

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

Introduction

The Visium Spatial Gene Expression for FFPE is designed to measure mRNA in tissue sections derived from formalin fixed & paraffin embedded (FFPE) tissue samples and requires a Visium Spatial slide with intact tissue sections as input. Immunostaining tissue sections with fluorescently labeled antibodies enables simultaneous protein detection. This protocol outlines deparaffinization, decrosslinking, immunofluorescence (IF) staining, and imaging of tissue for use with 10x Genomics Visium 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 Spatial Gene Expression for FFPE - Tissue Preparation Guide (Document CG000408) for complete information on sectioning FFPE tissue blocks and placing sections on Visium Spatial slides. Ensure that tissue sections have been placed onto the appropriate slide prior to starting this Demonstrated Protocol. Consult the Visium Spatial Gene Expression for FFPE Imaging Guidelines (Document CG000436) to verify imaging settings prior to starting this Demonstrated Protocol. After completing this Demonstrated Protocol (CG000410), proceed with the Visium Spatial Gene Expression for FFPE - User Guide (CG000407).

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Reagent Kits

Visium Spatial Gene Expression for FFPE Reagent Kits

Refer to SDS for handling and disposal information

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

Visium

Spatial Gene Expression Slide Kit

16 rxns, PN-1000185

store at ambient temperature

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

10x
genomics

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

Visium

Spatial Gene Expression Slide Kit

4 rxns, PN-1000188

store at ambient temperature

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

10x
genomics

*Visium Slide Seals may come in varying dimensions and quantities in different lots. Check the number of slide seals in the kit. Additional seals may be required. Refer to page 13 ([Visium Slide Seal Application & Removal](#)) of this Demonstrated Protocol for instructions on how to resize seals or cut additional seals...

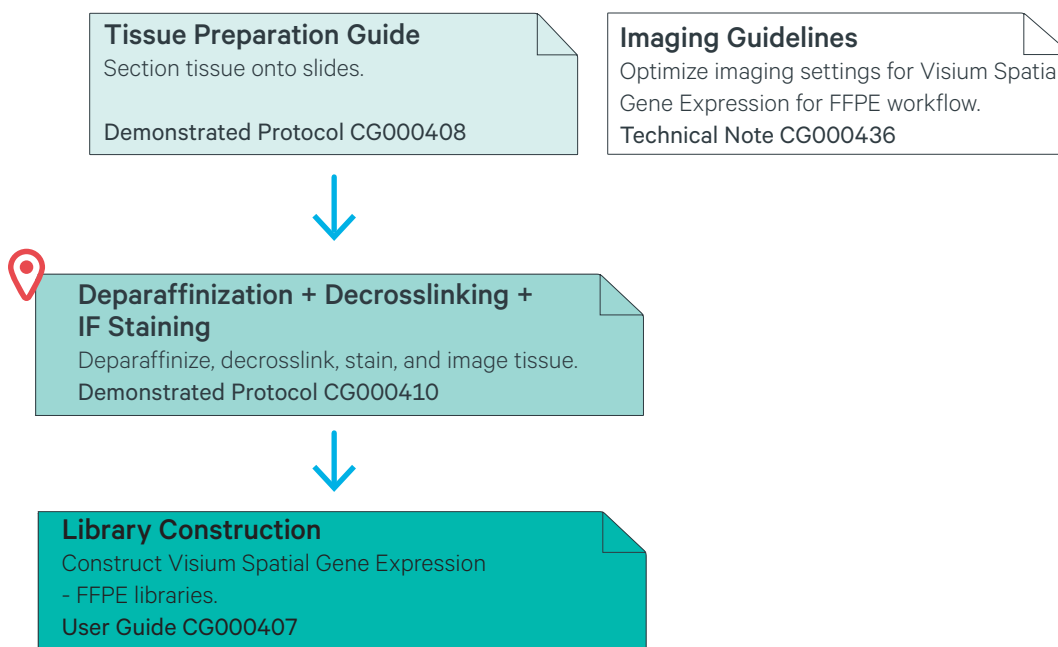
10x Genomics Accessories

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

Recommended Thermal Cyclers

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

Workflow Overview



Visit the 10x Genomics Support website for the most current documentation.

Specific Reagents & Consumables

For each item, a number of vendor options are listed. Choose item based on availability and preference.

Item	Alternatives/Options	Vendor	Part Number
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
TE Buffer (pH 9.0)	TE Buffer (pH 9.0) <i>Alternatively, prepare TE buffer using Tris and EDTA and adjust the pH to 9.0</i>	GeneMed	10-0046
Or	Tris Base <i>For preparing TE buffer (pH 9.0), alternative to Genemed product</i>	Fisher Scientific	BP152-500
Prepare using Tris and 0.5 M EDTA	UltraPure 0.5 M EDTA, pH 8.0 <i>For preparing TE buffer (pH 9.0), alternative to Genemed product</i>	Thermo Fisher Scientific	15575020
	Dilute to 1.0 M HCl stock <i>For preparing TE Buffer (pH 9.0), alternative to Genemed product</i>	Millipore Sigma	258148
PBS	PBS - Phosphate Buffered Saline (10X) pH 7.4, RNase-free	Thermo Fisher Scientific	AM9624
BSA	Albumin, Bovine Serum, 10% Aqueous Solution, Nuclease-Free	Millipore Sigma	126615-25ML
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
Antibody	-	-	-
RNase inhibitor	Protector RNase Inhibitor	Millipore Sigma	3335399001
Mounting medium	SlowFade Diamond Antifade Mountant	Thermo Fisher Scientific	S36967
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
Sealing film	Microseal 'B' PCR Plate Sealing Film, adhesive	Bio-Rad	MSB1001
Additional Materials			
Beakers		-	-
Ultrapure/Milli-Q Water, <i>from Milli-Q Integral Ultrapure Water System or equivalent</i>		-	-

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.

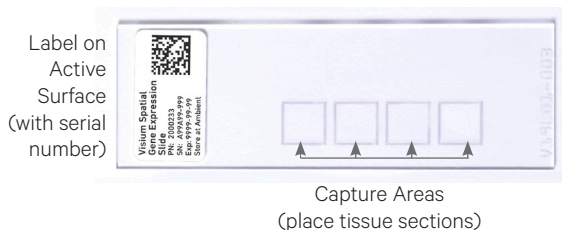
Pipette Calibration

- Follow manufacturer's calibration and maintenance schedules.

Visium Spatial Gene Expression Slide

- Visium slides include 4 Capture Areas (6.5 x 6.5 mm), each with ~5,000 unique gene expression spots.
- Each gene expression spot includes primers with a unique Spatial Barcode.
- The active surface of the slide is defined by a readable label that includes the serial number.
- The tissue sections are always placed on the active surface of the Capture Areas. For more information, consult the Visium Spatial Gene Expression for FFPE – Tissue Preparation Guide (Demonstrated Protocol CG000408).
- Always store slides in a cool, dry environment. After tissue placement, store the slides at room temperature in a low moisture environment such as a desiccator.

Visium Spatial Gene Expression Slide



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

Slide Handling

- Always wear gloves when handling slides.
- Ensure that the active surface of a slide faces up and is never touched. The orientation of the label on the slide defines the active surface.
- The tissue sections should always be on the active surface of the slide. DO NOT touch the tissue sections on the slide.
- Minimize exposure of the slides to sources of particles and fibers.
- When immersing slides in deparaffinization solutions and water, ensure that the tissue sections are completely submerged.
- Xylene and ethanol may cause the slide label to come off. Keep the label above the surface of the liquid when immersing in xylene and ethanol.
- Keep the slide flat on the bench when adding reagents to the active surface.
- Ensure that no absorbent surface is in contact with the reagents on the slide during incubation.

