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StrataMap Spatial Transcriptome Permeabilization Optimization Kit

User Guide

ILLUMINA PROPRIETARY

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Notice

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1. Overview

The StrataMap Spatial Transcriptome Permeabilization Optimization kit can be used to create six individually addressable libraries to determine the optimal permeabilization time for your tissue. Illumina recommends conducting a permeabilization time titration to determine the optimal permeabilization time for tissue sensitivity.

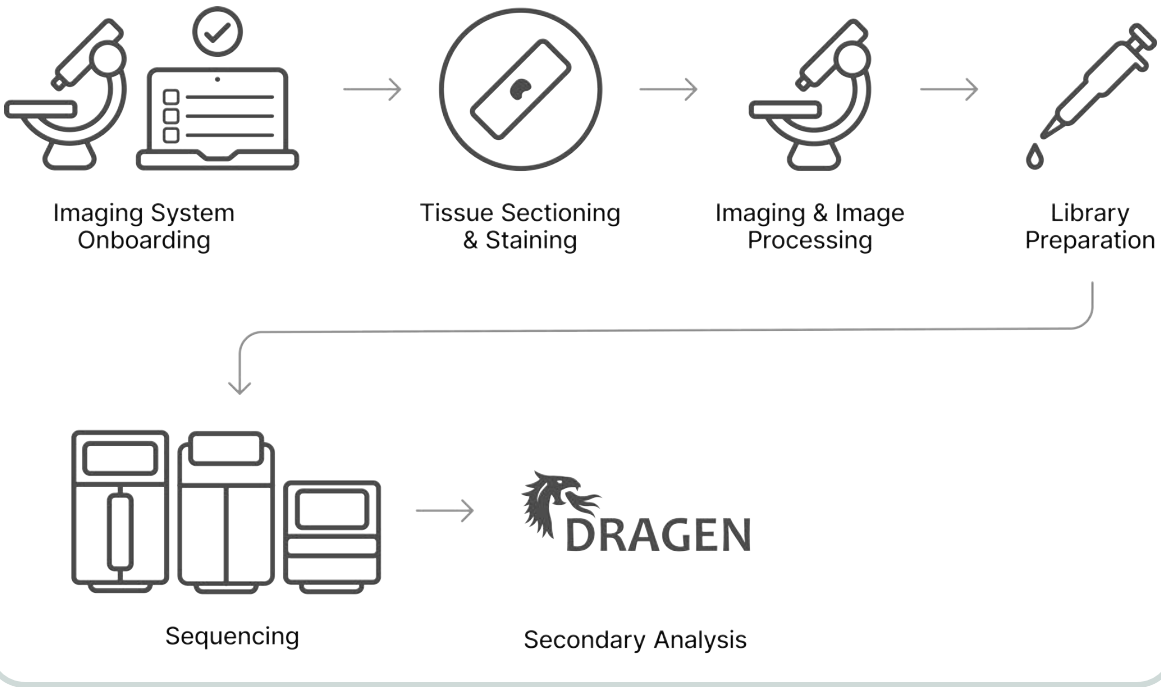
- For most tissues, 10 minutes is a suitable permeabilization time. For more information or recommendations for your tissue type of interest, contact Illumina Support.
- After the optimal permeabilization time is determined, the assay can be used to generate up to six final libraries for sequencing.
- You can also use this permeabilization time on the single-well assay. For more information, refer to the *StrataMap Spatial Transcriptome User Guide (document # 200073552)* on the Illumina support site.

This resource explains how to prepare libraries using the StrataMap Spatial Transcriptome Permeabilization Optimization kit. The entire assay workflow consists of the following actions:

- Assess your imaging system to confirm that it can work with the assay protocol
- Prepare and image tissue sections
- Create spatial libraries for sequencing
- Sequence using a NovaSeq X Series, NovaSeq 6000, or NextSeq 2000 system
- Secondary analysis using the DRAGEN StrataMap analysis software, BaseSpace, and Illumina BioInsight Platform Core (Platform Core)

Figure 1 shows the end-to-end workflow for the StrataMap Spatial Transcriptome Permeabilization Optimization kit.

Figure 1: End-to-End Workflow



Imaging Assessment

- Before you begin the protocol, evaluate your imaging system by performing *Imaging Assessment* in the *StrataMap Spatial Transcriptome Imaging Guide (document # 200081305)* on the Illumina support site.
  - This test confirms that fiducial markers are accurately imaged and compatible with analysis.
  - For more information on configuring Illumina StrataMap Image Tool (ISIT) for this test, refer to *Imaging Assessment* in the *Illumina StrataMap Spatial Software documentation*.

Protocol

- The protocol uses the StrataMap Spatial Transcriptome Permeabilization Optimization kit to prepare tissue sections for imaging and create spatial libraries for sequencing.
  - For more information on the StrataMap Spatial Transcriptome Permeabilization Optimization kit, refer to *2.1 Product Contents* on page 8.

  - For additional consumables and equipment needed for the protocol, refer to *2.2 User-Supplied Consumables and Equipment* on page 10 and *2.3 Illumina Consumables and Equipment* on page 14.
- The protocol is performed over the course of three days:
  - **Day 1**—The first day involves preparing the tissue and taking images of the tissue sections for analysis using your qualified imaging system. After imaging, you permeabilize the tissue, which releases the RNA from the cells, and then you can create cDNA that complements the RNA transcripts.
  - **Day 2**—The second day involves refining and isolating the cDNA to create the libraries needed for sequencing, which includes adding a second strand of cDNA. Then, you can elute and purify the cDNA before determining the optimal amplification cycle number.
  - **Day 3**—The third day is used to complete the sequencing preparation. Use the optimal amplification cycle number to amplify your library, which creates copies of each unique template for sequencing. After purifying the amplified library, check the quantity and quality of the library so that you can dilute it to the starting concentration needed for sequencing.

Sequencing

- The StrataMap Spatial Transcriptome Permeabilization Optimization kit is compatible with the following flow cells and workflows:
  - NovaSeq X Series 5B, 10B, and 25B flow cells using the NovaSeq X Series protocol
  - NovaSeq 6000 S2 and S4 flow cells using the Standard workflow
  - NovaSeq 6000 S4 flow cell using the Xp workflow
  - NextSeq 2000 P4 flow cell using the XLEAP-SBS reagents
- The sequencing system that should be used is dependent on the tissue amount and sequencing depth. To estimate the sequencing data output needed for your experiment, refer to *5.22 Sequencing and Analysis* on page 113.
- If you are using the NovaSeq X Series system, you can also do run planning per lane.
- StrataMap Spatial Transcriptome libraries can be sequenced across all lanes of a flow cell or in individually addressable lanes.
  - If the library originates from one slide, sequencing data from multiple flow cells can be combined into one analysis.
  - If combining with other library types on the same flow cell, refer to *Illumina StrataMap Spatial Software documentation* for specific instructions.
  - For sequencing depth recommendations, refer to *3.2 Sequencing Recommendations* on page 17.

Secondary Analysis

- Secondary analysis is done with the DRAGEN StrataMap analysis software, along with BaseSpace and Platform Core.
- Secondary analysis consists of the following steps in the *Illumina StrataMap Spatial Software documentation*:
  - **[Optional]** Cell segmentation can be initiated independently using the Illumina StrataMap Cell Segmentation Tool. This tool can be used after image acquisition and before proceeding with the assay protocol. You can confirm if the cell segmentation meets your experimental needs or if the images should be retaken.
  - Run setup and planning using auto or manual launch with Platform Core.

1.1 Warnings, Cautions, and Notes

Icons are used for warnings, cautions, and notes across this documentation and are defined in Table 1.

Table 1: Icons

Icon	Definition
	Warnings are used for actions that can result in harm to person or damage to the instrument.
	Cautions are used for actions that can lead to unexpected or detrimental results, or loss of data.
	Notes are used for supplemental information that is required for a step, including Illumina recommendations and references to related documentation.

1.2 Safety Considerations

- Before performing the protocol, review the following applicable safety documentation:
  - Safety Data Sheets (SDSs)
  - Laboratory safety procedures
  - Equipment manufacturer instructions
- Personnel performing the protocol must be trained on the applicable laboratory safety practices and the operation of the equipment used in the steps.
- If compressed gas is used to dry slides, ensure that the gas source is properly regulated and used in accordance with local compressed gas safety requirements.
- Cryostat operation involves hazardous sharp edges and cold surfaces. Follow all safety precautions provided by the cryostat manufacturer and your facility safety procedures.
- Adhere to the following warnings and cautions when using samples and the reagents with this protocol.

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Ventilation should be appropriate for handling of hazardous materials in reagents. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. *For additional environmental, health, and safety information, refer to the SDS at support.illumina.com/sds.html.*

Handle all samples as if they are potentially infectious agents.

Use routine laboratory precautions. Do not pipette by mouth. Do not eat, drink, or smoke in designated work areas. Wash hands thoroughly after handling specimens and assay reagents.

Exercise caution when handling sharps, including cryostat blades, razor blades, and scalpels. Follow institutional sharps safety procedures and dispose of used blades in approved sharps containers.

Cryostat operation involves hazardous sharp edges and cold surfaces. Follow all safety precautions provided by the cryostat manufacturer and your facility safety procedures.

Do not use any assay components beyond their stated expiration date on the assay box label. Do not interchange assay components from different assay lots. Assay lots are identified on the assay box label. Store the assay components at the specified temperature.

Failure to follow the procedures as outlined can result in erroneous results or significant reduction in library quality.

2. Consumables and Equipment

This section lists all components included in the StrataMap Spatial Transcriptome Permeabilization Optimization kit reagent kit, with storage conditions. This section also details the ancillary consumables, equipment, and other prerequisites needed to complete the protocol.

The protocol has been validated using the listed items. Before starting the protocol, make sure that you have the required items. Using alternate consumables and equipment can impact performance.

2.1 Product Contents

- The StrataMap Spatial Transcriptome Permeabilization Optimization kit protocol does not require index adapters.
- The StrataMap Spatial Transcriptome Permeabilization Optimization kit (catalog # 20146114) consists of the following contents:
  - StrataMap Spatial Transcriptome Box 1 (Table 2)
  - StrataMap Spatial Transcriptome Box 2 (Table 3)
  - StrataMap Spatial Transcriptome Box 3 (Table 4)
  - StrataMap Spatial 6-Well Cassette and Stencil Kit (Table 5)

Table 2: StrataMap Spatial Transcriptome Box 1

Quantity	Reagent
1	Enhanced PCR Mix (EPM)
1	First Strand Enzyme (FSE)
1	First Strand Reagent 1 (FSR1)
1	First Strand Reagent 2 (FSR2)
1	First Strand Reagent 3 (FSR3)
1	First Strand Supplement (FSS)
3	Spatial Amplification Mix (SAM)
1	Spatial Particle Mix (SPM)
1	Spatial Primer Cocktail (SPC)
1	Spatial PCR Reagent (SPR)
1	Spatial Surface Reagent (SSR)
1	Tissue Digestion Enzyme (TDE)
1	Tissue Permeabilization Enzyme (TPE)
1	Universal Cleave Mix (UCM)

Table 3: StrataMap Spatial Transcriptome Box 2

Quantity	Item
1	Illumina Purification Beads (IPB)
1	Resuspension Buffer (RSB)
1	Tissue Digestion Buffer (TDB)
20	Sealing tape

Table 4: StrataMap Spatial Transcriptome Box 3

Quantity	Item
1	StrataMap Spatial Slide (L)

Table 5: StrataMap 6-Well Cassette and Stencil Kit

Quantity	Item
1	Spatial cassette base
1	Spatial cassette lid
1	Stencil

Kit Storage

Kit contents must be stored at the following temperatures (Table 6):

Table 6: Storage Temperatures

Temperature	Kit Contents
-25°C to -15°C	StrataMap Spatial Transcriptome Box 1 (Table 2)
15°C to 30°C	StrataMap Spatial Transcriptome Box 2 (Table 3)
	6-Well Cassette and Stencil Kit (Table 5)
2°C to 8°C	StrataMap Spatial Transcriptome Box 3 (Table 4)

Store all reagent kit boxes in an upright position.

Make sure that the kit boxes are stored at the correct temperature.

The StrataMap Spatial slide ships at room temperature. After receiving the slide, it must be stored upright at 2°C to 8°C.

2.2 User-Supplied Consumables and Equipment

Table 7 and Table 8 provide information on the required user-supplied consumables and equipment for the StrataMap Spatial Transcriptome Permeabilization Optimization kit.

Unless otherwise specified, using substitute consumables and equipment can impact performance or cause failures.

Table 7: Consumables

Consumable	Supplier
0.1 N HCl	Fisher Scientific, catalog # SA541 (or equivalent)
10 N sodium hydroxide (NaOH), molecular biology grade	General lab supplier
Absolute ethanol (EtOH), molecular biology grade	• Decon, catalog # 2716 • Sigma, catalog # E7023 (or equivalent)
Absolute methanol (MeOH), molecular biology grade	Sigma, catalog # 34860-4L-R (or equivalent)
Alcoholic eosin Y[1]	• Sigma, catalog # HT110132 • Abcam, catalog # AB246824 • Leica, catalog # 3801615 or 3801616 • Epredia, catalog # 71204
Bluing reagent[1]	• Epredia, catalog # 6769001 • Sigma, catalog # S5134-6X100ML • Agilent, catalog # CS70230-2 • Leica, catalog # 3802901
Conical tubes, 50 ml (qty 20–25)	General lab supplier
Coverslip, no 1.5 (22 or 24 x 60 mm is recommended)	• Mercedes Scientific, catalog # MER R24601.5 • Fisher Scientific, catalog # 12541037 (or equivalent)
Dry ice	General lab supplier
Glycerol	General lab supplier

Consumable	Supplier
H&E Staining Kit with the following consumables[1]: • Eosin Y solution, 30 ml • Bluing reagent, 30 ml • Hematoxylin, 30 ml	Abcam, catalog # ab245880
Hematoxylin[1]	• Electron Microscopy Sciences, catalog # 26043-05 • Sigma, catalog # MHS1, MHS16, MHS32, MHS80, MHS128, or 51275 • Agilent, catalog # S330930-2 • Leica, catalog # 3801520 • Abcam, catalog # ab220365
High sensitivity D5000 ladder	Agilent, catalog # 5067-5594
High sensitivity D5000 reagents	Agilent, catalog # 5067-5593
High sensitivity D5000 screentape	Agilent, catalog # 5067-5592
High sensitivity RNA ladder	Agilent, catalog # 5067-5581
High sensitivity RNA sample buffer	Agilent, catalog # 5067-5580
High sensitivity RNA screentape	Agilent, catalog # 5067-5579
Isopropanol	Sigma, catalog # PX1835-6 (or equivalent)
Lint-free wipes	General lab supplier
KAPA Library Quantification Kit	Roche, catalog # 07960140001 or # 07960166001
KAPA SYBR FAST qPCR Master Mix (2X) Kit	Roche, catalog # 07959362001
Microcentrifuge tubes, 1.5 ml	General lab supplier
Microcentrifuge tubes, DNA LoBind, 2 ml	Eppendorf, catalog # 022431048 (or equivalent)
Nuclease-free water, molecular grade (700 ml)	General lab supplier
For OCT compound embedding, the following consumables: • Liquid nitrogen (with dewar) • Dry ice (with foam container) • Isopentane • Disposable cryomold	General lab supplier
PCR plate	General lab supplier

Consumable	Supplier
PCR plate seal	General lab supplier
Pipette tips, aerosol-resistant	General lab supplier
[Optional] QIAshredder	QIAGEN, catalog # 79656
qPCR plate	General lab supplier
Razor blade (or scalpel)	General lab supplier
RNase decontamination solution	General lab supplier
RNeasy Plus Mini Kit	QIAGEN, catalog # 74134
Saline-sodium citrate (SSC) buffer, RNAse-free, 20X	Sigma, catalog # S6639 (or equivalent)
Slide mailers, LockMailer	Simport, catalog # M9504MA
Tissue-Tek OCT compound	VWR, catalog # 25608-930
Trough, disposable plastic	General lab supplier

[1] Use either the H&E Staining Kit or the individual catalog numbers for alcoholic eosin Y, bluing reagent, and hematoxylin.

Table 8: Equipment

Equipment	Supplier
Benchtop mini-centrifuge	General lab supplier
Benchtop vortexer	General lab supplier
Cryostat, one of the following (or equivalent): • Epredia Cryostar NX70 • Epredia Cryostar NX50	Epredia, one of the following: • catalog # 957000K • catalog # 957260
Forceps	General lab supplier
Heat block (for microcentrifuge tubes)	General lab supplier
Magnetic stand (for PCR plates)	Invitrogen, one of the following: • catalog # 12027 • catalog # 12331D Sigma, catalog # 17-10071 (or equivalent)
Imaging system that meets minimum specifications in *StrataMap Spatial Transcriptome Imaging Guide (document # 200081305)*	Varies
Pipettor, Multichannel (200 µl)	General lab supplier
[Optional] Plate shaker (BioShake iQ) with 96-well PCR plate adapter	Q Instruments, catalog # 1808-0506 and 1808-1041

Equipment	Supplier
Plate spinner (or swinging bucket centrifuge), compatible with PCR plates	General lab supplier
[Optional] Pure compressed inert gas (for example, nitrogen gas)	General lab supplier
Real-time qPCR system, one of the following systems (or equivalent): • Bio-Rad CFX384 Touch Real-Time PCR System • Bio-Rad CFX Opus 96 Real-Time PCR System • Bio-Rad CFX Opus 384 Real-Time PCR System	Bio-Rad, one of the following: • catalog # 1855484 • catalog # 12011319 • catalog # 12011452
TapeStation, one of the following systems: • 4150 TapeStation • 4200 TapeStation	Agilent, one of the following: • catalog # G2992AA • catalog # G2991BA
Thermal cycler with 96-well block, any of the following (or equivalent) that can accommodate skirted PCR plates, has a minimum inside height of 14 mm, and a maximum lid pressure of 80 pounds of force: • Bio-Rad C1000 • Bio-Rad PTC Tempo • Analytik Jena Biometra TAdvanced 96 SG • Eppendorf Mastercycler x50	• Bio-Rad, catalog # 12015382 • Bio-Rad, catalog # 12015392 • Analytik Jena, catalog # 846-4-070-241 • Eppendorf, catalog # 6313000018
Timer	General lab supplier

2.3 Illumina Consumables and Equipment

Table 9 identifies the consumables that can be ordered from Illumina. The kit that should be used is dependent on the sequencing system ordered in Table 10.

Table 9: Sequencing Consumables

Consumable	Catalog Number
NovaSeq X Series 5B Reagent Kit (100 cycles)	20145965
NovaSeq X Series 10B Reagent Kit (100 cycles)	20085596
NovaSeq X Series 25B Reagent Kit (100 cycles)	20125967
NovaSeq 6000 S2 Reagent Kit v1.5 (100 cycles)	20028316
NovaSeq 6000 S4 Reagent Kit v1.5 (200 cycles)	20040719
NovaSeq 6000 Xp 4-lane Kit v1.5	20043131
NextSeq 2000 P4 XLEAP-SBS Reagent Kit (100 cycles)	20100994

Table 10 identifies the sequencing systems that can be ordered from Illumina. The sequencing system that should be used is dependent on the tissue amount and sequencing depth.

