STOmics

Stereo-seq TRANSCRIPTOMICS SET FOR CHIP-ON-A-SLIDE (0.5cm * 0.5cm)

USER MANUAL

Cat. No.: 211ST13004 (4 RXNs)
Kit Version: V1.3
Manual Version: B_1

STUM-TT002

REVISION HISTORY

Manual Version: A
Kit Version: V1.3
Date: Nov. 2024
Description: Initial release

Manual Version: B
Kit Version: V1.3
Date: Mar. 2025
Description:

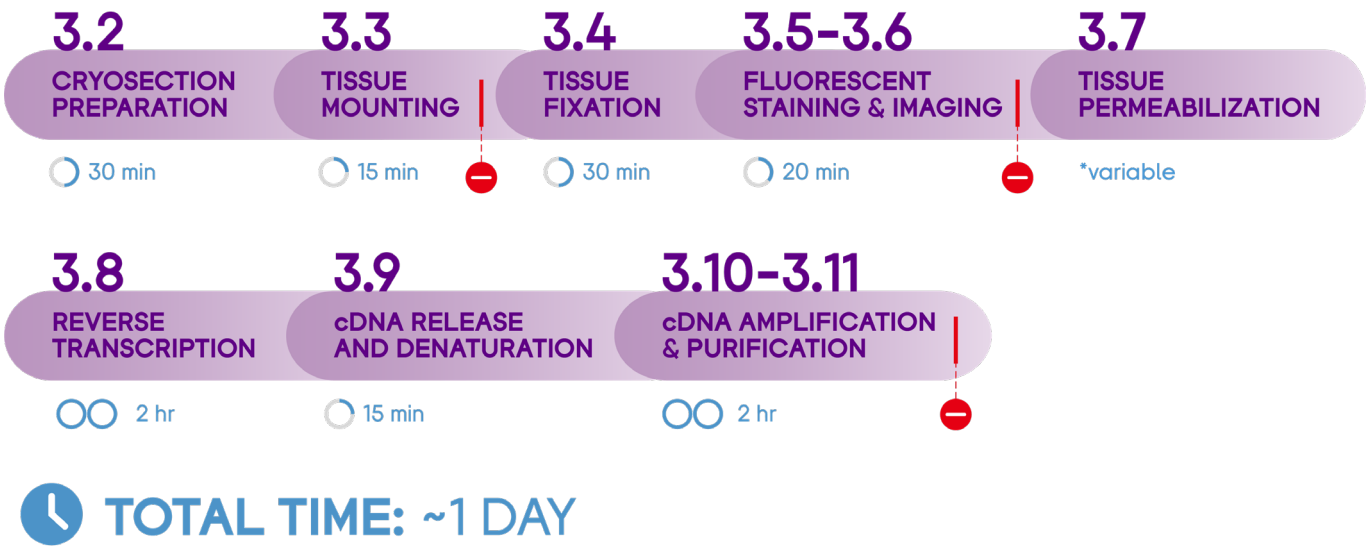
- Minor bugs fixed.
- Extended the transferring and storage temperature.
- Fixed magnetic bead purification to 1X of sample salvaging strategies in chapter 3.8.
- Added tissue sectioning and mounting instructions in this manual.

Manual Version: B_1
Kit Version: V1.3
Date: Jul. 2025
Description:

- Added US use only catalog numbers.
- Updated reference to PE001 with note on upcoming replacement by PE002.

Note: Please download the latest version of the manual and use it with the corresponding Stereo-seq Transcriptomics kit.

WORKFLOW



TOTAL TIME: ~1 DAY

TABLE OF CONTENTS

NOTE: Additional operation tips and guidance.

CRITICAL STEPS: Pay extra attention for these steps to avoid experimental setbacks or problematic results.

QUALITY CHECK POINT

CAUTION: Proceed with extra care; improper handling or carelessness may cause experimental failure or accidents.

STOP POINT: Here you may pause your experiment and store your sample.

CHAPTER 1
INTRODUCTION

1.1. Intended Use

STOmics Stereo-seq Transcriptomics Set for Chip-on-a-slide V1.3 is intended for generating a spatially-resolved 3′ mRNA library from biological tissue sections. Built upon DNA Nanoball (DNB) technology, STOmics Stereo-seq Transcriptomics Set for Chip-on-a-slide enables a "tissue-to-data" solution through *in situ* capture of the whole transcriptome at nanoscale resolution and centimeter-sized Field of View (FOV). This kit uses DNB-patterned array chips loaded with spatially-barcoded probes that capture and prime poly-adenylated mRNA from tissue sections *in situ*. Each cDNA synthesized from mRNA captured on a particular spot is linked to its spatially-barcoded probe, allowing subsequent gene expression mapping of a tissue section following sequencing and visualization analysis using the StereoMap visualization platform.

Stereo-seq workflow is also compatible with tissue H&E staining, which obtains better tissue morphological information, to assist with tissue type identification, to obtain a gene expression profile of a specific tissue region, and to conduct downstream differential analysis among selected regions of interests.

All reagents provided in this kit have passed stringent quality control and functional verification, ensuring performance stability and reproducibility.

1.2. Sequencing Guideline

Sequencing libraries produced using the Stereo-seq Transcriptomics Set require the DNBSEQ sequencing platform. For details, refer to the **Stereo-seq Transcriptome Library Preparation User Manual (Document No.: STUM-LP002).**

Required input parameters for Stereo-seq FF transcriptome libraries for the Stereo-seq Analysis Workflow (SAW) bioinformatics pipelines are as follows:

`--kit-version='Stereo-seq T FF V1.3'`

`--sequencing-type='PE75_50+100'`

1.3. List of Kit Components

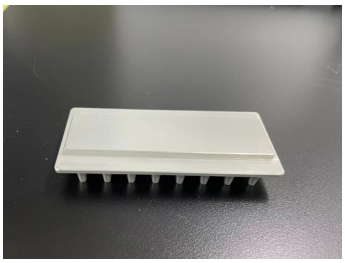
Each Stereo-seq Transcriptomics Set for Chip-on-a-slide (0.5cm * 0.5cm) consists of:

- Stereo-seq Transcriptomics T Kit *1 (4 RXN)
- Stereo-seq Chip T Slide (0.5cm * 0.5cm) *1 (4 EA)
- STOmics Stereo-seq Accessory Kit *2 (5 PCs)

Stereo-seq 16 RXN Library Preparation Kit is not included in the Stereo-seq Transcriptomics Set for Chip-on-a-slide and must be purchased separately. If you wish to construct Stereo-seq FF transcriptome libraries in-house, refer to the **Stereo-seq Transcriptome Library Preparation User Manual (Document No.: STUM-LP002)** for details.

Compatible auxiliary not included:

(Order separately) Stereo-seq PCR Adaptor *1 (2 EA)



Catalog numbers, kit components, and specifications are listed below (Table 1-1 to Table 1-4).

CAUTION: Upon receiving the Stereo-seq Chip T Slide (0.5cm * 0.5cm), follow the instructions in Stereo-seq Chip Slide Operation Guide For Receiving, Handling And Storing to properly store unused Stereo-seq Chip T Slides.

The performance of products may only be guaranteed before their expiration date. Proper performance is also subject to the products being transported, stored, and used in the appropriate conditions.

Table 1-1 Stereo-seq Transcriptomics T Kit Components

Stereo-seq Transcriptomics T Kit	Cat. No.: 211KT13114 / 211KT13114-CG		
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (tube)**
RI	1000028499		300 μL × 1
Glycerol	1000047910		100 μL × 1
H&E Mounting Medium	1000041969		50 μL × 1
RT Buffer Mix	1000047911	(transparent)	731 μL × 1
RT Plus	1000047912		18 μL × 1
RT Oligo	1000047913	(transparent)	44 μL × 1
PR Enzyme	1000028500		10 mg × 1
RT Enzyme Mix	1000047914	(transparent)	88 μL × 1
Elute Additive	1000048030		22 μL × 1
Neutralization Solution	1000047915		102 μL × 1
4X cDNA PCR Mix	1000047916		337 μL × 1
cDNA Primer	1000047917		53 μL × 1
Storage Temperature: -25°C ~ -15°C	Transportation Temperature: -25°C ~ -15°C	Expiration Date: refer to label	

This reagent is used solely for coverslip mounting on the H&E-stained tissue section and is not used if the H&E staining workflow is not implemented.

Table 1-2 Stereo-seq Chip T Slide (0.5cm * 0.5cm) Kit Components

Stereo-seq Chip T Slide (0.5cm * 0.5cm) Cat. No.: 210CT13004 / 210CT13004-CG	
Component	**Quantity (per kit)**
Stereo-seq Chip T Slide (0.5cm * 0.5cm)	4 EA
Storage Temperature: 2°C ~ 8°C	Transportation Temperature: -25°C ~ -15°C	Expiration Date: refer to label

Table 1-3 STOmics Accessory Kit Components

STOmics Accessory Kit	Cat. No.: 1100033700 / 1100033700-CG	
Component	**Reagent Cat. No.**	**Quantity (per kit)**
Cassette	1000033699	1 EA
Gasket	1000033698	4 EA
Sealing Tape	1000042970	6 EA
Storage Temperature: 18 °C ~ 25°C	Transportation Temperature: 0 °C ~ 30°C	Expiration Date: refer to label

Table 1-4 Stereo-seq PCR Adaptor Components

Stereo-seq PCR Adaptor	Cat. No.: 301AUX001 / 301AUX001-CG	
Component	**Quantity (per kit)**	
Stereo-seq PCR Adaptor	2 EA	
Storage Temperature: 18°C ~ 25°C	Transportation Temperature: 0 °C ~ 30°C	Expiration Date: refer to label

1.4. Additional Equipment and Materials

The table below lists the equipment and materials needed for this protocol. The user is expected to have access to common laboratory equipment not named in the document (equipment such as an ice maker, biological safety cabinet, freezers, and so on). For specific microscope requirements, refer to the **STOmics Microscope Assessment Guideline (Document No.: STUM-PE001)**, which will be superseded by **Stereo-seq Imaging Requirements and Guidelines (Document No.: STUM-PE002)** once available. Please refer to the same document link for the latest version.

Table 1-5 Additional Equipment and Materials

Equipment		
Brand	**Description**	**Cat. No.**
-	Cryostat	-
-	Benchtop Centrifuge	-
-	Pipettes	-
Leica*	Fluorescence Microscope	DM6M
STOmics*	Fluorescence Microscope	900-000586-0
-	Vortex Mixer	-
-	Metal Bath (or equivalent instrument)	-
Bio-Rad^	T100™ Thermal Cycler	1861096
Thermo Fisher Scientific^	ProFlex™ 3 x 32-well PCR System	4483636
Labnet	Slide Spinner (optional)	C1303-T
NEB	NEBNext® Magnetic Separation Rack for <200 µL tubes	S1515S
Thermo Fisher Scientific	Magnetic Rack: DynaMag™-2 Magnet for 1.5-2 mL Tubes	12321D
	Qubit™ 3 Fluorometer	Q33216 (or similar)
Agilent Technologies™	Agilent 2100 Bioanalyzer	G2939AA (or similar)

* Choose either one of the listed brands (marked with *).
^ Choose either one of the listed brands (mark with ^). A suitable PCR Adaptor will be needed.

