DEMONSTRATED PROTOCOL

Methanol Fixation, H&E Staining & Imaging for Visium Spatial Protocols

Overview

The Visium Spatial Gene Expression Solution measures the total mRNA in tissue sections and requires a Visium Spatial slide with intact tissue sections as input. This protocol outlines methanol fixation, Hematoxylin & Eosin (H&E) staining, and imaging of tissue for use with 10x Genomics Visium Spatial protocols. Fixed and stained tissue sections are inputs for the downstream Visium Spatial Tissue Optimization and Visium Spatial Gene Expression workflows.

Additional Guidance

Consult the Visium Spatial Protocols - Tissue Preparation Guide (Document CG000240) for Tips & Best Practices on freezing, embedding, and cryosectioning tissue and placing sections on Visium Spatial Slides. Consult the Visium Spatial Gene Expression Imaging Guidelines (Document CG000241) to verify imaging settings prior to starting this Demonstrated Protocol.

Perform this Demonstrated Protocol on tissue sections placed on the correct slide.

- Use a Visium Spatial Tissue Optimization slide if performing tissue optimization.
- Use a Visium Spatial Gene Expression slide if proceeding with library construction.

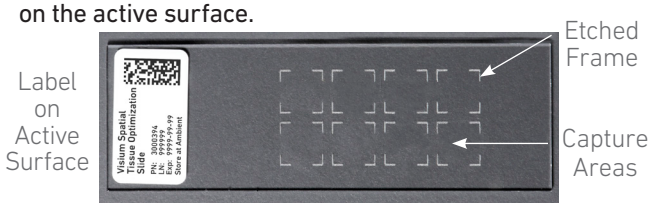
The Tissue Optimization workflow must be performed prior to the Gene Expression workflow to determine the ideal tissue section permeabilization time.

After completing this Demonstrated Protocol (CG000160), proceed with either the Visium Spatial Gene Expression Reagent Kits - Tissue Optimization User Guide (CG000238) or the Visium Spatial Gene Expression Reagent Kits User Guide (CG000239).

Visium Slide Selection

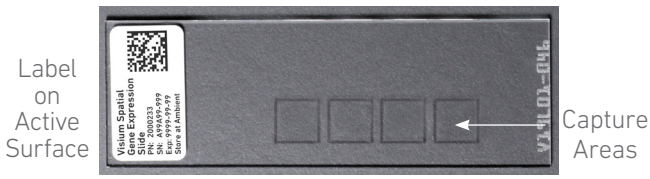
Visium Spatial Tissue Optimization Slide (PN-3000394)

- Used with Visium Spatial Gene Expression Reagent Kits – Tissue Optimization User Guide (CG000238) to identify optimal permeabilization time for a specific tissue type and section thickness.
- Includes 8 Capture Areas, each covered with oligonucleotides for mRNA capture.
- Each Capture Area is 8 x 8 mm and is surrounded by an etched frame.
- A readable label defines the active surface of the slide. Tissue sections are always placed on the Capture Areas on the active surface.



Visium Spatial Gene Expression Slide (PN-2000233)

- Used with Visium Spatial Gene Expression Reagent Kits User Guide (CG000239) to generate Visium Spatial Gene Expression libraries.
- Includes 4 Capture Areas, each with ~5,000 unique gene expression spots.
- Each Capture Area is 6.5 x 6.5 mm and is surrounded by a fiducial frame for a total area of 8 x 8 mm.
- A readable label with a serial number defines the active surface of the slide. Tissue sections are always placed on the Capture Areas on the active surface.



Visium Spatial Reagent Kits

Ensure that tissue sections have been placed onto the appropriate slide prior to starting this Demonstrated Protocol. Consult the Visium Spatial Protocols - Tissue Preparation Guide (CG000240) for more information.