> *To estimate the sequencing data output needed for your experiment, refer to 5.22 Sequencing and Analysis on page 113.*

Table 10: Equipment

Equipment	Catalog Number
NovaSeq X Sequencing System	20084803
NovaSeq X Plus Sequencing System	20084804
NovaSeq 6000 Sequencing System	20023471
NextSeq 2000 Sequencing System	20038897

3. Before You Begin

Before starting the protocol, review the following information:

- Tissue section requirements
- Sequencing recommendations
- Instructions for embedding tissue in Optimal Cutting Temperature (OCT) compound
- RNA quality assessment requirements
- Instructions for extracting RNA from tissue sections
- Information on RIN analysis and how to get the RIN value
- Specimen transportation and storage requirements

> *The StrataMap Spatial Transcriptome Permeabilization Optimization kit is compatible with high-quality fresh frozen tissue. The tissue must contain polyadenylated RNAs so that these transcripts can be captured during library preparation.*

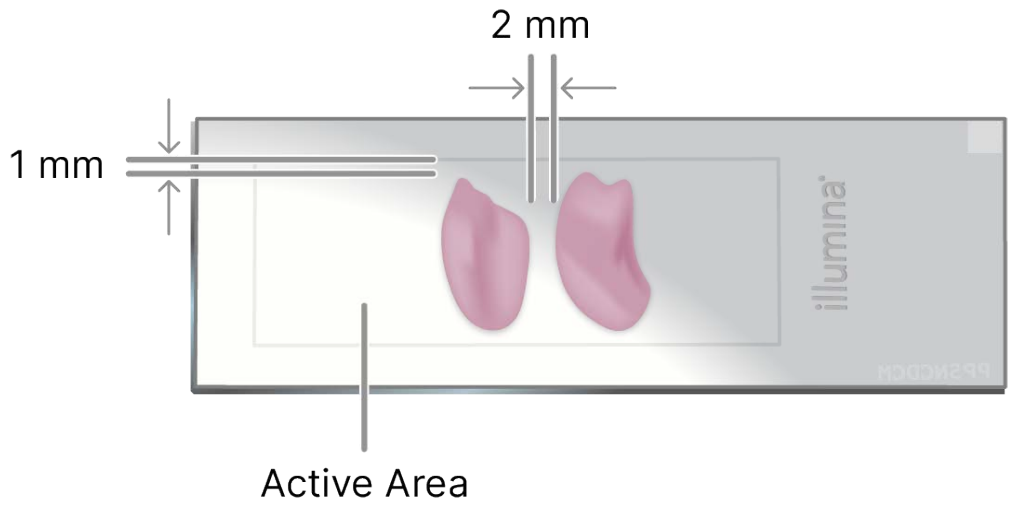
3.1 Tissue Placement Guidelines

- Illumina has currently demonstrated protocol performance with human, mouse, pig, and rat tissue.
  - Broader compatibility with many animal tissues is expected.
  - Untested species and tissue with rigid extracellular structures, such as those with cell walls (for example, plant tissue) or calcified matrices (for example, bone), have not been tested and may require customization and further optimization.

- Tissue sections should be placed at least 2 mm apart (Figure 2).

> *Illumina recommends having a margin of 1 mm between the tissue edge and active area in order to capture an adequate number of fiducials (Figure 2).*
>
> *For more information on handling and tissue placement, refer to 4.2 Handle Tissue and Slide on page 25.*

Figure 2: Tissue Section Spacing



- Illumina has successfully processed slides containing up to 12 tissue sections. For slides containing more than 12 tissue sections or tissue sections larger than 13 mm × 33 mm (or an equivalent area, such as 429 mm²), image processing in ISIT and downstream secondary analysis can take longer to complete.

> *For guidance in applications that exceed these recommendations, contact Illumina Support.*

- Illumina recommends that all tissue sections within a well are of a similar type and approximately the same size if equal sequencing representation across samples is required.
  - Large tissue naturally generates more sequencing output when placed with smaller tissue.
  - Similar type refers to tissues that originate from the same species and the same organ or anatomical region.

  - For permeabilization time optimization experiments, use the same tissue type and anatomical region in each well. Illumina recommends placing serial sections across the six wells.
- Individual images can be taken for each tissue section or images can contain multiple sections and samples separated during image processing. Memory requirements scale with image size and resolution. For example, a 10 mm x 10 mm image with 0.4 pixels/µm and three color channels occupies ~1.7 GB of memory. Recommended memory for image processing is 32 GB or higher with a minimum of 16 GB.

3.2 Sequencing Recommendations

- For permeabilization optimization libraries, Illumina recommends sequencing to a depth of 3.25 B raw reads per slide.
- For post-optimization libraries, Illumina recommends sequencing to a depth of 5000 raw reads per 10 x 10 µm tissue area.
  - This sequencing depth recommendation is a starting point and is provided as general guidance. It can be further adjusted based on your needs.
  - Actual reads per cell reported in the analysis output can vary based on tissue type and cell size.

For more information, refer to 5.22 Sequencing and Analysis on page 113.

3.3 Embed Tissue in OCT Compound

Tissue samples must be frozen and embedded in Optimal Cutting Temperature (OCT) compound for optimal section quality. The following instructions describe a recommended method for freezing and embedding tissue samples in OCT compound and are intended as general guidance.

1. Add liquid nitrogen to a dewar.
2. Fill a container (for example, a stainless steel beaker) with isopentane and place it in the liquid nitrogen.
3. Place the fresh tissue sample into a cryomold.
4. Fill the cryomold with OCT compound until the tissue sample is covered.
5. Using forceps, lower the cryomold into the isopentane. Hold until the OCT compound is opaque and solidified.

Do not let the isopentane enter the cryomold.

6. Remove the OCT compound-embedded tissue sample from the cryomold.
7. Store the tissue sample at -80°C until you are ready to start the protocol.

3.4 Extract RNA for Quality Assessment

This section describes the process for collecting tissue sections in preparation for RNA extraction and RIN analysis. Before placing tissue sections on a slide, assess RNA quality by extracting RNA from fresh tissue and calculating the RNA integrity number (RIN).

- RIN scores are indicative of RNA quality. Low scores indicate that RNA is degraded, which results in fewer RNA molecules available for capture.
- The RNA quality within a tissue block impacts the sensitivity of the StrataMap Spatial Transcriptome Permeabilization Optimization kit. For best results, use tissue blocks with RNA that has a RIN greater than or equal to 7.

> *For tissue-specific recommendations, refer to the RNA extraction kit protocol.*

Consumables

- QIAGEN RNeasy Plus Mini Kit
- **[Optional]** QIAGEN QIAshredder
- Nuclease-free water
- 2 ml DNA LoBind microcentrifuge tube
- Dry ice
- Forceps
- Razor blade or scalpel
- RNase decontamination solution

Preparation

1. Turn on the cryostat to cool the chamber.

> *Make sure that the cryostat reaches operating temperature before sectioning.*

2. Remove the tissue block from -80°C storage.
3. Place the tissue block into the cryostat for 30 minutes to equilibrate to the cryostat chamber temperature.

4. Clean forceps with RNase decontamination solution, and then rinse with nuclease-free water. Let the forceps air dry.
5. Place the 2 ml microcentrifuge tube and forceps on dry ice for at least 5 minutes.

> *The tube and forceps must be chilled to prevent thawing and degradation of tissue sections.*

Procedure

1. Adjust the temperature of the cryostat blade and specimen holder based on the tissue type.

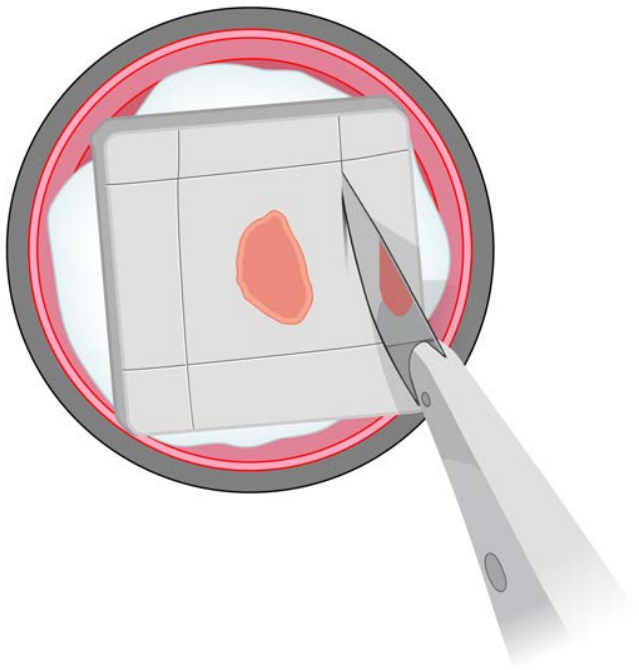
> *Illumina recommends -20°C for the blade holder and -10°C for the specimen head as starting points.*
>
> *For more information on adjusting temperature, refer to the cryostat manufacturer manual.*

2. Place the specimen chuck with your tissue block into the specimen head of the cryostat.

3. If the OCT compound extends more than ~1 mm beyond the tissue edge, use a razor blade or scalpel to trim around the tissue carefully to remove excess OCT (Figure 3).

> *Excess mounting media can negatively affect the RIN scores.*

Figure 3: OCT Trimming



4. Install the cryostat blade. Use a new blade for each tissue type.

> *To avoid injury when using the cryostat blade, make sure to follow all safety precautions and instructions in the cryostat manufacturer manual.*

5. Slice tissue into 10 µm sections.

For instructions, refer to the cryostat manufacturer manual.

6. Remove the forceps and microcentrifuge tube from the dry ice.
7. Using the chilled forceps, collect two 10 µm tissue sections.

Illumina recommends discarding the first three sections cut from the tissue block so that highest quality tissue is available for mounting.

For instructions, refer to the cryostat manufacturer manual.

8. Place the tissue sections in the chilled microcentrifuge tube.

Make sure that the tissue sections do not thaw or melt.

9. Place the microcentrifuge tube on dry ice.

If you are stopping here, refer to the *SAFE STOPPING POINT on page 22* *at the end of this procedure. Otherwise, proceed to step 10.*

10. Perform the Purification of Total RNA from Animal Tissues protocol from the QIAGEN RNeasy Plus Mini Kit.

If you are stopping here, refer to the *SAFE STOPPING POINT on page 22* *at the end of this procedure. Otherwise, proceed to* *3.5 RIN Analysis on page 23*.

SAFE STOPPING POINT

If you are stopping after placing the tissue sections in the microcentrifuge tube in step 9, then store the tube at -80°C until you are ready to proceed with step 10.

If you are stopping after extracting RNA in step 10, then you can store the extracted RNA at -80°C until you are ready to proceed to *3.5 RIN Analysis* on page 23.

3.5 RIN Analysis

This step quantifies the amount of RNA from two tissue sections and determines the RIN for the tissue block.

Consumables

- High sensitivity RNA screentape
- High sensitivity RNA screentape ladder
- High sensitivity RNA screentape sample buffer

Procedure

1. Quantify the extracted RNA and determine the RIN value for the tissue block.

> *For instructions, refer to the RNA screentape assay documentation on the Agilent website.*

3.6 Specimen Transport and Storage

- Transportation of samples must comply with all applicable governing regulations for the transport of etiologic agents.

> *Handle all samples as if they are potentially infectious agents.*

- Expedited shipping and transportation methods are recommended.

> *Exposure to elevated temperatures during transportation can negatively impact individual sample failure rates and sample performance.*

- Use a conical tube or slide mailer to transport the spatial slide.

If tissue is mounted, do not use a slide mailer to transport the spatial slide. Minimize movement during transport. Otherwise, slide damage can occur.

If you are transporting the spatial slide to another lab space after mounting tissue slices, but before tissue fixation, Illumina recommends transporting the slide in a pre-chilled, empty conical tube on dry ice.

If you are transporting the spatial slide to another lab space after tissue fixation, Illumina recommends placing the slide in a clean, empty conical tube at room temperature during transport.

4. Tips and Techniques

This section identifies tips and techniques that can be used to perform actions in the protocol.

General Tips and Techniques

- Follow the protocol steps precisely.

  Changes in volumes, timing, or steps can cause compromised results or assay failure.

- When sectioning tissue, embedding tissues in OCT compound, or sequencing, make sure that you are following the guidance outlined in *3. Before You Begin* on page 15.
- Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

4.1 Avoid Contamination

- Wear properly fitted gloves and change them frequently.
- Use filter tips only.
- If you are adding or transferring samples or reagent master mixes, change the tips.
- When you are between experiments, sterilize the bench and equipment to reduce the risk of carryover contamination as follows.

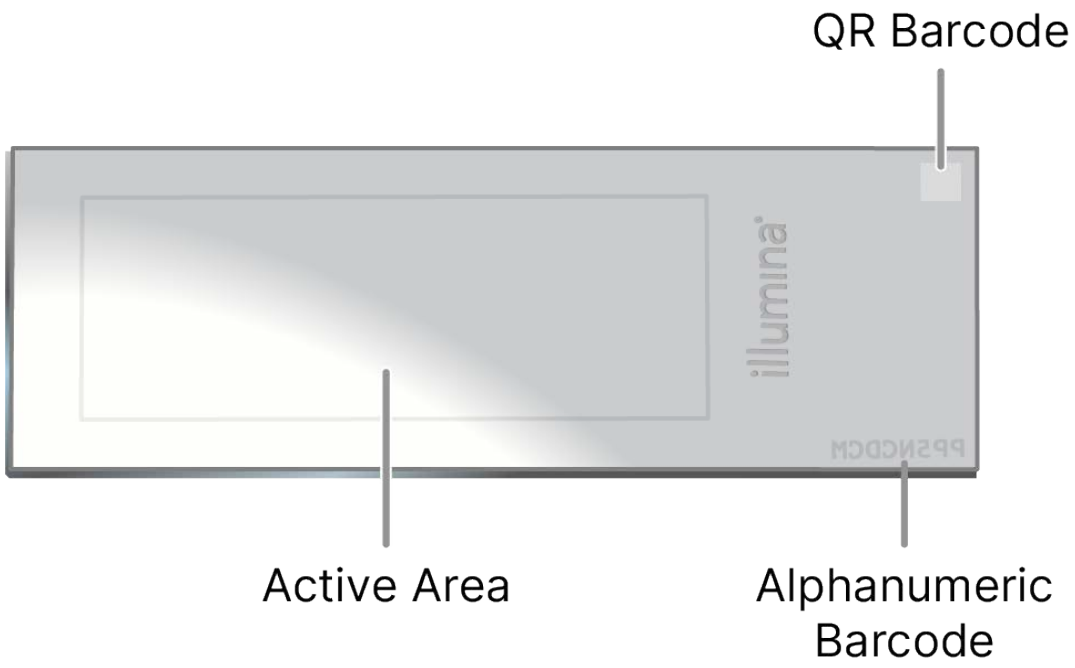
  1. Wipe surfaces with 70% EtOH and let dry.
  2. Wipe surfaces with 10% sodium hypochlorite and let stand for at least 1 minute.
  3. Wipe surfaces with sterilized, laboratory-grade water to remove any residual sodium hypochlorite, and then let dry.

4.2 Handle Tissue and Slide

- Identify the active side of the slide (Figure 4).
  - When the active side is facing up, the Illumina logo is legible.
  - The alphanumeric barcode is etched in the bottom-right corner of the slide. The barcode is not legible on the active side. Tissue sections are placed on this side of the slide.

- If the barcode is legible, turn the slide over so that the active area is facing up.
- If you are placing the slide on a surface, make sure that the active side is facing up.

Figure 4: Active Side of Slide



- When handling the slide, avoid touching the active area. Touch the edges of the slide only (Figure 5).

Figure 5: Slide Handling Example

- The slide is fragile. When handing the slide, do not apply excessive pressure.

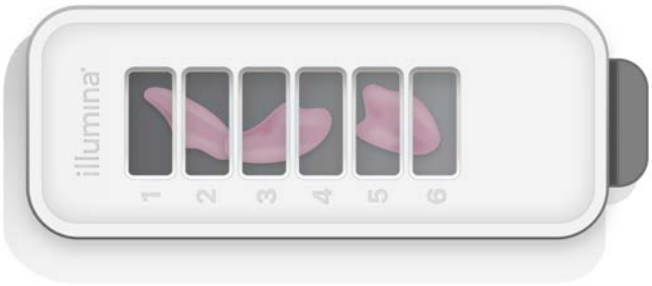
4.3 Mount Tissue

- For large tissue blocks, Illumina recommends shallowly scoring the block so that the tissue section fits within the well.

- Avoid mounting large tissue sections across multiple wells. During a permeabilization time course, a tissue section mounted across multiple wells undergoes different permeabilization times across the same tissue (Figure 6).

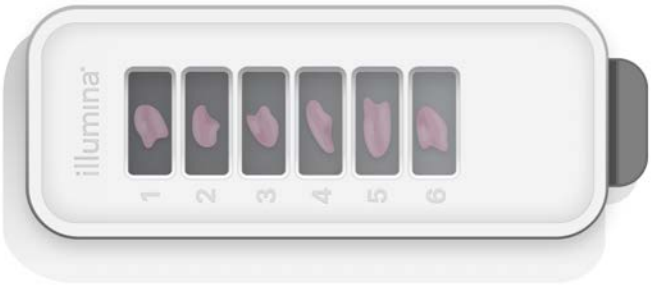
> *Placement errors can result in loss of usable area or sample.*

Figure 6: Incorrect Tissue Section Placement



- Tissue sections should be mounted within each spatial slide target area (Figure 7).

Figure 7: Correct Tissue Section Placement



- Mounting six tissue sections within the spatial slide target area requires precise placement and spacing. Tissue sections cannot be removed or repositioned once they are mounted on the spatial slide.
- To identify the positions of the six cassette wells, use the provided stencil.

– Place the slide into the stencil with the active side facing away from the stencil template (Figure 8).

Figure 8: Slide Placement in Stencil

– Use an alcohol-based marker to trace the stencil onto the non-active side of the slide. Fill in the entire stencil groove so that the separation between wells is visible (Figure 9).

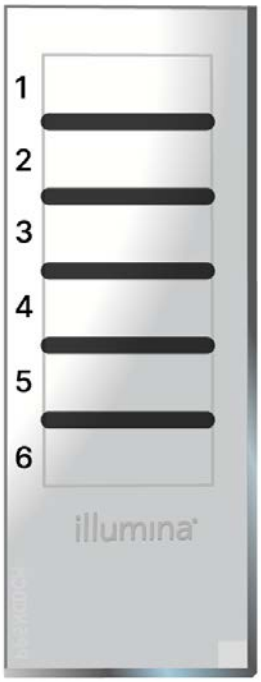
Tissue placed over the stenciled area is inaccessible to reagents.

Figure 9: Stencil Tracing

- Illumina recommends designating a tissue section with different permeabilization conditions as separate samples in ISIT.
- Sample tissue masks should comprise a region of tissue with a single permeabilization time.

- Illumina recommends practicing tissue placement on a standard glass slide using the provided stencil first to simulate the six spatial slide target areas (Figure 10).
  - This practice step can be performed using a less limited or non-critical tissue block that is approximately the same size as the tissue block used for your experiments.
  - The target area numbers in Figure 10 are for reference only and should not be marked on the slide.

Figure 10: Slide Target Area Numbering



4.4 Section Tissue

- Adjust the temperature of the cryostat blade and specimen holder based on the tissue type.

> *Illumina recommends -20°C for the blade holder and -10°C for the specimen head as starting points.*
>
> *For more information on adjusting temperature, refer to the cryostat manufacturer manual.*

- Avoid touching the active area of the slide to the surface of the cryostat. Sensitivity can be reduced.
- To avoid decreased performance, make sure that the shortest amount of time possible (less than one hour) is used between mounting the first tissue section and fixing tissue.
- If you are stopping at the **SAFE STOPPING POINT** after *5.2 Section and Place Tissue* on page 48, store the mounted slide at -80°C within one hour of mounting the first tissue section.

4.5 Apply and Remove Coverslip

- When placing a coverslip:
  - Use 75 µl 85% glycerol.
  - Pipette slowly to avoid bubbles.
  - If there are any bubbles on top of the tissue sections, gently press the coverslip onto the slide to remove them.

> *Do not shift the coverslip after mounting. Damage to the underlying tissue can occur.*

- Remove a coverslip as follows.
  1. Place the slide in a prepared 50 ml conical tube with 45 ml nuclease-free water.
  2. Incubate at room temperature for 5 minutes.
  3. Using forceps, slowly remove the slide from the conical tube. As you lift the slide, make sure that the coverslip slides off.

4. If the coverslip does not slide off, submerge the slide in the nuclease-free water as much as possible and gently pull the coverslip parallel to the slide surface to separate it from the slide (Figure 11).

> *Do not pry or lift the coverslip at an angle or scrape the tissue sample.*

Figure 11: Coverslip Removal

4.6 Wash Slide

- The slide must be washed in nuclease-free water at specific points in the protocol (for example, during steps in *5.3 Stain Tissue* on page 56).
- Wash the slide with nuclease-free water as follows.

