STOmics

Stereo-seq TRANSCRIPTOMICS SET FOR CHIP-ON-A-SLIDE

USER MANUAL

mIF COMPATIBLE

Cat. No.: 211ST114 (4 RXNs)
Kit Version: V1.2
Manual Version: A

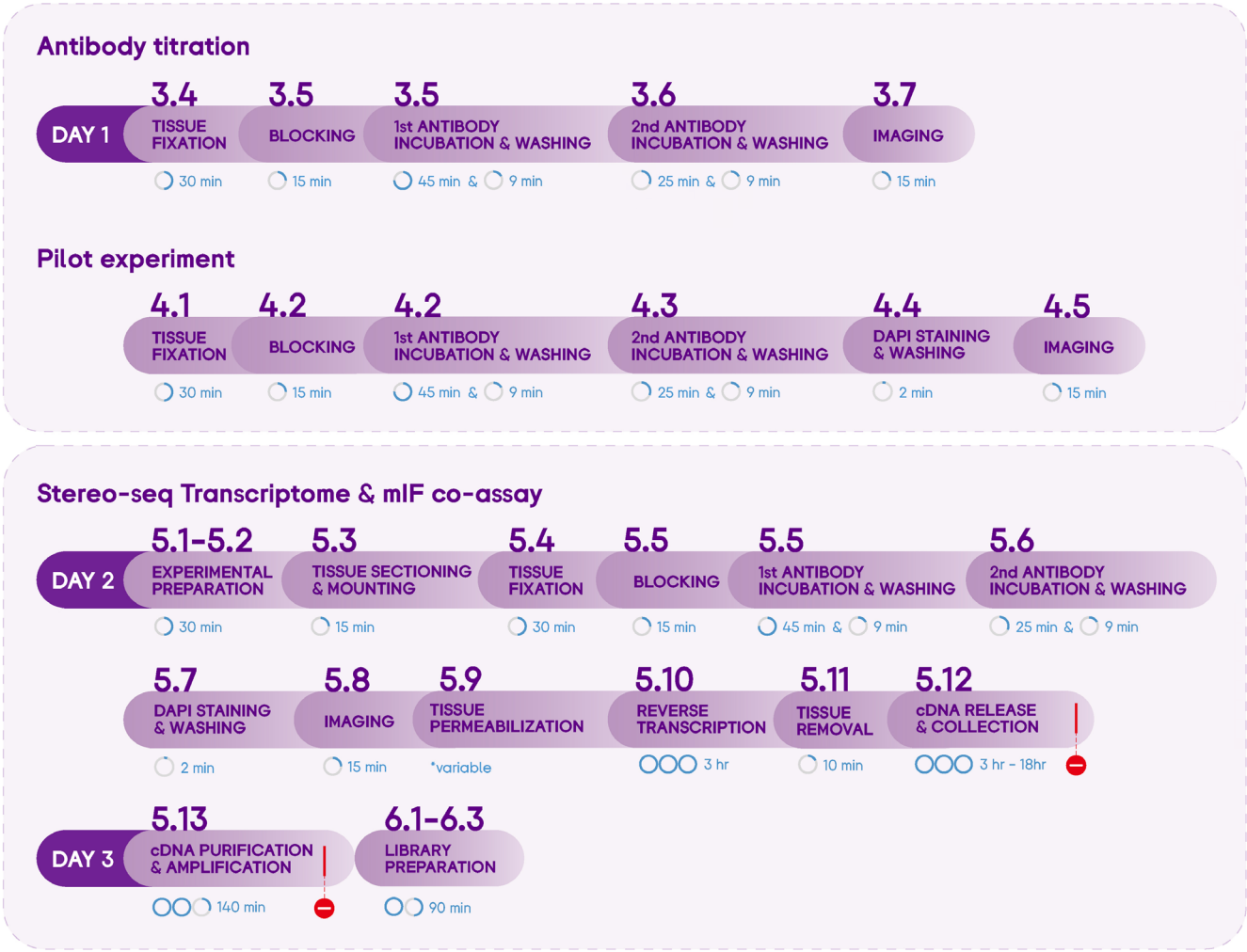
REVISION HISTORY

Manual Version: A
Kit Version: V1.2
Date: Mar. 2023
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: ~3 DAYS

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

1.1. Intended Use 1
1.2. Sequencing Guideline 1
1.3. List of Kit Components 2
1.4. Additional Equipment and Materials 5
1.5. Practice Tips 8
1.6. Precautions and Warnings 11

CHAPTER 2: SAMPLE PREPARATION

2.1. Sample Requirements 14
2.2. Sample Embedding 15
2.3. Sample Storage and Transportation 19

CHAPTER 3: ANTIBODY TITRATION

3.1. Experimental Preparation 21
3.2. Cryosection Preparation 22
3.3. Tissue Mounting 23
3.4. Tissue Fixation 24
3.5. Tissue Blocking and Primary Antibody Incubation 25
3.6. Secondary Antibody Incubation 27
3.7. Imaging 27
3.8. Determination of Optimal Dilution Concentration 28

CHAPTER 4: PILOT Stereo-seq mIF EXPERIMENT

4.1. Preparations for Tissue Blocking 30
4.2. Tissue Blocking & Primary Antibody Incubation 30
4.3. Secondary Antibody Incubation 32
4.4. DAPI staining 32
4.5. Imaging 33
4.6. mIF Pilot Experiment Results 33

TABLE OF CONTENTS

CHAPTER 5: Stereo-seq TRANSCRIPTOMICS SET FOR CHIP-ON-A-SLIDE STANDARD OPERATING PROCEDURE (mIF COMPATIBLE)

5.1. Experimental Preparation 35
5.2. Cryosection Preparation 37
5.3. Tissue Mounting 37
5.4. Tissue Fixation 40
5.5. Tissue Blocking & Primary Antibody Incubation 41
5.6. Secondary Antibody Incubation 43
5.7. DAPI Staining 43
5.8. Imaging 44
5.9. Tissue Permeabilization 47
5.10. Reverse Transcription 48
5.11. Tissue Removal 49
5.12. cDNA Release and Collection 50
5.13. cDNA Purification and Amplification 51

CHAPTER 6: LIBRARY PREPARATION

6.1. Experimental Preparation 57
6.2. cDNA Fragmentation and Amplification 57
6.3. PCR Product Size Selection 59

CHAPTER 7: LIBRARY CONSTRUCT & SEQUENCING 61

APPENDIX

Appendix A: PCR Barcode Primer Mix Use Rules 62
Appendix B: Stereo-seq Transcriptomics Set for Chip-on-a-slide Experimental Record 63

NOTE: Additional operation tips and guidance.

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

QUALITY CHECK POINT

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

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

CHAPTER 1
INTRODUCTION

1.1. Intended Use

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

By integrating mIF staining method into the process of Stereo-seq Transriptomics standard workflow, Stereo-seq transcriptome and multiplex immunofluorescence (mIF) co-detection technology enables spatial visualization of multiple proteins on top of the unbiased whole transcriptome information on the same tissue slice. Without affecting mRNA capturing, the additional detected protein information can be integrated with gene expression data to in-depth evaluate valuable samples, and to parse complex pathological and physiological processes. The amount of protein that can be detected depends on user's antibody selection and imaging configuration. In this user manual, we are showing DAPI with stainings of three antibodies as an example.