Visium Spatial Tissue Optimization Slide Kit PN-1000191 (store at ambient temperature)

Visium
Spatial Tissue Optimization Slide Kit

	PN
Visium Spatial Tissue Optimization Slide	3000394
*Slide Seals	3000279
*Slide Cassette	3000406
*Slide Gasket	3000426
*Tissue Removal Buffer	2000221
*Tissue Removal Enzyme	3000387

*Not used in this protocol

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Visium Spatial Gene Expression Slide Kit, 16 rxns PN-1000185, 4 rxns PN-1000188 (store at ambient temperature)

Visium
Spatial Gene Expression Slide Kit

	PN
Visium Spatial Gene Expression Slide	2000233
*Slide Seal	3000279
*Slide Cassette	3000406
*Slide Gasket	3000426

*Not used in this protocol

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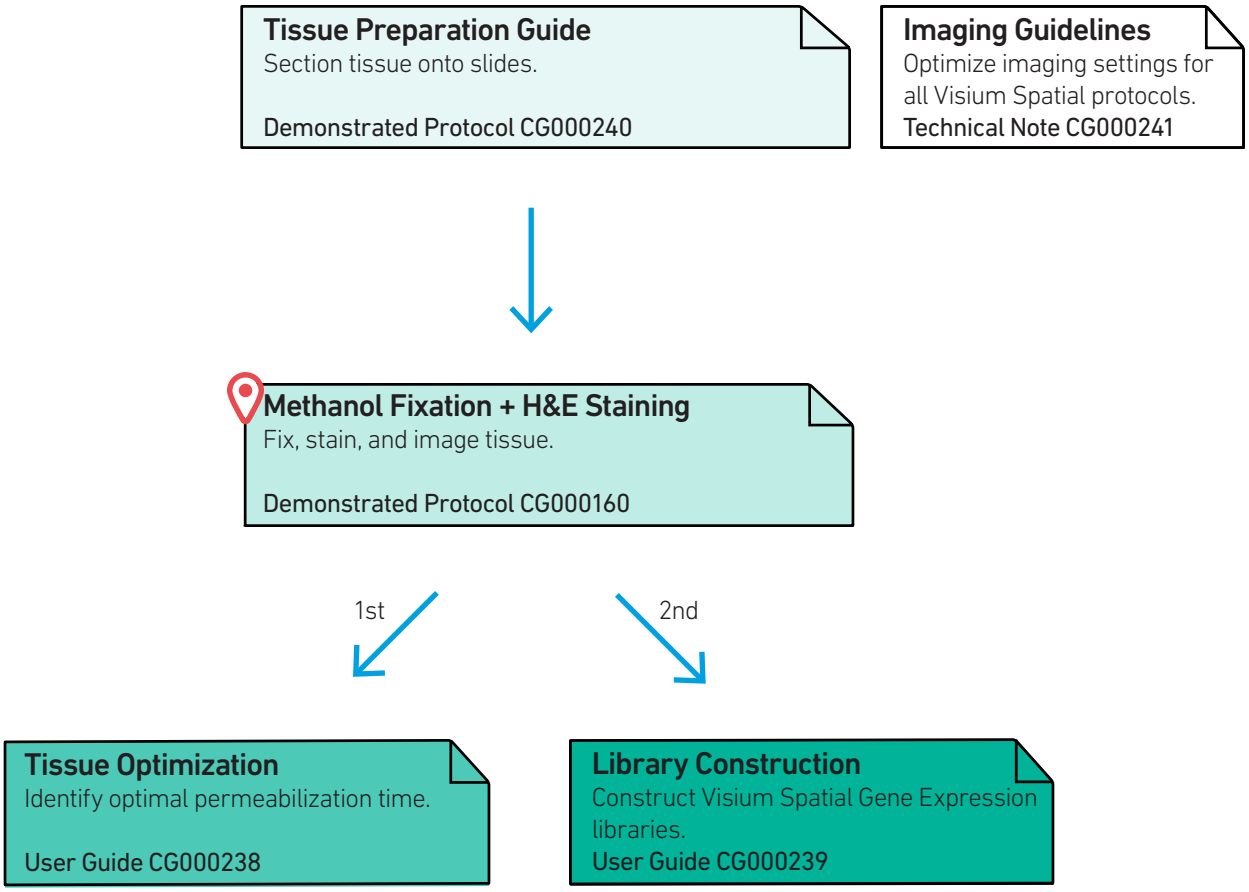
Visium Accessories

