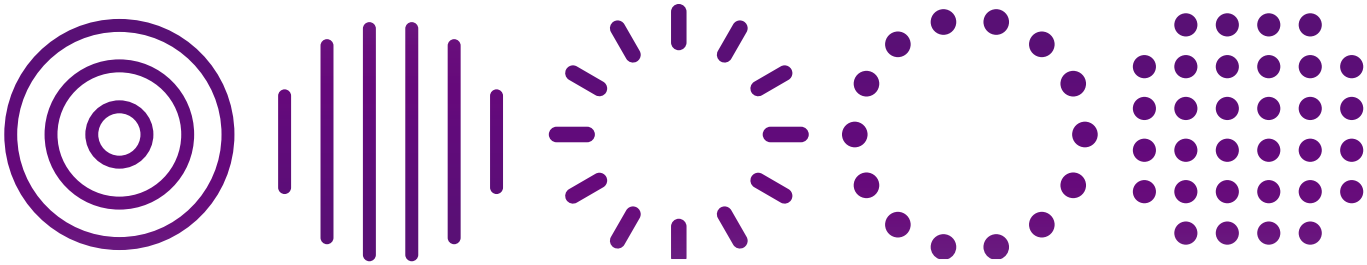
STOmics

STEREO-CITE PROTEO-TRANSCRIPTOMICS SET

USER MANUAL



Cat. No.: 211PT114(4 RXNs)
Kit Version: V1.0
Manual Version: A

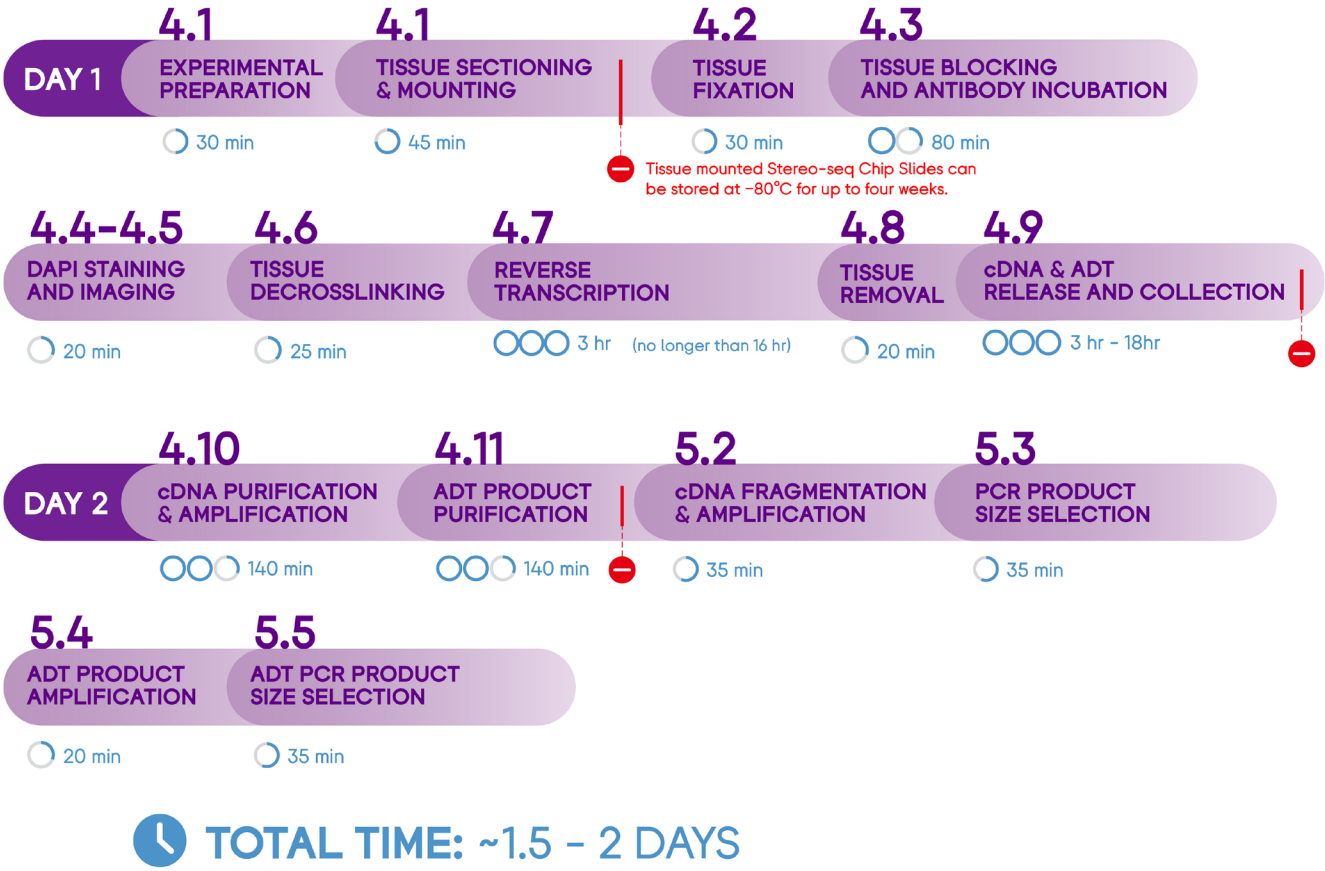
STUM-TT005

REVISION HISTORY

Manual Version:	A
Kit Version:	V1.0
Date:	Mar. 2024
Description:	Initial release

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

WORKFLOW



TOTAL TIME: ~1.5 - 2 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

STOmics Stereo-CITE Proteo-Transcriptomics Set is intended for simultaneous detection of 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, 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, perform 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 via the Stereo-CITE Proteo-Transcriptomics Set requires the DNBSEQ sequencing platform. For details refer to **Chapter 6: Library Construct and Sequencing** of this manual.

1.3. List of Kit Components

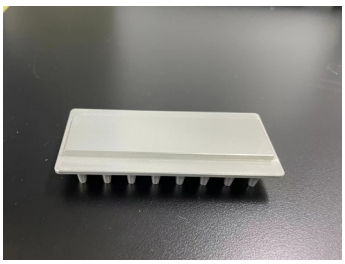
Each Stereo-seq Transcriptomics Set for Chip-on-a-slide consists of:

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

The Stereo-seq Library Preparation Kit is not included in the Stereo-seq Transcriptomics Set for Chip-on-a-slide kit and needs to be purchased separately. If you wish to construct a Stereo-seq library in-house refer to **Chapter 5: Library Preparation** for more detail.

Compatible auxiliary not included:

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



Further information on catalog numbers, kit components, and specifications are listed below (next page).

Upon receiving the Stereo-seq Chip T Slide (1cm*1cm), follow the instructions in Stereo-seq Chip P Slide_Stereo-seq Chip T Slide Operation Guide For Receiving, Handling And Storing to properly store unused Stereo-seq Chip T Slides.

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

Table 1-1 Stereo-seq Transcriptomics T Kit

Stereo-seq Transciptomics T Kit	Cat. No.:111KT114		
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (tube)**
RI	1000028499		300 μL × 1
PR Enzyme	1000028500		10 mg × 1
PR Rinse Buffer	1000042897		880 μL × 1
Glycerol	1000031615		50 μL × 1
RT Reagent	1000042898	(transparent)	720 μL × 1
RT Oligo	1000028508	(transparent)	1 OD × 1
RT Additive	1000028502	(transparent)	44 μL × 1
ReverseT Enzyme	1000042899	(transparent)	44 μL × 1
TR Buffer	1000028505		1725 μL × 2
cDNA Release Enzyme	1000028511		88 μL × 1
cDNA Release Buffer	1000028512		1725 μL × 2
cDNA Primer	1000028513		36 μL × 1
cDNA Amplification Mix	1000028514		220 μL × 1
Storage Temperature: -25°C~-18°C	Transported by cold chain	Expiration Date: refer to label	

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

Stereo-seq Chip T Slide (1cm*1cm) Cat. No.: 210CT114			
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (per kit)**
Stereo-seq Chip T (1cm * 1cm)	-	-	4 EA
Storage Temperature: -25°C~8°C	Transported by cold chain		Expiration Date: refer to label

Table 1-3 Stereo-seq Protein Assisted Kit

Stereo-seq Protein Assisted Kit Cat. No.: 212KA114			
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (per kit)**
Decrosslinking Reagents	1000043549	●	1725 µL x 2
ADT Amplification Kit	1000043547	●	220 µL x 1
ADT Primer Mix	1000043548	●	36 µL x 1
Blocking Reagents	1000044666	○ (transparent)	60 µL x 1
Storage Temperature: -25°C~ -18°C	Transported by cold chain		Expiration Date: refer to label

Table 1-4 STOmics Accessory Kit

STOmics Accessory Kit Cat. No.: 1000033700			
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (per kit)**
Cassette	1000033699	-	1 EA
Gasket	1000033698	-	4 EA
Sealing Tape	1000042970	-	6 EA
Storage Temperature: Room Temperature	Transported at room temperature		Expiration Date: refer to label

Table 1-5 Stereo-seq PCR Adaptor

Stereo-seq PCR Adaptor Cat. No.: 301AUX001			
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (per kit)**
Stereo-seq PCR Adaptor	-	-	2 EA
Storage Temperature: Room Temperature	Transported at room temperature		Expiration Date: refer to label

1.4. Additional Equipment and Materials

The tables below list equipment and materials needed for this protocol. Some common laboratory equipments not named in Table 1-6 are expected to be accessible by the user; for instance, an ice maker, biological safety cabinet, freezers, and so on. For specific microscope requirements, refer to **STOmics Microscope Assessment Guideline.**

Table 1-6 Additional equipment required

Equipment		
Brand	**Description**	**Cat. No.**
-	Cryostat	-
-	Benchtop centrifuge	-
-	Pipettes	-
-	pH meter	
-	Metal heating block dry bath (optional)	-
-	Vortex mixer	-
Bio-Rad*	T100 Thermal Cycler	1861096
ABI*	ProFlex 3 x 32-well PCR System	4483636
Labnet	Slide Spinner (optional)	C1303-T
NEBNext®	Magnetic Separation Rack for <200 µL tubes	S1515S
Thermo Fisher Scientific	Magnetic rack DynaMag™-2 for 1.5-2mL tubes	12321D
	Qubit™3 fluorometer	Q33216
Agilent Technologies™	Agilent 2100 bioanalyzer	G2939AA (or similar)

Choose either one of the listed brands (with * mark). Suitable PCR adaptor will be needed.

Reagents		
Brand	**Description**	**Cat. No.**
-	100% Ethanol (Analytical grade)	-
Gibco	PBS (10X), pH 7.4 - for PFA fixed tissue	70011044
BOSTER (or other brands)	4% Paraformaldehyde (with DEPC) - for PFA fixed tissue	AR1069
Ambion	Nuclease-free water	AM9937
	20X SSC	AM9770
	1X TE buffer, pH 8.0	AM9858

*Beckman Coulter	AMPure® XP	A63882
	SPRIselect	B23317/B23318/ B23319
*VAZYME	VAHTS™ DNA Clean Beads	N411-02
Sigma Aldrich	Hydrochloric acid, HCl (0.1 N)	2104-50ML
	Methanol	34860-1L-R
SAKURA	SAKURA Tissue-Tek® O.C.T. Compound	4583
Invitrogen	Salmon Sperm DNA (1mg/mL)	AM9680
	Qubit dsDNA HS Assay Kit	Q32854
Agilent Technologies™	High sensitivity DNA kit	5067-4626
	High sensitivity RNA kit	5067-1513
Thermo Fisher Scientific™	DAPI Solution (1 mg/mL)	62248
	Gibco™ Horse Serum*	26050070
	Gibco™ Goat Serum*	16210064
	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.

BioLegend	TotalSeq-A™ singular 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 Bloacking Reagent is for blocking of Fc receptors on the cell membrane. Please select either one according to the host species of your sample. Please choose Human TruStain FcX™ (Fc Receptor Blocking Solution) for human tissues and choose TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody for mouse tissues. However, TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody is not recommended if the host species of the primary antibody is Rat.

Choose either one of the listed brands (with * mark). Suitable PCR adaptor will be needed.

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. Please feel free to combine the singular TotalSeq-A™ antibody depending on your experimental design. All the Singular TotalSeq-A™ antibodies to be used must have their optimal concentrations determined by titration before combination. Please find more information in Appendix C. The species of most of the TotalSeq-A™ antibodies from Biolegend are humans and mice. For detailed information, please refer to the Biolegend website: https://www.biolegend.com/en-us/search-results?PageSize=25&Format=TOTALSEQ_A

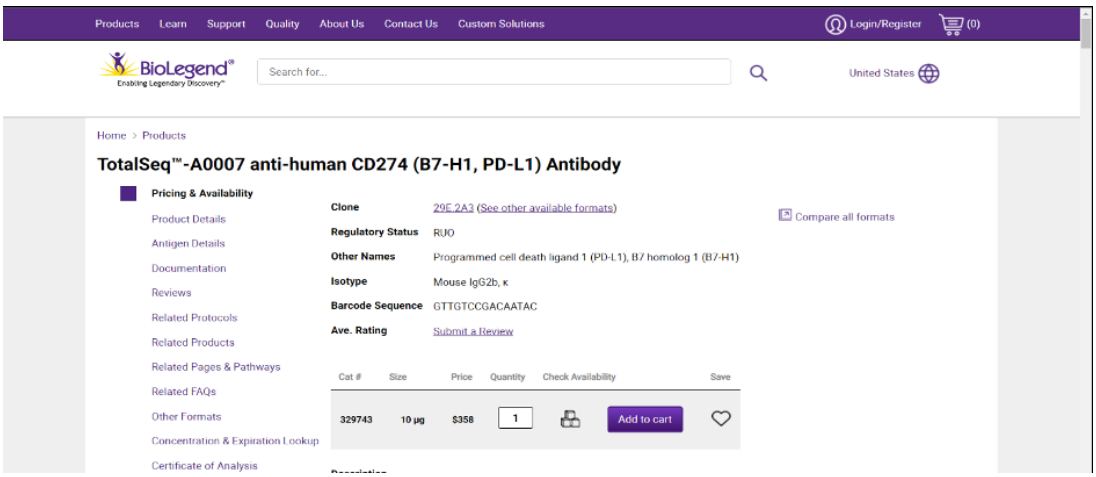
Special Notification:

Isotype control antibody

An isotype control antibody has similar characteristics 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 to add 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 as these cocktails already include isotype control antibodies.

The selection of isotype control antibodies can be found at this website: https://www.biolegend.com/en-us/search-results?PageSize=25&Category=ISO_CTRL&Format=TOTALSEQ_A



Consumables		
Brand	**Description**	**Cat. No.**
-	Stainless-steel base mold	-
-	Aluminum foil	-
-	Forceps	-
-	Slide staining rack	-
	Sterilized Syringe	-
	Slide Container	-
	Microscope Slide Storage Box/IHC wet box	-
	Microscope Slides	-
Millipore	Super PAP Pen (hydrophobic barrier pen)	SLGV033N
	Millex Syringe Filter, Durapore PVDF, 0.22 µm pore size	
-	Microscope glass coverslip (area: 18 mm x 18 mm, thickness: 0.13 - 0.16 mm)	-
Corning	Corning® 100 mm TC-treated Culture Dish	353003
	50 mL centrifuge tubes	430829
	15 mL centrifuge tubes	430791
Kimtech	KimWipes™ delicate task wipes	34155
MATIN	Power Dust Remover (can be substituted with other similar power dust remover)	M-6318
Axygen	1.5 mL centrifuge tubes	MCT-150-A
	0.2 mL PCR tubes*	PCR-02-C
	96-well PCR plate*	PCR-96M2-HS-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

Axygen	10 µL filtered tips	TXLF-10-L-R-S
	0.5 mL thin wall PCR tubes	PCR-05-C
Invitrogen	Qubit Assay Tubes	Q32856
BIOSHARP	Metal Block	-

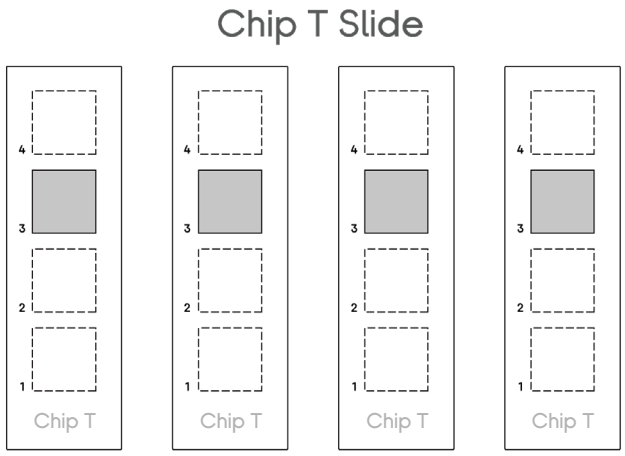
Choose either one of the listed brands (with * mark).

