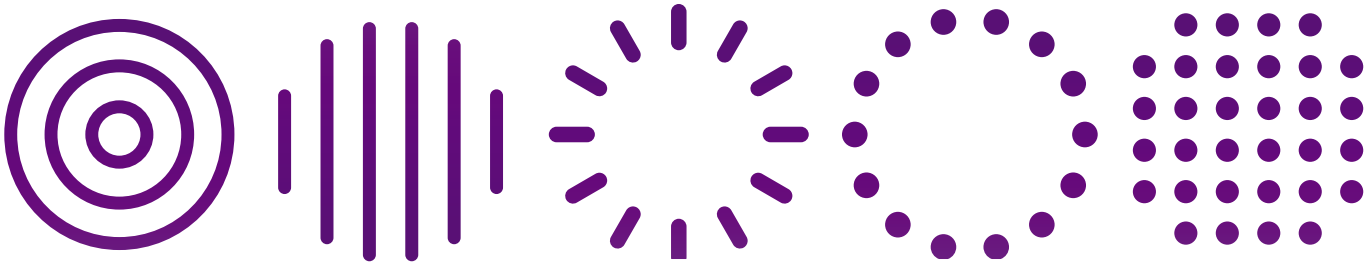
STOmics

STEREO-CITE PROTEO-TRANSCRIPTOMICS SET

USER MANUAL



Cat. No.: 211PT11114(4 RXNs)
Kit Version: V1.1
Manual Version: A

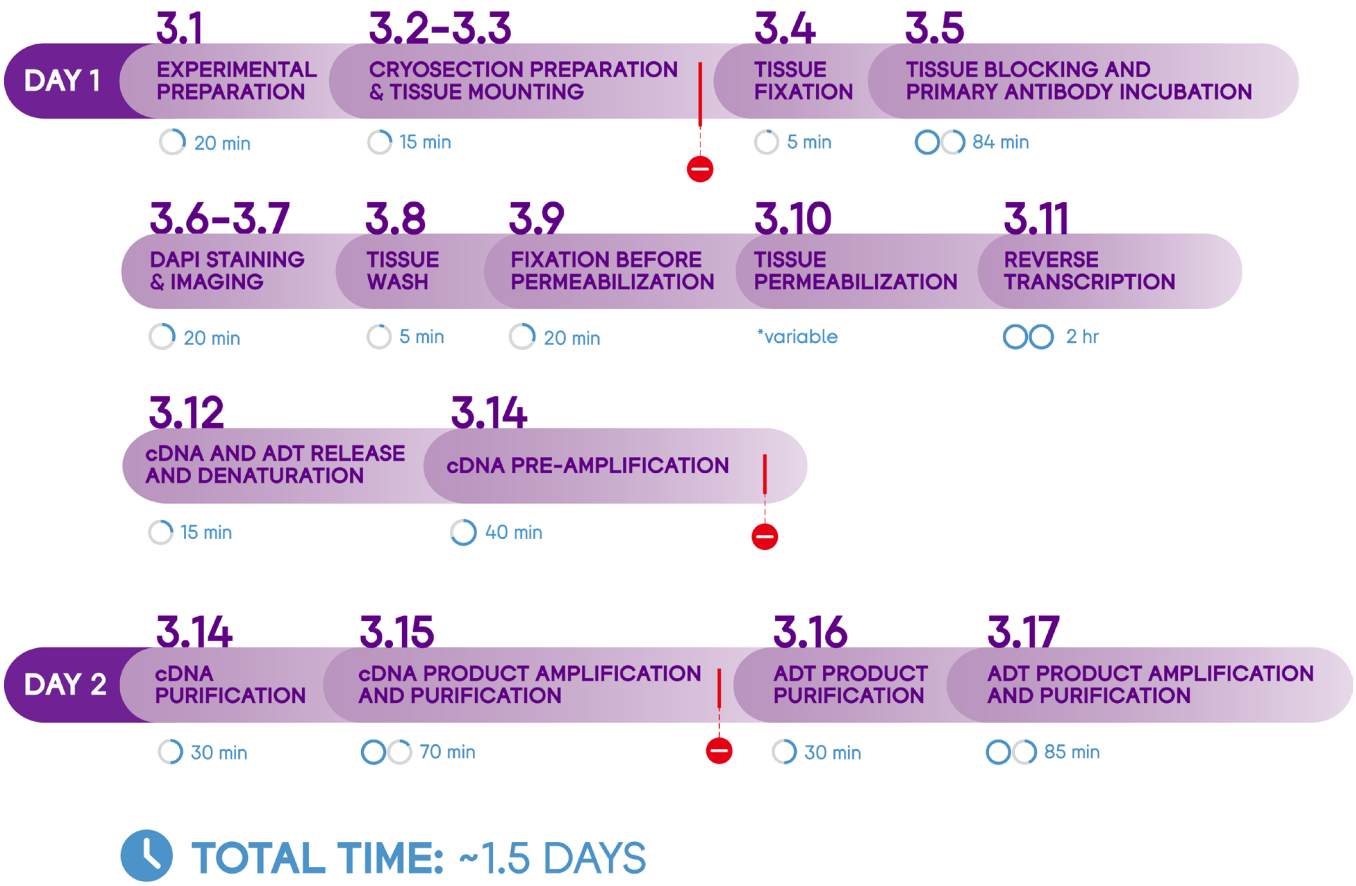
STUM-TT005

REVISION HISTORY

Manual Version:	A
Kit Version:	V1.1
Date:	Mar. 2025
Description:	Initial release

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

WORKFLOW



TOTAL TIME: ~1.5 DAYS

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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

This user manual provides experimental instructions specifically for the Stereo-CITE Proteo-Transcriptomics Set V1.1.
STOmics Stereo-CITE Proteo-Transcriptomics Set is intended to simultaneously detect the whole transcriptome and high-plex protein on the same tissue section. Built upon DNA Nanoball (DNB) technology, STOmics Stereo-CITE Proteo-Transcriptomics Set enables a “tissue-to-data” solution through in situ capture of the whole transcriptome and high-plex protein markers at nanoscale resolution and centimeter-sized field of view (FOV). The Stereo-seq Chip T (poly-T-based chip) is loaded with capture probes containing spatial coordinate information. Through a series of biochemical processes, the probes can capture mRNA molecules and antibody-derived tags (ADTs) in situ within the tissue, then through cDNA synthesis, and obtain transcriptome plus multi-protein spatial distribution information of the entire tissue through sequencing and a complementary visualization platform.
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-CITE Proteo-Transcriptomics Set require the DNBSEQ sequencing platform.

Compatible sequencing platforms:

- DNBSEQ-T7RS

  Pooling both Stereo-CITE transcriptome and ADT libraries on the same flowcell is recommended.

- DNBSEQ-G400

  It is recommended that you sequence Stereo-CITE transcriptome and ADT libraries separately on different flowcells. Pooling both Stereo-CITE transcriptome and ADT libraries on the same flowcell is not recommended.

For details, refer to the **Stereo-CITE Proteo-Transcriptomics Library Preparation User Manual (Document No.: STUM-LP003)**.

1.3. List of Kit Components

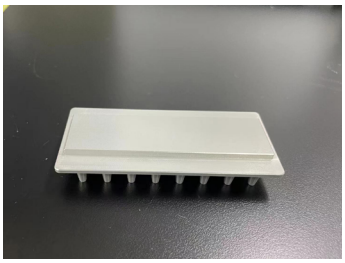
Each Stereo-CITE Proteo-Transcriptomics Set consists of:

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

Stereo-seq 16 RXN Library Preparation Kit is not included in the Stereo-CITE Proteo-Transcriptomics Set and must be purchased separately. If you wish to construct Stereo-CITE Proteo-transcriptome libraries in-house, refer to the **Stereo-CITE Proteo-Transcriptomics Library Preparation User Manual (Document No.: STUM-LP003)** 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 1-5).

Upon receiving the Stereo-seq Chip T Slide (1cm * 1cm), 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 the 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			
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	Transported by cold chain	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 Protein Assisted Kit Components

Stereo-seq Protein Assisted Kit Cat. No.: 212KA11114			
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (tube)**
Blocking Reagent	1000044666	○ (transparent)	66 μL x 2
ADT Primer Mix A	1000051059	●	36 μL x 1
cDNA Primer	1000028513	●	36 μL x 1
PCR Amplification Mix	1000028519	●	400 μL x 1
Storage Temperature: -25°C~ -15°C	Transported by cold chain	Expiration Date: refer to label	

Table 1-3 Stereo-seq Chip T Slide (1cm * 1cm) Kit Components

Stereo-seq Chip T Slide (1cm*1cm) Cat. No.: 210CT13114	
Component	**Quantity (per kit)**
Stereo-seq Chip T (1cm * 1cm)	4 EA
Storage Temperature: 2°C~8°C	Transported by cold chain / Expiration Date: refer to label

Table 1-4 STOmics Accessory Kit Components

STOmics Accessory Kit Cat. No.: 1100033700		
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	Transported at 0°C ~ 30°C	Expiration Date: refer to label

Table 1-5 Stereo-seq PCR Adaptor Kit Components

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

1.4. Additional Equipment and Materials

Table 1-6 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**.

Table 1-6 Additional Equipment and Materials

Equipment		
Brand	**Description**	**Cat. No.**
Leica	Cryostat	CM1950
Eppendorf	Refrigerated Centrifuge (for Stereo-CITE application)	5418R
-	Microcentrifuge	-
-	Pipettes	-
-	pH Meter	-
-	Metal Bath (or equivalent instrument)	-
-	Immunohistochemistry (IHC) Humidity Box	-
Leica*	Fluorescence Microscope	DM6M
STOmics*	Fluorescence Microscope	900-000586-00
-	Vortex Mixer	-
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™4 Fluorometer	Q33216
Agilent Technologies™	Agilent 2100 Bioanalyzer	G2939AA (or similar)

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

Reagents		
Brand	**Description**	**Cat. No.**
BOSTER (or other brands)	4% Paraformaldehyde (with DEPC)	AR1068
Invitrogen*	Nuclease-free Water	2186768
Ambion*	Nuclease-free Water	AM9937
	20X SSC	AM9770
	1X TE Buffer, pH 8.0 (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA)	AM9858
SAKURA	SAKURA Tissue-Tek® O.C.T. Compound	4583
-	100% Ethanol (Analytical grade)	-
Sigma Aldrich	Hydrochloric acid, HCl (0.1 N)	2104-50ML
	Methanol	34860-1L-R
	Triton X-100 Solution, 10%	93443-100ML
	DMSO	D4540
Invitrogen	Salmon Sperm DNA, sheared (10 mg/mL)	AM9680
Thermo Fisher Scientific™	RNase Inhibitor (RI)	EO0382
	DAPI Solution (1 mg/mL)	62248
	Gibco™ Horse Serum	26050070
	Gibco™ PBS, pH 7.4	10010031
	Alexa Fluor™ Plus anti-Rat secondary Antibody (for mouse samples)	A48270
	Alexa Fluor™ Plus anti-Mouse secondary Antibody (for human samples)	A32773
	Other fluorescent secondary antibodies should be tested before use.	
Beckman Coulter^	AMPure® XP	A63882
	SPRIselect	B23317/ B23318/ B23319
VAZYME^	VAHTS™ DNA Clean Beads	N411-02
Invitrogen	Qubit dsDNA HS Assay Kit	Q32854

Agilent Technologies™	High sensitivity DNA Kit	5067-4626
	High sensitivity RNA Kit	5067-1513
Aladdin#	Potassium Hydroxide Solution, 8M	P291842
Millipore Sigma#	Potassium Hydroxide Solution, 8M	P4494-50ML
BioLegend	TotalSeq-A™ Antibody	-
	TotalSeq-A™ Mouse Universal Cocktail, V1.0	199901
	TotalSeq-A™ Human Universal Cocktail, V1.0	399907
	Human TruStain FcX™ (Fc Receptor Blocking Solution)	422301
	TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody	156604

FcR Blocking Reagent is for blocking Fc receptors on the cell membrane. Select either one according to the host species of your sample. Choose Human TruStain FcX™ (Fc Receptor Blocking Solution) for human tissues and choose TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody for mouse tissues.