Table 1-6 Additional Rreagents

Reagent		
Brand	**Description**	**Cat. No.**
-	100% Ethanol (Analytical grade)	-
Ambion	Nuclease-free Water	AM9937
	1X TE Buffer, pH 8.0	AM9858
	20X SSC	AM9770
Beckman Coulter*	SPRIselect	B23317/ B23318/B23319
	AMPure® XP	A63882
VAZYME*	VAHTS™ DNA Clean Beads	N411-02

Reagent		
Brand	**Description**	**Cat. No.**
Sigma Aldrich	Hydrochloric Acid, HCl	2104-50ML
	Methanol	34860-1L-R
Invitrogen	Qubit ssDNA Assay Kit	Q10212
	Qubit dsDNA HS Assay Kit	Q32854
Aladdin^	Potassium Hydroxide Solution, 8M KOH	P291842
Millipore Sigma^	Potassium Hydroxide Solution, 8M	P4494-50ML
Agilent Technologies™	High Sensitivity DNA Kit	5067-4626
	High Sensitivity RNA Kit	5067-1513
SAKURA	SAKURA Tissue-Tek® O.C.T. Compound	4583
Sangon Biotech (or other brands)	Eosin Y, free Acid	A600190-0025
Sigma	Hematoxylin Solution (filter before use)	51275
Agilent (or other brands)	Bluing Buffer	CS70230- 2

*** Choose either one of the listed brands (marked with *).**

^ Choose either one of the listed brands (marked with ^).

The rows highlighted in purple are for Stereo-seq Transcriptomics H&E staining workflow only and will not be used if the H&E staining workflow is not implemented.

Table 1-7 Additional Consumables

Consumables		
Brand	**Description**	**Cat. No.**
-	Aluminum Foil	-
-	Forceps	-
-	Slide Staining Rack	-
-	Microscope Slides	-
-	Microscope Glass Coverslip (size: 18 mm x 18 mm, thickness: 0.13 - 0.16 mm)	-
Corning	Corning® 100 mm TC-treated Culture Dish	353003
	50 mL centrifuge tubes	430829
	15 mL centrifuge tubes	430791
Kimtech	KimWipes™ Delicate Task Wipes	34155
MATIN	Power Dust Remover	M-6318
Axygen	1.5 mL Centrifuge Tubes	MCT-150-A
	0.2 mL PCR Tubes*	PCR-02-C
	0.2 mL thin-wall 8 strip PCR tubes*	PCR-0208-CP-C
	200 µL Filtered Tips	TF-200-L-R-S
	100 µL Filtered Tips	TF-100-R-S
	10 µL Filtered Tips	TXLF-10-L-R-S
	0.5 mL Thin Wall PCR Tubes^	PCR-05-C
Invitrogen	**Qubit Assay Tubes^**	Q32856
-	Disposable Sterile Syringe	-
Millipore (or other brands)	Millex Syringe Filter, Durapore PVDF, 0.22 µm pore size	SLGV033N

* Choose either one of the listed materials (marked with *).

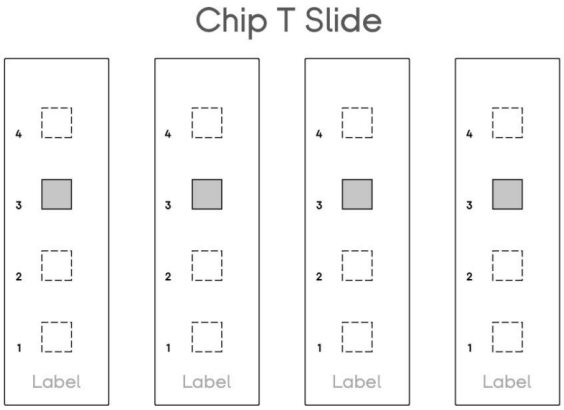
^ Choose either one of the listed brands (marked with ^).

The rows highlighted in purple are for Stereo-seq Transcriptomics H&E staining workflow only and will not be used if the H&E staining workflow is not implemented.

1.5. Stereo-seq Chip Slide Information

Stereo-seq Chip T Slide

Includes **4** Stereo-seq Chip T Slides containing **one** Chip T (0.5cm * 0.5cm) on each slide.

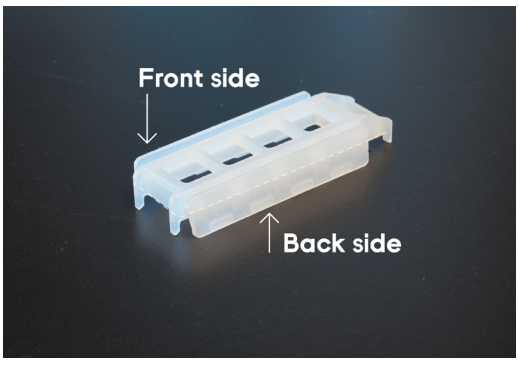


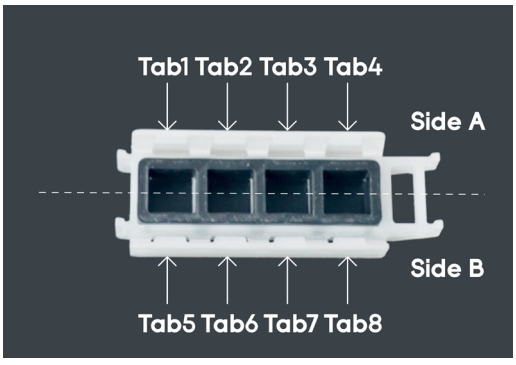
Stereo-seq Chip T Slide Storage

Always store unused slides in their original slide container and keep them sealed in a sealable aluminum bag at 2°C~8°C. Keep sealed with tape or another re-sealable bag. **Always KEEP the desiccant in the bag.**

Stereo-seq Slide Cassette

STOmics Stereo-seq Accessory Kit contains a Stereo-seq Cassette and removable Gaskets which need to be assembled prior to use.





For a demonstration video of Stereo-seq Slide Cassette assembling and removal, please refer to the link or by scanning the QR code:
https://en.stomics.tech/resources/videos/list.html

Refer to Appendix I: Stereo-seq Slide Cassette Assembly

1.6. Precautions and Warning

- This product is intended for research use only, not for use in diagnostic procedures. Read all instructions in this manual carefully before using the product.
- Before performing experiments with the kits, ensure that you are familiar with all related instruments and operate them according to the manufacturers' instructions.
- Instructions provided in this manual are intended for general use only; optimization may be required for specific applications.
- Thaw reagents in the kits properly prior to use. For enzymes, centrifuge briefly and keep them on ice until use. For other reagents, thaw them first at room temperature, invert several times to mix them properly, and centrifuge them briefly. Place them on ice for future use.
- RNA capture will be compromised or absent for any scratched areas on the front surface of the chip.
- We recommend using filtered pipette tips to prevent cross-contamination. Use a new tip each time for pipetting different solutions.
- We recommend using a thermal cycler with heated lids for PCR reactions. Unless otherwise stated, pre-heat the thermal cycler to reaction temperature before use.
- Improper handling of samples and reagents may contribute to aerosol contamination of PCR products, resulting in data inaccuracy. Therefore, for PCR reaction preparation and PCR product cleanup tests, we recommend working in two distinctly separate working areas in the laboratory. Use designated pipettes and equipment for each area, and perform regular cleaning (with 0.5% sodium hydrochloride or 10% bleach) to ensure a clean and sterile working environment.
- Do not consume any sample or reagent, and avoid direct contact of reagents with skin and eyes. In case of an accident, immediately wash the affected area thoroughly with a large amount of water. Seek emergency medical assistance if needed.

CHAPTER 2
SAMPLE AND EXPERIMENT PREPARATION

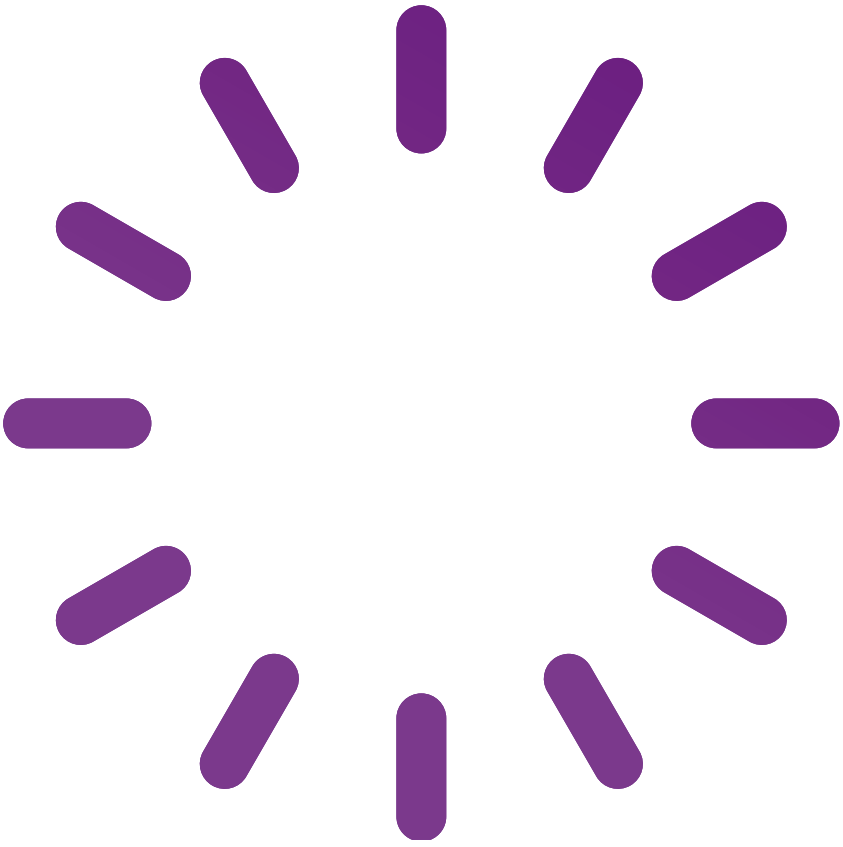
This guide describes how to check the RIN quality of a fresh frozen tissue sample before proceeding to the Stereo-seq experiment.

It is strongly recommended that you proceed only with tissue samples with a RIN value ≥4.0.

For frozen sample embedding guides, refer to the **Sample Preparation Guide for Fresh Frozen Samples on Stereo-seq Chip Slides (Document No.: STUM-SP001).**

CHAPTER 3

Stereo-seq TRANSCRIPTOMICS SET FOR CHIP-ON-A-SLIDE (0.5cm * 0.5cm) STANDARD OPERATING PROCEDURE



3.1. Experiment Preparation

Unless otherwise specified, use nuclease-free water for all reagents prepared prior to this experiment.

