Visium CytAssist Spatial Gene Expression for FFPE – Tissue Preparation Guide

Introduction

The Visium CytAssist Spatial Gene Expression for FFPE workflow is designed to analyze mRNA in tissue sections derived from formalin fixed & paraffin embedded (FFPE) tissue samples on the CytAssist instrument. A single CytAssist run accommodates up to two tissue slides (tissue placed on a standard glass slide) as sample input. Proper tissue handling and preparation techniques preserve the morphological quality of the tissue sections and the integrity of mRNA transcripts. Maintaining tissue adhesion and high-quality RNA is critical to assay performance.

The Tissue Preparation Guide provides guidance on:

- Best practices for handling tissue samples and tissue slides before and after sectioning.
- Removing hardset coverslips from archived tissue slides.
- Sectioning of tissue blocks and placement of sections on blank slides.
- Performing RNA quality assessment of FFPE tissue blocks or archived tissue sections on tissue slides.

Contents

Additional Guidance

This protocol is compatible with most human and mouse tissue types. Modifications to this protocol (e.g. section flotation time and water bath temperature) may be required for the preparation of certain tissue blocks and difficult tissue types, such as breast, colon, skin, and lungs. Refer to the 10x Genomics Support website for additional resources, including a list of tissues tested.

The tissue slides prepared using this Tissue Preparation Guide can be used with:

- Visium CytAssist Spatial Gene Expression for FFPE –Deparaffinization, H&E Staining, Imaging & Decrosslinking (CG000520)
- Visium CytAssist Spatial Gene Expression for FFPE – Deparaffinization, Decrosslinking, IF Staining & Imaging (CG000519)

Document Navigation

- If archived or freshly placed tissue sections are larger than the Visium Capture Area (Refer to Determining Allowable Area for dimensions), an area of interest will need to be defined. Refer to Optional Area of Interest Selection for Large Tissue Sections on page 8 for more information.
- If working with archived slides that have been hardset coverslipped, protocol steps begin on page 12.
- If working with FFPE tissue blocks, protocol steps begin on page 18.

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.

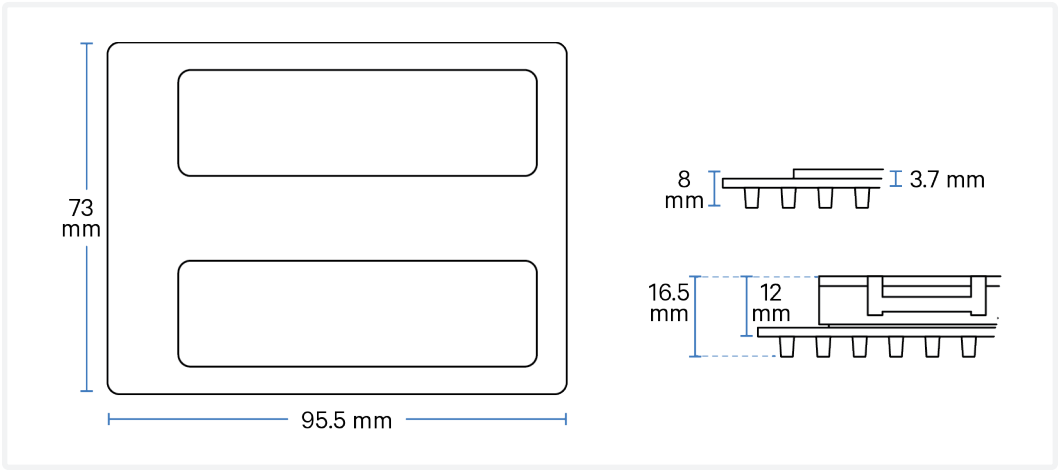
The following thermal cyclers should have their ramp rates adjusted for all steps:

- 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.



Specific Reagents & Consumables

Equipment For FFPE Tissue Sectioning & Section Placement			
Item	**Alternatives/Options**	**Vendor**	**Part Number**
Microtome	Epredia HM 355S Automatic Microtome *Or any standard histology grade microtome*	Fisher Scientific	23-900-672
Microtome blade	Epredia MX35 Premier Disposable Microtome Blades, Low Profile	Fisher Scientific	3052835
Section transfer system (STS)	Thermo Scientific Section Transfer System (STS), *Optional - If using Section Transfer System*	Fisher Scientific	771200
Probes	Fisherbrand Fine Precision Probe	Fisher Scientific	12-000-153
Forceps	Fisherbrand Curved Medium Point General Purpose Forceps	Fisher Scientific	16-100-110
Water bath	Premiere Tissue Floating Bath, Lighted *Or any equivalent water bath*	Fisher Scientific	76278-820
Section dryer oven	Epredia High Capacity Section Dryer	Fisher Scientific	A84600051
Slide drying rack *If using section dryer oven*	StatLab TISSUE-TEK VERT 24 SLIDE RACK	Fisher Scientific	LWS2124
Thermal cycler *Alternative to section dryer oven with thermocycler adapter*	C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module	Bio-Rad	1851197
Brushes	Camel Hair Brushes *Or any equivalent paintbrush*	Ted Pella	11859
Green Marker, *Optional if marking area of interest*	Sharpie Argyle Green Permanent Marker	Sharpie	1785396
Blank Slides	Epredia Shandon ColorFrost Plus Slides	Fisher Scientific	6776214
	Fisherbrand Superfrost Plus Microscope Slides	Fisher Scientific	12-550-15
	Poly-Prep Slides	Millipore Sigma	P0425
	VWR Superfrost Plus Micro Slides, Premium	VWR	48311-703
Additional Materials			
Razor blades		-	-
Ice bucket		-	-
Dry ice		-	-
Ultrapure/Milli-Q Water for Water Bath, *from Milli-Q Integral Ultrapure Water System or equivalent*		-	-

For RNA Quality Assessment			
Xylene	Xylene, Reagent Grade	Millipore Sigma	214736
	Xylene, Histological Grade	Millipore Sigma	534056
Ethanol	Ethyl Alcohol, Pure (200 Proof, anhydrous)	Millipore Sigma	E7023-500ML
	Ethanol absolute ≥99.5%, TechniSolv, pure (Europe Only)	VWR	83813.360DP
RNA extraction	RNeasy FFPE Kit (50)	Qiagen	73504
Deparaffinization solution	Deparaffinization Solution	Qiagen	19093
RNase decontamination solution	RNaseZap RNase Decontamination Solution	Thermo Fisher Scientific	AM9780
Nuclease-free water	Nuclease-free Water (not DEPC-Treated)	Thermo Fisher Scientific	AM9937
Staining dishes *If working with hardset coverslipped slides. Alternative to Coplin jars.*	Staining Dish, White	VWR	25608-906
Coplin jars *If working with hardset coverslipped slides. Alternative to staining dishes.*	Glass Coplin Staining Jar, Screw Cap	VWR	2100500-232
Metal Block *If working with hardset coverslipped slides*	Light Labs 96-WELL ALUMINUM BLOCK	Fisher Scientific	NC9820161
1.5-ml centrifuge tubes	DNA LoBind Tubes, 1.5 ml	Eppendorf	022431021
0.2 ml PCR 8-tube strips *Choose either Eppendorf, USA Scientific, or Thermo Fisher Scientific PCR 8-tube strips*	PCR Tubes 0.2 ml 8-tube strips	Eppendorf	951010022
	TempAssure PCR 8-tube strip	USA Scientific	1402-4700
	MicroAmp 8-TubeStrip, 0.2 ml	Thermo Fisher Scientific	N8020580
	MicroAmp 8-cap Strip, clear	Thermo Fisher Scientific	N8010535
Nanodrop/Qubit fluorometer	Nanodrop 2000c Spectrophotometers *Or any equivalent Nanodrop Alternative to Qubit Fluorometer*	Thermo Fisher Scientific	ND-2000C
	Qubit RNA BR Assay Kit	Thermo Fisher Scientific	Q10210
	Qubit Assay Tubes	Thermo Fisher Scientific	Q32856
	Qubit 4 Fluorometer	Thermo Fisher Scientific	Q33238
Bioanalyzer/Tapestation Choose Bioanalyzer or TapeStation based on availability & preference.	RNA 6000 Pico Kit	Agilent	5067-1513
	2100 Bioanalyzer Laptop Bundle	Agilent	G2953CA
	4200 TapeStation	Agilent	G2991AA
	High Sensitivity RNA ScreenTape	Agilent	5067-5579
	High Sensitivity RNA ScreenTape Ladder	Agilent	5067-5581
	High Sensitivity RNA ScreenTape Sample Buffer	Agilent	5067-5580

Tips & Best Practices

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

Sample Preparation

- It is recommended to store FFPE tissue blocks at 4°C and to avoid exposure to direct light to ensure even chilling and to preserve RNA integrity.

RNA Quality Assessment

- Assess RNA quality of the tissue block or archived sections before proceeding with sectioning by calculating the percentage of total RNA fragments >200 nucleotides (DV200) of RNA extracted from tissue sections.
- Various factors could lead to variations in DV200 scores, such as specific tissue types, diseased or necrotic tissues, sample preparation, handling, loading concentration errors or errors with ladder.

