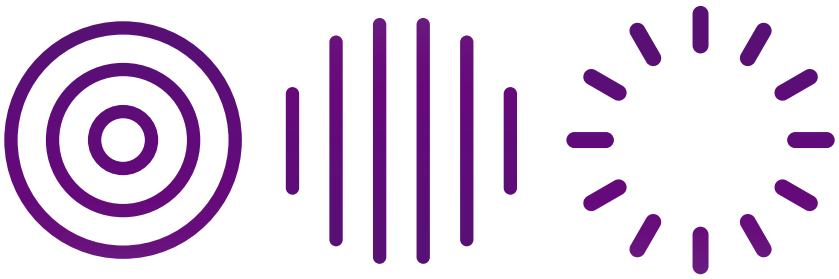
STOmics

Stereo-seq TRANSCRIPTOMICS SET FOR LARGE CHIP DESIGNS (≤ 2cm * 3cm)

USER MANUAL



Cat. No.:
111ST13122 (2 RXNs) / 111ST13221 (1 RXN) / 111ST13231 (1 RXN)
111ST13122-CG (2 RXNs) / 111ST13221-CG (1 RXN) / 111ST13231-CG (1 RXN)
Kit Version: V1.3
Manual Version: A

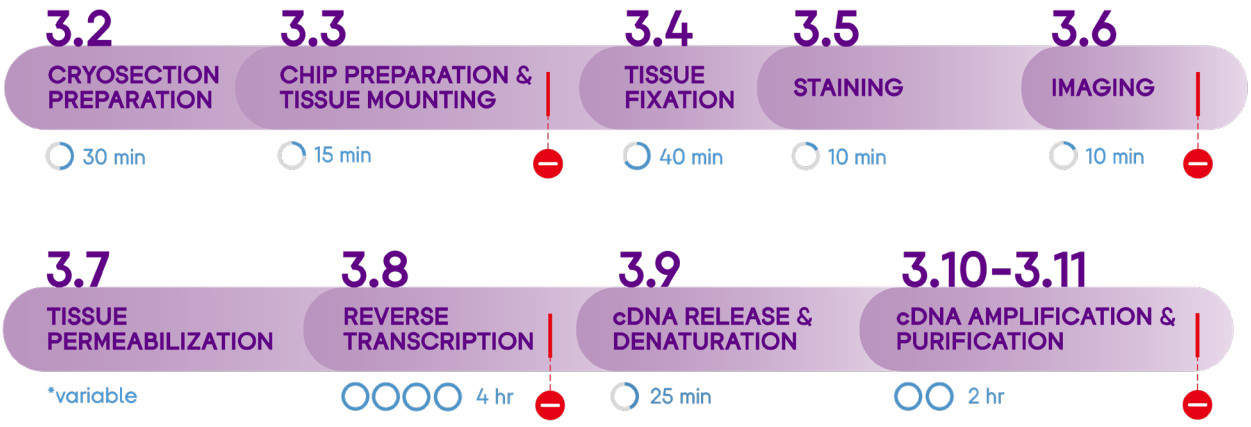
STUM-TT006

REVISION HISTORY

Manual Version:	A
Kit Version:	V1.3
Date:	Aug. 2025
Description:	Initial release

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

WORKFLOW



TOTAL TIME: ~1.5 DAY

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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

Stereo-seq Transcriptomics Set for Large Chip Designs 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 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.

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

1.2. Sequencing Guidelines

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

1.3. List of Kit Components

Each Stereo-seq Transcriptomics Set for Large Chip Designs consists of:

- Stereo-seq Transcriptomics T Kit *1 (2 RXN / 1 RXN / 1 RXN)
- **Two** Stereo-seq Chip T (1cm * 2cm) **OR One** Stereo-seq Chip T **OR One** Stereo-seq Chip T (2cm * 3cm)
- **Two** Stereo-seq Chip Reaction Plate (1cm * 2cm) **OR One** Stereo-seq Chip Reaction Plate (2cm * 2cm) **OR One** Stereo-seq Chip Reaction Plate (2cm * 3cm)

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

Catalog numbers, kit components, and specifications are listed in Table 1-1, Table 1-2, and Table 1-3.

Upon receiving the Stereo-seq Chip T, follow the instructions in Stereo-seq Large Chip Operation Guide for Receiving, Handling and Storing to properly store unused Stereo-seq Chips.

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.: 111KT13002 (2 RXNs) / 111KT13001 (1 RXN) / 111KT13002-CG (2 RXNs) / 111KT13001-CG (1 RXN)			
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (tube)**
RI	1000028499		300 μL × 1
PR Enzyme	1000028500		10 mg × 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
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 Kit Components

Stereo-seq Chip T (1cm * 2cm)	**Cat. No.: 110CT13122 / 110CT13122-CG**
Component	**Quantity (kit)**
Stereo-seq Chip T (1cm * 2cm)	2 EA × 1
Stereo-seq Chip T (2 cm * 2 cm)	**Cat. No.: 110CT131221 / 110CT131221-CG**
Component	**Quantity (kit)**
Stereo-seq Chip T (2cm * 2cm)	1 EA × 1
Stereo-seq Chip T (2 cm * 3 cm)	**Cat. No.: 110CT131231 / 110CT131231-CG**
Component	**Quantity (kit)**
Stereo-seq Chip T (2cm * 3cm)	1 EA × 1
Storage Temperature: 2°C ~ 8°C	Transportation Temperature: -25°C ~ -15°C
Expiration Date: refer to label	

Table 1-3 Stereo-seq Chip Reaction Plates

Stereo-seq Chip Reaction Plate (1cm * 2cm) Cat. No.: 1000059464 / 1000059464-CG	
Component	**Quantity (kit)**
Stereo-seq Chip Reaction Plate (1cm * 2cm)	2 EA × 1
Stereo-seq Chip Reaction Plate (2cm * 2cm) Cat. No.: 1000059153 / 1000059153-CG	
Component	**Quantity (kit)**
Stereo-seq Chip Reaction Plate (2cm * 2cm)	1 EA × 1
Stereo-seq Chip Reaction Plate (2cm * 3cm) Cat. No.: 1000059154 / 1000059154-CG	
Component	**Quantity (kit)**
Stereo-seq Chip Reaction Plate (2cm * 3cm)	1 EA × 1
Storage Temperature: 18°C ~ 25°C	Transportation Temperature: 0°C ~ 30°C
Expiration Date: refer to label	

1.4. Additional Equipment and Materials

Table 1-4 to 1-7 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 **Stereo-seq Imaging Requirements and Guidelines**.

Table 1-4 Additional Equipment and Materials

Equipment		
Brand	**Description**	**Cat. No.**
-	Cryostat	-
-	Fluorescence Microscope with image stitching capability	-
-	Metal Bath (or equivalent instrument)	-
-	Benchtop Centrifuge	-
-	Pipettes	-
-	Vortex Mixer	-
-	Slide Dryer	-
-	Constant Temperature Incubator	-
Thermo Fisher Scientific*	ProFlex™ 3 x 32-well PCR System	4483636
Bio-Rad*	T100™ Thermal Cycler	1861096
Thermo Fisher Scientific	Magnetic Rack: DynaMag™-2 Magnet for 1.5-2 mL Tubes	1861096
Thermo Fisher Scientific	Qubit™ 3 Fluorometer	Q33216 (or similar)
Agilent Technologies™	Agilent 2100 Bioanalyzer	G2939AA (or similar)

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

Table 1-5 Additional Reagent

Reagent		
Brand	**Description**	**Cat. No.**
-	100% Ethanol (Analytical grade)	-
Ambion	Nuclease-free Water	AM9937
	1X TE Buffer, pH 8.0	AM9858
	20X SSC	AM9770

Reagent		
Brand	**Description**	**Cat. No.**
Beckman Coulter*	SPRIselect	B23317/B23318/ B23319
Agencourt*	AMPure® XP DNA Cleanup Beads	A63882
VAZYME*	VAHTS™ DNA Clean Beads	N411-02
Sigma Aldrich	Hydrochloric Acid, HCl	2104-50ML
	Methanol	34860-1L-R
Invitrogen	Qubit ssDNA Assay Kit	Q10212
	Qubit dsDNA HS Assay Kit	Q32854
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