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

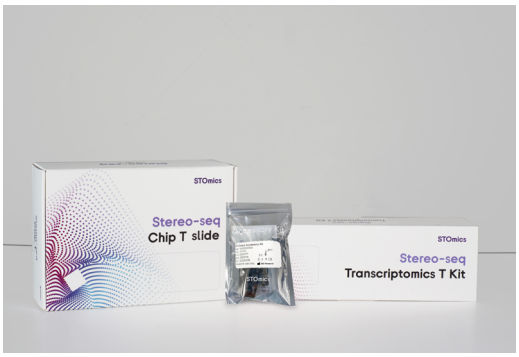
1.2. Sequencing Guideline

Sequencing libraries produced via the Stereo-seq Transcriptomics Set for Chip-on-a-slide requires the DNBSEQ sequencing platform. For details, please refer to **Chapter 7: 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:

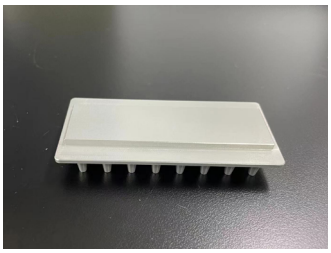
- Stereo-seq Transcriptomics Kit *1 (4 RXN)
- Stereo-seq Chip T Slide (1cm*1cm) *1 (4 EA)
- STOmics Stereo-seq Accessory Kit *2 (5 PCs)



Stereo-seq Library Preparation Kit is not included in the Stereo-seq Transcriptomics Set for Chip-on-a-slide and needs to be purchased separately. If you wish to construct a Stereo-seq library in-house, please refer to **Chapter 6: Library Preparation** for more detail.

Compatible auxiliary but not included:

- (Optional) 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), please 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.

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 1-1

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	1000033684		440 μL × 1
Glycerol	1000031615		50 μL × 1
RT Reagent	1000028507	(transparent)	360 μL × 1
RT Oligo	1000028508	(transparent)	1 OD × 1
RT Additive	1000028502	(transparent)	44 μL × 1
ReverseT Enzyme	1000028509	(transparent)	22 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: on dry ice	Expiration Date: refer to label	

Table 1-2

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 at room temperature	Expiration Date: refer to label	

Table 1-3

STOmics Accessory Kit Cat. No.: 1000033700			
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (per kit)**
Cassette	10000033699	-	1 EA
Gasket	10000033698	-	4 EA
Sealing Tape	-	-	1 EA
Storage Temperature: Room Temperature	Transported at room temperature	Expiration Date: refer to label	

Table 1-4

Stereo-seq PCR Adaptor Cat. No.: 301AUX001			
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (per kit)**
Stereo-seq PCR Adaptor	1000037666	-	2 EA
Storage Temperature: Room Temperature	Transported at room temperature	Expiration Date: refer to label	

1.4. Additional Equipment and Materials

Table below lists equipments and materials needed for this protocol. Some common laboratory equipments not named in Table 1-5 are expected to be accessible by the user, for instance, ice maker, biosafety cabinet, freezers, etc. For specific microscope requirements, please refer to **STOmics Microscope Assessment Guideline.**

Table 1-5

Equipment		
Brand	**Description**	**Catalog Number**
-	Cryostat	-
-	Benchtop centrifuge	-
-	Pipettes	-
	pH meter	-
-	Metal heating block dry bath (optional)	-
-	Vortex mixer	-
Eppendorf	Microcentrifuge	5418 R
*Bio-Rad	T100 Thermocycler	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 (or similar)
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**	**Catalog Number**
-	100% Ethanol (Analytical grade)	-
Ambion	Nuclease-free water	AM9937
	1X TE buffer, pH 8.0	AM9858
	20X SSC	AM9770
*Agencourt	AMPure® XP	A63882
*Beckman Coulter	SPRIselect	B23317/B23318/ B23319

Reagents		
Brand	**Description**	**Catalog Number**
*VAZYME	VAHTSTM DNA Clean Beads	N411-02
Sigma Aldrich	Hydrochloric acid, HCl	2104-50ML
	Methanol	34860-1L-R
	Triton X-100 Solution, 10%	93443-100ML
SAKURA	SAKURA Tissue-Tek® O.C.T. Compound	4583
Invitrogen	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
BioLegend	Human TruStain FcX™ (Fc Receptor Blocking Solution)	422301
	TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody	156604
FcR Blocking 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.		
Invitrogen	Alexa Fluor Plus Secondary Antibodies	-

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

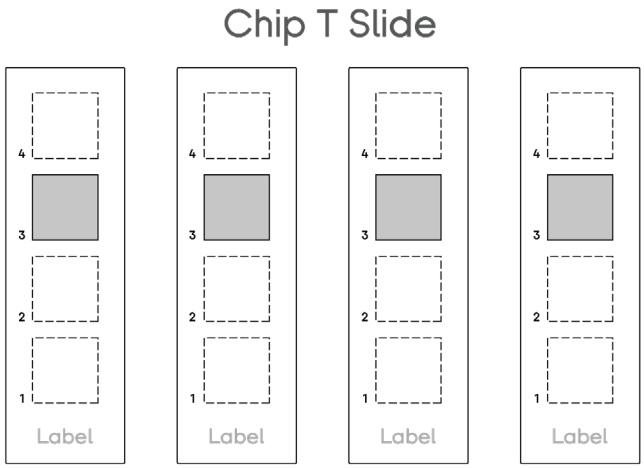
Consumbales		
Brand	**Description**	**Catalog Number**
-	Stainless-steel base mold	-
-	Aluminum foil	-
-	Forceps	-
-	Slide Staining Rack	
-	Microscope glass coverslip (area: 18 mm x 18 mm, thickness: 0.13 - 0.16 mm)	-
	Sterilized Syringe	
	Slide Container	
	Microscope Slide Storage Box	
	Microscope Slides or Adhesion Microscope Slides	
	Super PAP Pen (hydrophobic barrier pen)	
Millipore	Millex Syringe Filter, Durapore PVDF, 0.22 μm pore size	SLGV033N
Corning	Corning® 100 mm TC-treated Culture Dish	353003
	50 mL centrifuge tubes	430829
	15 mL centrifuge tubes	430791
Kimtech	Kimwipes™ delicate task wipes	34155
MATIN	Power dust remover	M-6318
Axygen	5.0 mL MaxyClear Snaplock Microcentrifuge Tube	MCT-500-C
	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
	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.

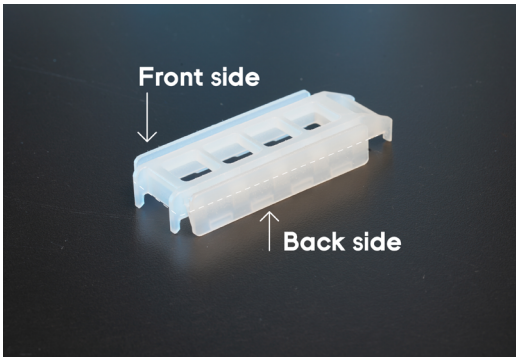


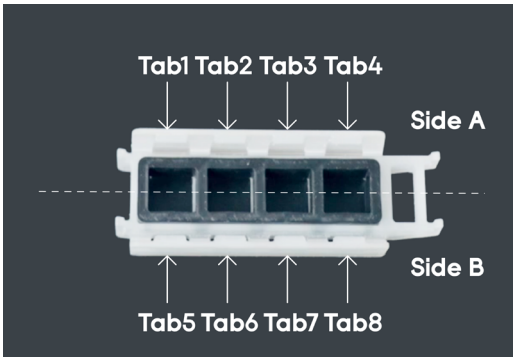
Stereo-seq Chip T Slide Storage

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

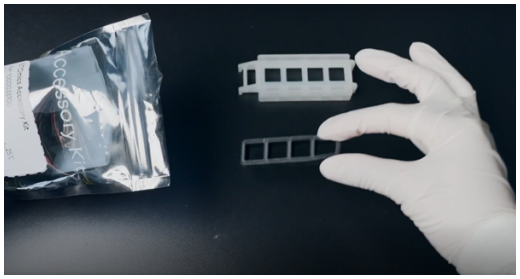




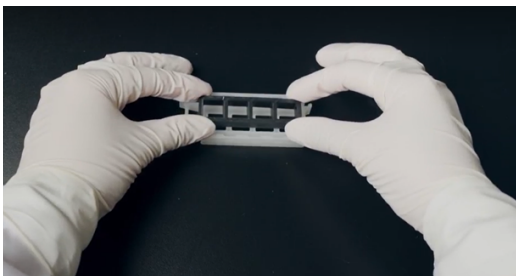
For a demonstration video of Stereo-seq Slide Cassette assembling and removal, please refer to the link or by scanning the QR code:
https://drive.google.com/drive/folders/1_ty31lBo03rK9ux0xMY8xVQKIlOaulfm?usp=sharing

Stereo-seq Slide Cassette Assemble

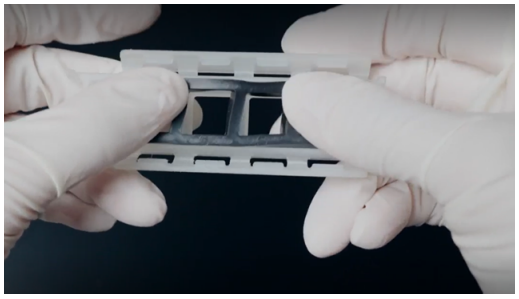
a. Take the Stereo-seq Slide Cassette and gasket out of the STOmics Stereo-seq Accessory Kit.