Section Thickness

- Recommended section thickness is 3-10 µm. Though the entire recommended range of thicknesses were tested internally, most sections were cut at 5 µm, the thickness reference throughout this protocol.

Water Bath Temperature & Section Floating Time

- Optimal water bath temperature and section floating time are critical for tissue section expansion. 42ºC is the recommended water bath temperature for most tissues. However, water bath temperature may need optimization based on the tissue type.
- If the tissue is taking too long to expand, turn the water bath temperature up by 1 or 2 degrees and let the section float for longer.
- If the tissue is expanding too quickly and dissociating, turn the water bath temperature down by 1 or 2 degrees and shorten the floating time.
- Clean water bath according to manufacturer recommendations, followed by an RNase decontamination solution.

Section Placement on Blank Slides

- After section placement, blank slides are referred to as tissue slides.
- Prior to section placement, draw an outline of the allowable area on the back of the blank slide to ensure downstream compatibility with the Visium CytAssist instrument (Refer to Determining Allowable Area).
- If working with multiple sections on multiple tissue slides, ensure that sections are placed in the same location on the tissue slides for improved imaging efficiency.
- If placing multiple sections on a blank slide, ensure that the paraffin and/or tissue do not overlap.
- If multiple sections are placed on a blank slide, only one section from each tissue slide should be used for each Visium CytAssist Spatial Gene Expression Slide Capture Area.

Practice Section Placement

- Practice correct section placement using non-experimental blocks.

Section Attachment

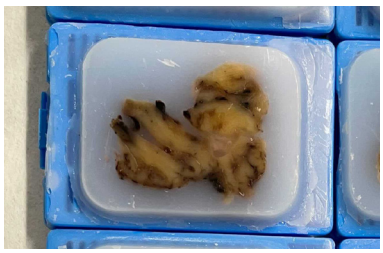
- Tissue block and section quality can affect the section attachment to blank slides.
- Carefully inspect the tissue block to gauge the extent of dehydration. Allow sufficient time in the ice bath to ensure proper hydration.
- Use a new, clean blade for sectioning each tissue type. Inspect the blade after every 20-25 sections and adjust to the blade areas that are not nicked or rough. Replace the blade after ~50 sections.
- Perform sectioning in a continuous motion to get a ribbon of sections. The sections should be separated during floating in the water bath. If floating multiple sections, monitor float time carefully.
- Ensure collected sections have the same thickness throughout experiments and replicates.
- Allow the section to float in the water bath until it is free of folds and wrinkles. Folds are associated with poor UMI capture and can be identified via H&E staining, or by eye or under the microscope prior to staining.

- Tissue detachment may occur due to factors such as the quality of paraffin used during the tissue embedding process, the age of the tissue block, tissue section thickness, and the length of time used to infiltrate a tissue in paraffin. Refer to Troubleshooting for more information.

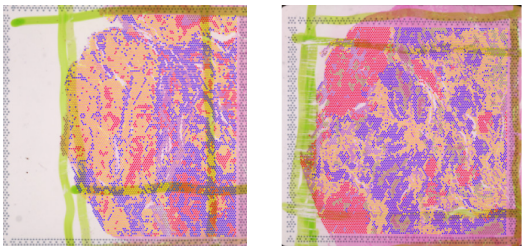
Optional Area of Interest Selection for Large Tissue Sections

- If a tissue section does not fit completely within the Visium CytAssist Spatial Gene Expression Slide Capture Area (6.5 mm or 11 mm), a smaller area of interest should be defined. Refer to Figure A in the Determining Allowable Area section or the Visium CytAssist for Accessory Kit Instruction Quick Reference Card (CG000548).
- The area of interest should be:
    - Small enough to fit inside a well in the Visium CytAssist Tissue Slide Cassette gasket (Refer to Determining Allowable Area, Figure B for allowable area sizing). Tissue outside of the gasket will not be processed during the CytAssist workflow.
    - Centered within the appropriate Capture Area size on the Tissue Slide Stage on the Visium CytAssist instrument
- Defining an area of interest can occur after section placement by examining the tissue under a microscope or after H&E staining and imaging.
- The area of interest can be annotated on the back of the tissue slide with a permanent marker to assist in alignment with the Tissue Slide Cassette gasket. Annotations should be removed prior to loading the tissue slide onto the instrument.
    - Any remaining 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 has the least impact on the automatic tissue registration process. 10x Genomics recommends using the Sharpie Argyle Green Permanent Marker (PN 1785396) for minimal impact on automatic tissue registration.

Large Tissue Section in Paraffin Block



Areas of Interest Annotated with Green Marker



Determining Allowable Area

Use the following diagrams to verify that freshly placed tissue sections are compatible. Reference the images below to draw the allowable area on the back of blank slides (remove after tissue placement). Images are to scale if scaling settings are not modified. To verify, ensure that the first block in section A measures 6.5 x 6.5 mm.

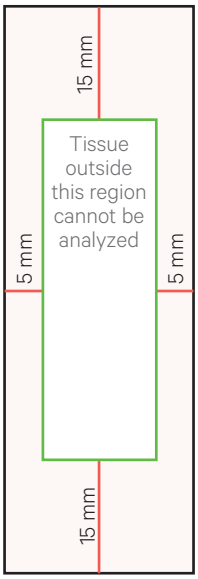
A **Ensure that the area of interest will fit within the Capture Area of a Visium CytAssist Spatial Gene Expression Slide.**

Tissue sections larger than these Capture Areas may be placed on the slide, but only the tissue within the Capture Area will be processed by the CytAssist instrument.

Overlay the slide, centering the tissue on either square.

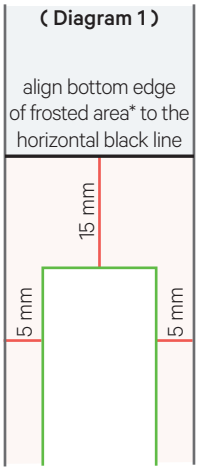
B **If slide has no frosted areas, overlay on this diagram.**

Tissue should lie within the green allowable area:
15 mm from top and bottom edges
5 mm from the sides

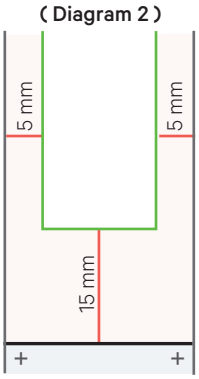


If slide has a frosted end and/or marks, overlay on diagram 1 and then on diagram 2.

Check the allowable area from both the top (1) and bottom (2) to ensure the tissue lies within the green allowable area (*area is variable due to variability in the dimensions of frosted areas across slide brands):*
15 mm from edge of frosted area/marks
5 mm from the sides



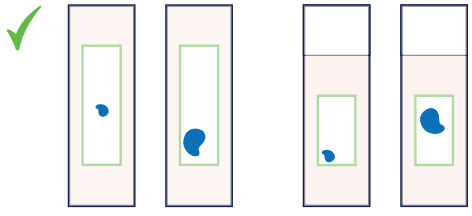
**If text is present below frosted area of slide, align bottom of the text to the line*



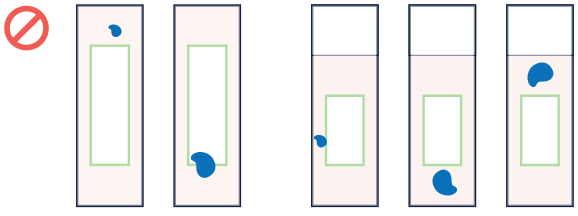
align bottom edge** to the horizontal black line

***If markings are present at bottom edge of slide, align markings to the + signs*

C **Examples of Good Tissue Placement**



Examples of Bad Tissue Placement



Visium CytAssist Tested Slides

The following slides have been tested for use with the Visium CytAssist Tissue Slide Cassette and instrument.