Table 3-1 Experiment Preparation Steps

Reagent	Preparation Steps	Maintenance
5X SSC	Dilute 5 mL of 20X SSC to 20 mL.	Room temperature
0.1X SSC	Dilute 250 µL of 20X SSC to 50 mL.	Room temperature
Methanol	Pre-cool at -20°C for no longer than 30 min before use.	-20°C
Wash Buffer	For ssDNA staining: Prepare at least 225 µL per chip (213.5 µL 0.1X SSC with 11.5 µL RI). For H&E staining: Prepare at least 300 µL per chip (285 µL 0.1X SSC with 15 µL RI).	On ice until use
0.01N HCl	Prepare at least 2 mL of 0.01N HCl per sample. Measure and ensure that the pH = 2.	Room temperature for 48 hr **(Storing longer than 48 hr will affect the desired pH. Use within 48 hr of preparation)**
ALWAYS use freshly prepared 0.01N HCl (pH = 2.0 ± 0.1). For pre-made 0.1N HCl and newly purchased HCl, check the pH prior to the experiments.		
10X Permeabilization Reagent (PR) Stock Solution	Add 1 mL of freshly prepared 0.01N HCl to dissolve PR Enzyme (red cap, in powder form), and thoroughly mix the reagent by pipetting.	On ice until use, up to 1 hr
DO NOT vortex the permeabilization enzyme. Mix by pipette before using. **Aliquot the 10X stock solution to avoid freeze-thaw cycles and keep it at -20°C for long term storage.**		
1X Permeabilization Reagent Solution	Prepare 1X PR Solution by diluting 10X PR stock solution with 0.01N HCl. Prepare 200 µL per chip.	On ice until use, up to 6 hr
0.1M KOH	Dilute 10 µL of 8M KOH to 800 µL. Prepare 195 µL per chip.	Room temperature
Always use freshly prepared 0.1M KOH. For newly purchased 8M KOH, first dilute to 1M and verify that the pH is 14 ± 0.3 before preparing the 0.1M KOH. **Do not prepare until you are ready to use it.**		

Reagent	Preparation Steps	Maintenance
Glycerol	Take it out and equilibrate to room temperature at least 5 min prior to use. Prepare 2 μL per chip.	Room temperature
Elute Additive	Take it out of -20°C, and thaw on ice in advance. Prepare 5 μL per chip.	On ice until use
Neutralization Solution	Take it out of -20°C, and thaw at room temperature. Prepare 23 μL per chip.	Room temperature
Eosin Solution	Dissolve 0.026g Eosin Y powder in 50 mL methanol and keep it sealed with a parafilm until use.	Room temperature up to 1 month
Hematoxylin (filtered)	Prepare and filter the Hematoxylin Solution using a 0.22 μm pore-sized filter (i.e., needle cartridge filter, matched with a disposable sterile syringe) and seal it with a parafilm until use; use 100 μL per chip.	Room temperature up to 7 days in the dark
H&E Mounting Medium	Equilibrate to room temperature 5 min in advance. Prepare 1.5 μL per chip.	Room temperature
The rows highlighted in purple are for Stereo-seq Transcriptomics H&E staining workflow only and will not be used if the H&E staining workflow is not implemented.		
80% Ethanol	Dilute 100% ethanol to 80%	Room temperature up to 1 day
Magnetic Beads	Take it out and equilibrate to room temperature at least 30 min prior to use.	Room temperature up to 6 hr

Other Preparation		
Equipment	**Set up**	**Notes**
Cryostat	Set the cryostat chamber temperature to -20°C and the specimen disc temperature (object temperature) to -10°C~-15°C.	The specimen disc temperature depends on the tissue type.

Other Preparation		
PCR Thermal Cycler	Set the temperature in the following order: 1. 37°C for slide drying and permeabilization (heated lid at 60°C). 2. 45°C for reverse transcription (heated lid at 60°C). 3. 55°C for cDNA release (heated lid at 60°C). 4. 95°C for denaturation (heated lid at 105°C).	Check the PCR Thermal Cycler for any abnormalities and replace it if necessary.
Metal Bath (or equivalent heating instrument)	37°C for preheating of 1X Permeabilization Reagent Solution.	Check the instrument for any abnormalities and replace it if necessary.
Fluorescence Microscope	Set the epi-fluorescence channel to FITC mode for imaging of fluorescent-stained tissue and BF-Epi channel for imaging of H&E-stained tissue.	Check the microscope for any abnormalities and replace it if necessary.

3.2. Cryosection Preparation

a. Set the PCR thermal cycler to 37°C with the heated lid to 60°C in advance with a PCR Adaptor.

Temperature	Time	Number of cycles	Step
(Heated lid) 60°C	on	-	-
37°C	**∞**	**1**	**Tissue Permeabilization**
45°C	∞	1	Reverse Transcription
55°C	∞	1	cDNA Release

b. Set cryostat chamber temperature to -20°C and specimen disc temperature (object temperature) to -10°C~-15°C.

If the specimen disc is over-cooled, it can result in tissue section cracking during sectioning.When the disc temperature is too high, sections will wrinkle. Optimal specimen disc temperature depends on the tissue type.

c. Place forceps, brushes, and razor blades inside the chamber for pre-cooling.

d. Take the OCT-embedded tissue sample out of the -80°C freezer and place it in the chamber for **30 min** to allow it to equilibrate to the cryostat chamber temperature.

e. Remove the sample outer covers (aluminum foil) and trim the embedded tissue block to the appropriate size (sectioning area smaller than 0.45 cm x 0.45 cm).

f. Using OCT, mount the embedded tissue block onto the specimen disc/holder of the cryostat chamber.

g. Trim again if necessary to ensure a good fit between the tissue section and the Stereo-seq Chip. Now, the specimen is ready for cryosection.

3.3. Tissue Mounting

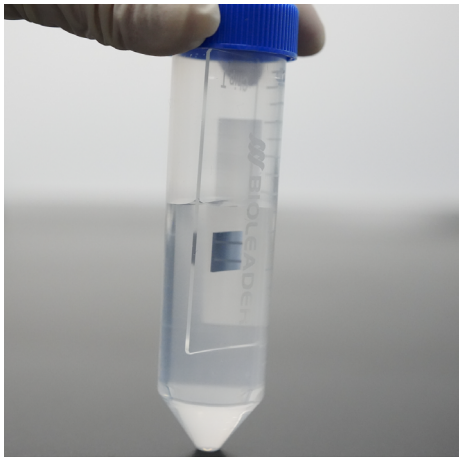
For a demonstration video of tissue mounting onto the Stereo-seq Chip Slide, refer to the link below or scan the QR code:
https://en.stomics.tech/resources/videos/list.html

a. Take the Stereo-seq Chip Slide out of the vacuum-sealed aluminum bag and record the Chip ID (SN) number located on the back of the slide. Do not touch the front of the chip.

After opening the bag, check all Stereo-seq Chip Slides in the slide container and make sure they are oriented front-side up. The front of the chip has a shiny surface that contains DNB-probes for RNA capture. DO NOT scratch the surface.

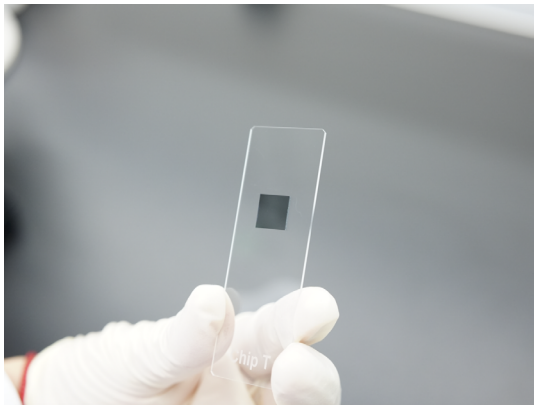
b. Make sure the PCR thermal cycler has been turned on and set to **37°C**.

c. Equilibrate the Stereo-seq Chip Slide to room temperature for **1 min** on the bench, then rinse with **100 µL** nuclease-free water **twice** with a pipette, or, rinse the slide twice in a 50 mL centrifuge tube by immersing it in sufficient water, using forceps for handling.



Store unused slides in the original packaging (first in the slide container and then in the sealable aluminum bag) and store at 2°C ~ 8°C. KEEP THE DESICCANT IN THE ALUMINUM BAG.

d. Gently blow off excess water from the chip with a power dust remover (MATIN, M-6318). Wipe off excess water from around the chip and on the slide with dust-free paper.



e. When the chip is completely dry and without wavy white stains is it ready for tissue mounting.

f. Prepare sufficient methanol in a 50mL centrifuge tube or a slide container, and ensure that all tissue sections are completely submerged.

Immerse a blank microscope slide in the 50mL centrifuge tube or slide container to ensure that there is sufficient methanol.

g. Keep the tube cap tightly and place it in the cryostat chamber. Allow it to equilibrate to the cryostat chamber temperature (-20°C) for **5-30 min**.

H&E applications only: Prepare a slide container or a 50 mL centrifuge tube, and add enough eosin solution at a volume that could submerge all the chips on the slide. Immerse a regular glass slide in the eosin-containing tube to check if the volume is enough. Close the lid and pre-cool the eosin solution for 5-30 min at -20°C. This step only applies if the H&E staining is intended for later use in the Stereo-seq workflow.

h. Place the tissue-mounted specimen disc/holder onto the cryostat head and adjust the angle accordingly.

i. Tissue mounting can be achieved using either the cold method (option A) or the warm method (option B). We recommend practicing tissue mounting and section placement on plain glass slides first. Select the appropriate section thickness according to the experiment needs; a section thickness of 10 μm is normally used.

A. Cold Method

1) Place the Stereo-seq Chip Slide inside the chamber with the front side facing up and pre-cool inside the cryostat chamber for **1-6 min**.

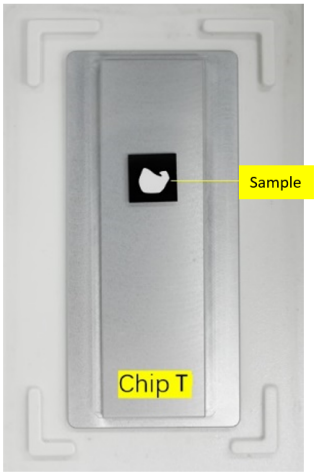
Prolonged cooling for longer than 6 min may cause mist to form on the chip surface.

2) Perform cryosection, then carefully flatten the tissue section by gently touching the surrounding OCT with cryostat brushes. Place the tissue section onto the chip center carefully with forceps and brushes. Ensure that the tissue section is complete and without wrinkles.

3) Immediately pick up the Stereo-seq Chip Slide and place a finger on the backside of the Stereo-seq Chip Slide directly under the chip for a few seconds to allow the section to adhere to the chip.

4) Place the tissue-mounted Stereo-seq Chip Slide back inside the chamber and move on to the second tissue slicing and mounting. Continue transferring sections on remaining chips.

5) When all tissue mounting is completed, immediately dry the Stereo-seq Chip Slide at 37°C on a PCR thermal cycler with a PCR adaptor for **5 min** (without heated lid).



When performing cold mounting, mind the time interval between each tissue section placement. Longer time intervals (>5 min) could result in tissue wrinkle formation.

B. Warm Method

1) Perform cryosection and obtain two or four consecutive tissue sections (depending on the number of chips on the Stereo-seq Chip Slide), carefully flatten the tissue sections by gently touching the surrounding OCT with cryostat brushes.

2) Move the tissue sections to the edge and place each tissue section at a distance greater than the chip spacing on the Stereo-seq Chip Slide, avoiding slide contact with other sections.

3) Flip the Stereo-seq Chip Slide and aim the tissue section within a chip area on the Stereo-seq Chip Slide by gently touching the section with the front side of the chip.

4) Repeat step 3) until all of the tissue sections have been mounted onto the chips of the Stereo-seq Chip Slide.

5) Flip the Stereo-seq Chip Slide over with the front facing up, and immediately dry it in the PCR thermal cycler at 37°C with PCR Adaptor for **5 min** (without heated-lid).