  1. Carefully place the slide into one of the prepared conical tubes with 45 ml nuclease-free water.

  > *When handling the slide, use gloves and touch the edges only. Do not touch the active area.*

  > *For more information on how to handle the slide, refer to 4.2 Handle Tissue and Slide on page 25.*

  2. Invert conical tube 15 times to wash.

  3. Using forceps, remove the slide from the conical tube.

- If you are having issues removing the slide, the following methods can help with removal:
  - Use the forceps to move the slide gently from side to side to loosen it from the tube wall.
  - Recap and invert the conical tube, and then gently tap the lid on the benchtop to release the slide from the bottom of the tube.

– Gently squeeze the sides of the conical tube to widen the area inside where the slide is stuck, and then use the forceps to remove the slide (Figure 12).

Make sure that the slide is not lodged in the cap before twisting to open the conical tube. Damage to the slide can occur.

Figure 12: Slide Removal

4.7 Handle Beads

Store and Prepare Beads

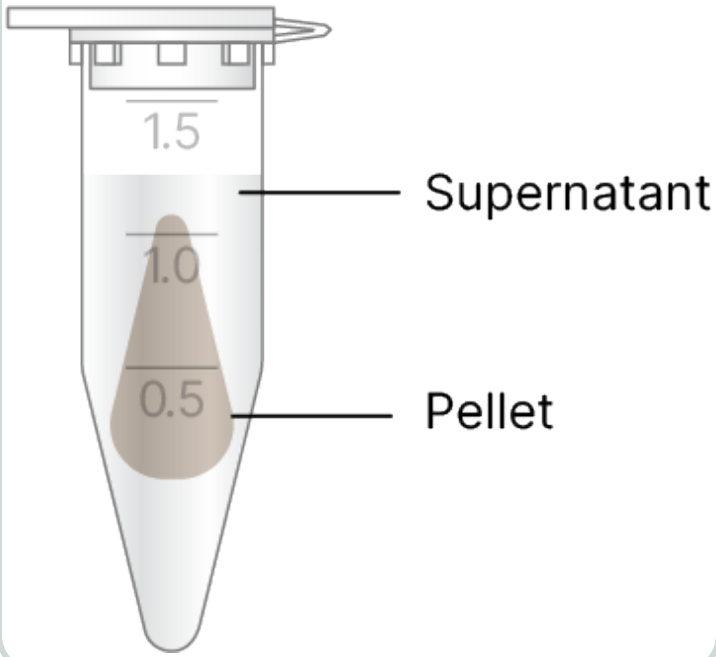
- Store purification beads upright so that beads are always submerged in the buffer.
- Vortex stock bead tubes for 1 minute until the beads are resuspended. To avoid resettling the beads, vortex before pipetting.

Use Beads During Cleanup

- Use the magnetic stand to pull bead pellets to the bottom of the tubes so that they are fully pelleted (Figure 13).

> *Do not agitate the tubes while they are on the stand. Remove them only when instructed.*

Figure 13: Pelleted Beads in Supernatant



- Pipette slowly to minimize bubbles.
- Do not disturb the bead pellets.
- If beads are aspirated into the pipette tips, dispense them back into the tube or onto the plate that is on the magnetic stand.
- Wait until the liquid is clear before proceeding.
- If the liquid adheres to the side or top of the tube, centrifuge at 280 × g for 3 seconds.

5. Protocol

This section describes the protocol for creating and sequencing six libraries using the StrataMap Spatial Transcriptome Permeabilization Optimization kit.

- Review the complete assay workflow.
- Make sure that products and experiment parameters are compatible with the assay.
- Before proceeding, confirm the kit contents and make sure that you have the required components, equipment, and consumables.
- Make sure that the consumables are fresh, sealed, and stored properly and that containers are clean and RNase-free.
- Follow the protocol steps in the order shown, using the specified volumes and incubation parameters. Changes in volumes, timing, or steps can cause compromised results or assay failure.

> *For a comprehensive overview of the protocol, refer to* *5.1 Permeabilization Time Workflow on page 42*.
>
> *For support videos, refer to the Spatial Transcriptomics playlist.*

- The StrataMap Spatial Transcriptome Permeabilization Optimization kit requires a third-party imaging system. The imaging system should be qualified before beginning the StrataMap Spatial Transcriptome Permeabilization Optimization kit workflow.

> *For more information, refer to the StrataMap Spatial Transcriptome Imaging Guide (document # 200081305) on the Illumina support site.*

- Permeabilization times from third-party spatial transcriptomics kits cannot be used with the StrataMap Spatial Transcriptome Permeabilization Optimization kit.

Determine Permeabilization Time

- If you need to determine the optimal permeabilization time for your tissue, Illumina recommends conducting a permeabilization time titration to determine the optimal permeabilization time.
- After the optimal permeabilization time has been determined, you can use the product and following steps to amplify and sequence the remainder of any of the six libraries:
  - *5.24 Amplify Post-Optimization Libraries* on page 118
  - *5.25 Clean Up Post-Optimization Libraries* on page 122

- Sequencing the remainder of the libraries can provide deeper sequencing coverage and reveal additional unique molecules.
- You can also use the permeabilization time to perform the StrataMap Spatial Transcriptome protocol using the StrataMap Spatial Transcriptome (L) or (S) kit.

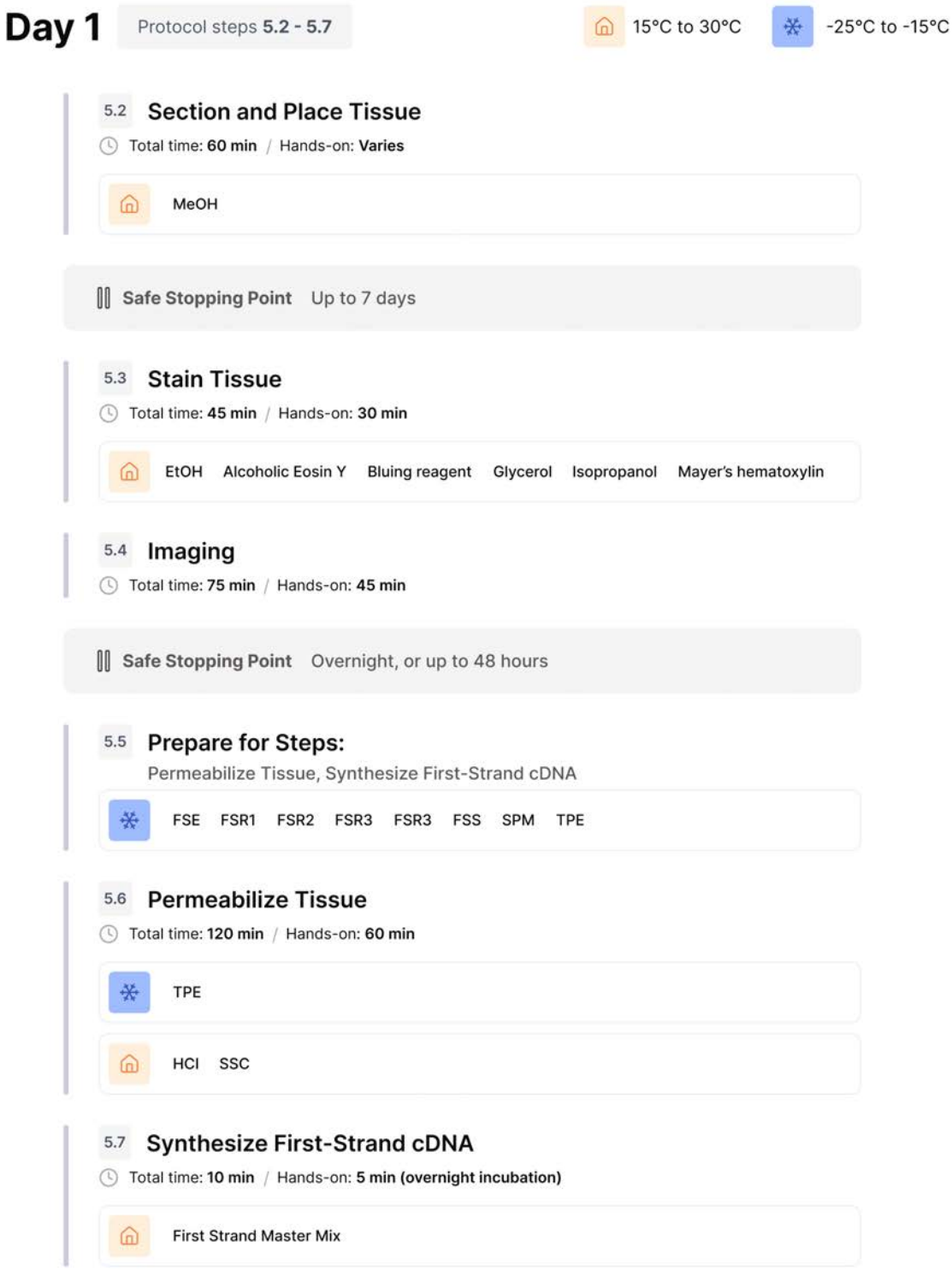
> *For more information, refer to StrataMap Spatial Transcriptome User Guide (document # 200073552) on the Illumina support site.*

- The following warnings and precautions must be followed when setting up or performing protocol steps.

5.1 Permeabilization Time Workflow

With the StrataMap Spatial Transcriptome Permeabilization Optimization kit, you can determine the optimal pemeabilization time for your tissue type of interest.

- Protocol steps are organized by day.
- Each step has a section number identified for navigation.
- The total and hands-on time estimates have been included.
- Applicable reagents for each protocol step are listed.
- Safe stopping points are included along with general instructions.


Day 1
Protocol steps 5.2 - 5.7
15°C to 30°C
-25°C to -15°C
5.2 Section and Place Tissue
Total time: 60 min / Hands-on: Varies
MeOH
Safe Stopping Point Up to 7 days
5.3 Stain Tissue
Total time: 45 min / Hands-on: 30 min
EtOH Alcoholic Eosin Y Bluing reagent Glycerol Isopropanol Mayer's hematoxylin
5.4 Imaging
Total time: 75 min / Hands-on: 45 min
Safe Stopping Point Overnight, or up to 48 hours
5.5 Prepare for Steps:
Permeabilize Tissue, Synthesize First-Strand cDNA
FSE FSR1 FSR2 FSR3 FSR3 FSS SPM TPE
5.6 Permeabilize Tissue
Total time: 120 min / Hands-on: 60 min
TPE
HCl SSC
5.7 Synthesize First-Strand cDNA
Total time: 10 min / Hands-on: 5 min (overnight incubation)
First Strand Master Mix

Day 2

Protocol steps **5.8 - 5.13**

15°C to 30°C

-25°C to -15°C

5.8 Prepare for Steps:

Digest Tissue, Remove RNA

- TDE
- RSB TDB

5.9 Digest Tissue

Total time: **75 min** / Hands-on: **15 min**

- TDE
- TDB

5.10 Remove RNA

Total time: **15 min** / Hands-on: **7 min**

- RSB NaOH

5.11 Prepare for Steps:

Synthesize Second-Strand cDNA, Elute cDNA

- SAM SSR UCM
- RSB

5.12 Synthesize Second-Strand cDNA

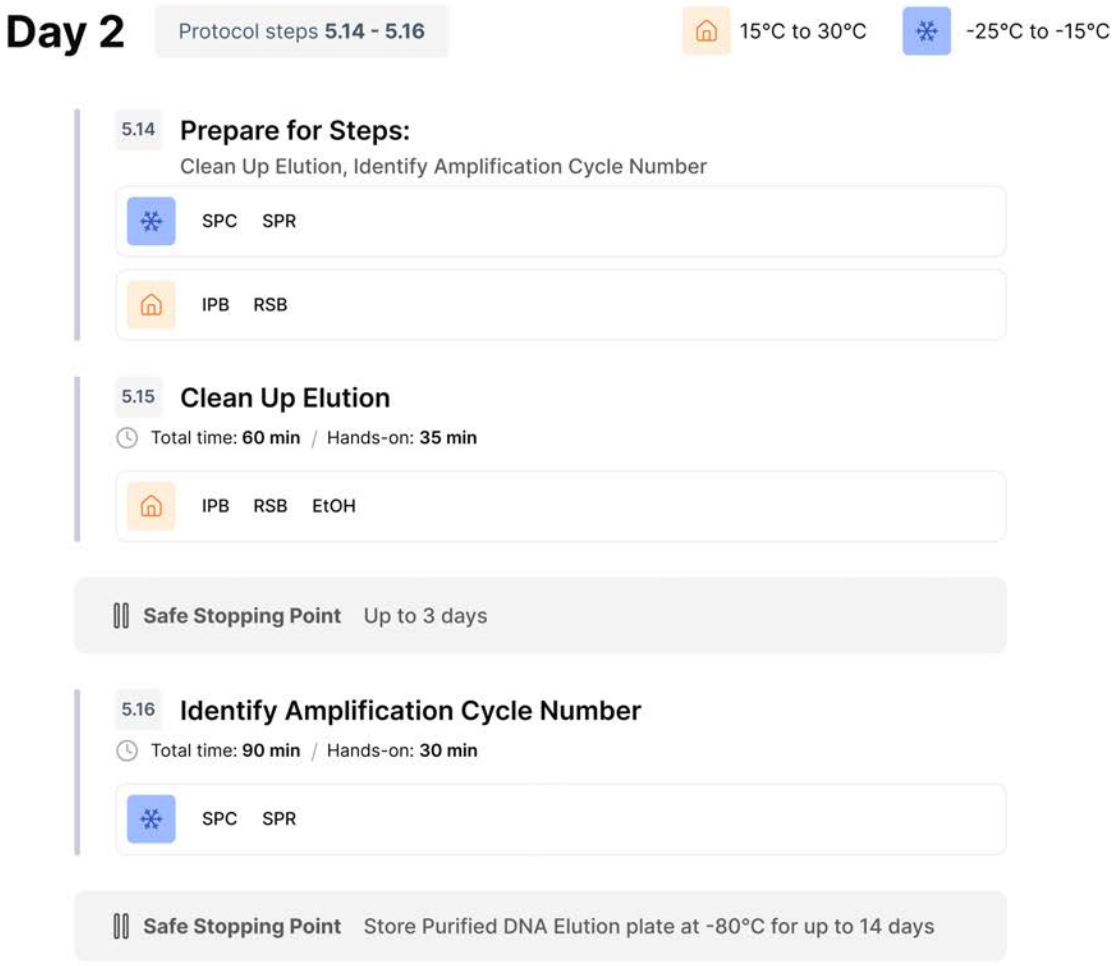
Total time: **80 min** / Hands-on: **30 min**

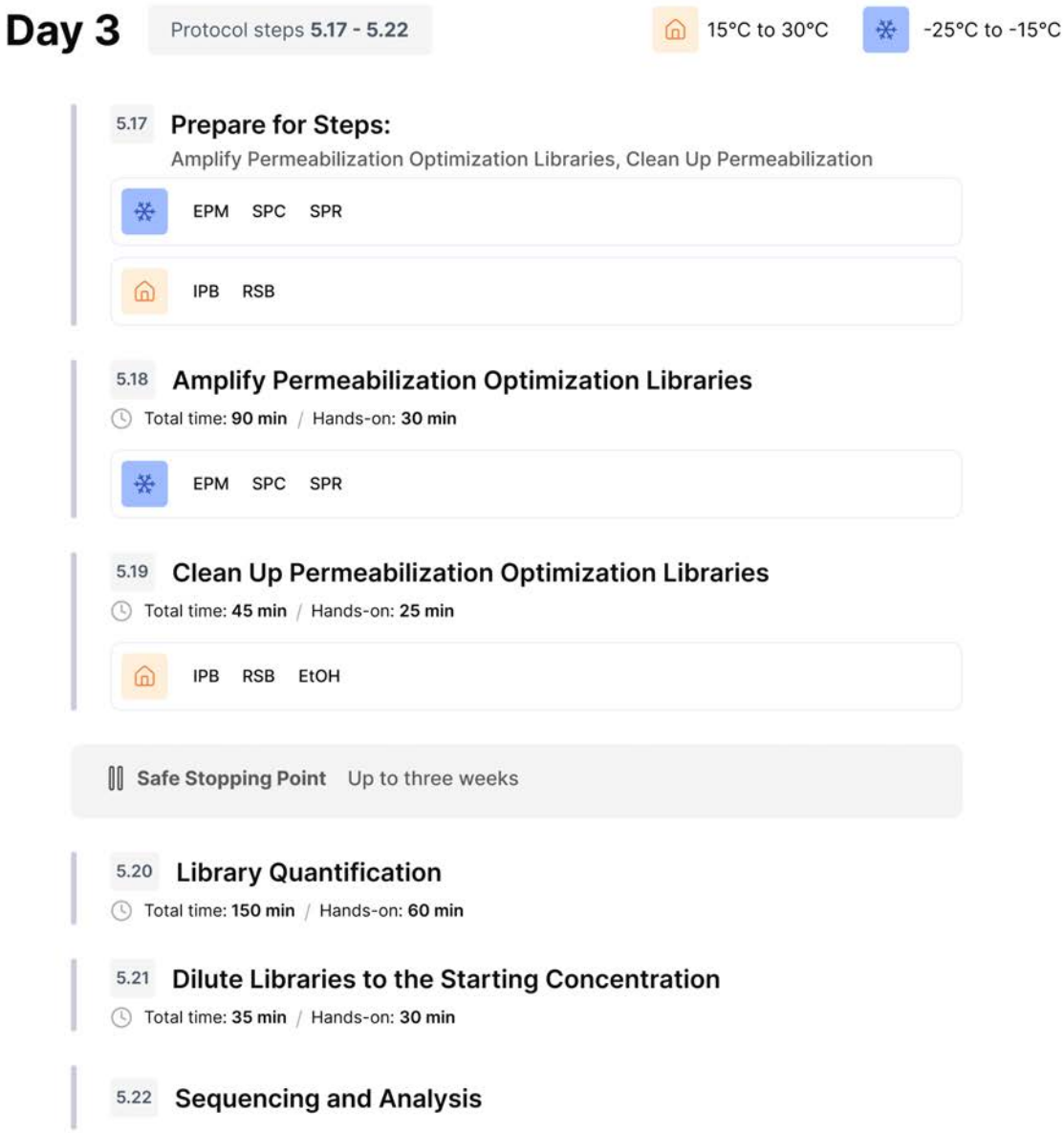
- SAM SSR
- RSB NaOH

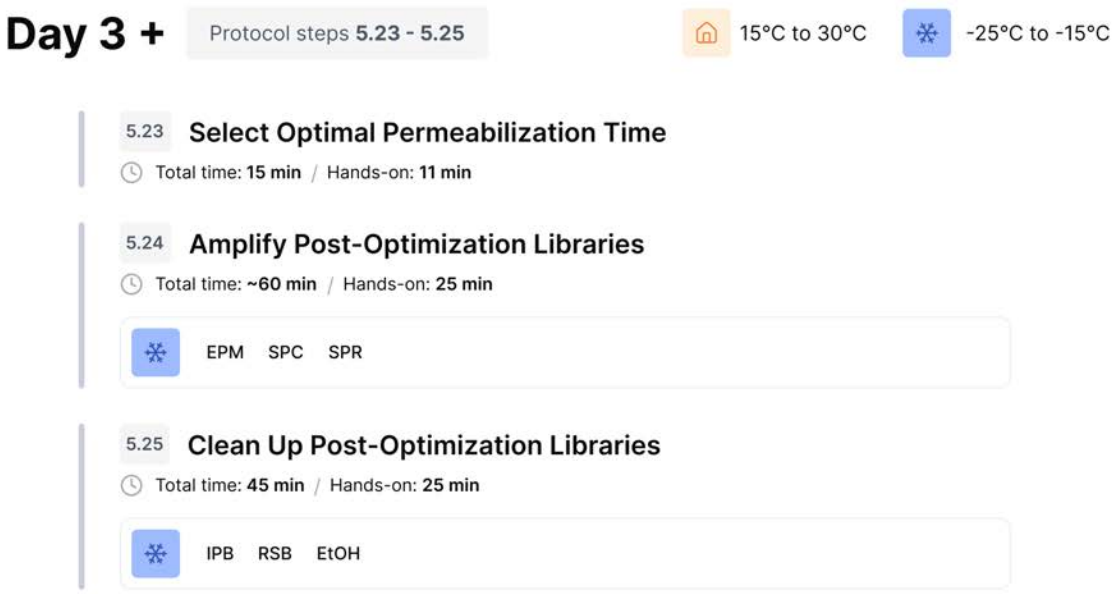
5.13 Elute cDNA

Total time: **20 min** / Hands-on: **10 min**

- SAM SSR
- RSB


Day 2
Protocol steps 5.14 - 5.16
15°C to 30°C
-25°C to -15°C
5.14
Prepare for Steps:
Clean Up Elution, Identify Amplification Cycle Number
SPC SPR
IPB RSB
5.15
Clean Up Elution
Total time: 60 min / Hands-on: 35 min
IPB RSB EtOH
Safe Stopping Point Up to 3 days
5.16
Identify Amplification Cycle Number
Total time: 90 min / Hands-on: 30 min
SPC SPR
Safe Stopping Point Store Purified DNA Elution plate at -80°C for up to 14 days


Day 3
Protocol steps 5.17 - 5.22
15°C to 30°C
-25°C to -15°C
5.17 Prepare for Steps:
Amplify Permeabilization Optimization Libraries, Clean Up Permeabilization
EPM SPC SPR
IPB RSB
5.18 Amplify Permeabilization Optimization Libraries
Total time: 90 min / Hands-on: 30 min
EPM SPC SPR
5.19 Clean Up Permeabilization Optimization Libraries
Total time: 45 min / Hands-on: 25 min
IPB RSB EtOH
Safe Stopping Point Up to three weeks
5.20 Library Quantification
Total time: 150 min / Hands-on: 60 min
5.21 Dilute Libraries to the Starting Concentration
Total time: 35 min / Hands-on: 30 min
5.22 Sequencing and Analysis


Day 3 +
Protocol steps 5.23 - 5.25
15°C to 30°C
-25°C to -15°C
5.23
Select Optimal Permeabilization Time
Total time: 15 min / Hands-on: 11 min
5.24
Amplify Post-Optimization Libraries
Total time: ~60 min / Hands-on: 25 min
EPM SPC SPR
5.25
Clean Up Post-Optimization Libraries
Total time: 45 min / Hands-on: 25 min
IPB RSB EtOH

5.2 Section and Place Tissue

This step sections and transfers fresh frozen tissue onto the slide.