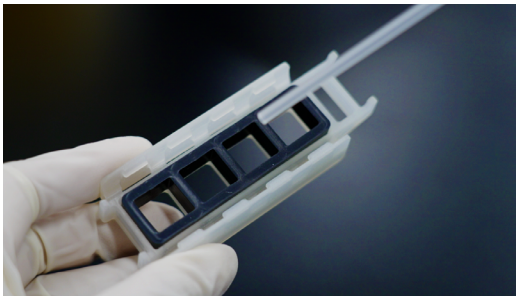
b. Pick up the Stereo-seq Slide Cassette and flip over. Insert the gasket into Stereo-seq Slide Cassette, ensuring the cutouts are aligned.



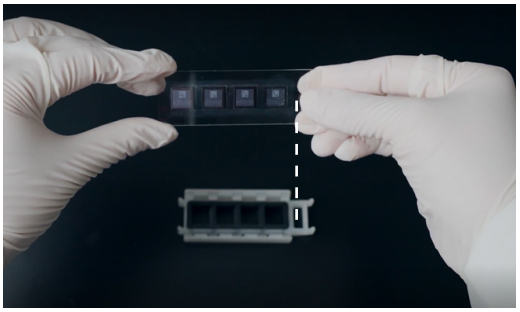
c. Press down the gasket to better fit the cassette.



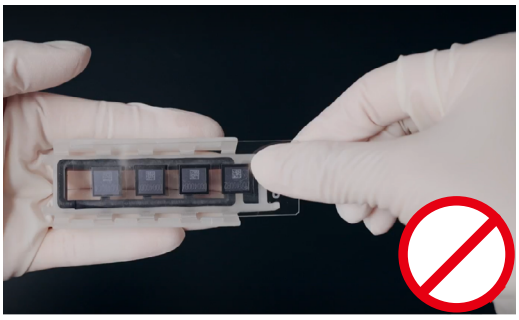
d. Use a power dust remover to blow off any debris on the gasket if necessary.



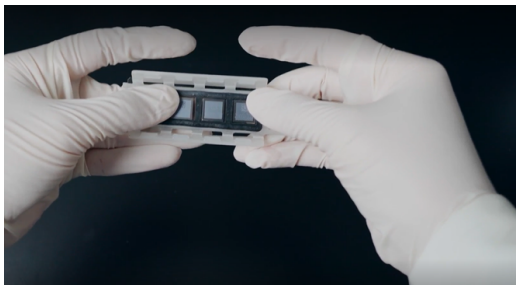
e. Pick up the Stereo-seq Chip Slide and flip over with the chip surface facing down. Align the engraved label with the long edge of the Stereo-seq Slide Cassette.



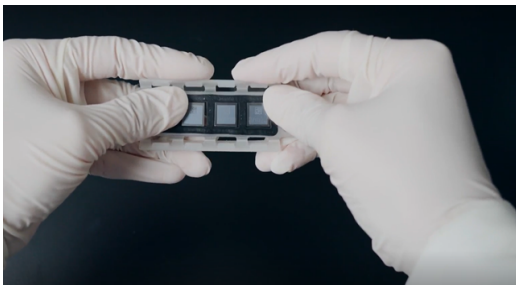
f. Make sure the chips are aligned within the empty space of the gasket and avoid touching the chip surface with the gasket or cassette during slide placement. Insert Stereo-seq Chip Slide under the bottom 4 tabs.



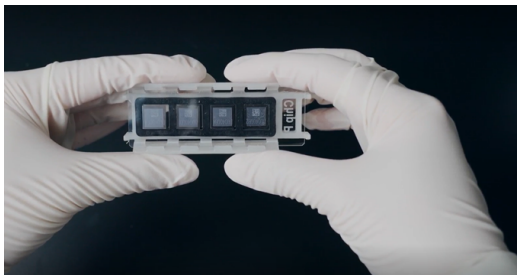
g. Support the back of the cassette with both middle fingers. Place left thumb between tab 1 and tab 2 while right thumb between tab 3 and tab 4.



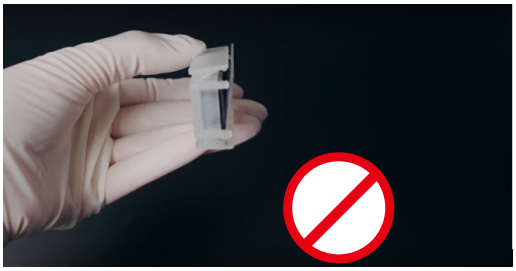
h. Press down evenly on the upper side (A side) of the slide (near the edge) and then simultaneously press down the top edge firmly with both index fingers to clip the slide in place until you hear a clicking sound.



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



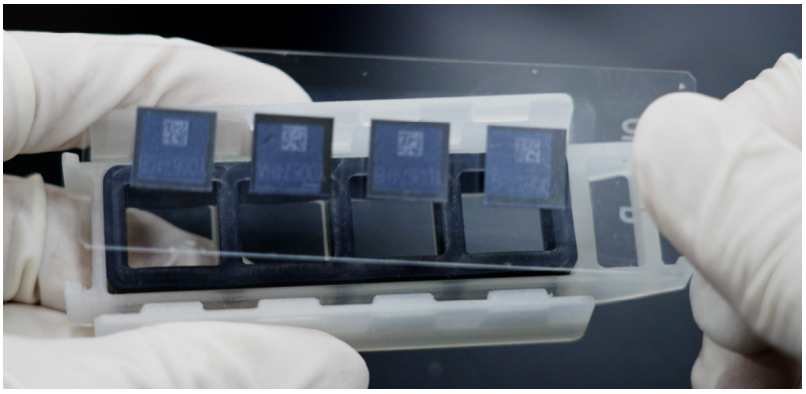
j. Take a final look at the Stereo-seq Slide Cassette to make sure the slide is clipped in place.



Stereo-seq Slide Cassette Removal

a. Flip the cassette over and firmly press down the upper side 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 side with the engraved label.



Stereo-seq Slide Cassette removal is not needed for the Stereo-seq Transcriptomics Set for Chip-on-a-slide.

1.6. Precautions and Warning

- This product is intended for research use only, not for use in diagnostic procedures. Please read all instructions in this manual carefully before using the product.
- Before performing experiments with the kits, users are recommended to 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 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-side surface of the chip.

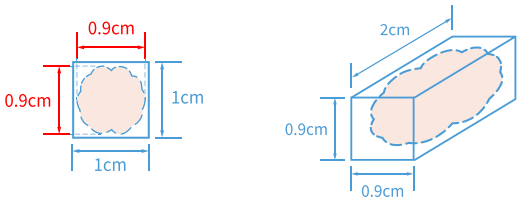
- To prevent cross-contamination, we recommend the use of filtered pipette tips. Use a new tip each time for pipetting different solutions.
- We recommend using thermal cycler with heated lids for PCR reactions. Pre-heat the thermal cycler to reaction temperature before use.
- Improper handling of samples 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 PREPARATION

2.1. Sample Requirements

To avoid RNA degradation, we recommend performing tissue embedding **within 30 min** upon harvesting.

The tissue size should not exceed **0.9 cm x 0.9 cm x 2 cm**, as the tissue section should not exceed 80% area coverage of the chip.