Product	Part Number (Kit)	Part Number (Item)
Thermocycler Adaptor	1000194	3000380
*Visium Imaging Test Slide		2000235
*Slide Alignment Tool		3000433

*Not used in this protocol

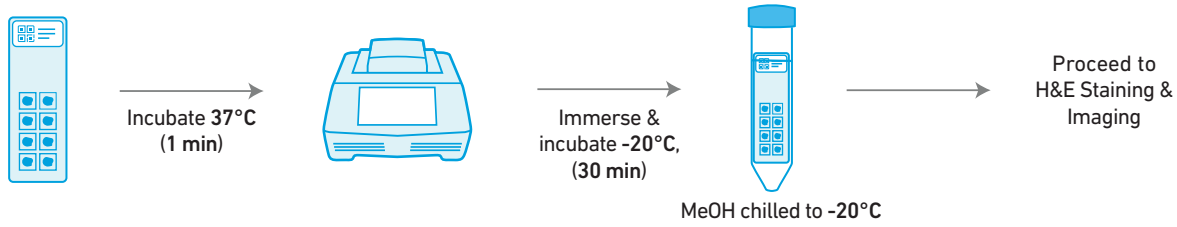
Workflow Overview



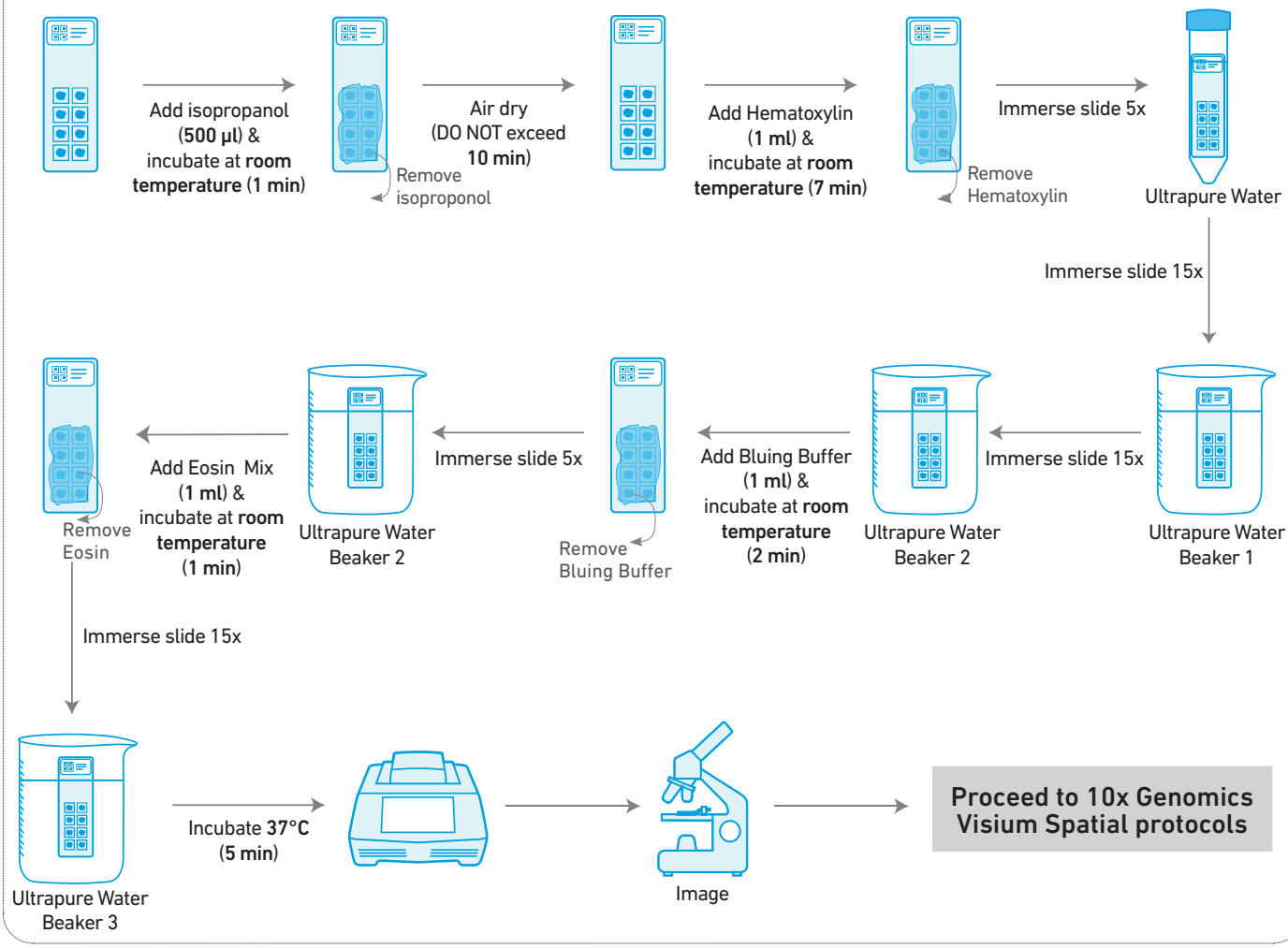
Visit the 10x Genomics Support website for the most current documentation.