Before beginning this step, Illumina recommends practicing placement on a standard glass slide. For more information, refer to *4.3 Mount Tissue* on page 27.

Consumables

- Absolute MeOH
- Nuclease-free water
- Slide mailer
- 50 ml conical tube (qty 3–4)
- Disposable trough
- **[Optional]** Pure compressed inert gas (for example, nitrogen gas)
- Spatial transcriptome slide
- Razor blade (or scalpel)

Preparation

1. Turn on the cryostat to cool the chamber.

   For instructions, refer to the cryostat manufacturer manual.

2. Remove the tissue block from -80°C storage.
3. Place the tissue block into the cryostat for 30 minutes to equilibrate to the cryostat chamber temperature.
4. Prepare the MeOH as follows.
   a. Add 14 ml of absolute MeOH to a slide mailer.
   b. Place the slide mailer in the -20°C freezer for at least 30 minutes.

5. Record the serial number of the slide packaging (Figure 14).

> *This serial number is used for image processing with ISIT and is identified as SN on the label. If you do not record the SN, issues with cloud upload and run planning can occur.*

Figure 14: Example Packing Labels with SN

6. Remove the slide from the storage buffer.

> *When handling the slide, make sure that you are using a sterile RNase-free technique to avoid contamination. Make sure that you are wearing gloves and only touch the edges of the slide to avoid the active area.*

7. Gently wash the slide three times in nuclease-free water. Use a new conical tube for each wash.

> *For instructions, refer to 4.6 Wash Slide on page 36.*

8. If you have pure compressed inert gas, use it to dry the slide.

9. If you do not have pure compressed inert gas, use a swinging bucket centrifuge to spin the slide in the conical tube at 250 x g for 30 seconds to remove excess water.

> *When using the swinging bucket centrifuge, do not exceed 250 x g. Otherwise, the slide can break or become damaged.*

10. Remove the slide from the conical tube and identify the active area.

> *For more information, refer to 4.2 Handle Tissue and Slide on page 25.*

11. Place the slide active side up and let it air dry until no water droplets remain.
12. Place the slide into the stencil with the active side facing away from the stencil template. Make sure that the slide is fully inserted into the stencil (Figure 15).

Figure 15: Slide Placement in Stencil

13. Using an alcohol-based marker, trace the stencil onto the non-active side of the slide (Figure 16).

- Fill in the entire stencil groove so that the separation between wells is visible.
- Make sure that the stencil dries before proceeding.

Tissue placed over the stenciled area is inaccessible to reagents.

Figure 16: Stencil Tracing

14. Remove the slide from the stencil.

Procedure

1. Set the temperature of the cryostat blade and specimen holder.

Make sure that the cryostat reaches operating temperature before sectioning.

Illumina recommends -20°C for the blade holder and -10°C for the specimen head as starting points.

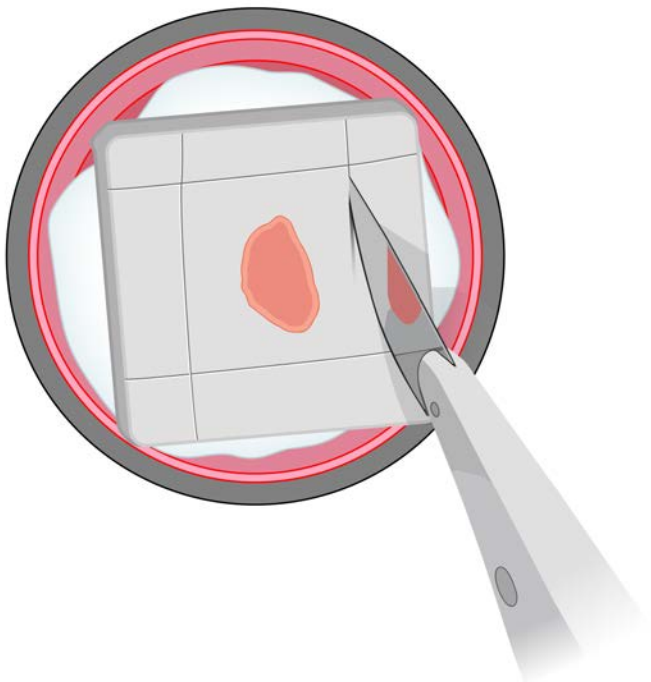
Adjust the temperatures as needed based on the tissue type or previous experience with using a cryostat for sectioning. For more information on adjusting temperature, refer to the cryostat manufacturer manual.

2. Place the specimen chuck with your tissue block into the specimen head of the cryostat.

3. Make sure that the OCT compound does not extend more than ~1 mm beyond the tissue edge. If it does, then use a razor blade or scalpel to carefully score so that excess OCT compound around the tissue can be removed (Figure 17).

> *Trimming excess OCT compound allows tissue to be positioned together closely, which maximizes the capture area.*
>
> *Illumina recommends scoring the tissue to fit within each of the six well areas of the slide. For more information, refer to* *4.3 Mount Tissue on page 27**.*

Figure 17: OCT Trimming



4. Install the cryostat blade.

> *To avoid injury when using the cryostat blade, make sure to follow all safety precautions and instructions in the cryostat manufacturer manual.*

> *Use a new blade for each tissue type.*

5. Slice tissue into 10 µm sections.

> *Make sure that the slide remains in the cryostat while working to avoid tissue thawing and degradation.*
>
> *To avoid decreased performance, make sure that the shortest amount of time possible (less than one hour) is used between mounting the first tissue section and fixing tissue.*
>
> *If you stop at the* *SAFE STOPPING POINT on page 55**, store the mounted slide at -80°C within one hour of mounting the first tissue section.*

> *Illumina recommends discarding the first three sections cut from the tissue block so that highest quality tissue is available for mounting. For instructions, refer to the cryostat manufacturer manual.*

6. Carefully place tissue sections in each of the six well areas on the slide surface as follows.

> *Make sure that you are placing the tissue section on the active side of the slide and avoid overlapping OCT or tissue with any neighboring tissue sections.*
>
> *Tissue sections cannot be adjusted or removed from the slide after placement. All tissue slices placed on the slide are part of the six final libraries.*

a. Pick up the slide and place a gloved finger on the back of the slide (the non-active side) where you are planning on placing the tissue section.
b. Hold your finger in place for at least 5 seconds to warm the slide before transferring the tissue section.

c. Lower the warmed active area of the slide onto the tissue section to start adhesion.

> *Do not touch the slide to the cryostat surface.*

> *The warmed area attracts the tissue section to the slide.*

d. Place a gloved finger on the back of the slide until the tissue section and OCT melts fully onto the slide.

e. Repeat steps a–d for the remaining tissue sections that must be added to the slide. Make sure that tissue sections are at least 2 mm apart.

> *If you are stopping here, refer to the SAFE STOPPING POINT on page 55. Otherwise, proceed to step 7.*

7. Using a lint-free wipe that is pre-moistened with alcohol, wipe the non-active side of the slide quickly to remove the drawn stencil lines.
8. Immediately place the slide into the slide mailer with pre-chilled MeOH. Make sure that the tissue sections are fully submerged.
9. Place the slide mailer upright in the -20°C freezer for 30 minutes for tissue fixation.

SAFE STOPPING POINT

If you are stopping before step 8, store the slide as follows.

- Pre-chill a 50 ml conical tube on dry ice or at -80°C.
- Place the slide in the pre-chilled, empty conical tube and seal tightly.
- Store the slide at -80°C for up to 7 days.

5.3 Stain Tissue

This step stains tissue with hematoxylin and eosin before imaging.

Consumables

- Absolute EtOH
- Alcoholic eosin Y
- Bluing reagent
- Glycerol
- Isopropanol
- Hematoxylin
- Nuclease-free water
- 1.5 ml microcentrifuge tubes
- Slide mailer (qty 4)
- 50 ml conical tube (qty 10)
- Lint-free wipes
- No 1.5 coverslip
- Disposable trough
- Cassette base

Preparation

1. If you stored the slide after *5.2 Section and Place Tissue* on page 48, prepare it as follows.
   a. Place the slide into the slide mailer with pre-chilled MeOH. Make sure that the tissue sections are fully submerged.
   b. Place the slide mailer upright in the -20°C freezer for 30 minutes for tissue fixation.
2. Prepare four slide mailers with reagents as follows.
   a. Add 14 ml isopropanol to a slide mailer.
   b. Add 14 ml hematoxylin to a slide mailer.
   c. Add 14 ml bluing reagent to a slide mailer.
   d. Add 14 ml alcoholic eosin Y to a slide mailer.
3. Add 45 ml nuclease-free water to six 50 ml conical tubes.

4. Combine the following volumes to create 100 ml fresh 70% EtOH, including overage. Vortex to mix.
   - Absolute EtOH (70 ml)
   - Nuclease-free water (30 ml)
5. Add 45 ml 70% EtOH to two conical tubes.
6. Combine the following volumes to create 100 ml fresh 50% EtOH, including overage. Vortex to mix.
   - Absolute EtOH (50 ml)
   - Nuclease-free water (50 ml)
7. Add 45 ml 50% EtOH to two conical tubes.
8. Combine the following volumes to create 300 µl 85% glycerol. Pipette slowly to avoid bubbles.
   - Absolute glycerol (255 µl)
   - Nuclease-free water (45 µl)
9. Preheat the thermal cycler to 37°C. Set the lid temperature to 47°C.
10. When prompted to select a reaction volume, set it to the maximum volume.

> *The volume is not applicable when using the cassette adapter.*

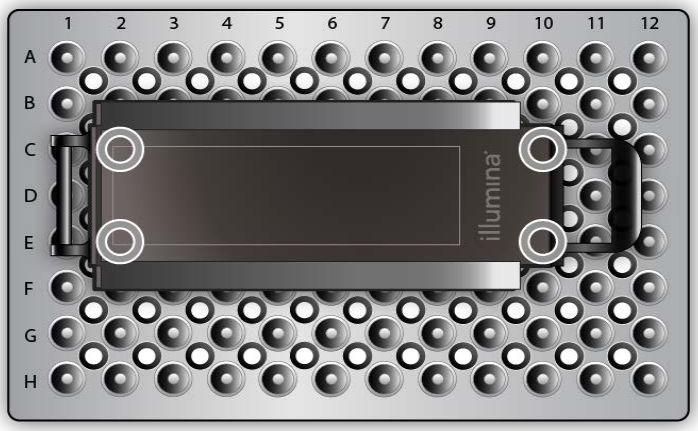
11. Remove the cassette from the packaging.

> *The cassette packaging includes the cassette base and lid. The cassette does not come pre-assembled.*

12. Place the cassette base onto the thermal cycler. Do not close the thermal cycler lid.

- The cassette base is used to dry the slide when it is placed in the thermal cycler.
- Make sure that the cassette is flat and that the cassette legs are within the wells of the thermal cycler (Figure 18).

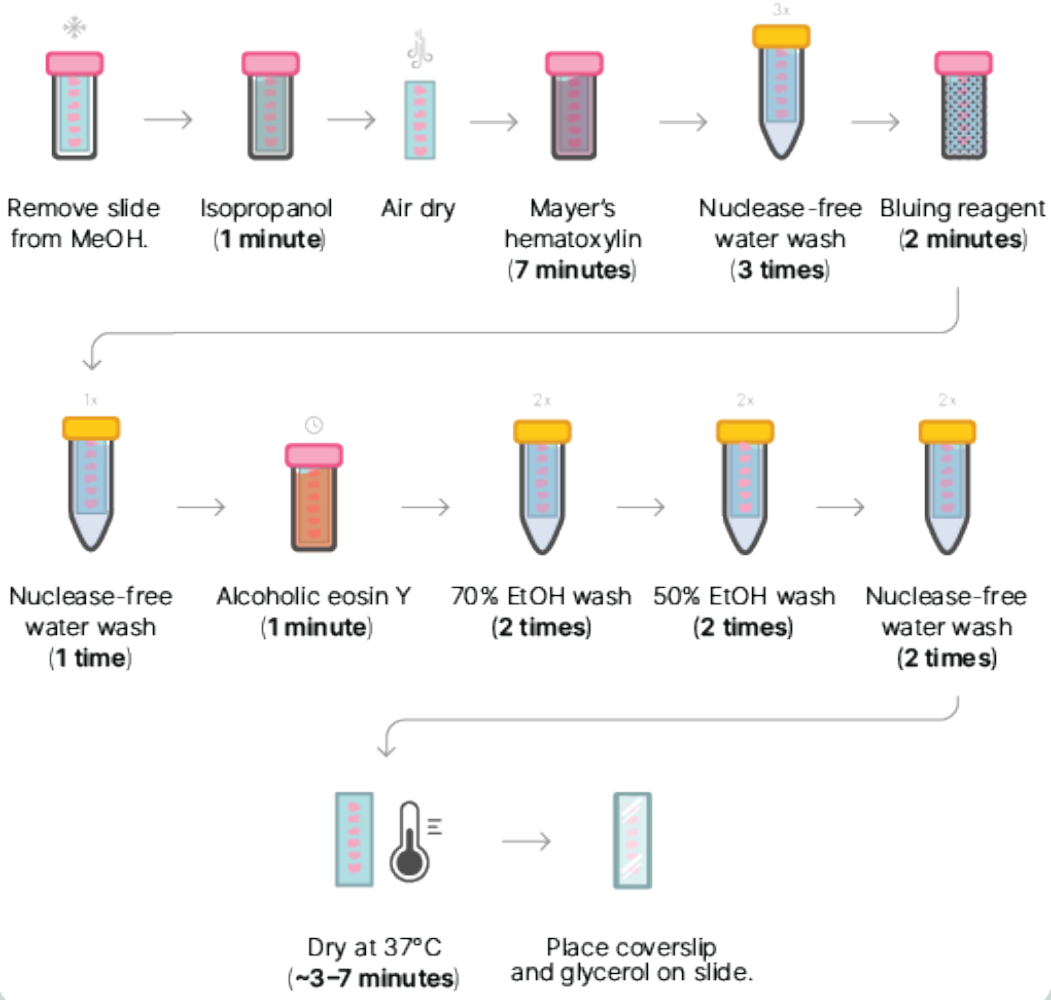
Figure 18: Cassette Placement onto Thermal Cycler



Procedure

This procedure involves incubating the slide in multiple reagents, as shown in Figure 19.

Figure 19: Stain Tissue Workflow



1. Remove the slide from the MeOH.

> *Do not touch the active surface. Damage to the sample or image quality issues can occur.*

If the slide has excess MeOH on the surface after removal, it is safe to proceed with staining.

2. Incubate the slide in isopropanol as follows.
   a. Carefully place the slide into the prepared slide mailer with 14 ml isopropanol.
   b. Incubate at room temperature for 1 minute.
   c. Using forceps, remove the slide from the slide mailer.
3. Place the slide active-side up on top of a trough and let air dry for 5–10 minutes. Inspect after 5 minutes.

Do not let the slide air dry for longer than 10 minutes. Damage to the sample or imaging quality issues can occur.

The tissue appears white when it is dried.

4. Incubate the slide in hematoxylin as follows.
   a. Carefully place the slide into the prepared slide mailer with 14 ml hematoxylin.
   b. Incubate at room temperature for 7 minutes.
   c. Using forceps, remove the slide from the slide mailer.

When using hematoxylin, avoid inhaling vapor or skin contact.

For more information, refer to the SDS at support.illumina.com/sds.html.

5. Wash the slide in nuclease-free water three times. Use a new conical tube for each wash.

For instructions, refer to 4.6 Wash Slide on page 36.

If there is excess water on the surface after washing, you can proceed.

6. Incubate the slide in bluing reagent as follows.
   a. Carefully place the slide into the prepared slide mailer with 14 ml bluing reagent.
   b. Incubate at room temperature for 2 minutes.
   c. Using forceps, remove the slide from the slide mailer.

7. Wash the slide in nuclease-free water one time.

   For instructions, refer to 4.6 Wash Slide on page 36.

8. Using forceps, remove the slide from the slide mailer.
9. Incubate the slide in alcoholic eosin Y as follows.
   a. Carefully place the slide into the prepared slide mailer with 14 ml alcoholic eosin Y.
   b. Incubate at room temperature for 1 minute.
   c. Using forceps, remove the slide from the slide mailer.

   When using alcoholic eosin Y, avoid inhaling vapor or skin contact.

   For more information, refer to the SDS at support.illumina.com/sds.html.

10. Wash the slide in 70% EtOH as follows.
    a. Carefully place the slide into the prepared conical tube with 45 ml 70% EtOH.
    b. Invert 15 times to wash.
    c. Using forceps, remove the slide from the conical tube.
    d. Repeat steps a–c one time for a total of two washes. Use a new conical tube for each wash.
11. Wash the slide in 50% EtOH as follows.
    a. Carefully place the slide into the prepared conical tube with 45 ml 50% EtOH.
    b. Invert 15 times to wash.
    c. Using forceps, remove the slide from the conical tube.
    d. Repeat steps a–c one time for a total of two washes. Use a new conical tube for each wash.
12. Wash the slide in nuclease-free water two times.

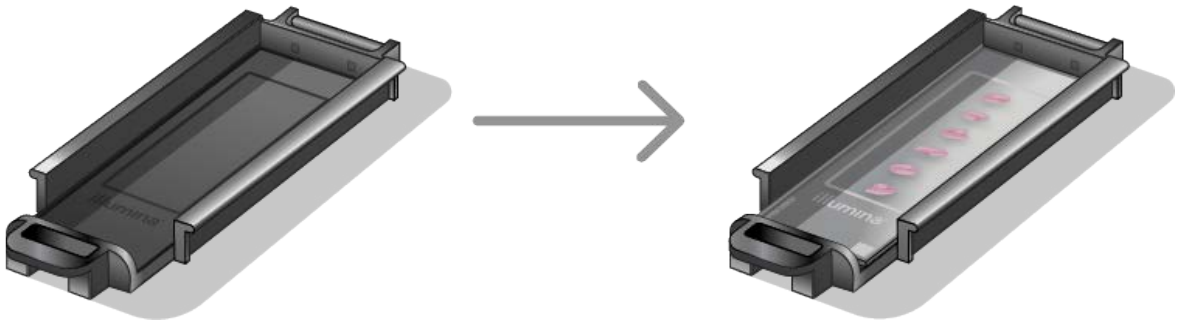
    For instructions, refer to 4.6 Wash Slide on page 36.