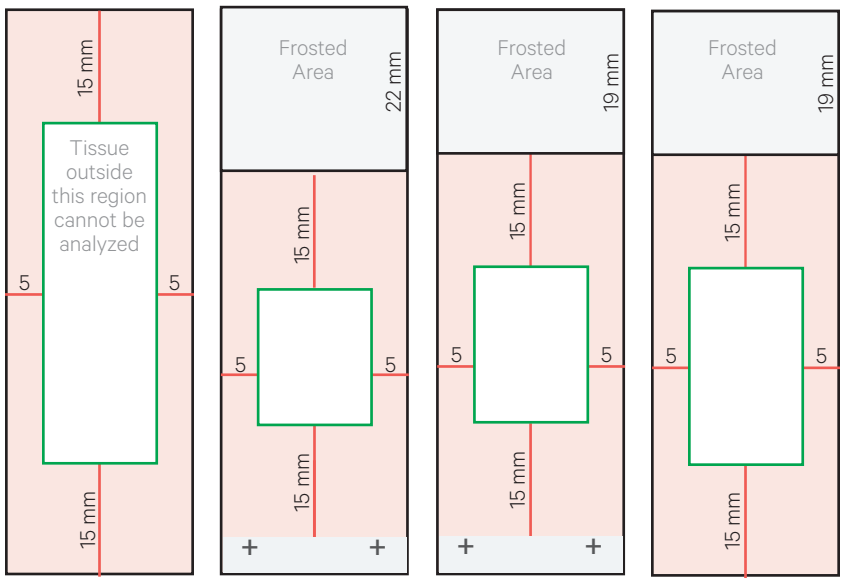
Item	Length (mm)	Width (mm)	Thickness (mm)
Epredia Shandon ColorFrost Plus Slides	75.0	25.0	1.0
Fisherbrand SuperFrost Plus Microscope Slides	75.0	25.0	1.0
Sigma-Aldrich Poly Prep Slides	75.0	25.0	1.0
VWR SuperFrost Plus Micro Slide, Premium	75.0	25.0	1.0

If unsure of slide part number, refer to "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 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



Handling Tissue Slides

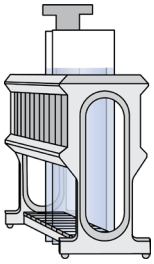
- When immersing slides in reagent, ensure all tissue sections are immersed.
- Maintain tissue slides in a low moisture environment such as a desiccator, avoid exposure to direct light, and keep at room temperature.
- Tissue slides that have been incubated at 42°C for 3 h and dried overnight at room temperature in a desiccator can be stored for up to two weeks at room temperature in a desiccator.

Tissue Slide Incubation (choose one method)

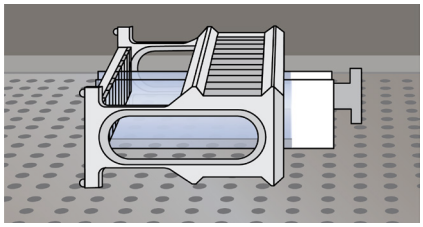
A. Incubation using a Section Dryer Oven:

- Place tissue slides in a slide drying rack on its side to prevent melted paraffin wax from disturbing adjacent tissue sections (if applicable).

Place Slide in a Rack



Place slide in a rack



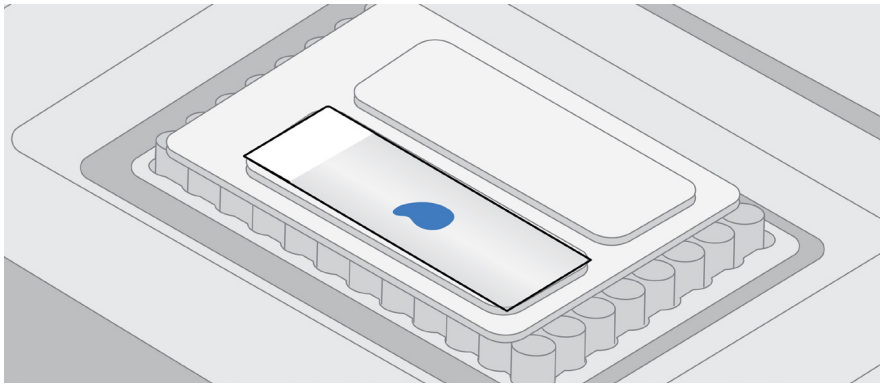
Place slide sideways and keep the oven door closed during incubation

- Close the lid when incubating the tissue slide in the oven.

B. Incubation using a Thermocycler:

- Position a Low Profile Thermocycler Adapter on a thermal cycler that is set at the incubation temperature. Low Profile Thermocycler Adapter must come to temperature before placing tissue slide.
- Ensure that the Low Profile Thermocycler Adapter is in contact with the thermal cycler surface uniformly.
- When incubating a tissue slide, position the tissue slide on the Low Profile Thermocycler Adapter with the side with tissue facing up.
- Ensure that the entire bottom surface of the tissue slide is in contact with Low Profile Thermocycler Adapter.
- DO NOT close the thermal cycler lid when incubating the tissue slide.
- Allow Low Profile Thermocycler Adapter to cool before removing from thermal cycler.

Incubation on a Thermal Cycler



1. Hardset Coverslip Removal and RNA Quality Assessment for Archived Slides

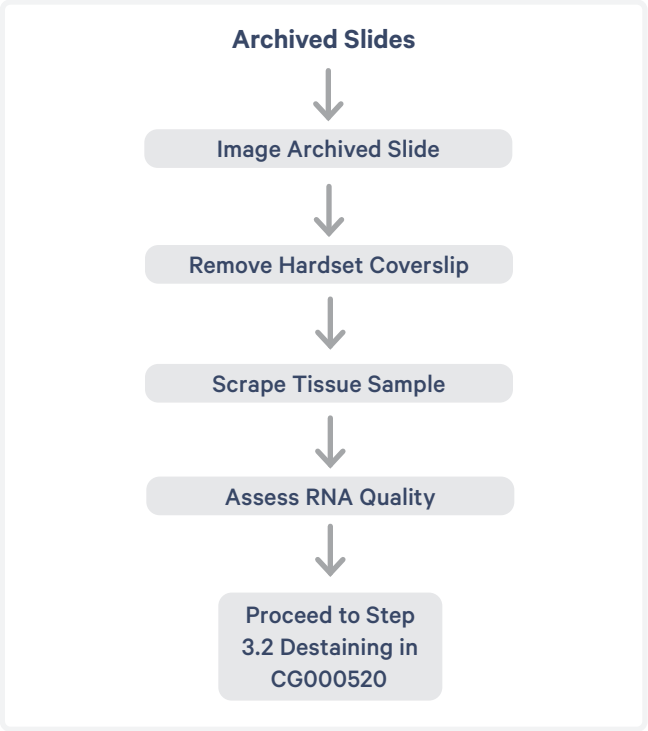
Overview

This chapter provides guidance on hardset coverslip removal (coverslips that have been sealed with a reagent such as CytoSeal) and assessing RNA quality for archived slides.

Archived slides are defined as glass slides containing FFPE tissue sections that were placed in the past, where mounting of a coverslip was performed with Cytoseal or nail polish (hardset). Archived slides should have been imaged and stored at room temperature or 4°C. Over time, archived slides may experience RNA degradation; thus, freshly placed FFPE tissue sections are preferred for the Visium CytAssist assay.

These protocols should be performed on archived slides derived from the same tissue block as the slide that will be used for the full CytAssist assay. If this is not possible, an unimportant area of the section for processing can be scraped to assess RNA quality.

Prior to removal of hardset coverslip from the slide, verify that images of archived slides meet the specifications required for analysis as specified in Document CG000521. If images are absent or incompatible, before hardset coverslip removal, image the archived slide according to guidelines described in the Visium CytAssist Spatial Gene Expression Imaging Guidelines Technical Note (CG000521).



After hardset coverslip removal, H&E stained and imaged sections proceed directly to Destaining in the Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, H&E Staining, Imaging & Decrosslinking Demonstrated Protocol (Document CG000520).

Remove Hardset Coverslip

Archived slides are dipped in xylene for a few minutes followed by rapid freezing to remove coverslips without damaging tissue sections.

Scrape Tissue Sample

Once hardset coverslip is removed, a small portion from a proximal section or an unimportant area of tissue from the section of interest is scraped to assess RNA quality.

Assess RNA Quality

RNA quality of the tissue on the archived slide is assessed by calculating the DV200 score of extracted RNA.

Mean RNA fragment size is a reliable determinant of RNA quality. This requires a measurement of the percentage of total RNA fragments >200 nucleotides (DV200) for RNA quality assessment upstream of library preparation. For more information on DV200, refer to Appendix 1: DV200 Performance and Recommendations and Appendix 2: Example DV200 Traces.

Before starting RNA extraction, apply RNaseZap to a paper towel and wipe work surface, centrifuge rotor and shafts of the pipettes. Rinse with RNase-free water and then wipe dry. The electrodes of the Bioanalyzer can also be cleaned with RNaseZap by following Agilent decontamination procedure.

Proceed to Document CG000520

After assessing RNA quality, proceed directly to Destaining (step 3.2) in the Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, H&E Staining, Imaging & Decrosslinking Demonstrated Protocol (Document CG000520).

1.1 Preparation - Buffers

Prepare fresh weekly, process two slides per jar	
Items	**Preparation & Handling**
☐ Xylene	Label two Coplin jars or staining dishes as Xylene Jar 1 and 2. Dispense 30 ml xylene in each.
☐ 100% Ethanol	Label two Coplin jars or staining dishes as 100% Ethanol Jar 1 and 2. Dispense 30 ml 100% ethanol in each.
☐ 96% Ethanol	Label two Coplin jars or staining dishes as 96% Ethanol Jar 1 and 2. Dispense 30 ml 96% ethanol in each.
☐ 70% Ethanol	Label one Coplin jar or staining dishes as 70% Ethanol Jar. Dispense 30 ml 70% ethanol.
Obtain	
Items	**Preparation & Handling**
☐ 100% Ethanol	
☐ Xylene	
☐ Nuclease-free water	
☐ Metal block	
☐ New Razor Blade	
☐ Dry Ice	
☐ 0.2 ml 8-tube Strip	
☐ RNeasy FFPE Kit	RNeasy MinElute Spin Column, Buffer PKD, Proteinase K, DNase Booster Buffer, DNase I Solution, RBC Buffer, and RPE Buffer are contained in this kit.