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

Table 1-6 Additional Comsumables

Consumables		
Brand	**Description**	**Cat. No.**
Thermo Fisher Scientific	Nunc™ EasYDish™ (6 cm)	150462
	ABsolute qPCR Seal (sealing tape)	AB-1170
Corning	Costar® Multiple Well Cell Culture Plates (6-well plates)	3516
	Corning® 100 mm TC-treated Culture Dish	353003
	50 mL Centrifuge Tubes	430829
	15 mL Centrifuge Tubes	430791
Kimtech	Kimwipes™ Delicate Task Wipes	34155
JONOSTICK	Forceps	815F-ST
MATIN	Power Dust Remover	M-6318
-	Microscope Glass Coverslip (size: 24 mm x 32 mm, thickness: 0.13-0.16 mm, for 2cm * 3cm chip use)	-

Consumables		
Brand	**Description**	**Cat. No.**
-	Microscope Glass Coverslip (size: 24 mm x 24 mm, thickness: 0.13-0.16 mm, for 1cm * 2cm and 2cm * 2cm chips use)	-
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
	1000 µL Filtered Tips	TF-1000-R-S
	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
Invitrogen	Qubit Assay Tubes	Q32856
-	5 mL Centrifuge Tubes	-
PARAFILM	Sealing Film	PM996
-	Aluminum Foil	-
-	Microscope Slide	-

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

Table 1-7 Reagents and Consumables for H&E Staining Workflow (Optional)

Reagent		
Brand	**Description**	**Cat. No.**
Sangon Biotech	Eosin Y, Free Acid	A600190-0025
Sigma	Hematoxylin Solution (filter before use)	51275
Agilent (or other brands)	Bluing Buffer	CS702
Consumables		
-	Disposable Sterile Syringe	-
Millipore (or other brands)	Millex Needle Filter	SLGV033N

1.5. Stereo-seq Large Chip Information

Stereo-seq Large Chip T

Each Stereo-seq Transcriptomics Set V1.3 for Large Chip Designs contains one Stereo-seq Chip T Kit. The Stereo-seq Chip T Kit (1cm * 2cm) contains two Stereo-seq Chip T. The Stereo-seq Chip T Kit (2cm * 2cm or 2cm * 3cm) each contains one Stereo-seq Chip T.

Stereo-seq Large Chip T Storage

- Stereo-seq large chips are packaged in vacuum-sealed aluminum foil bags and transported under cold-chain conditions. Upon receipt, promptly check the integrity of the aluminum foil bag and ensure it remains vacuum-sealed.
- Always store unopened Stereo-seq Chips T/P in their original chip container and keep them sealed in a sealable aluminum foil bag at 2°C ~ 8°C. Keep sealed with tape or another re-sealable bag. Always KEEP the desiccant in the bag.
- If the bag is opened but the chip is not used, refer to the re-packaging instructions to properly place the chip back into the chip box. Re-seal the bag with a desiccant and tape or another sealable method.
- Chips that are not vacuum-sealed should not be stored for more than two weeks. It is recommended to use them as soon as possible after opening.

1.6. Precautions and Warnings

- 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

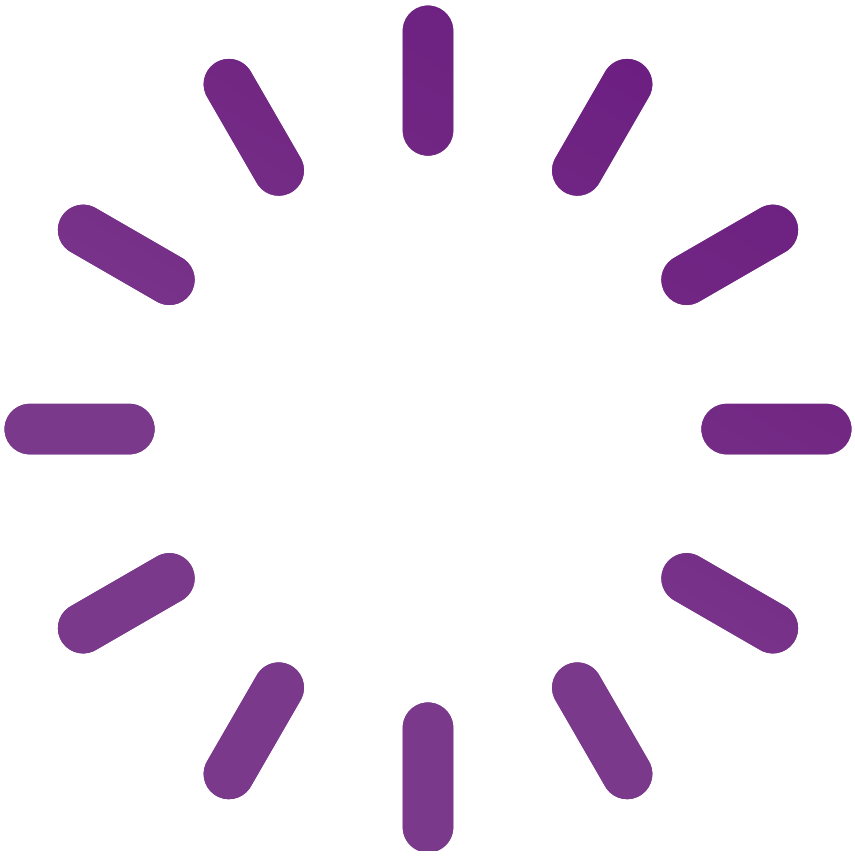
For frozen sample embeddings, refer to the **Sample Preparation Guide for Fresh Frozen Samples on Stereo-seq Chips or Stereo-seq Chip Slides (Document No.: STUM-SP001)**.

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 ≥6.0.

CHAPTER 3

Stereo-seq TRANSCRIPTOMICS SET FOR LARGE CHIP (≤ 2cm * 3cm) 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	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T	Maintenance
5X SSC	Dilute 1 mL of 20X SSC to 4 mL.			Room temperature
0.1X SSC	Dilute 250 µL of 20X SSC to 50 mL.			Room temperature
Methanol	Add 3-4 mL/chip to the 6-well cell culture plates	Add 3-4 mL/chip to the 6-well cell culture plates	Add 4-5 mL/ chip to the 6 cm culture dish	-20°C
Pre cool methanol at -20 °C for 5-30 min before use.				
RI	Take it out and keep on ice at -20°C.			On ice until use
Wash Buffer	Prepare at least 550 µL per chip (522.5 µL 0.1X SSC with 27.5 µL RI).	Prepare at least 900 µL per chip (855 µL 0.1X SSC with 45 µL RI).	Prepare at least 1500 µL per chip (1425 µL 0.1X SSC with 75 µL RI).	On ice until use
Glycerol	Equilibrate to room temperature at least 5 min prior to use			Room temperature
0.01N HCl	Prepare at least 1805 µL of 0.01N HCl per chip.	Prepare at least 2185 µL of 0.01N HCl per chip.	Prepare at least 2565 µL of 0.01N HCl per chip.	Room temperature for 48 hr
Measure and make sure the pH = 2. 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. Storing longer than 48 hr will affect the desired pH. Use within 48 hr of preparation.				
10X Permeabilization Reagent (PR) Stock Solution	Briefly centrifuge the PR Enzyme (red cap, in powder form) for 2-3 sec. Add 1 mL of freshly prepared 0.01N HCl 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 PR stock solution to avoid freeze-thaw cycles and keep it at -20°C for long-term storage.				

Reagent	1cm * 2cm Chip T	2cm * 2cm Chip T	2cm * 3cm Chip T	Maintenance
1X Permeabilization Reagent (PR) Solution	Dilute 45 µL of 10X PR stock solution to 450 µL with 0.01N HCl. (Prepare at least 400 µL per chip.)	Dilute 65 µL of 10X PR stock solution to 650 µL with 0.01N HCl. (Prepare at least 600 µL per chip.)	Dilute 85 µL of 10X PR stock solution to 850 µL with 0.01N HCl. (Prepare at least 800 µL per chip.)	On ice until use, up to 6 hr
0.1M KOH	Dilute 10 µL of 8M KOH to 800 µL.	Dilute 10 µL of 8M KOH to 800 µL.	Dilute 11 µL of 8M KOH to 880 µL.	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.				
Elute Additive	Thaw on ice in advance. Prepare 15 µL per chip.	Thaw on ice in advance. Prepare 20 µL per chip.	Thaw on ice in advance. Prepare 22.5 µL per chip.	On ice until use
80% Ethanol	Dilute 100% ethanol to 80%			Room temperature up to 1 day
Neutralization Solution	Equilibrate to room temperature at least 5 min prior to use			Room temperature
Magnetic Beads	Equilibrate to room temperature at least 30 min prior to use.			Room temperature up to 6 hr
Eosin Solution	Dissolve 0.026g Eosin Y powder in 50 mL methanol and keep sealed with sealing film.			Room temperature up to 1 month
Hematoxylin (filtered)	Prepare and filter the Hematoxylin Solution using a 0.22 µm pore-sized filter (a needle cartridge filter and a disposable sterile syringe) and seal it with sealing film until use			Room temperature in the dark up to 7 days
H&E Mounting Medium	Equilibrate to room temperature 5 min in advance			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.