Stereo-seq Transcriptomics T Kit has been validated to be compatible with the singular TotalSeq-A™ antibody mixture, the TotalSeq-A™ Mouse, and Human Universal Cocktails from Biolegend. Feel free to combine the singular TotalSeq-A™ antibody depending on your experiment design. All the Singular TotalSeq-A™ antibodies to be used must have their optimal concentrations determined by titration before combination. For more information, refer to Appendix II: Antibody Titration. The species of most of the TotalSeq-A™ antibodies from Biolegend are human and mice. For detailed information, refer to the Biolegend website: https://www.biolegend.com/en-us/search-results?PageSize=25&Format=TOTALSEQ_A.

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

Special Notification:

Isotype control antibody

An isotype control antibody has characteristics similar to the primary antibody but lacks specificity for the target of interest. Isotype control antibodies are often used as negative controls to help distinguish non-specific background signals from specific antibody signals. The selection of an isotype control antibody should match the species and class of the primary antibody, including the light chain.

For example, in Figure 1, the TotalSeq™-A0007 anti-human CD274 (B7-H1, PD-L1) antibody has a mouse IgG2b κ isotype. Therefore, when detecting this antibody, it is necessary to include the TotalSeq™-A0092 Mouse IgG2b, κ isotype control antibody. The specific type of isotype control antibody should be determined based on the type of TotalSeq™-A primary antibody used. Additionally, it is important to note that if the kit is used with TotalSeq™-A Mouse Universal Cocktail, V1.0 (Biolegend, Cat. No.: 199901) or TotalSeq™-A Human Universal Cocktail, V1.0 (Biolegend, Cat. No.: 399907), there is no need to add an isotype control antibody because these cocktails already include isotype control antibodies.

For a list of isotype control antibodies, visit:
https://www.biolegend.com/en-us/search-results?PageSize=25&Category=ISO_CTRL&Format=TOTALSEQ_A

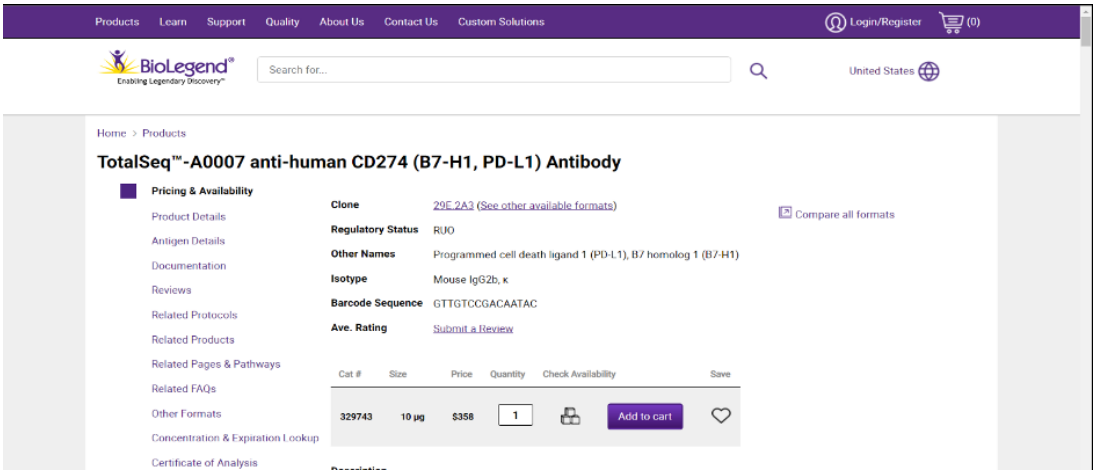


Figure 1 The isotype antibody of TotalSeq™-A0007 anti-human CD274 (B7-H1, PD-L1)

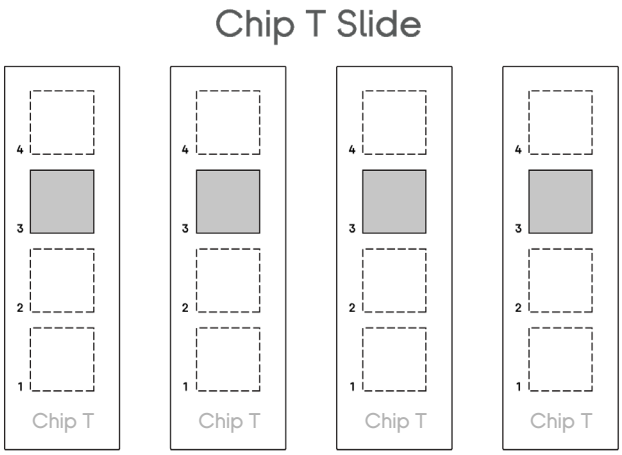
Consumables		
Brand	**Description**	**Cat. No.**
-	Aluminum Foil	-
-	Slide Container	-
-	Forceps	-
-	Slide Staining Rack	-
-	Microscope Glass Coverslip (area: 18 mm x 18 mm, thickness: 0.13 - 0.16 mm)	-
-	Sterilized Syringe	-
Millipore	Millex Syringe Filter, Durapore PVDF, 0.22 µm pore size	SLGV033N
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
	1,000 µL Filtered Tips	TF-1000-L-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
	0.5 mL Thin Wall PCR Tubes^	PCR-05-C
Axygen	Qubit Assay Tubes^	Q32856

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

1.5. Stereo-seq Chip Slide Information

Stereo-seq Chip T Slide

Includes 4 Stereo-seq Chip T Slides containing one Chip T (1cm * 1cm) on each slide.

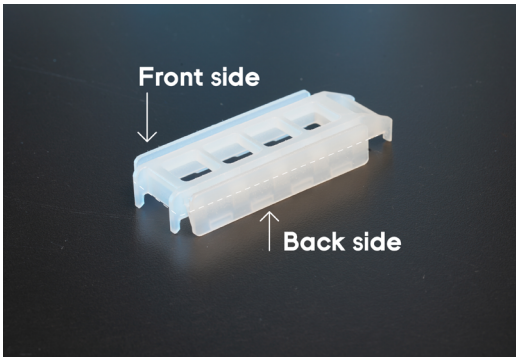


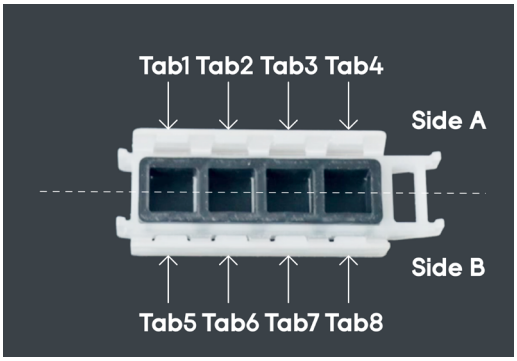
Stereo-seq Chip T Slide Storage

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 assembly and removal, refer to the link below or scan the QR code:

https://en.stomics.tech/resources/videos/list.html

For assembly instructions, refer to Appendix I: Stereo-seq Slide Cassette Assembly.

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 embedding, refer to the **Sample Preparation Guide for Fresh Frozen Samples on 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 ≥4.0.

CHAPTER 3

Stereo-CITE PROTEO-TRANSCRIPTOMICS SET STANDARD OPERATING PROCEDURE

Biolegend's TotalseqA antibodies are developed for CITE-seq technology and may not be suitable for tissue sections. For users who choose their own antibodies, we recommend that you first perform antibody titration on tissue sections of interest to select the most appropriate antibody concentration before proceeding with the standard operating procedure. For the detailed procedure, refer to Appendix II: Antibody Titration.

If the kit is used with TotalSeq™ -A Mouse Universal Cocktail, V1.0 (Biolegend, Cat. No.: 199901) or TotalSeq™ -A Human Universal Cocktail, V1.0 (Biolegend, Cat. No.: 399907), you can skip the antibody titration experiments and directly proceed to the standard operating procedure using our recommended dilution ratio.

Before the standard operating procedure can be performed, determine the optimal permeabilization time and permeabilization enzyme concentration using the permeabilization reagent kit. For details, refer to the Stereo-seq Permeabilization Set for Stereo-CITE Proteo-transcriptomics Application User Manual (Document No.: STUM-PR004).

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

Prep Day	Reagent	Preparation Steps	Maintenance
Day 1	4% PFA	Mix well after thawing at 4°C. Aliquot to 2 mL/tube for storage. Equilibrate to room temperature before use.	4°C for 1 week
	Keep it at -20°C for long-term storage.		
	0.1X SSC	Dilute 150 µL of 20x SSC to 30 mL.	Room Temperature
	Wash Buffer	Prepare 3000 µL per chip (2850 µL 0.1X SSC with 150 µL RI).	On ice until use, up to 12 hr
	5X SSC	Dilute 1 mL of 20X SSC to 4 mL.	Room Temperature
	Methanol	Pre-cool at -20°C for no longer than 30 min before use.	-20°C

Prep Day	Reagent	Preparation Steps	Maintenance
Day 1	TotalSeq™-A Mouse Universal Cocktail, V1.0 Or TotalSeq™-A Human Universal Cocktail, V1.0 (Optional)	a. Equilibrate the lyophilized powder tubes at room temperature for 5 min. b. Place the lyophilized powder tube in an empty 2 mL EP tube at 10,000 g for 30 sec at room temperature. c. Add 27.5 µL of 1X PBS and incubate for 5 min at room temperature. d. Vortex and centrifuge at 10,000 g for 30 sec at room temperature. e. Transfer the entire volume (27.5 µL) of the reconstituted antibody cocktail solution to a new EP tube and centrifuge at 14,000 g for 10 min at 4°C. f. Transfer 25 µL of the supernatant to a new EP tube, and place on ice for later use.	On ice until use, up to 6 hr
	Filtered Serum Aliquot	Thaw the horse serum, then filter it with a 0.22 µm pore-sized filter membrane and a sterilized syringe. For one round of permeabilization and proteo-transcriptomics workflow, aliquot the filtered serum in 200 µL per tube and store at -20°C. Thaw the aliquoted serum on ice and centrifuge at 14,000 g for 10 min at 4°C. Place on ice until use. Approximately 20 µL of serum is needed for one chip, and any remaining serum can be reused.	On ice until use, up to 6 hr
	Do not freeze and thaw the aliquot more than 3 times. Keep the aliquots at -20°C for long-term storage.		
	Blocking Reagent	Thaw on ice before use, 15 µL/chip.	On ice until use, up to 6 hr
	Sheared Salmon Sperm DNA	Thaw on ice before use, 15 µL/chip.	On ice until use, up to 6 hr
	RI	Take RI from -20°C and place it on ice until use.	On ice until use, up to 12 hr
	50X Diluted DAPI Solution	Dilute DAPI (1 mg/mL) with 5X SSC at a ratio of 1:50.	On ice in the dark until use, up to 6 hr
	Primary and Secondary Antibody	Thaw on ice following the original product instructions, and centrifuge for 10 min at 14,000 g. The Primary Abs should include the isotype Abs.	On ice until use, up to 6 hr

Prep Day	Reagent	Preparation Steps	Maintenance
Day 1	Diluted Primary and Secondary (Optional)	Dilute the antibodies if needed.	On ice until use, up to 6 hr
	10% Triton X-100	Use 10% Triton X-100 or dilute 100% Triton X-100 with nuclease-free water.	Room temperature
	FcR Blocking Reagent	For mouse samples, use TruStain Fcx™ PLUS (anti-mouse CD16/32) Antibody; for human samples, use Human TruStain Fcx™.	On ice until use, up to 6 hr
	Glycerol	Equilibrate to room temperature 5 min in advance.	Room temperature
	0.01N HCl	Prepare at least 2 mL of 0.01N HCl per sample. Configure HCl to 0.01N. Measure and make sure 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 through 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. Keep the aliquots at -20°C for long-term storage.**		
	0.5X Permeabilization Reagent Solution	Dilute 7.5 µL of 10X Permeabilization Reagent Stock Solution to 150 µL with 0.01N HCl (at least 150 µL/chip)	On ice until use, up to 6 hr
	0.1M KOH	Dilute 10 µL of 8M KOH to 800 µL; use 195 µL per chip.	Room temperature
	Always use freshly prepared 0.1M KOH, and check the pH prior to the experiments for newly purchased 8M KOH.(8M KOH diluted to 1 M KOH, pH 14±0.3). **Do not prepare until you are ready to use it.**		
	Elute Additive	Thaw on ice 5 min prior to use. Use 5 µL per chip.	On ice until use
	Neutralization Solution	Equilibrate to room temperature 5 min prior to use; use 23 µL per chip.	Room temperature

Prep Day	Reagent	Preparation Steps	Maintenance
Day 2	Magnetic Beads	Equilibrate to room temperature at least 30 min prior to use.	Room temperature up to 6 hr
	80% Ethanol	Dilute 100% ethanol to 80%.	Room temperature up to 1 day

Other Preparation		
Equipment	**Setup**	**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.
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 and ADT 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	37°C for preheating the PR Enzyme.	Check the instrument for any abnormalities and replace it if necessary.
Refrigerated Centrifuge	Centrifuge the cocktail lyophilized powder at room temperature, then set the centrifuge temperature to 4°C in advance.	4°C is used for centrifugation of serum, primary antibodies, secondary antibodies, and reconstituted antibody cocktails.
Fluorescence Microscope	Make sure the microscope is equipped with at least DAPI, FITC, TRITC, CY5	Select the channels according to the fluorescent emission wavelengths of your secondary antibodies.