Stop Point:

- **After drying the tissue containing Stereo-seq Chip Slides on a PCR Thermal Cycler, transfer the Stereo-seq Chip Slide into a slide container or 50 mL centrifuge tube, then place it in a sealable plastic bag. Place one desiccant pack per Stereo-seq Chip Slide into a sealable bag, push out as much air as possible and seal the bag tightly. The sealed Stereo-seq Chip Slide can be transferred to a -80°C freezer on dry ice.**
- **Store the sealed plastic bag containing Stereo-seq Chip Slides with tissue at -80°C for up to four weeks.**
- **When retrieving Stereo-seq Chip Slides with tissue from the freezer, transfer out the slide container on dry ice, and take out the tissue containing Stereo-seq Chip Slides then immediately incubate at 37°C with PCR Adaptor for 5 min.**

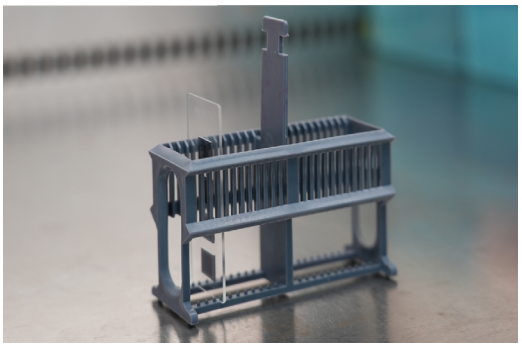
3.4. Tissue Fixation

For tissue samples that are intended for Stereo-seq Transcriptomics H&E workflow, tissue fixation & eosin staining (at -20°C), Hematoxylin solution staining & bluing, coverslip mounting and imaging steps need to be done prior to Tissue Permeabilization. Skip Sections 3.4 to 3.6, and refer to Appendix II for detailed procedures.

a. After drying the tissue-mounted Stereo-seq Chip Slide, immediately immerse it in pre-cooled methanol for a **30-min** fixation at -20°C **(do not exceed 1 hr)**. When immersing the Stereo-seq Chip Slide in methanol, ensure that all tissue sections are completely submerged.

b. After fixation is completed, move the 50 mL centrifuge tube or slide container to a sterile fume hood. Take out the Stereo-seq Chip Slide and wipe off excess methanol from around the edges and the back of the slide with dust-free paper without touching the chips. Ensure that there is no methanol residue between chips.

c. Place the Stereo-seq Chip Slide on a slide staining rack and leave it in the fume hood for **4-6 min** to allow the methanol to evaporate completely.



d. Prepare tissue fluorescent staining solution according to Table 3-2 and store it **in the dark**. **[PREPARE AHEAD]**

Table 3-2 Tissue fluorescent staining solution

Components	1X (μL)
5X SSC	94.5
Qubit ssDNA Reagent	0.5
RI	5
Total	100

e. When the methanol is completely evaporated, transfer the Stereo-seq Chip Slide onto a flat and clean bench.

3.5. Fluorescent Staining

Equilibrate the glycerol at room temperature for 5 min in advance.

a. Add **25 μL** of tissue fluorescent staining solution per chip by first pipetting one droplet at each corner of the chip and then adding the rest of the staining solution to the middle to merge all droplets.

b. Incubate it for **5 min** at room temperature in the dark.

Ensure that the chip is completely covered by tissue fluorescent staining solution.

c. Prepare **25 μL** Wash Buffer per chip during staining.

d. Set aside the 2 mL of 0.01N HCl and 1X Permeabilization Reagent Solution that you prepared in **3.1 Experiment Preparation**.

e. Set the temperature of a metal bath or equivalent heating instrument to 37 °C, and set the PCR program on hold at 37 °C.

f. Take out one gasket and one cassette from the STOmics Accessory Kit and blow any impurities off of the gasket with a power dust remover and then assemble only the Stereo-seq Cassette and the Gasket.

g. Ensure that the PCR thermal cycler has been switched on and set to 37°C. Pre-warm the assembled cassette and the gasket in the PCR thermal cycler for **10 min**.

h. Warm the aliquoted 1X Permeabilization Reagent Solution in the 37°C PCR thermal cycler or metal bath for **>10 min (no longer than 30 min)**.

i. Slightly tilt the Stereo-seq Chip Slide and gently remove the staining solution from the corner of the chip using a pipette. Try to remove as much solution as possible.

j. Add **25 μL** of Wash Buffer per chip.

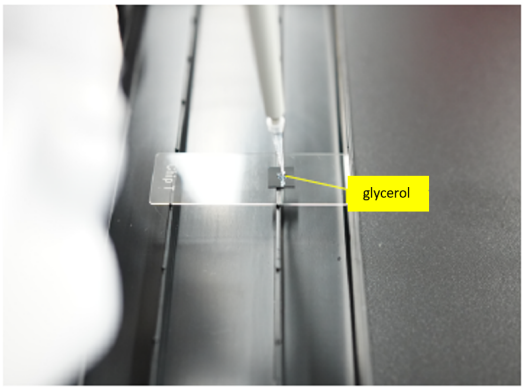
k. Slightly tilt the Stereo-seq Chip Slide while gently removing the Wash Buffer from the corner of the chip using a pipette. Try to remove as much solution as possible.

l. Transfer the Stereo-seq Chip Slide onto dust-free paper. Hold the slide with one hand and completely dry the chips using a power dust remover held in the other hand at an approximate distance of 2-3 cm from the chip surface. Blow gently from one side of the chip at a 30-degree angle horizontal to the plane of the chip. Ensure that there is no liquid residue around the chips.

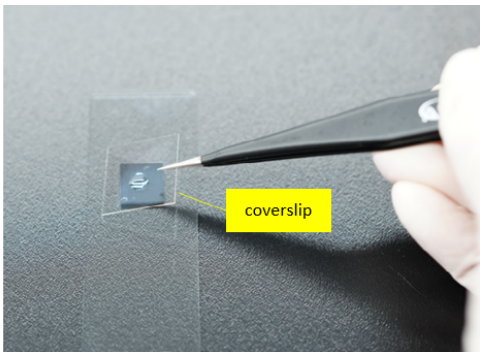
Alternatively, centrifuge the Stereo-seq Chip Slide for 10 sec in a slide spinner to completely dry the chips.

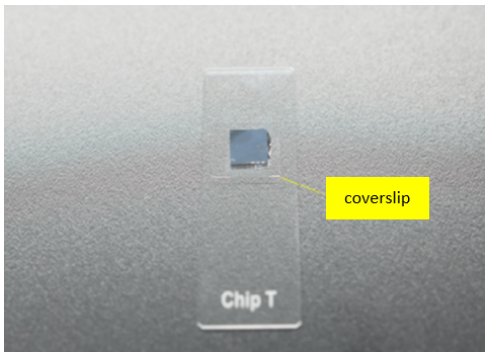
Ensure that the coverslip is clean and free of any dust or debris. Wipe with an alcohol swab or blow the debris off with a power dust remover.

m. Before using the glycerol tube, centrifuge it to remove any bubbles. Using a pipette, carefully add **5 μL** of glycerol to the center of the tissue on the chip without introducing air bubbles.



n. Using clean forceps, place one end of the coverslip onto the chip while holding the other end and then gradually lower the coverslip onto the chips. Ensure that the tissue is completely covered with glycerol and the coverslip. To avoid fluorescent bleaching, IMMEDIATELY proceed to **3.6 Imaging**.





3.6. Imaging

Ensure that your microscope has been turned on, switched to FITC-mode and is ready for imaging.

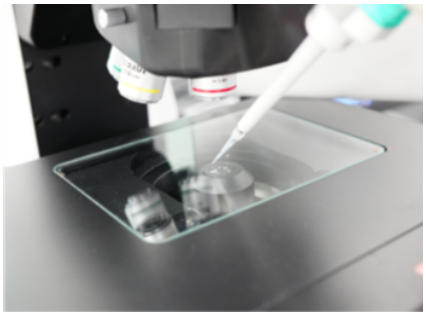
During the imaging process, ensure that the tracklines on the chip and the tissue area are both clear and within focus, and ensure that the tissue area is not overexposed.

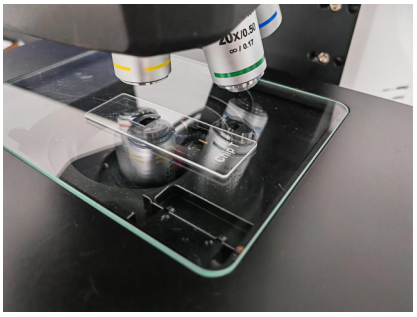
a. Create a new folder in the microscope imaging software, name it with the chip ID number and other essential information.

Use only letters, numbers, and underscores in the folder name. Special characters and spaces are not allowed.

Example chip ID number: B00249A1

b. Place the Stereo-seq Chip Slide in the slide holder on the microscope imaging platform. If a slide holder is not available, place **1-2 μL** of water on the imaging platform, then transfer and place the Stereo-seq Chip Slide onto the water drop. Water surface tension will grab onto the slide and adhere it to the imaging platform.





c. Be sure to place the Stereo-seq Chip Slide horizontally on the imaging platform with the engraved label on the right. The maximum tilting angle allowed is less than 15°.

CAUTION!
The Stereo-seq Chip Slide must be positioned with the engraved label on your right.

d. Turn on the fluorescence microscope and select the epifluorescence mode (FITC channel for ssDNA staining).

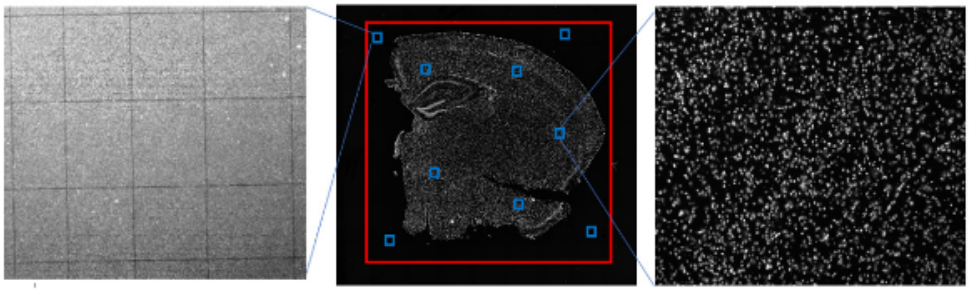
e. Determine the tissue location: Select the 4X objective lens, move the Field of View(FOV) to the tissue area on the chip, adjust Brightness, Gain, and Exposure, then use the focusing knob to adjust the focus until the tissue and cell boundaries are clear and within focus (the light intensity should be kept low to prevent fluorescence quenching).

f. Scan the map: Box-select the chip area to fully enclose the chip boundaries (should be slightly larger than the chip), and then scan the map under the 4X objective lens (if the microscope does not have a map scanning function, skip this step).

g. Adjust the microscope magnification: Switch to the 10X objective lens, then further adjust the box-selected area to ensure that the four corners of the chip are within the selected area and overlap the edge of the box-selected area as much as possible.

h. Focus plane determination: Use the focusing knob to adjust until the tissue and cell boundaries within the FOV are clear and within focus.

i. Manual focusing method (skip this step if the microscope you are using supports the autofocusing only): Shift the focus to a blank area on the chip depending on the situation, and then, if needed, adjust Brightness and Exposure until the tracklines on the chip are clear and within focus. Manually select and establish the model points. It is recommended that you first establish 3 to 5 model points in the blank areas of the chip (four corners of the chip). Shift the focus back on the tissue, and then, if needed, adjust Brightness and Exposure until the tissue and cell boundaries can be clearly visualized. Establish multiple model points on the regions of interest within the tissue area. It is recommended that you establish 3 to 5 model points per square centimeter in different places within the tissue.



j. Perform a full scan using the 10X objective lens, then save the original tile (FOV) image files and stitched images.

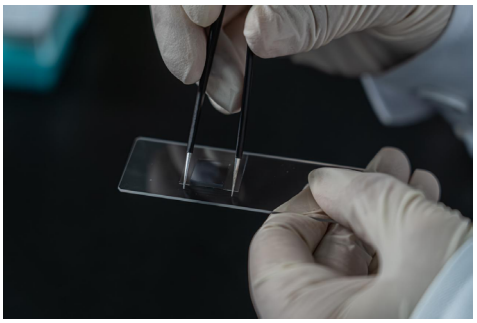
k. Open the StereoMap software and the Image Quality Control functional module in the software. Upload your nuclei-stained (ssDNA) image and run Image QC according to the instructions in the **StereoMap User Manual** in the software.