Table 3-2 Equipment Preparation

Other Preparation		
Equipment	**Preparation Steps**	**Notes**
Cryostat	Set the cryostat chamber temperature to -20°C and the specimen disc temperature (object temperature) to -15°C ~ -10°C	The specimen disc temperature depends on the tissue type.
Fluorescence Microscope	Set the epifluorescence channel to FITC mode for imaging fluorescent-stained tissue and BF-Epi channel for imaging H&E-stained tissue.	Check the microscope for any abnormalities and replace it if necessary.
Slide Dryer	37°C for chip drying	Check the slide dryer for any abnormalities and replace it if necessary.
Metal Bath (Instruments of equivalent functionality)	37°C for preheating 1X Permeabilization Reagent Solution.	Check the instrument for any abnormalities and replace it if necessary.
Constant Temperature Incubator	1. 37°C for Permeabilization 2. 45°C for Reverse Transcription 3. 55°C for cDNA Release	Check the incubator for any abnormalities and replace it if necessary.
PCR Thermal Cycler	95°C for denaturation (heated lid at 105°C).	Check the PCR Thermal Cycler for any abnormalities and replace it if necessary.

Table 3-3 Reaction Container Overview

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
Chip Placement	10 cm culture dish (with sealing film)	10 cm culture dish (with sealing film)	10 cm culture dish (with sealing film)
Chip Cleaning	6-well cell culture plates	6-well cell culture plates	6 cm culture dish
Methanol Fixation	6-well cell culture plates	6-well cell culture plates	6 cm culture dish
ssDNA Staining	10 cm culture dish (with sealing film)	10 cm culture dish (with sealing film)	10 cm culture dish (with sealing film)
Glycerol Cleaning	6-well cell culture plates	6-well cell culture plates	6 cm culture dish
Permeabilization	Stereo-seq Chip Reaction Plate (1cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 3cm)

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
Reverse Transcription	Stereo-seq Chip Reaction Plate (1cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 3cm)
cDNA Release	Stereo-seq Chip Reaction Plate (1cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 3cm)

Table 3-4 Reaction Container Overview for H&E Staining Workflow (Optional)

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
Chip Placement	10 cm culture dish (with sealing film)	10 cm culture dish (with sealing film)	10 cm culture dish (with sealing film)
Chip Cleaning	6-well cell culture plates	6-well cell culture plates	6 cm culture dish
Methanol Fixation	6-well cell culture plates	6-well cell culture plates	6 cm culture dish
Eosin Staining	6-well cell culture plates	6-well cell culture plates	6 cm culture dish
Hematoxylin Staining and Cleaning	Stereo-seq Chip Reaction Plate (1cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 3cm)
Permeabilization	Stereo-seq Chip Reaction Plate (1cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 3cm)
Reverse Transcription	Stereo-seq Chip Reaction Plate (1cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 3cm)
cDNA Release	Stereo-seq Chip Reaction Plate (1cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 2cm)	Stereo-seq Chip Reaction Plate (2cm * 3cm)

3.2. Cryosection Preparation

a. Set the Slide Dryer temperature to 37°C.

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

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

Due to variations in tissue size, equilibration times may differ. The benchmark is achieving smooth, continuous slices without chatter or section artifacts during sectioning. For reference, a 2 cm × 3 cm × 0.7 cm tissue is equilibrated for 1 hr.

e. Trim the embedded tissue block to the appropriate size (sectioning area smaller than 0.9 cm x 1.8 cm for a 1cm * 2cm chip, sectioning area smaller than 1.8 cm x 1.8 cm for a 2cm * 2cm chip, and sectioning area smaller than 1.8 cm x 2.7 cm for a 2cm * 3cm chip). Trim off the excess OCT surrounding the tissue block while retaining a portion of it to facilitate tissue transfer.

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. The specimen is ready for cryosection.

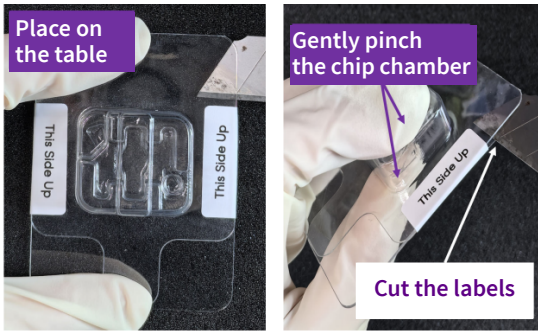
3.3. Chip Preparation and Tissue Mounting

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

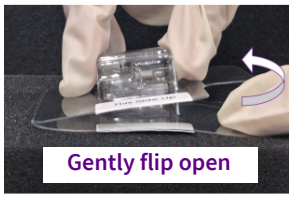
Ensure that the Chip ID on the back of the chip corresponds to the one on the packaging, and accurately document the Chip ID (SN). The information is crucial for image quality control and bioinformatic analysis. Please record the correct ID before you continue.

1) Remove the chip out of the vacuum-sealed aluminum bag, position the chip container on the table with the "This Side Up" labels facing upwards.

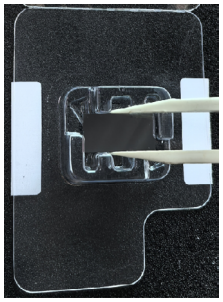
2) Slice the labels with razor blades along the opening gap of the container on each side (when handling the chip container, carefully pinch the chip chamber to prevent the chip from shifting).



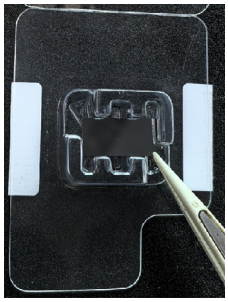
3) Hold the exposed section of the bottom cover on the table, then carefully lift the upper cover open.



4) Grip the middle of the chip's long edge using blunt-end forceps to transfer the chip out of the container.
Or use blunt-end forceps to grip the lower-right corner of the chip, maintaining a distance of approximately **2-3 mm** both in length and width.



or

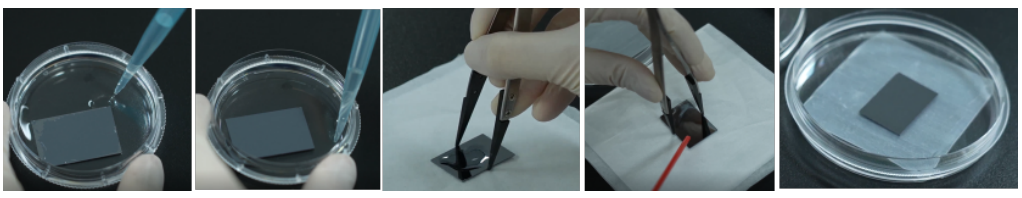


Refer to this step for the proper handling of Stereo-seq Large Chips throughout the experiment.

The front of the chip has a shiny surface that contains probes for mRNA capture. DO NOT scratch the surface.

Seal the unused chips following the Stereo-seq Large Chip Operation Guide For Receiving, Handling and Storing. KEEP the desiccant in the aluminum bag. Store at 2°C ~ 8°C for up to two weeks.

b. Place the chip in a 10 cm culture dish (with a sealing film on the bottom). Equilibrate the chip at room temperature for **1 min** and look for impurities on the chip surface. If there are impurities on the chip, clean the 1cm * 2cm and 2cm * 2cm chips **twice** with **3000 µL** nuclease-free water in a 6-well cell culture plate (clean the 2cm * 3cm chip twice with **4000 µL** nuclease-free water in a 6 cm culture dish). After cleaning, gently blow off excess water from the chip with a power dust remover. When the chip is completely dry and void of wavy white stains, it is ready for tissue mounting.



Retain a small volume of liquid in the dish before removing the chip to facilitate handling.

c. Pre-cool the methanol: For 1cm * 2cm and 2cm * 2cm chips, add **3-4 mL** methanol to the 6-well cell culture plates. For 2 cm * 3 cm chips, add **4-5 mL** methanol to the 6 cm culture dish to ensure that the methanol completely covers the chip. Pre-cool the methanol for **5-30 min** at -20°C.