3.2. Cryosection Preparation

a. Set the PCR Thermal Cycler to 37°C with heated lid set to 60°C in advance with a PCR Adaptor.

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

b. Set the cryostat chamber temperature to -20°C and the 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. Transfer the OCT-embedded tissue sample from the -80°C freezer and place it in the cryostat chamber. Allow it to equilibrate to the cryostat chamber temperature for **30 min**.

e. Meanwhile, use sufficient 4% PFA solution (at least 400 µL 4% PFA solution per chip) and equilibrate to room temperature in a fume hood.

f. While waiting for the cryostat temperature to equilibrate to the target temperature, prepare the blocking solution according to Table 3-2, and place it on ice until use.

Table 3-2 Blocking Solution

Component	1X (µL)	1X + 10% (µL)	2X + 10% (µL)	3X + 10% (µL)	4X + 10% (µL)
5X SSC	180	198	396	594	793
10% Triton X-100	3	3.3	6.6	9.9	13.2
Blocking Reagent	15	16.5	33	49.5	66
Sheared Salmon Sperm DNA	30	33	66	99	132
FcR Blocking Reagent*	15	16.5	33	49.5	66
RI	15	16.5	33	49.5	66
Filtered Serum Aliquot	30	33	66	99	132
Nuclease-free Water	12	13.2	26.4	39.6	52.8
Total	300	330	660	990	1320

***FcR Blocking Reagent is for blocking Fc receptors on the cell membrane. Select either one according to the host species of your sample. For mouse tissue, use Human TruStain FcX™ (Fc Receptor Blocking Solution, Cat. No.: 156604), and for human tissue, use TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody (Cat. No.: 422031).**

The blocking solution prepared for one tissue/one slide (1X) is enough for two usages: tissue blocking and primary antibody solution preparation.

g. Remove the sample outer covers (aluminum foil) and trim the embedded tissue block to the appropriate size (sectioning area smaller than 0.9 cm x 0.9 cm).

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

i. 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.

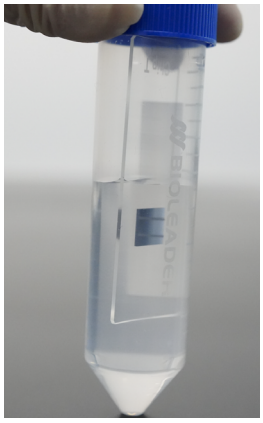
3.3. Tissue Mounting

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..

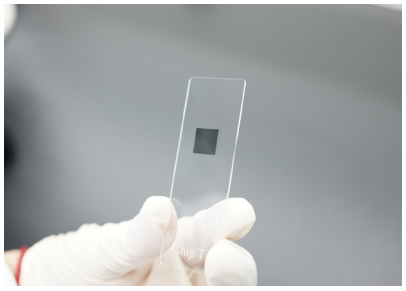
After opening the bag, check all Stereo-seq Chip Slides in the slide container and make sure they are oriented front-side up. The front of the chip has a shiny surface that contains DNB probes for RNA capture. DO NOT scratch the surface.

b. 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 in a 50 mL centrifuge tube or slide container with sufficient nuclease-free water by holding the slide with forceps and pulling it out of the solution and then immersing it twice to wash.



Seal unused slides in the original packaging (first in the slide container and then in the sealable aluminum bag) and store at -25°C ~ 8°C. KEEP the desiccant in the aluminum bag.

c. Gently blow off excess water from the chip with a power dust remover. Wipe off excess water from around the edges of the chip and on the slide with dust-free paper.



d. When the chip is completely dry and void of wavy white stains, it is ready for tissue mounting.

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

f. Tissue mounting can be achieved using either the warm method (option A) or the cold 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. Warm Method

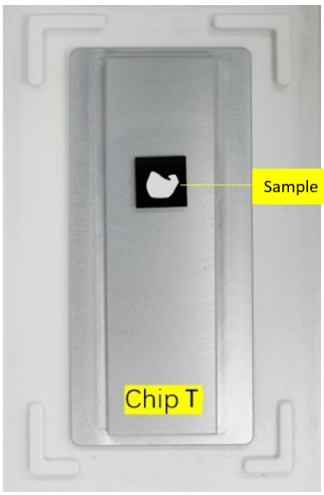
1) Perform cryosection and obtain two or four consecutive tissue sections (depending on the number of chips on the Stereo-seq Chip Slide), and carefully flatten the tissue sections out by gently touching the surrounding OCT with cryostat brushes.

2) Move the tissue sections to the edge and place each tissue section such that the space between each is greater than the chip spacing on the Stereo-seq Chip Slide, avoiding slide contact with other sections.

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

4) Repeat **step 3)** until all 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 a PCR Adaptor for **5 min** (without heated lid).



B. Cold Method

1) Place the Stereo-seq Chip Slide inside the cryostat chamber with the front facing up and pre-cool the slide 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 out by gently touching the surrounding OCT with cryostat brushes. Carefully place a tissue section onto the chip center using forceps and brushes. Make sure the tissue section is complete and without wrinkles.
3) Immediately pick up the Stereo-seq Chip Slide and place a finger on the back of the Stereo-seq Chip Slide directly under the chip for a few sec to allow the section to adhere to the chip.
4) Place the tissue-mounted Stereo-seq Chip Slide back inside the cryostat chamber. Continue by mounting the remaining tissue sections on the 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) can result in tissue wrinkle formation

Stop Point:

- **After drying the tissue containing Stereo-seq Chip Slides in the PCR Thermal Cycler, transfer the Stereo-seq Chip Slide into a slide container or a 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. Transfer the sealed Stereo-seq Chip Slide to a -80°C freezer on dry ice.**
- **Store the sealed plastic bag containing the Stereo-seq Chip Slides with tissue at -80°C for up to 21 days.**
- **When retrieving Stereo-seq Chip Slides with tissue from the freezer, transfer out the slide container on dry ice, 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

a. Refer to **Appendix I: Stereo-seq Slide Cassette Assembly** for assembling the cassette and gasket onto the Stereo-seq Chip Slide.

b. Assemble the Stereo-seq Chip Slide onto the cassette, forming a handheld Stereo-seq Slide Cassette. Make sure the 8 tabs are locked in place and the cassette is tightly securing the sides of the cassette.

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

c. Place the Stereo-seq Slide Cassette in a fume hood, and add **400 µL** of 4% PFA solution per well. Place **unpeeled** sealing tape on top of the cassette and incubate for **5 min at room temperature**.

d. After fixation, remove the **unpeeled** sealing tape and place it on the work surface for later use.

e. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20°, and remove 4% PFA solution from the corner of the well using a pipette, keeping the chip and tissue surface moist.

f. Immediately add Wash Buffer (**400 µL** per well) and incubate at room temperature for **1 min**.

g. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20°, and remove the Wash Buffer from the corner of the well using a pipette, keeping the chip and tissue surface moist.

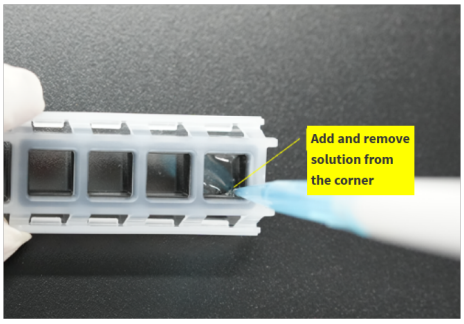
Do not allow the tissue to dry out during the liquid exchange process. Make sure that all the tissue sections are completely submerged.

h. Repeat wash **step f**.

i. During incubation, transfer the Stereo-seq Slide Cassette from the fume hood to a bench. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20°, and remove the Wash Buffer from a corner of the well using a pipette. Keep the chip and tissue surface moist.

3.5. Tissue Blocking and Primary Antibody Incubation

a. Immediately add **150 µL** blocking solution per well, place unpeeled sealing tape on top of the Slide Cassette, and incubate at room temperature **for 20 min**.



b. During incubation, prepare the primary antibody incubation solution according to Table 3-3 based on the actual number of individual TotalSeq™-A antibodies used. Vortex to mix, centrifuge briefly, then leave the primary antibody incubation solution on ice until use.

For TotalSeq-A™ Mouse Universal Cocktail, V1.0 (Biolegend, Cat. No.: 199901) and TotalSeq-A™ Human Universal Cocktail, V1.0 (Biolegend, Cat. No.: 399907), prepare the primary antibody incubation solution according to Table 3-4.

Table 3-3 Primary Antibody Incubation Solution
(For freely combined individual TotalSeq™-A antibodies of your choosing)

Component	1X (µL)
Blocking Solution	150-(V1+V2+...+Vn)
Primary Antibody #1	V1
Primary Antibody #2	V2
...	...
Primary Antibody #N	Vn
Total	150

Antibody 1~N should contain the corresponding isotype control antibody. Centrifuge the TotalSeq™-A antibody before use, and try not to allow the pipette tip to directly contact the bottom of the tube.

Ensure that the primary antibodies have been centrifuged before use.

Table 3-4 Primary Antibody Incubation Solution (For TotalSeq™-A Cocktails)

Component	1X (µL)
Blocking Solution	137.5
Universal Cocktail	12.5
Total	150

c. After the incubation is completed, remove the unpeeled sealing tape and place it on the laboratory bench for later use.

d. Pipette to remove the blocking solution from one corner of the well. Ensure that the chip and tissue surface remain moist. Immediately add **150 µL** of the primary antibody incubation solution into each well.

Do not allow the tissue to dry out during the liquid exchange process. It is recommended that you perform this process one chip at a time.

e. Place unpeeled sealing tape on top of the Slide Cassette and incubate for **45 min** at room temperature. During primary antibody incubation, prepare the secondary antibody incubation solution according to Table 3-5. Vortex the mixture and leave it on ice **in the dark** until use.

Table 3-5 Secondary Antibody Incubation Solution

Component	1X (µL)
5X SSC	90
RI	7.5
Secondary Antibody	0.3
Nuclease-free Water	52.2
Total	150

The solution should be stored in the dark. We recommend using Alexa Fluor Plus Series Fluorescent Secondary Antibodies at a 1:500 dilution concentration. If fluorescent secondary antibodies from other vendors are used, adjust the dilution ratio according to the manufacturer's instructions.