1.2 Hardset Coverslip Removal

Xylene incubation steps should be performed in a fume hood. Either Coplin jars or staining dishes may be used. If archived slides have been H&E stained, image the archived slide according to guidelines described in the Visium CytAssist Spatial Gene Expression Imaging Guidelines Technical Note (CG000521) and ensure they are compatible with the Space Ranger analysis pipeline. If archived slides have not been imaged, image per the above guidelines before removing the hardset coverslip.

a. Cool a metal block on dry ice for **5-10 min**.

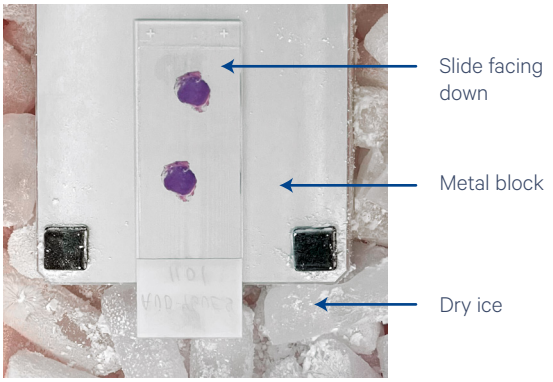
b. Gently immerse archived slide in Xylene Jar 1. Secure the jar cap to prevent xylene loss.

c. Incubate for **5 min**.

d. Remove excess xylene from archived slide with a laboratory wipe and place on pre-cooled metal block with the coverslipped tissue sections facing down.

e. Wait **1 min**.

Archived Slide on Metal Block

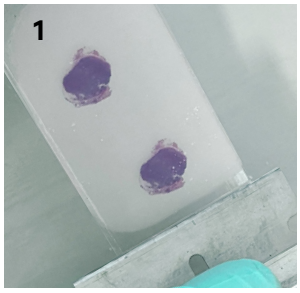


f. Insert a clean blade a short distance between coverslip and archived slide on the shorter edge of the archived slide (see image below). Work slowly in small steps, keeping the archived slide on the cold metal block in between steps. Avoid touching tissue with the blade.

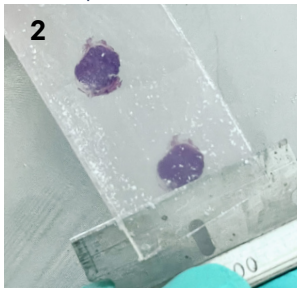
Exercise caution, as blade is sharp.

Removing Coverslip with Razor Blade

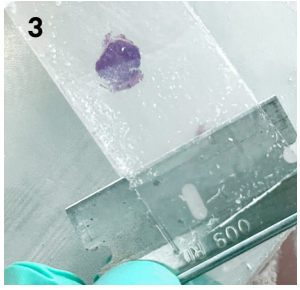
Align edge of razor blade to right most edge of coverslip



Slowly wedge razor blade between slide and coverslip until coverslip releases from slide



Holding razor blade towards coverslip, slowly work blade to the left to lift coverslip off slide



g. Immerse archived slide without coverslip 2x in Xylene Jar 1, then immerse for **10 min**.

h. Immerse archived slide 2x in Xylene Jar 2, then immerse for **10 min**.

i. Immerse archived slide 2x in 100% Ethanol Jar 1, then immerse for **3 min**.

j. Immerse archived slide 2x in 100% Ethanol Jar 2, then immerse for **3 min**.

k. Immerse archived slide 2x in 96% Ethanol Jar 1, then immerse for **3 min**.

l. Immerse archived slide 2x in 96% Ethanol Jar 2, then immerse for **3 min**.

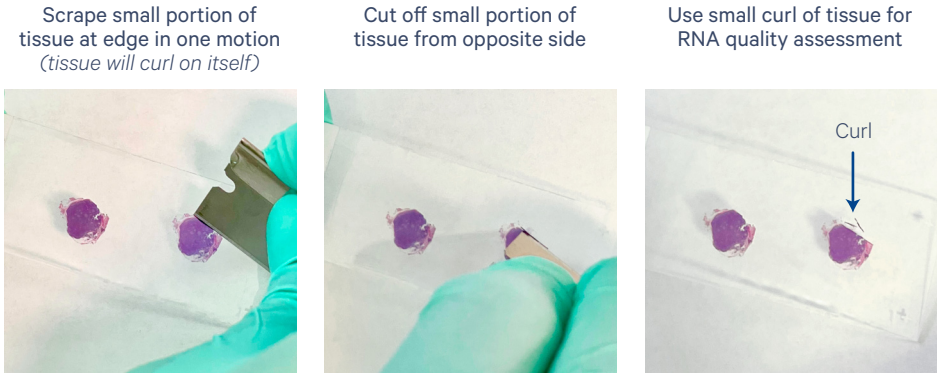
m. Immerse archived slide 2x in 70% Ethanol Jar 1, then immerse for **3 min**.

n. Remove excess ethanol from archived slide carefully with a laboratory wipe. Do not touch tissue.

o. Select a small portion of the section that can be scraped for RNA quality assessment. Sections for RNA quality assessment should have a minimum size of 2 x 2 mm and minimum thickness of 5 µm. Practice scraping sections from test tissues.

p. Using a clean blade, scrape the small portion for RNA quality assessment in one motion, resulting in one curl.

Scrape Tissue for Assessing RNA Quality



q. Lift the curl using the blade, and use a clean pipette tip to transfer curl to one 0.2-ml tube in a tube strip on ice. Store at **-80°C** for long term storage or proceed immediately to RNA Extraction. If processing multiple archived slides, curls can be kept in tube strips on ice until ready for RNA extraction.

r. The remaining section on the archived slide may be used for the Visium CytAssist workflow, or stored in a sealed slide mailer in a desiccator kept in the dark at **4°C** for up to **one week**.

1.3 RNA Extraction

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

Lid Temperature	Reaction Volume	Run Time
80°C	160 µl	30 min
Step	**Temperature**	**Time**
Pre-equilibrate	56°C	Hold
Incubation 1	56°C	00:15:00
Incubation 2	80°C	00:15:00

b. Add **150 µl** of Buffer PKD to sample tube and pipette mix.

c. Add **10 µl** of Proteinase K to sample tube and pipette mix.

d. Add sample tube to thermal cycler and skip prequilibrate step.

e. Pipette mix every **5 min** during Incubation 1 and 2 steps. Pipette mix without removing tube from block to prevent burns.

f. Incubate a 2-ml microcentrifuge tube on ice for **3 min**.

g. Transfer sample to the pre-cooled 2-ml microcentrifuge tube after Incubation 2.

h. Add **16 µl** of DNase Booster Buffer to tube.

i. Add **10 µl** of DNase I Solution to tube. Pipette mix.

j. Incubate at room temperature for **15 min**.

k. Add **320 µl** of RBC Buffer to tube and pipette mix.

l. Add **720 µl** of 100% Ethanol to tube and pipette mix.

m. Transfer sample to RNeasy MinElute column. Do not allow sample to overflow in the column.

n. Centrifuge column for **15 sec** at **8,000 rcf**.

o. Repeat steps m-n until all sample has passed through the column.

p. Add **500 µl** of RPE Buffer to column.

q. Centrifuge column for **2 min** at **8,000 rcf**.

r. Transfer column to a new 2-ml microcentrifuge tube.

s. Centrifuge column for **5 min** at **maximum speed** with column lid open.

t. Transfer column to a new 2-ml microcentrifuge tube.

u. Add **12 µl** of nuclease-free water to column.

v. Centrifuge column for **1 min** at maximum speed.

w. Place RNA on ice or store at **-80°C** until ready to assess RNA quality.

x. Use **1 µl** of sample for DV200 evaluation using either Agilent RNA 6000 Pico Kit or TapeStation (High Sensitivity RNA ScreenTape, RNA ScreenTape Sampler Buffer, RNA ScreenTape Ladder). Follow manufacturer's instructions for DV200 evaluation.

- For more information on DV200, refer to Appendix 1: DV200 Performance and Recommendations and Appendix 2: Example DV200 Traces.

After DV200 evaluation, proceed directly to Destaining (step 3.2) in the Visium CytAssist Spatial Gene Expression for FFPE - Deparaffinization, H&E Staining, Imaging & Decrosslinking Demonstrated Protocol (Document CG000520).

2. FFPE Tissue Sectioning & Section Placement

Overview

This chapter provides guidance on sectioning FFPE tissue blocks using a microtome and section placement on blank slides using a water bath. A Section Transfer System (STS) can also be used for section placement.