1.5. Practice Tips

Stereo-seq Chip T Slide

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

Stereo-seq Chip P Slides and Stereo-seq Chip T Slides are differentiated by a laser-engraved label at the end of the slide.

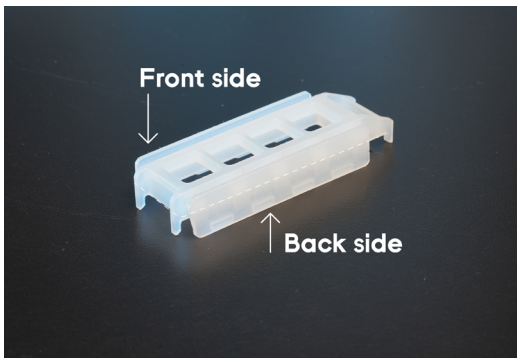


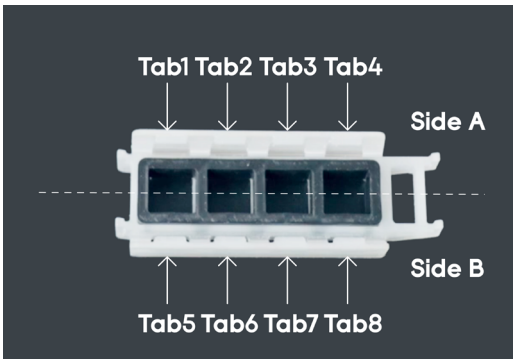
Stereo-seq Chip T Slide Storage

Always store unused slides in their original slide container and keep them sealed with a sealable aluminum bag at -25°C ~ 8°C. Keep sealed with tape or another re-sealable bag. **Always KEEP the desiccant in the bag.**

Stereo-seq Slide Cassette

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

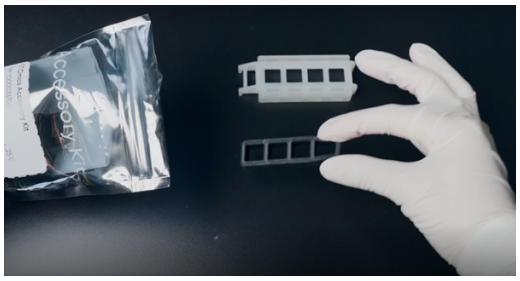




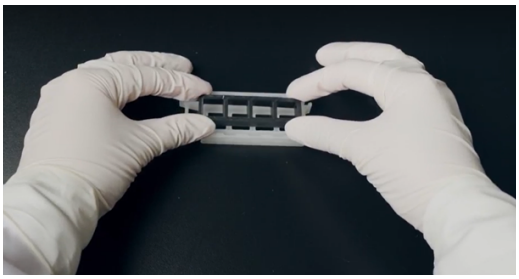
For a demonstration video of Stereo-seq Slide Cassette assembling and removal, please refer to the link or by scanning the QR code:
https://en.stomics.tech/resources/videos/list.html

Stereo-seq Slide Cassette Assembly

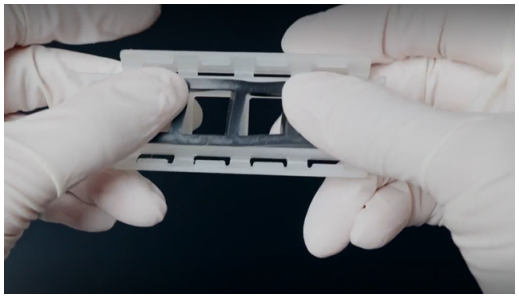
a. Take the Stereo-seq Slide Cassette and gasket out of the STOmics Stereo-seq Accessory Kit.



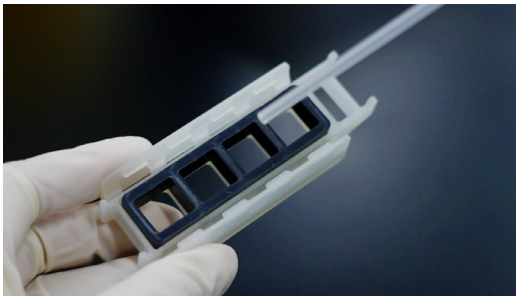
b. Pick up the Stereo-seq Slide Cassette and flip it over. Insert the gasket into the Stereo-seq Slide Cassette, ensuring that the cutouts are aligned.



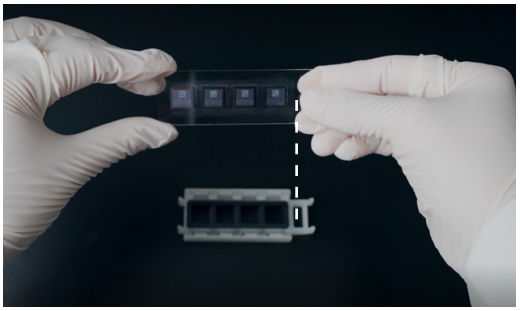
c. Press the gasket down to secure it in the cassette.



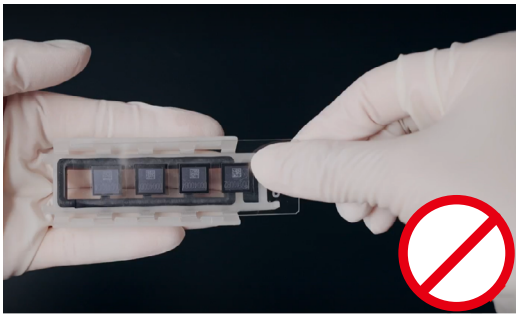
d. Use a power dust remover to blow off any debris on the gasket if necessary.



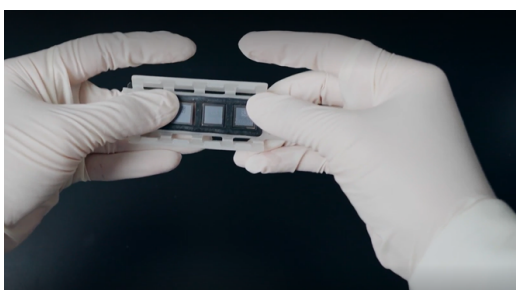
e. Pick up the Stereo-seq Chip Slide and flip it over with the chip surface facing down. Align the engraved label with the long edge of the Stereo-seq Slide Cassette.



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

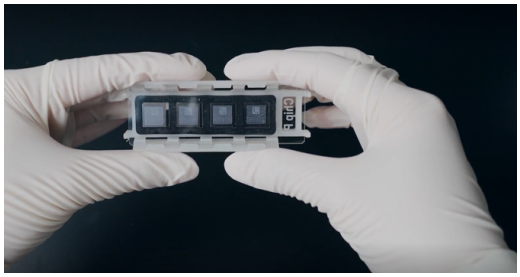


g. Support the back of the cassette with both middle fingers. Place your left thumb between tab 1 and tab 2, and place your right thumb between tab 3 and tab 4.

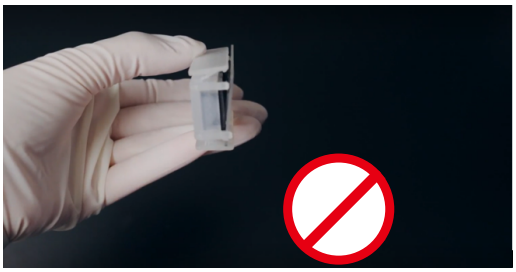


h. Press the upper side (A side) of the slide (near the edge) evenly and then simultaneously press the top edge down firmly with both index fingers to clip the slide in place until you hear it click.

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



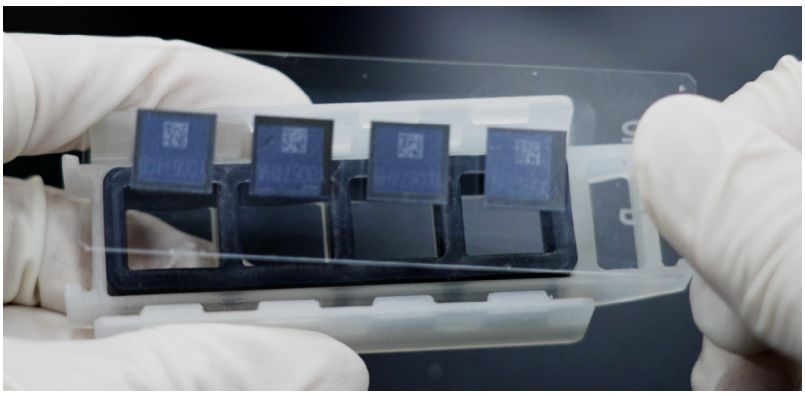
j. Look closely at the Stereo-seq Slide Cassette and confirm that the slide is clipped in place.



Stereo-seq Slide Cassette Removal

a. Flip the cassette over and firmly press the upper side down to release the slide from the tabs, while gently supporting the back of the Stereo-seq Chip Slide with both thumbs to prevent the Stereo-seq Chip Slide from falling off.

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



1.6. Precautions and Warning

- This product is intended for research use only, not for use in diagnostic procedures. Read all instructions in this manual carefully before using the product.
- Before performing experiments with the kits, users are recommended that you ensure that they are familiar with related instruments, and operate them according to manufacturer's instructions.
- Instructions provided in this manual are intended for general use only, and optimization may be required for specific applications.
- Thaw the reagents in the kits properly prior to use. For enzymes, centrifuge briefly and keep them on ice until further use. For other reagents, thaw them first at room temperature followed by inverting several times to mix them properly, and centrifuge them briefly before placing on ice for further use.
- mRNA capture will be compromised or absent for any scratched areas on the front surface of the chip.
- To prevent cross-contamination, we recommend using filtered pipette tips. 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, we recommend two distinctly separated working areas in the laboratory for PCR reaction preparation and PCR product cleanup tests. Use designated pipettes and equipments for each area and execute 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 guidelines on sample embedding, sectioning, and mounting, please refer to Sample Preparation, Sectioning, and Mounting Guide for Fresh Frozen Samples on Stereo-seq Chip Slides (Document No.: STUM-SP001).

CHAPTER 3
PILOT STEREO-CITE EXPERIMENT

TotalSeq™-A antibodies from Biolegend are developed for single-cell CITE-seq technology and may not be suitable for all tissue sections. For users selecting their antibodies, it is recommended to conduct an initial immunofluorescence (IF) test on slides with the tissue type of interest to ensure that the selected TotalSeq™-A antibodies are well suited for the tissue and an optimal dilution ratio of the antibodies can be determined. Once the concentration of each antibody is determined, the formal Stereo-CITE experiment can be performed.

This chapter can be skipped if TotalSeq™-A Mouse Universal Cocktail, V1.0 (Biolegend, Cat. No. 199901) or TotalSeq™-A Human Universal Cocktail, V1.0 (Biolegend, Cat. No. 399907) are being used. Please directly proceed to Chapter 4, the standard operating procedure for the Stereo-CITE workflow, and use the recommended dilution ratios following the instructions from Biolegend.

3.1. Experiment Preparation

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

Reagent	Preparation Steps	Maintenance
4% PFA	Mix well after thawing and aliquot to a 2 mL/tube for storage. Equibrate to room temperature before use.	-20°C for no more than 1 month
5X SSC	Dilute 5 mL of 20X SSC to 20 mL.	Room Temperature
0.1X SSC	Dilute 100 µL of 20X SSC to 20 mL; dilute 250 µL of 20x SSC to 50 mL.	Room Temperature
Filtered Serum Buffer	Take the serum out in advance and thaw it. Mix horse serum and goat serum in a 1:1 ratio. Filter the mixture using a 0.22 µm filter membrane (compatible with a syringe-style sterile disposable syringe). Recommend aliquoting 200 µL per tube (for one round of permeabilization and proteo-transcriptomics workflow). Before the experiment, thaw the aliquoted serum at -20°C. Centrifuge at 4°C, 14000g for 10 min and keep it for later use. Approximately 20 µL of serum is needed for one chip, and any remaining serum can be reused.	-20°C
Do not freeze and thaw the aliquot more than 3 times.		
Salmon Sperm DNA	Thaw on ice before use, 20µL/chip.	-20°C

Primary & Secondary Antibody	Thaw on ice following the original product instructions, centrifuge for 10 min at 14000g. The Primary Abs should include the isotype Abs	4°C or -20°C
Dilute the antibodies if needed.	Dilute the antibodies if needed.	Place on ice until ready to use
10% Triton X-100	Dilute 100% Triton X-100 into 10% Triton X-100 with Nuclease-free water if there is no ready-to-use 10% Triton X-100	Room Temperature
FcR Blocking Reagent	For mouse samples, use TruStain Fcx™ PLUS (CD16/32) Antibody; for human samples, use TruStain Fcx™	4°C
Glycerol	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.	Room Temperature
Decrosslinking Reagent	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.	Room Temperature

Other Preparation

Equipments	Set up	Note
Cryostat	Set the cryostat chamber temperature to -20°C and specimen disc temperature (object temperature) to -10°C ~ -15°C.	The specimen disc temperature depends on the tissue type.
Centrifuge	Set the centrifuge to cool to 4°C in advance	-
PCR Thermal Cycler	Set the PCR module to 37°C (heating lid at 42°C)	Check if there is any abnormality with the PCR Thermal Cycler and replace it if necessary.
Fluorescence Microscope	Ensure that the microscope is equipped with at least DAPI, FITC, TRITC, CY5	Please select the channels according to the fluorescent emission wavelengths of your secondary antibodies.

3.2. Cryosection Preparation

a. Set PCR thermal cycler to 37°C with heated lid set to 42°C in advance with a PCR adaptor.

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

If the specimen disc is over-cooled, it could lead to tissue section cracking during sectioning, while sections would wrinkle when the disc temperature is too high. Optimal specimen disc temperature depends on the tissue type.

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

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

e. During temperature equilibration, prepare the blocking buffer (2 sections for every antibody), please refer to Table 3-1 and put it on ice.

Table 3-1 Blocking Buffer

Components	1X (μL)	2X + 10% (μL)	3X + 10% (μL)	4X + 10% (μL)
5X SSC	120	252	372	492
10% Triton X-100	2	4.2	6.2	8.2
Salmon Sperm DNA, sheared	20	42	62	82
FcR Blocking Reagent*	10	21	31	41
Filtered Serum	20	42	62	82
Nuclease-free water	28	58.8	86.8	114.8
Total	200	420	620	820

FcR Bloacking Reagent is for blocking of Fc receptors on the cell membrane. Please select either one according to the host species of your sample. For human tissue, please use Catalog# 422031 and for mouse tissue, please use Catalog# 156604.

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

f. 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).

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

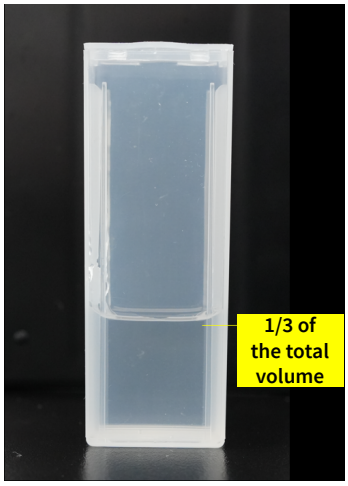
h. Do a final trimming if necessary to ensure a good fit between the tissue section and Stereo-seq Chip T. Now, the specimen is ready for cryosection.

3.3. Tissue Mounting on Microscopic Slides

a. Add over 1/3 Volume of 4% PFA to the slide container and ensure that the volume can cover the tissue on the chip and slide. Prepare enough 4% PFA in a 50 mL corning tube or an empty slide container at a volume that could submerge the entire microscopic slide (about 1/3 of the container).

b. Tissue mounting: 1 section per 1 slide is recommended (2 sections per antibody, as 1 section is for the negative control).

c. Tissue mounting can be achieved via either the cold method (option A) or warm method (option B).



A. Cold Method

1) Place microscope slide inside the chamber with the front-side facing up and pre-cool inside the cryostat chamber for **1~6 min**.

Prolonged cooling for over 6 min may cause mist formation on the chip surface.

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

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

4) Once all tissue mounting is complete, immediately dry the microscope slide at 37°C on a PCR thermal cycler with PCR adaptor for **5 min**.

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

B. Warm Method

1) Perform cryosection and 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 and aim the tissue section onto the center of the slide by gently touching the section with the microscope slide.

3) Check and see if the tissue section has been mounted on to the microscope slide.

4) Turn the microscope slide over, and immediately dry it at 37°C on a PCR thermal cycler with PCR adaptor front-side up for **5 min**.