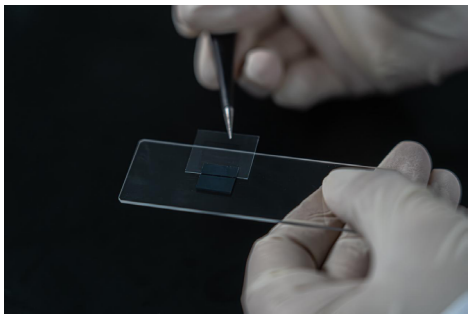
The captured ssDNA-stained image need to pass Image QC before you can proceed to further image analysis (register).

CAUTION! The captured nuclei-stained image need to pass Image QC before further image analysis (image "register") can be performed in Stereo-seq Analysis Workflow (SAW) pipelines. If Image QC fails, continue with the experiment procedures and perform optimal image analysis later under the guidance of your local Field Application Scientist.

Glycerol-mounted chips can not be stored longer than 2 hr after imaging at room temperature. For tissues prone to RNA degradation, such as pancreas, proceed to the next step immediately to avoid RNA degradation.

l. After imaging, use clean forceps to grip the coverslip, and then slowly pull and slide the coverslip over the Stereo-seq Chip Slide edge until the chips and the coverslip are completely separated.





m. Place the Stereo-seq Chip Slide in a 50 mL centrifuge tube filled with at least 30 mL of 0.1X SSC and immerse it for **3-5 sec**.

Ensure that all the chips on the Stereo-seq Chip Slide have been submerged in the solution.

n. Take out the Stereo-seq Chip Slide and wipe off excess solution from around the edges and the back of the slide with dust-free paper without touching the chips. Ensure that there is no liquid residue around the chips.

3.7. Tissue Permeabilization

a. Thaw RT Plus and RT Oligo at room temperature, thaw RT Buffer Mix on ice, and place the RT Enzyme Mix on ice until use.

b. Assemble the cassette and gasket and then place the Stereo-seq Chip Slide in the cassette according to the instructions in Appendix I: Stereo-seq Slide Cassette Assembly. It is recommended that you practice with a regular blank glass slide. Grip along the Stereo-seq Cassette to ensure that the Stereo-seq Chip Slide has been locked in place.

Do not touch the front of the chip while assembling the Stereo-seq Slide Cassette.

c. Ensure that the PCR thermal cycler has been set to the desired temperature (**highlighted in bold** below).

Temperature	Time	Number of cycles	Step
(Heated lid) 60°C	on	-	-
37°C	**∞**	**1**	**Tissue Permeabilization**
45°C	∞	1	Reverse Transcription
55°C	∞	1	cDNA Release

d. Place the Stereo-seq Slide Cassette in the 37°C PCR thermal cycler, add **200 µL** of 1X Permeabilization Reagent Solution onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all droplets.

Ensure that the chip is completely covered with 1X Permeabilization Reagent Solution.

e. Apply **unpeeled** sealing tape on top of the Stereo-seq Slide Cassette and let the chip incubate inside the PCR thermal cycler at 37°C for the optimal permeabilization time.

Optimal permeabilization time is determined by the Stereo-seq Permeabilization Kit (211KP11118). Refer to the Stereo-seq Permeabilization Set for Chip-on-a-slide User Manual (Document No.: STUM-PR002) for more information.

f. While waiting for permeabilization to be completed, prepare RT Mix according to Table 3-3, then leave it on ice until use. **[PREPARE AHEAD]**

Table 3-3 RT Mix

Component	1X (μL)	2X + 10% (μL)	3X + 10% (μL)	4X + 10% (μL)
RT Buffer Mix	166	365.2	547.8	730.4
RT Plus	4	8.8	13.2	17.6
RT Oligo	10	22	33	44
RT Enzyme Mix	20	44	66	88
Total	200	440	660	880

g. When incubation is completed, remove the Stereo-seq Slide Cassette from the PCR Adaptor (37°C).

h. PCR thermal cycler: Skip the 37°C step and continue to the 45°C step (**highlighted in bold** below).

Temperature	Time	Number of cycles	Step
(Heated lid) 60°C	on	-	-
37°C	∞	1	Tissue Permeabilization
45°C	**∞**	**1**	**Reverse Transcription**
55°C	∞	1	cDNA Release

i. Slightly tilt the Stereo-seq Slide Cassette at an angle of less than 20°. Pipette to remove the 1X Permeabilization Reagent Solution from the corner of the well; do not touch the chip surface.

j. Add **200 μL** of Wash Buffer per chip by first pipetting one droplet at one corner of the chip and then add the remaining liquid along the edge of the chip from the gap between the chip and the gasket.

k. Then slightly tilt the Stereo-seq Slide Cassette to remove the solution from the corner of each well. Keep the chip surface moist.

CAUTION! Do not dry the chip completely.

To prevent RNA degradation, proceed immediately to 3.8 Reverse Transcription.

3.8. Reverse Transcription

a. Make sure the PCR thermal cycler with PCR Adaptor has been set to 45°C.

b. Mix the prepared RT Mix by pipetting it up and down, then centrifuge briefly. Gently add **200 µL** of RT Mix per chip along the side of each well, ensuring that the well surface is uniformly covered with RT Mix.

c. Apply a new sealing tape to the Stereo-seq Slide Cassette and seal it tightly.

d. Incubate the Stereo-seq Slide Cassette at 45°C for **2 hr** or longer (no longer than **5 hr**).

3.9. cDNA Release and Denaturation

a. Five (5) min before the Reverse Transcription Program is completed, prepare the cDNA Release Mix according to Table 3-4, and then place the mix at room temperature.

Table 3-4 cDNA Release Mix

Component	1X (µL)	2X (µL)	3X (µL)	4X (µL)
0.1M KOH	195	390	585	780
Elute Additive	5	10	15	20
Total	200	400	600	800

b. When incubation is completed, remove the Stereo-seq Slide Cassette from the 45°C PCR Adaptor.

c. PCR thermal cycler: Skip the 45°C step and continue to the 55°C step (**highlighted in bold** below).

Temperature	Time	Number of cycles	Step
(Heated lid) 60°C	on	-	-
37°C	∞	1	Tissue Permeabilization
45°C	∞	1	Reverse Transcription
55°C	**∞**	**1**	**cDNA Release**

d. Remove the sealing tape. Slightly tilt the Stereo-seq Cassette and, using a pipette, remove the RT Mix from the corner of each well without touching the chip surface.

When removing the sealing tape, hold on to the Stereo-seq Slide Cassette with one hand without applying force to Side A and Side B of the cassette. This prevents the Stereo-seq Chip Slide from falling off the cassette.

e. Add **200 µL** 0.1X SSC into each well.

f. Slightly tilt the Stereo-seq Cassette and, using a pipette, remove 0.1X SSC from the corner of each well.

g. Add **180 µL** cDNA Release Mix into each well.

h. Apply sealing tape to the Stereo-seq Slide Cassette and seal it tightly. Incubate the Stereo-seq Slide Cassette at 55°C on the PCR Adaptor for **10 min**.

i. When incubation is completed, remove the Stereo-seq Slide Cassette from the PCR Adaptor and remove the sealing tape.

Do not allow the cDNA Release Mix to spill.

j. Remove the tissue from the chip by pipetting up and down about **10 times**. Do not touch the tissue with the pipette tips. If a small amount of tissue residue remains, the subsequent steps can be continued without treatment.

Do not pipette the cDNA Release Mix onto the gasket.

k. Completely collect the cDNA Release Mix from each well into a new 1.5 mL tube.

l. Add **23 µL** Neutralization Solution directly into the 1.5 mL tube with the collected cDNA, and then vortex and mix well. The final volume of the combined solution should be about **198 µL**. If the total volume is less than 198 µL after mixing, top it up with nuclease-free water.

m. Aliquot the collected cDNA into three PCR tubes, **66 µL** in each tube. Place them into the PCR thermal cycler, and incubate at 95°C for **5 min** using the following incubation protocol.

Temperature	Time	Number of cycles
(Heated lid) 105°C	on	-
95°C	5 min	1
12°C	∞	-

Ensure that all of the chip ID numbers have been recorded as required for downstream analysis. And ensure that all chip numbers accurately correspond to product collection tube numbers.

CAUTION! Temporarily store the post-collected Stereo-seq Chip in the 4°C refrigerator and do not discard it until the entire experiment and data processing have been completed.

3.10. cDNA Amplification

a. Prepare PCR Mix according to Table 3-5. Pipette up and down to mix, then centrifuge briefly. Store it on ice until use.

Table 3-5 PCR Mix

Component	1X (μL)	2X + 10% (μL)	3X + 10% (μL)	4X + 10% (μL)
4X cDNA PCR Mix	76.5	168.3	252.5	336.6
cDNA Primer	12	26.4	39.6	52.8
Nuclease-free water	13.5	29.7	44.5	59.4
Total	102	224.4	336.6	448.8

b. Aliquot **34 μL** PCR Mix into each PCR tube with **66 μL** cDNA products (from **step m.** in section 3.9). Vortex to mix well.

c. Briefly spin the three PCR tubes then proceed to cDNA Amplification based on the PCR program shown in Table 3-6.

Table 3-6 PCR Program for Amplification (for 100 μL)

Temperature	Time	Number of cycles
(Heated lid) 105°C	on	-
95°C	5 min	1
98°C	20 sec	13
58°C	20 sec	
72°C	3 min	
72°C	5 min	1
12°C	∞	-

PCR Mix can be left in the PCR thermal cycler overnight at 12°C. Alternatively, PCR Mix can be temporarily stored at 4°C for no longer than 16 hr after amplification completes.

d. Prepare Qubit dsDNA Mix according to Table 3-7.

Table 3-7 Qubit dsDNA Mix

Component	1X (μL)
Invitrogen™ Qubit dsDNA HS Buffer	199
Qubit dsDNA HS Reagent 200X	1
Total	200

e. Vortex the Qubit dsDNA Mix, then take 199 μL of the Qubit dsDNA Mix and place it in the Qubit detection tube (part of the Qubit dsDNA HS Assay Kit).

f. Add **1 μL** of the PCR product into the detection tube and mix it with the **199 μL** Qubit dsDNA Mix by vortexing. Measure the concentration of the PCR product using the Qubit dsDNA HS Assay Kit.

QC

QC **The DNA concentration is usually more than 5 ng/μL.**

For troubleshooting purposes, we recommend leaving approximately 2 μL of the PCR product in a PCR tube.

Strategies for salvaging samples with DNA concentrations below 5 ng/μL:

a. Comfirm that the previous operation follows the instructions in this manual.

b. Proceed to 3.11 cDNA Purification and perform the **1X** magnetic bead purification steps from **steps b.1) to b.7)**.

c. Resuspend the dried beads in **72 μL** of TE Buffer (instead of 100 μL), then vortex to mix. Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube onto a magnetic separation rack for 3-5 min until the liquid is clear.

d. Transfer **70.5 μL** of supernatant (instead of 98 μL) to a new 0.2 mL PCR tube.

e. Add **25.5 μL** of 4X cDNA PCR Mix and **4 μL** cDNA Primer, mix well and centrifuge, then place the PCR tube back into the PCR thermal cycler for **8 cycles** (follow Table 3-6 PCR Program for Amplification in 3.10 cDNA Amplification, **step c**, but for 8 cycles only).

f. Continue to perform 0.8X magnetic bead purification according to the instructions in 3.11 cDNA Purification, starting from **step a**.