13. Tilt the edge of the slide onto a lint-free wipe to remove excess water. Wipe the back of the slide until it is dry.

    Make sure that the lint-free wipe does not touch the active area of the slide.

14. Place the slide active-side up into the base of the cassette so that the Illumina logos on the slide and cassette base align. Make sure that the slide is flat and level in the base of the cassette. (Figure 20).
    - Make sure that you are wearing gloves and only touch the edges of the slide to avoid the active area.
    - Make sure that the slide is flat and level in the base of the cassette.

Figure 20: Slide Placement in Cassette Base



15. If the cassette base was removed from the thermal cycler, place the cassette base with the slide onto the thermal cycler.

> *Do not close the thermal cycler lid.*

16. Dry the slide at 37°C for 3–7 minutes or until there is no visible moisture.

> *Monitor the slide closely during the drying process. Insufficient drying can lead to bubble formation when applying the coverslip. Excessive drying can damage the tissue and affect image quality.*

17. Remove the cassette base with the slide from the thermal cycler.
18. Remove the slide from the cassette base and place it on a lint-free wipe. Set the cassette base aside for later use.
19. Using glycerol, place the coverslip on the slide.

For instructions, refer to 4.5 Apply and Remove Coverslip on page 34.

20. **[Optional]** If a significant amount of bubbles form on the area covered by tissue, remove the coverslip, wash the slide two times in nuclease-free water, and apply a new coverslip.

    For instructions, refer to 4.5 Apply and Remove Coverslip on page 34 and 4.6 Wash Slide on page 36.

21. Incubate the slide at room temperature for at least 10 minutes to allow the glycerol to settle evenly.
22. Pick up the slide and lightly press it at an angle against a lint-free wipe to remove any excess glycerol that is seeping out from under the coverslip.
23. Using a lint-free wipe moistened with absolute EtOH, clean the back of the slide.

Excess glycerol must be removed before imaging. If glycerol is not removed, damage to the imaging system or image quality issues can occur.

After staining, proceed directly to 5.4 Imaging on page 64.

For imaging requirements and recommendations, refer to the StrataMap Spatial Transcriptome Imaging Guide (document # 200081305) on the Illumina support site.

5.4 Imaging

Imaging is done with a third-party imaging system that is used to take images of the stained tissue on the slide. Before imaging, make sure that your system has been assessed and that the same setup (including the objective lens) during the assessment is used for this step. After imaging, the Illumina StrataMap Image Tool (ISIT) is used to assess images.

For more information, including example imaging systems, assessment instructions, and guidance on good image quality, refer to the StrataMap Spatial Transcriptome Imaging Guide (document # 200081305) on the Illumina support site.

For setup instructions and imaging guidance, refer to the manufacturer instructions for your system.

For more information on ISIT, refer to the Illumina StrataMap Image Tool section of the Illumina StrataMap Spatial Software documentation.

Preparation

1. Make sure that the imaging system is set to 20X magnification.
2. Position the slide in the slide holder so that it is properly oriented for the imaging system.
   - For more information, refer to the imaging system manufacturer instructions.
   - If the objective is in the top of the imaging system, place the slide active side up.
   - If the objective is in the bottom of the imaging system, place the slide active side down.

   If you are using a spring-loaded slide holder, release the spring slowly so that it does not crack the slide.
3. Make sure that the imaging system is stabilized.

   Do not move or shake the table or touch the imaging system during imaging. Imaging errors can occur.

For more information, refer to the manufacturer instructions.

Procedure

1. Image the slide using the imaging procedure from your imaging system manufacturer.
   - If the image contains more than one tissue section, crop the image so that there are individual images for each tissue section.
   - Include one Field of View (FOV) border around the tissue edge.
2. Using the software provided with your imaging system, stitch the FOVs together.

   You can also use a third-party application for FOV stitching.

3. Save the brightfield colored image as a single-page, uncompressed TIFF file with at least 8 bit depth.
4. Assign a unique name for the image (for example, slide ID_RepX). Make sure that the well number in the sample plate is included.

   This name is used across all workflow steps. After the name is assigned, it cannot be changed.

5. Record the image pixel size in microns for use when processing the image with ISIT.
6. After imaging, assess the image visually and make sure that they have the following attributes:
   - In focus with no excessive blur
   - Clearly defined nuclear borders and cellular/tissue structures
   - Balanced exposure

   Good image quality is necessary for accurate image alignment and downstream analysis.

For more information and examples of good image quality, refer to Image Quality Standards in StrataMap Spatial Transcriptome Imaging Guide (document # 200081305) on the Illumina support site.

7. After visually inspecting the image, process the image using ISIT.

Multiple tissue sections within the same well must be registered as separate samples in ISIT. For more information, refer to Image Processing in the Illumina StrataMap Spatial Software documentation.

A tissue section that spans multiple wells must be registered as separate samples in ISIT. For more information, refer to 4.3 Mount Tissue on page 27.

8. Make sure that the image passes the Image QC step in ISIT, and then proceed to *5.6 Permeabilize Tissue* on page 69.

Always visually inspect the image. An image passing the QC step in ISIT does not guarantee high-quality downstream analysis.

9. **[Optional]** Use the Illumina StrataMap Cell Segmentation Tool to initiate cell segmentation before proceeding with the remainder of the assay protocol.

The Illumina StrataMap Cell Segmentation Tool can confirm if the cell segmentation meets your experimental needs or if the images should be retaken.

For more information, refer to the Illumina StrataMap Spatial Software documentation.

SAFE STOPPING POINT

- Slides can be stored in the following conditions:
  - Room temperature (~20–22°C) overnight (up to 16 hours)
  - 4°C for up to 48 hours
- Slides must be kept flat.
- Keep the coverslip and glycerol on the slide.

5.5 Prepare for Steps

Reagents from the StrataMap Spatial Transcriptome Permeabilization Optimization kit must be prepared for the following protocol steps:

- *5.6 Permeabilize Tissue* on page 69
- *5.7 Synthesize First Strand cDNA* on page 80

During these steps, make sure that reagents are pipetted onto and off of the slide promptly to minimize drying on the slide surface.

For support videos, refer to the Spatial Transcriptomics playlist.

1. Prepare the applicable reagents for each step (Table 11).

Table 11: Reagent Preparation Instructions

Step	Reagent	Box Number	Instructions
5.6 Permeabilize Tissue on page 69	Tissue Permeabilization Enzyme (TPE)	1	Thaw at room temperature.
5.7 Synthesize First Strand cDNA on page 80	First Strand Enzyme (FSE)	1	Do not thaw. Store on ice.
	First Strand Reagent 1 (FSR1)	1	Thaw at room temperature.
	First Strand Reagent 2 (FSR2)	1	Do not thaw. Store on ice.
	First Strand Reagent 3 (FSR3)	1	Thaw at room temperature.
	First Strand Supplement (FSS)	1	Thaw at room temperature.
	Spatial Particle Mix (SPM)	1	Thaw at room temperature.

2. Prepare the First Strand Master Mix for *5.7 Synthesize First Strand cDNA* on page 80 as follows.
   a. Prepare the following consumables:
      - FSE—Centrifuge briefly, pipette to mix, and centrifuge briefly again. Store on ice.

- FSR1—Vortex for 3 seconds to mix, and then centrifuge briefly.
- FSR2—Centrifuge briefly, pipette to mix, and centrifuge briefly again. Pipette slowly to avoid bubbles. Store on ice.
- FSS—Vortex for 3 seconds to mix, and then centrifuge briefly.
- FSR3—Centrifuge briefly, pipette to mix, and centrifuge briefly again.
- SPM—Vortex for up to 30 seconds. Make sure that particles are homogenous immediately before preparing the First Strand Master Mix.

Make sure that FSR1, FSS, and the 1.5 ml microcentrifuge tube are at room temperature before proceeding.

Make sure that the SPM particles are homogenous immediately before preparing the First Strand Master Mix.

b. Combine volumes in the following order into a 1.5 ml microcentrifuge tube to prepare 935 µl First Strand Master Mix (Table 12). Pipette 10 times to mix.

Table 12: First Strand Master Mix Preparation

Reagent	Volume (µl)
FSR1	520
FSS	25
FSR2	18
FSR3	44
FSE	108
SPM	220

The First Strand Master Mix can appear light brown after mixing. This coloration does not affect assay performance.

FSR2 can contain minor particulates that do not affect assay performance.

An excess of FSS is expected after the required volume is dispensed.

c. Store the First Strand Master Mix at room temperature and set aside for *5.7 Synthesize First Strand cDNA* on page 80.

5.6 Permeabilize Tissue

This step permeabilizes tissue so that the RNA is released from cells.

> *Previously determined permeabilization times from third-party transcriptomics kits are not applicable to the StrataMap Spatial Transcriptome Permeabilization Optimization kit and can result in suboptimal performance.*

Consumables

- Tissue Permeabilization Enzyme (TPE)
- 0.1 N HCl
- 20x SSC buffer
- Nuclease-free water
- 50 ml conical tube (qty 3)
- 1.5 ml microcentrifuge tube
- Lint-free wipes
- Cassette base
- Cassette lid
- PCR tube (qty 6)
- Sealing tape

Preparation

1. If the slide was stored at 4°C, let the slide equilibrate to room temperature for a minimum of 5 minutes.
2. Preheat the thermal cycler to 37°C. Set the lid temperature to 47°C.
3. Add 45 ml nuclease-free water to three 50 ml conical tubes.
4. Combine the following volumes to prepare 7 ml 0.1x SSC wash buffer. Vortex to mix.
   - 20x SSC buffer (35 µl)
   - Nuclease-free water (6.97 ml)
5. Place the following consumables and equipment near the thermal cycler:

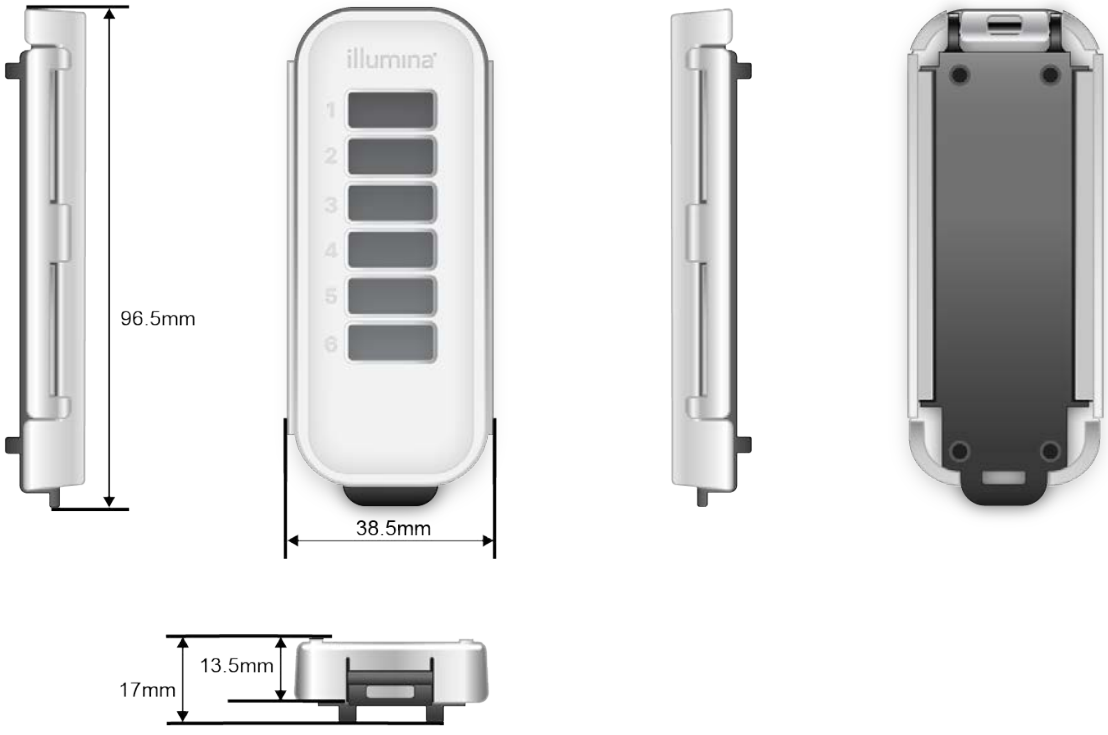
- Sealing tape
- 200 µl pipettor with tips
- Timer (set to 60 minutes)
- Tip waste container (non-biohazardous)

Procedure

Prepare Slide and Assemble Cassette

Use the following instructions to wash the slide and assemble the cassette for permeabilization. Figure 21 shows the assembled cassette and its dimensions.

Figure 21: Assembled Cassette with Dimensions



1. Remove the coverslip from the slide.

   For instructions, refer to 4.5 Apply and Remove Coverslip on page 34.

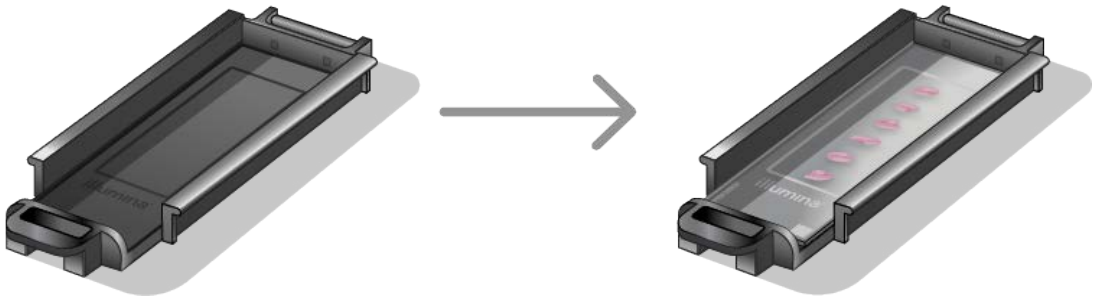
2. Wash the slide two times in nuclease-free water. Use a new conical tube for each wash.

   For instructions, refer to 4.6 Wash Slide on page 36.

3. Using a lint-free wipe, remove any excess liquid from the back of the slide.
4. Place the slide into the base of the cassette so that the active area is facing up and the Illumina logo on the cassette base and slide are aligned (Figure 22).

   Make sure that you are wearing gloves and only touch the edges of the slide to avoid the active area. Make sure that the slide is flat and level in the base of the cassette.

Figure 22: Slide Placement in Cassette Base

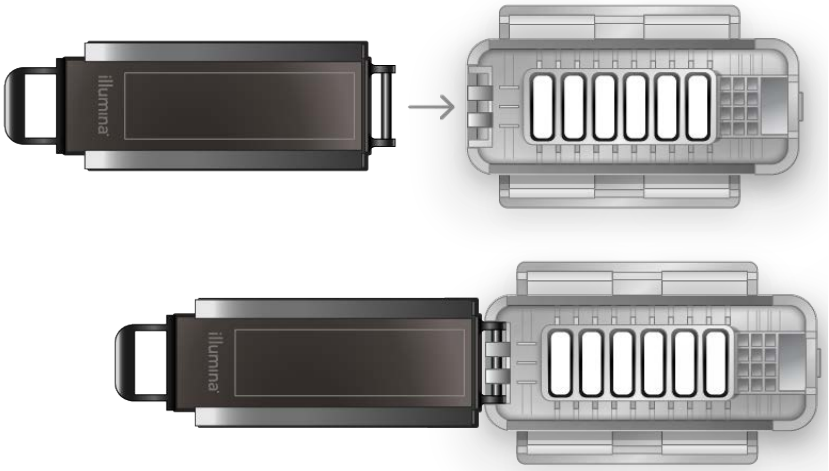


5. Place the cassette base with the slide into the thermal cycler. Do not close the thermal cycler lid.
6. Dry the slide at 37°C for 5–10 minutes or until there is no visible moisture.
7. Remove the cassette base with the slide from the thermal cycler.
8. Remove the slide from the cassette base and place it on top of a clean lint-free wipe or a trough.
9. Place the cassette base and white cassette lid flat on the benchtop.

For optimal performance, use the cassette as intended. To prevent damage, avoid excessive handling when assembling and using the cassette.

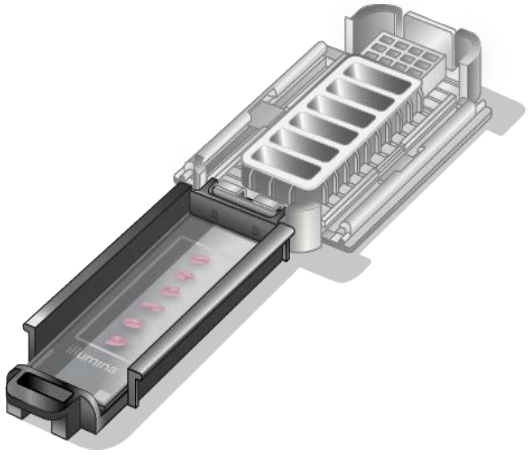
10. Attach the cassette lid to the cassette base at the hinge (Figure 23).

Figure 23: Cassette Hinge Attachment



11. Place the slide into the cassette so that the active area is facing up and the barcode aligns with the Illumina logo on the cassette base (Figure 24).

Figure 24: Slide Placement in Cassette



12. Close the cassette lid as follows.

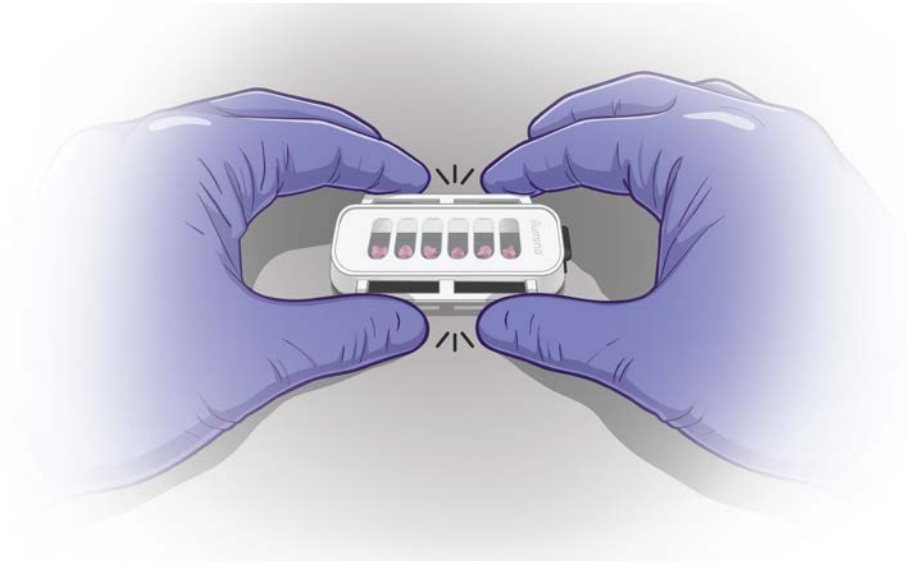
a. Place the cassette flat on the workbench.

b. Close the cassette lid. Press down on top of the closed lid so that the lid is properly seated.

c. While pressing down on the lid, close the two clips on each side of the cassette lid so that the lid is secured to the base and the cassette is sealed (Figure 25).