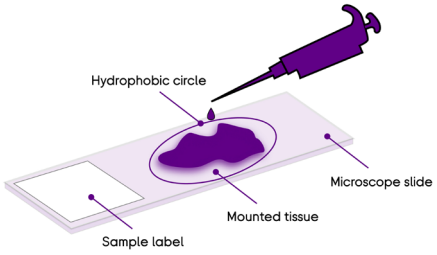
3.4. Tissue Fixation

a. After drying, immediately immerse the tissue-mounted microscope slide in 4% PFA solution at room temperature and fix for 10 min.

b. After fixation, move the 4% PFA container to a fume hood. Meanwhile, prepare a 50mL centrifuge tube filled with at least 30mL of 0.1X SSC.

c. Take the microscope slide out of the 4% PFA solution and immediately immerse the slide in a 50 mL centrifuge tube with at least 30 mL of 0.1X SSC for 10 sec.

d. Take out the slide and immediately wipe off excess SSC solution from around and the back of the slide with Kimwipes 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.

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

f. Transfer the processed slides to an immunohistochemistry wet cassette.

All the liquid must be added after the hydrophobic isolation residue dries.

3.5. Tissue Blocking and Primary Antibody Incubation

a. Vortex the blocking solution that was prepared in Table 3-1 and add no more than 100 µL/section of blocking solution dropwise on the tissue surface within the hydrophobic zone. 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 0.5cm × 0.5cm, the recommended blocking solution volume is 30 µL/section.

b. During incubation, prepare the Primary Antibody Incubation Buffer according to Table 3-2. Vortex the buffer and leave it on ice until use.

Table 3-2 Primary Antibody Incubation Buffer

Components	1X (µL)
Primary Antibody	X
Blocking Buffer	100-X
Total	100

The singular Biolegend TotalSeq™-A can be diluted at a ratio of 1:250 for the initial attempt. If the fluorescent staining does not meet the requirements, it is recommended to titrate the antibody specifically to determine the final antibody dilution ratio by referring to Appendix C.

c. Aspirate the blocking solution, slowly add the primary antibody incubation solution (no primary antibody in the negative control group) with no more than 100 µL/section, and incubate the primary antibody for **45 min** at room temperature.

Avoid tissue drying, as it may lead to the generation of non-specific signals that interfere with the final results.

d. During primary antibody incubation, prepare the secondary antibody incubation solution detailed in Table 3-3 according to the optimal dilution ratio of the secondary antibody. After vortex mixing and brief centrifugation, leave the secondary antibody solution on ice **in the dark** until use.

Table 3-3 Secondary Antibody Incubation Buffer

Components	1X (μL)
5X SSC	60
Secondary Antibody	X
Nuclease- free water	40-X
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 are used, please adjust the dilution ratio according to the manufacturer's instructions.

3.6. Secondary Antibody Incubation

a. Remove the primary antibody incubation solution of the experimental group and the blocking solution of the negative control group.

b. Add **100 μL** 0.1X SSC per section. Incubate at room temperature for **1 min** and then remove.

Avoid tissue drying, as it may lead to the generation of non-specific signals that interfere with the final results.

c. Repeat step b. once.

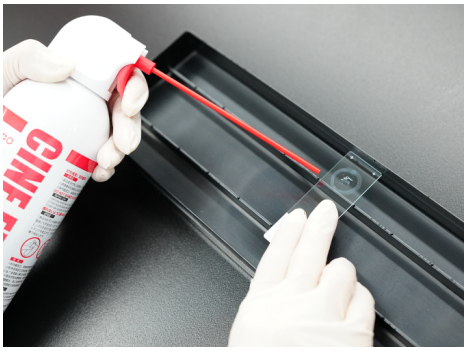
d. Slowly add no more than **100 μL** secondary antibody incubation solution per section from the non-tissue area in both the experimental group and the negative control group. Incubate for **15 min** at room temperature **in the dark**.

e. Remove the secondary antibody incubation solution.

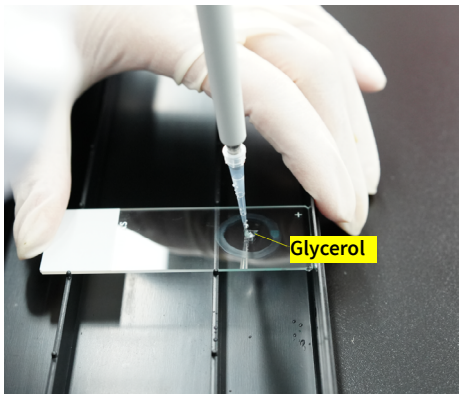
f. Add 100 μL 0.1X SSC per section. Incubate at room temperature for **1 min** and then remove.

g. Repeat step f. once.

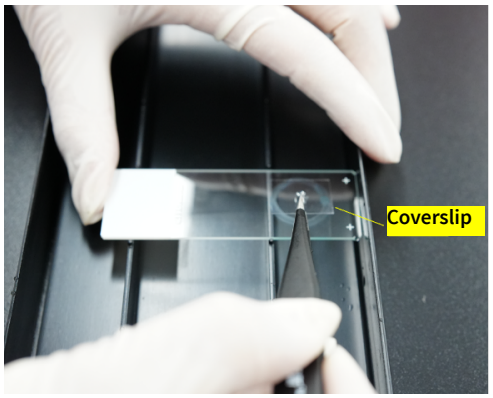
h. Hold on to the slide with one hand and completely dry the tissue using a power dust remover at a distance 2-3 cm away from the tissue surface by blowing gently from one side at a 30-degree angle horizontal to the plane of the slide.

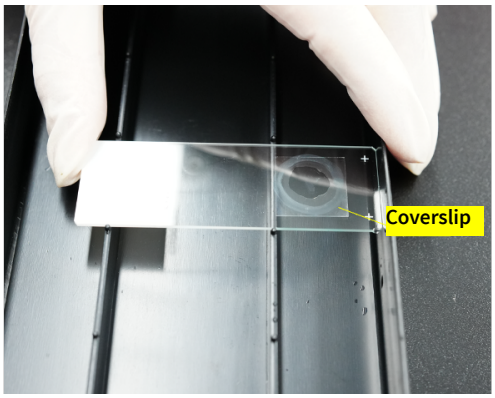


i. Pipette **5 µL** glycerol gently onto the center of the tissue on each slide without introducing air bubbles.



j. With a pair of forceps, place one end of the coverslip onto the tissue edge while holding the other end and then gradually lower the coverslip onto the tissue. Ensure that the tissue is completely covered by glycerol and the coverslip. To avoid fluorescent bleaching, **IMMEDIATELY proceed to imaging.**





Ensure that the coverslip is clean without any dust or debris. Wiping with an alcohol swab or blowing with a power dust remover could be used for cleaning.

3.7. Imaging

a. Fluorescent images are taken using a fluorescence microscope with an image stitching function, and the fluorescence configuration is recommended as follows:

- Light source wavelength range: 380-680 nm
- Monochrome camera (≥ 12 bit)
- DAPI filter cube (ref. Excitation 375/28 nm, Emission 460/50 nm)
- FITC filter cube (Reference: Excitation 480/30 nm, Emission 525/50 nm)
- TRITC filter cube (ref. Excitation 545/25 nm, Emission 605/70 nm)
- CY5 filter cube (Reference: Excitation 620/50 nm, Emission 690/50 nm)

- Maximum pixel size: 5 μm/pixel
- Exposure time: 1 ms~2 s (depending on the characteristics of the antibody used)

b. Transfer the slides to the microscope stage and remove the light shield.

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

3.8. Stereo-CITE Pilot Experiment Results

As shown in Figure 2, taking the negative control Ctrl and CD68 staining of mouse thymus tissue as an example, it is confirmed that using the primary antibody at the ratio of 1:250 yielded the desired staining results. If you do not get the expected results, it will be necessary to determine the optimal antibody dilution by antibody titration.

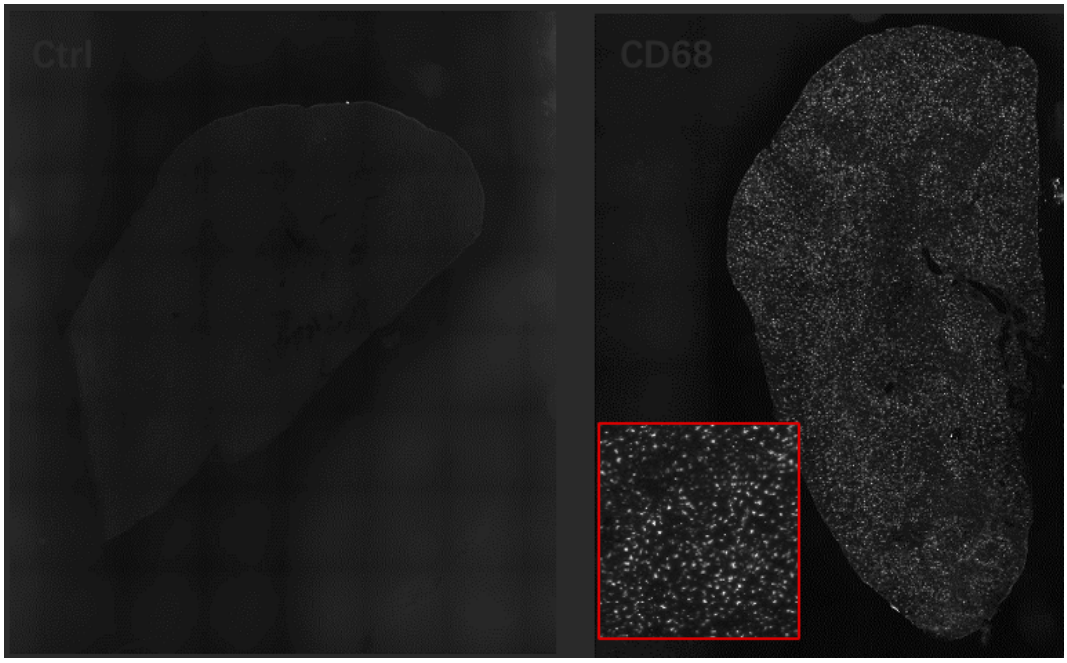
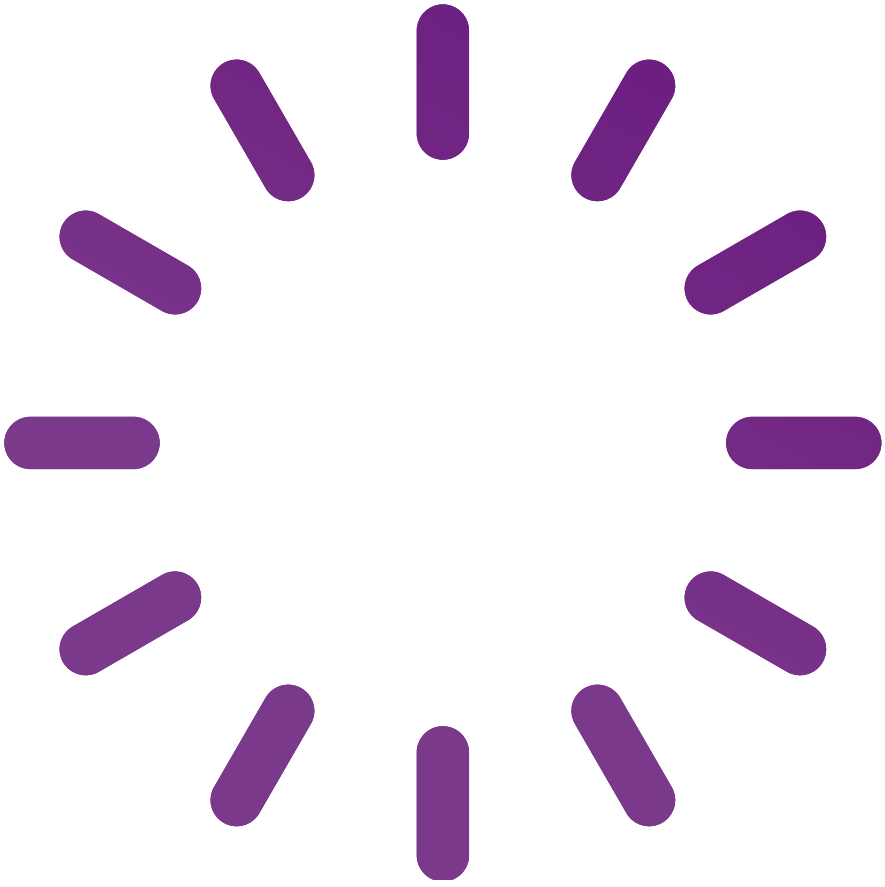


Figure 1 Immunofluorescent staining of the control and CD68 in mouse thymus

CHAPTER 4

Stereo-CITE PROTEO-TRANSCRIPTOMICS SET STANDARD OPERATING PROCEDURE



4.1. Experimental Preparation

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

Reagent	Preparation Steps	Maintenance
4% PFA	Mix well after thawing and aliquot to 2mL/tube for storage. Equibrate to room temperature before use.	-20°C for no more than 1 month
5X SSC	Dilute 5 mL of 20x SSC to 20 mL	Room Temperature
0.1X SSC	Dilute 100 µL of 20x SSC to 20 mL; Dilute 250 µL of 20x SSC to 50 mL	Room Temperature
Wash Buffer	Add 160µL RI into 3040 µL of 0.1X SSC for one tissue section	Room Temperature
Filtered Serum Buffer	Take the serum out in advance and thaw it. Mix horse serum and goat serum in a 1:1 ratio. Filter the mixture using a 0.22 µm filter membrane (compatible with a syringe-style sterile disposable syringe). Recommend aliquoting 200 µL per tube (for one round of permeabilization and proteo-transcriptomics workflow). Before the experiment, thaw the aliquoted serum at -20°C. Centrifuge at 4°C, 14000g for 10 min and keep it for later use. Approximately 20 µL of serum is needed for one chip, and any remaining serum can be reused.	-20°C
Do not freeze and thaw the aliquot more than 3 times.		
Salmon Sperm DNA	Thaw on ice before use, 20µL/chip	-20°C
Blocking Reagent	Thaw on ice before use, 15 µL/chip	-20°C
RI	Thaw on ice before use, 210 µL/chip	Place on ice until ready to use
50X Diluted DAPI	Dilute DAPI with 5X SSC at the ratio of 1:50	4°C in the dark for 1 day
Primary & Secondary Antibody	Thaw on ice following the original product instructions, centrifuge for 10 min at 14000g. The Primary Abs should include the isotype Abs	4°C or -20°C

Diluted Primary & Secondary (Optional)	Dilute the antibodies if needed.	Place on ice until ready to use
10% Triton X-100	Dilute 100% Triton X-100 into 10% Triton X-100 with Nuclease-free water if there is no ready-to-use 10% Triton X-100	Room Temperature
FcR Blocking Reagent	For mouse samples, use TruStain Fcx™ PLUS (CD16/32) Antibody; for human samples, use TruStain Fcx™	4°C
Glycerol	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.	Room Temperature
Decrosslinking Reagent	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.	Room Temperature
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 2mL EP tube at 10,000 g for 30 S at room temperature; c. Add 27.5 µL of 1XPBS 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. Aspirate 25 µL of the supernatant, transfer to a new EP tube, and place on ice for later use.	4°C
0.01N HCl	Prepare at least 2mL of 0.01N HCL per sample. Configure HCL to 0.01N. Measure and ensure 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 conducting experiments.		
10X Permeabilization Reagent Stock Solution	Add 1 mL of freshly prepared 0.01N HCl to dissolve PR Enzyme (red cap, in powder form), and thoroughly mix the reagent by pipetting.	-20°C
Do not vortex the permeabilization enzyme. Mix by pipetting before use. **Aliquot this 10X stock solution to prevent freeze-thaw cycles.**		

1X Permeabilization Reagent Solution	Make 1X PR solution (150 µL / chip) by diluting 10X PR stock solution with 0.01N HCl.	Place on ice until ready to use. Can be on ice for up to 6 hr.
RT Oligo	Short spin the primer tube, dissolve RT Oligo in 79 µL TE buffer. Close the lid, vortex the tube for 15 sec at highest speed and short spin the tube.	-20°C
Aliquot the unused RT Oligo to avoid freeze-thaw cycles and store at -80°C.		
PR Rinse Buffer	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.	Room Temperature
PR Rinse Buffer (with 5% RI)	Prepare at least 100 µL per chip (95 µL PR Rinse Buffer with 5 µL RI)	Place on ice until ready to use

Other Preparation

Equipment	Set up	Note
Cryostat	Set the cryostat chamber temperature to -20°C and specimen disc temperature (object temperature) to -10°C ~ -15°C.	The specimen disc temperature depends on the tissue type.
Metal Bath	70°C for decrosslinking 37°C for pre-heating the permeabilization enzyme	-
PCR Thermal Cycler	Set the temperature in the following order: 37°C for slide drying and permeabilization (heating lid at 42°C); 42°C for reverse transcription (heating lid at 47°C); 55°C for tissue removal and cDNA release (heating lid at 60°C).	Check the PCR Thermal Cycler for abnormalities. If necessary, replace it.
Fluorescence Microscope	Ensure that the microscope is equipped with at least DAPI, FITC, TRITC, CY5	Please select the channels according to the fluorescent emission wavelengths of your secondary antibodies.
Centrifuge	Centrifuge cocktail lyophilized powder at room temperature, then adjust the centrifuge temperature to 4 °C in advance.	4°C is used for centrifugation of serum, primary antibodies, secondary antibodies, and reconstituted antibody cocktails.