Ensure that the secondary antibodies have been centrifuged before use.

f. After the incubation is completed, remove the unpeeled sealing tape and place it on the laboratory bench for later use.

g. Pipette to remove the primary antibody incubation solution from one corner of the well. Ensure that the chip and tissue surface remain moist.

h. Add **200 μL** Wash Buffer per well and incubate the chip at room temperature for **1 min**.

i. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20°, remove the Wash Buffer from the corner of the well using a pipette, and keep the chip and tissue surface moist.

j. Repeat wash **steps h.** and **i**.

Do not allow the tissue to dry out during the liquid exchange process. It is recommended that you perform this process one chip at a time.

k. Immediately add **150 μL** secondary antibody incubation solution per well onto the chip. Place unpeeled sealing tape on to the Slide Cassette and incubate at room temperature **in the dark** for **15 min**.

3.6. DAPI Staining

Reagent	Preparation Steps	Maintenance
50X Diluted DAPI Solution	Dilute DAPI stock solution with 5X SSC then keep it on ice in the dark. Mix and centrifuge briefly. Do not pipette the precipitation at the bottom.	On ice in the dark until use, up to 6 hr

a. During secondary antibody incubation, prepare DAPI staining solution according to Table 3-6. Vortex, centrifuge briefly, and then leave it on ice in the dark until use.

Table 3-6 DAPI Staining Solution

Component	1X (μL)	1X + 10% (μL)	2X + 10% (μL)	3X + 10% (μL)	4X + 10% (μL)
5X SSC	90	99	198	297	396
50X Diluted DAPI Solution	1.5	1.65	3.3	4.95	6.6
RI	7.5	8.25	16.5	24.75	33
Nuclease-free Water	51	56.1	112.2	168.3	224.4
Total	150	165	330	495	660

b. Pipette to remove the secondary incubation solution from one corner of the well. Keep the chip and tissue surfaces moist.

c. Add **200 μL** Wash Buffer per well and incubate the chip at room temperature for **1 min**.

d. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20°. Pipette to remove the Wash Buffer from one corner of the well, keeping the chip and tissue surface moist.

e. Repeat wash **steps c.** and **d**.

Do not let the tissue dry out during the liquid exchange process. It is recommended that you perform the step one chip at a time.

f. Slowly add **150 μL** DAPI Staining Solution per well onto the chip from the non-tissue area. Place unpeeled sealing tape on top of the Slide Cassette and incubate at room temperature **in the dark** for **2 min**.

g. Remove the unpeeled sealing tape from the Slide Cassette and place it on the laboratory bench for later use.

h. Pipette to remove the solution from one corner of the well. Keep the chip and tissue surface moist.

i. Add **200 μL** Wash Buffer per well and incubate the chip at room temperature for **1 min**.

j. Slightly tilt the Stereo-seq Slide Cassette at an angle of less than 20°. Pipette to remove the Wash Buffer from the corner of the well; do not touch the chip surface, and keep it moist.

k. Repeat wash **steps i.** and **j**.

l. Remove the slide from the Stereo-seq Slide Cassette according to the instructions in Appendix I: Stereo-seq Slide Cassette Assembly.

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

m. 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.

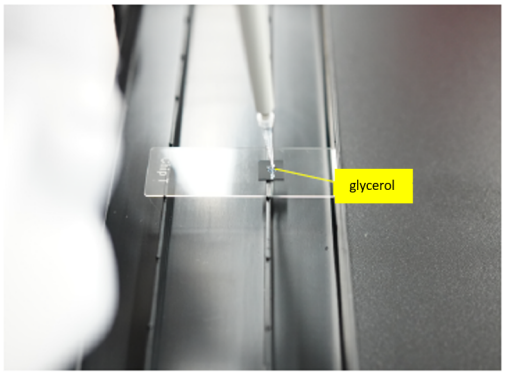
Alternatively, centrifuge the Stereo-seq Chip Slide for 10 sec in a slide spinner to completely dry the chip.

Ensure that there is no residual solution on the chip.

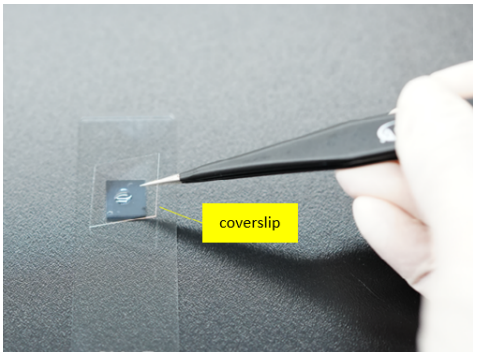
Equilibrate the glycerol at room temperature for 5 min in advance.

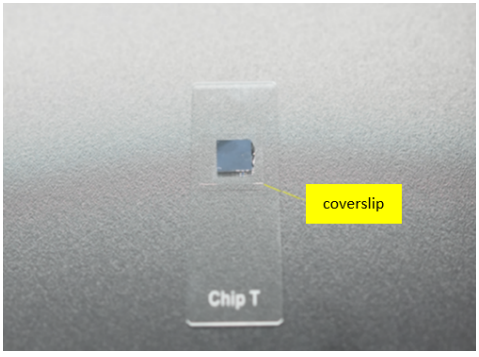
Ensure that the coverslip is clean and free of any dust or debris. Wipe with an alcohol swab or blow the debris off with a power dust remover.

n. 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.



o. 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 prevent fluorescent bleaching, IMMEDIATELY proceed to **3.7 Imaging**.





3.7. 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.

Note that a general-purpose microscope is used for example purposes. For specific requirements for microscopes, refer to the **STOmics Microscope Assessment Guideline**.

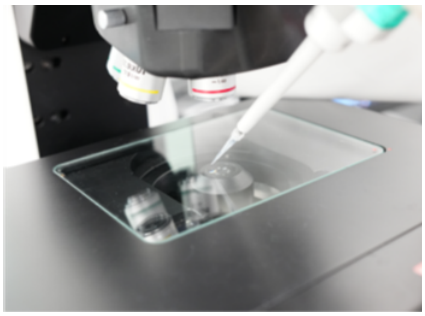
Required fluorescence channels depend on antibody selection. The recommended fluorescence configuration is as follows:

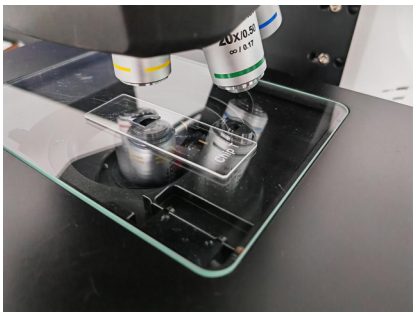
- Light source with a wavelength range of 380 nm - 680 nm
- Monochrome camera (≥8 bit)
- DAPI filter cube (Excitation 375/28 nm, Emission 460/50 nm)
- FITC filter cube (Excitation 480/30 nm, Emission 525/50 nm)
- TRITC filter cube (Excitation 545/25 nm, Emission 605/70 nm)
- CY5 filter cube (Excitation 620/50 nm, Emission 690/50 nm)
- Maximum pixel size: 5 μm
- Exposure time: 1 ms - 2 sec

a. **[DAPI Imaging]** Create a new folder in a fluorescent microscope-connected PC, name the folder with the chip ID number, and include other essential information.

Use only letters, numbers, and underscores in the folder name. Special characters and spaces are not allowed.
Example chip ID number: Y00035N1

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 onto 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°.

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

d. Turn on the fluorescence microscope and select epifluorescence scanning mode, DAPI channel.

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 (which 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 the focus 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 only supports autofocusing): Shift the focus to a blank area on the chip, 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.

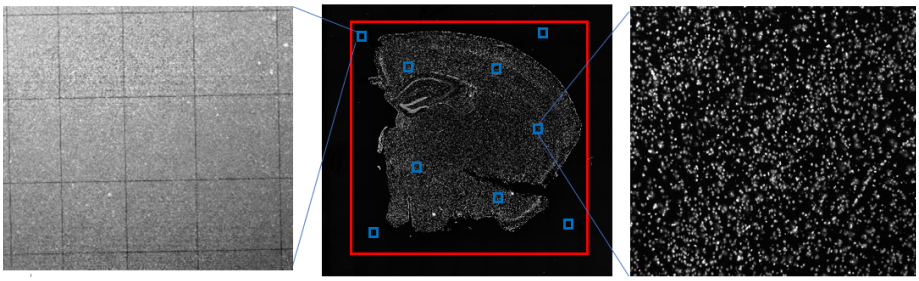


Figure 2 DAPI staining of mouse brain

j. Final Imaging: After the model point is completed, the gain is adjusted to the minimum, and the scan is carried out. After taking the photo, save the entire folder (original FOV small image and stitched large image).

k. **[IF Imaging]** Create a new folder, name and save it with the chip number_IF format (e.g., Y00035N1_All_IF) using the computer connected to the fluorescence microscope. Select the appropriate fluorescent channel (FITC channel, TRITC channel, or CY5 channel), depending on the secondary antibody selected **WITHOUT** moving the Stereo-seq Chip Slide, re-scanning the map, re-adding the focal points, or changing the red box of the selected tissue area. Then adjust the focus and exposure until the stained tissue is clearly displayed. Finally, complete the full scan on the capturing area with the 10X objective lens.

Use only letters, numbers, and underscores in the folder name. Special characters and spaces are not allowed.
Example folder name: Y00035N1_All_IF

l. Based on the results of the immunofluorescence staining image, whether the TotalSeq™-A primary antibody has been successfully bound to the antigen can be determined. The judging criteria are dependent on the type of antibody added, and the images obtained should be consistent with prior knowledge of immunofluorescent staining. Using Figure 3 as an example, due to the addition of multiple primary antibodies, fluorescent signals are almost across the thymus tissue (Figure 3, left image). The cell membrane staining can be clearly visualized after magnification, indicating that primary antibodies are successfully bound to the target antigen (Figure 3, right image).

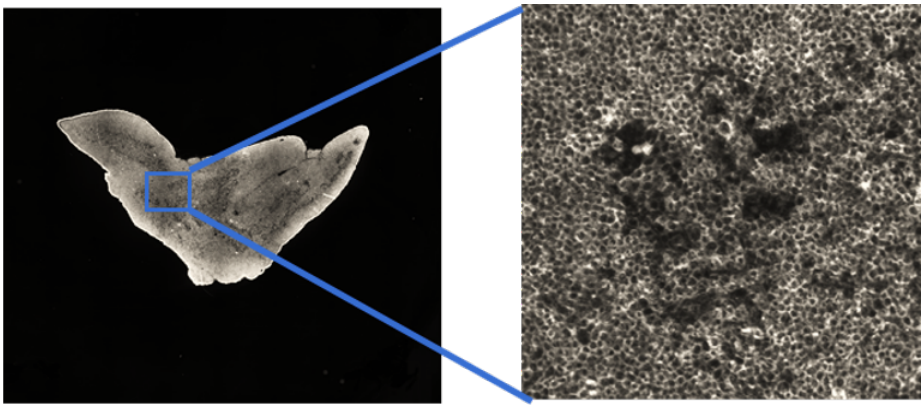


Figure 3. IF staining of the mouse thymus

The staining of the secondary antibody can be used to determine whether the primary antibodies are successfully bound to the target antigen. If unsuccessful, conduct troubleshooting and contact your local Field Application Scientist for more assistance.

m. Save the original tile (FOV) image files (stitching large and small image folders).

n. Open the StereoMap software and the Image Quality Control functional module in the software. Upload your nuclei-stained (DAPI) image, then run Image QC according to the StereoMap User Manual in the software.

The entire procedure from the coverslip mounting to the end of the imaging should be completed within 1 hr. To avoid photobleaching, do not expose the tissue under the fluorescence for a long time while waiting.

For tissues that are prone to RNA degradation, such as pancreas, proceed to thenext step immediately to avoid RNA degradation.

The captured nuclei-stained image needs to pass Image QC before further image analysis (image "register") in Stereo-seq Analysis Workflow (SAW) pipelines can be performed.

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

o. After imaging, remove the Stereo-seq Chip Slide from the microscope platform, keep the coverslip mounted, and place it on the laboratory bench for later use.