Sample Types

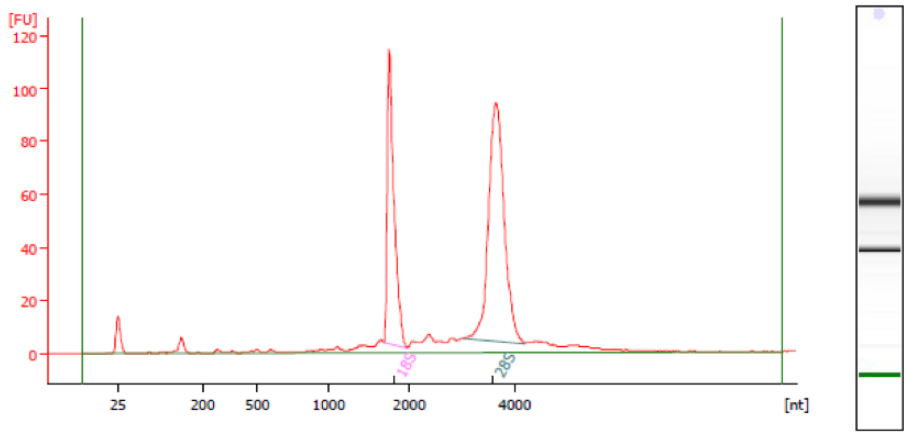
This set of kits can be used for samples from all common animals, including but not limited to human, monkey, and mouse.

For details, please refer to the list:
https://drive.google.com/drive/folders/1ZWWZ-bdNhnjKQiwotX4a_CbTx-4Ew2fh

Sample RNA Integrity Number (RIN) Value

It is recommended to check the RNA quality (RIN value) of a tissue sample before proceeding to Stereo-seq transcriptomics experiment. Total RNA can be extracted from 10-20 slices of 10 μm-thick tissue sections and stored at -20°C in a pre-cooled 1.5mL EP tube. Please refer to the figure below (Figure 1) for the peak of RNA RIN value in mouse brain tissue sections.

It is strongly recommended to proceed only with tissue samples with a RIN value >7.



Overall Results for sample 6 : 8522203007050

RNA Area:	568.4	RNA Integrity Number (RIN):	9.8 (B.02.11, Anomaly Threshold(s) manually adapted)
RNA Concentration:	281 ng/µl		
rRNA Ratio [28s / 18s]:	1.6	Result Flagging Color:	
		Result Flagging Label:	RIN: 9.80

Fragment table for sample 6 : 8522203007050

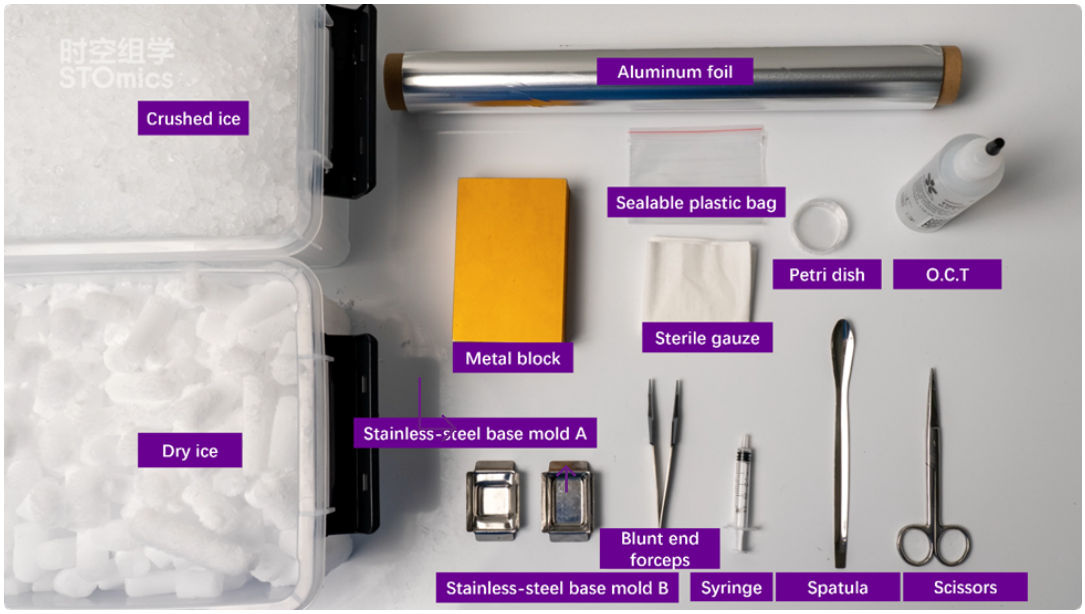
Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,675	1,964	151.3	26.6
28S	3,006	4,187	238.4	41.9

Figure 1. Example of RNA size distribution and RIN value measurement of mouse brain tissue sections.

2.2. Sample Embedding

For a demonstration video of tissue embedding, please refer to the link or by scanning the QR code:
https://drive.google.com/drive/folders/1O138SbfP8lKkYLaScnPkU3pOvwMf0NTW?usp=sharing

a. Prepare these apparatuses/materials in advance:



Materials		
Brand	**Description**	**Quantity**
-	Crushed ice in a box	1
-	Dry ice in a box	1
-	Aluminum foil	1
-	Sealable plastic bag	1
BIOSHARP/Metal Coolbox/ BC032	Metal Block	1
-	Sterile gauze	2
Corning	Corning® 35 mm TC-treated Culture Dish (353001)	1
Sakura/Base Molds/4583	O.C.T	1
Sakura/Base Molds/4162	Stainless-steel base mold A	2
Sakura/Base Molds/7055	Stainless-steel base mold B (slightly larger than mold A)	
-	Blunt end forceps	1
-	Syringe	1
-	Spatula	1
-	Scissors	1

a1. A box of crushed ice and pre-cool OCT on ice for **10 min** in advance.

a2. **2** pieces of stainless-steel base molds slightly larger than the tissue of your interest - mold A and mold B (slightly larger than mold A).

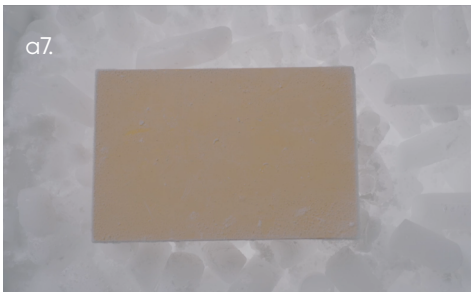
a3. Add a few drops of pre-cooled OCT in the mold A until it reaches approximately 2/3 of the mold and pre-cool on ice for **> 10 min** (remove introduced air bubble using a syringe).

a4. A petri dish filled with OCT and pre-cool it on ice for **> 10 min** (remove introduced air bubble using a syringe).

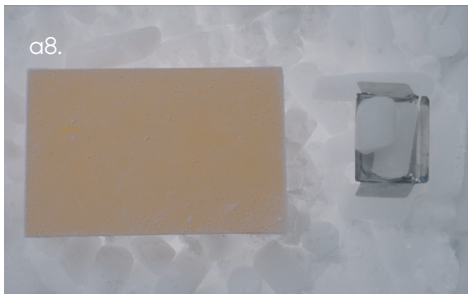
a5. A box of dry ice.

a6. A metal block that has a flat surface to support the stainless-steel base mold when placed on dry ice. The size of the metal block should be larger than the stainless-steel base mold.

a7. Place the metal block on dry ice and pre-cool for **> 5 min** with the flat surface facing up.

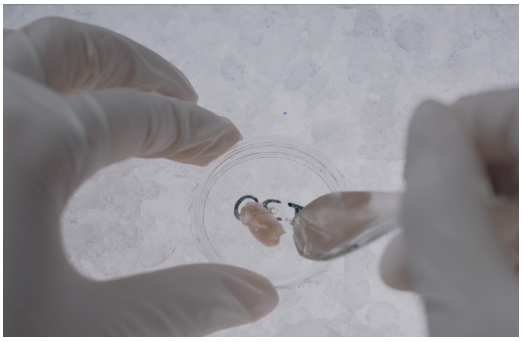


a8. Place mold B on dry ice and pre-cool for **> 5 min**.