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

During the temperature equilibration step at cryosection preparation, prepare the blocking buffer by referring to Table 4-1 and put it on ice. Meanwhile, take enough 4% PFA solution (at least 400 µL 4% PFA solution per chip) from 4°C to equilibrate to room temperature.

Table 4-1 Blocking Buffer

Components	1X (µL)	2X + 10% (µL)	3X + 10% (µL)	4X + 10% (µL)
5X SSC	180	378	558	738
10% Triton X-100	3	6.3	9.3	12.3
Blocking Reagent	15	31.5	46.5	61.5
Salmon Sperm DNA, sheared	30	63	93	123
FcR Blocking Reagent*	15	31.5	46.5	61.5
RI	15	31.5	46.5	61.5
Filtered Serum	30	63	93	123
Nuclease-free water	12	25.2	37.2	49.2
Total	300	630	930	1230

FcR Bloacking Reagent is for blocking of Fc receptors on the cell membrane. Please select either one according to the host species of your sample. For human tissue, please use Catalog# 422031 and for mouse tissue, please use Catalog# 156604.

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

4.2. Tissue Fixation

a. Assemble the Cassette and Gasket then place the Stereo-seq Chip Slide in the Cassette according to guidance written in **1.5 Practice Tips**. It is recommended to practice with a regular blank glass slide.

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

b. Place the Stereo-seq Slide Cassette in a fume hood, and add 400 µL of 4% PFA solution per well. Seal the cassette with sealing tape, and incubate for 10 min at room temperature.

c. After fixation, remove the sealing tape, and tilt the Stereo-seq Slide Cassette slightly at an angle less than 20°. Remove the 4% PFA solution from a corner of the well using a pipette. Keep the chip surface moist.

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

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

Avoid drying out the tissue during the liquid exchange process. Ensure that that all the tissue sections are completely submerged.

f. Repeat wash **steps d.** and **e.** one more time.

4.3. Tissue Blocking and Primary Antibody Incubation

a. Blocking: Transfer the Stereo-seq Slide Cassette out of the fume hood to a bench top and add **150 µL** blocking buffer per chip onto the tissue surface immediately. Incubate the chip at room temperature for **20 min**.

b. While waiting, prepare the primary antibody incubation solution according to Table 4-2 with the actual number of actual number of individual TotalSeq™-A antibodies used. After vortex mixing and brief centrifugation, 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. 399907), please refer to Table 4-3 for preparing the primary antibody incubation solution.

Table 4-2 Primary Antibody Incubation Solution

(For freely combined individual TotalSeq™-A antibodies of your interest)

Components	1X (μL)
Blocking Buffer	150-(V1+V2+...+Vn)
Primary Antibody #1	V1
Primary Antibody #2	V2
...	...
Primary Antibody #N	30
Nuclease-free water	Vn
Total	150

Antibody 1~N should contain the corresponding isotype control antibody. Ensure that to centrifuge the TotalSeq™-A antibody before use, and try to avoid the tip of the pipette coming in direct contact with the bottom of the tube.

Table 4-3 Primary Antibody Incubation Solution

(For TotalSeq™-A Cocktails)

Components	1X (μL)
Blocking Buffer	137.5
Universal Cocktail	12.5
Total	150

c. Pipette to remove the blocking buffer from one corner of the well. Ensure the chip and tissue surface remain moist.

Avoid drying out the tissue during the liquid exchange process. It is recommended to perform the procedure one chip at a time.

d. Slowly add **150 μL** of the primary antibody incubation solution per well onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets. Incubate for 45 min at room temperature.

e. During primary antibody incubation, prepare the secondary antibody incubation solution according to Table 4-4. Vortex the mixture and leave it on ice **in the dark** until use.

Table 4-4 Secondary Antibody Incubation Solution

Components	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, please adjust the dilution ratio according to the manufacturer's instructions.

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

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

h. 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.

i. Repeat wash **steps f.** and **g.** one more time.

Avoid drying out the tissue during the liquid exchange process. It is recommended to perform the step one chip at a time.

j. Slowly add **150 μL** secondary antibody incubation solution per well onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets., and incubate **in the dark** at room temperature for **15 min**.

4.4. DAPI Staining

a. During the secondary antibody incubation, prepare DAPI staining solution according to Table 4-5. Vortex and centrifuge briefly then leave it on ice in the dark until use.

Table 4-5 DAPI Staining Solution

Components	1X (μL)
5X SSC	90
50X-diluted DAPI solution	1.5
RI	7.5
Nuclease-free water	51
Total	150

b. Pipette to remove the secondary incubation solution from one corner of the well, and keep the chip and tissue surface 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 less than 20°, remove the Wash Buffer from one corner of the well using a pipette, and keep the chip and tissue surface moist.

e. Repeat **step c.** and **d.** once to achieve 2 washes.

Avoid drying out the tissue during the liquid exchange process. It is recommended to perform the step one chip at a time.

f. Slowly add **150 μL** DAPI Staining Solution per well onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets. Incubate **in the dark** at room temperature for **2 min**.

g. Pipette to remove the solution from one corner of the well. Keep the chip and tissue surface 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.** one more time.

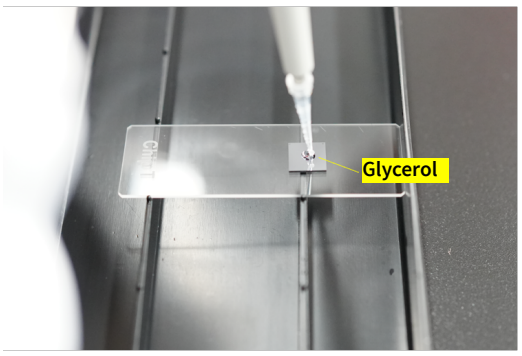
k. Remove the slide from the Stereo-seq Slide Cassette according to instructions in **1.5 Practice Tips.**

l. Hold on to the slide with one hand and completely dry the tissue further with a power dust remover in the other hand at a distance 2-3 cm away from the tissue surface by blowing gently from one side at a 30-degree angle horizontal to the plane of the slide.

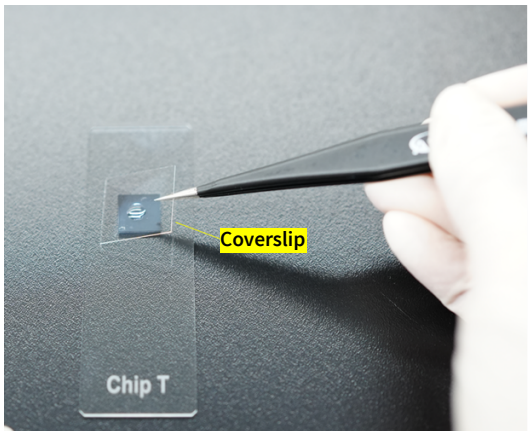
Alternatively, centrifuge the Stereo-seq Chip Slide for 10 sec in a slide spinner to completely dry the chips (Labnet Slide Spinner, C1303-T).

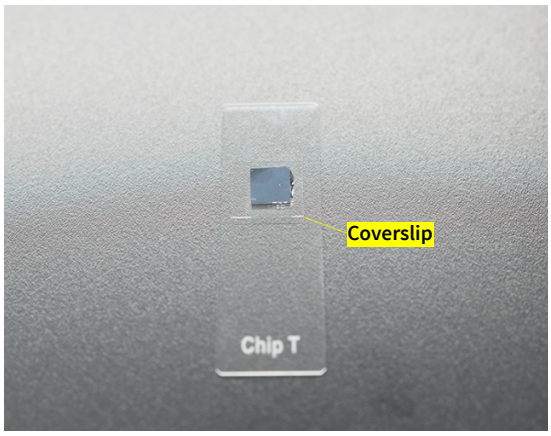
Ensure no residual solution is left on the chip.

m. Pipette 5 μL glycerol gently onto the center of the tissue on each chip without introducing air bubbles.



n. With a pair of forceps, place one end of the coverslip onto the chip while holding the other end and then gradually lower the coverslip onto the chips. Ensure that the chips are completely covered by glycerol and the coverslip. To avoid fluorescent bleaching, IMMEDIATELY proceed to imaging.





Ensure that the coverslip is clean without any dust or debris. Wiping with an alcohol swab or blowing with a power dust remover could be used for cleaning.

4.5. Imaging

a. Fluorescence images are taken using a fluorescence microscope with image stitching functions, and the fluorescence configuration is recommended as following:

- Light source wavelength range: 380-680 nm
- Monochrome camera (≥ 12 bit)
- DAPI filter cube (ref. Excitation 375/28 nm, Emission 460/50 nm)
- FITC filter cube (Reference: Excitation 480/30 nm, Emission 525/50 nm)
- TRITC filter cube (ref. Excitation 545/25 nm, Emission 605/70 nm)
- CY5 filter cube (Reference: Excitation 620/50 nm, Emission 690/50 nm)
- Maximum pixel size: 5 μm/pixel
- Exposure time: 1 ms~2 s (depending on the characteristics of the antibody used)

b. Create a new folder in a fluorescent microscope-connected PC, name it with the chip ID number and other essential information.

Note: Only use letters, numbers, and underscores in folder naming. Special characters and spacings are not allowed.

Example: Y00035N1

c. Take fluorescence images from the chip with the following microscope setting: DAPI channel, 4X and 10X objective lenses, with stitching function.

d. Add a few drops of water (~2μL) to the microscope loading platform, then place the Stereo-seq Chip Slide on the water to better adhere to the platform and avoid the displacement of the Stereo-seq Chip Slide during image scanning.

e. Remove the light shield, switch to 4X objective lens, select the DAPI channel and select the chip area of interest. Adjust Brightness and Gain. The specific parameters will vary according to different microscopes, as long as the tissue can be imaged clearly. But the light intensity should be kept in the lower range to prevent fluorescence quenching.

f. After scanning with 4X objective lens, take fluorescence images from the chip with the following microscope setting: DAPI channel, 10X objective lens, full scan on capture area.

As shown in Figure 4, taking the DAPI channel image of mouse thymus tissue as an example, the red box in the middle picture is the selected tissue area, and the small blue box is the added focal points. The picture on the left shows a screenshot of a focal field window selected outside the tissue, which should ensure that the tracklines are clear and distinct. The picture on the right is a screenshot of a focal field window selected in the tissue area, which should ensure that the tissue outline and contour is clear.

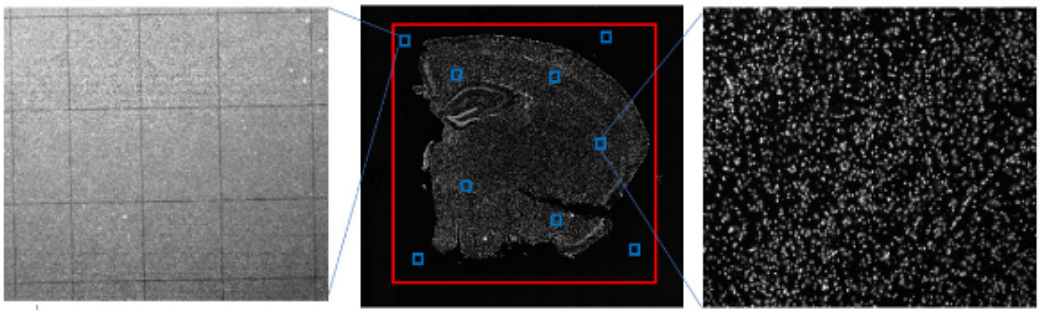


Figure 2 DAPI staining of mouse brain

g. Once you complete the first scan of the DAPI channel, switch directly to the next 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. Save original tile (FOV) image files and stitched images. Simply create a new folder, name and save it with the chip number_IF format name (e.g., Y00035N1_IF) in the computer connected to the fluorescence microscope. Then adjust the focus and exposure until the stained tissue is clearly displayed. Finally, complete the full scan on the capturing area with a 10X objective lens.

h. Based on the results of the immunofluorescence staining image, whether the TotalSeq™-A primary antibody has been successfully conjugated 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. For example, taking Figure 4 as an example, due to the addition of multiple primary antibodies, all areas of the thymus tissue have shown a fluorescent signal (Figure 4, left image), while the cell membrane can be identified when zoomed into a specific cell membrane primary antibody (Figure 4, right image). The cell membrane staining can be clearly visualized after magnification, indicating that there is no occurrence of unwanted antibody binding.

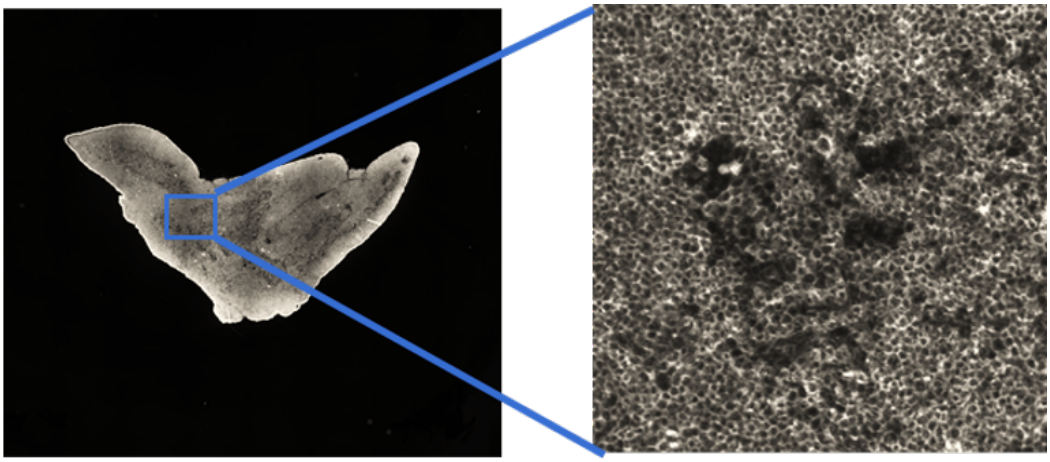


Figure 3 IF staining of the mouse thymus

The staining of the secondary antibody can be used to determine whether the primary antibody has been successfully conjugated. If unsuccessful, it is not recommended to continue with subsequent experiments.

i. Save original tile (FOV) images files and stitched images.

j. j. Open the ImageStudio software and the Image Quality Control functional module within the software. Upload your nuclei-stained (DAPI) image then run Image QC according to the **ImageStudio User Manual** within the software.

The captured nuclei-stained image needs to pass ImageQC in order to proceed to further image analysis (image "register") in Stereo-seq Analysis Workflow (SAW) pipelines.

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

k. After imaging, remove the Stereo-seq Chip Slide from the microscope platform and keep the coverslip mounted for the next steps.

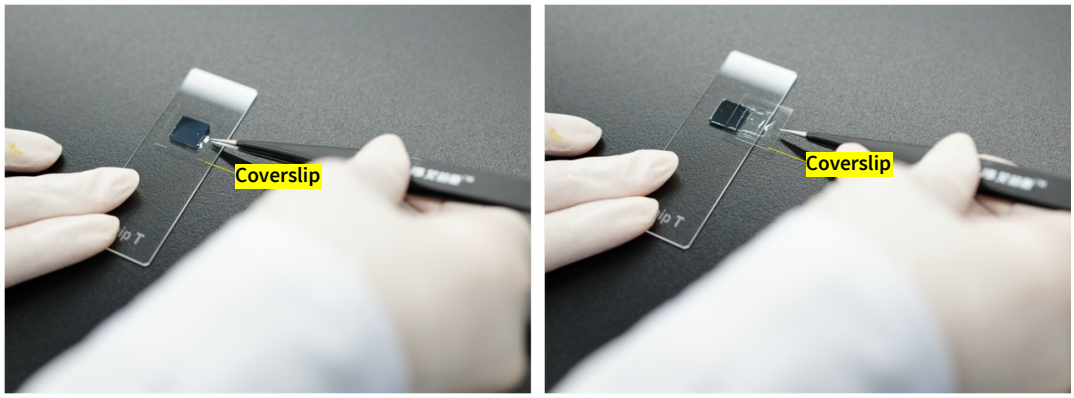
4.6. Tissue Decrosslinking

a. Set aside the Decrosslinking Reagent you prepared in [Experimental Preparation].

b. Place the PCR adaptor into the PCR thermal cycler and set one PCR module to 70°C with the lid set to 75°C and another PCR module to 37°C with the lid set to 42°C

c. Warm up the Decrosslinking reagent to 70°C for at least 10 min.

d. Grip onto the coverslip with a pair of forceps and pull it to slide over the Stereo-seq chip Slide slowly until the chips and the coverslip are fully separated.



e. Wipe off the excess solution from around and the back of the slide with Kimwipes without touching the chips. Ensure that there is no liquid residue around the chip.

f. Assemble the Cassette and a new Gasket then place the Stereo-seq chip Slide in the Cassette according to guidance written in **1.5 Practice Tips**. It is recommended to practice with a regular blank glass slide.

g. Add **400 µL** Wash Buffer per well and incubate the chip at room temperature for **1 min**.