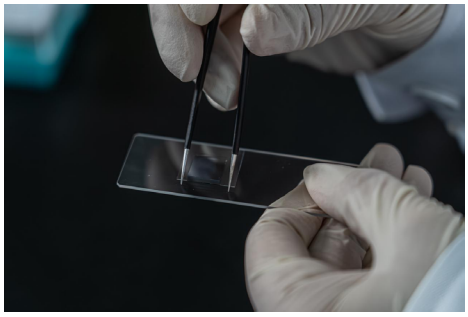
p. Prepare 70% DMSO solution in a fume hood according to Table 3-7. Vortex gently to mix thoroughly, and equilibrate to room temperature before use. Prepare it fresh before each experiment and use within 30 min after preparation.

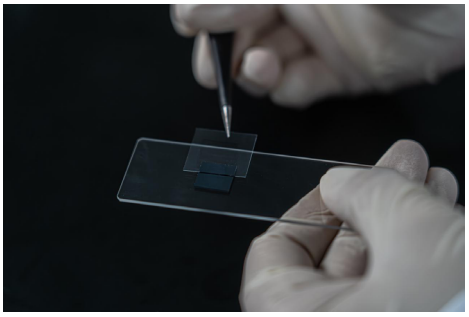
Table 3-7 70% DMSO

Component	1X (μL)	1X + 10% (μL)	2X + 10% (μL)	3X + 10% (μL)	4X + 10% (μL)
1X PBS	120	132	264	396	528
DMSO	280	308	616	924	1232
Total	400	440	880	1320	1760

3.8. Tissue Wash

a. Use clean forceps to grip the coverslip and then pull and slide the coverslip over the Stereo-seq Chip Slide edge slowly until the chips and the coverslip are completely separated.





b. Wipe off excess solution from around the edges and the back of the slide with Kimwipes delicate wipes without touching the chips. Make sure there is no solution residue.

c. There is no need to replace the gasket. Assemble the cassette and gasket, 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 glass slide.

d. Add **400 μL** Wash Buffer per well. Incubate **1 min** at room temperature.

e. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20°, and remove the buffer from the corner of the well using a pipette, keeping the chip and tissue surface moist.

f. Repeat wash **step d**.

g. Place the Stereo-seq Slide Cassette in a fume hood. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20°, and remove the Wash Buffer from the corner of the well using a pipette, keeping the chip and tissue surface moist.

h. Add **400 µL** 70% DMSO per well. Place **unpeeled** sealing tape on to the Slide Cassette and incubate for **5 min** at room temperature.

i. During incubation, prepare sufficient methanol to 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.

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

3.9. Fixation before Permeabilization

a. Discard the sealing tape and discard the 70% DMSO solution with a pipette.

b. Remove the slide from the Stereo-seq Slide Cassette according to the instructions in Appendix I: Stereo-seq Slide Cassette Assembly. Discard the used gasket and place the cassette on the bench for later use.

Do not touch the front of the chip.

c. Wash the slide by immersing it in a 50 mL centrifuge tube or a slide container with sufficient 0.1X SSC for **5 sec**, ensuring that all tissue sections are completely submerged. Discard the solution.

Take out the Stereo-seq Slide and wipe off excess solution on the slide with Kimwipes to ensure no liquid remaining.

d. After washing, immediately place the Stereo-seq Chip Slide in the -20°C precooled methanol that you prepared in **step i**. in section 3.8 and allow it to fix for **20 min**. Ensure that all tissue sections are completely submerged in methanol.

e. During the fixation process, set aside the 2 mL of 0.01N HCl and 0.5X Permeabilization Reagent (PR) Solution that you prepared in 3.1 Experiment Preparation.

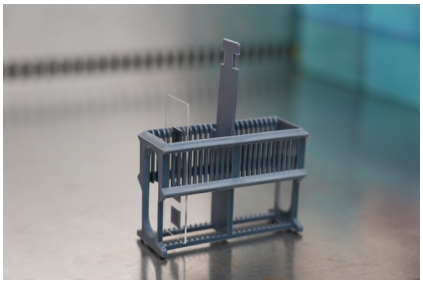
Table 3-8 0.5X Permeabilization Reagent Solution

Component	1X (µL)	1X + 10% (µL)	2X + 10% (µL)	3X + 10% (µL)	4X + 10% (µL)
0.01N HCl	142.5	156.8	313.5	470.25	627
10X Permeabilization Reagent Stock Solution	7.5	8.2	16.5	24.75	33
Total	150	165	330	495	660

f. Prewarm the 0.5X Permeabilization Reagent (PR) Solution in the 37°C PCR Thermal Cycler or metal bath for **10 min (no longer than 30 min)**.

g. After fixation, take the Stereo-seq Chip Slide out of the 50 mL centrifuge tube or the slide container. Wipe off the excess methanol from around the edges and the back of the slide with dust-free paper without touching the tissue. Ensure that there is no methanol residue between chips.

h. 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.



i. Assemble a new cassette and gasket, then place the Stereo-seq Chip Slide in the cassette according to the instructions in Appendix I: Stereo-seq Slide Cassette Assembly. Press along both edges of the Stereo-seq Cassette to ensure that the Stereo-seq Chip Slide is locked in place. Proceed to 3.10 Tissue Permeabilization immediately.

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

3.10. Tissue Permeabilization

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

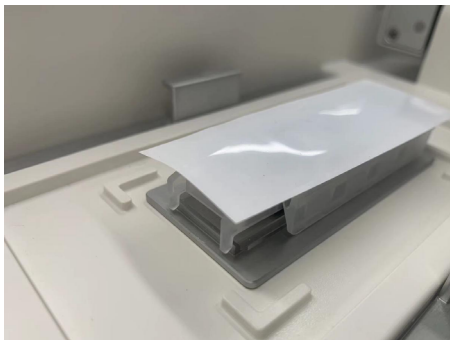
b. Ensure that the PCR Thermal Cycler has been set to the desired temperature (**the rows highlighted in bold below**).

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

c. Place the Stereo-seq Slide Cassette in the 37°C PCR Thermal Cycler with heated lid set to 60°C. Add **150 µL** of 0.5X Permeabilization Reagent Solution onto the chip at each corner of the chip.

Make sure the chip is completely covered with 0.5X Permeabilization Reagent (PR) Solution.

d. Apply a new unpeeled sealing tape onto the Stereo-seq Slide Cassette and let the chip incubate inside the PCR Thermal Cycler at 37°C with the heated lid closed for optimal permeabilization time.



The optimal permeabilization time and permeabilization enzyme concentration are pre-determined by permeabilization test experiments. In Stereo-CITE Proteo-transcriptomic experiments, it is recommended that you use 0.5X Permeabilization Reagent (PR) Solution to determine the permeabilization time. For more information, refer to the Stereo-seq Permeabilization Set for Stereo-CITE Proteo-transcriptomics Application User Manual (Document No.: STUM-PR004).

e. During the permeabilization, prepare RT mix according to Table 3-9 then leave it on ice until use.

Table 3-9 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	20	44	66	88
Total	200	440	660	880

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

g. Tilt the cassette slightly at an angle of less than 20°. Pipette to remove the Permeabilization Reagent (PR) Solution from a corner of the well.

Do not touch the chip surface.

h. Add **200 μL** Wash Buffer per well.

i. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20°, remove the Wash Buffer from the corner of the well using a pipette, and keep the chip and tissue surface moist.

It is recommended that you perform the process one chip at a time. Do not dry the chip.

3.11. Reverse Transcription

a. Set the temperature of the PCR Thermal Cycler with PCR Adaptor to 45°C in advance.

Temperature	Time	Number of cycles	Step
(Heated lid) 60°C	on	-	-
37°C	∞	1	Slide drying and permeabilization
45°C	**∞**	**1**	**Reverse Transcription**
55°C	∞	1	cDNA and ADT release

b. 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)**.

3.12. cDNA and ADT Release and Denaturation

a. Prepare cDNA Release Mix according to Table 3-10, and then place the mix at room temperature for later use.

Table 3-10 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	Slide drying and permeabilization
45°C	∞	1	Reverse Transcription
55°C	**∞**	**1**	**cDNA and ADT release**

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

When removing the sealing tape, hold on to the Stereo-seq Slide Cassette gently 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 of the cassette.

e. Add **200 µL** 0.1X SSC per well.

f. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20°, remove the 0.1X SSC from the corner of the well using a pipette, and keep the chip and tissue surface moist.

g. Add **180 µL** cDNA Release Mix per well.

The volume is 180 µL/chip, not 200 µL/chip.

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

i. When the incubation is competed, remove the Stereo-seq Slide Cassette from the PCR Thermal Cycler, place it on the bench, and carefully remove the sealing tape.

Do not allow the cDNA Release Mix to spill.

j. Pipette on the surface of the chip 10 times. The tissue on the surface of the chip will fall off. If a small amount of tissue residue remains, the subsequent steps can be continued without treatment.

Try not to allow the pipette tip to directly contact the bottom of the tube.

Do not pipette the cDNA Release Mix onto the gasket.

k. Tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20°. Use a pipette to completely collect the cDNA Release Mix from each well into a new 1.5 mL tube.

l. Use a pipette to measure the total volume of collected cDNA Release Mix. 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	5min	1
12°C	∞	-

Ensure that all the chip ID numbers on the slide have been recorded; this is required for downstream analysis.

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.

3.13. Precautions for Handling Magnetic Beads

Background Information

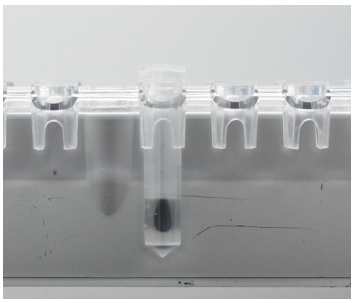
For bead-based purification, we recommend using AMPure® XP DNA Cleanup Beads (Beckman Coulter, Cat. No.: A63882), SPRIselect DNA Cleanup Beads (Beckman Coulter, Cat. No.: B23317/B23318/B23319), or VAHTS™ DNA Cleanup Beads (Vazyme, Cat. No.: N411-02). If magnetic beads from other sources are used, optimize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

Before Use

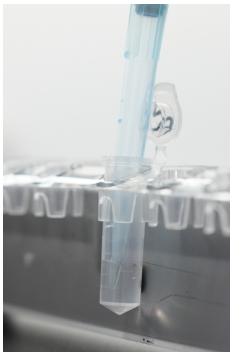
- To ensure the DNA capture efficiency of the magnetic beads, equilibrate the beads to room temperature 30 min before use.
- Vortex or pipette up and down to ensure that the beads are thoroughly mixed every time before use.
- The number of magnetic beads directly affects the distribution of purified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

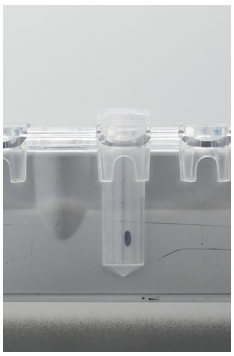
Operation Notes

- 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 take longer or shorter depending on the type of magnetic separation rack being used.

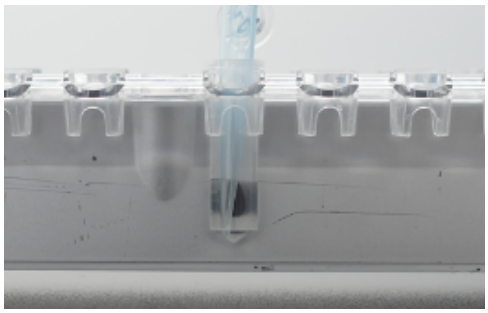


- When collecting the supernatant with a pipette after magnetic separation, avoid taking up the beads. Instead of collecting the entire supernatant fraction, leave 2-3 µL in the tube to prevent the pipette from directly contacting the beads. If the beads are accidentally taken up, dispense everything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

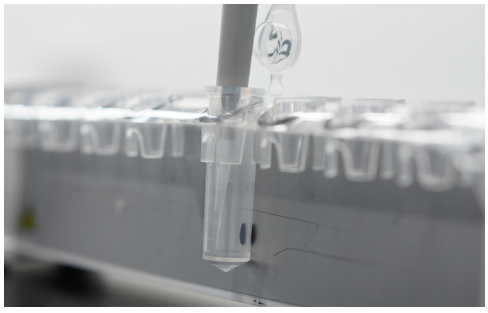




- Use freshly prepared 80% ethanol (at room temperature) to wash the beads. Keep the sample tube on the magnetic separation rack during the wash step. Do not shake or disturb the beads.