Exposing the Tissue

FFPE tissue block is placed in a microtome and cut to expose the tissue or face the block.

Sectioning

A section is taken from the block for RNA quality assessment.

RNA Quality Assessment

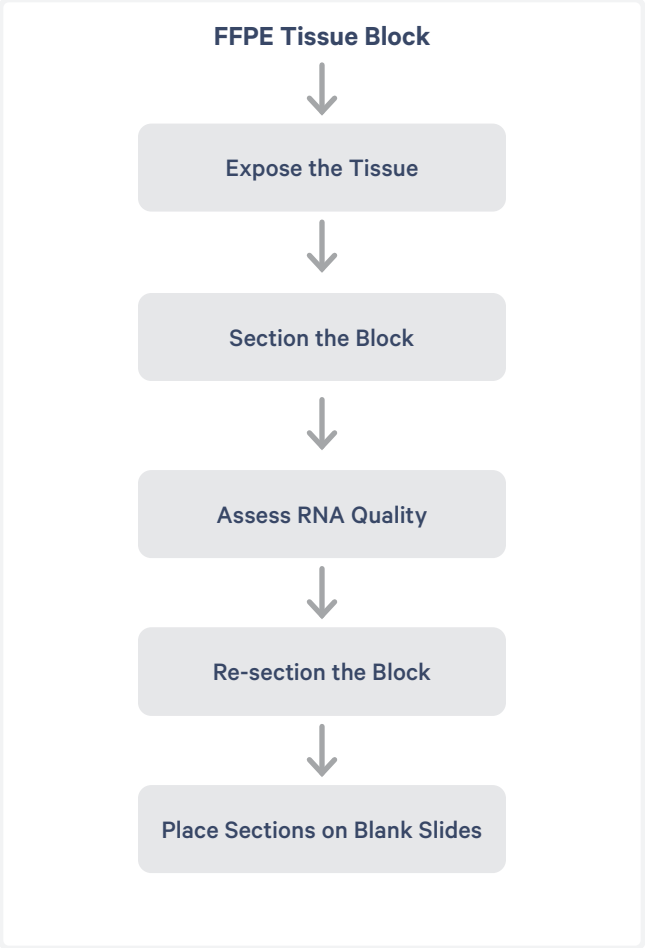
RNA quality of the tissue is assessed by calculating DV200 of RNA extracted from freshly collected tissue sections.

Re-sectioning

The tissue block is then sectioned by a microtome to generate sections for the blank slides.

Section Placement

Sections are collected in a water bath and are allowed to float on the water surface until they are flat and free from wrinkles and folds. The optimal floating time depends upon the specific tissue type and should be empirically determined when working with new tissue types. Expanded sections are then placed on blank slides.



Practice section placement using a non-experimental block before proceeding with tissue slides for the Visium workflow. Each tissue slide must only be used for only one CytAssist experimental run. DO NOT re-run tissue slides as this could affect assay performance.

2.1 Exposing the Tissue or Facing the Block

Before starting, wipe down all the surfaces and work areas with RNaseZap RNase decontaminating solution.

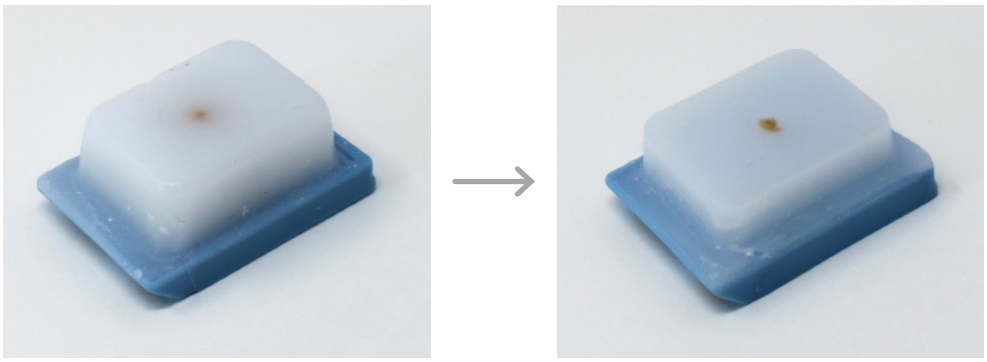
a. Remove tissue blocks from storage. For a tissue block with already exposed tissue, proceed directly to RNA Quality Assessment (step 2.2).

b. Set the microtome to the 15 µm setting.

c. Place tissue block on the specimen clamp.

d. Cut the tissue block at 15 µm until all of the edges of the tissue are exposed or until the area of interest is exposed. The block should be at **room temperature** during cutting.

Exposing the Tissue Block



2.2 RNA Quality Assessment of FFPE Tissue Block

This section provides guidance on determining the RNA quality of the tissue block by calculating DV200 of RNA extracted from freshly collected tissue sections.

Mean RNA fragment size is a reliable determinant of RNA quality. This requires a measurement of the percentage of total RNA fragments >200 nucleotides (DV200) for RNA quality assessment upstream of library preparation. For more information on DV200, refer to Appendix 1: DV200 Performance and Recommendations and Appendix 2: Example DV200 Traces.

Before starting RNA extraction, apply RNaseZap to a paper towel and wipe work surface, centrifuge rotor and shafts of the pipettes. Rinse with RNase-free water and then wipe dry. The electrodes of the Bioanalyzer can also be cleaned with RNaseZap by following Agilent decontamination procedure.

a. Set the microtome to the 10 µm setting and collect tissue sections of 10 um thickness for RNA extraction. Discard the first few sections if the block was already exposed. The number of sections needed depends upon the tissue size. Refer to the RNA extraction kit manufacturer instructions to determine the appropriate number of sections. See below for guidance:

- Collect ~4 sections for smaller tissues (≤6.5 x 6.5 mm)
- Collect 1-2 sections for larger tissues (≥6.5 x 6.5 mm)

b. Place the sections inside a **pre-cooled** microcentrifuge tube. Sections may be stored at **–80°C** for **long-term** storage. For sections stored at **–80°C,** equilibrate to **room temperature** for **5 min** prior to adding the deparaffinization solution.

c. Proceed to RNA extraction using RNeasy FFPE Kit and follow manufacturer's instructions.

d. Using a Nanodrop or Qubit Fluorometer, measure RNA concentration to determine the appropriate dilution for DV200 evaluation using the RNA 6000 Pico Kit. RNA outside of the recommended concentration may lead to inaccurate DV200 evaluation.

e. Store purified RNA at **–80°C** for **long-term** storage or **immediately** proceed to DV200 evaluation using either the Agilent RNA 6000 Pico Kit or TapeStation High Sensitivity Kit. Follow manufacturer's instructions (Agilent) for DV200 evaluation.

2.3 Sectioning

Section Collection:

a. Place blocks in the ice bath, ensuring that the tissue part is fully submerged.

b. Incubate on the ice bath for **10-30 min**. The incubation time depends upon the tissue type and the extent of dehydration.

Monitor the exposed tissue every 5-10 min during the ice bath incubation. The tissue surface should be smooth and shiny and free of bumps at the end of the incubation. For more information on tissue hydration, see Troubleshooting section.

FFPE Tissue Block in Ice Bath

c. Carefully wipe off the excess oils from a 35X Ultra disposable blade using a laboratory wipe with 100% ethanol. Let the ethanol evaporate before proceeding. Always use new blades for sectioning each tissue type.

d. Secure blade in disposable blade holder of the microtome and place knife guard over the blade to minimize injury. Ensure that the clearance angle is set to 10°.

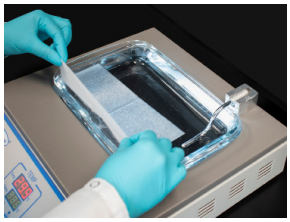
e. After hydration is complete, place the tissue block in the specimen clamp and align it with the blade.

f. Fill up a water bath with Milli-Q or ultrapure water and ensure that the temperature is set at **42°C** and free from bubbles & particulates by gliding a laboratory wipe over the water surface. Repeat this step between sectioning if necessary.

TIPS

42°C is the recommended water bath temperature for most tissues. However, water bath temperature may need optimization based on the tissue type. Determine optimal water bath conditions before tissue placement on the blank slides by practicing section placement on a blank slide with non-experimental blocks. See Tips & Best Practices for guidance on optimizing water bath temperature. To better visualize the tissue sections, a Lighted Tissue Floating Bath with LED illumination can be used instead of standard water bath.

Remove Bubbles



g. Set the microtome to 5 µm for tissue sectioning and begin sectioning. For tissue blocks with exposed tissue, discard the first few sections and start collection on the subsequent sections.

h. To collect sections, place the paintbrush tip slightly above and parallel to the blade. Lift the section by lightly touching the edge with the paintbrush while rotating the wheel handle.

i. With the help of the brush, pick the section up. Immediately place it on the water surface of the water bath, making sure that the brush tip goes underneath and away from the section.

j. Proceed directly to Section Placement.