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

i. Repeat **Step h.** and **i.** once.

j. Place the PCR adaptor in the PCR thermal cycler first. Ensure that your PCR thermal cycler has been switched on and set to 70°C with the lid set to 75°C. Place the Stereo-seq Slide Cassette on the PCR adaptor and add **400 µL** Decrosslinking Buffer per well. Seal the cassette with new sealing tape. Close the heated lid and incubate at 70°C for **15 min**.

k. While waiting, pre-warm the 1X Permeabilization Reagent Solution you prepared in Experimental Preparation to 37°C in advance (no more than 30 min).

l. Transfer the Stereo-seq Slide Cassette to the bench and gently remove the sealing tape. Do not remove the decrosslinking solution. Equilibrate the cassette to room temperature for **10 min**.

4.7. Tissue Permeabilization

a. Set aside the 2 mL of HCl and 1X Permeabilization Reagent Solution you prepared in **Experimental Preparation**.

b. Ensure that your PCR thermal cycler has been switched on and set to 37°C and the heating lid has been set to 42°C. Place the PCR adaptor in the PCR thermal cycler, then close the heated lid to pre-heat the adaptor for **3 min**.

Temperature	Time	Cycle
(Heated lid) 42°C	on	-
37°C	60 min	1
37°C	hold	-

c. Thaw RT Reagent, RT Additive and RT Oligo on ice.

d. Once the cassette equilibrates to room temperature, tilt the Stereo-seq Slide Cassette slightly at an angle of less than 20°, remove the Decrosslinking Solution from a corner of the well using a pipette, and keep the chip and tissue surface moist.

e. Add **400 µL** Wash Buffer per well.

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

g. Add **150 µL** 1X Permeabilization Reagent Solution per chip onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets.

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

h. Seal the Stereo-seq Slide Cassette with a new sealing tape and place it on the PCR Adaptor. Close the hot lid and let the chip incubate inside the PCR thermal cycler at 37°C with the optimal permeabilization time.

Optimal permeabilization time is pre-determined by Stereo-seq Permeabilization Kit (111KP118). For more information, refer to the user manual of Stereo-seq Permeabilization Set for Stereo-CITE Proteo-Transcriptomics Application User Manual.

i. While waiting for permeabilization to be done, prepare RT mix according to Table 4-6 and leave it on ice until use.

Table 4-6 RT Mix

Components	1X (μL)	2X + 10% (μL)	3X + 10% (μL)	4X + 10% (μL)
RT Reagent	160	336	496	656
RT Additive	10	21	31	41
RI	10	21	31	41
RT Oligo	10	21	31	41
ReverseT Enzyme	10	21	31	41
Total	200	420	620	820

j. Keep the Stereo-seq Slide Cassette in the PCR thermal cycler and remove the sealing tape. Tilt the cassette slightly at an angle of less than 20°. Pipette to remove the 1x Permeabilization Reagent Solution from a corner of the well, and avoid touching the chip surface.

k. Add **200 μL** PR Rinse Buffer (with 5%RI) per well.

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

Ensure that to not dry the chip completely.

m. Continue with reverse transcription immediately to avoid RNA degradation.

4.8. Reverse Transcription

a. Ensure that the temperature of the PCR Thermal Cycler with PCR Adaptor has been set to 42°C with the heated lid set to 47°C in advance.

b. Pipette up and down, then short spin the prepared RT Mix. 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 sealing tape to Stereo-seq Slide Cassette and ensure that it is sealed tightly. Incubate the Stereo-seq Slide Cassette at 42°C for **3 hr** or longer (no longer than 16 hr) with the following incubation protocol.

Temperature	Time	Cycle
(Heated lid) 47°C	on	-
42°C	3 hr - 16 hr	1
42°C	hold	-

4.9. Tissue Removal

Prepare		
Reagent	**Preparation Steps**	**Maintenance**
TR buffer	Heat the buffer for **5 min** at 55 °C to dissolve the precipitate. Equilibrate it to room temperature prior to use.	Room Temperature
cDNA Release Buffer	Heat the buffer for **5 min** at 55 °C to dissolve the precipitate. Equilibrate it to room temperature prior to use.	Room Temperature

If white precipitate is observed in the buffer, dissolve them by heating the buffer at 55 °C again and equilibrate to room temperature before mixing.

a. Check and ensure that the PCR Thermal Cycler with PCR Adaptor has been set to 55°C and the heated lid has been set to 60°C.

b. Remove the Stereo-seq Slide Cassette from the 42°C PCR Adaptor and then remove the sealing tape.

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

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

d. Add **400 µL** TR Buffer into each well and incubate the Stereo-seq Slide Cassette at 55°C on the PCR Adaptor for **20 min** with the following incubation protocol.

Temperature	Time	Cycle
(Heated lid) 60°C	on	-
55°C	20 min	1
55°C	Hold	-

e. While waiting, prepare cDNA Release Mix according to Table 4-7.

Table 4-7 cDNA Release Mix

Components	1X (µL)	2X + 10% (µL)	3X + 10% (µL)	4X + 10% (µL)
cDNA Release Buffer	380	836	1254	1672
cDNA Release Enzyme	20	44	66	88
Total	400	880	1320	1760

f. Slightly tilt the Stereo-seq Slide Cassette, remove TR Buffer with a pipette from the corner of each well without touching the chip surface.

If tissue removal is not complete, add 400 µL of 0.1X SSC and pipette up and down the well to remove the remaining tissue on the chip. Slightly tilt the Stereo-seq Slide Cassette, remove 0.1X SSC solution from the corner of each well without touching the chip surface.

4.10. cDNA & ADT Release and Collection

a. Add **400 µL** of cDNA Release Mix per chip prepared in 4.8-e into each well of the Stereo-seq Slide Cassette.

b. Apply sealing tape to Stereo-seq Slide Cassette and ensure that it is sealed tightly. Incubate the Stereo-seq Slide Cassette at 55°C for **3 hr** or longer (no longer than 18 hr) with the following incubation protocol.

Temperature	Time	Cycle
(Heated lid) 60°C	on	-
55°C	3 hr - 18 hr	1
55°C	Hold	-

Stop Point:

DNA collection step may be left overnight. If it is left overnight, ensure that the Stereo-seq Slide Cassette has been sealed tightly with the sealing tape.

c. After the reaction, completely collect the cDNA Release Mix from each well into a new 1.5 mL tube.

d. Add **100 µL** of nuclease-free water per chip into each well. Pipette up and down to wash the chip surface thoroughly and then collect it into the same 1.5 mL tube with the cDNA Release Mix.

Ensure that to collect as completely as possible to retrieve enough cDNA on the chip. cDNA Release Mix should be about 400 µL after incubation (the volume might be less than 400 µL). It is required to combine the collected cDNA Release Mix with the 100 µL nuclease-free water before proceeding to the next step.

Ensure all the chip ID numbers on the Slide have been recorded, as it is required for downstream analysis.

4.11. cDNA Purification and Amplification

Background Information

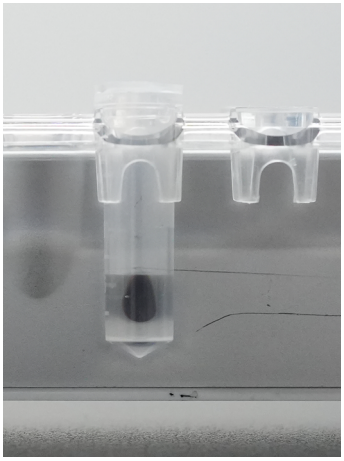
For bead-based purification, we recommend using DNA Cleanup Beads AMPure® XP(Agencourt, Cat. No. A63882), SPRIselect (Beckman Coulter, B23317/B23318/B23319) or VAHTS™ DNA Clean Beads (Vazyme, Cat. No. N411-02). *If magnetic beads from other sources are used, please optimize the cleanup conditions before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

Before Use

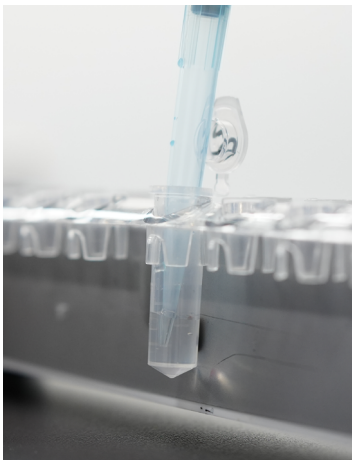
- To ensure the DNA capture efficiency of the magnetic beads, equilibrate the beads to room temperature **30 min before use**.
- Vortex or pipette up and down to ensure the beads are thoroughly mixed every time before use.
- The volume of magnetic beads directly affects the distribution of purified DNA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

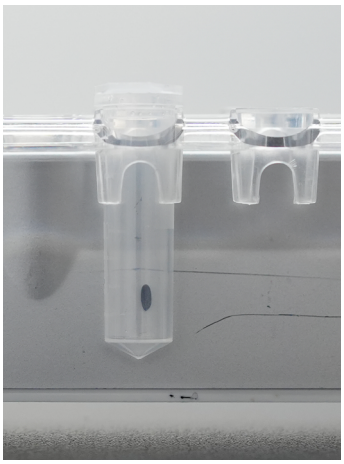
Operation Notes

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

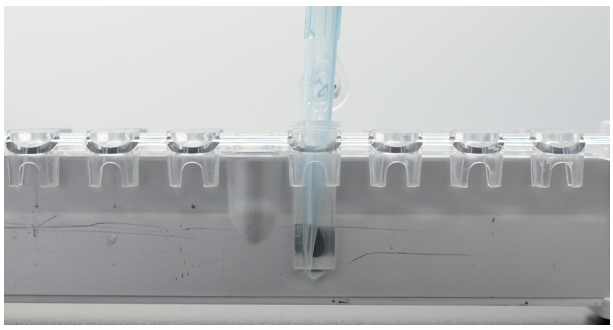


2. When collecting the supernatant with a pipette after magnetic separation, avoid taking up the beads. Instead of collecting the entire supernatant fraction, leave 2-3 µL in the tube to avoid the pipette from directly contacting the beads. If the beads are mistakenly taken up, dispense everything and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

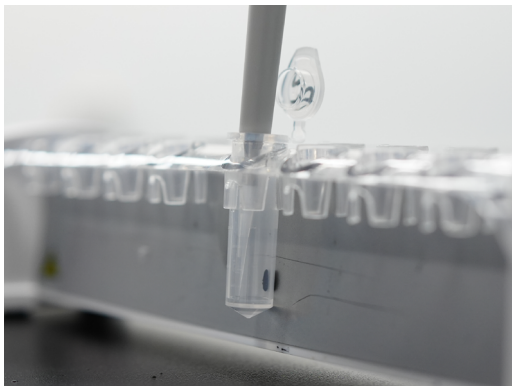




3. Use freshly prepared 80% ethanol (at room temperature) to wash the beads. Keep the sample tube on the magnetic separation rack during the washing step. Do not shake or disturb the beads.

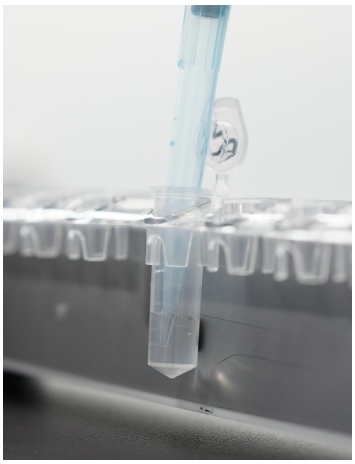


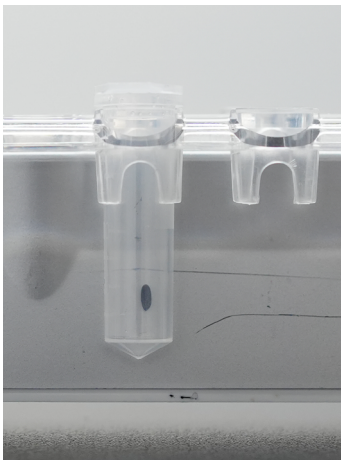
4. After the 2nd wash of beads with ethanol, try to remove all the liquid within the tube. You may centrifuge briefly to accumulate any remaining liquid at the bottom of the tube, then separate beads magnetically, and remove the remaining liquid by using a small volume pipette.



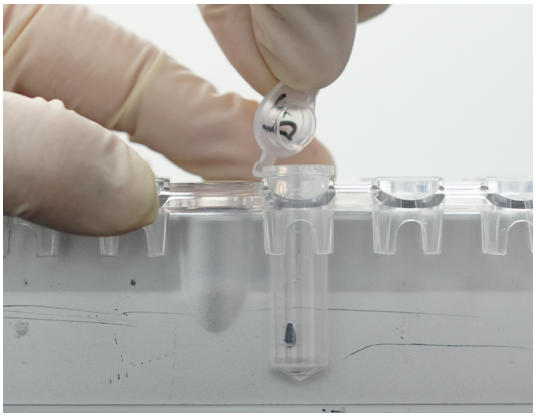
5. After washing twice with ethanol, air-dry the beads at room temperature. Drying usually takes approximately **5-10 min** depending on the lab temperature and humidity. Watch closely until the pellet appears sufficiently dry with a matte appearance, then continue to the elution step with TE Buffer.

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





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



a. Equilibrate the magnetic beads to room temperature for at least **30 min**.

b. cDNA Purification Procedures with 1X Magnetic Bead

1) Mix the collected cDNA (450-490 µL) with the beads in a ratio of 1 : 1. Vortex the mix then incubate it at room temperature for **10 min**.

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

3) Carefully transfer the supernatant with a pipette to a new 2.0 mL tube. The supernatant contains ADT products and can be stored at room temperature temporarily. Refer to 4.11 for ADT product purification).

4) Keep the tube on the magnetic separation rack and add 1 mL of freshly prepared 80% ethanol to 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 (If foam is seen on the cap, clean the cap with 80% ethanol).

5) Repeat step 4) one more time.

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 observed. Drying times will vary but will be approximately **5-8 min**.

7) Add **22 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 onto a magnetic separation rack for **3-5 min** until the liquid becomes clear.

8) Transfer the supernatant (**21 µL**) into a new 0.2 mL PCR tube.

9) Add another **22 µL** of nuclease-free water to the dried beads in step 7) for a second elution. Mix the beads and nuclease-free water by vortexing. Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube onto a magnetic separation rack for **3-5 min** until the liquid becomes clear.

10) Transfer the supernatant (**21 µL**) into the 0.2 mL PCR tube in step 8) and obtain a combined eluted cDNA (~**42 µL**)

c. If collected eluted cDNA is less than 42 µL, simply top it up with nuclease-free water.

Resuspend the beads with 42 µL of nuclease-free after cDNA collection. Store resuspended beads at 4°C until the cDNA final product has passed QC.

d. Prepare PCR Mix by referring to Table 4-8. The total volume for the PCR reaction is 100 µL.

Table 4-8 PCR Mix

Components	1X (µL)	2X + 10% (µL)	3X + 10% (µL)	4X + 10% (µL)
cDNA Amplification Mix	50	110	165	220
cDNA Primer	8	17.6	26.4	35.2
Eluted cDNA	42	2 x 42	3 x 42	4 x 42
Total	100	2 x 100	3 x 100	4 x 100

e. Mix gently and short spin before placing the reaction tube in a thermal cycler. Amplify the eluted cDNA based on the PCR program stated in Table 4-9.

Table 4-9 PCR Program for Amplification (for 100 µL)

Temperature	Time	Cycle
(Heated lid) 105°C	on	-
95°C	5 min	1
98°C	20 sec	15
58°C	20 sec	
72°C	3 min	
72°C	5 min	1
12°C	Hold	-

While waiting, refer to section 4.11 for purification and amplification of ADT product.

f. Prepare Qubit dsDNA Mix and record the concentration of PCR product according to Table 4-10.

Table 4-10 Qubit dsDNA Mix

Components	1X (μL)
Invitrogen™ Qubit dsDNA HS Buffer	198
Qubit dsDNA HS Reagent 200X	1
PCR Product	1
Total	200

g. Vortex the mix and then take 1 μL of the PCR product and measure its concentration using Qubit dsDNA HS Kit. The DNA concentration is usually more than 5 ng/μL.