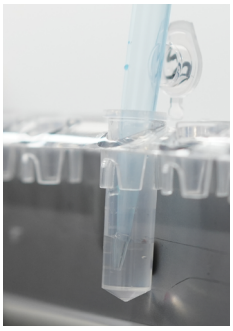


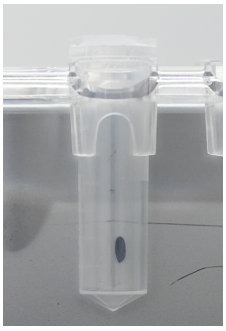
- After the second washing of the beads with ethanol, try to remove all liquid from the tube. You may centrifuge briefly to accumulate any remaining liquid at the bottom of the tube, then separate the beads magnetically, and then remove the remaining liquid by using a small volume pipette.



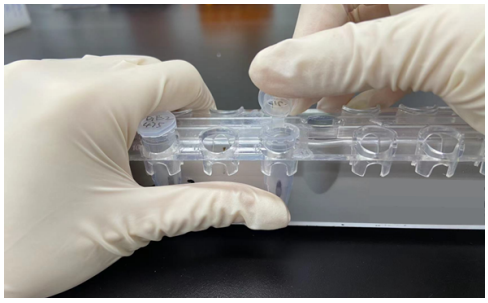
- After washing twice with ethanol, air-dry the beads at room temperature. Drying usually takes approximately **5-10 min** depending on the lab temperature and humidity level. Watch closely until the pellet appears sufficiently dry and has a matte appearance, then continue to the elution step with TE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

- During the elution step, do not touch the beads with the pipette tip when removing the supernatant. Contaminating 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.





- Be careful 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.



3.14. cDNA Pre-amplification and Purification

a. Prepare PCR Mix according to Table 3-11. After mixing by pipetting, centrifuge briefly, and then store PCR Mix on ice.

Table 3-11 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** of PCR Mix into each 0.2 mL PCR tube containing 66 µL of cDNA (from **step m.** in section 3.12). Vortex to mix well.

c. Briefly spin before placing the reaction tube in the PCR Thermal Cycler. Pre-amplify the cDNA according to the following PCR program.

Temperature	Time	Number of cycles
(Heated lid) 105°C	on	-
95°C	5min	1
98°C	20 sec	6
58°C	20 sec	
72°C	3 min	
72°C	5 min	1
12°C	Hold	-

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 according to Table 3-12.

Table 3-12 Qubit dsDNA Mix

Component	1X (µL)	1X + 10% (µL)	2X + 10% (µL)	3X + 10% (µL)	4X + 10% (µL)
Invitrogen™ Qubit dsDNA HS Buffer	199	218.9	437.8	656.7	875.6
Qubit dsDNA HS Reagent 200X	1	1.1	2.2	3.3	4.4
Total	200	220	440	660	880

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 The cDNA concentration is usually greater than 2 ng/µL.

g. After cDNA pre-amplification, perform cDNA Purification (**0.8X**):

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

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 on a magnetic rack for **3-5 min** until the liquid is clear.

4) Using a pipette, carefully transfer the supernatant to a new 1.5 mL tube. **The supernatant contains ADT products** and can be stored at room temperature temporarily. Refer to section 3.16 ADT Product Purification.

Do not discard the supernatant!

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 then 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 be approximately **5-8 min**.

8) Add **44 µL** of nuclease-free water to the dried beads. Mix the beads and nuclease-free water by vortexing. Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube on a magnetic rack for **3-5 min** until the liquid is clear.

9) Transfer the supernatant (**~42 µL**) to a new 0.2 mL PCR tube.

h. If the amount of collected cDNA is less than **42 µL**, simply top it up with nuclease-free water.

Store the beads with 40 µL of nuclease-free water at 4°C after collecting the eluted cDNA, until your final cDNA product has passed QC.

3.15. cDNA Product Amplification and Purification

a. Prepare cDNA PCR Mix according to Table 3-13. The total volume for the PCR reaction is 100 μL.

Table 3-13 cDNA PCR Mix

Component	1X (μL)
PCR Amplification Mix	50
cDNA Primer	8
Purified cDNA Products	42
Total	100

b. Mix gently using a pipette. Briefly spin the cDNA PCR Mix and amplify the eluted cDNA according to the following PCR program.

Temperature	Time	Number of cycles
(Heated lid) 105°C	on	-
95°C	5min	1
98°C	20 sec	7
58°C	20 sec	
72°C	3 min	
72°C	5 min	1
12°C	Hold	-

While waiting for the eluted cDNA amplification to be completed, refer to section 3.16 ADT Product Purification.

c. Prepare Qubit dsDNA Mix according to Table 3-12 in **step d.** in section 3.14.

d. Vortex the Qubit dsDNA Mix, then take **199 μL** of the Qubit dsDNA Mix and place it in a Qubit detection tube (from the Qubit dsDNA HS Assay Kit).

e. 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 The cDNA concentration is usually greater than 10 ng/μL.

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

f. cDNA purification (**0.8X**):

1) Mix the cDNA PCR product (**100 µL**) with 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 sample tube onto a magnetic rack for **3 min** until the liquid is clear. Carefully remove and discard the supernatant with a pipette.

4) Keep the tube on the magnetic separation rack and add **200 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.

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) is visible. Drying times will vary but will take approximately **5-8 min**.

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

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 final cDNA product has passed QC.

g. Take **1 µL** of the cDNA sample, measure the concentration of the purified cDNA using the Qubit dsDNA HS Kit, and then record it.

h. 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), or Fragment Analyzer™ (Advanced Analytical).

A qualified cDNA sample should have fragment distribution of 200- 2,000 bp (Figure 4) and a yield greater than 100 ng. The distribution of cDNA fragments is related to tissue type and RNA quality. It is normal for cDNA fragments to be shorter in some tissues, and subsequent experiments can be continued.

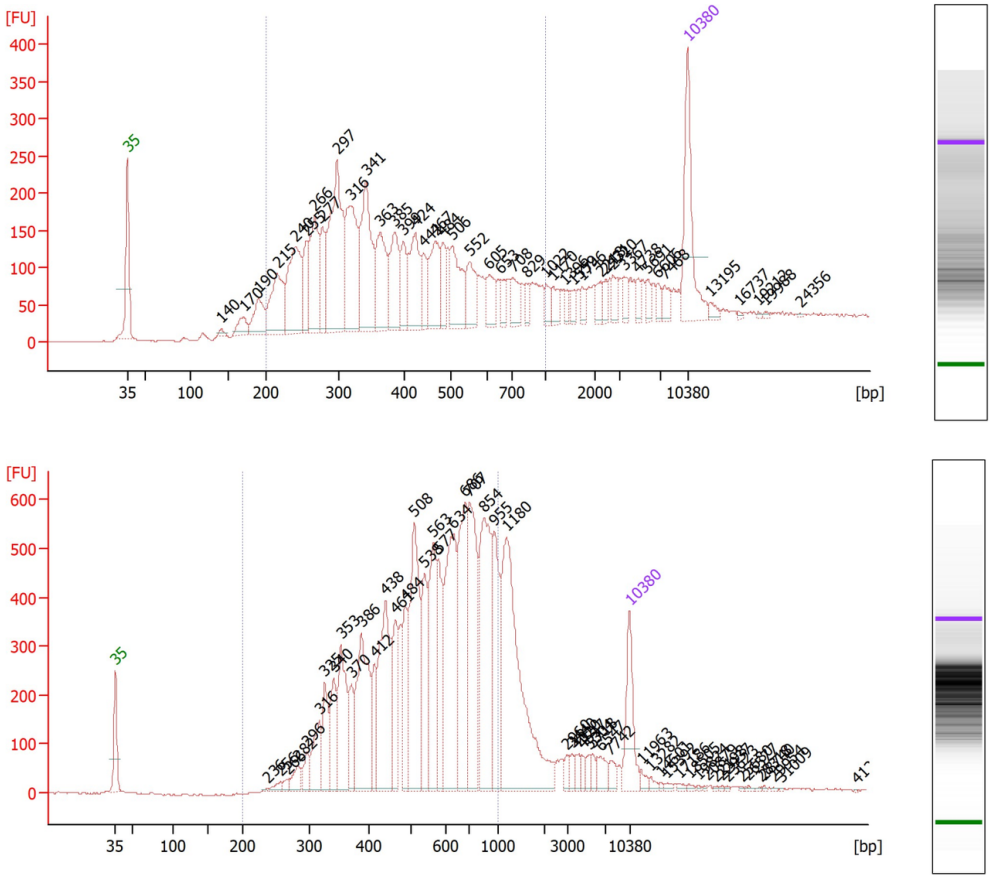


Figure 4 Representative Agilent Bioanalyzer 2100 analysis results of cDNA amplified products. Mouse thymus tissue (top) Human tonsil tissue (bottom)

Refer to the **Stereo-CITE Proteo-Transcriptomics Library Preparation User Manual (Document No.:STUM-LP003)** for details on subsequent library preparation.

3.16. ADT Product Purification

a. Ensure that the magnetic beads have been equilibrated to room temperature for at least 30 min, and prepare fresh 80% ethanol.

b. ADT products purification with 2.0X magnetic bead:

1) Mix the collected supernatant in the 1.5 mL tube collected in **section 3.14, step d., substep 4)** with 360 µL beads.

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

3) Spin down and place the tube on a magnetic rack for **5 min** until the liquid is clear.

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

5) Keep the 1.5 mL tube on the magnetic separation rack and add **1 mL** of freshly prepared 80% ethanol. Wash the beads by rotating the tube on the magnetic rack. Incubate for **30 sec**, and then 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 **44 µL** of nuclease-free water to the dried beads. Mix the beads and nuclease-free water by vortexing. Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube on a magnetic rack for **3-5 min** until the liquid is clear.

9) Transfer the supernatant (**~42 µL**) into a new 0.2 mL PCR tube.

c. If the amount of collected eluted ADT is less than **42 µL**, top it up with nuclease-free water.

Store the beads with 40 µL of nuclease-free water at 4°C after collecting the eluted ADT until your final ADT product has passed QC.

3.17. ADT Product Amplification and Purification

a. Prepare PCR Mix according to Table 3-14. The total volume for the PCR reaction is **100 µL**.

Table 3-14 ADT PCR Mix

Component	1X (µL)
PCR Amplification Mix	50
ADT Primer Mix A	8
Eluted ADT Product	42
Total	100

b. Mix gently and briefly spin before placing the reaction tube in the PCR Thermal Cycler. Amplify the eluted ADT product according to the following PCR protocol.

Temperature	Time	Number of cycles
(Heated lid) 105°C	on	-
95°C	5min	1
98°C	20 sec	18
58°C	20 sec	
72°C	1 min	
72°C	5 min	1
12°C	Hold	-

c. Prepare Qubit dsDNA Mix and record the concentration of PCR product according to Table 3-12 in **step d.** in section 3.14.

d. After the preparation is completed, vortex and mix well, and then transfer **199 μL** into a new Qubit tube.

e. After vortexing, take **1 μL** of the PCR product, measure and record the concentration using Qubit dsDNA HS Assay Kit.

QC

The DNA concentration is usually greater than 5 ng/μL.
For troubleshooting purposes, we recommend saving 2 μL of the ADT PCR products.

f. ADT PCR product purification (**2.0X**):

1) Transfer the ADT PCR product (**100 μL**) to a new 1.5 mL centrifuge tube, and mix the product with beads in a ratio of **1:2**.

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

3) Spin down and place the sample tube on a magnetic rack for **5 min** until the liquid is clear.