Active Surface with Tissue Sections



Immersing Slide

Correct



Incorrect



Reagent on Slide

Correct



Incorrect



Slide Incubation Guidance

Incubation at a specified temperature

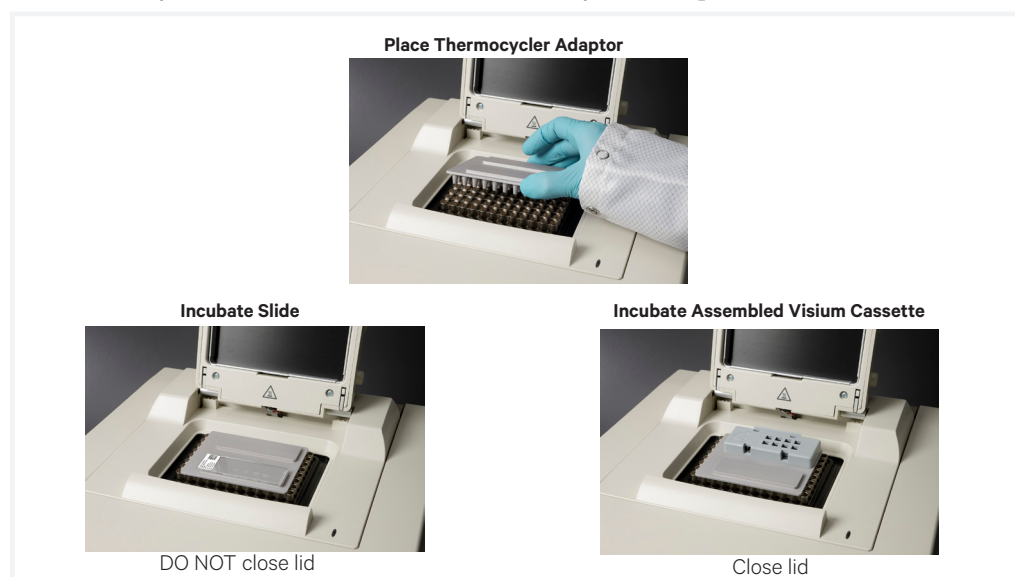
Incubation using a Section Dryer Oven:

- Place the slides in a slide drying rack.
- Close the lid when incubating the slide in the oven.



Incubation using a Thermal Cycler:

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

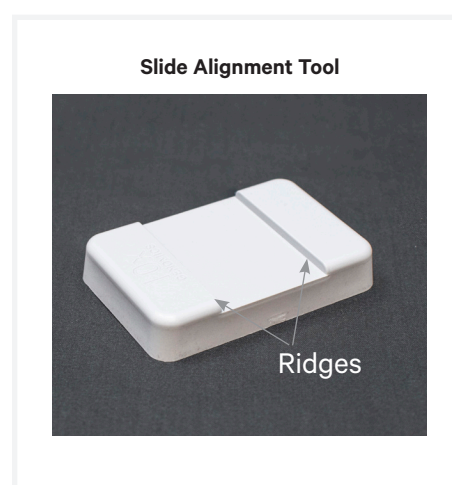
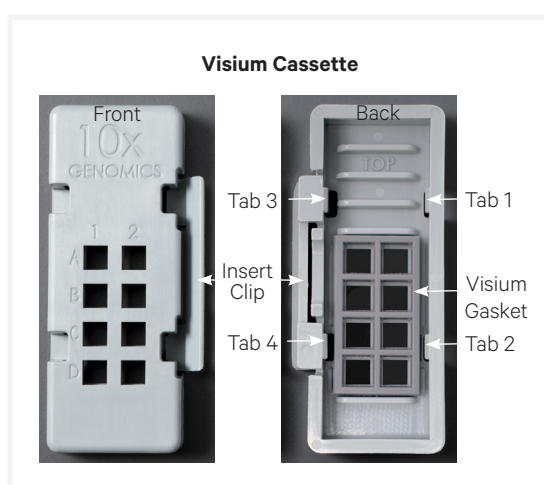


Incubation at room temperature

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

Visium Cassette

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

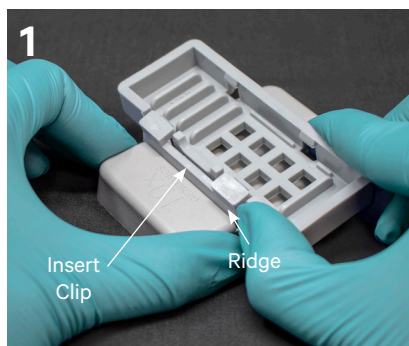


Visium Cassette Assembly



Exercise caution when handling slide edges to prevent injury.

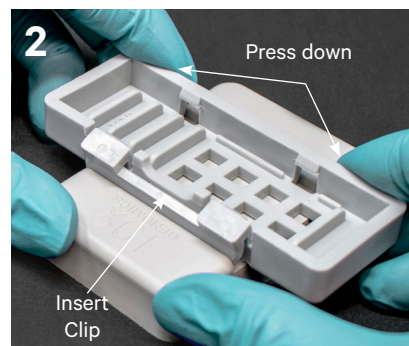
Position Visium Cassette along alignment tool ridges



Visium Cassette secured on alignment tool



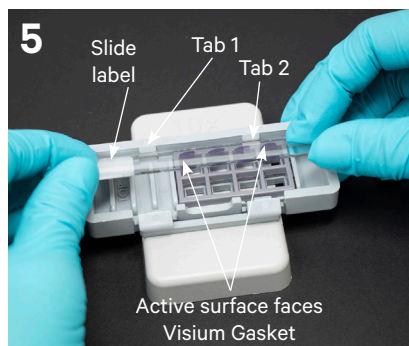
Push Insert Clip along the ridge & press Visium Cassette down



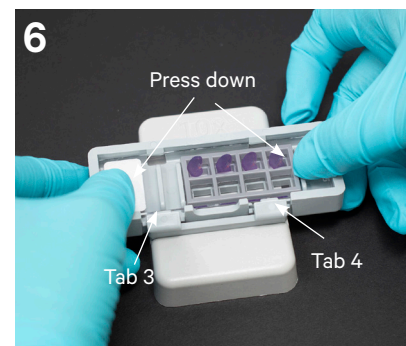
Position Visium Gasket to align with Visium Cassette cutouts



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



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



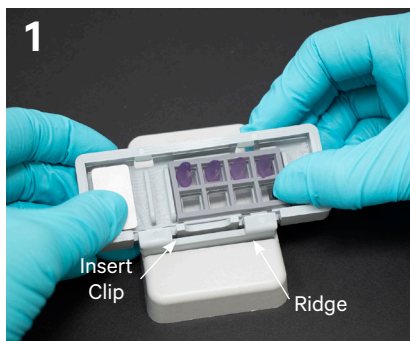
Remove Visium Cassette while pressing slide against the Visium Gasket



Slides in images are representative.

Visium Cassette Removal

Position Visium Cassette along alignment tool ridges



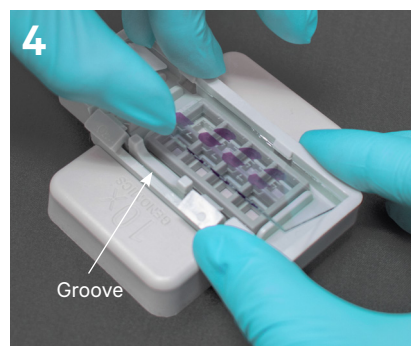
Push Insert Clip along the ridge & press down



Visium Cassette sits securely on alignment tool



Lift slide at Visium Cassette groove



Slides in images are representative.