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

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

1) Mix the cDNA PCR product (100 μL) with beads in a ratio of 1 : 1. Vortex the mixture then incubates it at room temperature for **10 min**.

2) Spin down and place the sample tube onto a magnetic separation rack for **3 min** until the liquid becomes clear. Carefully remove and discard the supernatant with a pipette.

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

4) Repeat step 3) one more time.

5) Keep the tube on the magnetic rack, and open the lid to air-dry the beads at room temperature until no wetness (reflectiveness) is observed. Drying times will vary but will be approximately **5-8 min.**

6) Vortex the dried beads with **42 μL** of TE buffer. Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube onto a magnetic rack for **3-5 min** until the liquid becomes clear. Transfer **40 μL** supernatant 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 till your cDNA final product has passed QC.

i. Take 1 µL of the cDNA sample and measure and record the concentration of the purified cDNA with Qubit dsDNA HS Kit.

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

A qualified cDNA sample should have a main fragment distribution peak appearing at around 500-1,200 bp (Figure 4), and a yield higher than 20 ng.

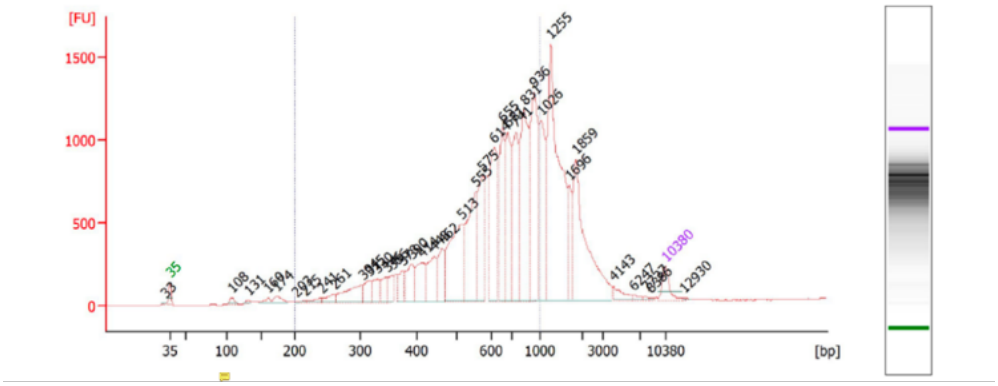


Figure 4. A representative Agilent Bioanalyzer 2100 analysis result of a PCR amplified cDNA sample

4.12. ADT Product Purification

a. Equilibrate the magnetic beads to room temperature for at least **30 min**. Prepare 80% ethanol freshly and equilibrate to room temperature before use.

b. ADT Product Purification Procedures with 2.0X Magnetic Bead:

1) Mix the collected supernatant in the 2.0 mL tube collected in section **4.10 b-3)** with 500 µL beads. Vortex the mix then incubate it at room temperature for **10 min**.

2) Spin down and place the tube onto a magnetic separation rack for **5 min** until the liquid becomes clear.

3) Carefully remove and discard the supernatant with a pipette (If foams are seen on the cap, discard them with a pipette).

4) Keep the 2.0 mL tube on the magnetic separation rack and add **1.5 mL** of freshly prepared 80% ethanol to 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 (If foams are seen on the cap, clean the cap with 80% ethanol).

5) Repeat step 4) one more time.

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 observed. Drying times will vary but will be approximately **5-8 min**.

7) Add **22 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 onto a magnetic separation rack for **3-5 min** until the liquid becomes clear.

8) Transfer the supernatant **(~21 µL)** into a new 0.2 mL PCR tube.

9) Add another **22 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 onto a magnetic separation rack for **3-5 min** until the liquid becomes clear.

10) Transfer the supernatant **(~21 µL)** into the 0.2 mL PCR tube in step 9) and obtain a combined eluted ADT product.

c. If the collected eluted ADT 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 till your cDNA final product has passed QC.

d. Prepare PCR Mix by referring to Table 4-11. The total volume for the PCR reaction is 100 µL.

Table 4-11 ADT PCR Mix

Components	1X (µL)	2X + 10% (µL)	3X + 10% (µL)	4X + 10% (µL)
ADT Amplification Mix	50	110	165	220
ADT Primer	8	17.6	26.4	35.2
Eluted ADT	42	2 x 42	3 x 42	4 x 42
Total	100	2 x 100	3 x 100	4 x 100

e. Mix gently and short spin before placing the reaction tube in a thermal cycler. Amplify the eluted ADT product based on the PCR program stated in Table 4-12.

Table 4-12 PCR Program for Amplification (for 100 μL)

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

While waiting, refer to section 4.11 for purification and amplification of ADT product.

f. Prepare Qubit dsDNA Mix and record the concentration of PCR product according to Table 4-13.

Table 4-13 Qubit dsDNA Mix

Components	1X (μL)
Invitrogen™ Qubit dsDNA HS Buffer	198
Qubit dsDNA HS Reagent 200X	1
PCR Product	1
Total	200

g. Vortex the mix and then take 1 μL of the PCR product and measure its concentration using Qubit dsDNA HS Kit. The DNA concentration is usually more than 5 ng/μL.

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

h. ADT PCR product Purification Procedures with 2.0X Magnetic Bead.

1) Mix the ADT PCR product (100 μL) with beads in a ratio of **1 : 2**. Vortex the mixture then incubates it at room temperature for **10 min.**

2) Spin down and place the sample tube onto a magnetic separation rack for **5 min** until the liquid becomes clear. Carefully remove and discard the supernatant with a pipette.

3) Keep the tube on the magnetic separation 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.

4) Repeat step 3) one more time.

5) Keep the tube on the magnetic rack, and open the lid to air-dry the beads at room temperature until no wetness (reflectiveness) is observed. Drying times will vary but will be approximately **5-8 min**.

6) Vortex the dried beads with **42 µL** of TE buffer. Incubate at room temperature for **5 min**. Spin down briefly and place the sample tube onto a magnetic rack for **3-5 min** until the liquid becomes clear. Transfer **40 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 till your ADT PCR final product has passed QC.

i. Take 1 µL of the ADT PCR product sample, measure and record the concentration of the purified ADT PCR product with Qubit dsDNA HS Kit.

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

A qualified ADT PCR product sample should have a main fragment distribution peak appearing at around 170- 220 bp (Figure 5), and the concentration is above 10 ng/µL.

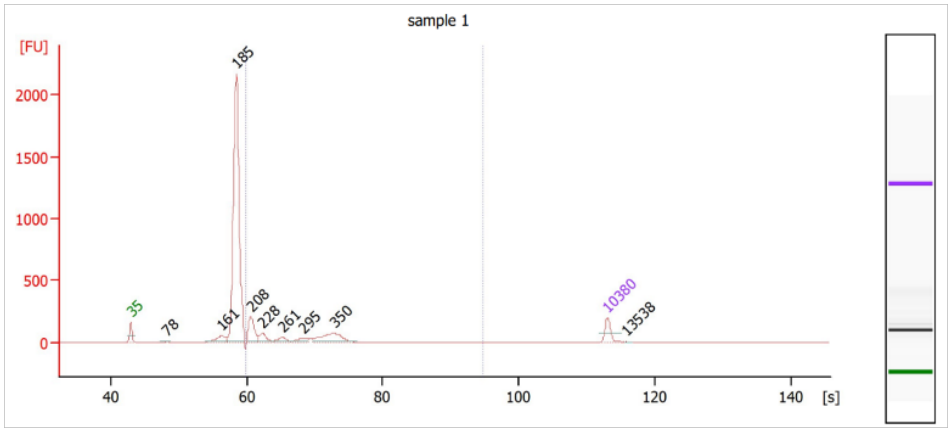
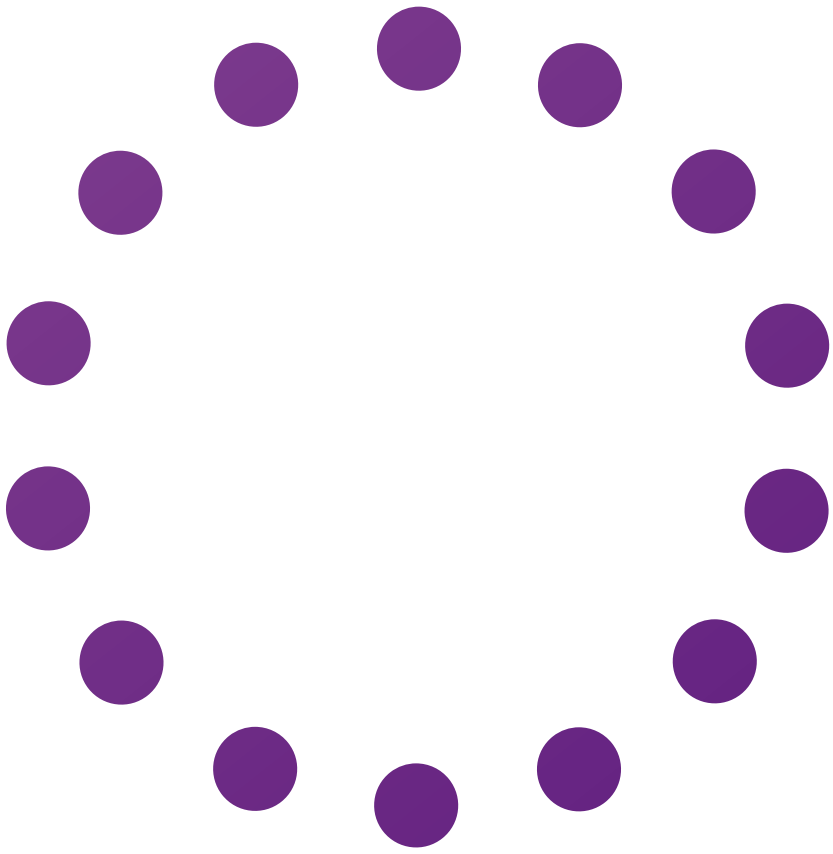


Figure 5. A representative Agilent Bioanalyzer 2100 analysis results in a PCR amplified ADT product

CHAPTER 5
LIBRARY PREPARATION



The later steps will require the Stereo-seq Library Preparation kit. Please ensure that you have purchased the kit separately and prepare the reagents in advance prior to library construction.

Table 5-1 Library Preparation Kit

Stereo-seq Library Prep Kit Cat. No.: 111KL114			
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (tube)**
TME	1000028515	○ (white)	4 μL × 1
Stop Buffer	1000028516	○ (white)	40 μL × 1
TMB	1000028517	○ (white)	40 μL × 1
PCR Barcode Primer Mix (Barcode 57-64)	1000028519	●	50 μL × 1
PCR Barcode Primer Mix (Barcode 81-88)	1000029088	●	50 μL × 1
PCR Barcode Primer Mix (Barcode 89-96)	1000029089	●	50 μL × 1
PCR Barcode Primer Mix (Barcode 97-104)	1000029180	●	50 μL × 1
PCR Amplification Mix	1000029181	●	400 μL × 1

Storage Temperature: -25°C~-18°C | Transported by cold chain | Expiration Date: refer to label

Please ensure that a substantial amount of dry ice remains with the kits upon arrival.

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

Table 5-2 Barcode Amplification Kit

Stereo-seq 16 Barcode Amplification Kit Cat. No.: 111KB016			
Component	Reagent Cat. No.	Cap Color	Quantity (tube)
PCR Barcode Primer Mix 1	1000043201	🔴	25 μL × 1
PCR Barcode Primer Mix 2	1000043202	🔴	25 μL × 1
PCR Barcode Primer Mix 3	1000043203	🔴	25 μL × 1
PCR Barcode Primer Mix 4	1000043204	🔴	25 μL × 1
PCR Barcode Primer Mix 5	1000043205	🟠	25 μL × 1
PCR Barcode Primer Mix 6	1000043206	🟠	25 μL × 1
PCR Barcode Primer Mix 7	1000043207	🟠	25 μL × 1
PCR Barcode Primer Mix 8	1000043208	🟠	25 μL × 1
PCR Barcode Primer Mix 9	1000043209	🟡	25 μL × 1
PCR Barcode Primer Mix 10	1000043210	🟡	25 μL × 1
PCR Barcode Primer Mix 11	1000043211	🟡	25 μL × 1
PCR Barcode Primer Mix 12	1000043212	🟡	25 μL × 1
PCR Barcode Primer Mix 13	1000043213	🟢	25 μL × 1
PCR Barcode Primer Mix 14	1000043214	🟢	25 μL × 1
PCR Barcode Primer Mix 15	1000043215	🟢	25 μL × 1
PCR Barcode Primer Mix 16	1000043216	🟢	25 μL × 1
PCR Amplification Mix	1000043217	🔵	25 μL × 1
Storage Temperature: -25°C~-18°C		Transported by cold chain	Expiration Date: refer to label

This kit does not contain TME, Stop Buffer, TMB, etc. For fresh frozen samples, please use the Stereo-seq Library Preparation Kit (4 RXN, Cat. No.: 111KL114).

5.1. Experimental Preparation

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

Reagent	Preparation Steps	Maintenance
80% Ethanol	Dilute 100% ethanol to 80%	Room temperature up to 1 day
Magnetic beads	Take it out in advance and equilibrate to room temperature at least 30 min prior to use.	4°C
10-fold diluted TME	Dilute 1 μL of TME to 10 μL with TE buffer	On ice up to 1 hr
DO NOT dilute all the TME at once. Volume provided should be enough for 4 dilutions.		
Stop Buffer	Take it out in advance and equilibrate to room temperature at least 30 min prior to use	Room temperature up to 1 day

5.2. cDNA Fragmentation and Amplification

a. Use 20 ng cDNA sample prepared in section 4.10. for the following fragmentation reaction.

b. Prepare the Fragmentation Reaction Mix on ice through gentle pipetting according to Table 5-3. Pipette 10-fold diluted TME up and down before mixing with the rest. After a short spin, gently mix the solution through pipetting while keeping the tube on ice.

Avoid vortexing TME.

Table 5-3 Fragmentation Reaction Mix

Components	1X (μL)
TMB	4
10-fold diluted TME	1
cDNA Product	X
Nuclease-free water	15-X
Total	20

cDNA Input: X (μL) = 20 ng/Concentration of cDNA (ng/μL)

c. Program a thermocycler according to Table 5-4. When the module starts to heat up, put the reaction tube into the thermocycler.

Please DO NOT leave the reaction tube on ice after taking it out of the thermocycler.

Table 5-4 Fragmentation Reaction Program

Temperature	Time
(Heated lid) 60°C	on
55°C	10 min
12°C	Hold

d. After the fragmentation reaction program is done, take out the reaction tube, short spin it to collect all the liquid to the bottom and then leave it at room temperature. Add 5 µL of Stop Buffer to the fragmentation reaction mix to terminate the fragmentation process, and then pipette to mix thoroughly. Incubate the mix at room temperature for **5 min**.

e. Set up PCR Library Mix according to Table 5-5 to start the amplification process of fragmented cDNA.

Table 5-5 PCR Library Mix

Components	1X (µL)
Fragmentation product	25
PCR Barcode Primer Mix	25
PCR Amplification Mix	50
Total	100

Selection principle of PCR Barcode Primer Mix:

- **1-4 Libraries per sequencing run, choose the kit with Cat. No.: 111KL114 and refer to Appendix A for guidelines for using PCR Barcode Primer Mix;**
- **4 < libraries <16 per sequencing run, choose the kit with Cat. No.: 111KB016 and refer to Appendix B for guidelines for using PCR Barcode Primer Mix;**

f. Vortex and spin down briefly the reaction mix prepared above. Incubate it in a thermocycler with the following incubation protocol (Table 5-6) and start the program.

Table 5-6 PCR Amplification Program (for 100μL)

Temperature	Time	Cycle
(Heated lid) 105 °C	on	-
95°C	5 min	1
98°C	20 sec	13
58°C	20 sec	
72°C	30 sec	
72°C	5 min	1
12°C	Hold	-

g. Take 1 μL of the PCR product and use the Qubit dsDNA HS Kit to measure the concentration. The concentration is usually around 10-100 ng/μL.

5.3. PCR Product Size Selection

a. Mix the PCR product obtained above with the magnetic beads in a volume ratio of 1 : 0.55 (PCR product : beads = 100 μL : 55 μL) in a PCR tube. Vortex the mixture then incubate it at room temperature for **5 min**.

b. Short spin the reaction mix and place the tube onto a magnetic separation rack for **3 min** until it becomes clear. Then, carefully transfer the supernatant to a new PCR tube.