3.11. cDNA Purification and Amplification

Recommended Magnetic Beads

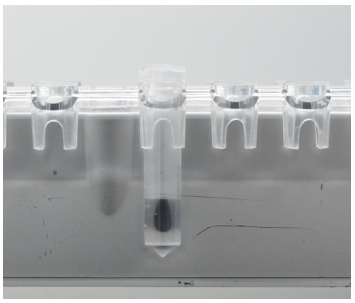
For bead-based purification, we recommend using DNA Cleanup Beads AMPure® XP(Beckman Coulter, Cat. No.: A63882), SPRIselect (Beckman Coulter, Cat. No.: B23317/B23318/B23319) or VAHTSTM™ DNA Clean Beads (VAZYME, Cat. No.: N411-02). *If magnetic beads from other sources are used, please optimize the cleanup conditions before getting started.*

Before Using the Beads

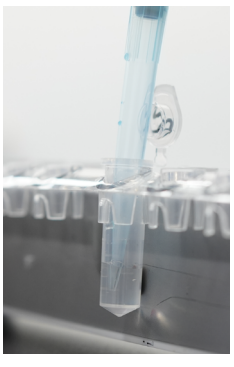
- To ensure the DNA capture efficiency of the magnetic beads, equilibrate the beads to room temperature **30 min before use**.
- Vortex or pipette up and down to ensure that the beads are thoroughly mixed every time before use.
- The number of magnetic beads directly affects the distribution of purified DNA fragments.

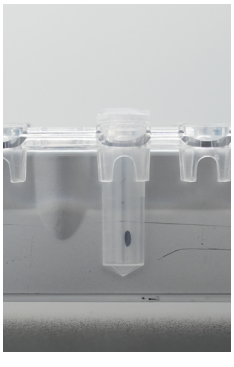
Operation Notes

- In the magnetic separation step, allow the solution to become completely clear before removing the supernatant. This process usually takes approximately **2-3 min**, but it can be longer or shorter, depending on the type of magnetic separation rack in use.

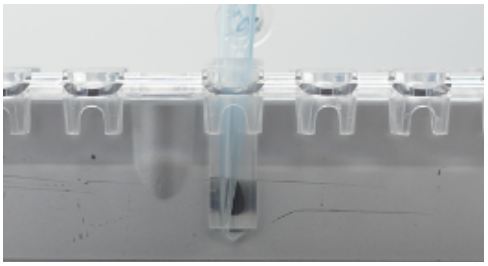


- When collecting the supernatant after magnetic separation, avoid taking up the beads in the pipette. Instead of collecting the entire supernatant fraction, leave 2-3 μL in the tube to prevent the pipette from directly contacting the beads. If the beads are accidentally taken up, dispense everything and redo the magnetic separation.

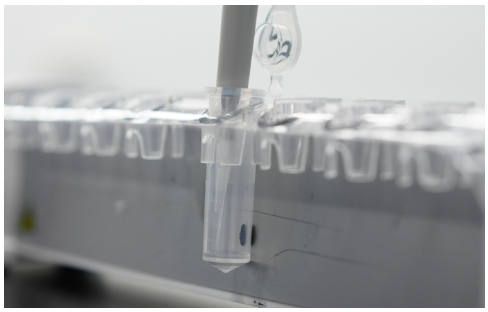




- Use freshly prepared 80% ethanol (at room temperature) to wash the beads. Keep the sample tube on the magnetic separation rack during the washing step. Do not shake or disturb the beads.

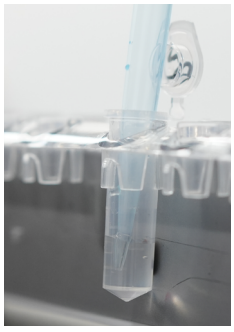


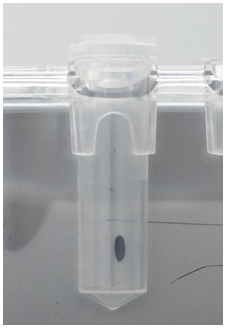
- After the second washing of beads with ethanol, try to remove all of the liquid in the tube. You may centrifuge briefly to accumulate any remaining liquid at the bottom of the tube, then separate the beads magnetically, and remove the remaining liquid by using a small-volume pipette.



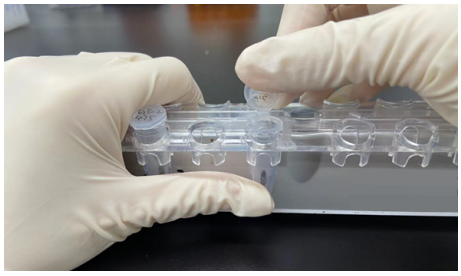
- After washing twice with ethanol, air-dry the beads at room temperature. Drying usually takes approximately **5-10 min** depending on the lab temperature and humidity level. Watch closely until the pellet appears sufficiently dry with a matte appearance, then continue to the elution step with TE Buffer.

- During the elution step, do not touch the beads with the pipette tip when removing the supernatant. Contamination of a DNA sample with beads may affect subsequent purification steps. Therefore, to prevent the pipette tip from directly contacting the beads, always collect the eluate in 2 μL less than the initial volume of TE Buffer used for the elution.





- **Pay attention when opening/closing the lid of a sample tube on a separation rack. Strong vibrations may cause samples or beads to spill from the tubes. Hold the body of the tube while opening the lid.**



a. Ensure the magnetic beads have been equilibrated to room temperature for at least **30 min**.

b. Use the magnetic beads to purify the PCR product in a volume ratio of 1:0.8 (DNA:beads).

1) Mix the three tubes of PCR products of the same cDNA (**300 µL**) in a 1.5 mL centrifuge tube, then mix the combined PCR products with the magnetic beads in a ratio of **1:0.8**.

2) Vortex the mixture, then incubate it at room temperature for **10 min**.

3) Spin down and place the tube onto a magnetic rack for **3 min** until the liquid becomes clear.

4) Carefully remove and discard the supernatant with a pipette.

5) Keep the tube on the magnetic rack and add **600 µL** of freshly prepared 80% ethanol. Wash the beads by rotating the tube on the magnetic rack. Incubate for **30 sec** and carefully remove and discard the supernatant.

Always place the pipette tips on the tube wall and away from the magnetic beads. Do not disturb the beads while transferring the supernatant.

6) Repeat **step 5)**.

7) Keep the tube on the magnetic rack, and open the lid to air-dry the beads at room temperature until no wetness (reflectiveness) or cracks are visible. Drying times will vary but will take approximately **5-8 min**.

8) Add **100 µL** of TE Buffer to the dried beads. Mix the beads and TE Buffer by vortexing. Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube onto a magnetic rack for **3-5 min** until the liquid is clear. Transfer the supernatant (~ **98 µL**) into a new 1.5mL centrifuge tube.

The purified cDNA sample can be stored at -20°C for up to 1 month.

For troubleshooting purposes, we recommend storing the beads with 40 µL of nuclease-free water at 4°C after purification until your cDNA final product has passed QC.

c. Take 1 µL of the cDNA sample and measure and record the concentration of the purified cDNA using the Qubit dsDNA HS Assay Kit.

d. Analyze the sample (dilution might be required) on an Agilent Bioanalyzer High Sensitivity chip or other library quality control platform such as TapeStation (Agilent Technologies), LabChip® GX, GXII, GX Touch (PerkinElmer) and Fragment Analyzer™ (Advanced Analytical).

A qualified cDNA sample should have fragment distribution appearing at approximately 200-2,000 bp (Figure 1) and a yield that is greater than 100 ng.

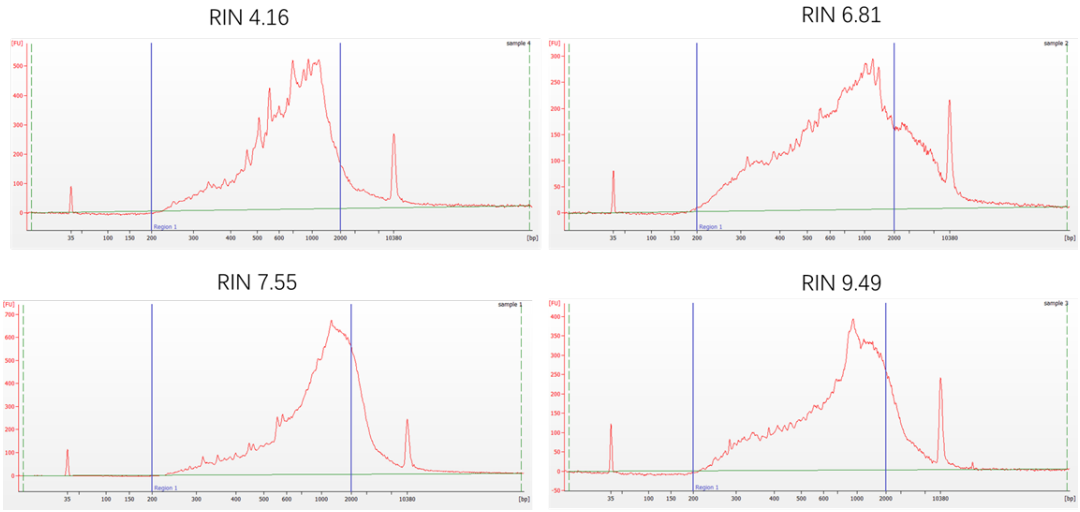


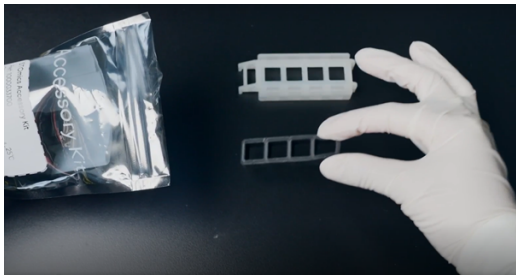
Figure 1. Representative Agilent Bioanalyzer 2100 analysis results of PCR-amplified cDNA samples with different RIN values

Refer to the Stereo-seq Transcriptome Library Preparation User Manual (Document No.: STUM-LP002) for details on subsequent library preparation.

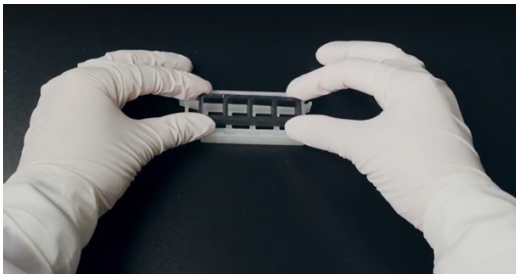
Appendix I: Stereo-seq Slide Cassette Assembly

Stereo-seq Slide Cassette Assembly

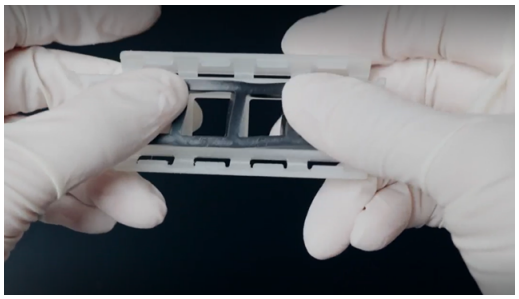
a. Take the Stereo-seq Slide Cassette and Gasket out of the STOmics Stereo-seq Accessory Kit.



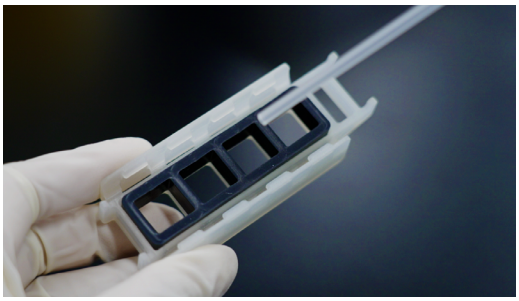
b. Pick up the Stereo-seq Slide Cassette and flip it over. Insert the gasket into the Stereo-seq Slide Cassette, ensuring that the cutouts are aligned.