> *When closing the clips, apply even pressure with all fingers simultaneously. Otherwise, twisting at the hinge and damage to the cassette and slide can occur.*
>
> *Make sure that you close the side clips to seal the cassette. Otherwise, leaks can occur.*

Figure 25: Cassette Side Clips



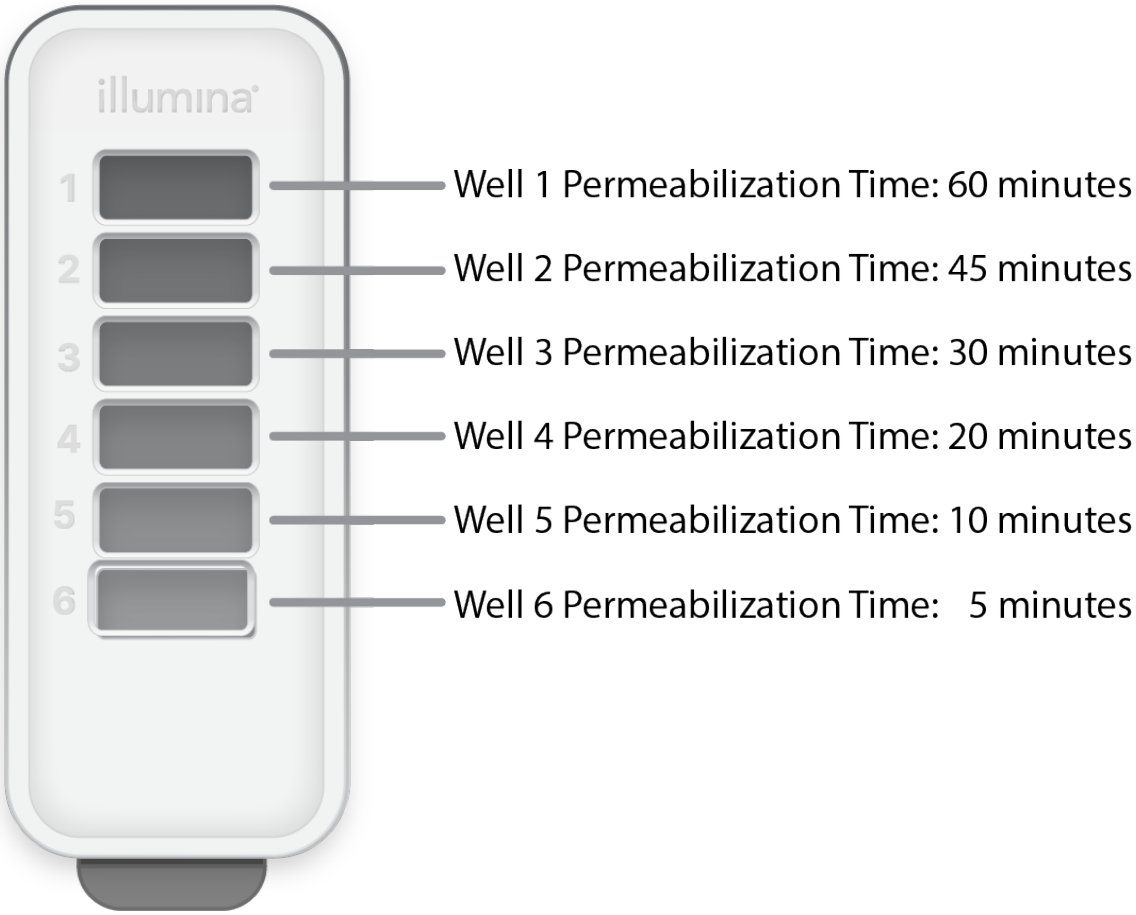
Permeabilize Tissue

1. Resuspend the TPE as follows.
   a. Centrifuge TPE briefly.
   b. Add 1.67 ml 0.1 N HCl to the TPE tube.
   c. Vortex for 1 minute and centrifuge briefly.
   d. Pipette resuspended TPE into individual PCR tubes until there are six 250 µl aliquots.
   e. Store at room temperature until you are ready to preheat.

2. Use the permeabilization time course to determine the optimal permeabilization time (Figure 26).

> *The time course can be modified based on the tissue type. Permeabilization time is the amount of time that tissue sections are exposed to TPE.*

Figure 26: Permeabilization Times



3. For well 1, permeabilize the tissue as follows.
   a. Using the thermal cycler, warm one of the aliquoted tubes of resuspended TPE at 37°C for 2 minutes.

> *The resuspended TPE aliquot must be preheated for at least 2 minutes before use. Do not warm the TPE for more than 20 minutes.*

b. Place the cassette with the slide onto the thermal cycler used in step a.

c. Pipette 150 µl nuclease-free water into the cassette well to wet the slide surface. Make sure that the tissue is covered.

d. Using a pipettor, remove and discard the water wash from the cassette well.

> *Do not touch the tissue with the pipette tip.*

e. Pipette 180 µl warmed resuspended TPE into well 1. If needed, tap the cassette so that the TPE covers the tissue.

> *Avoid pipetting bubbles from the TPE tube.*

f. Start the timer to begin the 60-minute incubation period.

g. Seal the cassette with sealing tape to prevent evaporation and close the thermal cycler lid.
   - Make sure that the sealing tape is applied lightly so that removal and resealing can happen during the permeabilization time course.
   - Make sure that you are handling the cassette properly when applying the sealing tape (Figure 27).

Figure 27: Cassette Handling

4. For wells 2–6, permeabilize the tissue as follows.

> *These steps must be completed for each well in order, starting with well 2 and ending with well 6.*

a. Two minutes before starting each permeabilization condition, incubate a fresh aliquot of resuspended TPE in the same thermal cycler at 37°C for 2 minutes. For example, if you have a 45-minute permeabilization condition, begin incubating the TPE with 47 minutes left on the timer.

> *Each resuspended TPE aliquot must be preheated for at least 2 minutes before use. Do not warm the TPE for more than 20 minutes.*

b. Peel back the sealing tape on the cassette.

c. Pipette 150 µl nuclease-free water into the cassette well to wet the slide surface. Make sure that the tissue is covered.

d. Using a pipettor, remove and discard the water wash from the cassette well.

> *Do not touch the tissue with the pipette tip.*

e. When the timer reaches the permeabilization point, pipette 180 µl warmed resuspended TPE into the cassette well. If necessary, tap the cassette until the TPE covers the tissue.

> *Avoid pipetting bubbles from the TPE tube.*

f. Reseal the cassette with the same sealing tape from step b and close the thermal cycler lid.

g. Repeat steps a–f until all of the wells have been permeabilized.

> *For an overview of the permeabilization optimization timeline, refer to Table 13.*

Table 13: Permeabilization Optimization Timeline

Well Number	Timer Reading (minutes)	Action	Step Number
1	Before timer starts	Warm aliquot of TPE. Pipette 150 µl water into well. Remove and discard.	3a 3c
	60	Pipette 180 µl warmed TPE into well.	3e
2	47	Warm new aliquot of TPE.	4a
	46	Pipette 150 µl water into well. Remove and discard.	4c
	45	Pipette 180 µl warmed TPE into well.	4e
3	32	Warm new aliquot of TPE.	4a
	31	Pipette 150 µl water into well. Remove and discard.	4c
	30	Pipette 180 µl warmed TPE into well.	4e
4	22	Warm new aliquot of TPE.	4a
	21	Pipette 150 µl water into well. Remove and discard.	4c
	20	Pipette 180 µl warmed TPE into well.	4e
5	12	Warm new aliquot of TPE.	4a
	11	Pipette 150 µl water into well. Remove and discard.	4c
	10	Pipette 180 µl warmed TPE into well.	4e
6	7	Warm new aliquot of TPE.	4a
	6	Pipette 150 µl water into well. Remove and discard.	4c
	5	Pipette 180 µl warmed TPE into well.	4e

5. After permeabilization, remove the cassette from the thermal cycler. Keep the cassette near the thermal cycler and proceed to *Wash Cassette Wells* on page 78.

> *Do not transport the cassette and minimize cassette movement while TPE is in the cassette well.*

Wash Cassette Wells

1. Remove the sealing tape from the cassette.
2. Using a pipettor, remove the TPE from the cassette wells. Discard the TPE and pipette tips.
3. Immediately wash the cassette wells with 0.1x SSC wash buffer as follows.
   a. Pipette 200 µl 0.1x SSC wash buffer into each of the cassette wells. Pipette from the corners of the cassette wells.
   b. Using a pipettor, remove and discard the 0.1x SSC wash buffer. Tilt the cassette so that the liquid pools in the corner of the cassette wells (Figure 28).

Figure 28: Pipetting Location

   c. Repeat steps a and b two times for a total of three washes.
4. Leave the last 0.1x SSC wash in the cassette wells until you are ready to add the First Strand Master Mix in *5.7 Synthesize First Strand cDNA* on page 80.

5.7 Synthesize First Strand cDNA

This step uses reverse transcriptase to synthesize DNA that complements the RNA transcripts on the surface of the slide.

Consumables

- First Strand Master Mix
- Sealing tape

Procedure

1. Preheat the thermal cycler to 20°C. Set the lid temperature to 20°C.

   If your thermal cycler lid cannot be set to 20°C, then set it to the lowest temperature setting available.

2. Place the cassette with the 0.1x SSC wash onto the thermal cycler.
3. Using a pipettor, remove the 0.1x SSC wash buffer from the cassette wells. Discard the buffer and pipette tips.
4. Pipette First Strand Master Mix prepared in *5.5 Prepare for Steps* on page 67 10 times to mix and homogenize.
5. Pipette 140 µl First Strand Master Mix into the cassette wells.

   After adding First Strand Master Mix, do not tilt or move the cassette.

6. Seal the cassette thoroughly with sealing tape to prevent evaporation.
7. Close the thermal cycler lid.
8. Incubate the cassette at 20°C for 5 minutes.
9. While the cassette is in the thermal cycler, change the temperature to 42°C. Set the lid temperature to 52°C.

10. Incubate the cassette at 42°C overnight (approximately 12–18 hours).

Do not exceed 18 hours.

5.8 Prepare for Steps

Reagents from the StrataMap Spatial Transcriptome Permeabilization Optimization kit must be prepared for the following protocol steps:

- *5.9 Digest Tissue* on page 83
- *5.10 Remove RNA* on page 85

For support videos, refer to the Spatial Transcriptomics playlist.

1. Prepare the applicable reagents for each step (Table 14).

Table 14: Reagent Preparation Instructions

Reagent	Box Number	Instructions
Tissue Digestion Enzyme (TDE)	1	Do not thaw. Store on ice.
Resuspension Buffer (RSB)	2	Store at room temperature.
Tissue Digestion Buffer (TDB)	2	Store at room temperature.

5.9 Digest Tissue

This step digests and removes tissue and cell debris from the surface of the slide.

Consumables

- Resuspension Buffer (RSB)
- Tissue Digestion Buffer (TDB)
- Tissue Digestion Enzyme (TDE)
- 1.5 ml microcentrifuge tube
- Sealing tape

Preparation

1. Prepare the following consumables:
   - TDB—Vortex for 3 seconds to mix, and then centrifuge briefly.
   - TDE—Centrifuge briefly, pipette to mix, and centrifuge briefly again. Store on ice.
2. Combine the following volumes into a 1.5 ml microcentrifuge tube to prepare 760 µl Tissue Digestion Master Mix, including overage (Table 15). Pipette mix 10 times.

Table 15: Tissue Digestion Master Mix Preparation

Reagent	Volume (µl)
TDB	745
TDE	15

3. Store Tissue Digestion Master Mix at room temperature.

Procedure

1. After overnight incubation, remove the sealing tape from the cassette.
2. With the cassette still on the thermal cycler, use a pipettor to remove the First Strand Master Mix. Discard the pipette tips and First Strand Master Mix.

> *Even though the tissue slices can appear less visible, avoid touching the pipette tips to the area where they were placed. The tips can damage the library surface.*

3. Wash the cassette wells with RSB as follows.
   a. Pipette 150 µl RSB into the cassette wells.
   b. Using a pipettor, remove the RSB from the cassette wells. Discard the RSB and pipette tips.
   c. Repeat steps a and b two times for a total of three RSB washes.

> *Particles can adhere to the cassette surface during the second day of the protocol. These particles do not affect assay performance.*

4. Pipette 115 µl Tissue Digestion Master Mix into the cassette wells.
5. Seal the cassette with sealing tape to prevent evaporation and close the thermal cycler lid.
6. Change the thermal cycler temperature to 56°C and the lid temperature to 66°C.
7. Incubate at 56°C for 1 hour.

5.10 Remove RNA

This step removes RNA from the surface of the slide.

Consumables

- Resuspension Buffer (RSB)
- 10 N NaOH
- Nuclease-free water

Preparation

1. Combine the following volumes to prepare 5 ml 0.1 N NaOH:
   - Nuclease-free water (4.95 ml)
   - Stock 10 N NaOH (50 µl)

Procedure

1. Remove the cassette from the thermal cycler.
2. Remove sealing tape from the cassette.
3. Using a pipettor, remove and discard Tissue Digestion Master Mix from the cassette wells.
4. Wash the cassette wells with RSB as follows.
   a. Pipette 150 µl RSB into the cassette wells.
   b. Using a pipettor, remove the RSB from the cassette wells. Discard the RSB and pipette tips.
   c. Repeat steps a and b two times for a total of three RSB washes.
5. Wash the slide in 0.1 N NaOH as follows.
   a. Pipette 150 µl 0.1 N NaOH into the cassette wells.
   b. Incubate at room temperature for 5 minutes.

> *While you are incubating, you can start preparing the reagents for 5.12 Synthesize Second Strand cDNA on page 88. For more information, refer to 5.11 Prepare for Steps on page 87.*

c. Using a pipettor, remove the 0.1 N NaOH from the cassette wells. Discard the pipette tips and 0.1 N NaOH.

6. Pipette 180 µl RSB into the cassette wells to wash away residual NaOH. Leave the RSB on the slide until you are ready to begin .

7. Store the remaining 0.1 N NaOH at room temperature.

> *This NaOH is used during* *5.12 Synthesize Second Strand cDNA on page 88*.

5.11 Prepare for Steps

Reagents from the StrataMap Spatial Transcriptome Permeabilization Optimization kit must be prepared for the following protocol steps:

- *5.12 Synthesize Second Strand cDNA* on page 88
- *5.13 Elute cDNA* on page 92

> *For support videos, refer to the Spatial Transcriptomics playlist.*

1. Prepare the applicable reagents for each step (Table 16).

Table 16: Reagent Preparation Instructions

Reagent	Box	Instructions
Spatial Amplification Mix (SAM)	1	Thaw three tubes at room temperature.
Spatial Surface Reagent (SSR)	1	Thaw at room temperature.
Universal Cleave Mix (UCM)	1	Thaw at room temperature.
Resuspension Buffer (RSB)	2	Store at room temperature.

5.12 Synthesize Second Strand cDNA

This step adds a second strand of cDNA.

For workflow videos, refer to the Spatial Transcriptomics support playlist.

Consumables

- Spatial Amplification Mix (SAM)
- Spatial Surface Reagent (SSR)
- Resuspension Buffer (RSB)
- Universal Cleave Mix (UCM)
- 0.1 N NaOH
- Sealing tape

Preparation

1. Prepare the following consumables:
   - SSR—Pulse vortex three times to mix, and then centrifuge briefly. Store on ice.
   - SAM—Centrifuge briefly, pipette to mix, and centrifuge briefly again. Store on ice.
2. Program the thermal cycler to run the SAM program as follows.
   a. Set the temperature and time for each cycle (Table 17).

Table 17: SAM Program Cycles

Number of Cycles	Temperature	Time
1	65°C	10 minutes
1	22°C	1 minute

   b. Set the lid temperature to 75°C.
   c. When prompted to select a reaction volume, set it to the maximum volume.
   d. Save the SAM program.
3. Preheat the thermal cycler to 40°C. Set the lid temperature to 50°C.

4. Preheat the heat block for microcentrifuge tubes to 37°C.

> *The heat block is used to preheat the UCM tube used in 5.13 Elute cDNA on page 92.*

Procedure

Surface Conditioning

1. Using a pipettor, remove the RSB from the cassette wells. Discard the RSB and pipette tips.
2. Pipette 115 µl SSR into each of the cassette wells.
3. Seal the cassette with sealing tape.
4. Place the cassette onto the thermal cycler. Close the thermal cycler lid.
5. Incubate at 40°C for 10 minutes.
6. Remove the cassette from the thermal cycler.
7. Remove the sealing tape from the cassette.
8. Using a pipettor, remove the SSR from the cassette wells. Discard the SSR and pipette tips.
9. Pipette 180 µl RSB into each of the cassette wells.

First SAM Incubation

1. Using a pipettor, remove the RSB from the cassette wells. Discard the RSB and pipette tips.
2. Pipette 115 µl SAM into each of the cassette wells.
3. Seal the cassette with sealing tape.
4. Place the cassette onto the thermal cycler and close the lid.
5. Start the SAM program that was saved on the thermal cycler in *Preparation* on page 88.
6. After the SAM program ends, remove the cassette from the thermal cycler.
7. Remove the sealing tape from the cassette.
8. Using a pipettor, remove SAM from the cassette wells. Discard SAM and the pipette tips.

First NaOH Incubation

1. Add 115 µl 0.1 N NaOH into each of the cassette wells.
2. Incubate at room temperature for 5 minutes.
3. Using a pipettor, remove 0.1 N NaOH from the cassette wells. Discard NaOH and the pipette tips.
4. Pipette 180 µl RSB into each of the cassette wells to wash.

Second SAM Incubation

1. Using a pipettor, remove the RSB from the cassette wells. Discard the RSB and pipette tips.
2. Pipette 115 µl SAM into each of the cassette wells.
3. Seal the cassette with sealing tape.
4. Place the cassette onto the thermal cycler and close the lid.
5. Start the SAM program that was saved on the thermal cycler in *Preparation* on page 88.
6. After the SAM program ends, remove the cassette from the thermal cycler.
7. Remove the sealing tape from the cassette.
8. Using a pipettor, remove SAM from the cassette wells. Discard SAM and pipette tips.

Second NaOH Incubation

1. Add 115 µl 0.1 N NaOH into each of the cassette wells.
2. Incubate at room temperature for 5 minutes.
3. Using a pipettor, remove 0.1 N NaOH from the cassette wells. Discard NaOH and the pipette tips.
4. Pipette 180 µl RSB into each of the cassette wells to wash.

Third SAM Incubation

1. Using a pipettor, remove the RSB from the cassette wells. Discard the RSB and pipette tips.
2. Pipette 115 µl SAM into each of the cassette wells.
3. Seal the cassette with sealing tape.
4. Place the cassette onto the thermal cycler and close the lid.
5. Start the SAM program that was saved on the thermal cycler in *Preparation* on page 88.
6. While the SAM program is running, use the heat block to warm the UCM tube at 37°C for 15 minutes.

The UCM tube is used in 5.13 Elute cDNA on page 92.

7. After the SAM program ends, remove the cassette from the thermal cycler.
8. Remove the sealing tape from the cassette.
9. Using a pipettor, remove SAM from the cassette wells. Discard SAM and pipette tips.
10. Pipette 180 µl RSB into each of the cassette wells. Leave the RSB on until you are ready to start *5.13 Elute cDNA* on page 92.

5.13 Elute cDNA

This step elutes the cDNA from the slide surface.

Consumables

- Universal Cleave Mix (UCM)
- PCR plate
- Sealing tape

Preparation

1. Label the PCR plate as UCM Elution.
2. Prepare UCM by centrifuging briefly, pipetting to mix, and then centrifuging briefly again. Make sure that UCM is at 37°C before use.

Procedure

1. Using a pipettor, remove RSB from the cassette wells. Discard RSB and pipette tips.
2. Pipette 120 µl warmed UCM into each of the cassette wells.

   An excess of UCM is expected after the required volume is dispensed.

3. Incubate at room temperature for 5 minutes.
4. Gently move the cassette from side to side to mix the liquid across the surface for 5 seconds.
5. Pick up and carefully tilt the cassette so that the elution moves to the corners.
6. Using a pipettor, remove 58 µl elution from each well and transfer it to the individual wells of the UCM Elution plate. Place the plate on ice.

   Do not combine elutions from different cassette wells.

   Do not discard the elution in the UCM Elution plate. This plate contains the assay libraries.

7. Using a pipettor, remove an additional 58 µl from each cassette well and transfer the second aliquot from each well into an unused well on the UCM Elution plate. Place the plate on ice.

> *Do not combine eluates. Each 58 µl transfer should be placed into separate PCR plate wells.*

> *Each cassette well elution must be separated into two wells on the UCM Elution plate. This separation results in 12 total wells containing eluted cDNA.*

8. Discard the assembled slide and cassette.

5.14 Prepare for Steps

Reagents from the StrataMap Spatial Transcriptome Permeabilization Optimization kit must be prepared for the following protocol steps:

- *5.15 Clean Up Elution* on page 95
- *5.16 Identify Amplification Cycle Number* on page 98

For support videos, refer to the Spatial Transcriptomics playlist.

Prepare the applicable reagents for each step (Table 18).