2.4 Section Placement

a. Before proceeding with tissue slides intended for the Visium CytAssist workflow, practice section placement using non-experimental blocks. Consider the following:

- If placing multiple sections on the blank slide, ensure that sections do not overlap (only one tissue section can be analyzed per tissue slide).
- If placing a large section, refer to Optional Area of Interest Selection for Large Tissue Sections for information on selecting an area of interest.

  Trace allowable area onto back of the blank slide with a laboratory marker before section placement to ensure compatibility. Refer to Section Placement on Blank Slides and Visium CytAssist Validated Slides sections for more information. Markings will need to be removed prior to alignment on the Visium CytAssist instrument.

b. Allow sections to float for the time determined previously determined to be optimal.

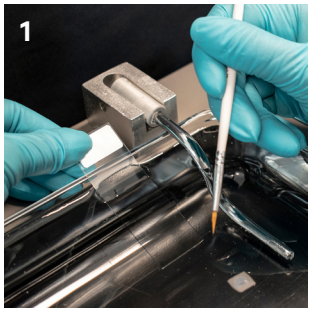
c. Hold the top of the blank slide and insert the blank slide into the water, aligning the allowable area with the surface of the water while keeping the blank slide straight.

d. Using the paintbrush or the probe, maneuver the section to the allowable area.

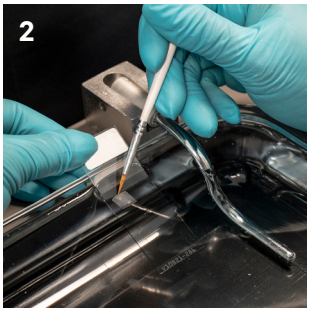
TIPS

If sections float away from the blank slide, the blank slide can also be dipped into the water bath before sections are placed in the water.

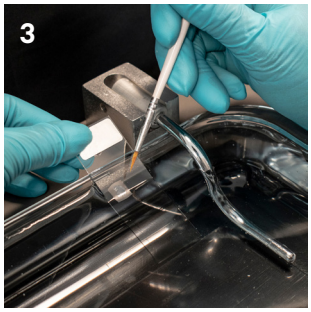
Section floating in the water bath



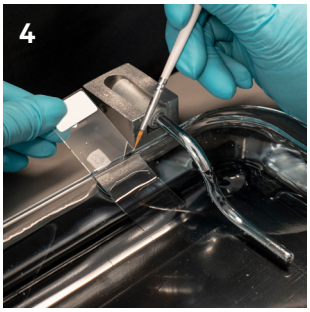
Blank slide inserted below surface of water



Align section edge with desired location



Pull the blank slide out of the water



e. Pull the blank slide up and out of the water, ensuring there are no air bubbles trapped underneath and set aside in a standing rack.

f. Place the tissue slides in a slide drying rack in a section dryer and incubate for **3 h** in an oven at **42°C.** Alternatively, a thermal cycler set at **42°C** can be used for drying.

See Tips & Best Practices for guidance on slide incubation.

g. Place in a desiccator and keep overnight at **room temperature** to ensure proper drying.

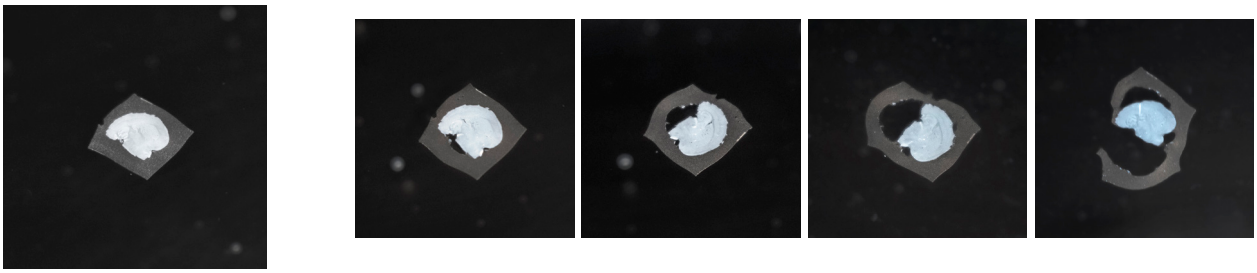
h. After overnight drying, proceed to deparaffinization and staining protocols (see References) or store the tissue slide containing dry tissue sections at **room temperature** in a desiccator for up to **2 weeks.**

Troubleshooting

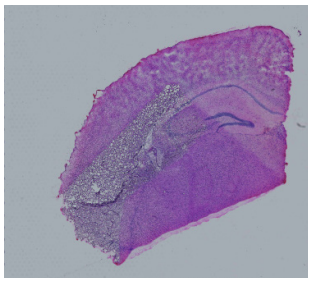
Ideal Floating Time Determination

Ideal Floating Time

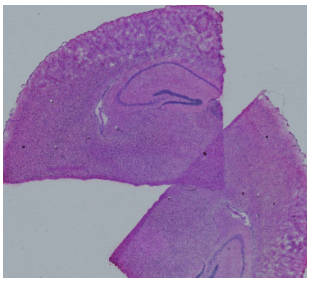
Section disintegration due to increased floating time



Incorrect Placement of Tissue Sections



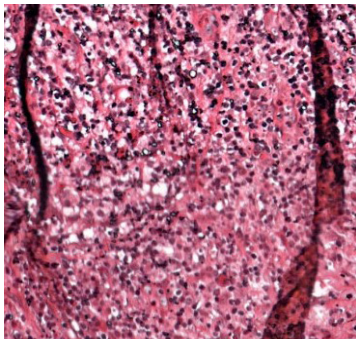
Folded tissue section



Overlapping sections

Common Artifacts that cause Detachment or Misleading Spatial Data

Wrinkles



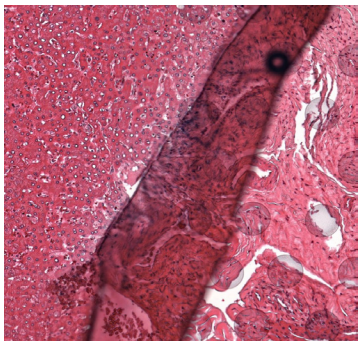
Causes

- Section compression (due to warm block or dull blade) during sectioning leads to wrinkle formation. These wrinkles become permanent when placed in the water bath.
- Accumulated wax or static electricity on microtome parts also contribute to section compression.
- Incorrect blade/clearance angle may cause compression.

Troubleshooting

- Ensure that the block is well hydrated.
- Adjust temperature down and increase float time.
- Gently and gradually lay FFPE sections onto water bath surface, lengthwise.
- Utilize a new blade.
- Ensure microtome is cleaned with 100% ethanol to minimize static and section compression (bunching on blade).
- Ensure blade/clearance angle is correct prior to sectioning.

Folds



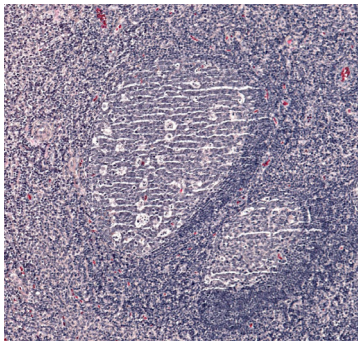
Causes

- Mostly happens when placing the section on the water bath especially when the section is wavy.
- If the fold is at the edge this most likely can happen during sectioning or mounting on the slide.

Troubleshooting

- Gently and gradually lay FFPE ribbons or sections onto water bath surface, lengthwise.
- If sections curl during sectioning, gently flatten them with a brush before floating.

Venetian Blinds or Shatter



Causes

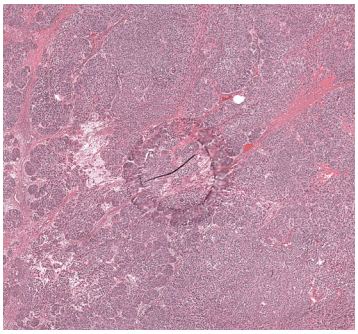
- Parallel lines in the section mostly appear due to dry tissue because of under-hydration of the block in the ice bath.
- Less likely due to dull blade or loose parts of the microtome.

Troubleshooting

- Increase incubation time of the block in ice bath.
- Tighten down components of microtome and make sure the blade is at a correct angle.

Common Artifacts that cause Detachment or Misleading Spatial Data

Air Bubbles



Causes

- Air bubbles from the bottom of the water bath can rise and stick under the section.

Troubleshooting

- Using a brush, gently remove the bubbles from the bottom of the water bath before floating the sections.

Waves

May be block-specific and easily observed under the microscope right after the section is picked up from the water bath. Minor waves can disappear after longer flotation in the water bath. Larger waves can create wrinkles or folds that are permanent after drying of the section.