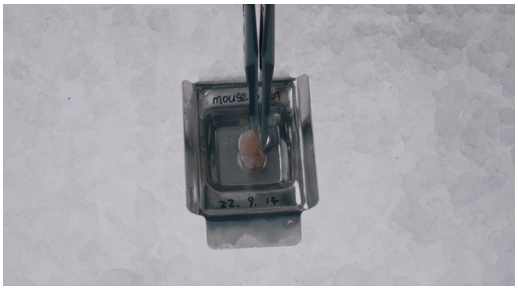
b. Upon harvesting within **30 min**, use sterile gauze or dust-free paper to absorb excess liquid on the tissue surface to avoid ice formation in later steps.

c. Place the tissue in pre-cooled OCT and wrap the tissue evenly with OCT using a spatula without introducing air bubbles.

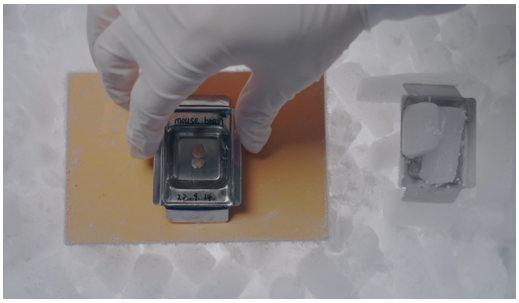


d. Remove any air bubbles using a syringe.

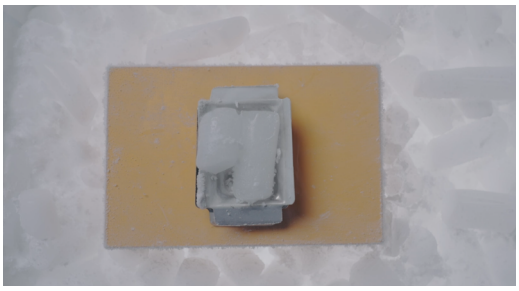
e. Orient the tissue to have the side intended to be sectioned facing downwards and then place into mold A. Make sure the tissue is at the bottom of mold A and fill the mold with chilled OCT without introducing bubbles until the tissue is fully covered.



f. Place the tissue containing mold A onto the metal block that was placed on dry ice.



g. Use mold B as a lid with opening facing up, place on top of mold A gently and then place a few dry ice cubes on top of mold B. Make sure the two stainless-steel base molds can be covered with enough dry ice cubes.



h. After **5 min**, remove mold B and check if the OCT is completely frozen and turns opaque, otherwise repeat f.

i. If the tissue block has solidified and turned opaque, grip the two edges of mold A and press down the edges to detach the tissue block from the mold.

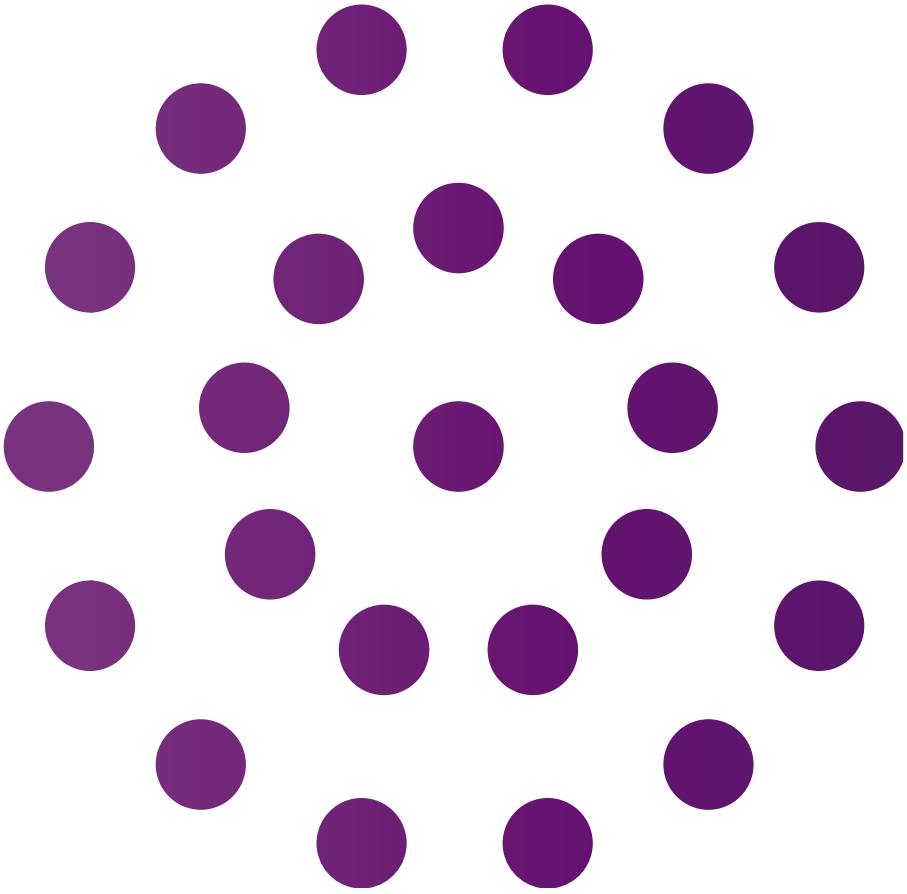
j. Check if the sectioning side of the tissue has been completely covered by OCT. If not, place the tissue block on the metal block, sectioning side facing up, add a few drops of the OCT and then wait till it solidifies and turns opaque.

k. Label the tissue block to mark the orientation of the tissue.

2.3. Sample Storage and Transportation

For storing, wrap the tissue block with aluminum foil and keep it in a properly labeled sealable plastic bag to prevent dehydration and damage then store at -80°C. For transportation, please ship samples on dry ice according to local policy.

CHAPTER 3
ANTIBODY TITRATION



Conventional multiple immunofluorescence (mIF) technology allows for simultaneous detection of multiple protein markers on a single tissue section by mixing different primary antibodies of different species. The location of the corresponding primary antibody was detected by tagging with different fluorescent-labeled secondary antibodies to realize the spatial localizations of multiple targets.

Antibody selection is the key component of the mIF experiment, and antibody performance can directly affect data quality. For the Stereo-seq mIF 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 Transcriptomics - mIF co-assay.

3.1. Experimental Preparation

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 above 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, 1:10000 and a negative control group (same procedures but without the addition of the primary antibody). A total of 6 slides will be required.

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.
PCR Thermal Cycler	Set the temperature to 37°C.	Check if there is any abnormality with the PCR thermal cycler and replace it if necessary.
Fluorescence Microscope	Make sure the microscope is equipped with at least DAPI, FITC, TRITC, CY5 channels.	Please select the channels according to the fluorescent emission wavelengths of your secondary antibodies.
Microcentrifuge	Set the temperature to 4°C.	Check if there is any abnormality with the microcentrifuge and replace it if necessary.

3.2. Cryosection Preparation

a. Set PCR Thermal Cycler to 37°C and place the PCR adaptor for pre-heating in advance.

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 to the chamber and allow it to equilibrate to the cryostat chamber temperature for **30 min**.

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

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

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

3.3. Tissue Mounting on Microscope Slides

a. Prepare enough methanol in a 50 mL corning tube or an empty slide container at a volume that could submerge the entire microscope slide. Close the lid and pre-cool methanol for **10-30 min** at -20°C.

b. Tissue mounting could be achieved via either cold method (option A) or warm method (option B). We recommend placing one tissue slice on one microscope slide. Perform cryosectioning and obtain a standard 10 μm-thick tissue slices.

c. Number of sections required: The number of tissue sections required for titration tests is related to the antibody dilution gradient set to be tested.

It is recommended to set 2 concentrations below and above 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, 1:10000 and a negative control group (same procedures only without the addition of the primary antibody). A total of 6 slides will be required.