H&E application only - Eosin solution preparation: For 1cm * 2cm and 2cm * 2cm chips, add 3-4 mL eosin solution to the 6-well cell culture plates. For 2cm * 3cm chips, add 4-5 mL eosin solution to the 6 cm culture dish to ensure that the eosin solution can completely cover the chip. Pre-cool the eosin solution for 5-30 min at -20°C.

d. Anchor the sample chuck with tissue block onto the chuck holder.

e. Tissue mounting can be achieved via either the warm method (option A) or the cold method (option B). Select the appropriate section thickness according to the experiment needs; a section thickness of 10 μm is normally used.

A. Warm Method

1) Perform cryosection and carefully flatten the tissue sections out by gently touching the surrounding OCT with cryostat brushes. Move the tissue sections to the right side near the edge.

2) Hold a corner of the chip with a pointed forceps held in a reverse grip to position the chip face down and aim for the tissue section.

3) Gently touch the section with the front of the chip.

It is recommended that you perform tissue mounting within 1 min after sectioning.

4) Flip the Stereo-seq Chip over with the front facing up, and immediately dry it in the Slide Dryer at 37°C according to the drying times shown in Table 3-5.

Table 3-5 Drying Time for Different Chip Sizes

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
Drying time	8 min	8 min	10 min

B. Cold Method

1) Place the Stereo-seq Chip inside the cryostat chamber with the front facing up and pre-cool the chip inside the chamber for **3~10 min**.

Prolonged cooling may cause mist formation on the chip surface. An insufficient cooling time may cause the chip to fail to reach the expected temperature.

2) Perform cryosection, then carefully flatten the tissue section out by gently touching the surrounding OCT with cryostat brushes. Carefully place a tissue section onto the chip center using forceps and brushes.

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

It is recommended that you perform tissue mounting within 5 min.

4) Immediately dry it in the Slide Dryer at 37°C according to the drying time in Table 3-5.

When using the cold method for tissue mounting, it is necessary to control the time of each section. If the interval is too long, the tissue section will shrink.

Optional Stop Point:

- **After drying the tissue containing Stereo-seq Chip, transfer the Stereo-seq Chip into a container (6-well cell culture plates or 6 cm culture dish), seal it with sealing film, then place it in a sealable bag. Place one desiccant pack per Stereo-seq Chip into the sealable bag, push out as much air as possible and seal the bag tightly. The sealed Stereo-seq Chip can be transferred to a -80°C freezer on dry ice. Store the sealed plastic bag containing Stereo-seq Chip with tissue at -80°C for up to 1 month.**
- **When continuing the experiment, transfer out the chip container on dry ice, take out the Stereo-seq Chip with tissue then immediately incubate in the Slide Dryer at 37°C according to the drying time in Table 3-5.**

3.4. Tissue Fixation

For tissue samples that are intended for the Stereo-seq Transcriptomics H&E workflow, the tissue fixation & eosin staining (at -20°C), hematoxylin solution staining and bluing, coverslip mounting, and imaging steps need to be completed prior to Tissue Permeabilization. Skip sections 3.4 to 3.6 and refer to Appendix I: H&E Staining Operating Procedure for detailed procedures.

a. After drying the tissue-mounted Stereo-seq Chip, immediately immerse it in pre-cooled methanol for a **40-min** fixation at -20°C (for tissues that may detach easily, the fixation time can be extended, but **do not exceed 1 hr**). When immersing the Stereo-seq Chip in methanol, ensure that all tissue sections are completely submerged.

During fixation, refer to table 3-6 in 3.5. Fluorescent staining. Prepare the tissue fluorescent staining solution in advance, and store it at room temperature in the dark for later use.

b. After fixation is completed, transfer the 6-well cell culture plates/6 cm culture dish to a sterile fume hood.

c. Take out the Stereo-seq Chip and transfer it to a dust-free paper, and wipe off excess methanol from around the edges and the back of the chip with dust-free paper without touching the chips surface.

d. Place the chip in a 10 cm culture dish with sealing film, and leave it in the fume hood for **4-6 min** to allow the methanol to evaporate completely.

e. When the methanol is completely evaporated, the tissue turns white and visible to the naked eye. Transfer the Stereo-seq Chip onto a flat and clean bench.

3.5. Fluorescent Staining

a. Prepare tissue ssDNA fluorescent staining solution according to Table 3-6, and store it at room temperature in the dark for later use.

Table 3-6 Tissue Fluorescent Staining Solution

Volume per Component (μL)	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
5X SSC	189	378	756
Qubit ssDNA Reagent	1	2	4
RI	10	20	40
Total	200	400	800

b. Add the tissue fluorescent staining solution 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. The volumes are listed in Table 3-6. Ensure that the chip is completely covered by tissue fluorescent staining solution. Incubate for **5 min** at room temperature in the dark.

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

c. Prepare Wash Buffer during staining, place it on ice for later use; the Wash Buffer volumes are listed in Table 3-7.

d. Prepare 0.01N HCl and 1X PR Solution in advance according to Table 3-11 in 3.7 Tissue Permeabilization.

e. Set the temperature of a metal bath or equivalent heating instrument to 37°C. Warm the aliquoted 1X PR Solution in the metal bath or equivalent heating instrument for **10 min (no longer than 30 min)**.

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

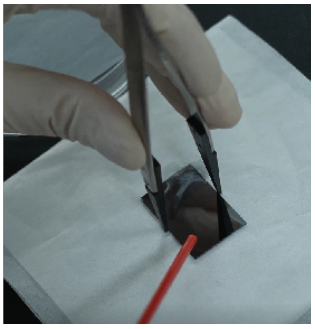
g. Add Wash Buffer; the volumes are listed in Table 3-7.

Table 3-7 Cleaning Volume for Different Chip Sizes

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
Wash Buffer	150 μL/chip	300 μL/chip	700 μL/chip

h. Slightly tilt the Stereo-seq Chip and gently remove Wash Buffer from the corner of the chip using a pipette. Try to remove as much liquid as possible.

i. Transfer the Stereo-seq Chip onto dust-free paper. Hold forceps with one hand to fix the chip 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 corner of the chip at a 30-degree angle horizontal to the plane of the chip, avoiding excessive airflow.

Ensure that there is no liquid residue around the chips.

j. Apply **1-2 μL** water to a glass slide, then mount the chip onto the droplet to anchor it.

Before glycerol addition, prepare a 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.

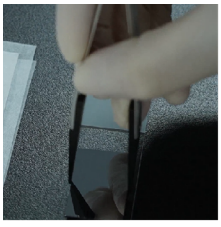
k. Before using the glycerol, centrifuge the glycerol to remove any bubbles. Using a pipette, carefully add glycerol to the center of the tissue on the chip without introducing air bubbles. A few drops can be added evenly to the surface of the chip; the volumes are listed in Table 3-8.

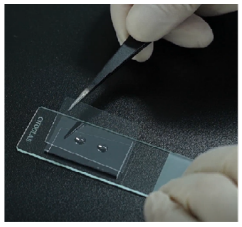
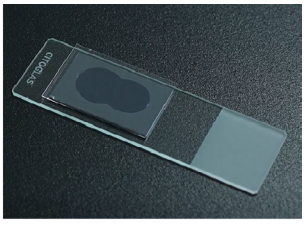
If the sample is large, more glycerol can be added as needed. Glycerol should not be dispensed into too many drops, as this can easily lead to incomplete coverage and bubbles. Ensure that the glycerol fully covers the chip without the formation of bubbles.

Table 3-8 Glycerol Volume for Different Chip Sizes

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
Glycerol	10-15 μL/chip	20-28 μL/chip	30-40 μL/chip

l. 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 chip. 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

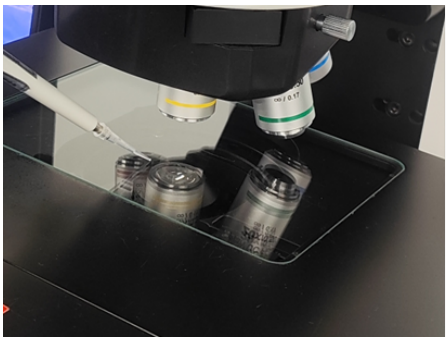
During the imaging process, ensure that the tracklines on the chip and the tissue area are both clear and within focus, and ensure that the tissue area is not overexposed.

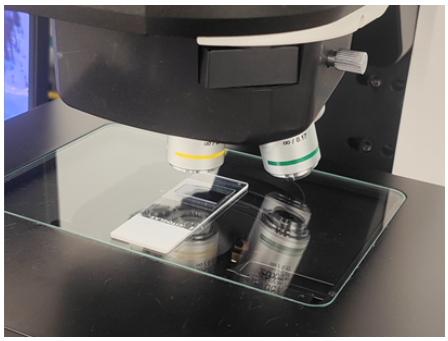
a. Create a new folder in the microscope imaging software, and name it with the chip ID number and other essential information.