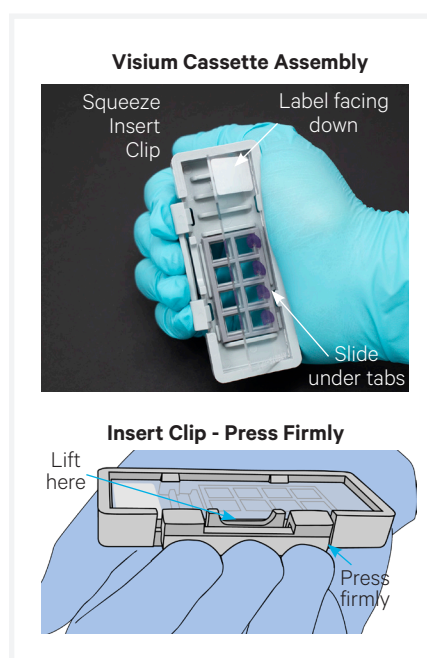
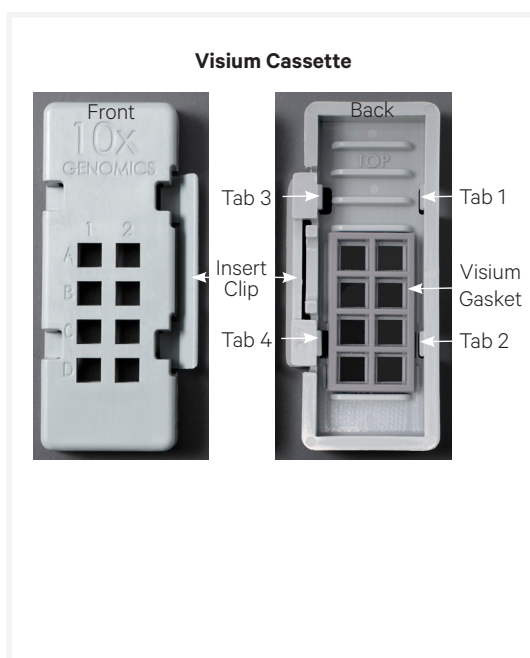
Manual Visium Cassette Assembly & Removal

Assembly

- Remove the Visium Gasket from the Visium Cassette and re-insert the Visium Gasket, ensuring that the Visium Gasket and Visium Cassette cutouts are aligned.
- Align the label on top of the slide to the top of the Visium Cassette, as shown.
- Insert the slide under tabs 1 and 2. Ensure that the long edge of the slide is flush with the side of the Visium Cassette.
- Press the insert clip very firmly by applying even force on the lower part of the insert clip.
- Place a finger in between tab 3 and the top of the cassette, and one finger between tab 4 and the bottom of the cassette. Press down on the slide evenly until the slide is under each tab and release the insert clip.

Removal

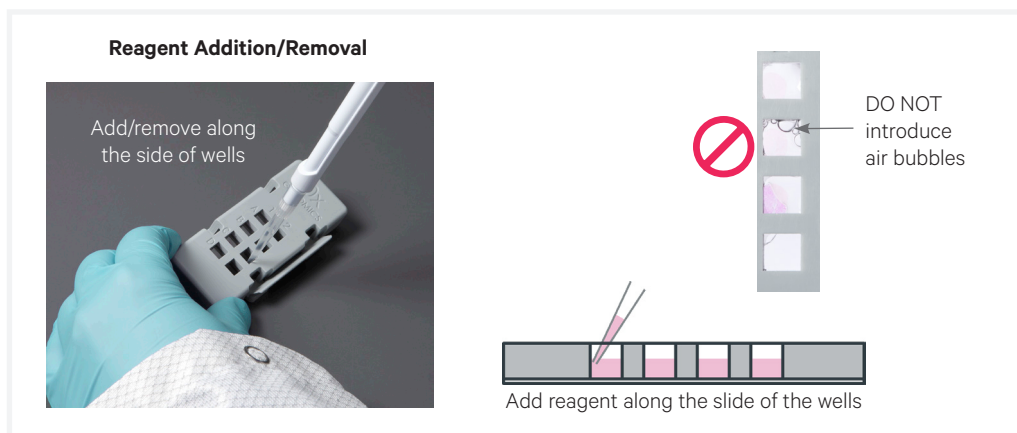
- Press the insert clip very firmly to release the slide from the cassette.
- Lift slide at Visium Cassette groove between tabs 3 and 4 until the slide can be removed.



Reagent Addition to Wells

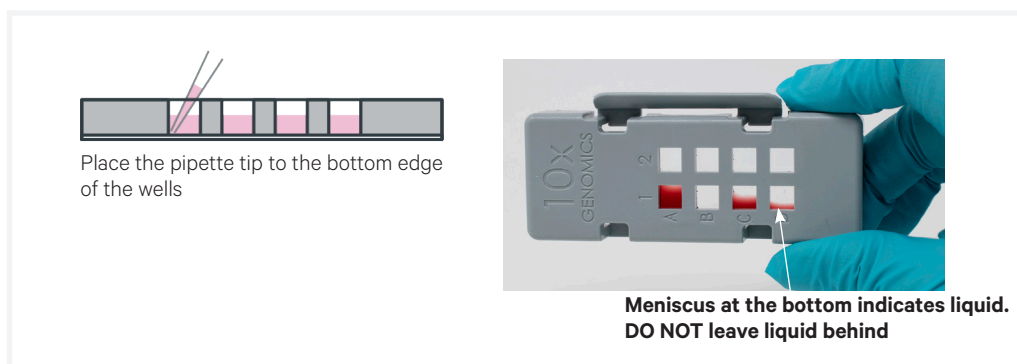


- Place the assembled slide in the Visium Cassette flat on a clean work surface.
- Dispense reagents along the side of the wells without touching the tissue sections and without introducing bubbles.
- Always cover the tissue section completely when adding reagents to the well. A gentle tap may help spread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



Reagent Removal from Wells

- Place the assembled slide in the Visium Cassette flat on a clean work surface.
- Slightly tilt the Visium Cassette while removing the reagent.
- Place the pipette tip to the bottom edge of the wells.
- Remove reagents along the side of the wells without touching the tissue sections and without introducing bubbles.
- Ensure that no bubbles are introduced in the process.
- Remove all liquid from the wells in each step. To ensure complete removal, check the bottom of the well by tilting the cassette slightly. A meniscus at the bottom of the well will indicate the presence of liquid in the well.



Visium Slide Seal Application & Removal

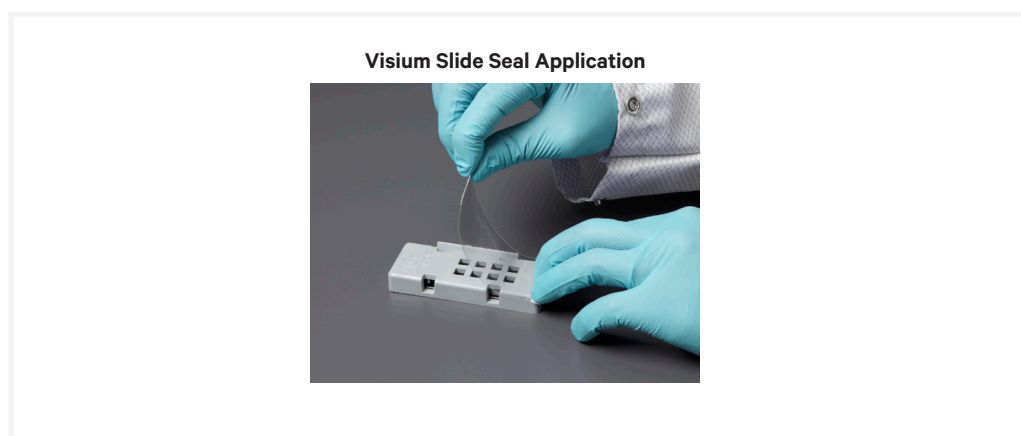
To generate new or resize Visium Slide Seals, use one of the provided seals (PN-2000283/2000284) as a template to cut additional seals from MicroSeal 'B' PCR Plate Sealing Film (PN-MSB1001; listed in Specific Reagents & Consumables). Contact support@10xgenomics.com if assistance is required.

Application

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

Removal

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



Tissue Detachment on Visium Slides



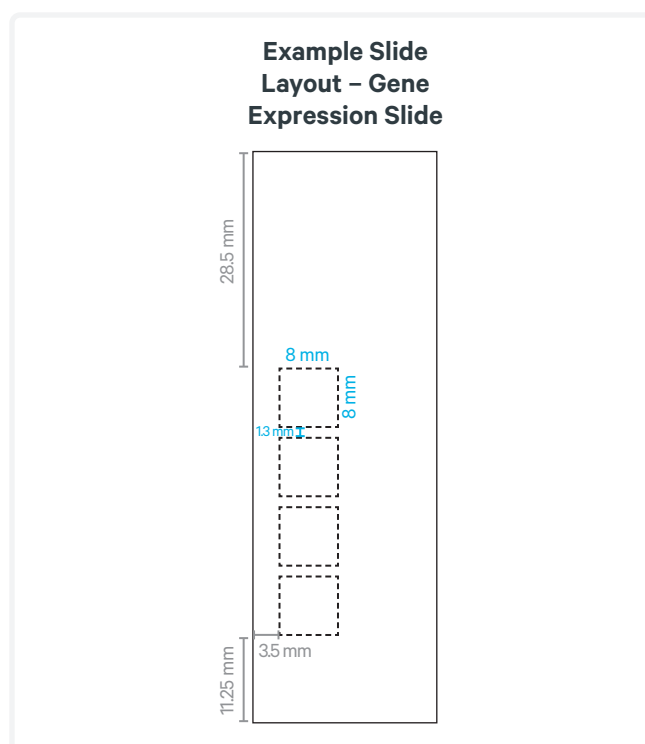
- Monitor section adhesion on the Visium slides throughout the workflow. For more information, consult the Visium Spatial Gene Expression for FFPE – Tissue Paraffin Guide (Demonstrated Protocol CG000408).
- Tissue detachment during the workflow can impact performance.