Table 18: Reagent Preparation Instructions

Reagent	Box	Instructions
Spatial Primer Cocktail (SPC)	1	Thaw at room temperature.
Spatial PCR Reagent (SPR)	1	Thaw at room temperature.
Illumina Purification Beads (IPB)	2	Store at room temperature.
Resuspension Buffer (RSB)	2	Store at room temperature.

5.15 Clean Up Elution

This step purifies the eluted DNA with a 1.5x bead cleanup using Illumina Purification Beads (IPB).

For more information on using IPB, refer to 4.7 Handle Beads on page 38.

Consumables

- Illumina Purification Beads (IPB)
- Resuspension Buffer (RSB)
- Absolute EtOH
- Nuclease-free water
- 50 ml conical tube
- PCR plate

Preparation

1. Combine the following volumes into a conical tube to prepare 18 ml 80% EtOH wash solution, including overage. Vortex to mix.
   - Absolute EtOH (14.4 ml)
   - Nuclease-free water (3.6 ml)

   This solution can be used now and in later parts of the protocol. If you are continuing past the SAFE STOPPING POINT on page 97 and proceeding immediately to 5.16 Identify Amplification Cycle Number on page 98, you can retain the solution for use in that protocol step.

2. Vortex IPB for one minute to disperse the beads. Make sure that the IPB is mixed thoroughly for bead binding efficiency.
3. Label a new PCR plate as Purified DNA Elution.

Procedure

Bind

1. Add 87 µl IPB to each of the 12 wells in the UCM Elution plate.
2. Using the same pipette tip, pipette at least 10 times to mix.
3. Incubate at room temperature for 8 minutes.

Wash

1. Place the plate on a magnetic stand for 5 minutes or until the beads are fully pelleted.
2. Remove and discard the supernatant.
3. While the plate is on the magnetic stand, add 150 µl 80% EtOH to each well.

> *Do not disturb the bead pellet.*

4. After 30 seconds, remove and discard 80% EtOH.

> *Make sure that the beads are not disrupted during this process.*

5. Repeat steps 3 and 4 one time for a total of two 80% EtOH washes.
6. Centrifuge at 280 × g for 10 seconds to collect the remaining EtOH.
7. Place the plate back on the magnetic stand for 1 minute or until the beads are fully pelleted.
8. Using a P20 pipettor with fine tips, remove the residual supernatant.
9. While the plate is on the magnetic stand, let the beads dry at room temperature for up to 5 minutes until they are no longer glossy.

> *Do not let the beads dry to the point where they crack or become lighter brown.*

Elute

1. Remove the plate from the magnetic stand.
2. Add 31 µl RSB to the well.
3. Pipette at least 10 times to mix. Make sure that the beads are completely resuspended.

4. If any liquid remains on the walls, centrifuge at 280 × g for 5 seconds.
5. Incubate at room temperature for 5 minutes.
6. Place the plate on the magnetic stand for 2 minutes or until the beads are fully pelleted.
7. Transfer the first aliquot to the Purified DNA Elution plate as follows.
   a. For each cassette well, identify the first corresponding UCM Elution plate well.
   b. Transfer 29 µl elution from the UCM Elution plate well identified in step a into a single well on the Purified DNA Elution plate. Place the plate on ice.

> *When transferring elution, make sure that there is one destination well for each cassette well.*

8. Immediately transfer the second aliquot to the Purified DNA Elution plate as follows.
   a. For the same cassette well, locate the second corresponding PCR plate well.
   b. Transfer 29 µl from the second PCR plate well to the same well of the Purified DNA Elution plate. Place the plate on ice.

> *This transfer combines both aliquots that originate from the cassette well.*

9. Store the remaining 80% EtOH at room temperature.

> *This EtOH can be used in 5.19 Clean Up Permeabilization Optimization Libraries on page 106.*

SAFE STOPPING POINT

If you are stopping, Illumina recommends determining your optimal cycle number within three days of elution.

Store the Purified DNA Elution plate in -80°C freezer for up to 14 days.

5.16 Identify Amplification Cycle Number

This step identifies the optimal amplification cycle number for library amplification.

Consumables

- Spatial Primer Cocktail (SPC)
- Spatial PCR Reagent (SPR)
- KAPA SYBR FAST qPCR Master Mix (2X) kit
- Nuclease-free water
- 1.5 ml microcentrifuge tube
- PCR plate seal
- qPCR plate

Preparation

1. Prepare the following consumables:
   - SPC—Pulse vortex three times to mix, and then centrifuge briefly. Store at room temperature.
   - SPR—Pulse vortex three times to mix, and then centrifuge briefly. Store at room temperature.
2. Combine the following volumes to prepare 72 µl Spatial qPCR Master Mix (Table 19). Pipette to mix thoroughly.

Table 19: Spatial qPCR Master Mix Preparation

Reagent	Volume (µl)
KAPA SYBR FAST qPCR Master Mix (2X) kit	40
Nuclease-free water	21.5
SPR	8
SPC	2.5

3. Place master mix on ice until it is ready to use.
4. Store SPR and SPC until the amplification cycle number is identified.

These reagents are used again in 5.18 Amplify Permeabilization Optimization Libraries on page 104.

For storage information, refer to SAFE STOPPING POINT on page 102.

Procedure

1. Set up the No Template Control (NTC) as follows.
   a. Aliquot 9 µl Spatial qPCR Master Mix into one well of the qPCR plate.
   b. Add 1 µl nuclease-free water to the well with the Spatial qPCR Master Mix.
2. Combine the following volumes for each of the six wells in a qPCR plate to prepare the six qPCR reactions.
   - Spatial qPCR Master Mix (9 µl)
   - Eluted DNA (1 µl)
3. Store the remaining eluted DNA until the amplification number is identified.

For storage information, refer to SAFE STOPPING POINT on page 102.

4. Seal the qPCR plate and shake at 2000 rpm for 1 minute.
5. Using a plate spinner, briefly centrifuge the plate.
6. Place the qPCR plate in the thermal cycler.
7. Run the following program on the thermal cycler:
   a. Choose the preheat lid option and set to 105°C.
   b. Set the reaction volume to 10 µl.
   c. Set the temperature and time for each set of cycles (Table 20).

Table 20: Thermal Cycler Program Cycles

Number of Cycles	Temperature	Time
1	95°C	3 minutes
25	95°C	10 seconds
	60°C	1 minute

   d. Use the SYBR channel to read the signal.
8. Identify the optimal amplification cycle number as follows.

a. Calculate the average RFUs at the plateau phase.
b. Calculate where 30% of the plateau average is.
c. Identify the cycle number that corresponds with the 30% plateau average for each of the six samples.

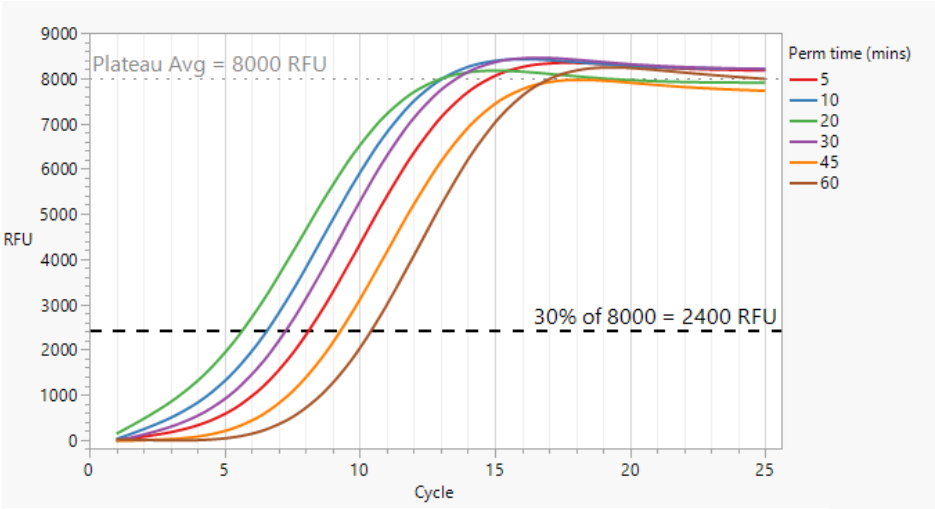
d. Adjust the identified C_q. Refer to the example in Figure 29.

- If you are amplifying permeabilization optimization libraries to determine the optimal permeabilization time, then add 2 to the identified C_q.
- If you are amplifying the final post-permeabilization optimization libraries after determining the optimal permeabilization time, then subtract 1 from the identified C_q. Proceed to *5.24 Amplify Post-Optimization Libraries* on page 118.
- If the calculated optimal value is less than 1, use 1 so that there is sufficient amplification of the final library.
- For example, if the C_q at 30% threshold is 5.6, then round that value up to 6 and add 2 to determine the optimal cycles:

> *If the calculated optimal value is less than 1, use 1 so that there is sufficient amplification of the final library.*

$$Cq + 2 = Optimal\ Cycle\ Number$$
$$6 + 2 = 8\ optimal\ cycles$$

Figure 29: Cycle Number Identification Example



e. If the C_q values vary significantly between the wells, amplify wells with C_q values within 2 cycles of each other using the same PCR program.
   - For each group, use the highest C_q value to calculate the amplification cycle number.
   - For example, if the C_q values for wells 1–6 are 6, 7, 8, 9, 10, and 11, then wells 1–3 (C_q 6–8) and wells 4–6 (C_q 9–11)can be amplified together.
   - For wells 1–3 (C_q 6–8), use the highest C_q in the group (8) to calculate the cycle number:

$$Cq + 2 = Optimal\ Cycle\ Number$$
$$8 + 2 = 10\ optimal\ cycles$$

   - For wells 4–6 (C_q 9–11), use the highest C_q in the group (11) to calculate the cycle number:

$$Cq + 2 = Optimal\ Cycle\ Number$$
$$11 + 2 = 13\ optimal\ cycles$$

SAFE STOPPING POINT

Store the Purified DNA Elution plate at -80°C for up to 14 days.

If you are stopping here, store SPR and SPC at -20°C.

If you are amplifying the libraries immediately after identifying the amplification number, then store the eluted DNA, SPR, and SPC at 4°C for up to 2 hours during qPCR.

5.17 Prepare for Steps

Reagents from the StrataMap Spatial Transcriptome Permeabilization Optimization kit must be prepared for the following protocol steps:

- *5.18 Amplify Permeabilization Optimization Libraries* on page 104
- *5.19 Clean Up Permeabilization Optimization Libraries* on page 106

> *For support videos, refer to the Spatial Transcriptomics playlist.*

Prepare the applicable reagents for each step (Table 21).

Table 21: Reagent Preparation Instructions

Reagent	Box	Instructions
Enhanced PCR Mix (EPM)	1	Thaw at room temperature.
Spatial Primer Cocktail (SPC)	1	Thaw at room temperature.
Spatial PCR Reagent (SPR)	1	Thaw at room temperature.
Illumina Purification Beads (IPB)	2	Store at room temperature.
Resuspension Buffer (RSB)	2	Store at room temperature.

5.18 Amplify Permeabilization Optimization Libraries

This step amplifies a portion of all six libraries to create copies of each unique template. These libraries are used to determine the optimal permeabilization time.

The remaining eluted cDNA can be used to create the final post-optimization libraries. Amplifying the remaining input can provide deeper sequencing coverage and reveal additional unique molecules.

For more information, refer to 5.24 Amplify Post-Optimization Libraries on page 118.

Consumables

- Enhanced PCR Mix (EPM)
- Resuspension Buffer (RSB)
- Spatial Primer Cocktail (SPC)
- Spatial Primer Reagent (SPR)
- 1.5 ml microcentrifuge tube
- PCR strip tube

Preparation

1. Combine volumes in the following order in a 1.5 ml microcentrifuge tube on ice to prepare 310 µl PCR Master Mix, including overage (Table 22). Pipette mix thoroughly.

Table 22: PCR Master Mix Preparation

Reagent	Volume (µl)
EPM	132
RSB	135
SPC	10
SPR	33

2. Store SPR, SPC, and EPM at -20°C.

These reagents are used again in 5.24 Amplify Post-Optimization Libraries on page 118.

Procedure

1. Pipette 47 µl PCR Master Mix into six PCR strip tubes.
2. Pipette 3 µl eluted DNA into one of the six PCR strip tubes.
   - Each of the six samples are used to set up a separate PCR reaction, which results in six 50 µl PCR reactions.
   - Pipette mix each well thoroughly.
3. Run the following PCR program:
   a. Choose the preheat lid option and set to 105°C.
   b. Set reaction volume to 50 µl.
   c. Set the temperature and time for each set of cycles (Table 23).

Table 23: PCR Program Cycles

Number of Cycles	Temperature	Time
1	98°C	30 seconds
4	98°C	30 seconds
	58°C	1 minute
	72°C	1 minute
X[1]	98°C	30 seconds
	70°C	1 minute
	72°C	1 minute
1	72°C	2 minutes
1	10°C	Infinite hold[2]

[1] The number of cycles (X) is the optimal cycle number identified in *5.16 Identify Amplification Cycle Number* on page 98. If the calculated optimal amplification cycle number is less than 1, then use 1 so that there is sufficient amplification of the final library.
[2] Press the ∞ key to set infinite hold.

4. After running the PCR program, centrifuge the PCR strip tubes at 280 x g for 10 seconds and proceed immediately to *5.19 Clean Up Permeabilization Optimization Libraries* on page 106.

5.19 Clean Up Permeabilization Optimization Libraries

This step purifies the amplified permeabilization optimization library with a 0.6x bead cleanup using Illumina Purification Beads (IPB).

Consumables

- Illumina Purification Beads (IPB)
- Resuspension Buffer (RSB)
- Absolute EtOH
- Nuclease-free water
- 50 ml conical tube
- PCR plate

Preparation

1. **[Optional]** Combine the following volumes in a 50 ml conical tube to prepare 6 ml 80% EtOH, including overage. Vortex to mix.
   - Absolute EtOH (4.8 ml)
   - Nuclease-free water (1.2 ml)

   > *If you retained 80% EtOH during 5.15 Clean Up Elution on page 95 and did not stop at the SAFE STOPPING POINT, you can use that for this step and do not have to prepare additional 80% EtOH.*

2. Vortex IPB for 1 minute to disperse the beads fully. Make sure that IPB is mixed thoroughly for bead-binding efficiency.

Procedure

Bind

1. Using a pipettor, transfer 45 µl of each PCR reaction into separate wells in a PCR plate.
2. Add 45 µl RSB to bring the total volume of each well to 90 µl.

3. Add 54 µl IPB to each of the PCR products in the PCR plate. Pipette at least 10 times to mix.
4. Incubate at room temperature for 8 minutes.

Wash

1. Place the PCR plate on the magnetic stand for 5 minutes or until the beads are fully pelleted.
2. Remove and discard the supernatant.
3. Wash the beads as follows.
   a. While the PCR plate is on the magnetic stand, add 150 µl 80% EtOH.
   b. After 30 seconds, remove and discard the supernatant.

   Make sure that the beads are not disrupted during this process.

   c. Repeat steps a and b one time for a total of two 80% EtOH washes.
4. Centrifuge at 280 × g for 10 seconds to collect the remaining EtOH.
5. Return the PCR plate to the magnetic stand for one minute or until the beads are fully pelleted.
6. Using a P20 pipettor with fine tips, remove the residual supernatant.
7. While the PCR plate is on the magnetic stand, let the beads dry at room temperature for up to 5 minutes until they are no longer glossy.

   Do not let beads dry to the point where they crack or become lighter brown.

Elute

1. Remove the PCR plate from the magnetic stand.
2. Add 27 µl RSB to each sample. Pipette mix at least 10 times and make sure that the beads are completely resuspended before proceeding.
3. If any liquid remains on the walls, centrifuge at 280 × g for 5 seconds.
4. Incubate at room temperature for 5 minutes.
5. Place the PCR plate on the magnetic stand for 2 minutes or until the beads are fully pelleted.
6. Transfer 25 µl elution into a new PCR plate to create the final permeabilization optimization library.

SAFE STOPPING POINT

If you are stopping, store at -20°C for up to three weeks.

5.20 Library Quantification

This step checks the quantity and quality of the purified library in preparation for library dilution and sequencing.

When performing library quantification, expect a wide distribution of fragment sizes and library concentrations that are greater than or equal to 2 nM. The average fragment size and concentration varies based on various factors, such as tissue type and the initial RNA quality in the tissue block.

Consumables

- KAPA Library Quantification Kit
- High sensitivity D5000 screentape
- High sensitivity D5000 reagents
- High sensitivity D5000 ladder

Procedure

qPCR Library Quantification

1. Use the KAPA Library Quantification Kit to quantify your library.

> *For instructions, refer to the KAPA Library Quantification Kit documentation.*
>
> *Library quantity cannot be determined using the TapeStation quantification values.*

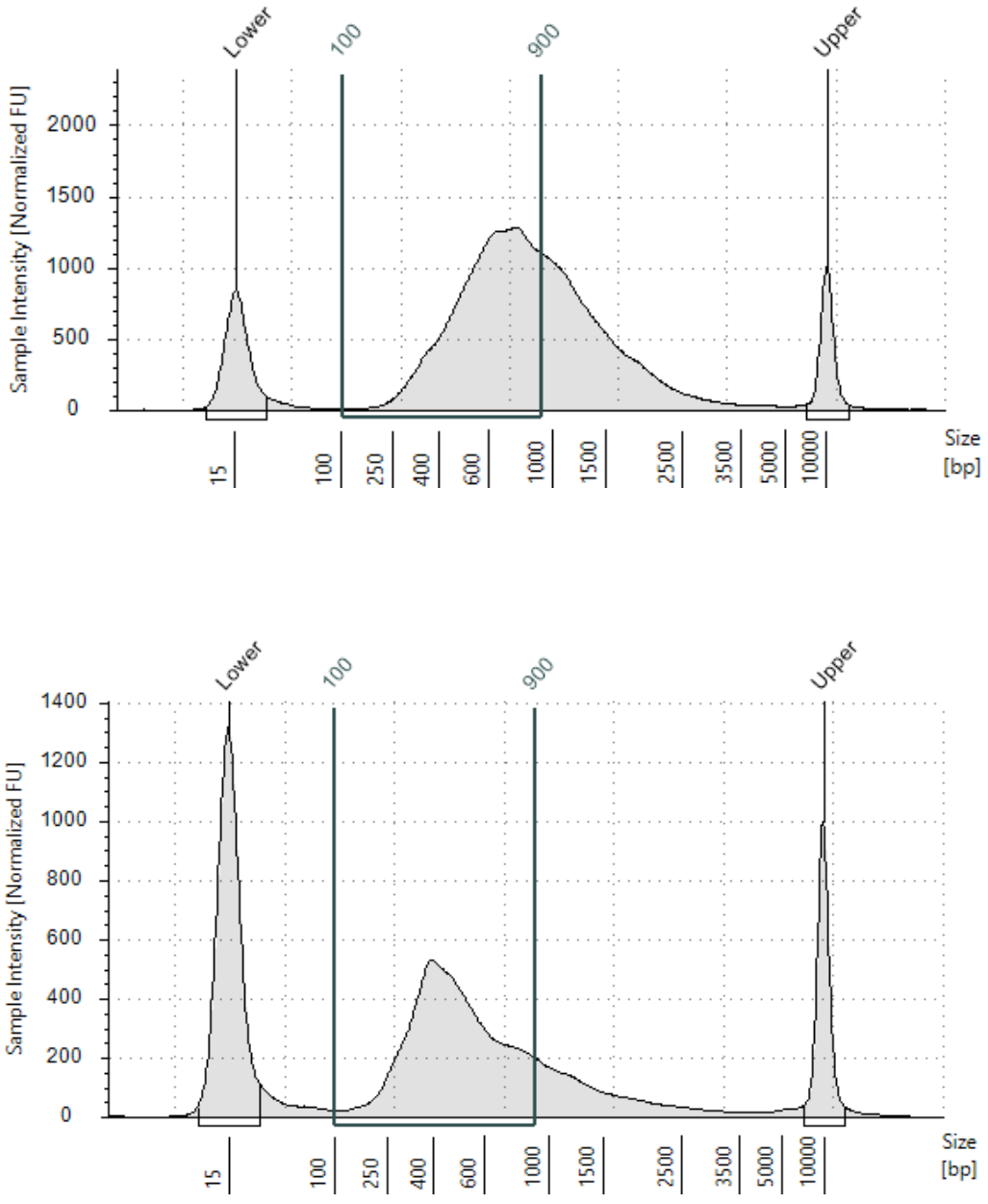
TapeStation Library Analysis

1. Prepare the high sensitivity D5000 reagents according to the manufacturer recommendations.
2. Using the high sensitivity D5000 screentape, analyze 2 µl of each library.