Keep the supernatant and discard the beads.

c. Add **15 μL** of beads to the new PCR tube with the supernatant from step b. Vortex to mix thoroughly. Incubate at room temperature for **5 min**.

d. Spin down and place the tube onto a magnetic separation rack for **3-5 min** until it becomes clear. Carefully discard the supernatant with a pipette.

e. Keep the tube on the magnetic separation rack and add **200 μL** of freshly prepared 80% ethanol to wash the beads by rotating the tube on the magnetic rack. Incubate for **30 sec** and carefully remove the supernatant with a pipette.

f. Repeat step e. one more time.

g. Spin-down the tube and put it on the magnetic rack to collect beads on the sidewall. Use a smaller pipette tip to remove the remaining liquid and discard it.

h. Air-dry the beads for **3-5 min** until the bead surface is not reflective. Do not allow the beads to overdry and crack.

i. Mix the dried beads with **20 μL** of TE buffer, vortex to mix and incubate at room temperature for **5 min**. Spin down briefly and place the centrifuge tube onto a magnetic separation rack for **3 min** until the liquid becomes clear. Transfer the supernatant to a new 1.5 mL tube.

Keep the supernatant.

j. Take 1 μL of purified PCR product and measure the concentration with Qubit dsDNA HS Kit. Use Bioanalyzer, Tapestation (Agilent Technologies), LabChip® GX, GXII, GX Touch (PerkinElmer), Fragment Analyzer™ Advanced Analytical) or other equipment based on the principle of electrophoretic separation to detect the fragment distribution of the purified PCR products. The main peak of fragment distribution is required to be 200-600 bp (Figure 6). Normally, the PCR yield is higher than 100 ng.

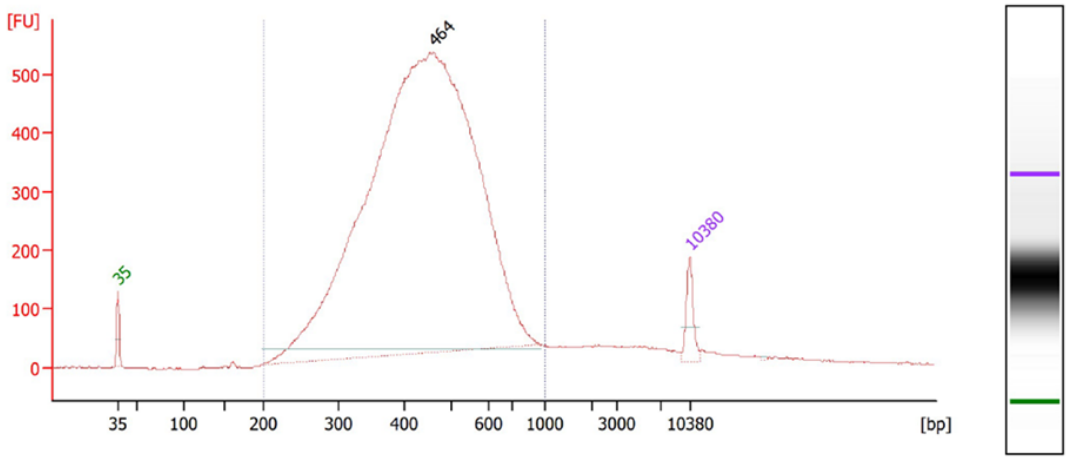


Figure 6. Agilent 2100 Bioanalyzer fragment size distribution of the purified PCR product

5.4. ADT Product Amplification

It is required to use different sample barcodes when sequencing ADT Libraries and transcriptome libraries together. Please refer to Appendix A and Appendix B for details of the usage rules.

If not specified, all the reagents should use Nuclease-free water for dilution.

a. Use 20 ng ADT product prepared in section 4.11. for the following fragmentation reaction.

b. Set up PCR Library Mix according to Table 5-5 to start the amplification process of ADT product.

Table 5-7 ADT Product PCR Library Mix

Components	1X (μL)
ADT product (20ng)	X
Nuclease-free water	25 - X
PCR Barcode Primer Mix	25
PCR Amplification Mix	50
Total	100

Selection principle of PCR Barcode Primer Mix:

- **1-4 Libraries per sequencing run, choose the kit with Cat. No.: 111KL114 and refer to Appendix A for guidelines for using PCR Barcode Primer Mix;**
- **4 < libraries <16 per sequencing run, choose the kit with Cat. No.: 111KB016 and refer to Appendix B for guidelines for using PCR Barcode Primer Mix;**

c. Vortex and spin down briefly the reaction mix prepared above. Incubate it in a thermocycler with the following incubation protocol (Table 5-6) and start the program.

Table 5-8 PCR Amplification Program (for 100μL)

Temperature	Time	Cycle
(Heated lid) 105 °C	on	-
95°C	5 min	1
98°C	20 sec	8
58°C	20 sec	
72°C	1 min	
72°C	5 min	1
12°C	Hold	-

d. Take 1 μL of the PCR product and use the Qubit dsDNA HS Kit to measure the concentration. The concentration is usually around 10-100 ng/μL.

5.5. ADT PCR Product Size Selection

a. Mix the PCR product obtained above with the magnetic beads in a volume ratio of 1 : 2 (PCR product : beads = 100 µL : 200 µL) in a PCR tube. Vortex the mixture then incubates it at room temperature for **10 min**.

b. Short spin the reaction mix and place the tube onto a magnetic separation rack for **3-5 min** until it becomes clear. Then, carefully transfer the supernatant to a new PCR tube.

Keep the supernatant and discard the beads.

c. Keep the tube on the magnetic separation rack and add **400 µL** of freshly prepared 80% ethanol to wash the beads by rotating the tube on the magnetic rack. Incubate for **30 sec** and carefully remove the supernatant with a pipette.

d. Repeat step c. one more time.

e. Spin-down the tube and put it on the magnetic rack to extract the beads out of the liquid. Use a smaller pipette tip to remove the remaining liquid and discard it.

f. Air-dry the beads for **5-8 min** until the bead surface is not reflective or cracked.

g. Mix the dried beads with **24 µL** of TE buffer, vortex to mix and incubate at room temperature for **5 min**. Spin down briefly and place the centrifuge tube onto a magnetic separation rack for **3-5 min** until the liquid becomes clear. Transfer the supernatant to a new 1.5 mL tube.

Keep the supernatant.

h. Take 1 µL of purified PCR product and measure the concentration with Qubit dsDNA HS Kit. Use Bioanalyzer, Tapestation (Agilent Technologies), LabChip® GX, GXII, GX Touch (PerkinElmer), Fragment Analyzer™ (Advanced Analytical) or other equipment based on the principle of electrophoretic separation to detect the fragment distribution of the purified PCR products. The main peak of fragment distribution is required to be 200-250 bp (Figure 7). Normally the PCR yield is higher than 100 ng.

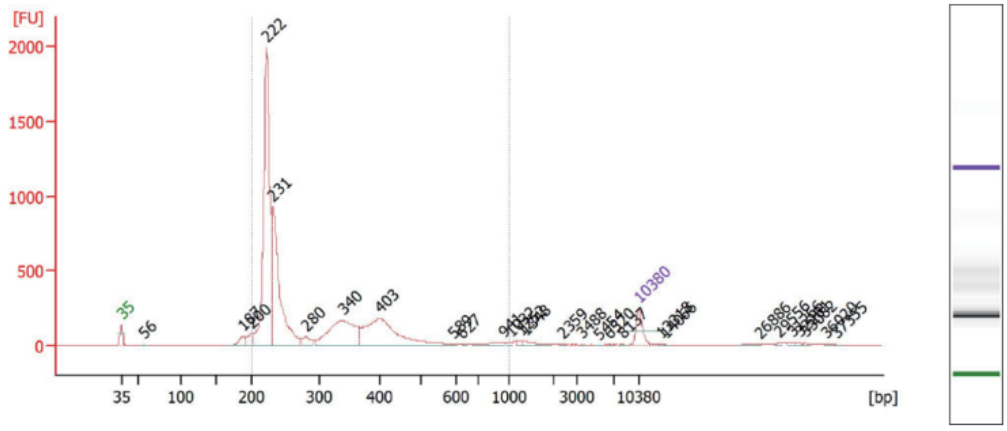
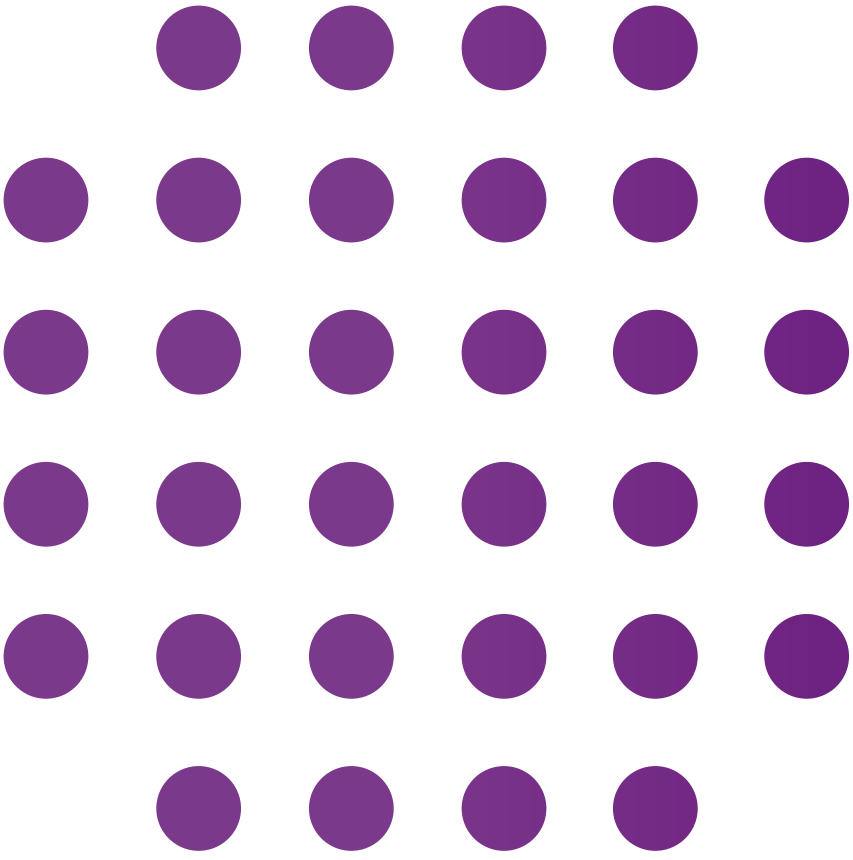


Figure 7. Agilent 2100 Bioanalyzer fragment size distribution of the ADT PCR product

CHAPTER 6
LIBRARY CONSTRUCT & SEQUENCING



This chapter introduces the compatible sequencing instruments and sequencing reagents for the Stereo-seq system. The cDNA and ADT library constructs are illustrated in Figure 8 and Figure 9.

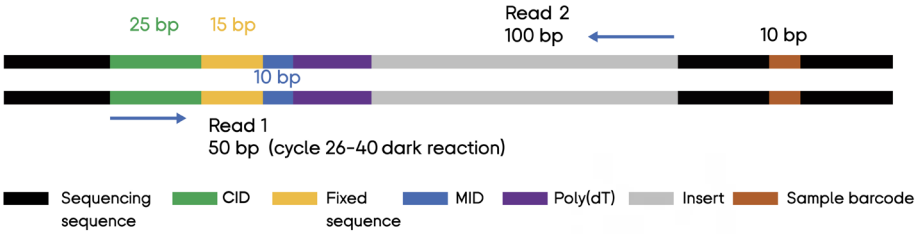


Figure 8. Stereo-CITE Transcriptome Library Construct

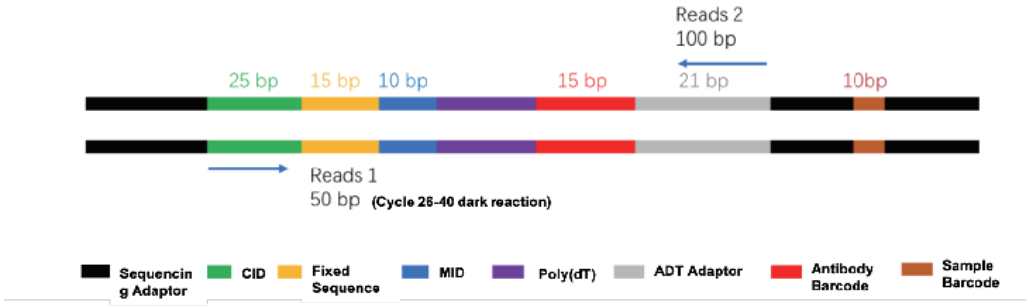


Figure 9. Stereo-CITE ADT Library Construct

Please refer to the user manual of High-throughput Sequencing Primer Kit (STOmics), Cat. no. 940-000037-00 for DNB preparation.

Use the following parameters to perform the sequencing run:

- Without sample barcode sequenced (for only one sample): choose paired-ended mode with 50 cycles of Read 1 and 100 cycles of Read 2. Use dark cycles on Read 1 from 26 to 40 cycles.
- With sample barcode sequenced (for two or more samples): choose paired-ended mode with 50 cycles of Read 1 and 100 cycles of Read 2 and an additional 10 cycles of sample barcode. Use dark cycles on Read 1 from 26 to 40 cycles.

Please read the corresponding user manual High-throughput Sequencing Primer Kit (STOmics), Cat. no. 940-000037-00 carefully before performing sequencing and strictly follow the instructions. If you have any questions about sequencing, please contact your local MGI account manager or technical support.

Appendix A: PCR Barcode Primer Mix Use Rules

The PCR Barcode Primer Mix in this kit is a pre-mixed barcode combination with a balanced set of bases, which can be randomly selected for use by the customer. Splitting barcode is required in sequencing for two or more samples in the same lane (in order to distinguish your sequencing samples). If there is only one sample, do not split barcode for sequencing. The following table is the barcode sequence number in each pre-set PCR Barcode Primer Mix.

PCR Barcode Primer Mix Name	Contains the Barcode Sequence Number							
PCR Barcode Primer Mix (Barcode 57~64)	57	58	59	60	61	62	63	64
PCR Barcode Primer Mix (Barcode 81~88)	81	82	83	84	85	86	87	88
PCR Barcode Primer Mix (Barcode 89~96)	89	90	91	92	93	94	95	96
PCR Barcode Primer Mix (Barcode 97~104)	97	98	99	100	101	102	103	104

Appendix B: 16 PCR Barcode Primer Mix Use Rules

The Stereo-CITE 16 Barcode Amplification Kit provides 16 types of PCR Barcode Primer Mix. It is designed for a large number of samples batched library construction and multi-sample mixed sequencing. This kit is based on the design principle of base balance, and has been tested by repeated experiments. The best Barcode combinations have been selected for use with the Stereo-CITE Library Preparation Kit. To ensure the best results, please read the following usage rules carefully.

Any sample with the same barcode cannot be sequenced in the same lane.

Based on the base balancing principle, the PCR Barcode Primer Mix should be used in groups with the following grouping rules:

4 PCR Barcode Primer Mixes in groups: 1~4, 5~8, 9~12, 13~16, a total of 4 sets.

Ensure that to centrifuge the PCR Barcode Primer Mix thoroughly before use. Gently open the lid during use, to prevent the liquid from splashing and avoid cross-contamination. Pipetting to mix well and shortly centrifuge while using. Close the lid in time after use.

N types of PCR Barcode Primer Mix Method: take the same volume of each; mix them and add the mixture to the sample.

