X (µl)	2X+ 10% (µl)	4X + 10% (µl)
TrueBlack Lipofuscin Quencher	-	-	7.0	15.4	30.8
70% Ethanol	-	-	133.0	292.6	585.2
Total	**-**	**-**	**140.0**	**308.0**	**616.0**

f. Remove all 2X SSC from the wells.

g. Add **■100 µl or ▲200 µl** 1X Blocking Buffer along the side of the wells.

h. Re-apply Visium Slide Seal on the Visium CytAssist Tissue Slide Cassette.

i. Incubate for **30 min** at **room temperature**.

j. Peel back slide seal from cassette and remove all Blocking Buffer from the wells.

k. Add **■70 µl or ▲140 µl** Primary Antibody Solution along the side of the wells. Tap gently to ensure uniform coverage.

l. Re-apply slide seal on the cassette.

m. Incubate for **1h** at **room temperature** in the dark.

n. Peel back slide seal and remove Primary Antibody Solution.

o. Add **■150 µl or ▲300 µl** Wash Buffer along the side of the wells.

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

q. Remove Wash Buffer from the wells.

r. **Repeat** n-p two more times for a total of three washes.

s. Add **■70 µl or ▲140 µl** Secondary Antibody Solution along the side of the wells. Tap gently to ensure uniform coverage.

t. Re-apply slide seal on the cassette.

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

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

w. Add ■**150 µl or** ▲**300 µl** 1X Wash Buffer along the side of the wells.

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

y. Remove 1X Wash Buffer from the wells.

z. Repeat v-x three more times for a total of four washes.

aa. *Optional* - TrueBlack Quenching

- Add ■**70 µl or** ▲**140 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 or** ▲**300 µl** 1X PBS along the side of the wells.
- Remove all PBS from the wells.
- Repeat PBS washes three more times for a total of four washes.

ab. Remove slide from cassette.



See Tips & Best Practices for removal instructions.

ac. Gently immerse slide 5x in 1X PBS in 50-ml centrifuge tube. If processing multiple slides, use a separate 50-ml centrifuge tube filled with PBS for each slide.

ad. Remove slide from the PBS and proceed **immediately** to Coverslipping.



DO NOT let the slide dry.

1.5 Coverslipping

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

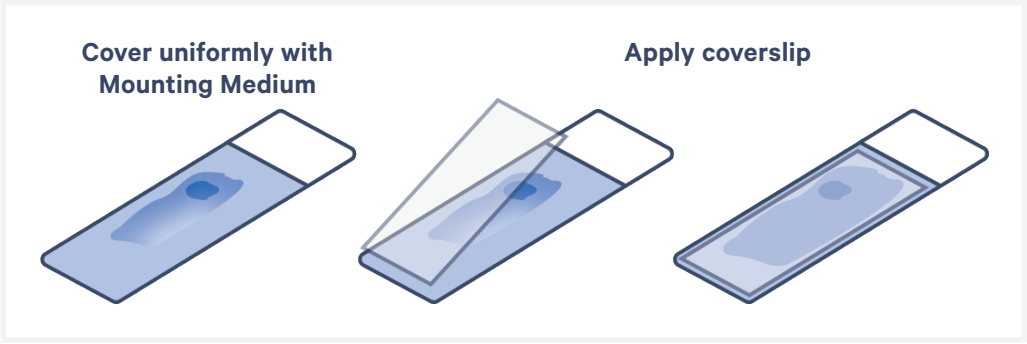
b. Using a **wide-bore** pipette tip, add **100 µl** Mounting Medium to uniformly cover all tissue sections on slides. Avoid generating bubbles.

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. If needed, remove any large excess of mounting media by carefully wicking away from the edge of the coverslip with a laboratory wipe. Be careful not to move 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.

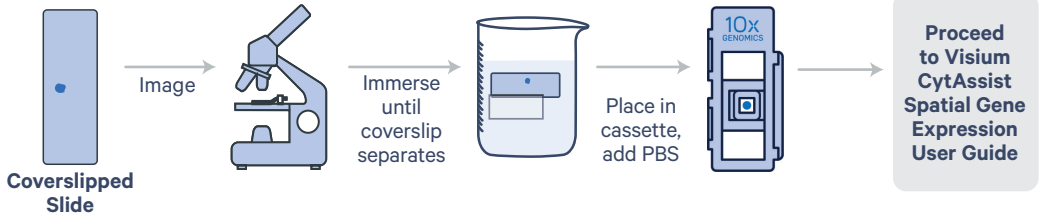


2. Tissue Imaging

2.0 Overview

This chapter provides guidance on imaging tissue slides containing immunofluorescent stained FFPE sections and coverslip removal.

Tissue Imaging & Coverslip Removal



2.1 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.

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
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
Exposure times 100 milli sec-2 sec

2.2 Imaging

a. Image all tissue sections individually at the desired magnification using fluorescence imaging settings.

b. Consult the Visium CytAssist Spatial Applications Imaging Guidelines Technical Note (CG000521) for additional information.

c. After imaging, proceed **immediately** to the Coverslip Removal.