Antibody Optimization

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

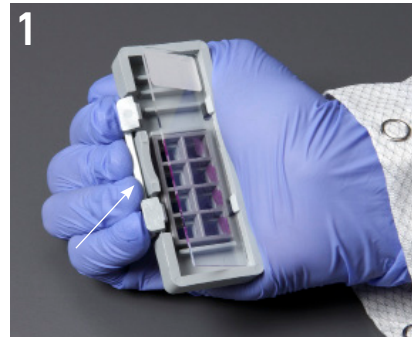
- Optimal antibody concentrations for this Demonstrated Protocol may differ from other applications. Composition of reagents and buffers may also differ from other immunofluorescence applications.
- To optimize antibody concentration, draw representative frames on the back of a 75 x 25 x 1 mm plain glass slide using the example slide layout. Place tissue sections in the frames on the front of the slide for compatibility with the Visium Cassette. Ensure that tissue sections used during optimization are similar in size to tissue sections used for Visium Spatial Gene Expression for FFPE workflow.
- Execute the Deparaffinization, Decrosslinking & Immunofluorescence Staining protocol using a range of antibody concentrations. A starting concentration of 0.01 µg/µl (0.5 µg/sample) is recommended.
- Select the antibody concentration that results in the specific staining of desired cells, while minimizing nonspecific background staining.
- When optimizing the antibody, ensure that stained slides can be imaged according to the imaging guidelines listed in Visium Spatial Gene Expression for FFPE Imaging Guidelines Technical Note (CG000436).
- Wash Visium Cassette and Visium Gasket after immunofluorescence staining. See Visium Cassette & Visium Gasket Cleaning for more information.

TIPS

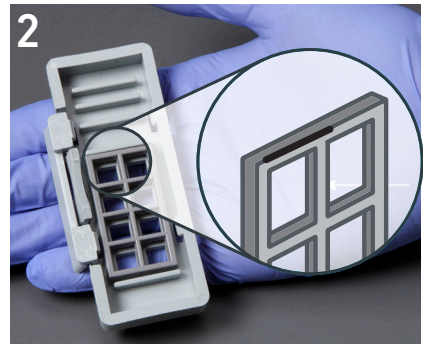


Visium Cassette and Visium Gasket Cleaning

Remove slide from Visium Cassette



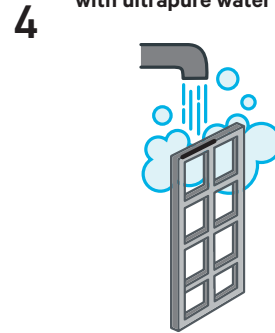
Mark the top portion of the Visium Gasket that faced the slide with an alcohol resistant marker



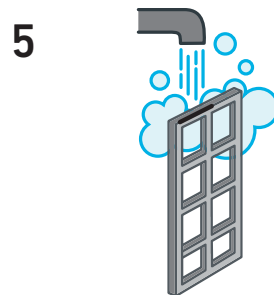
Remove Visium Gasket



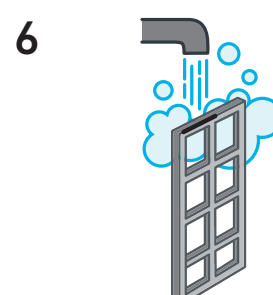
Rinse Visium Cassette and Gasket with ultrapure water



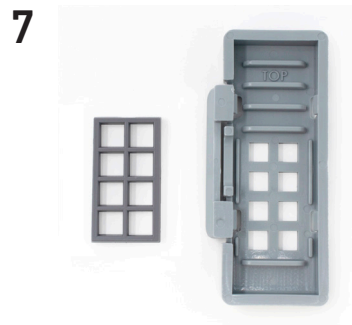
Spray with 70% isopropanol, then rinse with ultrapure water



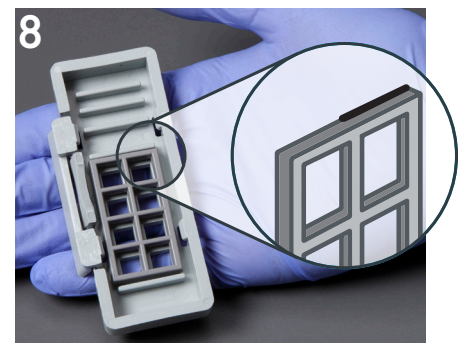
Spray with 70% isopropanol a second time, then rinse with ultrapure water



Air dry





Re-insert the Visium Gasket such that the marked portion is at the top of the Visium Cassette and now faces the Visium Cassette.



Protocol Steps & Timing

~5.5 hours

	Steps		Timing	Stop & Store
	Step 1 – Deparaffinization			
	1.2	Deparaffinization	60 min	
	Step 2 – Decrosslinking			
	1.3	Decrosslinking	60 min	
	Step 3 – Immunofluorescence Staining			
	1.4	Immunofluorescence Staining		
		<i>60-150 min depending upon the type of immunofluorescence staining performed, see below.</i>		
		1.4A Fluorophore Conjugated Primary Antibodies	60 min	
		OR	150 min	
	Step 4 – Tissue Imaging			
	2.2	Imaging	variable	
	2.3	Coverslip Removal	5 min	 4°C ≤2 weeks

1. Deparaffinization, Decrosslinking & Immunofluorescence (IF) Staining

1.0 Overview

This chapter provides guidance on deparaffinization, decrosslinking, and immunofluorescence (IF) staining of Visium slides containing FFPE tissue sections that are dried overnight in a desiccator. Ensure that microscope settings have been verified and imaging programs have been created prior to starting this program. Consult the Visium Spatial Gene Expression for FFPE Imaging Guidelines Technical Note (CG000436) 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).