> *If the library is not within the quantification range of the D5000 high sensitivity kit, then you can dilute the library until it is in the range.*

3. Using the TapeStation analysis software, edit the region boundaries to define an analysis window of 100–900 base pairs (bp). Refer to the examples in Figure 30.

Figure 30: TapeStation Trace Examples



4. Within the 100–900 bp analysis region, calculate the average fragment size.

> *If you do not use the 100-900 bp region for size normalization, inaccurate library quantifications and reduced sequencing performance can occur.*
>
> *A long tail of fragments with less than 300 bp can indicate an error in size selection during cleanup, which can result in reduced performance.*

> *Spatial libraries have fragments that extend beyond 900 bp that should not be included when determining the average fragment size for loading.*

5.21 Dilute Libraries to Starting Concentration

This step dilutes the libraries to the starting concentration for the sequencing system and is the first step in a serial dilution. After diluting to the starting concentration, the libraries are ready to be denatured and diluted to the final loading concentration.

The final loading concentration is a starting point and is provided as general guidance. Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

Consumables

- RSB
- 1 N NaOH
- PhiX Control v3

Procedure

1. Calculate the molarity value of each library using the following formula:

$$qPCR\ concentration \times \frac{452\ bp}{average\ fragment\ length\ in\ 100–900\ bp\ region} \times dilution\ factor = Adjusted\ library\ molarity$$

> *Use the qPCR KAPA library quantification kit and TapeStation to analyze each library. For instructions, refer to* *5.20 Library Quantification on page 108*.

2. Normalize each of the six libraries to the same target molar concentration based on the quantification results.
3. Pool equal volumes of each normalized library to create an equimolar six-plex pool.

4. Using the final pooled molarity value, calculate the volumes of RSB, PhiX Control v3, and library needed to dilute the library to the starting concentration for your system (Table 24).

> *The following final loading concentrations are used as a general guideline. Determine the optimal loading concentrations for your workflow using the preferred quantification method with sequencing runs or through flow cell titration.*

Table 24: Final Loading Concentrations

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)	PhiX (%)
NovaSeq X Series 5B flow cell	2	250	1
NovaSeq X Series 10B flow cell	2	250	1
NovaSeq X Series 25B flow cell	2	250	1
NovaSeq 6000 S2 and S4 flow cells	0.5	100	1
NextSeq 2000 P4 flow cell	2	800	1

5. Follow the denature and dilute instructions from the Denature and Dilute Protocol Generator to dilute each library to the starting concentration.
6. Set up a single-end run for your system.

> *Illumina recommends setting up a single-end run with 130 cycles for Read 1 and 8 cycles for Index Read 1.*
>
> *For NovaSeq X Series, NovaSeq 6000, and NextSeq 2000, Illumina recommends a single library (single slide) with the library loaded across one flow cell.*
>
> *For NovaSeq 6000 and NovaSeq X Series, Illumina recommends multiple spatial transcriptome libraries (multiple slides) with individual libraries loaded by lane across one flow cell. The libraries are separated by individual lanes.*

5.22 Sequencing and Analysis

After diluting your libraries to the starting concentration, you can start sequencing using a NovaSeq 6000, NovaSeq X Series, or NextSeq 2000 system.

For more information on the sequencing systems, refer to the following documentation:

- *NovaSeq X Series Product Documentation*
- *NovaSeq 6000 Sequencing System Guide*
- *NextSeq 1000/2000 Product Documentation*

StrataMap Spatial Transcriptome Permeabilization Optimization kit libraries can be sequenced across all lanes of a flow cell or in individually addressable lanes.

- If the library originates from one slide, sequencing data from multiple flow cells can be combined into one analysis.
- If combining with other library types on the same flow cell, refer to the *Illumina StrataMap Spatial Software documentation* for specific instructions.

Sequencing Planning

The spatial transcriptome assay is compatible with the following flow cells and workflows:

- NovaSeq X Series 10B and 25B flow cells using the NovaSeq X Series protocol
- NovaSeq 6000 S2 and S4 flow cells using the Standard workflow
- NovaSeq 6000 S4 flow cell using the Xp workflow
- NextSeq 2000 P4 flow cell using the XLEAP-SBS reagents

If you are using the NovaSeq X Series instrument, you can also do per-lane loading run planning.

Recommended Sequencing Depth

- For permeabilization optimization libraries, Illumina recommends sequencing to a depth of 3.25 B raw reads.
- For post-optimization libraries, Illumina recommends sequencing to a depth of 5000 raw reads per 10 x 10 µm tissue area.
  - This sequencing depth recommendation is a starting point and is provided as general guidance. It can be further optimized based on your needs.
  - Actual reads per cell reported in the analysis output can vary based on tissue type and cell size.

Calculate Sequencing Amount Using Tissue Area

The area of each tissue section (in mm²) can be found in the local ISIT export file (for example, `tissue_area_for_substrate_[Slide ID].csv`). The area for each tissue section is also shown on the Refine tissue masks page. Use the following formula to calculate the recommended sequencing depth in number of raw sequencing reads using the total area:

$$sum\ of\ sample\ area\ (mm^2) \times 10{,}000 \times 5000\ raw\ reads \times 1.10$$

This equation assumes that there is a 10% overage for conservative sequencing estimation. The unit conversion is 10,000 for converting mm² to a 10 x 10 µm tissue area.

For example, if there are three samples with tissue areas of 41.8, 38.8, and 38.7 mm², then ~6.56 billion reads are needed to achieve ~5,000 raw reads per 10 x 10 µm tissue area in each sample:

$$119.3 \times 10{,}000 \times 5{,}000 \times 1.10 = 6{,}561{,}500{,}000$$

Primary and Secondary Analysis

The DRAGEN StrataMap analysis software is used to process sequencing data along with the images taken during the protocol. Secondary analysis with this software and the DRAGEN StrataMap pipeline consists of the following steps:

- **[Optional]** Cell segmentation can be initiated independently using the Illumina StrataMap Cell Segmentation Tool. This tool can be used after image acquisition and before proceeding with the assay protocol. You can confirm if the cell segmentation meets your experimental needs or if the images should be retaken.
- Run setup, including run planning, and manual launch with Platform Core.
- If you are using the NovaSeq X Series system, you can also do run planning per lane.
- Uploading the following analysis inputs:
  - BCL folders
  - Sample sheets
  - Reference genomes
  - OME-TIFF files from ISIT
  - **[Optional]** Analysis configuration file
  - **[Optional]** Cell segmentation file from the Illumina StrataMap Cell Segmentation Tool

- Generating the outputs from the Intermediates folder.

  For a list of the files with descriptions, refer to the Illumina StrataMap Spatial Software documentation.

- Generating the Run Summary report and information (for example, contour CSV files and binned data) that is used by ICM for downstream analysis.

  For more information, refer to the DRAGEN StrataMap in the Illumina StrataMap Spatial Software documentation.

Tertiary Analysis

ICM is used to create and update studies that can be used for spatial analysis. ICM also generates the Spatial Report, which contains graphs and plots of the bin and cluster data. In this report, you can do the following actions:

- Navigate and adjust the spatial image
- Configure the cluster and bin plots
- Highlight clusters across plots

For more information, refer to the Illumina Connected Multiomics in the Illumina StrataMap Spatial Software documentation.

5.23 Select Optimal Permeabilization Time

Analyzing the sensitivity data generated during the permeabilization optimization experiment enables you to determine the optimal permeabilization time. The analysis can be done with any third-party software.

After you have determined the optimal permeabilization time for your tissue type, you can proceed with the following steps:

- *5.24 Amplify Post-Optimization Libraries* on page 118
- *5.25 Clean Up Post-Optimization Libraries* on page 122
- *5.20 Library Quantification* on page 108
- *5.21 Dilute Libraries to Starting Concentration* on page 111
- *5.22 Sequencing and Analysis* on page 113

You can also use the permeabilization time to run the StrataMap Spatial Transcriptome assay.

> *For instructions, refer to the StrataMap Spatial Transcriptome User Guide (document # 200073552) on the Illumina support site.*
>
> *To determine the optimal permeabilization time, use the grid transcript saturation data instead of the cell transcript saturation data.*

Retrieve your analysis file in Platform Core and analyze it as follows.

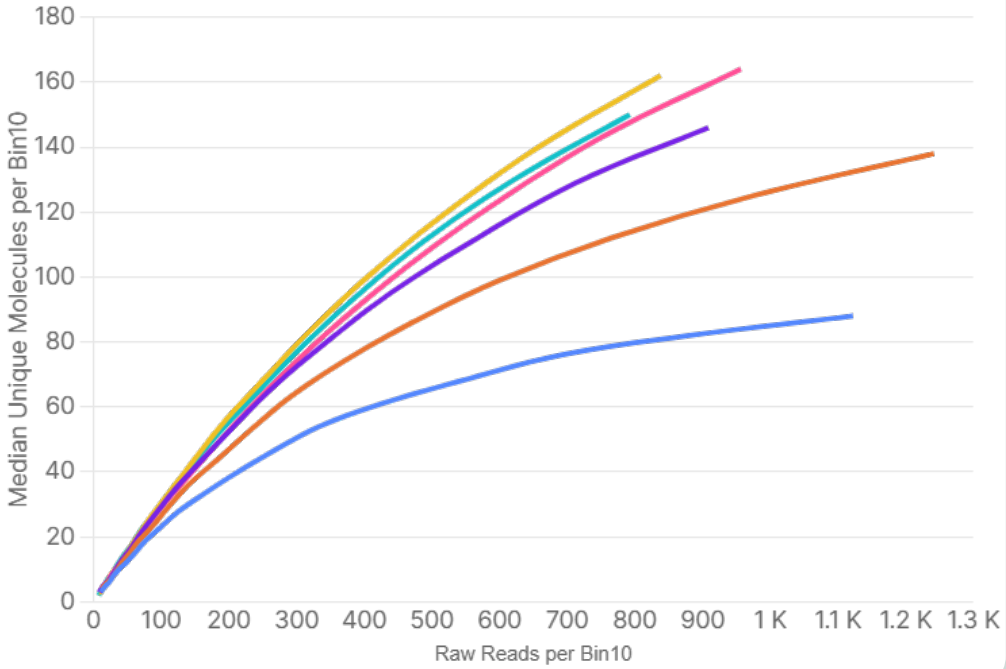
1. In Platform Core, select your project.
2. From the Flow drop-down menu, select **Analyses**.
3. In Analysis, select the Report folder.

4. Using the Grid Transcript Saturation plot diagram in the DRAGEN report, identify the following information:
   - Highest shared Raw Reads per Bin10 for the six samples
   - Sample with the highest Median Unique Molecules per Bin10 at that shared read depth

The permeabilization time for this sample is the optimal permeabilization time for your tissue of interest.

Figure 31 shows that the sample represented by the yellow curve achieved the highest Median Unique Molecule per Bin10 at that highest shared read depth. The permeabilization time represented by the yellow saturation curve is the optimal permeabilization time.

Figure 31: Grid Transcript Saturation Plot Diagram Example



5.24 Amplify Post-Optimization Libraries

After sequencing the permeabilization optimization libraries and completing the analysis to determine the optimal permeabilization time, you can amplify the remaining cDNA to create your final post-optimization libraries. This amplification can be done on the library corresponding to the optimal permeabilization condition or on any subset of the six libraries, depending on your experimental goals. Amplifying the remaining input can provide deeper sequencing coverage and reveal additional unique molecules.

Consumables

- Enhanced PCR Mix (EPM)
- Resuspension Buffer (RSB)
- Spatial Primer Cocktail (SPC)
- Spatial Primer Reagent (SPR)
- 1.5 ml microcentrifuge tube (qty 2)
- PCR plate seal
- PCR plate

Preparation

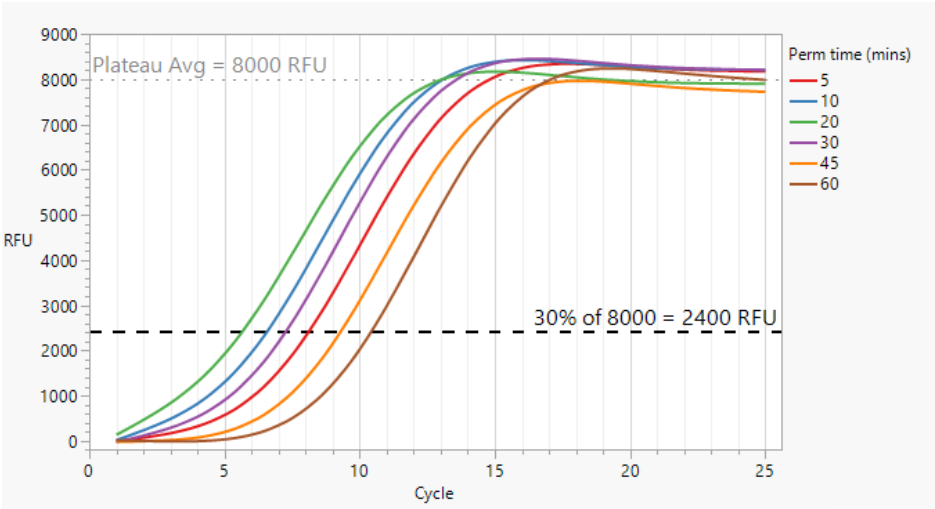
1. Determine the number of final post-optimization libraries to amplify using the eluted cDNA from the Purified DNA Elution plate.
2. Determine the amplification cycle number for each of the post-optimization libraries that you want to amplify as follows.
   a. Calculate the average RFUs at the plateau phase.
   b. Calculate where 30% of the plateau average is.
   c. Identify the cycle number that corresponds with the 30% plateau average for each of the six samples.

d. Subtract 1 from the identified C_q. Refer to the example in Figure 32.

- If the calculated optimal value is less than 1, use 1 so that there is sufficient amplification of the final library.
- For example, if the Cq at 30% threshold is 5.6, then round that value up to 6 and subtract 1 to determine the optimal cycles:

$$Cq - 1 = Optimal\ Cycle\ Number$$
$$6 - 1 = 5\ optimal\ cycles$$

Figure 32: Cycle Number Identification Example



3. Based on the number of samples, combine volumes in the following order in a 1.5 ml microcentrifuge tube on ice to prepare the PCR Master Mix. Pipette mix thoroughly.
   - The following table shows the formulation for amplifying one or six libraries. Scale the volumes based on the number of samples that are being amplified.
   - The volume for one library produces 107 µl PCR Master Mix for three reactions, including overage.
   - The volume for six libraries produces 640 µl PCR Master Mix for 18 PCR reactions, including overage.

   The spatial transcriptomics assay can be used with up to six amplified final post-optimization libraries.

Table 25: PCR Master Mix Preparation

Reagent	Volume for 1 Library (µl)	Volume for 6 Libraries (µl)
EPM	66	396
RSB	19	115
SPC	5	30
SPR	17	99

4. Combine the following volumes in a 1.5 ml microcentrifuge tube to produce 150 µl library amplification mix for each of the chosen libraries.

Table 26: Library Amplification Mix Preparation

Reagent	Volume (µl)
PCR Master Mix	97
Eluted DNA	53

Procedure

1. For each of the chosen library amplification mixes, pipette three 50 µl amplification mix replicates into separate wells of the PCR plate.
   - There should be three replicates for each of the chosen DNA libraries.
   - Pipette mix each well thoroughly.
2. Seal the plate with the PCR plate seal.
3. Run the following PCR program:
   a. Choose the preheat lid option and set to 105°C.
   b. Set reaction volume to 50 µl.

c. Set the temperature and time for each set of cycles.

Table 27: PCR Program Cycles

Number of Cycles	Temperature	Time
1	98°C	30 seconds
4	98°C	30 seconds
	58°C	1 minute
	72°C	1 minute
X[1]	98°C	30 seconds
	70°C	1 minute
	72°C	1 minute
1	72°C	2 minutes
1	10°C	Infinite hold[2]

[1] The number of cycles (X) is the optimal cycle number identified in *5.16 Identify Amplification Cycle Number* on page 98. If the calculated optimal amplification cycle number is less than 1, then use 1 for the number of cycles so that there is sufficient amplification of the final post-optimization libraries.
[2] Press the ∞ key to set infinite hold.

4. After running the PCR program, centrifuge the plate at 280 x g for 10 seconds and proceed immediately to *5.25 Clean Up Post-Optimization Libraries* on page 122.

5.25 Clean Up Post-Optimization Libraries

This step purifies the amplified post-optimization libraries with a 0.6x bead cleanup using Illumina Purification Beads (IPB).

Consumables

- Illumina Purification Beads (IPB)
- Resuspension Buffer (RSB)
- Absolute EtOH
- Nuclease-free water
- 50 ml conical tube
- PCR plate

Preparation

1. [Optional] Combine the following volumes in a conical tube to prepare 6 ml 80% EtOH, including overage. Vortex to mix.
   - Absolute EtOH (4.8 ml)
   - Nuclease-free water (1.2 ml)
2. Prepare the following consumables:
   - IPB—Vortex for 1 minute to disperse the beads fully. Make sure that IPB is mixed thoroughly for bead-binding efficiency.
   - RSB—Vortex to mix.

Procedure

Bind

1. Using a pipettor, transfer and combine 45 µl from the three PCR replicates into the number of PCR plate wells required to create the chosen amount of 135 µl amplified libraries.
2. Add 81 µl IPB to the PCR product in the PCR plate. Pipette at least 10 times to mix.
3. Incubate at room temperature for 8 minutes.

Wash

1. Place the PCR plate on the magnetic stand for 5 minutes or until the beads are fully pelleted.
2. Remove and discard the supernatant.
3. Wash the beads as follows.
   a. While the PCR plate is on the magnetic stand, add 150 µl 80% EtOH.
   b. After 30 seconds, remove and discard the supernatant.

   Make sure that the beads are not disrupted during this process.

   c. Repeat steps a and b one time for a total of two 80% EtOH washes.
4. Centrifuge at 280 × g for 10 seconds to collect the remaining EtOH.
5. Return the PCR plate to the magnetic stand for one minute or until the beads are fully pelleted.
6. Using a P20 pipettor with fine tips, remove the residual supernatant.
7. While the PCR plate is on the magnetic stand, let the beads dry at room temperature for up to 5 minutes until they are no longer glossy.

   Do not let them dry to the point where they crack or become lighter brown.

Elute

1. Remove the PCR plate from the magnetic stand.
2. Add 81 µl RSB to each sample. Pipette mix at least 10 times and make sure that the beads are completely resuspended before proceeding.
3. If any liquid remains on the walls, centrifuge at 280 × g for 5 seconds.
4. Incubate at room temperature for 5 minutes.
5. Place the PCR plate on the magnetic stand for 2 minutes or until the beads are fully pelleted.
6. Transfer 75 µl elution into a new PCR plate. This 75 µl elution is the final purified library.
7. After transferring the elution, perform the following steps on your post-optimization libraries:
   - *5.20 Library Quantification* on page 108
   - *5.21 Dilute Libraries to Starting Concentration* on page 111
   - *5.22 Sequencing and Analysis* on page 113

SAFE STOPPING POINT

If you are stopping, store at -20°C for up to three weeks.

6. Resources and References

This section contains reference documentation and the revision history.

6.1 Reference Documentation

Table 28 identifies documentation that is used with the StrataMap Spatial Transcriptome Permeabilization Optimization kit or can be referred to for additional information on Illumina software.

Table 28: Reference Documentation

QR Code	Title
	*StrataMap Spatial Transcriptome Imaging Guide*
	*StrataMap Spatial Transcriptome User Guide*
	*Illumina StrataMap Spatial Software documentation*

6.2 Revision History

Document	Date	Description of Change
Document # 200073553 v00	August 2026	Initial release.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website:	www.illumina.com
Email:	techsupport@illumina.com

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download from support.illumina.com.

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