When the data amount requirements for each sample are the same:

For different sample sizes, please refer to the recommended Barcode combinations shown below:

Sample/lane	Method 1	Method 2	Method 3	Method 4
1	1 to 4	5 to 8	9 to 12	13 to 16
2	Sample 1: 1 and 2 Sample 2: 2 and 4	Sample 1: 5 and 6 Sample 2: 7 and 8	Sample 1: 9 and 10 Sample 2: 11 and 12	Sample 1: 13 and 14 Sample 2: 15 and 16
3	Sample 1: 1 Sample 2: 2 Sample 3: 3 and 4	Sample 1: 5 Sample 2: 6 Sample 3: 7 and 8	Sample 1: 9 Sample 2: 10 Sample 3: 11 and 12	Sample 1: 13 Sample 2: 14 Sample 3: 15 and 16
4	Sample 1: 1 Sample 2: 2 Sample 3: 3 Sample 4: 4	Sample 1: 5 Sample 2: 6 Sample 3: 7 Sample 4: 8	Sample 1: 9 Sample 2: 10 Sample 3: 11 Sample 4: 12	Sample 1: 13 Sample 2: 14 Sample 3: 15 Sample 4: 16
5	Sample 1: 1 Sample 2: 2 Sample 3: 3 Sample 4: 4 Sample 5: select any group from the remaining three groups	Sample 1: 5 Sample 2: 6 Sample 3: 7 Sample 4: 8 Sample 5: select any group from the remaining three groups	Sample 1: 9 Sample 2: 10 Sample 3: 11 Sample 4: 12 Sample 5: select any group from the remaining three groups	Sample 1: 13 Sample 2: 14 Sample 3: 15 Sample 4: 16 Sample 5: select any group from the remaining three groups

Sample/lane	Method 1	Method 2	Method 3	Method 4
6	Sample 1: 1 Sample 2: 2 Sample 3: 3 Sample 4: 4 Sample 5 and 6: select any two groups from the remaining three groups	Sample 1: 5 Sample 2: 6 Sample 3: 7 Sample 4: 8 Sample 5 and 6: select any two groups from the remaining three groups	Sample 1: 9 Sample 2: 10 Sample 3: 11 Sample 4: 12 Sample 5 and 6: select any two groups from the remaining three groups	Sample 1: 13 Sample 2: 14 Sample 3: 15 Sample 4: 16 Sample 5 and 6: select any two groups from the remaining three groups
7	Sample 1: 1 Sample 2: 2 Sample 3: 3 Sample 4: 4 Sample 5 and 7: select groups by referencing the methods used for 3 samples/lane	Sample 1: 5 Sample 2: 6 Sample 3: 7 Sample 4: 8 Sample 5 and 7: select groups by referencing the methods used for 3 samples/lane	Sample 1: 9 Sample 2: 10 Sample 3: 11 Sample 4: 12 Sample 5 and 7: select groups by referencing the methods used for 3 samples/lane	Sample 1: 13 Sample 2: 14 Sample 3: 15 Sample 4: 16 Sample 5 and 7: select groups by referencing the methods used for 3 samples/lane
8	Select any two groups from the four groups.			
N= 9~16 **(N, number of mixed samples)**	Perform the following steps: 1. Classify samples 1 to 8 as a group, and add the PCR Barcode Primer Mix by referencing the methods used for 8 samples/lane. 2. Classify the remaining samples as a group, and correspondingly add different groups of PCR Barcode Primer Mix based on a value of (N-8) by referencing the methods used for 1 to 8 samples/lane.			

Examples of the mixture with different PCR Barcode Primer Mix:

Example one, 2 samples /lane (refer to Method 1):

1. Take 12.5 μL of PCR Barcode Primer Mix 1 and 2, mix them in equal volumes, and add them to Sample 1.
2. Add 12.5 μL of PCR Barcode Primer Mix 3 and 4 to Sample 2.

Example two, 13 samples/lane:

1. Add 25 μL of PCR Barcode Primer Mix 1 to Sample 1, 25 μL of PCR Barcode Primer Mix 2 to Sample 2, ..., and 25 μL of PCR Barcode Primer Mix 12 to Sample 12.
2. Take 6.25 μL of PCR Barcode Primer Mix 13, 14, 15, and 16, respectively, and mix them in equal volumes and add them to Sample 13.

When the library data amount requirements are different:

Libraries that require more than 20% data in a lane are required to use grouped PCR Barcode Primer Mix

Example:

If there are 9 samples pooled in a lane, and 1 of them requires 30% data, the following scheme is required: if the other 8 samples each use PCR Barcode Primer Mix 1~8, this sample should not use a single PCR Barcode Primer Mix, but use a non-duplicative and grouped PCR Barcode Primer Mix 9~12 or 13~16.

Appendix C: Antibody Titration

Singular TotalSeq™-A Antibody selection is the key component for combining antibodies of your interest in proteo-transcriptome co-detection experiment, and antibody performance can directly affect data quality. For the Stereo-seq proteo-transcriptome co-detection experiment, the selection of antibodies follows the selection principle of conventional mIF method, which requires considerations of host sources, specificities and species reactivities of added antibodies. We recommend users to first perform antibody titration for each antibody on tissue-mounted glass slides individually to find the optimal antibody concentration, then perform a pilot experiment with the selected antibody concentrations before proceeding to Stereo-seq proteo-transcriptome co-detection assay.

Experimental Preparation

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

Reagent/ Consumables	Preparation Steps	Maintenance
5X SSC	Dilute 5 mL of 20x SSC to 20 mL	Room Temperature
0.1X SSC	Dilute 100 µL of 20x SSC to 20 mL	Room Temperature
Aliquot Serum	Thaw serum then filter it with a 0.22µm pore-sized filter and a sterilized syringe. Aliquot the filtered serum and store at -20°C.	20°C
Microscope Slides	Prepare 6 glass slides for each antibody (5 different concentrations and 1 negative control).	Room Temperature

It is recommended to set 2 concentrations below and over the recommended dilution ratio according to manufacturer's instruction of each antibody. Take the primary antibody of NeuN (abcam, ab104224) as an example. If the instruction recommended 1:1000, then set the experimental group with 5 dilution ratios, 1:100, 1:500, 1:1000, 1:5000, 1:1000 and a negative control group (same procedures only without the addition of the primary antibody). A total of 6 slides will be required.

Reagent	Preparation Steps	Maintenance
4% PFA	Mix well after thawing and aliquot to 2mL/tube for storage. Equibrate to room temperature before use.	-20°C for no more than 1 month
5X SSC	Dilute 5 mL of 20x SSC to 20 mL	Room Temperature
0.1X SSC	Dilute 100 µL of 20x SSC to 20 mL; Dilute 250 µL of 20x SSC to 50 mL	Room Temperature
Filtered Serum Buffer	Take the serum out in advance and thaw it. Mix horse serum and goat serum in a 1:1 ratio. Filter the mixture using a 0.22 µm filter membrane (compatible with a syringe-style sterile disposable syringe). Recommend aliquoting 200 µL per tube (for one round of permeabilization and proteo-transcriptomics workflow). Before the experiment, thaw the aliquoted serum at -20°C. Centrifuge at 4°C, 14000g for 10 min and keep it for later use. Approximately 20 µL of serum is needed for one chip, and any remaining serum can be reused.	-20°C
Do not freeze and thaw the aliquot more than 3 times.		
Salmon Sperm DNA	Thaw on ice before use, 20µL/chip	-20°C
Primary & Secondary Antibody	Thaw on ice following the original product instructions, centrifuge for 10 min at 14000g. The Primary Abs should include the isotype Abs	4°C or -20°C
Diluted Primary & Secondary (Optional)	Dilute the antibodies if needed.	Place on ice until ready to use
10% Triton X-100	Dilute 100% Triton X-100 into 10% Triton X-100 with Nuclease-free water if there is no ready-to-use 10% Triton X-100	Room Temperature
FcR Blocking Reagent	For mouse samples, use TruStain Fcx™ PLUS (CD16/32) Antibody; for human samples, use TruStain Fcx™	4°C
Glycerol	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.	Room Temperature
Decrosslinking Reagent	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.	Room Temperature

Other Preparation		
Equipment	**Set up**	**Note**
Cryostat	Set the cryostat chamber temperature to -20°C and 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 and hot lid to 42°C.	Check if there is any abnormality with the PCR thermal cycler and replace it if necessary.
Fluorescence Microscope	Ensure that the microscope is equipped with at least DAPI, FITC, TRITC, CY5	Please select the channels according to the fluorescent emission wavelengths of your secondary antibodies.
Centrifuge	Cool the centrifuge temperature to 4 °C in advance.	-

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

During the temperature equilibration step at cryosection preparation, prepare the blocking buffer by referring to Table C-1 and put it on ice. Meanwhile, take enough 4% PFA solution (at least 400 µL 4% PFA solution per chip) from 4°C to equilibrate to room temperature.

Table C-1 Blocking Buffer

Components	1X (µL)	2X + 10% (µL)	3X + 10% (µL)	4X + 10% (µL)
5X SSC	180	252	372	492
10% Triton X-100	120	4.2	6.2	8.2
Blocking Reagent	2	42	62	82
Salmon Sperm DNA, sheared	20	21	31	41
FcR Blocking Reagent*	10	42	62	82
RI	20	58.8	86.8	114.8
Filtered Serum	28	420	620	820
Nuclease-free water	200	25.2	37.2	49.2
Total	300	630	930	1230

FcR Bloacking Reagent is for blocking of Fc receptors on the cell membrane. Please select either one according to the host species of your sample. For human tissue, please use Catalog# 422031 and for mouse tissue, please use Catalog# 156604.

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

Tissue Mounting on Microscopic Slides

a. Add over 1/3 Volume of 4% PFA to the slide container and ensure that the volume can cover the tissue on the chip and slide. Prepare enough 4% PFA in a 50 mL corning tube or an empty slide container at a volume that could submerge the entire microscopic slide (about 1/3 of the container).

b. b. Tissue mounting: 1 section per 1 slide is recommended (2 sections per antibody, as 1 section is for the negative control).

c. c. Tissue mounting can be achieved via either the cold method (option A) or warm method (option B).

A. Cold Method

1) Place microscope slide inside the chamber with the front-side facing up and pre-cool inside the cryostat chamber for **1~6 min**.

Prolonged cooling for over 6 min may cause mist formation on the chip surface.

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

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

4) Once all tissue mounting is complete, immediately dry the microscope slide at 37°C on a PCR thermal cycler with PCR adaptor for **5 min**.

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

B. Warm Method

1) Perform cryosection and 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 and aim the tissue section onto the center of the slide by gently touching the section with the microscope slide.

3) Check and see if the tissue section has been mounted on to the microscope slide.

4) Turn the microscope slide over, and immediately dry it at 37°C on a PCR thermal cycler with PCR adaptor front-side up for 5 min.

Tissue Fixation

a. After drying, immediately immerse the tissue-mounted microscopic slide in 4% PFA solution at room temperature and fix for 10 min.

b. After fixation, move the 4% PFA container to a fume hood. Meanwhile, prepare a 50mL centrifuge tube filled with at least 30mL of 0.1X SSC.

c. Take the microscopic slide out of the 4% PFA solution and immediately immerse the slide in a 50 mL centrifuge tube with at least 30 mL of 0.1X SSC for 10 sec.

Slide transfer should be done as soon as possible to avoid tissue from over drying.

d. Take out the slide and immediately wipe off excess SSC solution from around and the back of the slide with Kimwipes 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.

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

f. Transfer the processed slides to an immunohistochemistry wet cassette.

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

Tissue Blocking and Primary antibody incubation

a. Vortex the blocking solution that was prepared in Table 3-1 and add no more than 100 µL/section of blocking solution dropwise on the tissue surface within the hydrophobic zone. 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 0.5cm × 0.5cm, the recommended blocking solution volume is 30 µL/section.

b. While waiting for the incubation to be done, prepare the Primary Antibody Incubation Buffer according to Table C-2. Vortex the buffer and leave it on ice until use.

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

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

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

^The amount of primary antibody required is dependent on the dilution ratio.

*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.

c. Discard the blocking solution with a pipette.

- For experimental groups: Slowly add the primary antibody solution from the non-tissue area until the solution covers the tissue section. Do not exceed 100 µL/slide. Ensure that to label the dilution ratio on the microscope slide. Incubate at room temperature for **45 min**.
- For negative control group: Add 100 µL/slide of blocking solution. Incubate at room temperature for **45 min.**

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

d. While waiting for the primary antibody incubation to be done, prepare secondary antibody solution according to Table C-3. After vortex mixing and brief centrifugation, leave the secondary antibody solution on ice **in the dark** until use.

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

Components	1X (µL)
Secondary antibody	0.2
Blocking Solution	99.8
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, please adjust the dilution ratio according to the manufacturer's instructions.

Secondary Antibody Incubation

a. Aspirate the primary antibody incubation solution of the experimental group and the blocking solution of the negative control group.

b. Add **100 µL** 0.1X SSC per section. Incubate at room temperature for **1 min** and then aspirate.

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

c. Repeat step b. once.

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

e. Aspirate the secondary antibody incubation solution.

f. Add **100 µL** 0.1X SSC per section. Incubate at room temperature for **1 min** and then aspirate.

g. Repeat step f. once.

h. Hold on to the slide with one hand and completely dry the tissue further with a power dust remover in the other hand at a distance 2-3 cm away from the tissue surface by blowing gently from one side at a 30-degree angle horizontal to the plane of the slide.

i. Pipette **5 µL** glycerol gently onto the center of the tissue on each chip without introducing air bubbles.

j. With a pair of forceps, place one end of the coverslip onto the tissue edge while holding the other end and then gradually lower the coverslip onto the tissue. Ensure that the tissue is completely covered by glycerol and the coverslip. To avoid fluorescent bleaching, **IMMEDIATELY proceed to imaging**.

Make sure the coverslip is clean without any dust or debris. Wiping with an alcohol swab or blowing with a power dust remover could be used for cleaning.

Imaging

a. Fluorescence images are taken using a fluorescence microscope with image stitching function, and the fluorescence configuration is recommended as following:

- Light source wavelength range: 380-680 nm
- Monochrome camera (≥ 12 bit)
- DAPI filter cube (ref. Excitation 375/28 nm, Emission 460/50 nm)
- FITC filter cube (Reference: Excitation 480/30 nm, Emission 525/50 nm)
- TRITC filter cube (ref. Excitation 545/25 nm, Emission 605/70 nm)
- CY5 filter cube (Reference: Excitation 620/50 nm, Emission 690/50 nm)
- Maximum pixel size: 5 µm/pixel
- Exposure time: 1 ms~2 s (depending on the characteristics of the antibody used)

b. Transfer the slides to the microscope stage and remove the light shield.

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

Image all experimental groups with the same imaging parameters to compare the signal differences across groups.

Guidelines for Selecting Optimal Antibody Concentration

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

Taking the CD68 antibody titration sample data as an example, as shown in the figure below, select the lowest concentration that does not significantly reduce the fluorescence intensity based on the fluorescence signal. In this example, 1:250 should be selected as the optimal antibody dilution concentration for subsequent IF pilot experiments and the official Stereo-CITE Proteo-Transcriptoics experiments.

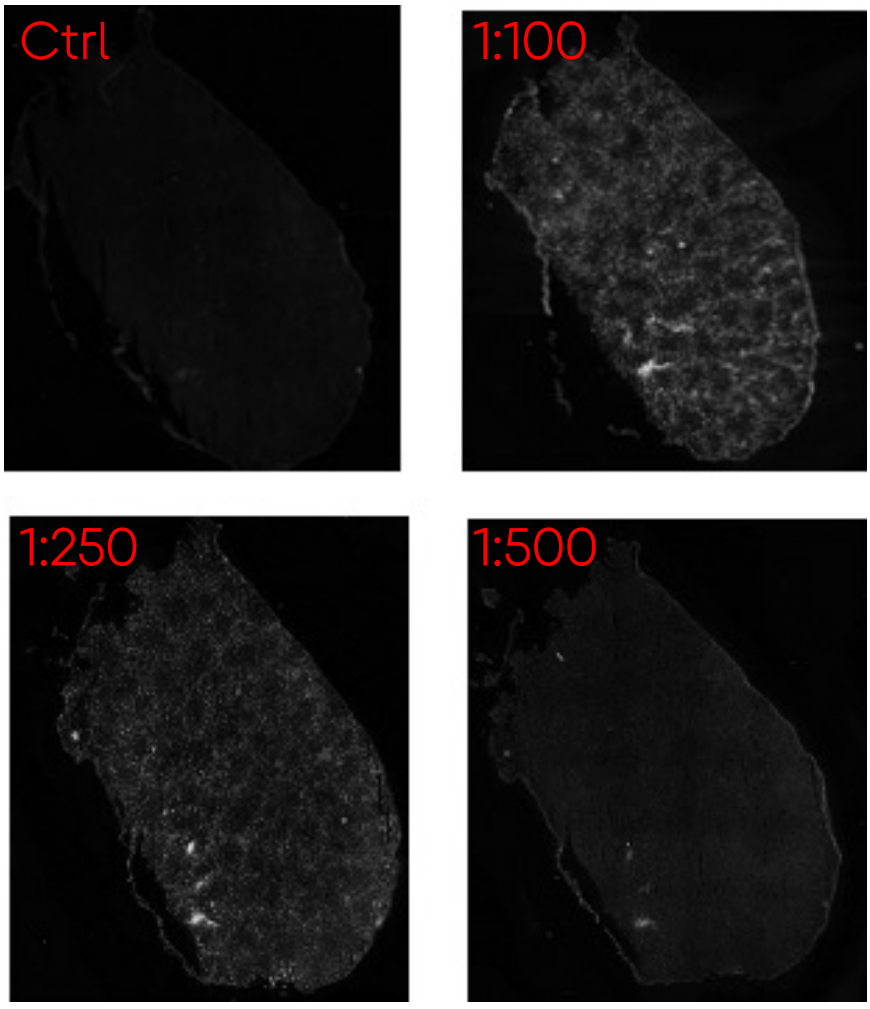


Figure 10. Immunofluorescent staining of the control and anti-CD68 antibody in mouse thymus