1.1 Preparation - Buffers

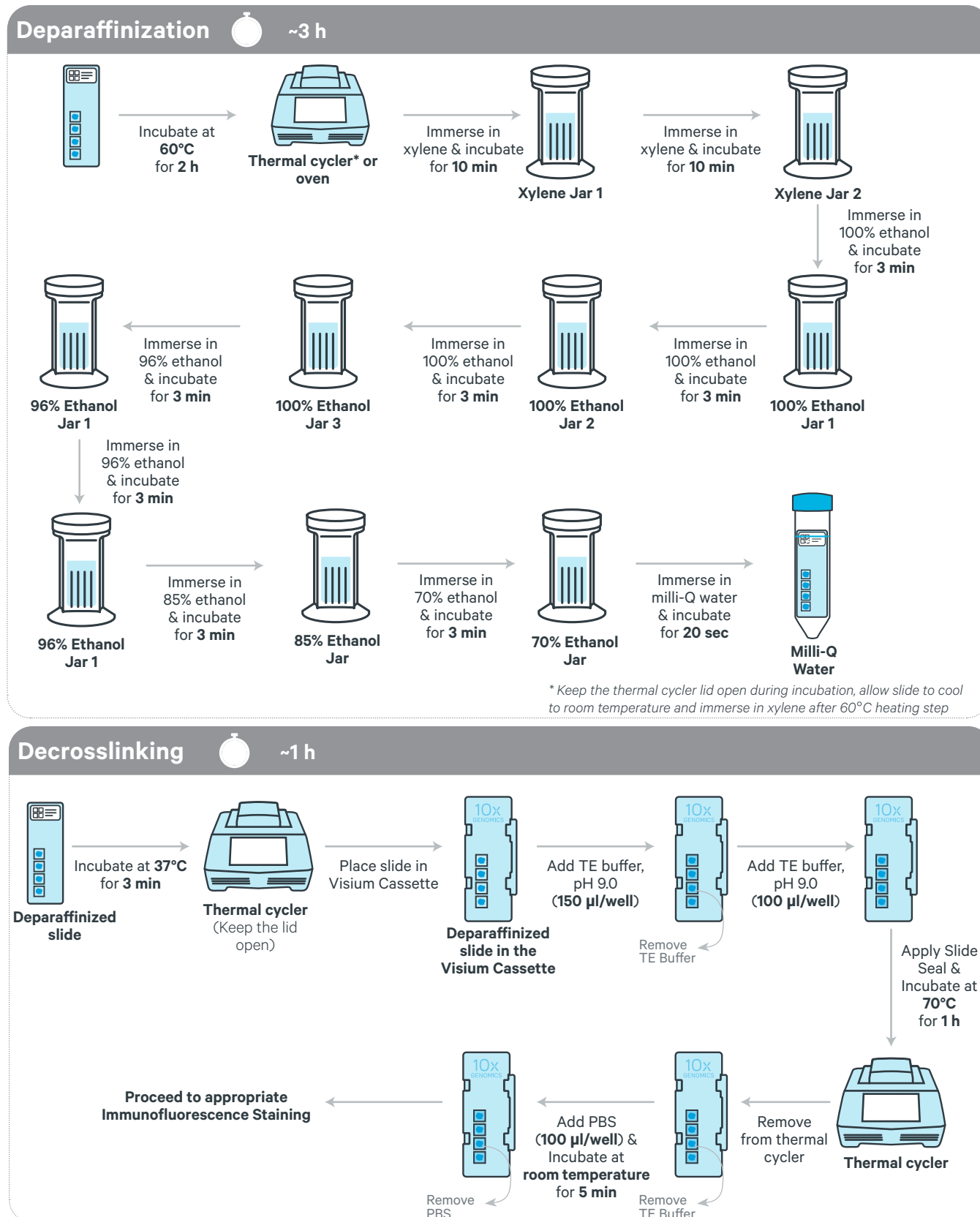
For Deparaffinization		
Items	Preparation & Handling	
<input type="checkbox"/> Xylene	Label two coplin jars as Xylene Jar 1 and 2. Dispense 30 ml xylene in each.	
<input type="checkbox"/> 100% Ethanol	Label three coplin jars as 100% Ethanol Jar 1, 2, and 3. Dispense 30 ml 100% ethanol in each.	
<input type="checkbox"/> 96% Ethanol	Label two coplin jars as 96% Ethanol Jar 1 and 2. Dispense 30 ml 96% ethanol in each.	
<input type="checkbox"/> 70% Ethanol	Label one coplin jar as 70% Ethanol Jar. Dispense 30 ml 70% ethanol.	
<input type="checkbox"/> Milli-Q Water	Label one coplin jar as Milli-Q Water Jar. Dispense 30 ml milli-Q water. Alternatively, use a 50-ml centrifuge tube or a beaker for each individual slide. Clean beaker with Milli-Q Water then RNase decontamination solution in between use.	

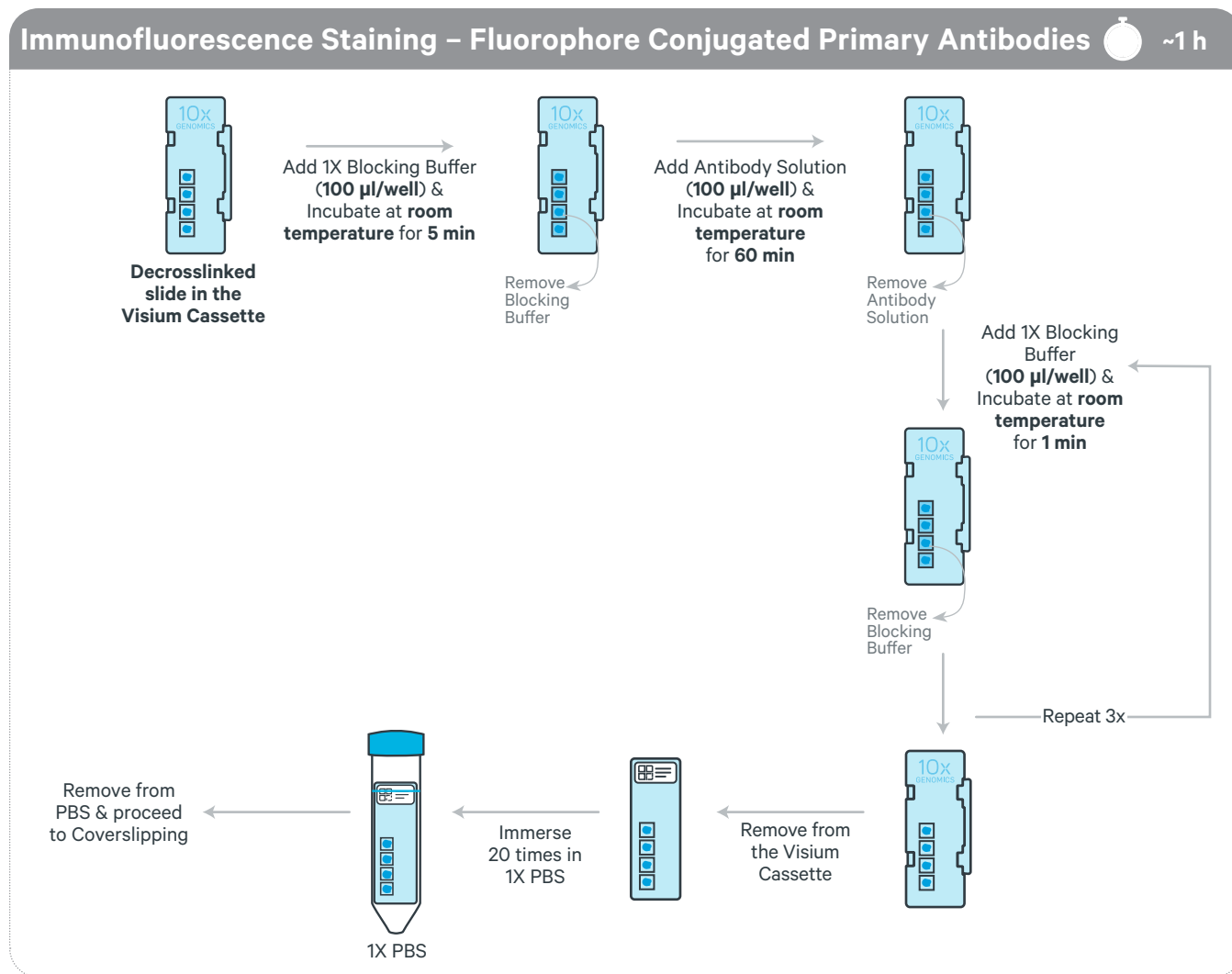


Alternatively, a slide staining dish can also be used in place of a coplin jar. Adjust the volumes of deparaffinization solutions and water, accordingly. Use xylene-resistant dishes, for immersion in xylene. Use xylene-resistant gloves or forceps for deparaffinization. Prepare fresh reagents every week. Use fresh reagents after every 20 slides or every month (whichever comes first).