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 slide 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 microscope slide center carefully with forceps and brushes. Make sure the tissue section is complete and without wrinkles.

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

4) Immediately dry the tissue-mounted microscope slide at 37°C on a PCR thermal cycler with PCR adaptor at 37°C for **5 min**.

B. Warm Method

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

2) Move the tissue section to the edge, flip the microscope slide and aim the tissue section onto the microscope 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 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 pre-cooled methanol prepared in section 3.3-a for a **30-min** fixation at -20°C. When immersing in methanol, ensure that the tissue section on the microscope slide is completely submerged.

b. While waiting for the fixation to be done, prepare the reagents required for tissue blocking and antibody incubation according to Table 3-1 and prepare the blocking solution according to Table 3-2 then leave it on ice until use.

Table 3-1 Reagent Preparation for Tissue Blocking and Primary Antibody Incubation (for Antibody Titration)

Reagent	Preparation Steps	Maintenance
Primary Antibodies	Take them out of -20°C or 4°C (depending on the manufacturer's instruction) and centrifuge at 14,000g, 4°C for 10 min then leave them on ice.	On ice until use
Secondary Antibodies	Take them out of -20°C or 4°C (depending on the manufacturer's instruction) and centrifuge at 14,000g, 4°C for 10 min then leave them on ice.	On ice until use
Post-centrifuged serum	Take aliquoted serum out of the -20°C freezer and thaw. Then centrifuge at 14,000g, 4°C for 10 min, then leave it on ice.	On ice until use
Each antibody dilution requires about 200 μL of serum. The rest of the aliquoted serum can be reused.		
10% Triton X-100	If without readily available 10% Triton X-100, prepare by diluting 100% Triton X-100 with nuclease-free water	Room Temperature
FcR Blocking Reagent	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.	On ice until use
FcR Blocking Reagent is for blocking of Fc receptors on the cell membrane. Please select either one according to the host species of your sample. Store at 4°C. TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody is not recommended if the host species of the primary antibody is rat. If a primary antibody of rat host species is required for the experimental design, an alternative solution is to skip adding FcR Blocking Reagent and replenish with nuclease-free water to a total volume of 330 μL.		
Glycerol	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.	Room Temperature
Diluted primary antibody (optional)	Primary antibodies can be diluted with blocking solution to a desired concentration if needed.	On ice until use

Table 3-2 Blocking solution (for Antibody Titration)

Components	1X (μL) for one slide	6X (μL) for six slides
5X SSC	198	1,188
Post-centrifuged serum (use the supernatant and pipette away from the bottom)	33	198
10% Triton X-100	3.3	19.8
*FcR Blocking Reagent	16.5	99
Nuclease-free water	79.2	475.2
Total	330	1980

***TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody is not recommended if the host species of the primary antibody is rat. If a primary antibody of rat host species is required for the experimental design, an alternative solution is to skip adding FcR Blocking Reagent and replenish with nuclease-free water to a total volume of 330 μL.**

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

c. After fixation, move the 50 mL corning tube or slide container to a sterile fume hood.

d. Take out the microscope slide and wipe off excess methanol from around and the back of the slide with dust-free paper without touching the tissue.

e. Place the microscope slide on a slide staining rack or dust-free paper and leave it in the fume hood to let the methanol fully evaporate.

f. Use a Super PAP Pen (hydrophobic barrier pen) on the microscope slide to draw a circle around the tissue to create a hydrophobic exclusion zone to prevent the subsequent addition of fluid from escaping.

g. Store the treated microscope slide in a microscope slide storage box.

3.5. Tissue Blocking & Primary Antibody Incubation

a. Vortex the blocking solution that was prepared in Table 3-2 and add no more than 100 μL/slide of blocking solution drop wise on the tissue surface then incubate at room temperature for **15 min**.

The amount of blocking solution used per slide 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/slide.

b. While waiting for the incubation to be done, prepare primary antibody solution according to Table 3-3. Take the primary antibody of NeuN (abcam, ab104224) as an example. The dilution ratio of NeuN was set to be 1:100, 1:500, 1:1000, 1:5000, 1:10000 and a negative control group (without the addition of the primary antibody). A total of 6 groups for antibody titration. After vortex mixing and brief centrifugation, leave the primary antibody solution on ice until use.

Table 3-3 Primary antibody solution (for Antibody Titration)

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

a) 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.

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

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. Make sure 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.

b. While waiting for the primary antibody incubation to be done, prepare secondary antibody solution according to Table 3-4. After vortex mixing and brief centrifugation, leave the secondary antibody solution on ice **in the dark** until use.

Table 3-4 Secondary antibody solution (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.

3.6. Secondary Antibody Incubation

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

b. Wash by adding 100 µL/slide of 0.1X SSC. Incubate for **3 min** then discard.

c. Repeat **b.** twice for a total three-time wash.

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. Slowly add 100 µL/slide of secondary antibody solution to the tissue. Incubate for **25 min** at room temperature **in the dark**.

e. Discard the secondary antibody solution with a pipette.

f. Wash by adding 100 µL/slide of 0.1X SSC. Incubate for **3 min** then discard.

g. Repeat **f.** twice for a total three-time wash.

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.

h. Air dry the microscope glass slides or dry with a hand-held fan until no residual solution is left on the tissue.

i. Pipette 5 µL glycerol/slide gently onto the center of the tissue without introducing air bubbles. With a pair of forceps, place one end of the coverslip onto the glycerol covered tissue while holding the other end and then gradually lower the coverslip. Proceed to imaging immediately.

3.7. Imaging

a. Take fluorescent images with microscopes that have stitching functions and both brightfield and fluorescence capacity. Scan using 10X lens.

b. Required fluorescence channels will depend on antibody selection.

Recommended fluorescence configuration:

- Light source with a wavelength range of 380 - 680 nm
- Monochrome camera (≥ 12 bit)
- DAPI filter cube (Excitation 375/28nm, Emission 460/50nm)
- FITC filter cube (Excitation 480/30nm, Emission 525/50nm)
- TRITC filter cube (Excitation 545/25nm, Emission 605/70nm)
- CY5 filter cube (Excitation 620/50nm, Emission 690/50nm)
- Maximum pixel size of 5 µm
- Exposure time 1 milli sec - 2 sec

Exposure times will depend on antibody sensitivity and fluorophores.

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

3.8. 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 serial dilution of Anti-NeuN antibody (abcam, ab104224) as an example, shown in the figure below. Select a concentration without significant reduction in fluorescence intensity. If two concentrations show similar results, choose the lower concentration. A 1:1000 dilution was selected as the optimal antibody dilution concentration in this example. Please determine the optimal concentrations for all antibodies before proceeding to subsequent mIF pilot experiment and Stereo-seq Transcriptomics experiment on the Stereo-seq Chip.