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

5) Keep the tube on the magnetic rack and add **300 μL** of freshly prepared 80% ethanol to wash the beads by rotating the tube on the magnetic separation rack. Incubate for **30 sec**, then 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) Vortex the dried beads with **100 µL** of TE Buffer. Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube on a magnetic rack for **3-5 min** until the liquid is clear. Transfer **98 µL** supernatant to a new 1.5 mL centrifuge tube.

Stop Point: **The purified ADT PCR product 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 purified ADT PCR product has passed QC.

g. Take **1 µL** of the purified ADT PCR product and measure the concentration of the purified ADT PCR product using the Qubit dsDNA HS Kit, and then record it.

h. 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), or Fragment Analyzer™ (Advanced Analytical).

Purified ADT PCR product should have a fragment distribution of 180- 230 bp (Figure 5) with a yield greater than 100 ng.

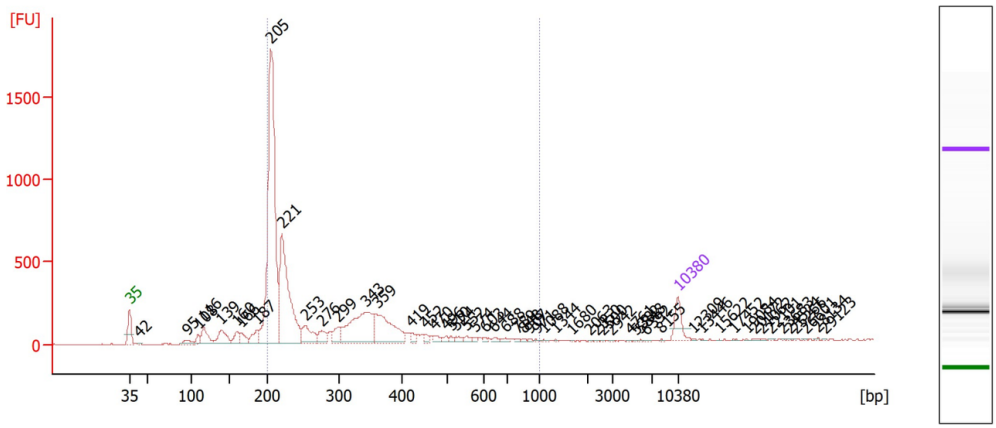


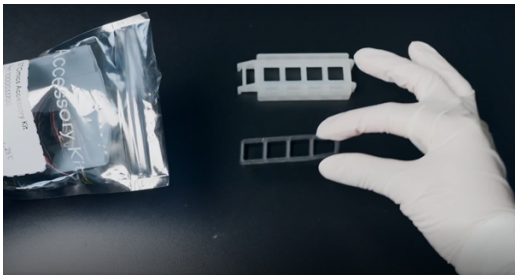
Figure 5 A representative Agilent Bioanalyzer 2100 analysis results of purified ADT PCR product

Refer to the Stereo-CITE Proteo-Transcriptomics Library Preparation User Manual (Document No.: STUM-LP003) for details on subsequent library preparation.

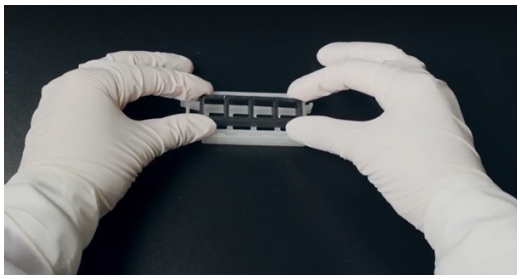
Appendix I: Stereo-seq Slide Cassette Assembly

Stereo-seq Slide Cassette Assembly

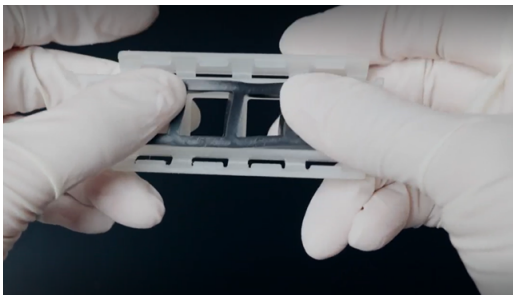
a. Take the Stereo-seq Slide Cassette and Gasket out of the STOmics Stereo-seq Accessory Kit.



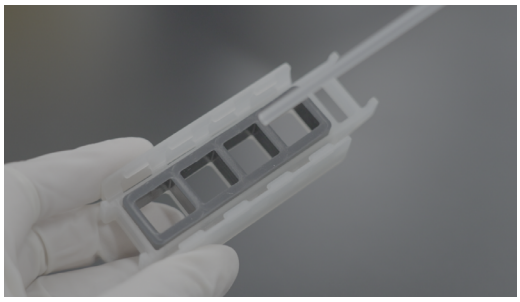
b. Pick up the Stereo-seq Slide Cassette and flip it over. Insert the gasket into the Stereo-seq Slide Cassette, ensuring that the cutouts are aligned.



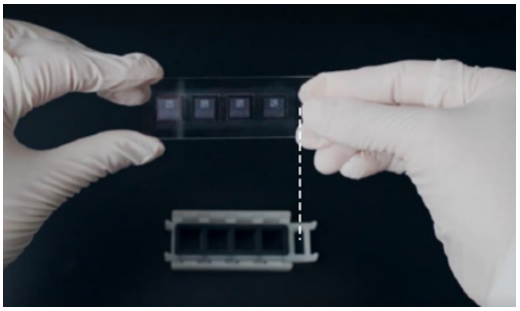
c. Press the gasket down to secure it in the cassette.



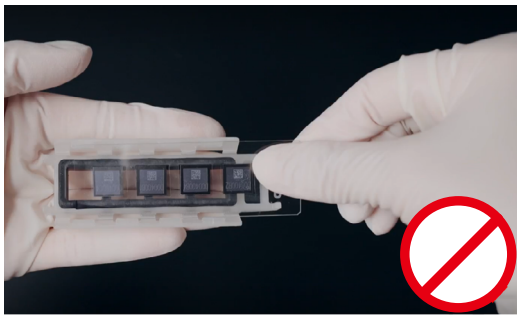
d. If necessary, use a power dust remover to blow any debris off the gasket.



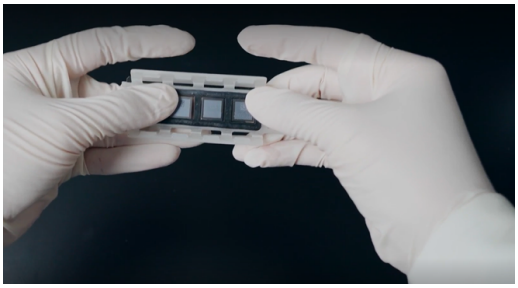
e. Pick up the Stereo-seq Chip Slide and flip it over with the chip surface facing down. Align the engraved label with the long edge of the Stereo-seq Slide Cassette.



f. Ensure that the chips are aligned within the empty space of the gasket, and do not touch the chip surface with the gasket or cassette during slide placement. Insert the Stereo-seq Chip Slide under the bottom 4 tabs.

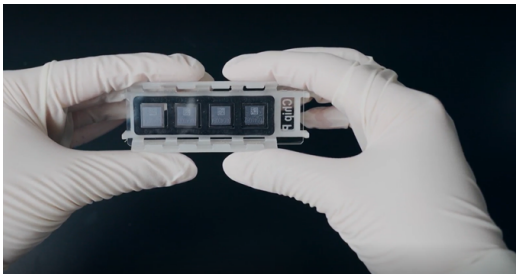


g. Support the back of the cassette with both middle fingers. Place your left thumb between tab 1 and tab 2 and place your right thumb between tab 3 and tab 4.

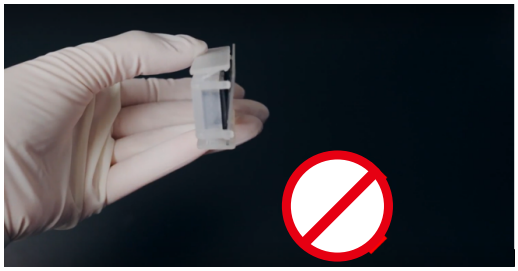


h. Press the upper side (A side) of the slide (near the edge) evenly and then simultaneously press the top edge down firmly with both index fingers to clip the slide in place until you hear it click.

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



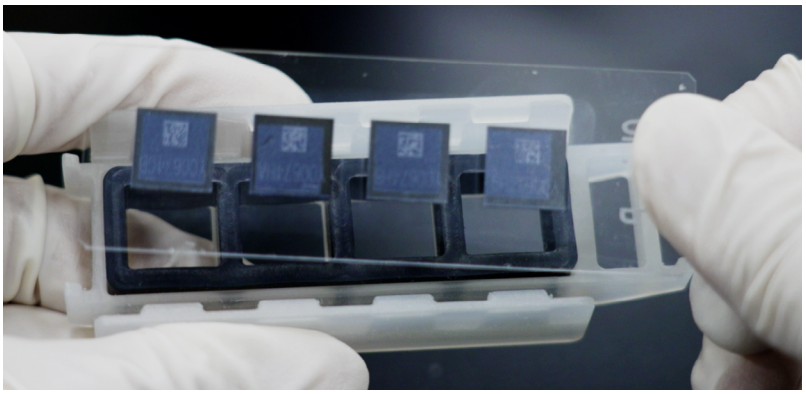
j. Recheck the Stereo-seq Slide Cassette and verify that the slide is clipped in place.



Stereo-seq Slide Cassette Removal

1. 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.

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



Appendix II: Antibody Titration

WORKFLOW

TOTAL TIME: ~3 HRS

Biolegend's TotalseqA antibodies are developed for single-cell CITE-seq technology and may not be suitable for tissue sections. For users who choose their own antibodies, we recommend that you first perform antibody titration on tissue sections of interest to select the most appropriate antibody concentration before proceeding to the standard operating procedure.

Experiment Preparation

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

Reagent	Preparation Steps	Maintenance
4% PFA	Mix well after thawing at 4°C. Prepare at least 30 mL and equilibrate to room temperature before use.	4°C up to 1 week
5X SSC	Dilute 1 mL of 20X SSC to 4 mL	Room Temperature
0.1X SSC	Dilute 500 µL of 20X SSC to 100 mL	Room Temperature
Filtered Serum Aliquot	Thaw the horse serum, then filter it with a 0.22 µm pore-sized filter membrane and a sterilized syringe. For convenience, aliquot the filtered serum in 200 µL per tube and store at -20°C. Thaw the aliquoted serum on ice and centrifuge at 14,000 g for 10 min at 4°C. Place on ice until use. Approximately 20 µL of serum is needed for one section, and any remaining serum can be reused.	On ice until use, up to 6 hr

Do not freeze and thaw the aliquot more than 3 times.

Reagent	Preparation Steps	Maintenance
10% Triton X-100	Use 10% Triton X-100 or dilute 100% Triton X-100 with nuclease-free water.	Room temperature
Sheared Salmon Sperm DNA	Thaw on ice before use, 20 µL/section.	On ice until use, up to 6 hr
Primary and Secondary Antibody	Thaw on ice following the original product instructions, and centrifuge for 10 min at 14,000 g. The Primary Abs should include the isotype Abs.	On ice until use, up to 6 hr
Diluted Primary and Secondary (Optional)	Dilute the antibodies if needed.	On ice until use, up to 6 hr
FcR Blocking Reagent	For mouse samples, use TruStain Fcx™ PLUS (anti-mouse CD16/32) Antibody; for human samples, use TruStain Fcx™.	On ice until use, up to 6 hr
Glycerol	Equilibrate to room temperature 5 min in advance, use 5 µL per section.	Room temperature

Equipment	Set up	Note
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.
PCR Thermal Cycler	Set the temperature to 37°C for slide drying.	Check the PCR Thermal Cycler for any abnormalities and replace it if necessary.
Refrigerated Centrifuge	Centrifuge the cocktail lyophilized powder at room temperature, then set the centrifuge temperature to 4°C in advance.	4°C is used for centrifugation of serum, primary antibodies, secondary antibodies, and reconstituted antibody cocktails.
Fluorescence Microscope	Make sure the microscope is equipped with at least DAPI, FITC, TRITC, CY5	Select the channels according to the fluorescent emission wavelengths of your secondary antibodies.