Protocol Overview

Fixation 35 min



H&E Staining & Tissue Imaging 30 min*



*Time excludes imaging steps

Tips & Best Practices

Icons

TIPS		
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.
- Promptly move reagents back to the recommended storage.
- Use a pH meter to adjust pH as necessary during buffer preparation.

Pipette Calibration

- Follow manufacturer's calibration and maintenance schedules.

Slide Storage

- Always store unused slides in a cool, dry environment.
- Store unused slides in original packaging and keep sealed. DO NOT remove dessicant. If necessary, place the sealed container in a secondary container, such as a resealable bag.
- After tissue placement, store the slides at **–80°C** in a sealed container for up to **4 weeks**.

Slide Storage

Slide Handling

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

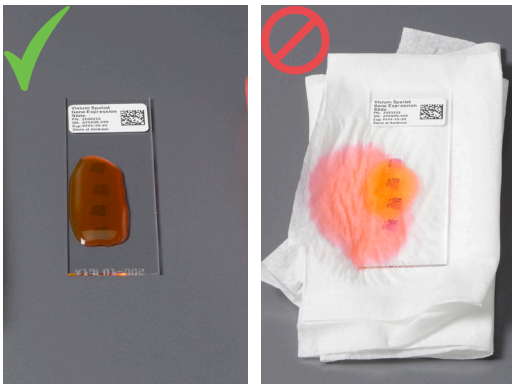
Active Surface with Tissue Sections

Immersing Slide

Correct Incorrect

Reagent on Slide

Correct Incorrect



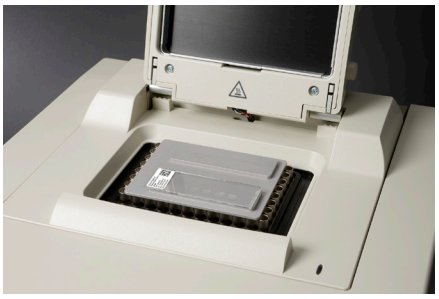
Slide Incubation Guidance

Incubation at a specified temperature

- 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.