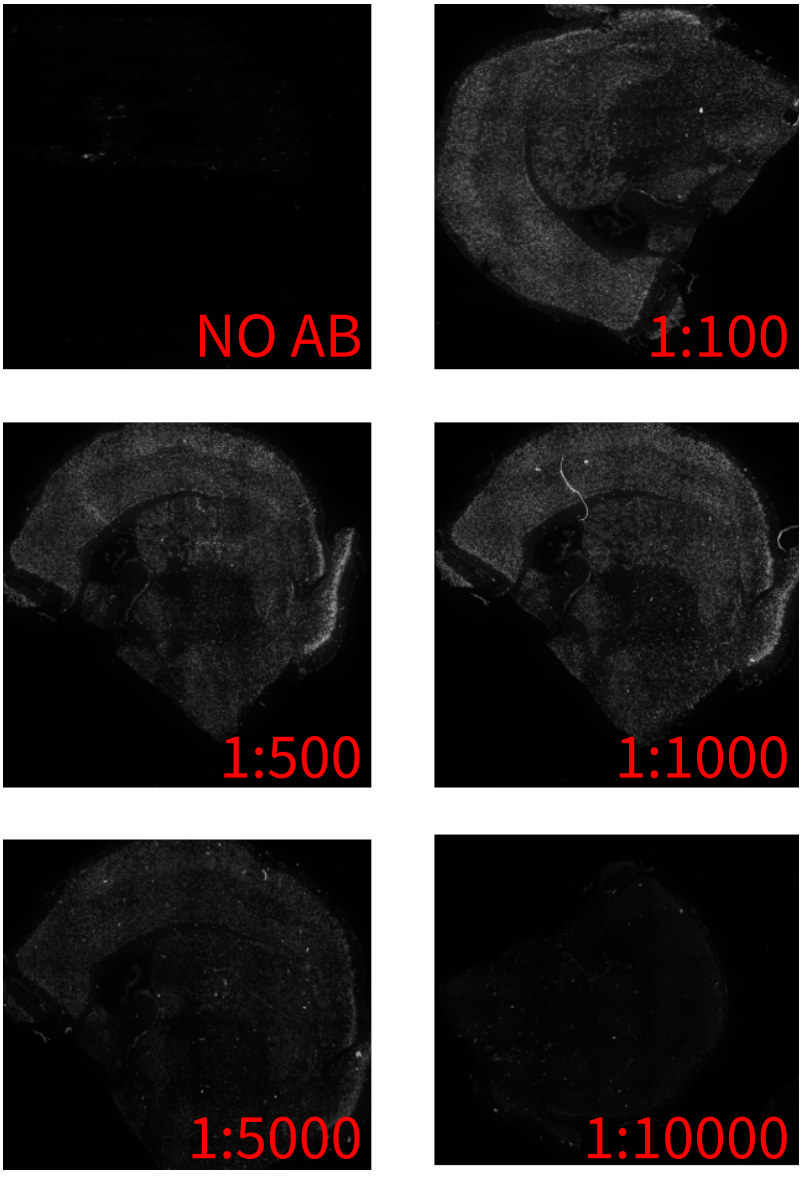
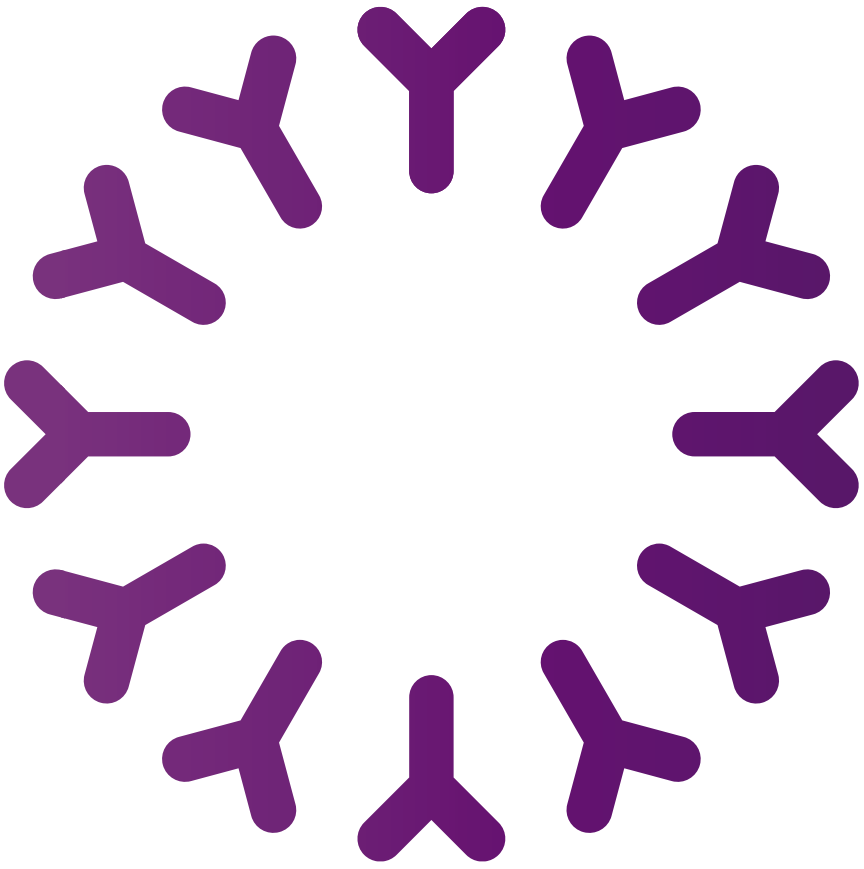


Figure 2. Serial Anti-NeuN antibody titration results of mouse brain (hemisphere)

CHAPTER 4
mIF PILOT EXPERIMENT



Once the optimal dilution ratio for each antibody has been determined, it is recommended to perform the mIF pilot experiment on a microscope glass slide with the optimal dilution concentration of all antibodies. The purpose of the mIF pilot experiment is to ensure that co-staining of all antibodies on the same tissue section can be clearly visualized under each corresponding fluorescent channel before proceeding to formal STOmics multiple immunofluorescence (mIF) and Stereo-seq Transcriptomics co-detection experiment on Stereo-seq Chip.

4.1. Preparations for Tissue Blocking

Please refer to **section 3.1 - 3.4 in Chapter 3** for procedures of experimental preparations, cryosection preparation, tissue mounting on microscope glass slide and tissue fixation and blocking solution preparation. DAPI solution (Thermo 62248) is required in this chapter. Prepare:

Reagent	Preparation Steps	Maintenance
50X-diluted DAPI solution	Dilute with 5X SSC then keep it on ice in the dark	On ice until use (in the dark)

4.2. Tissue Blocking & Primary Antibody Incubation

a. Vortex the blocking solution that was prepared in Table 3-2 and add no more than 100 μL/slide of blocking solution drop wise on the tissue surface then incubate at room temperature for **15 min**.

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

Table 4-1 Blocking solution (for mIF pilot experiment)

Components	1X (μL)
5X SSC	198
Post-centrifuged serum (use the supernatant and pipette away from the bottom)	33
10% Triton X-100	3.3
*FcR Blocking Reagent	16.5
Nuclease-free water	79.2
Total	330

***TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody is not recommended if the host species of the primary antibody is rat. If a primary antibody of rat host species is required for the experimental design, an alternative solution is to skip adding FcR Blocking Reagent and replenish with nuclease-free water to a total volume of 330 μL.**

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

b. While waiting for the incubation to be done, prepare primary antibody solution according to Table 4-2. Prepare the volume for all antibodies according to the optimal dilution ratio selected for each primary antibody during antibody titration. After vortex mixing and brief centrifugation, leave the primary antibody solution on ice until use.

Table 4-2 Primary Antibody Solution (for mIF pilot experiment)

Components	1X (μL)
Blocking Solution	100-(V1+V2+...+Vn)
Primary Antibody #1	V1
Primary Antibody #2	V2
...	...
Primary Antibody #N	Vn
Total	100

Before mixing multiple primary antibodies, antibody titration should be performed for each antibody. To avoid cross-reactivity of secondary antibodies, please choose primary antibodies of different host species.

c. Discard the blocking solution with a pipette. Slowly add the primary antibody solution from the non-tissue area until the solution covers the tissue section. Do not exceed 100 μL/slide. 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 in Table 4-3 according to the recommended dilution or manufacturer's instruction for each secondary antibody. After vortex mixing and brief centrifugation, leave the secondary antibody solution on ice **in the dark** until use.

Table 4-3 Secondary antibody solution (for mIF pilot experiment)

Components	1X (μL)
Blocking Solution	100-(V1+V2+...+Vn)
Secondary Antibody #1	V1
Secondary Antibody #2	V2
...	...
Secondary Antibody #N	Vn
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.

4.3. Secondary Antibody Incubation

a. Discard the primary antibody solution with a pipette.

b. Wash by adding 100 μL/slide of 0.1X SSC. Incubate for **3 min** then discard.

c. Repeat **b.** twice for a total three-time wash.

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. Slowly add 100 μL/slide of secondary antibody solution to the tissue. Incubate for **25 min** at room temperature **in the dark**.