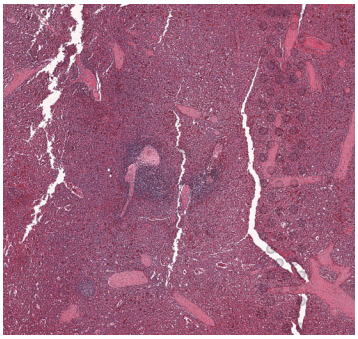
Causes

- Tissue incompletely infiltrated with wax absorbs water faster during hydration step. When sections from such blocks are floated, the parts that absorbed enough water will have difficulty flattening and become wavy.

Troubleshooting

- Chill the block on a cold block or on ice avoiding contact with water.
- Submerge the block for 5-15 min in the ice bath for gentle hydration.
- Increase flotation times and/or temperature of the water bath.

Cracks



Causes

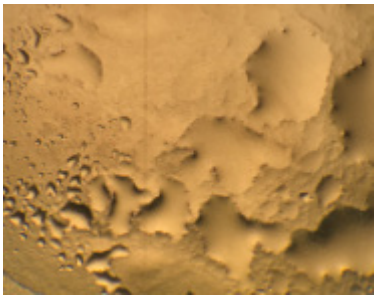
- Dry and over-processed tissue can crack during sectioning.
- The cracks that are created before tissue embedding will be filled with wax when section is observed under the microscope after sectioning and wax will be washed away after deparaffinization or H&E staining.

Troubleshooting

- Prolonged hydration on the ice bath will most likely reduce the cracks.
- There is no solution for cracks created before tissue was embedded in wax.

Common Artifacts that cause Detachment or Misleading Spatial Data

Sweating



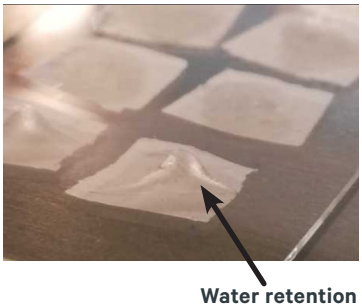
Causes

- Inadequate dehydration or under-processing of the tissue causes parts of the section to attract water and create this blistering effect. Such sections can disintegrate in the water bath.
- The blisters consist of:
  - Xylene or xylene substitutes if the cause is under-processing and insufficient removal of xylene or
  - Water droplets if the cause is inadequate dehydration.

Troubleshooting

- Be cautious about how long the block is kept in ice bath. Long incubation time in ice bath can impact section quality and thus should be avoided.
- Long flotation time in the water bath should be avoided.

Water Retention



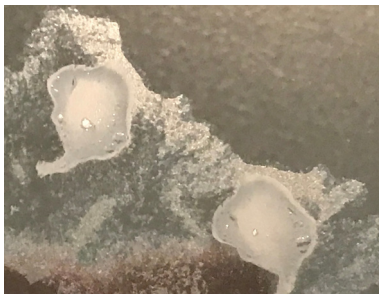
Causes

- Sections from tissues that are under-processed or excessively hydrated before sectioning may retain water under the section mounted on a slide.
- Water will accumulate under section and cause uneven drying ultimately leading to detachment.

Troubleshooting

- Gently flicking the slide will help to get rid of the water. This is one of the most overlooked cause of detachment.

Disintegrating/Exploding Section



Causes

- Sections from tissues that are under-processed and not sufficiently infiltrated with wax can rapidly absorb water and explode or gradually disintegrate in the water bath.
- Tissues that are not sufficiently dehydrated can show similar phenotypes.
- Floating at high temperatures (42-50°C) can exacerbate the disintegration.

Troubleshooting

- Chilling of the block should be mostly performed on ice or cold block (30-60 min).
- Exposure to water in ice bath during chilling should be kept to a minimum (5-10 min).
- Flotation water bath temperature should be lowered to 38-40°C.

Appendix

A1: DV200 Performance and Recommendations

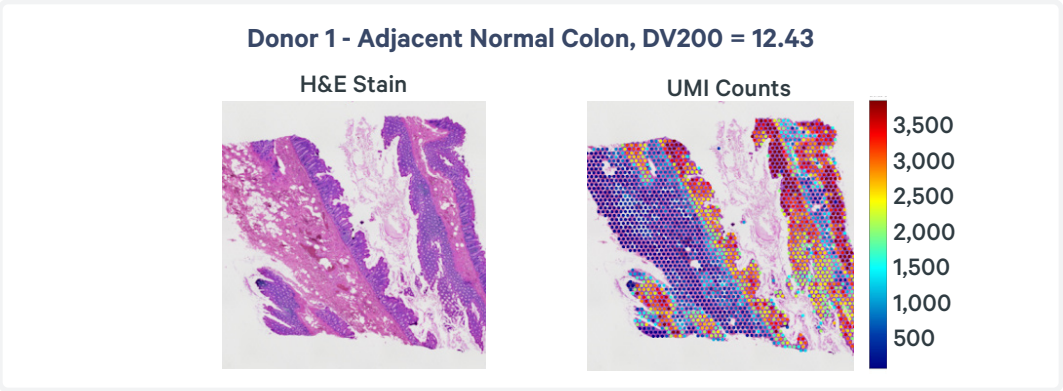
DV200 is a broad measurement of RNA quality and is influenced by factors including:

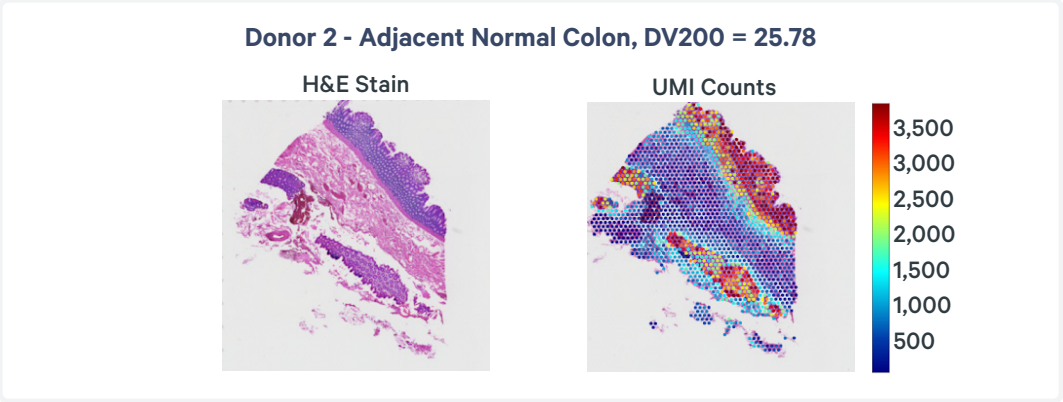
- Tissue block age, type and composition
- Region selected for RNA extraction
- Presence of diseased or necrotic regions
- Depth of section
- Fixation method
- Miscellaneous upstream tissue handling and processing

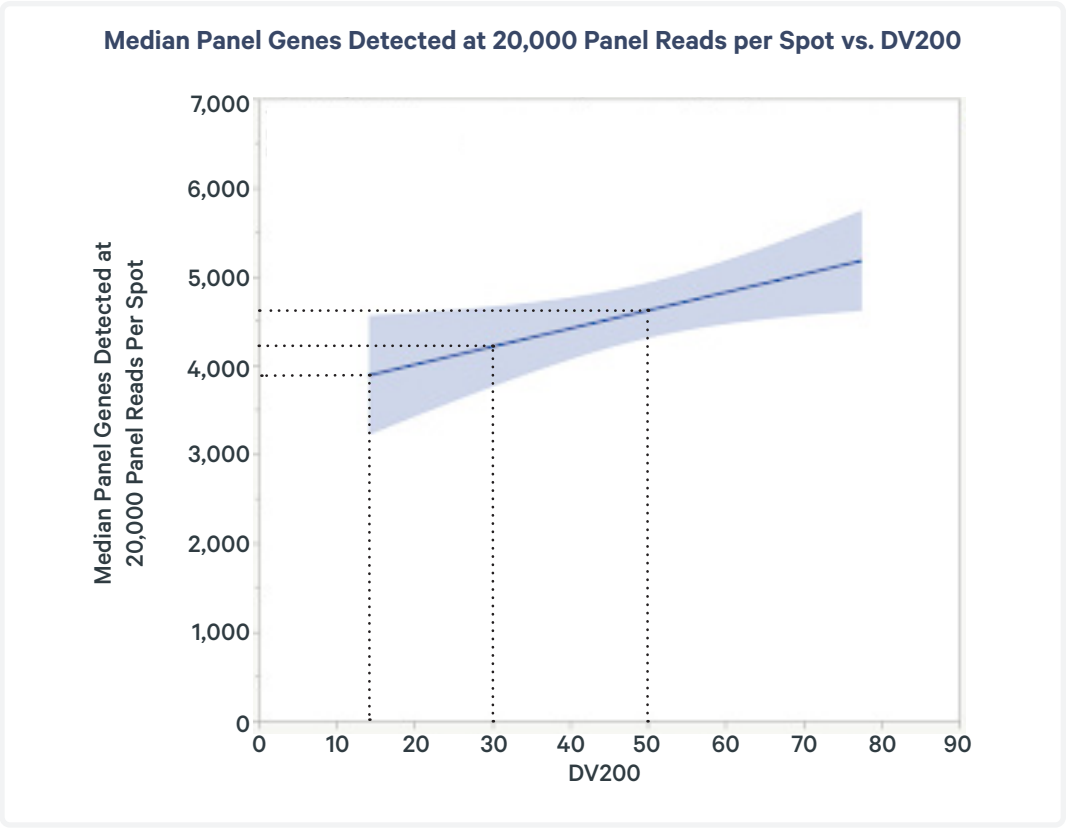
Assessment of multiple healthy and cancer tissue samples demonstrate a positive correlation between DV200 score and median UMI counts and panel genes detected with the Visium Spatial Gene Expression for FFPE workflow at 20,000 raw reads per spot. Based on these observations, tissue samples with a DV200 of >30% are more likely to yield robust and reproducible results.