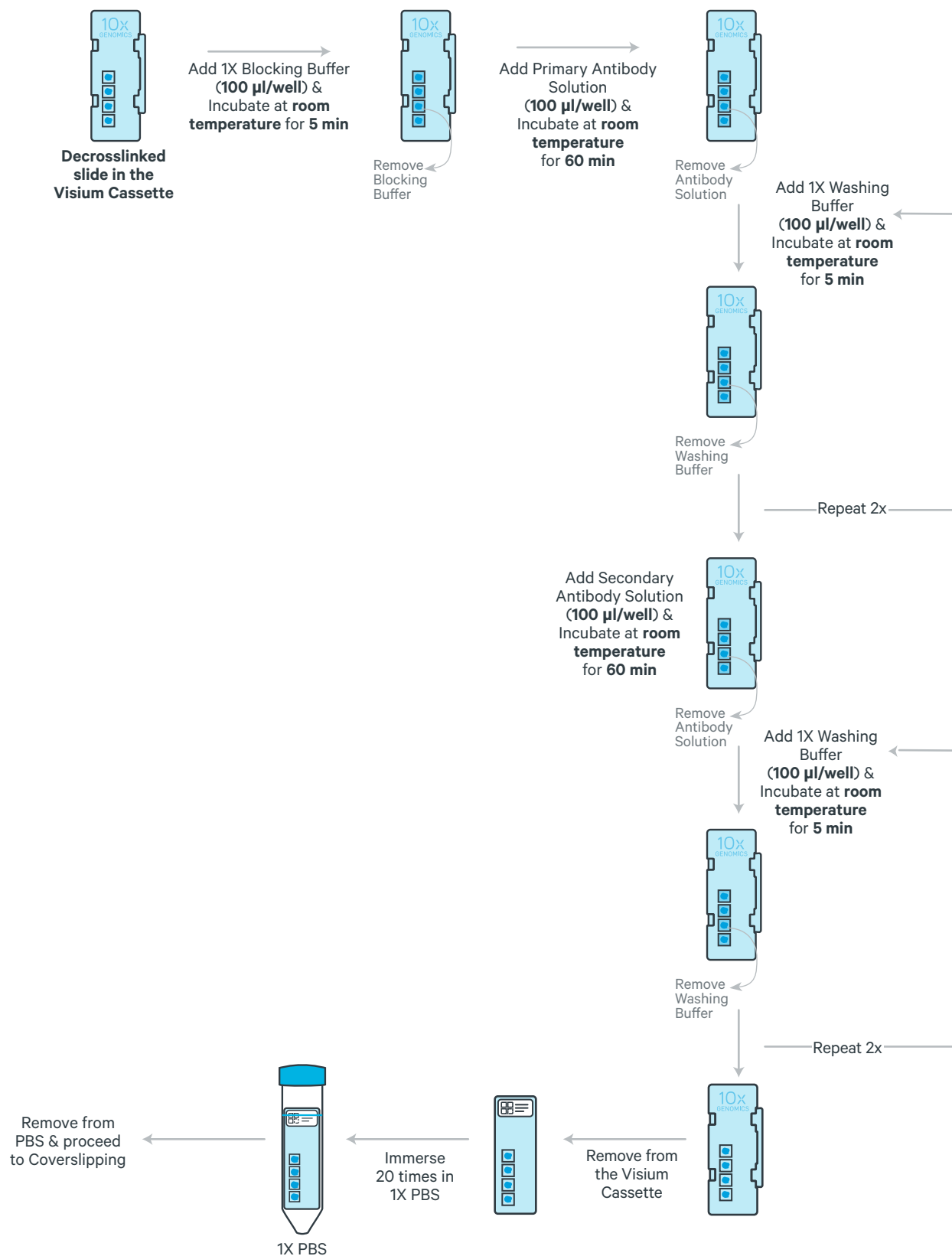
For Decrosslinking		
Items	Preparation & Handling	
<input type="checkbox"/> 1X PBS	Prepare 1X PBS using nuclease-free water.	
<input type="checkbox"/> TE Buffer, pH 9.0 Or		
<input type="checkbox"/> Prepare TE Buffer, pH 9.0	TE Buffer Preparation (Prepare fresh and maintain at room temperature) <ul style="list-style-type: none"> Dissolve 1.21 g Tris base in 950 ml nuclease-free water. Adjust the pH to 9.0 with 1.0 M HCl. Add 2 ml of 0.5 M EDTA and adjust the pH to 9.0 using 1.0 M HCl. Bring the volume to 1000 ml using nuclease-free water. 	

Protocol Overview





Immunofluorescence Staining – Primary and Secondary Antibodies ~2.5 h



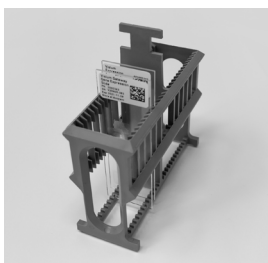
1.2 Deparaffinization

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

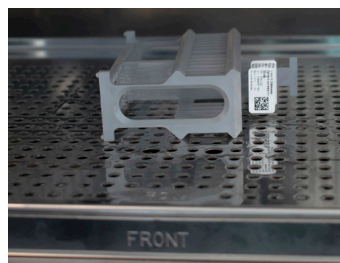
Visium Spatial slide contains a readable label with a serial number on the active surface of the slide. The label may come off during deparaffinization steps. In such cases, the serial number etched on the slide can be used.

- a. Retrieve the slide 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 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 Thermocycler Adaptor on a thermal cycler set at **60°C**. Place slide on the Thermocycler Adaptor with the active surface facing up and incubate **2 h** at **60°C**. **DO NOT** close the thermal cycler lid.

Incubation in a Thermal Cycler



Place Thermocycler Adaptor



Incubate Slide for 2 h at 60°C

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

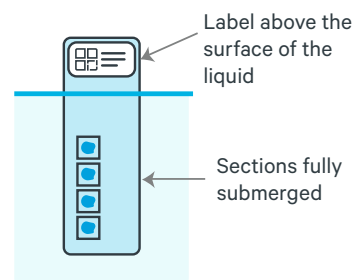
- d. Gently immerse the slide in the xylene in Xylene Jar 1. Secure the jar cap to prevent xylene loss.



When immersing slides in xylene, ensure that the tissue sections are completely submerged and the xylene doesn't reach the label.

- e. Incubate for **10 min.**
- f. During incubation, set up a thermal cycler to **37°C**. Place a Thermocycler Adaptor on the thermal cycler.
- g. Gently immerse slide in the xylene in Xylene Jar 2 and incubate for **10 min.**
- h. Gently immerse slide in the 100% Ethanol Jar 1 for **3 min.**
- When immersing slides in ethanol, ensure that the tissue sections are completely submerged and the ethanol doesn't reach the label.*
- i. Gently immerse slide in the 100% Ethanol Jar 2 for **3 min.**
- j. Gently immerse slide in the 100% Ethanol Jar 3 for **3 min.**
- k. Gently immerse slide in the 96% Ethanol Jar 1 for **3 min.**
- l. Gently immerse slide in the 96% Ethanol Jar 2 for **3 min.**
- m. Gently immerse slide in the 85% Ethanol Jar for **3 min.**
- n. Gently immerse slide in the 70% Ethanol Jar for **3 min.**
- o. Gently immerse slide in the water in the tube/beaker and incubate for **20 sec.**
- p. Let the slide air dry and proceed to Decrosslinking.

Submerge in Xylene/Ethanol



1.3 Decrosslinking

Reagent addition and removal should be done carefully. Remove reagents along the side of the wells without touching the tissue sections and without introducing bubbles.



- a. Place a Thermocycler Adaptor on a thermal cycler set at **37°C**. Place slide on the Thermocycler Adaptor with the active surface facing up and incubate **3 min** at **37°C**. DO NOT close the thermal cycler lid.
- b. Prepare the thermal cycler with the following incubation protocol and start the program.

Lid Temperature	Reaction Volume	Run Time
70°C	-	1 h
Step	Temperature	Time
Pre-equilibrate	70°C	Hold
Decrosslinking	70°C	00:60:00
Hold	22°C	Hold



- c. Place the slide in the Visium Cassette.
See Tips & Best Practices for assembly instructions.
Practice assembly with a blank slide.
- d. Add **150 µl** TE Buffer (pH 9.0) along the side of the wells.
- e. Remove all TE Buffer from the wells.
- f. Add **100 µl** TE Buffer (pH 9.0) along the side of the wells.
- g. Apply Visium Slide Seal on the Visium Cassette and place the cassette on the Thermocycler Adaptor at **70°C**.
- h. Close the thermal cycler lid. Skip pre-equilibrate step and initiate Decrosslinking.
- i. After decrosslinking is complete, remove the cassette from the Thermocycler Adaptor and place on a flat, clean work surface.
- j. Remove the slide seal and using a pipette, remove all TE Buffer from the well corners.
- k. Add **100 µl** 1X PBS along the side of the wells to uniformly cover the tissue sections, without introducing bubbles.
- l. Incubate for **5 min** at **room temperature**.
- m. Remove all PBS from the wells.
- n. Proceed **immediately** to appropriate Immunofluorescence Staining.

1.4 Immunofluorescence Staining

Choose appropriate staining protocol depending upon the antibodies used

1.4A Immunofluorescence Staining – Fluorophore Conjugated Primary Antibodies

- a. Prepare 2X Blocking Buffer on ice. Pipette mix 10x and centrifuge briefly.