Place Thermocycler Adaptor

Incubate Slide

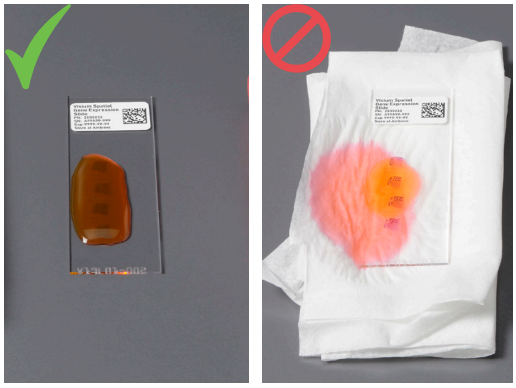


Incubation at room temperature

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

Slide Incubation

Correct Incorrect



1. Tissue Fixation & H&E Staining

1.0 Overview

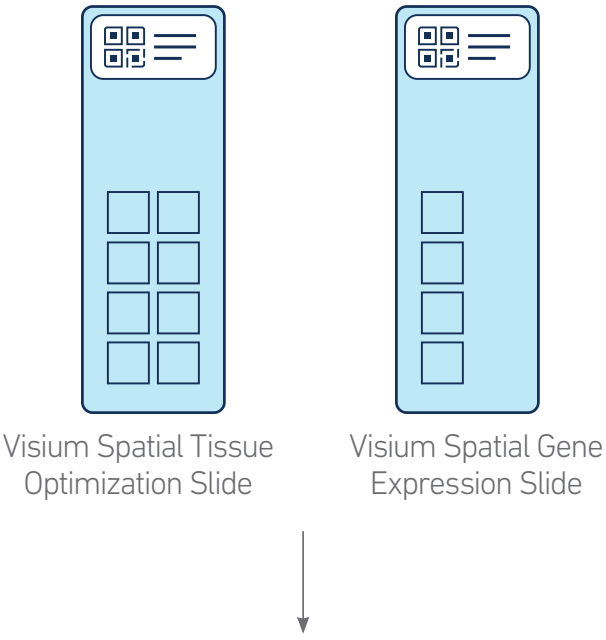
1.1 Specific Reagents & Consumables

1.2 Tissue Fixation

1.3 Tissue H&E Staining

1.0 Overview

Ensure that this protocol is performed on tissue sections placed on the correct slide. Refer to the Introduction and Workflow Overview sections for more information.



Ensure that microscope settings have been verified and imaging programs have been created prior to starting this protocol. Consult the Visium Spatial Gene Expression Imaging Guidelines Technical Note (CG000241) for more information.

1.1 Specific Reagents & Consumables

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

Ensure that tissue sections have been placed on the appropriate slide and stored at -80°C. The slide will be retrieved in step 1.2.

Tissue Fixation		
Vendor	**Item**	**Part Number**
Millipore Sigma	Methanol, for HPLC, ≥99.9%	34860
Tissue H&E Staining		
Vendor	**Item**	**Part Number**
Millipore Sigma	Acetic Acid, ≥99.9% 2-Propanol (Isopropanol), ≥99.5% Eosin Y solution, aqueous Eosin Y-solution, 0.5% aqueous (alternative to HT110216-500ML) Blueing Reagent (alternative to Agilent product) Hematoxylin Solution, Mayer's (alternative to Agilent product) Hematoxylin solution according to Mayer (alternative to Agilent product) Protector RNase Inhibitor	A6283 I9516-25ML HT110216-500ML 1098441000 65354-85 MHS16-500ML 51275-100ML 3335399001
Agilent	Hematoxylin, Mayer's (Lillie's Modification) Bluing Buffer, Dako Eosin, Dako (alternative to Millipore Sigma product)	S30930-2 CS70230-2 CS70130-2
Thermo Fisher Scientific	Tris Base (White Crystals or Crystalline Powder/Molecular Biology) Shandon Bluing Reagent (alternative to Agilent product)	BP152-500 6769001
Corning	Corning 250 mL Vacuum System, 0.2 µm Pore 19.6cm² NY Membrane Self-Standing Polypropylene Centrifuge Tubes, 50 ml, sterile	430771 430921

Additional Materials		
-	Dry Ice	-
-	Ultrapure/Milli-Q water (from Milli-Q Integral Ultrapure Water System or equivalent)	-

Prepare	
Methanol	• Dispense 40 ml/slide in a 50-ml centrifuge tube. Chill to **-20°C** before use.
Tris-Acetic Acid Buffer (0.45 M, pH 6.0) pH meter will be required. pH paper not recommended.	Prepare **200 ml** (200 slides), store at **room temperature**. • Dissolve **11 g** Tris base in **100 ml** nuclease-free water. • Adjust pH to 6.0 using 100% Acetic Acid. • Bring volume to **200 ml** with nuclease-free water. • Filter through **0.2 µm** nylon membrane filter system.