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

Example chip ID number: C05479C3D3

b. Fix the slide with the slide clamp on the microscope. If there is no slide clamp, place **1-2 μL** of water on the imaging platform first, then transfer and place the chip onto the water droplet. Water surface tension will grab onto the chip and adhere it onto the imaging platform, as shown in the following figure.

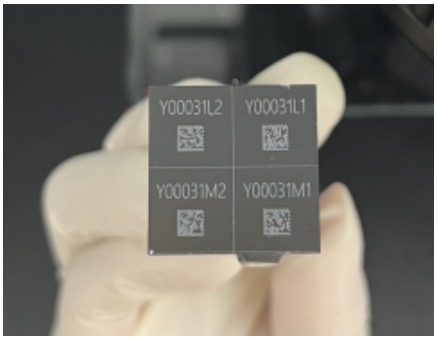




c. Place the slide gently on the stage, and try to keep the chip parallel to the stage.

The tilting angle relative to the stage edge should not exceed 15°.

d. When placing the chip, ensure that the orientation is correct, with the chip number (SN) on the back located at the top and the QR code at the bottom, as shown in the figure below:



Chip placement diagram (back view): The chip number is at the top, and the QR code is at the bottom.

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

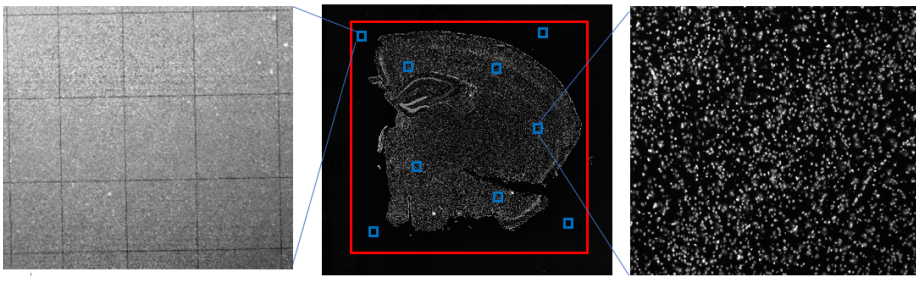
f. **Determine the tissue location:** Select the 4X objective lens, move the FOV to the tissue area on the chip, adjust Brightness, Gain, and Exposure, then use the focusing knob to adjust the focus until the tissue and cell boundaries are clear and within focus (the light intensity should be kept low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

g. **Scan the map:** Box-select the chip area to fully enclose the chip boundaries (the area 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.)

h. **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.

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

j. **Manual focusing method (skip this step if the microscope you are using supports autofocusing only):** Move the FOV to the non-tissue area on the chip and then, if necessary, 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.



k. **Final imaging:** After modeling, adjust the gain to the minimum. Perform a full scan using the 10X objective lens, then save the original tile (FOV) image files and stitched images.

l. 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.

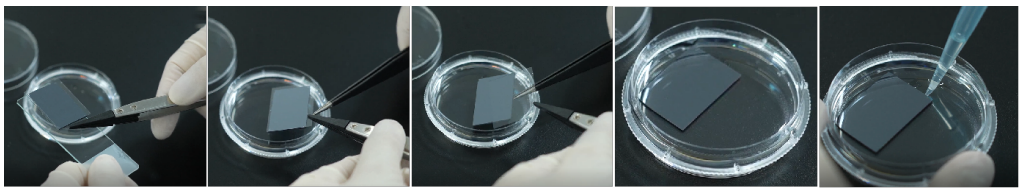
For comprehensive technical specifications and operational precautions, refer to the Stereo-seq Imaging Requirements and Guidelines.

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

If Image QC fails, carefully confirm the image clarity and retake the photos, ensuring that both tissue and tracklines are clear. If the second Image QC fails, continue with the experiment and perform optimal image analysis later under the guidance of your local Field Application Scientist, or perform manual image processing in StereoMap.

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.

m. After imaging, clamp the coverslip at a corner with forceps, transfer the chip and let it lean against the edge of the well plates or culture dish. Gently slide the coverslip parallel with forceps until the chip and the coverslip are completely separated.



n. Place 1cm * 2cm and 2cm * 2cm chips in a 6-well plate, and place 2cm * 3cm chips in a 6 cm culture dish. Add 0.1X SSC; the volumes are listed in Table 3-9. Pipette 0.1X SSC up and down **5 times** (wash it 3 times from left to right along the long edge of the chip, and wash it 2 times on the short edge of the chip).

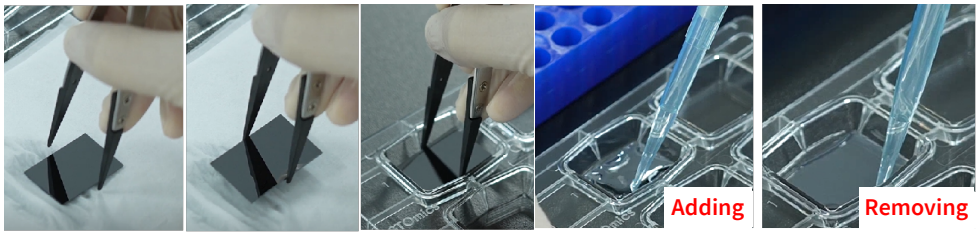
o. Aspirate the 0.1X SSC from the 6-well plate or the culture dish, then add 0.1X SSC (the volumes are listed in Table 3-9) and incubate for **3-5 sec**.

Table 3-9 0.1X SSC Volume for Different Chip Sizes after Glycerol Mounting

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
0.1X SSC	1500 μL/chip	2000 μL/chip	3000 μL/chip

Ensure that the chip is completely immersed in the solution.

p. Take out the chip and place it on dust-free paper to absorb the liquid from the back. Subsequently, place the chip into a Chip Reaction Plate of the corresponding size.



q. Slowly add 0.01N HCl solution to the chip; the volumes are listed in Table 3-10. After the liquid covers the entire chip, aspirate the liquid from the gap at the center of the long edge of the reaction well.

Table 3-10 0.01N HCl Volume for Different Chip Sizes

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
0.01N HCl	400 μL/chip	600 μL/chip	800 μL/chip

3.7. Tissue Permeabilization

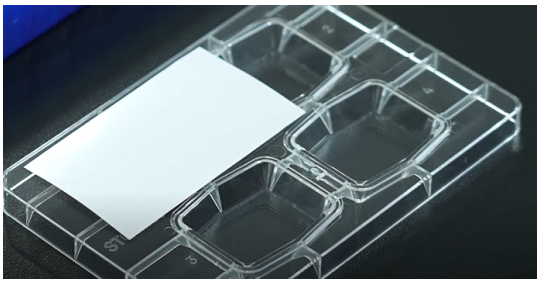
a. Thaw RT Buffer Mix, RT Plus, and RT Oligo in advance and thaw them at room temperature. After thawing, place them on ice for later use.

Table 3-11 1X PR Solution Volume for Different Chip Sizes

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
1X PR Solution	400 μL/chip	600 μL/chip	800 μL/chip

b. Using the 1X PR Solution that has completed incubation as described in **step e. of 3.5 Fluorescent Staining**, add the solution onto the chip (volumes in Table 3-11) by pipetting one droplet at each corner first, then adding the rest to the center to merge all droplets.

c. Place the **unpeeled sealing tape** on the Chip Reaction Plate to cover the reaction well, and incubate at 37°C.



Optimal permeabilization time is determined by the Stereo-seq Permeabilization Kit. Refer to the Stereo-seq Permeabilization Set for Large Chip Designs (≤ 2cm * 3cm) User Manual for more information.

d. While waiting for permeabilization to be completed, prepare the RT Mix according to Table 3-13, then leave it on ice until use.

e. When incubation is completed, remove the Stereo-seq Chip Reaction Plate from the 37°C incubator.

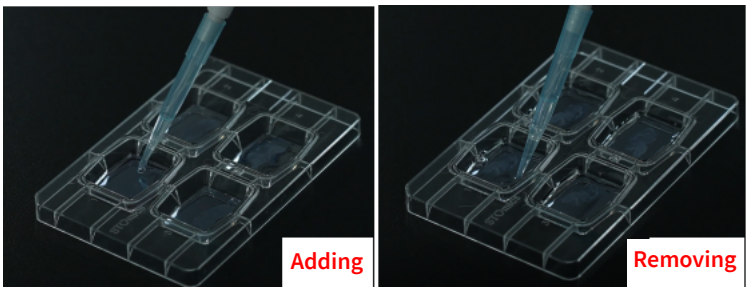
f. Aspirate the 1X PR Solution from the gap at the center of the long edge of the reaction well; do not touch the chip surface.

g. Add Wash Buffer to the chip; the volumes are listed in Table 3-12.