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

2X Blocking Buffer <i>Add reagents in the order listed. Maintain on ice</i>	Stock	Final	1X (μl)	4X+ 10% (μl)	8X + 10% (μl)
PBS	10X	2X	80.0	352.0	704.0
BSA	10%	4%	160.0	704.0	1408.0
Tween-20	10%	0.2%	8.0	35.2	70.4
Protector RNase Inhibitor	40 U/ μ l	2 U/ μ l	20.0	88.0	176.0
Nuclease-free Water	-		132.0	580.8	1161.6
Total	-		400.0	1760.0	3520.0

- b. Prepare Antibody Solution on ice. Pipette mix 10x and centrifuge briefly.

Antibody Solution <i>Add reagents in the order listed. Maintain on ice</i>	Stock	Final	1X (μl)	4X+ 10% (μl)	8X + 10% (μl)
2X Blocking Buffer	2X	1X	50.0	220.0	440.0
Nuclease-free Water	Variable	Variable	Variable	Variable	Variable
Diluted DAPI Dilute 1:100 in nuclease-free water before using.	10 μ g/ml	0.2 μ g/ml	2.0	8.8	17.6
Antibody #1	Variable	Variable	Variable	Variable	Variable
Antibody #2 (Optional)	Variable	Variable	Variable	Variable	Variable
Antibody #3 (Optional)	Variable	Variable	Variable	Variable	Variable
Total	-	-	100.0	440.0	880.0

*Antibody dilution can change depending on the antibody, ranging from 1:50 up 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.

- c. Prepare 1X Blocking Buffer on ice. Pipette mix 10x and centrifuge briefly.

1X Blocking Buffer <i>Add reagents in the order listed. Maintain on ice</i>	Stock	Final	1X (μl)	4X+ 10% (μl)	8X + 10% (μl)
2X Blocking Buffer	2X	1X	250.0	1100.0	2200.0
Nuclease-free Water	-	-	250.0	1100.0	2200.0
Total	-	-	500.0	2200.0	4400.0

- d. Add **100 μ l** 1X Blocking Buffer along the side of the wells.
- e. Incubate for **5 min** at **room temperature**.
- f. Remove all Blocking Buffer from the wells.
- g. Add **100 μ l** Antibody Solution along the side of the wells. Tap gently to ensure uniform coverage.
- h. Incubate for **60 min** at **room temperature**.
- i. Remove Antibody Solution
- j. Add **100 μ l** 1X Blocking Buffer along the side of the wells.
- k. Incubate for **1 min** at **room temperature**.
- l. Remove all Blocking Buffer from the wells.
- m. **Repeat** j-l three more times for a total of four washes.
- n. Remove the slide from Visium Cassette.
See Tips & Best Practices for removal instructions.
- o. Gently immerse slide 20 times in 1X PBS in 50-ml centrifuge tube.
- p. Remove slide from the PBS and proceed **immediately** to Coverslipping.
DO NOT let the slide dry.

TIPS


1.4B Immunofluorescence Staining – Primary and Secondary Antibodies

- a. Prepare 2X Blocking Buffer on ice. Pipette mix 10x and centrifuge briefly.

2X Blocking Buffer <i>Add reagents in the order listed. Maintain on ice</i>	Stock	Final	1X (μl)	4X+ 10% (μl)	8X + 10% (μl)
PBS	10X	2X	80.0	352.0	704.0
BSA	10%	4%	160.0	704.0	1408.0
Tween-20	10%	0.2%	8.0	35.2	70.4
Protector RNase Inhibitor	40 U/ μ l	2 U/ μ l	20.0	88.0	176.0
Nuclease-free Water	-		132.0	580.8	1161.6
Total	-		400.0	1760.0	3520.0

- b. Prepare 1X Blocking Buffer on ice. Pipette mix 10x and centrifuge briefly.

1X Blocking Buffer <i>Add reagents in the order listed. Maintain on ice</i>	Stock	Final	1X (μl)	4X+ 10% (μl)	8X + 10% (μl)
2X Blocking Buffer	2X	1X	250.0	1100.0	2200.0
Nuclease-free Water	-	-	250.0	1100.0	2200.0
Total	-	-	500.0	2200.0	4400.0

- c. Prepare Primary Antibody Solution on ice. Pipette mix 10x and centrifuge briefly.

Primary Antibody Solution <i>Add reagents in the order listed. Maintain on ice</i>	Stock	Final	1X (μl)	4X+ 10% (μl)	8X + 10% (μl)
2X Blocking Buffer	2X	1X	50.0	220.0	440.0
Antibody #1	Variable	Variable	Variable	Variable	Variable
Antibody #2 (Optional)	Variable	Variable	Variable	Variable	Variable
Antibody #3 (Optional)	Variable	Variable	Variable	Variable	Variable
Total	-	-	100.0	440.0	880.0

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

- d. Prepare 1X Washing Buffer. Pipette mix 10x and centrifuge briefly.

1X Washing Buffer <i>Add reagents in the order listed. Maintain on ice</i>	Stock	Final	1X (μl)	4X+ 10% (μl)	8X + 10% (μl)
PBS	10X	1X	100.0	440.0	880.0
Tween-20	10%	0.1%	10.0	44.0	88.0
Nuclease-free Water	-		890.0	3916.0	7832.0
Total	-		1000.0	4400.0	8800.0

- e. Prepare Secondary Antibody Solution on ice. Pipette mix 10x and centrifuge briefly.

Secondary Antibody Solution <i>Add reagents in the order listed. Maintain on ice</i>	Stock	Final	1X (μl)	4X+ 10% (μl)	8X + 10% (μl)
2X Blocking Buffer	2X	1X	50.0	220.0	440.0
Diluted DAPI Dilute 1:100 in nuclease-free water before using.	10 μ g/ml	0.2 μ g/ml	2.0	8.8	17.6
Antibody #1	Variable	Variable	Variable	Variable	Variable
Antibody #2 (Optional)	Variable	Variable	Variable	Variable	Variable
Antibody #3 (Optional)	Variable	Variable	Variable	Variable	Variable
Total	-	-	100.0	440.0	880.0

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

- f. Add **100 μ l** 1X Blocking Buffer along the side of the wells.
- g. Incubate for **5 min** at **room temperature**.
- h. Remove all Blocking Buffer from the wells.
- i. Add **100 μ l** Primary Antibody Solution along the side of the wells. Tap gently to ensure uniform coverage.
- j. Incubate for **1h** at **room temperature**.

- k.** Remove Primary Antibody Solution.
- l.** Add **100 µl** 1X Washing Buffer along the side of the wells.
- m.** Incubate for **5 min** at **room temperature**.
- n.** Remove 1X Washing Buffer from the wells.
- o.** **Repeat** l-n two more times for a total of three washes.
- p.** Add **100 µl** Secondary Antibody Solution along the side of the wells. Tap gently to ensure uniform coverage.
- q.** Incubate for **60 min** at **room temperature**.
- r.** Remove Secondary Antibody Solution.
- s.** Add **100 µl** 1X Washing Buffer along the side of the wells.
- t.** Incubate for **5 min** at **room temperature**.
- u.** Remove 1X Washing Buffer from the wells.
- v.** **Repeat** s-u two more times for a total of three washes.
- w.** Remove the slide from Visium Cassette.
See Tips & Best Practices for removal instructions.
- x.** Gently immerse slide 20 times in 1X PBS in 50-ml centrifuge tube.
- y.** Remove slide from the PBS and proceed **immediately** to Coverslipping.
DO NOT let the slide dry.

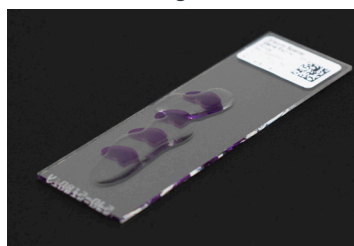


1.5 Coverslipping

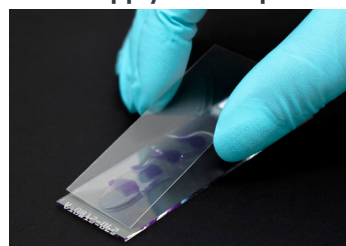
- a. Place slide on a flat, clean, non-absorbent work surface. Some residual droplets may remain.
- b. Add **5 drops** of SlowFade Diamond Antifade Mountant to uniformly cover all tissue sections on the slide.
- c. Apply the coverslip at an angle on one end of the slide. Slowly lower the coverslip, without 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.