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

1.2 Tissue Fixation

If fixing tissue on a Visium Spatial Gene Expression Slide - Note the serial number on the slide label; will be required for downstream analysis.

Ensure that the methanol (40 ml/slide) dispensed in a 50-ml centrifuge tube is chilled to **-20°C**.

a. Place a Thermocycler Adaptor on a thermal cycler set at **37°C** and equilibrate for **5 min**. Heating the thermal cycler lid is not required.

b. Remove slide from **-80°C** and place on dry ice in a sealed container.

Delay in transferring slides to dry ice may result in condensation, which may cause tissue damage and/or shifting of tissue sections on the slide.

c. Place slide on the Thermocyler Adaptor with the active surface facing up and incubate **1 min** at **37°C**. DO NOT close the thermal cycler lid. Maintain thermal cycler at **37°C** for step 1.2.

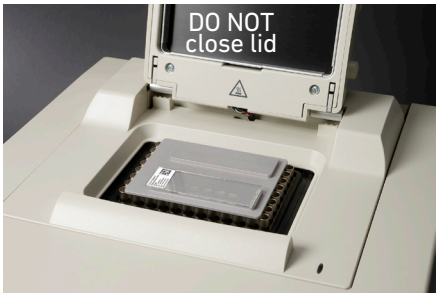
d. Remove slide from Thermocycler Adaptor and if necessary, wipe excess liquid from the back of the slide, without touching the tissue sections.

e. Completely immerse the slide in the pre-chilled methanol. Secure the tube cap to prevent methanol loss.

f. Incubate upright for **30 min** at **-20°C**.

Place Thermocycler Adaptor

Incubate Slide for 1 min at 37°C



Incubate in Methanol for 30 min at –20°C

1.3 Tissue H&E Staining

a. Dispense the following volumes of Milli-Q water.
50 ml in one 50-ml centrifuge tube/slide
800 ml in Beaker 1
800 ml in Beaker 2
800 ml in Beaker 3
Dispensed volume in each beaker can be used for two slides.

b. Prepare Eosin Mix. DO NOT add pure eosin to tissue sections.

Eosin Mix	Volume/slide (µl)
Eosin Y Solution	100
Tris-Acetic Acid Buffer (0.45 M, pH 6.0)	900
Total	**1,000**

c. Remove slide from methanol and wipe excess liquid from the back of the slide, without touching the tissue sections. Place on a flat, clean, nonabsorbant work surface. Some residual droplets may remain.

d. Add **500 µl** isopropanol to uniformly cover all tissue sections on the slide. See Tips & Best Practices.

e. Incubate **1 min** at **room temperature**.
When incubating the slide with reagents, ensure that the slide is not in contact with any absorbent surface, like laboratory wipes, which may absorb the reagents.

f. Discard reagent by draining and/or holding the slide at an angle with the bottom edge in contact with a laboratory wipe.

g. Wipe excess liquid from the back of the slide, without touching the tissue sections. Place on a flat, clean, nonabsorbant work surface.

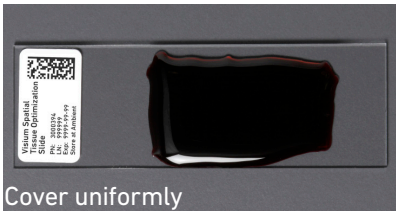
h. Air dry the slide. To prevent tissue section from over drying, inspect slide after **5 min**. DO NOT exceed **10 min**.

i. Add **1 ml** Hematoxylin to uniformly cover all tissue sections on the slide.

j. Incubate **7 min** at **room temperature**.

k. Discard reagent by draining and/or holding the slide at an angle with the bottom edge in contact with a laboratory wipe.