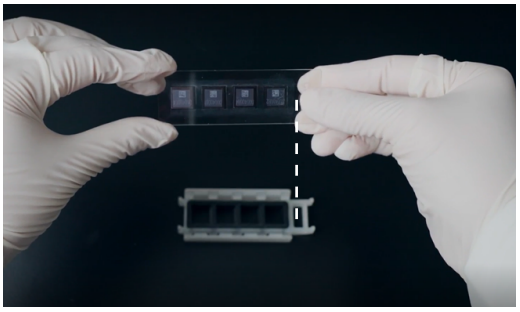
c. Press down on the gasket to secure it in the cassette.



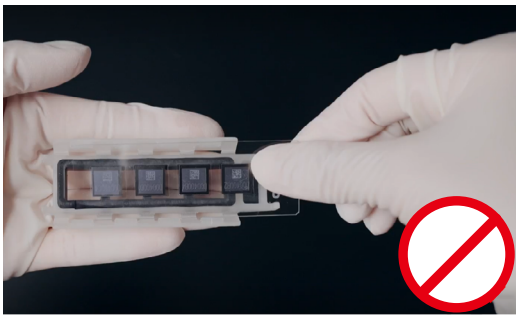
d. If necessary, use a power dust remover to blow any debris off the gasket.



e. Pick up the Stereo-seq Chip Slide and flip it over with the chip surface facing down. Align the engraved label with the long edge of the Stereo-seq Slide Cassette.

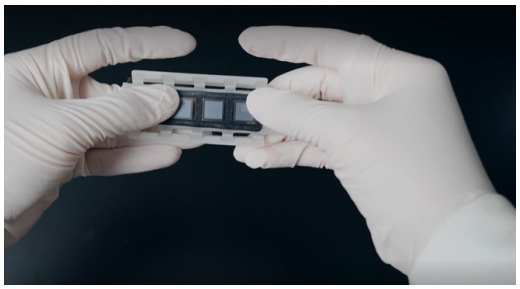


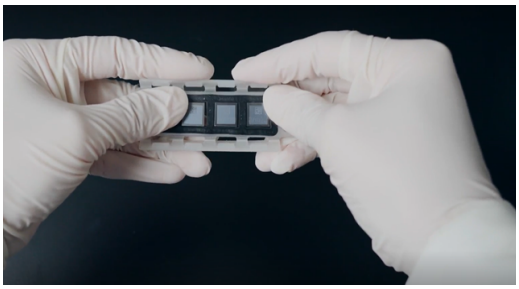
f. Ensure that the chips are aligned within the empty space of the gasket and avoid touching the chip surface with the gasket or cassette during slide placement. Insert the Stereo-seq Chip Slide under the bottom 4 tabs.



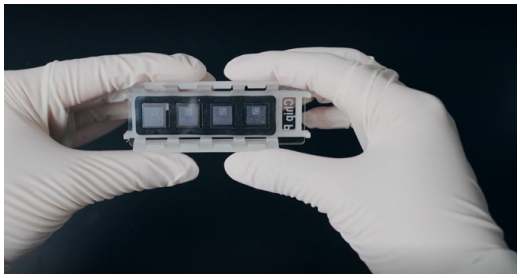
g. Support the back of the cassette with both middle fingers. Place your left thumb between tab 1 and tab 2, and place your while right thumb between tab 3 and tab 4.

h. Press the upper side (A side) of the slide (near the edge) evenly and then simultaneously press the top edge down firmly with both index fingers to clip the slide in place until you hear it click.

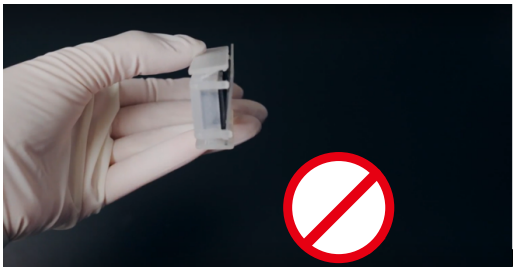




i. Press along both edges of the Stereo-seq Slide Cassette to ensure that the Stereo-seq Chip Slide is locked in place.

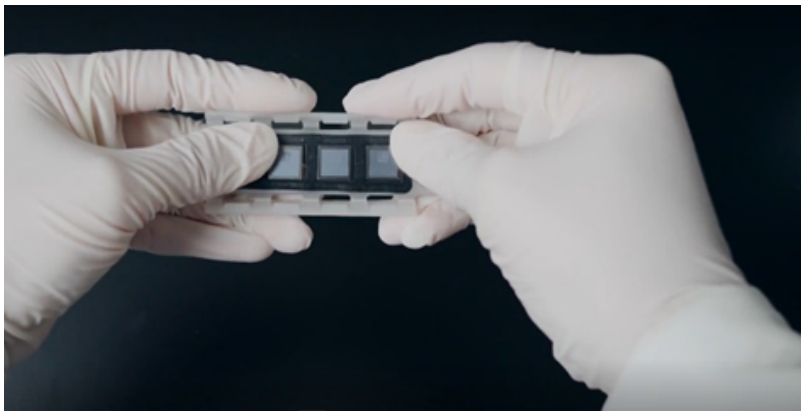


j. Recheck the Stereo-seq Slide Cassette and verify that the slide is clipped in place.

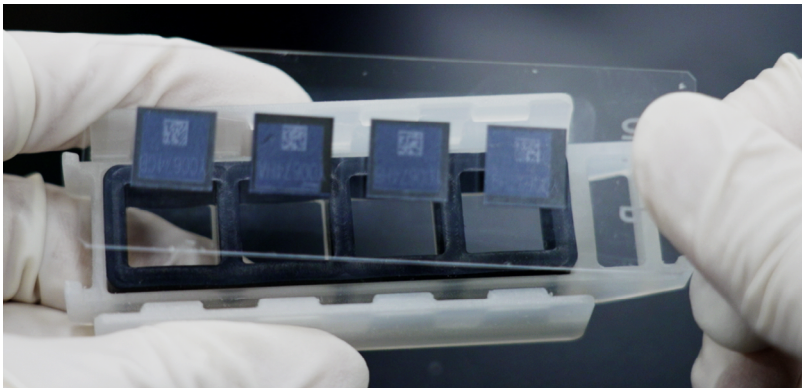


Stereo-seq Slide Cassette Removal

a. To release the slide from the tabs, first flip the cassette over, and then as you gently support the back of the Stereo-seq Chip Slide with both thumbs to prevent the Stereo-seq Chip Slide from falling, firmly press the upper side down.



b. Lift the Stereo-seq Chip Slide from the engraved label end.



Appendix II: H&E Staining Operating Procedures

Table II-1 Experiment Preparation Steps

Reagent Required for Appendix II	Purpose	Preparation
Methanol	Tissue fixation & eosin staining	Prepare 30-50mL of methanol in a centrifuge tube or a slide container. Pre-cool at -20°C for **5-30 min**.
Eosin Solution (H&E application)	Tissue fixation & eosin staining	Dissolve 0.026g Eosin Y powder in 50 mL methanol and keep sealed with a parafilm. Pre-cool at -20°C for **5-30 min**.
0.1X SSC	Washing	Dilute 250 µL of 20X SSC to 50 mL, then place it at room temperature.
Wash Buffer	Washing	Prepare at least 400 µL per chip.
Hematoxylin Solution (with 5% RI)	Hematoxylin staining	Prepare at least 100 µL per chip (95 µL Hematoxylin Solution with 5 µL RI).
Filter before use. **Do not add RI until you are ready to use it.**		
Bluing Buffer (with 5% RI)	Bluing	Prepare at least 100 µL per chip (95 µL Bluing Buffer with 5 µL RI).
Do not add RI until you are ready to use it.		
H&E Mounting Medium	Mounting	Equilibrate to room temperature 5 min in advance. Prepare 3.5 µL per chip.

Tissue Fixation & Eosin Staining (performed at -20 °C)

a. After drying the tissue-mounted Stereo-seq Chip Slide, immediately immerse it in pre-cooled methanol for a **30-min** fixation at -20°C (do not exceed 1 hr). When immersing the Stereo-seq Chip Slide in methanol, ensure that all tissue sections are completely submerged.

b. Transfer the Stereo-seq Chip Slide to the pre-cooled eosin solution, and ensure that all the tissue sections are completely submerged. Stain for **3 min** at -20 °C.

The staining duration should be adjusted to achieve uniform coloring of the tissue and controlled within a range of 3-5 min. It is important to maintain a consistent staining time for the same tissue block.

c. When eosin staining is completed, transfer the Stereo-seq Chip Slide back to the methanol-containing tube and incubate at -20°C for another **1 min**.

d. Move the methanol container to a sterile fume hood. Take out the Stereo-seq Chip Slide and wipe off excess methanol from around the edges and the back of the slide with dust-free paper without touching the chips. Ensure that there is no methanol residue between chips.

e. Place the Stereo-seq Chip Slide on a slide staining rack and leave it in the fume hood for **4-6 min** to allow the methanol to evaporate completely.

f. When the methanol is completely evaporated, transfer the Stereo-seq Chip Slide onto a flat and clean bench for further staining.

Hematoxylin Staining and Bluing

a. Set aside the 2 mL 0.01N HCl you prepared in 3.1 Experiment Preparation. Prepare the following reagents.

DO NOT leave them on ice. Add RI 5 min before the incubation, and then mix and vortex the reagents right before use.

Table II-2 Reagents Preparation Steps

Prepare		
Reagent	**Preparation Steps**	**Storage**
Hematoxylin Solution (with 5% RI)	Filter before use and prepare at least 100 µL per chip (95 µL Hematoxylin Solution with 5 µL RI).	Room temperature up to 5 min **in the dark**
Bluing Buffer (with 5% RI)	Prepare at least 100 µL per chip (95 µL Bluing Buffer with 5 µL RI).	Room temperature up to 5 min

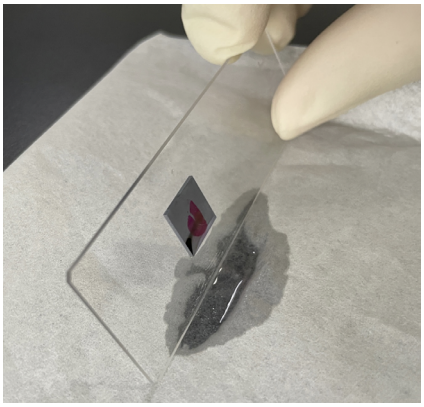
b. Add **25 µL** of Hematoxylin Solution (with 5% RI) onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all droplets, ensuring uniform solution coverage on the chip. Incubate at room temperature for **7 min** (Hematoxylin Solution from Sigma Aldrich) or **1-2 min** (Hematoxylin Solution from Solarbio).

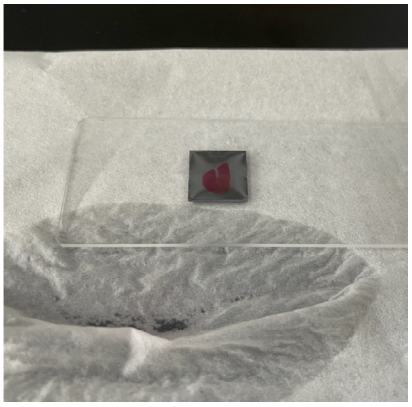
The incubation time needs to be adjusted according to the reagent manufacturer's protocol.

Equilibrate the H&E Mounting Medium to room temperature 5 min prior to use.

c. Discard Hematoxylin Solution by turning the Stereo-seq Chip Slide sideways at an angle of less than 60°, gently touch the edge of the chip with dust-free paper and allow the Hematoxylin Solution to pour onto dust-free paper. Remove as much solution as possible.

d. Add **25 µL** Wash Buffer per chip then discard it by turning the Stereo-seq Chip Slide sideways at an angle of less than 60° and allowing the Wash Buffer to pour onto dust-free paper.





e. Repeat **step d.** twice.

f. Add **25 μL** of Bluing Buffer (with 5% RI) onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets, ensuring uniform staining solution coverage on the chip. Incubate at room temperature for **2 min** (bluing reagent from Agilent).