Cover uniformly with
mounting medium



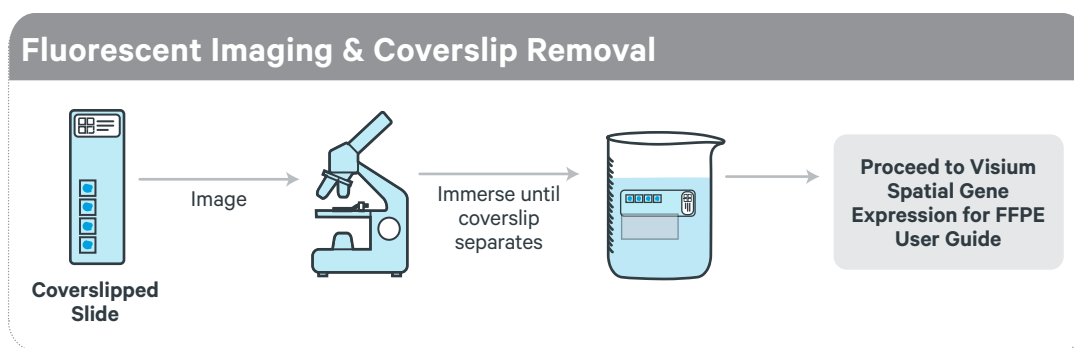
Apply coverslip



2. Tissue Imaging

2.0 Overview

This chapter provides guidance on imaging Visium slides containing immunofluorescent stained FFPE sections and 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

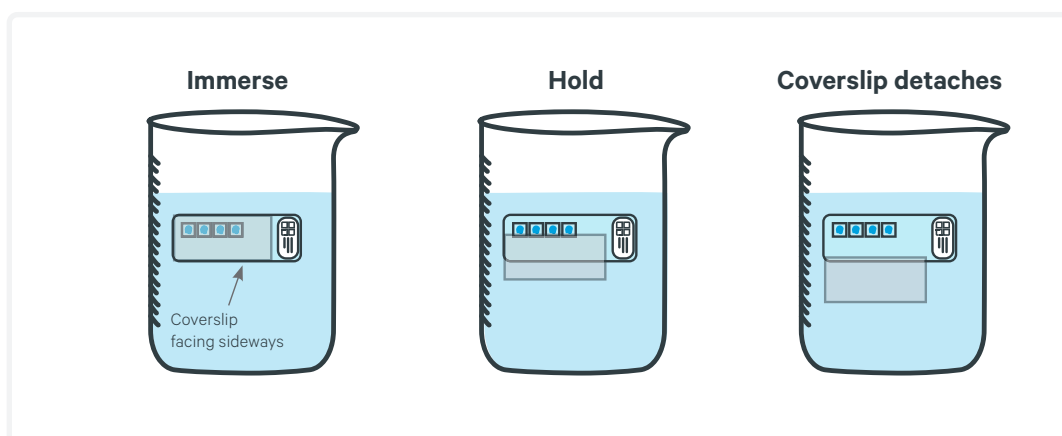
Fluorescence Recommended Configuration	
Light source (or equivalent) with a wavelength range of 380-680 nm	
Monochrome camera (14 bit, 2,424 x 2,424 pixel resolution)	
DAPI filter cube (Excitation 392/23, Emission 447/60)	
FITC filter cube (Excitation 480/40, Emission 535/50)	
TRITC filter cube (Excitation 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 Capture Areas individually at the desired magnification using fluorescence imaging settings. Ensure that fiducial frames are captured.
- b. Consult the Visium Spatial Gene Expression for FFPE Imaging Guidelines Technical Note (CG000436) for additional information.
- c. After imaging, proceed **immediately** to the Coverslip Removal.

2.3 Coverslip Removal

- a. Dispense **800 ml** Milli-Q water in a beaker.
- b. Remove the coverslip immediately after imaging is complete.
- c. Immerse the slide sideways/horizontal in the beaker containing **800 ml** Milli-Q water with the coverslipped surface fully sideways.
- d. Hold the slide in water until the coverslip slowly separates away from the slide.
To avoid damaging the tissue sections and Capture Areas or causing tissue detachment, DO NOT move the slide up and down, shake forcibly or manually move the coverslip.



- e. Gently immerse 15x in the Milli-Q water to ensure all mounting medium is removed.
- f. Wipe the back of the slide with a laboratory wipe. Place on a flat, clean, non-absorbent work surface and air dry.
- g. Incubate slide on the Thermocycler Adaptor with the thermal cycler lid open for **3 min** at **37°C**.
- h. Remove from the thermal cycler.
- i. Store the slide in a slide mailer with a desiccator or 50 -ml tube at **4°C** for up to **2 weeks**.
- j. Place the slide in the Visium Cassette. See Tips & Best Practices for assembly instructions.
- k. Add **100 µl** 1X PBS along the side of the wells and proceed **immediately** to Visium Spatial Gene Expression for FFPE User Guide (CG000407).



Troubleshooting

STEP	Notes
Tissue detachment	<ul style="list-style-type: none"> If tissue detachment is observed during the workflow, contact support@10xgenomics.com.
2.2 Weak or no signal	<ul style="list-style-type: none"> 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 prior to immunofluorescence staining. Verify imaging system filter cubes and wavelength. Ensure that fluorophores and filter cubes match. Protein of interest may have low expression. Test antibodies on tissues of interest prior to working with Visium Spatial slides.
2.2 High background	<ul style="list-style-type: none"> 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.

Results

Performing the Deparaffinization, Decrosslinking, Immunofluorescence Staining & Imaging protocol will likely result in a decrease in the number of unique transcripts detected for many tissue types with no impact on the fraction of reads mapped confidently to the transcriptome, as compared to the Deparaffinization, H&E Staining, Imaging & Decrosslinking protocol (CG000409). However, this should not affect interpretation of experimental results.

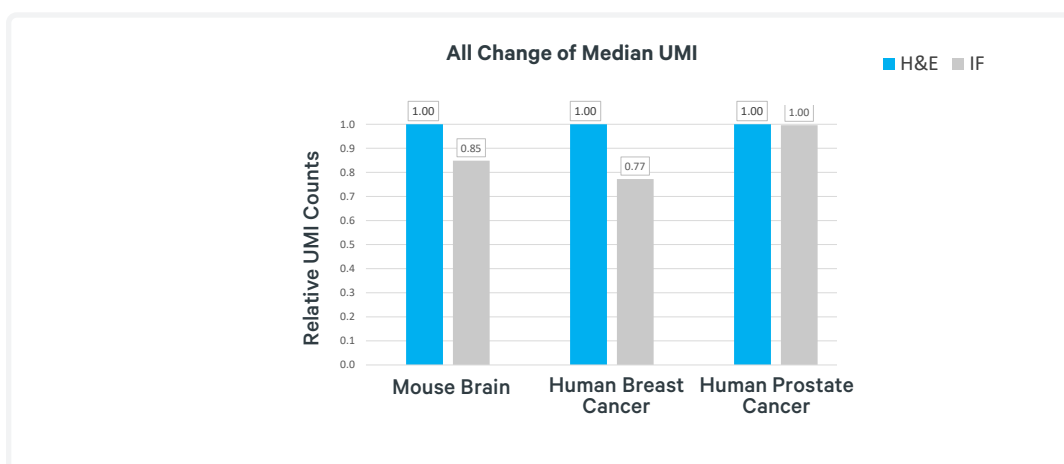


Figure 1. All change in UMI counts when downsampled to the same sequencing depth of 50,000 reads per spot.

Document Revision Summary

Document Number	CG000410
Title	Visium Spatial Gene Expression for FFPE – Deparaffinization, Decrosslinking, IF Staining & Imaging
Revision	Rev C to Rev D
Revision Date	July 2022
General Changes	Updated for general minor consistency of language and terms throughout
Specific Changes	<ul style="list-style-type: none">• Updated step p on Deparaffinization (page 22).• Updated 2X Blocking Buffer volume for Immunofluorescence staining (page 24, 26).

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