Incubate with Reagent



Discard Reagent

Slides in images are representative.

l. Immerse the slide 5x in the water in centrifuge tube.

m. Immerse the slide 15x in the water in Beaker 1.

n. Immerse the slide 15x in the water in Beaker 2.

o. Wipe excess liquid from the back of the slide without touching the tissue section. Place on a flat, clean, nonabsorbant work surface. Some droplets may remain

Each immersion is ~1 sec

Correct | Incorrect

p. Add **1 ml** Bluing Buffer to uniformly cover all tissue sections.

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

r. Discard reagent by draining and/or holding the slide at an angle with the bottom edge in contact with a laboratory wipe.

s. Immerse the slide 5x in the water in Beaker 2.

t. Wipe excess liquid from the back of the slide without touching the tissue section. Place on a flat, clean, nonabsorbant work surface. Some droplets may remain.

u. Add **1 ml** Eosin Mix to uniformly cover all tissue sections.

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

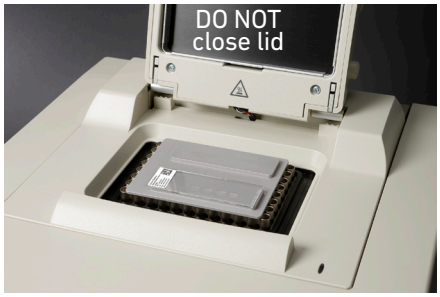
w. Discard reagent by draining and/or holding the slide at an angle with the bottom edge in contact with a laboratory wipe.

x. Immerse the slide 15x in the water in Beaker 3.

y. Wipe the back of the slide with a laboratory wipe. Place on a flat, clean, nonabsorbant work surface and air dry until tissue is opaque.

z. Incubate slide on the Thermocycler Adaptor with the thermal cycler lid open for **5 min** at **37°C**.

Incubate Slide



Proceed to tissue imaging.

OPTIONAL: A coverslip may be mounted on the slide before imaging. See Appendix for Coverslip Application & Removal protocol.

2. Tissue Imaging

2.0 Imaging System Recommendations

2.1 Tissue Imaging

2.0 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. Imaging systems should have both brightfield and fluorescence capacity.

Supplier	Description
Nikon	Nikon Eclipse Ti2
Molecular Devices	ImageXpress Nano Automated Cell Imaging System
Hamamatsu	NanoZoomer S60
Keyence	Keyence BZX800
BioTek	Cytation 7
Thermo Fisher Scientific	EVOS M7000
Leica	Leica DMi8 Versa 8
Brightfield Recommended Configuration	
Color camera (3 x 8 bit, 2424 x 2424 pixel resolution)	
White balancing functionality	
2.18 µm/pixel minimum capture resolution	
Exposure times 2-10 milli sec	
Fluorescence Recommended Configuration (Only required for Tissue Optimization and Imaging Test Slides)	
Light source (or equivalent) with a wavelength range of 380-680 nm	
Monochrome camera (14 bit, 2,424 x 2,424 pixel resolution)	
TRITC filter cube (Excitation 542/20, Emission 620/52)	
2.18 µm/pixel minimum capture resolution	
Exposure times 100 milli sec-2 sec	

2.1 Tissue Imaging

- If imaging a Visium Spatial Tissue Optimization Slide, image all Capture Areas together at the desired magnification using brightfield imaging settings.
- If imaging a Visium Spatial Gene Expression Slide, image all Capture Areas individually at the desired magnification using brightfield imaging settings. Ensure that fiducial frames are captured.
- Consult the Visium Spatial Gene Expression Imaging Guidelines Technical Note (CG000241) for additional information.
- After imaging, proceed immediately to the Visium Spatial Tissue Optimization User Guide (CG000238) or the Visium Spatial Gene Expression user Guide (CG000239).

Appendix

Coverslip Application & Removal

A coverslip may be mounted on the slides before imaging to enhance optical quality. Although imaging without a coverslip is sufficient to visualize the tissue morphology, some imaging systems or higher imaging magnifications require the use of coverslips.