Table 3-12 Wash Buffer Volume for Different Chip Sizes

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
Wash Buffer	400 μL/chip	600 μL/chip	800 μL/chip

h. Aspirate the liquid from the gap at the center of the long edge of the reaction well. Keep the chip surface moist.



⚠ **Do not dry the chip completely.**

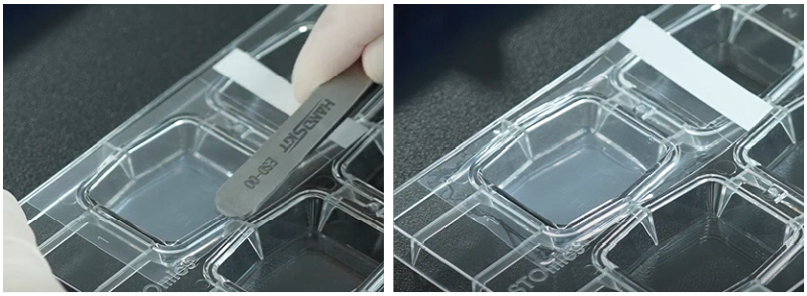
3.8. Reverse Transcription

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

Table 3-13 RT Mix

Volume per Component (μL)	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
RT Buffer Mix	373.5	539.5	705.5
RT Plus	9	13	17
RT Oligo	22.5	32.5	42.5
RT Enzyme Mix	45	65	85
Total	450	650	850
Volume	400	600	800

b. Apply sealing tape to the Stereo-seq Chip Reaction Plate and seal it tightly. Press the heighted edge firmly to prevent liquid evaporation. Incubate the Stereo-seq Chip Reaction Plate at 45°C for **4 hr** or longer (no longer than **16 hr**).



⚠ **The sealing tape seals the plate to prevent the evaporation of the reaction liquid!**

3.9. cDNA Release and Denaturation

a. **Five minutes (5 min)** before Reverse Transcription is completed, prepare the cDNA Release Mix according to Table 3-14 (an additional 150μL should be prepared to facilitate the subsequent addition of the recovery solution), and then place the mix at room temperature.

Table 3-14 cDNA Release Mix Volume for Different Chip Sizes

Volume per Component (μL)	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
0.1M KOH	585	780	877.5
Elute Additive	15	20	22.5
Total	600	800	900
Volume	450	650	750

b. When Reverse Transcription is completed, remove the Stereo-seq Chip Reaction Plate from the 45°C incubator. Carefully remove the sealing tape and aspirate the RT Mix from the gap at the center of the long edge of the reaction well.

c. Add 0.1X SSC to the chip and evenly cover the entire chip; the volumes are listed in Table 3-15.

Table 3-15 0.1X SSC Volume for Different Chip Sizes after Reverse Transcription

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
0.1X SSC	400 μL/chip	600 μL/chip	800 μL/chip

d. Do not discard the 0.1X SSC. Use forceps to transfer the chip to dust-free paper and wipe off any residual liquid that may be on the back of the chip and around the chip.

e. **Transfer the chip to a new reaction well**, and add cDNA Release Mix; the volumes are listed in Table 3-14. Seal the Chip Reaction Plate with sealing tape to prevent evaporation. Place the Chip Reaction Plate in the incubator at 55°C for **20 min in total** and **pipette 20 times every 5 minutes**.

1) After **5 min** of incubation, pause the timer (there is no need to record the time when pipetting the chip).

2) Take the Chip Reaction Plate out of the incubator and place it on the laboratory bench.

3) Carefully remove the sealing tape and pipette up and down 20 times on the surface of the chip (4 times in the center of the tissue and at the four corners, respectively). For a 1cm * 2cm chip, it is recommended to use a 200 μL pipette tip for pipetting, and for other size chips, use a 200 μL or 1000 μL pipette tip for pipetting. Note that there should be no liquid remaining on the pipette tip after pipetting.

Carefully remove the sealing film to prevent the liquid from splashing out. Take care when pipetting to avoid displacing liquid and causing liquid loss.

4) Seal the reaction well again with the sealing tape and place it in the incubator set at 55°C for another 5 minutes of incubation.

5) Repeat **steps 1) - 4) 3 more times** until the 20-minute reaction is completed.

f. When incubation is completed, position the Chip Reaction Plate on the laboratory bench and carefully peel the sealing tape off. Remove the tissue from the chip by pipetting up and down. 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.

g. Completely collect the cDNA Release Mix from each well into a new 1.5-mL tube. Slightly tilt the Chip Reaction Plate to collect all liquid from the reaction wells.

h. Measure the aspirated liquid volume using a pipette. If the collected volume is less than the target volume (see Table 3-16), calculate the **top-up volume X (μL)** by subtracting the **collected volume (μL)** from the **loaded volume of cDNA Release Mix (μL)** specified in Table 3-14. Subsequently, add the **calculated top-up volume X (μL)** of cDNA Release Mix to the chip for rinsing. Collect the top-up solution to reach the target volume.

i. Use a pipette to measure the volume of collected solution. Add Neutralization Solution according to **collected volume:Neutralization Solution = 180:23**, and then vortex and mix well.(Refer to Table 3-16 for the target volume of cDNA Release Mix and Neutralization Solution volume)

Table 3-16 Expected Number of PCR Tubes

Volume per Component (μL)	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
The target volume of cDNA Release Mix	370	570	670
Neutralization Solution	47.3	72.8	85.6
Expected number of PCR tubes	6 tubes	10 tubes	12 tubes

j. Aliquot the collected cDNA into PCR tubes, **66 μL** in each tube. For the expected number of PCR tubes, refer to Table 3-16.

If the volume of the final tube is less than 66 μL but greater than 20 μL, it can be directly topped up with nuclease-free water after the 5-minute incubation at 95°C. If it is less than 20 μL, it can be evenly distributed into each tube.

k. Place the tubes into the PCR thermal cycler and incubate at 95°C for **5 min** using the following incubation program.

Table 3-17 PCR Incubation Program

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

Ensure that all of the chip ID numbers have been recorded as required for downstream analysis.

Please place the released chip in the Chip Reaction Plate, seal it with the sealing tape, and store it in a 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 3-18. Pipette up and down to mix, then centrifuge briefly. Store it on ice until use.

Table 3-18 PCR Mix

Component	1X (μL)	6X + 10% (μL)	10X + 10% (μL)	12X + 10% (μL)
4X cDNA PCR Mix	25.5	168.3	280.5	336.6
cDNA Primer	4	26.4	44	52.8
Nuclease-free Water	4.5	29.7	49.5	59.4
Total	34	224.4	374	448.8

b. After 95°C incubation, aliquot **34 μL** of PCR Mix into each PCR tube with **66 μL** cDNA products that you prepared in **step k.** in 3.9 cDNA Release and Denaturation, then vortex and mix well.

c. Briefly spin the PCR tubes, then proceed to cDNA Amplification based on the PCR program in Table 3-19.

Table 3-19 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	∞	-

Stop Point: 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.

d. Prepare Qubit dsDNA Mix in a PCR tube according to Table 3-20.

Table 3-20 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 (from the Qubit dsDNA HS Assay Kit).

f. Combine PCR products of the same cDNA into the corresponding centrifuge tubes (about **600 μL** for a 1cm * 2cm chip, combine into a 1.5 mL centrifuge tube; about **1000 μL** for a 2cm * 2cm chip, combine into a 5 mL centrifuge tube; about **1200 μL** for a 2cm * 3cm chip, combine into a 5 mL centrifuge tube).

g. Take **1 μL** PCR product and add it into the detection tube with **199 μL** Qubit dsDNA Mix. Vortex, mix, and centrifuge briefly. Measure the concentration of the PCR product using the Qubit dsDNA HS Assay Kit.