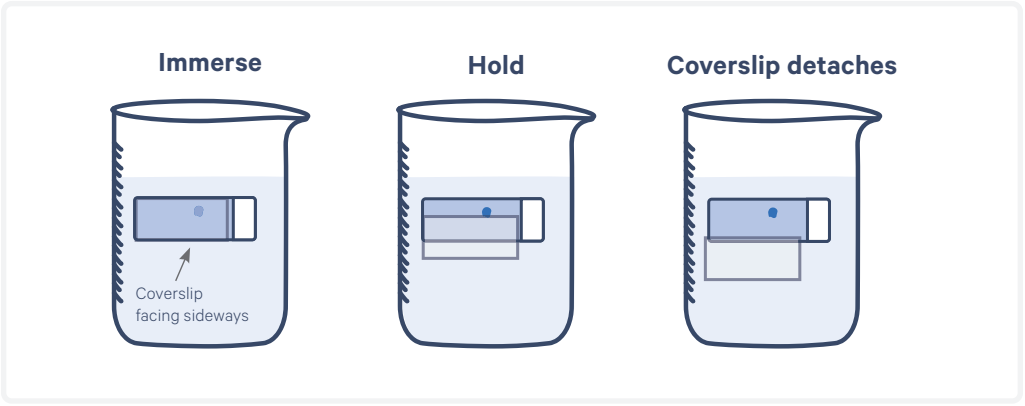
2.3 Coverslip Removal

a. Dispense **200 ml** 1X PBS in a beaker for every two slides being processed.

b. Immerse the slide sideways/horizontal in the beaker containing **200 ml** 1X PBS with the coverslipped surface fully sideways.

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 5x in the 1X PBS to ensure all mounting medium is removed.

e. Wipe the back of the slide with a laboratory wipe.

f. Place the slide in a new Visium CytAssist Tissue Slide Cassette. See Tips & Best Practices for assembly instructions.

g. Add ■**100 µl or 200 µl** 1X PBS along the side of the wells.

h. Apply a new pre-cut slide seal to the cassette.

i. Proceed **immediately** to Visium CytAssist Spatial Gene Expression User Guide (CG000495).

Troubleshooting

STEP	Notes
Tissue detachment	• Gently add and remove reagents from wells. Forceful removal or addition of reagents can impact adhesion. • Using a validated slide can improve tissue adhesion. • Consult the Troubleshooting section of the Visium CytAssist Spatial Gene Expression for FFPE - Tissue Preparation Guide (Document CG000518) for more information on sample preparation errors that may cause detachment.
2.2 Weak or no signal	• Verify that samples were not exposed to light after staining with fluorescent antibodies. • Verify antibody compatibility with decrosslinking conditions. • Verify antibody dilutions. Ensure that antibody optimization is performed. • Verify imaging system filter cubes and wavelength. Ensure that fluorophores and filter cubes match. • Protein of interest may have low expression. Use a positive control. Optimize antibody concentrations on tissues of interest prior to working with Visium Spatial slides. • If the signal from the primary conjugated antibodies are absent or weak, consider inclusion of secondary antibody staining.
2.3 High background	• Ensure that samples properly follow all indicated number of washes and incubation times. • Ensurer Blocking Buffer was made correctly and that blocking step was performed accurately. • Verify that samples did not dry out during the staining protocol. Ensure that samples always remain covered in liquid. • To prevent non-specific antibody binding, compare chosen antibody with antibodies that target the same cell type. If possible, compare staining results to cells known to express higher or lower levels of the target protein. • Using TrueBlack may reduce background fluorescence.

Document Revision Summary

Document Number	CG000519
Title	Visium CytAssist Spatial Gene Expression for FFPE – Deparaffinization, Decrosslinking, IF Staining & Imaging
Revision	Rev E
Revision Date	April 2025
General Changes	Updated for general minor consistency of language and terms throughout
Specific Changes	• Added 10x Genomics RNase Inhibitor 40X (PN-2001488) to page 3.

LEGAL NOTICE © 2025 10x Genomics, Inc. (10x Genomics). All rights reserved. Duplication and/or reproduction of all or any portion of this document without the express written consent of 10x Genomics, is strictly forbidden. Nothing contained herein shall constitute any warranty, express or implied, as to the performance of any products described herein. Any and all warranties applicable to any products are set forth in the applicable terms and conditions of sale accompanying the purchase of such product. 10x Genomics provides no warranty and hereby disclaims any and all warranties as to the use of any third-party products or protocols described herein. The use of products described herein is subject to certain restrictions as set forth in the applicable terms and conditions of sale accompanying the purchase of such product. A non-exhaustive list of 10x Genomics' marks, many of which are registered in the United States and other countries can be viewed at: www.10xgenomics.com/trademarks. 10x Genomics may refer to the products or services offered by other companies by their brand name or company name solely for clarity, and does not claim any rights in those third-party marks or names. 10x Genomics products may be covered by one or more of the patents as indicated at:www.10xgenomics.com/patents. The use of products described herein is subject to 10x Genomics Terms and Conditions of Sale, available at www.10xgenomics.com/legal-notices, or such other terms that have been agreed to in writing between 10x Genomics and user. All products and services described herein are intended FOR RESEARCH USE ONLY and NOT FOR USE IN DIAGNOSTIC PROCEDURES.
The use of 10x Genomics products in practicing the methods set forth herein has not been validated by 10x Genomics, and such non-validated use is NOT COVERED BY 10X GENOMICS STANDARD WARRANTY, AND 10X GENOMICS HEREBY DISCLAIMS ANY AND ALL WARRANTIES FOR SUCH USE. Nothing in this document should be construed as altering, waiving or amending in any manner 10x Genomics terms and conditions of sale for the Chromium Controller or the Chromium Single Cell Controller, consumables or software, including without limitation such terms and conditions relating to certain use restrictions, limited license, warranty and limitation of liability, and nothing in this document shall be deemed to be Documentation, as that term is set forth in such terms and conditions of sale. Nothing in this document shall be construed as any representation by 10x Genomics that it currently or will at any time in the future offer or in any way support any application set forth herein.

Contact:
support@10xgenomics.com
10x Genomics, Inc.
6230 Stoneridge Mall Road
Pleasanton, CA 94588 USA