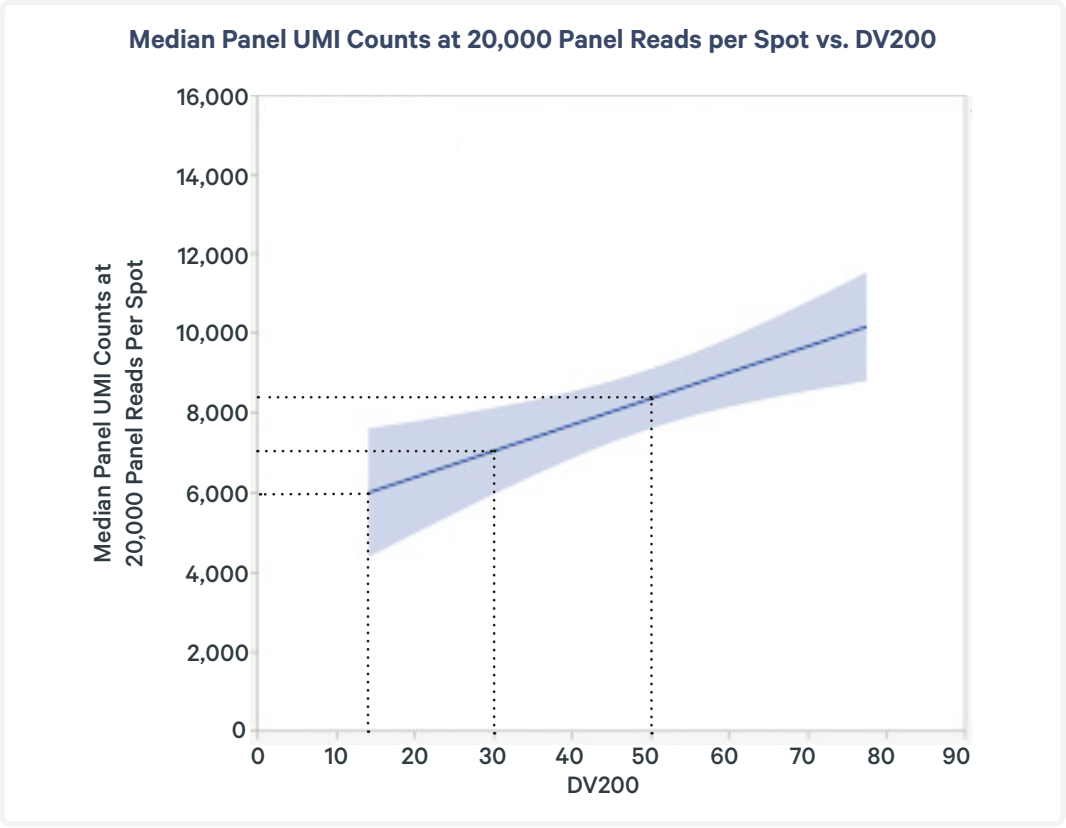
However, tissue samples with a low DV200 score have demonstrated compatibility with the assay with lower UMI and gene counts. For example, a DV200 of ~15% may yield ~6,000 UMI counts and ~3,800 panel genes detected at 20,000 raw reads per spot. Below are examples data with low DV200 score adjacent colon tissue sections from two different donors. In this example, the low DV200 score is likely due to the heterogeneous composition of the tissue, where ~70% of the section is comprised of low UMI count subcutaneous tissue.

These data were generated from freshly placed tissue sections; however, similar results may be found with archived sections.


Donor 1 - Adjacent Normal Colon, DV200 = 12.43
H&E Stain
UMI Counts
3,500
3,000
2,500
2,000
1,500
1,000
500


Donor 2 - Adjacent Normal Colon, DV200 = 25.78
H&E Stain
UMI Counts
3,500
3,000
2,500
2,000
1,500
1,000
500

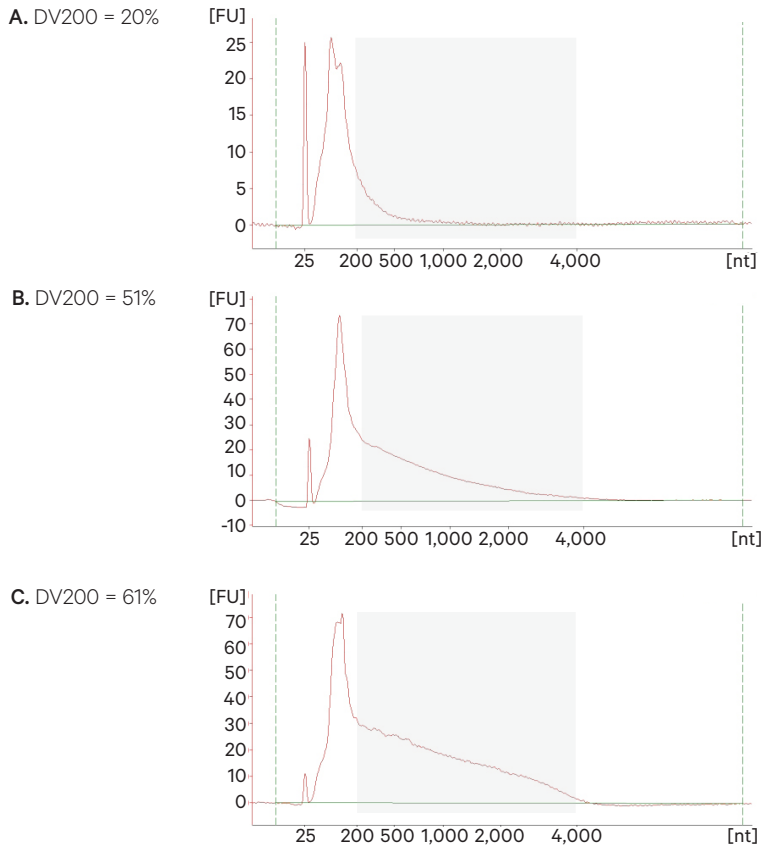

Median Panel Genes Detected at 20,000 Panel Reads per Spot vs. DV200
Median Panel Genes Detected at 20,000 Panel Reads Per Spot
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
0
10
20
30
40
50
60
70
80
90
DV200


Median Panel UMI Counts at 20,000 Panel Reads per Spot vs. DV200
Median Panel UMI Counts at 20,000 Panel Reads Per Spot
16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
0
10
20
30
40
50
60
70
80
90
DV200

A2: Example DV200 Traces

Bioanalyzer traces of RNA extracted from different tissue types for which DV200 was calculated are shown below.

Bioanalyzer Traces for Samples with Different DV200 Counts



Samples displayed are from DV200 <30% (A) and DV200 >30% (B and C). The Agilent RNA 6000 Pico kit was used for preparing the samples.

References

- Visium CytAssist Spatial Gene Expression for FFPE – Deparaffinization, H&E Staining, Imaging & Decrosslinking (CG000520).
- Visium CytAssist Spatial Gene Expression for FFPE – Deparaffinization, Decrosslinking, IF Staining & Imaging (CG000519).
- Visium CytAssist for Accessory Kit Instruction Quick Reference Card (CG000548)
- Karigoudar, M, et al. Alternative Rapid Methods for Coverslip Removal: A Comparitive Study. Journal of Clinical and Diagnostic Research 13:1-2, 2019

Document Revision Summary

Document Number	CG000518
Title	Visium CytAssist Spatial Gene Expression for FFPE – Tissue Preparation Guide
Revision	Rev D
Revision Date	May 2024
General Changes	Updated for general minor consistency of language and terms throughout
Specific Changes	• Updated magnetic separator part number on page 2. • Added PTC Tempo, VeritiPro, and Analytik Jena Biometra thermal cyclers to list on page 2. • Updated Low Profile Thermocycler Adapter image for clarity and added additional guidance for adapter removal on page 3. • Removed reference to cool-cut on page 4. • Removed redundant section on Allowable Area on page 11. • Added additional tips for incubation using a thermocycler on page 11. • Replaced photos with illustrations on pages 11 and 12 for clarity. • Removed references to archived IF slides on pages 12, 13, and 19.

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