If using a coverslip, follow this application and removal protocol to ensure that the tissue sections and the Capture Areas are not damaged.

Items

- ☐ **Large Coverslip** (Thermo Scientific 24 x 60 mm PN:22-050-233; Alternative, 24 x 50mm PN:22-050-232)
- ☐ **3X SSC** (prepare **500 ml** - add **75 ml** 20X SSC to **425 ml** Ultrapure water)
- ☐ **Mounting Medium** (prepare **200 µl** - add **22.5 µl** RNase Inhibitor (PN 3335399001), **7.5 µl** Nuclease-free water to **170.0 µl** Glycerol)
- ☐ **Laboratory Wipes**
- ☐ **Thermocycler Adaptor** (pre-equilibrated to **37°C** on a thermal cycler; may be used for drying)

Application

Prior to mounting the coverslip, ensure that the sample and the slide with the tissue sections are dry. Moisture on the surface of the slide may result in faulty mounting.

If necessary, incubate the slide for **1 min** at **37°C** by placing on the pre-equilibrated Thermocycler Adaptor placed on a thermal cycler with the lid open.

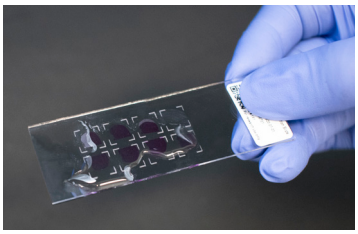
a. Add **200 µl** Mounting Medium to cover the tissue sections on the slide uniformly. If necessary, hold the slide at an angle for uniform coverage.

b. Apply the coverslip at an angle on one end of the slide. Slowly lower the coverslip, pressing down gently with forceps, without introducing bubbles.

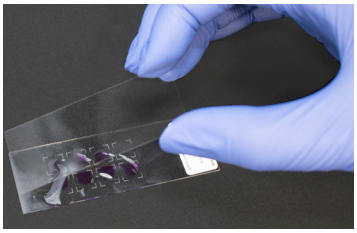
c. Remove excess Mounting Medium by placing one long edge of the slide on a laboratory wipe, and gently tilt the slide back and forth. Repeat with the second long edge of the slide. Repeat the process until the coverslip is secured.

d. After the coverslip is secured, **immediately** proceed with imaging.
DO NOT let the attached coverslip dry.
DO NOT use Cytoseal or nail polish for securing the coverslip.

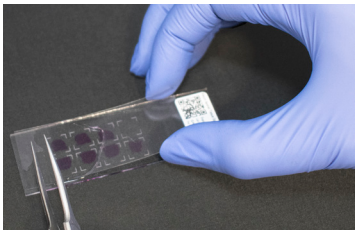
Cover uniformly with Mounting Medium



Apply coverslip



Press down



Remove excess Mounting Medium

Coverslip Application & Removal

Removal

Remove the coverslip immediately after imaging is complete.

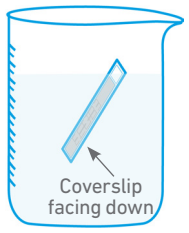
a. Immerse the slide at ~45° angle in the 3X SSC Buffer with the coverslipped surface fully submerged and facing down.

b. Hold the slide in 3X SSC Buffer until the coverslip slowly separates away from the slide.
DO NOT move the slide up and down or shake forcibly to prevent damaging the tissue sections and the Capture Areas.

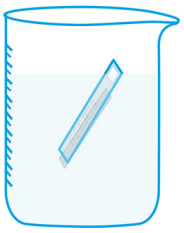
c. Remove the slide from the 3X SSC Buffer and immerse 1x in the 3X SSC Buffer to ensure all Mounting Medium is removed.

Proceed immediately to the Visium Spatial Tissue Optimization User Guide (CG000238) or the Visium Spatial Gene Expression User Guide (CG000239).

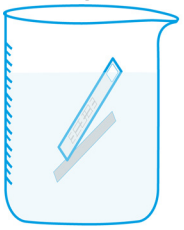
Immerse in 3X SSC Buffer



Hold in 3X SSC Buffer



Coverslip detaches



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