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

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

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

b. Proceed to 3.11 cDNA Purification and perform the 1X magnetic bead purification in **steps b-1) to b-5)**. Specifically, for 1cm * 2cm chip PCR products, mix into one tube and then purify. For a 2cm * 2cm or 2cm * 3cm chip, mix first, and then divide into two tubes for magnetic bead purification.

c. Resuspend the dried beads in **72 μL** of TE Buffer in each tube (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 the incubation protocol in **step c.** in 3.10 cDNA Amplification, 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

Recommended Magnetic Beads

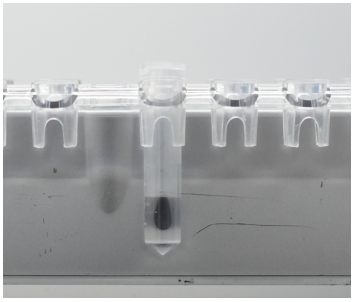
For bead-based purification, we recommend using DNA Cleanup Beads AMPure® XP (Beckman Coulter, Cat. No.: A63882), SPRIselect (Beckman Coulter, Cat. No.: B23317/B23318/B23319), or VAHTS™ DNA Clean Beads (VAZYME, Cat. No.: N411-02). *If magnetic beads from other sources are used, please optimize the cleanup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

Before Using the Beads

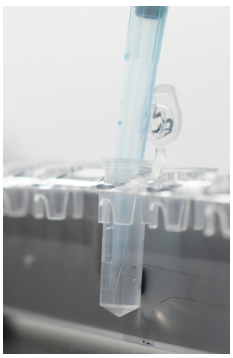
- To ensure the DNA capture efficiency of the magnetic beads, equilibrate the beads to room temperature **30 min before use**.
- Vortex or pipette up and down to ensure that the beads are thoroughly mixed every time before use.
- The number of magnetic beads directly affects the distribution of purified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

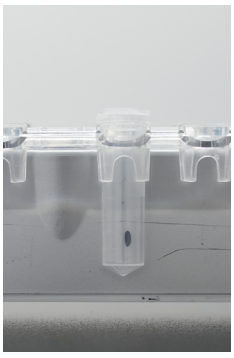
Operation Notes

- 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.

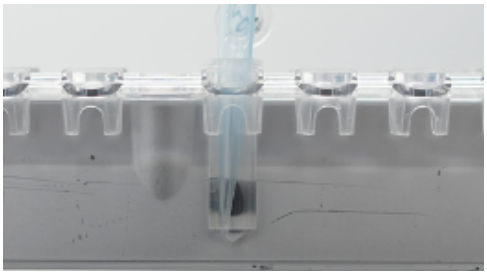


- When collecting the supernatant after magnetic separation, avoid taking up the beads in the pipette. Instead of collecting the entire supernatant fraction, leave **2-3 µL** in the tube to prevent the pipette from directly contacting the beads. If the beads are accidentally taken up, dispense everything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

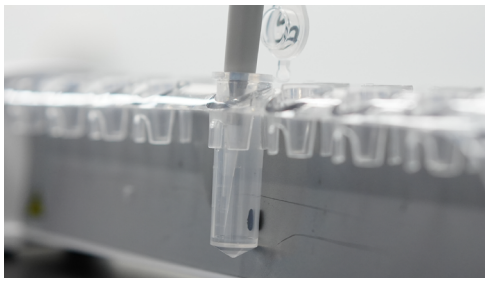




- Use freshly prepared 80% ethanol (at room temperature) to wash the beads. Keep the sample tube on the magnetic separation rack during the washing step. Do not shake or disturb the beads.

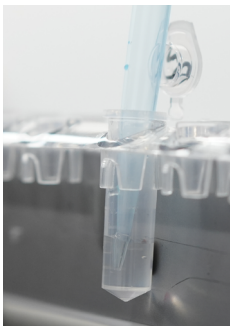


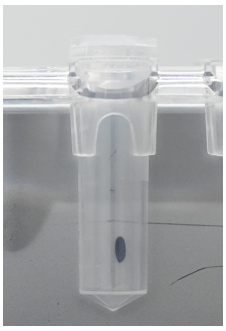
- After the second washing of beads with ethanol, try to remove all of the liquid in the tube. You may centrifuge briefly to accumulate any remaining liquid at the bottom of the tube, then separate the beads magnetically, and remove the remaining liquid by using a small-volume pipette.



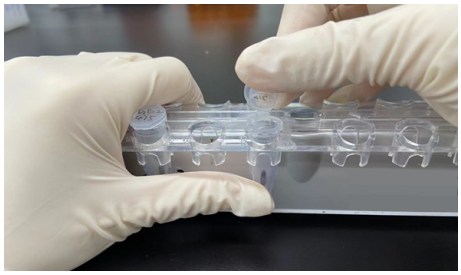
- After washing twice with ethanol, air-dry the beads at room temperature. Drying usually takes approximately **5-10 min**, depending on the lab temperature and humidity level. Watch closely until the pellet appears sufficiently dry with a matte appearance, then continue to the elution step with TE Buffer.

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

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

1) Mix the tubes of PCR products of the same cDNA in a 1.5 mL or a 5 mL centrifuge tube (about **600 μL** for a 1cm * 2cm chip, about **1000 μL** for a 2cm * 2cm chip, and about **1200 μL** for a 2cm * 3cm chip), then mix the combined PCR products with the magnetic beads balanced at room temperature in a ratio of **1:0.8**. 2cm * 2cm and 2cm * 3cm are mixed evenly and then aliquoted into two 1.5 mL centrifuge tubes. Vortex the mixture, then incubate it at room temperature for **10 min**.

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

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

4) Keep the tube on the magnetic rack and add **1-2 mL** 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.

5) Repeat **step 4)**.

6) 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**.

7) 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 (for 2cm * 2cm or 2cm * 3cm chip, mix the supernatant of the two tubes together, which will be approximately 196 μL).

Stop Point: **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 is expected to have fragments primarily distributed within the 200 - 2000 bp range (Figure 1) and a yield that is greater than 100 ng.

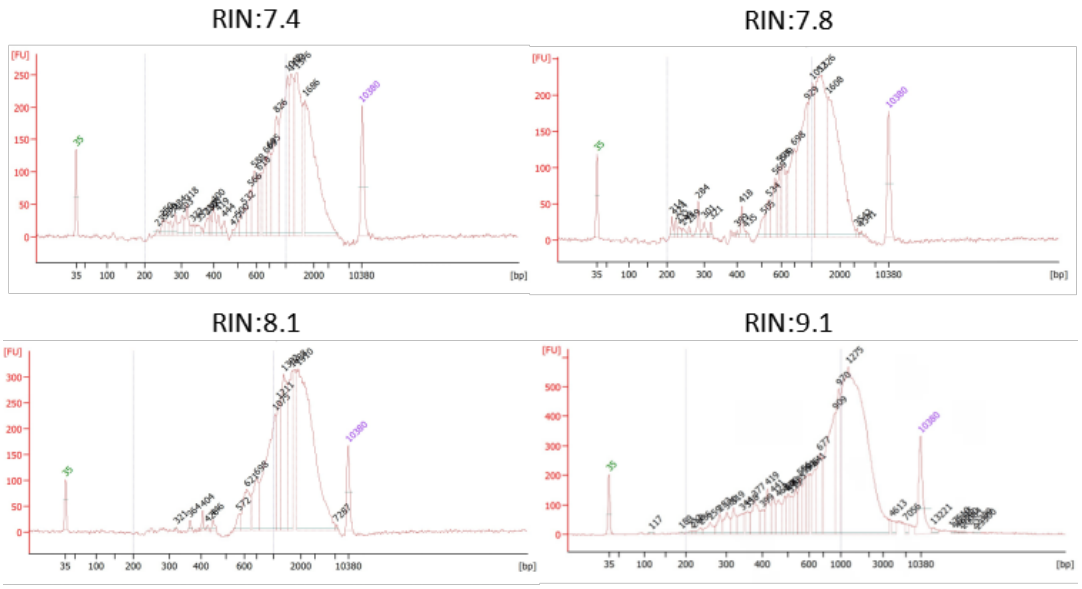


Figure 1. Representative Agilent Bioanalyzer 2100 analysis results of PCR-amplified cDNA samples with different RIN values

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

Appendix I: H&E Staining Operating Procedure

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

a. After drying the tissue-mounted Stereo-seq Chip, immediately immerse it in pre-cooled methanol for a **40-min** fixation at -20°C (for tissues that may detach easily, the fixation time can be extended, but **do not exceed 1 hr**). Ensure that all tissue sections are completely submerged.

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

The staining duration should be adjusted to achieve uniform coloring of the tissue and should be controlled within a range of 3-5min.