Cryosection Preparation

a. Set the PCR Thermal Cycler to 37°C with heated lid set to 60°C in advance with a PCR Adaptor.

Temperature	Time	Number of cycles
(Heated lid) 60°C	on	-
37°C	∞	1

b. Set cryostat chamber temperature to -20°C and the 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. Transfer the OCT-embedded tissue sample from the -80°C freezer, and place it in the cryostat chamber. Allow it to equilibrate to the cryostat chamber temperature for **30 min**.

e. Meanwhile, take enough 4% PFA solution in a slide container, ensure that all tissue sections can be completely submerged. Equilibrate the 4% PFA solution to room temperature in a fume hood.

f. During the temperature equilibration step at cryosection preparation, prepare the blocking solution according to Table B-1 and place it on ice until use.

Table B-1 Blocking Solution

Component	1X (µL)	1X + 10% (µL)	2X + 10% (µL)	3X + 10% (µL)	4X + 10% (µL)
5X SSC	120	132	264	396	528
10% Triton X-100	2	2.2	4.4	6.6	8.8
Sheared Salmon Sperm DNA	20	22	44	66	88
FcR Blocking Reagent*	10	11	22	33	44
Filtered Serum Aliquot	20	22	44	66	88
Nuclease-free water	28	30.8	61.6	92.4	123.2
Total	200	220	440	660	880

*FcR Blocking Reagent is for blocking Fc receptors on the cell membrane. Select either one according to the host species of your sample. For mouse tissue, use Human TruStain FcX™ (Fc Receptor Blocking Solution, Cat. No.: 156604), and for human tissue, use TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody (Cat. No.: 422031).

1X blocking solution is enough for blocking and preparation of primary antibody incubation buffer of one tissue section.

g. Remove the sample outer covers (aluminum foil) and trim the embedded tissue block to the appropriate size (sectioning area smaller than 0.9 cm x 0.9 cm).

h. By using OCT, mount the embedded tissue block onto the specimen disc/holder of the cryostat chamber.

i. Trim again if necessary. Now, the specimen is ready for cryosection.

Tissue Mounting on Microscope Slides

a. Tissue mounting can be achieved using either the cold method (option A) or the warm method (option B). We recommend placing one tissue slice on one microscope slide (if validating one antibody, four tissue sections are minimally required, one of which is used as a negative control).

b. Choose proper slide thickness according to the experiment needs, normally use 10 μm.

A. Cold Method

1) Place the microscope slide inside the chamber with the front facing up and pre-cool the slide inside the cryostat chamber for no less than 1 min.

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. Make sure the tissue section is complete and without wrinkles.

3) Immediately pick up the microscope slide and place a finger on the back of the microscope slide directly under the tissue for a few sec to allow the section to adhere to the surface.

4) Immediately dry the microscope slide at 37°C in a PCR Thermal Cycler with PCR Adaptor for **5 min**.

B. Warm Method

1) Perform cryosection and carefully flatten the tissue sections out by gently touching the surrounding OCT with cryostat brushes.

2) Move the tissue section to the edge, flip the microscope slide over and align the tissue section onto the center of the slide by gently touching the section with the microscope slide.

3) Check if the section has been successfully mounted.

4) Flip the microscope slide over, front-side up, and immediately dry it at 37°C in a PCR Thermal Cycler with PCR Adaptor for **5 min (without heated lid)**.

Tissue Fixation

a. Remove the microscope slide from the PCR Adapter, immerse it in the previously prepared 4% PFA solution that has been equilibrated to room temperature, and allow it to fix for **5 min** in the fume hood.

b. During fixation, prepare enough 0.1X SSC in a slide container, ensure that all tissue sections can be completely submerged.

c. When the fixation is completed, take the microscope slide out of the 4% PFA solution and immediately immerse it in the slide container with sufficient 0.1X SSC for **10 sec**.

Slide transfer should be done as soon as possible to prevent the tissue from over-drying.

d. Take out the slide and immediately wipe off excess 0.1X SSC from around the edges and the back of the slide with Kimwipes delicate wipes, without touching the tissue, to ensure that there is no residual liquid surrounding the tissue.

e. Use a Super PAP Pen (hydrophobic barrier pen) on the microscope slide to draw a circle around the tissue on the slide, creating a hydrophobic isolation zone that prevents subsequent additional fluid outflow.Transfer the processed slides to an immunohistochemistry wet cassette.

Subsequent fluid changes are carried out in the hydrophobic isolation area.

f. Transfer the processed slides to an immunohistochemistry (IHC) humidity box.

All the liquid must be added until the hydrophobic isolation area dries.

Tissue Blocking and Primary antibody incubation

a. Vortex the blocking solution that you prepared according to Table B-1 and add no more than **100 µL**/section of blocking solution drop-by-drop on the tissue surface within the hydrophobic area. Incubate for **20 min** at room temperature.

The amount of blocking solution used per section is dependent on the size of the hydrophobic area. For a hydrophobic area size of 0.5 cm × 0.5 cm, the recommended blocking solution volume is 30 µL/section.

b. During the incubation, prepare the Primary Antibody Incubation Buffer according to Table B-2. Vortex the buffer and leave it on ice until use.

Using CD68 antibody as an example, antibody titration tests are set for 1:100, 1:250, 1:500 and negative control without primary antibody.

Table B-2 Primary Antibody Incubation Buffer (for Antibody Titration)

Component	1X (µL)
Primary antibody or diluted primary antibody*	V^
Blocking Solution	100-V
Total	100

*If the volume required for the primary antibody is lower than the lowest nominal capacity of the pipette, the primary antibody should be diluted in advance with the blocking solution.
^The amount of primary antibody required is dependent on the dilution ratio.

c. Discard the blocking solution with a pipette.

- For experiment groups: Slowly add no more than **100 µL/slide** of the primary antibody solution from the non-tissue area until the solution covers the tissue section. Be sure to label the dilution ratio on the microscope slide. Incubate at room temperature for **45 min**.
- For negative control group: Add no more than **100 µL/slide** of blocking solution. Incubate at room temperature for **45 min**.

Do not allow the tissue to dry out during the solution addition process. Dried tissue might generate non-specific signals that interfere with the final results.

d. During primary antibody incubation, prepare the secondary antibody solution according to Table B-3. After vortexing and brief centrifugation, leave the secondary antibody solution on ice **in the dark** until use.

Table B-3 Secondary antibody Incubation Buffer (for Antibody Titration)

Component	1X (µL)
5X SSC	60
Secondary Antibody	0.2
Nuclease-free Water	39.8
Total	100

We recommend using Alexa Fluor Plus Series Fluorescent Secondary Antibodies at a 1:500 dilution concentration. If fluorescent secondary antibodies from other vendors were used, adjust the dilution ratio according to the manufacturer's instructions.

Secondary Antibody Incubation

a. Discard the primary antibody incubation solution (experiment groups) and the blocking solution (negative control group) with a pipette.

b. Wash by adding no more than **100 µL/slide** of 0.1X SSC. Incubate at room temperature for **1 min** and then discard.

c. Repeat **step b**.

Do not dry the tissue during the solution addition process. Otherwise, dried tissue might generate non-specific signals which interferes with the final results.

d. Slowly add no more than **100 µL/section** of secondary antibody incubation solution from the non-tissue area in both the experiment groups and the negative control group. Incubate at room temperature **in the dark** for **15 min**.

e. Discard the secondary antibody incubation solution using a pipette.

f. Wash the slide by immersing it in a slide container with enough 0.1X SSC for 10s, ensure that all tissue sections are completely submerged. Discard the solution.

g. Repeat **step f**.

h. Hold the slide with one hand and completely dry it using a power dust remover held in the other hand at an approximate distance of 2-3 cm from the slide surface. Blow gently from one side of the slide at a 30-degree angle horizontal to the plane of the slide.

i. Using a pipette, carefully add **5 µL** of glycerol onto the center of the tissue on the slides without introducing air bubbles.

Imaging

a. Fluorescence images are taken using a fluorescence microscope with image stitching function. Refer to the **STOmics Microscope Evaluation Handbook for specific microscope requirements**.

b. Select the epifluorescence scanning mode, adjust the fluorescence channel according to the fluorescence excitation light of the secondary antibody used, and scan using the 10X objective lens. After scanning, save both the FOV images and the stitched images.

Image all experiment groups with the same imaging parameters to compare thesignal differences across groups.

If there is any region with extremely high signal in the field of view, wash the slide by immersing it in a slide container with enough 0.1X SSC for 10s, ensure that all tissue sections are completely submerged. Discard the solution. Repeat the washing one more time.

Guidelines for Selecting Optimal Antibody Concentration

The principle of optimal antibody concentration selection is to select the optimal concentration that results in the best fluorescent signal of desired cells while minimizing nonspecific background staining.

Using the CD68 antibody titration sample data as an example, as shown in Figure 6, select the lowest concentration that does not significantly reduce the fluorescence intensity. In this example, 1:250 should be selected as the optimal antibody dilution concentration for subsequent official Stereo-CITE Proteo-Transcriptomics experiments.

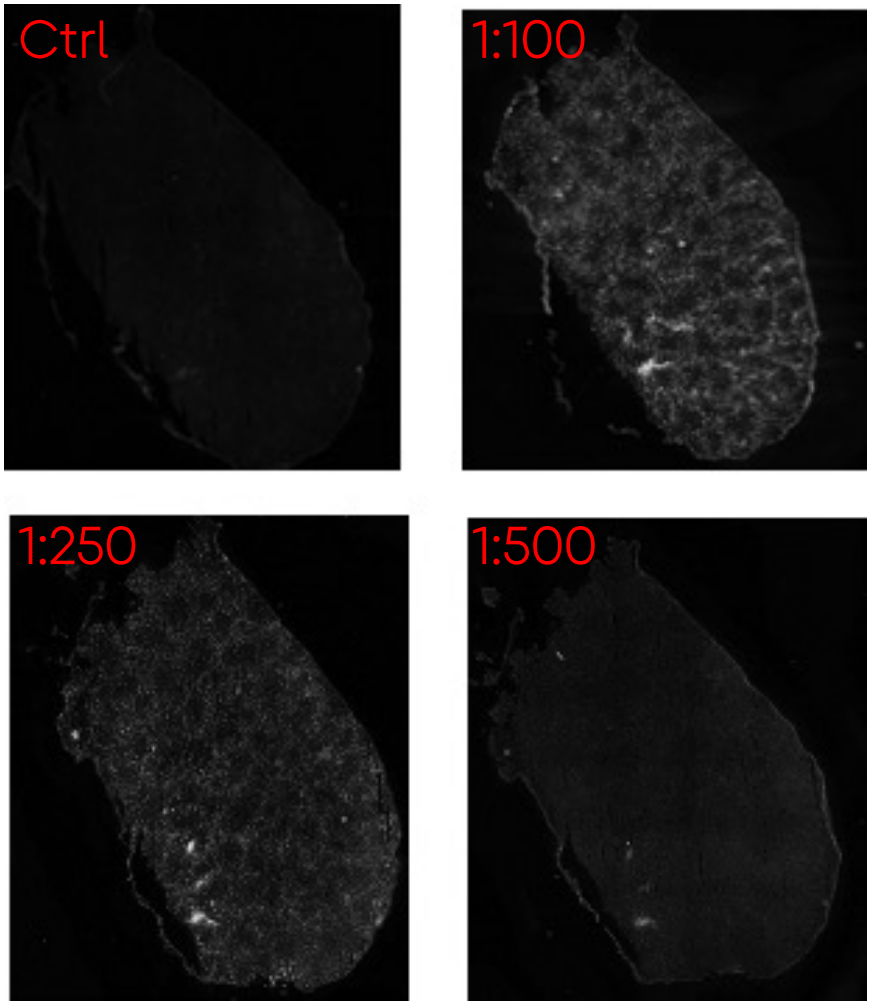


Figure 6. Immunofluorescent staining of anti-CD68 antibody titration in mouse liver