The incubation time needs to be adjusted according to the reagent manufacturer's protocol.

g. Discard Bluing Buffer by turning the Stereo-seq Chip Slide sideways at an angle of less than 60°, gently touch the edge of the chip with dust-free paper and allow the Bluing Buffer to pour onto dust-free paper. Remove as much solution as possible.

h. Add **25 μL** Wash Buffer per chip, then discard it by turning the Stereo-seq Chip Slide sideways at an angle of less than 60° and allowing the Wash Buffer to pour onto dust-free paper. Try to remove as much solution as possible during the final wash: gently touch the edge of the chip with dust-free paper to absorb the residual liquid.

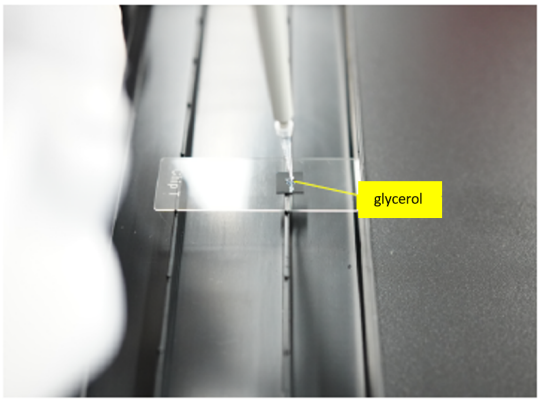
i. Transfer the Stereo-seq Chip Slide onto dust-free paper. Hold on to the slide with one hand and completely dry the chips further using a power dust remover held in the other hand at an approximate distance of 2-3 cm from the chip surface. Blow quickly from one side of the chip at a 30-degree angle horizontal to the plane of the chip. Ensure that there is no liquid residue around the chips.

Be sure to quickly dry the chip and the surrounding surfaces completely, especially the crevices between the chip and the slide. If there is residual liquid on the chip and surrounding surfaces, eosin staining on the tissue might get "smudgy".

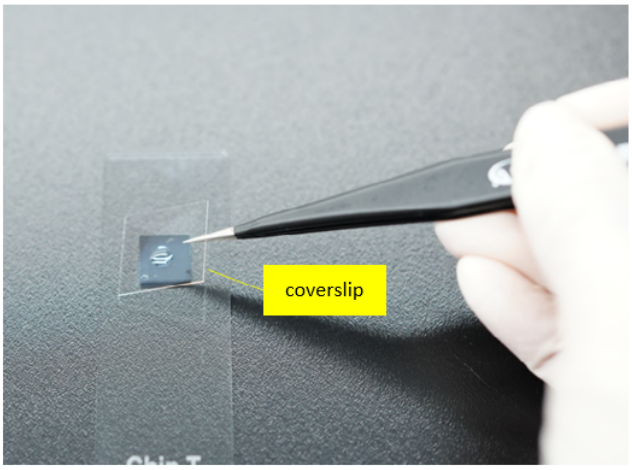
Ensure that the coverslip is clean and free of any dust or debris. Wipe with an alcohol swab or blow the debris off with a power dust remover.

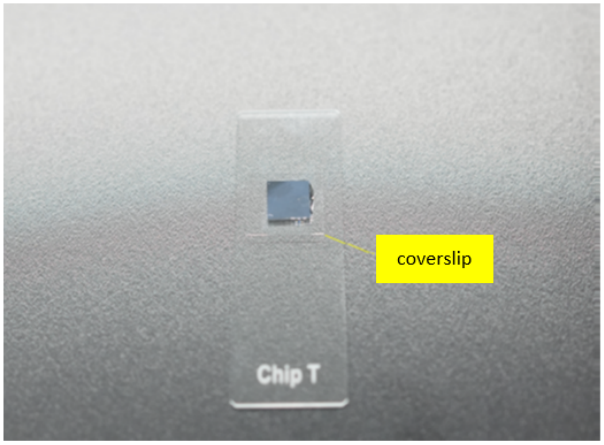
j. Gently pipette **1.5 μL** H&E Mounting Medium onto the center of the tissue on each chip without introducing air bubbles.

The cap color of the H&E Mounting Medium reagent is identical to that of glycerol. Identify the reagent label with caution before use.



k. Using clean forceps, place one end of the coverslip onto the chip while holding the other end and then gradually lower the coverslip onto the chips. Ensure that the chips are completely covered by H&E Mounting Medium and the coverslip. To ensure good image quality, IMMEDIATELY proceed to Imaging.





CAUTION! H&E Mounting Medium mounted chips can not be stored longer than 2 hrs at room temperature. For tissues that are prone to RNA degradation, such as pancrease, proceed to the next step immediately to avoid RNA degradation.

Imaging

a. Create a new folder in the microscope imaging software, name it with the chip ID number and other essential information.

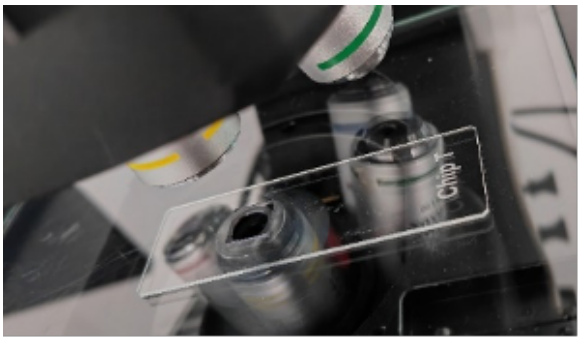
Use only letters, numbers, and underscores in the folder name. Special characters and spaces are not allowed.

Example chip ID number: B00249A1

b. Place the Stereo-seq Chip Slide in the slide holder on the microscope imaging platform. If a slide holder is not available, add **1-2 µL** of water on the imaging platform, then transfer and place the Stereo-seq Chip Slide onto the water drop. Water surface tension will grab onto the Slide and adhere it onto the imaging platform.

c. Be sure to place the Stereo-seq Chip Slide horizontally on the imaging platform with the engraved label on the right. The maximum tilting angle allowed is less than 15°.

CAUTION! The Stereo-seq Chip Slide must be positioned with the engraved label on the right.



d. Turn on the fluorescence microscope and select the epi-bright field (color camera) mode.

e. Determine the tissue location: Select the 4X objective lens, move the field of view to the tissue area on the chip, adjust Brightness and Exposure, then use the focusing knob to adjust focus until the tissue and cell boundaries are clear and within focus.

f. Scan the map: Box-select the chip area to fully enclose the chip boundaries (should be slightly larger than the chip), and scan the map under the 4X objective lens (if the microscope does not have a map scanning function, skip this step).

g. Adjust the microscope magnification: Switch to the 10X objective lens, then further adjust the box-selected area to ensure that the four corners of the chip are within the selected area and are overlapping with the edge of the box-selected area as much as possible.

h. Focus plane determination: Use the focusing knob to adjust focus until the tissue and cell boundaries within the field of view are clear and within focus.

i. White balance: Check whether the color of the H&E staining is correct; if there is an issue, select an area outside of the tissue area that is clean and free of impurities, then perform white balance correction.

j. Background balance: Shift the focus to a blank area on the chip, and perform background balancing. Adjust the focus to find a chip field of view without any tissue coverage or obvious impurities. Use the focusing knob to zoom out for defocusing. If there are small impurities in the previously selected area, they should be barely visible after defocusing. Finally, select "Background balancing".

k. Refocusing: Use the focusing knob to adjust focus until the tissue and cell boundaries are clear and within focus.

l. Manual focusing method (skip this step if the microscope you are using only supports the autofocusing method): Shift the focus to a blank area on the chip depending on the situation, then, if needed, adjust Brightness and Exposure until the tracklines on the chip are clear and within focus. Manually select and establish the model points. It is recommended that you first establish 3 to 5 model points in the blank areas of the chip (four corners of the chip). Shift the focus back on the tissue, then, if needed, adjust Brightness and Exposure until the tissue and cell boundaries can be clearly visualized. Establish multiple model points on the regions of interest within the tissue area. It is recommended that you establish 3 to 5 model points per square centimeter in different places within the tissue.

m. Perform a full scan using the 10X lens, then save the original tile (FOV) image files and stitched images.

n. Open the StereoMap software and the Image Quality Control functional module in the software. Upload your H&E-stained image and run Image QC according to the StereoMap User Manual in the software.

CAUTION! The captured H&E-stained image needs to pass Image QC before you can proceed to further image analysis (image “register”) in Stereo-seq Analysis Workflow (SAW) pipelines.

CAUTION! If the image fails to pass Image QC, continue with the experimental procedures and perform optimal image analysis later under the guidance of your local Field Application Scientist.

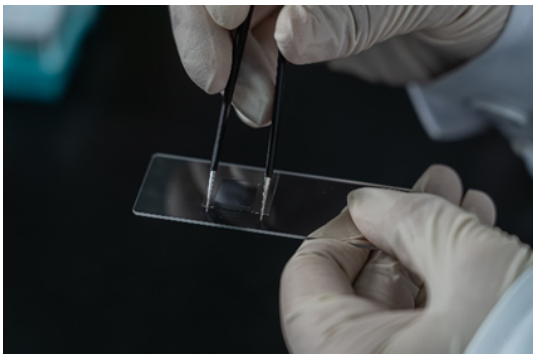
o. Set aside the 2 mL of 0.01N HCl and 1X Permeabilization Reagent Solution you prepared in 3.1 Experiment Preparation.

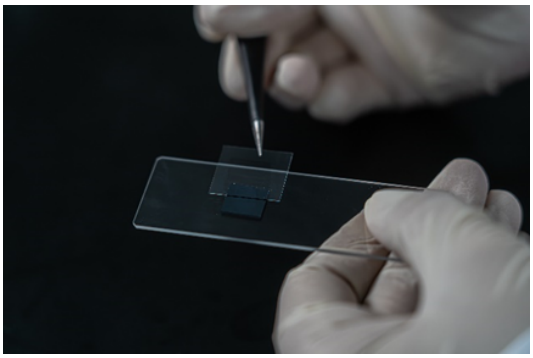
p. Set the temperature of a metal bath or equivalent heating instrument to 37°C, and set the PCR program on hold at 37°C.

q. Take out one gasket and one cassette from the STOmics Accessory Kit and blow any impurities off the gasket with a power dust remover, and then assemble only the Cassette and the Gasket.

r. Ensure that the PCR thermal cycler has been switched on and set to 37°C. Pre-warm the assembled Cassette and Gasket in the PCR thermal cycler for **10 min**. Warm up the aliquoted 1X Permeabilization Reagent Solution inside the 37°C PCR thermal cycler or metal bath for **>10 min (no longer than 30 min)**.

s. About 3 min before finishing the pre-warming of the 1X Permeabilization Reagent Solution, grip the coverslip with a pair of forceps, and then slowly pull and slide the coverslip over the Stereo-seq Chip Slide edge until the chips and the coverslip are fully separated.





t. Place the Stereo-seq Chip Slide in a centrifuge tube filled with at least 30 mL of 0.1X SSC and immerse it for **3-5 sec**.

Ensure that all the chips on the Stereo-seq Chip Slide have been submerged in the solution.

u. Take out the Stereo-seq Chip Slide and wipe off excess solution from around the edges and the back of the slide with dust-free paper without touching the chips. Ensure that there is no liquid residue between chips.

v. Add **25 µL** 0.01N HCl onto the chip, then remove it from the corner of the chip using a pipette.

CAUTION! Proceed to 3.7 Tissue Permeabilization to continue the procedure.