It is important to maintain a consistent staining time for the tissues from the same sample block.

c. When eosin staining is completed, transfer the Stereo-seq Chip back to the methanol-containing tube (6-well cell culture plates or 6 cm culture dish) and incubate at -20°C for another **1 min**.

d. After fixation is completed, transfer the 6-well cell culture plates/6 cm culture dish to a sterile fume hood. Take out the Stereo-seq Chip and wipe off excess methanol from around the edges and the back of the chip with dust-free paper without touching the chips. Ensure that there is no methanol residue between chips.

e. Place the chip in a 10 cm culture dish with sealing film, and leave it in the fume hood for **4-6 min** to allow the methanol to evaporate completely.

Prepare the reagent required in Hematoxylin Staining and Bluing, calculate the volume for standby, and dispense the reagent in advance. Confirm that the microscope has been opened and switched to epi-bright field, color photography, BF-Epi channel mode.

f. When the methanol is completely evaporated, the tissue turns white and is visible to the naked eye. Transfer the Stereo-seq Chip onto a flat and clean bench for further staining.

Hematoxylin Staining and Bluing

a. Set aside the 0.01N HCl you prepared in 3.1 Experiment Preparation. During the staining process, refer to Table 3-11 in 3.7 Tissue Permeabilization to prepare the 1X PR Solution prior to use.

b. Prepare the staining reagent solutions in advance according to Table I-1.

Table I-1 Volumes for Staining and Cleaning Solutions for Different Chip Sizes

Volume per Component (μL)	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
Hematoxylin Solution	600	800	1000
0.1X SSC	500	700	800
Bluing Buffer	500	700	800

Before use, vortex the pre-aliquoted staining solution to mix thoroughly. Do not place it on ice.

c. Place the chip in the Chip Reaction Plate of the corresponding size, add Hematoxylin Solution (the volumes are listed in Table I-1) 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.

d. Discard the Hematoxylin Solution by tilting the Stereo-seq Chip Reaction Plate at an angle of less than 60°, and gently touch the edge of the chip. Remove as much liquid as possible.

e. Add 0.1X SSC to the Stereo-seq Chip Reaction Plate; the volumes are listed in Table I-1. Then discard it by tilting the Stereo-seq Chip Reaction Plate at an angle of less than 60°. Remove as much liquid as possible.

f. Repeat **step e. twice (for a total of 3 times)**.

g. Add Bluing Buffer 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 that the solution uniformly cover the entrie chip. Adjust the incubation time according to the reagent manufacturer's protocol. The volumes are listed in Table I-1. 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.

h. Discard Bluing Buffer by tilting the Stereo-seq Chip Reaction Plate at an angle of less than 60°. Remove as much liquid as possible.

i. Add 0.1x SSC to the Stereo-seq Chip Reaction Plate and refer to Table I-1 for the volume. Then discard it by turning the Stereo-seq Chip Reaction Plate at an angle of less than 60°. Remove as much liquid as possible.

j. Transfer the Stereo-seq Chip onto dust-free paper. Hold forceps with one hand to fix the chip 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 quickly from one corner of the chip at a 30-degree angle horizontal to the plane of the chip.

k. Apply **1-2 μL** water to a glass slide, then mount the chip onto the droplet to anchor it.

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.

l. Using a pipette, carefully add H&E Mounting Medium to the center of the tissue on the chip without introducing air bubbles. A few drops can be added evenly to the surface of the chip; the volumes are listed in Table I-2.

Table I-2 H&E Mounting Medium Volume for Different Chip Sizes

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
H&E Mounting Medium	8-12 μL/chip	16-24 μL/chip	24-36 μL/chip

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

After dropping H&E Mounting Medium, it is recommended that you proceed immediately to coverslip mounting.

m. 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 chip. Ensure that the chip are completely covered by H&E Mounting Medium and the coverslip. To ensure good image quality, IMMEDIATELY proceed to Imaging.

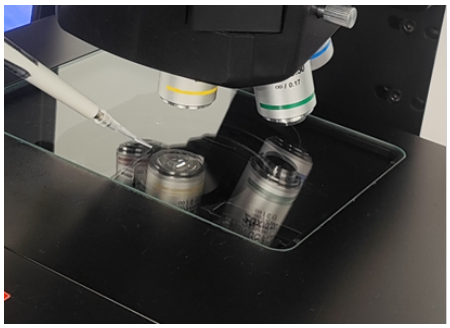
Imaging

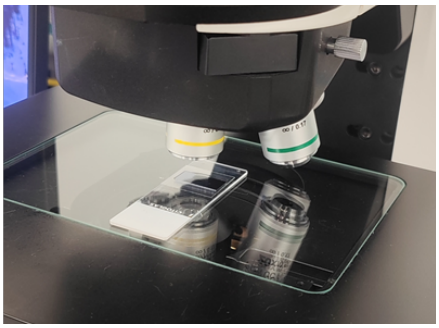
a. Create a new folder in the microscope imaging software, name it with the chip ID number and other essential information.

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

Example chip ID number: C05479C3D3

b. Fix the slide with the slide clamp of the microscope. If there is no slide clamp, place **1-2 μL** of water on the imaging platform first, then transfer and place the chip onto the water droplet. Water surface tension will grab onto the chip and adhere it onto the imaging platform, as shown in the following figure.

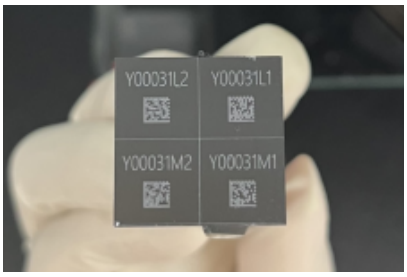




c. Place the slide gently on the stage, and try to keep the chip parallel to the stage.

The tilting angle relative to the stage edge should not exceed 15°.

d. When placing the chip, ensure that the orientation is correct, with the chip number (SN) on the back located at the top and the QR code at the bottom, as shown in the figure below:



Chip placement diagram (back view): The chip number is at the top, and the QR code is at the bottom.

Chip placement diagram (back view): The chip number is at the top, and the QR code is at the bottom.

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

f. Determine the tissue location: Select the 4X objective lens, move the FOV 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.

g. 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.

h. 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.

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

j. 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.

k. Background balance: Shift the focus to a blank area on the chip, and perform background balancing. Adjust the focus to find a chip FOV 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".

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

m. Manual focusing method (skip this step if the microscope you are using only supports the autofocusing method): Move the FOV to the non-tissue area on the chip, 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.

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

o. 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 instructions in the **StereoMap User Manual** in the software.

For comprehensive technical specifications and operational precautions, refer to the **Stereo-seq Imaging Requirements and Guidelines**.

The captured H&E-stained image must pass Image QC before you can proceed to further image analysis (image "register") in the Stereo-seq Analysis Workflow (SAW) pipelines.

If Image QC fails, carefully confirm the image clarity and retake the photos, ensuring that both tissue and tracklines are clear. If the second Image QC fails, continue with the experiment and perform optimal image analysis later under the guidance of your local Field Application Scientist, or perform manual image processing in StereoMap.

Stop Point:

H&E Mounting Medium-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 prevent RNA degradation.

p. Set the temperature of a metal bath or equivalent heating instrument to 37°C. Warm the aliquoted 1X PR Solution in the metal bath or equivalent heating instrument for **10 min (no longer than 30 min)**.

q. About 3 min before the end of 1X PR Solution incubation, hold the edge of the coverslip with forceps and gently slide the coverslip parallel with forceps until the chip and the coverslip are completely separated.

r. Place the chip in the Chip Reaction Plate of the new corresponding size, and add 0.1X SSC solution to the chip; the volumes are listed in Table I-3. Pipette 0.1X SSC up and down 5 times (wash it 3 times from left to right along the long edge of the chip, and wash it twice on the short edge of the chip).

Table I-3 0.1X SSC Volume for Different Chip Sizes after H&E Mounting Medium

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
0.1X SCC	1500 μL/chip	2000 μL/chip	3000 μL/chip

Ensure that the chip is completely immersed in the solution.

s. Aspirate the 0.1X SSC from the corner of the Chip Reaction Plate, then add 0.1X SSC (the volumes are listed in Table I-3) and immerse it for **3-5 sec**.

t. Aspirate the 0.1X SSC from the corner of the Chip Reaction Plate, then slowly add 0.01N HCl solution on the chip; the volumes are listed in Table I-4. After the liquid covers the entire chip, aspirate the liquid from the gap at the center of the long edge of the reaction well.

Table I-4 0.01N HCl Volume for Different Chip Sizes

Chip Size	Stereo-seq Chip T (1cm * 2cm)	Stereo-seq Chip T (2cm * 2cm)	Stereo-seq Chip T (2cm * 3cm)
0.01N HCl	400 μL/chip	600 μL/chip	800 μL/chip

CAUTION! Proceed to 3.7 Tissue Permeabilization.