4.4. DAPI Staining

a. While waiting for the secondary antibody incubation to be done, prepare DAPI staining solution according to Table 4-4. Mix with pipette then leave it on ice **in the dark** until use.

Table 4-4 DAPI staining solution

Components	1X (μL)
5X SSC	60
50X-diluted DAPI solution	1
Nuclease-free water	39
Total	100

b. Discard the secondary antibody solution with a pipette.

c. Wash by adding 100 μL/slide of 0.1X SSC. Incubate for **3 min** then discard.

d. Repeat **c.** twice for a total three-time wash.

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.

e. Slowly add 100 μL/slide of DAPI staining solution to the tissue. Incubate for **2 min** at room temperature i**n the dark**.

f. Discard the DAPI staining solution with a pipette. Wash by adding 100 μL/slide of 0.1X SSC then discard.

g. Air dry the microscope glass slides or dry with a hand-held fan until no residual solution is left on the tissue.

h. Pipette 5 μL glycerol/slide gently onto the center of the tissue without introducing air bubbles. With a pair of forceps, place one end of the coverslip onto the glycerol covered tissue while holding the other end and then gradually lower the coverslip. Proceed to imaging immediately.

4.5. Imaging

a. Take fluorescent images with microscopes that have stitching functions and both brightfield and fluorescence capacity. Scan using 10X lens.

b. Required fluorescence channels will depend on antibody selection.

Recommended fluorescence configuration:

- Light source with a wavelength range of 380 - 680 nm
- Monochrome camera (≥ 12 bit)
- DAPI filter cube (Excitation 375/28nm, Emission 460/50nm)
- FITC filter cube (Excitation 480/30nm, Emission 525/50nm)
- TRITC filter cube (Excitation 545/25nm, Emission 605/70nm)
- CY5 filter cube (Excitation 620/50nm, Emission 690/50nm)
- Maximum pixel size of 5 μm
- Exposure time 1 milli sec - 2 sec

Exposure times will depend on antibody sensitivity and fluorophores.

4.6. mIF Pilot Experiment Results

A successful mIF pilot experiment should ensure that each channel can obtain the specific staining result as expected, the signal-to-noise ratio is maintained at a reasonable intensity, and there is no significant fluorescence bleed-through among different channels which can affect the subsequent analysis.

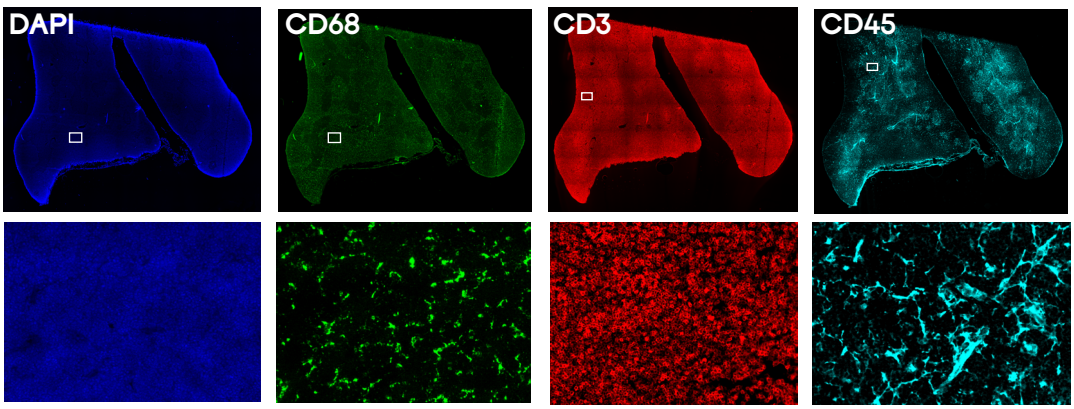
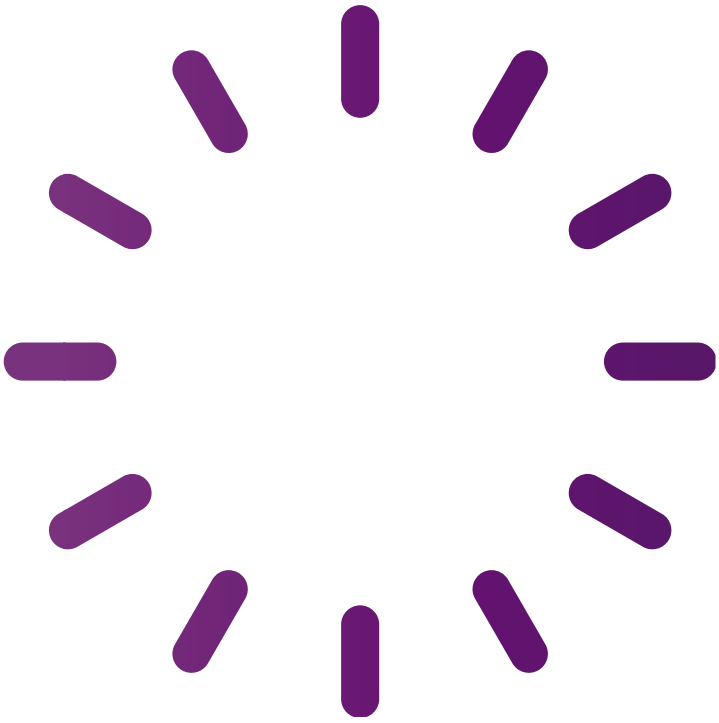


Figure 3. mIF pilot experiment results of mouse thymus with co-staining of Anti-CD68, Anti-CD3, and Anti CD45 Antibodies

CHAPTER 5

Stereo-seq TRANSCRIPTOMICS SET FOR CHIP-ON-A-SLIDE STANDARD OPERATING PROCEDURE (mIF COMPATIBLE)



5.1. Experimental Preparation

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

Prep Day	Reagent	Preparation Steps	Maintenance
Day 1	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
	0.01N HCl	Prepare at least 2 mL of 0.01N HCl per sample. Configure HCl to 0.01N. Measure and make sure the pH = 2 (Prepare at least 2 mL / sample).	Room temperature for 48 hr **(Storing longer than 48 hr will affect the desired pH. Please use WITHIN 48 hr of preparation)**
	ALWAYS use freshly prepared 0.01N HCl (pH = 2.0 ± 0.1). For pre-made 0.1N HCl and newly purchased HCl, check the pH prior to the experiments.		
	10X Permeabilization Reagent Stock Solution	Add 1 mL of freshly prepared 0.01N HCl to dissolve PR Enzyme (red cap, in powder form), and thoroughly mix the reagent through pipetting.	-20°C
	DO NOT vortex the permeabilization enzyme. Mix with pipette before using. Aliquot this 10X stock solution to avoid freeze-thaw cycles.		
	1X Permeabilization Reagent Solution	Make 1X PR solution (150 µL / chip) by diluting 10X PR stock solution with 0.01N HCl.	On ice until use, 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.		
	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

Day 1	Glycerol	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.	Room Temperature
	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)	On ice until use
	50X-diluted DAPI solution	Dilute with 5X SSC then keep it on ice in the dark	On ice until use (in the dark)
	50X-diluted DAPI solution can be stored at 4°C up to 5 days in the dark		
Day 2	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

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.
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 if there is any abnormality with the PCR thermal cycler and replace it if necessary.
Fluorescence Microscope	Make sure the microscope is equipped with at least DAPI, FITC, TRITC, CY5 channels.	Room Temperature
Microcentrifuge	Set the temperature to 4°C.	Check if there is any abnormality with the microcentrifuge and replace it if necessary.

5.2. Cryosection Preparation

a. Set PCR Thermal Cycler to 37°C in advance and place the PCR adaptor for pre-heating in advance.

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 to the chamber and allow it to equilibrate to the cryostat chamber temperature for **30 min**.

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

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

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

5.3. Tissue Mounting

For a demonstration video of tissue mounting onto the Stereo-seq Chip Slide, please refer to the link or by scanning the QR code:
https://drive.google.com/drive/folders/1mKOipoJdY_uDF-VA9I-githgVdrFeBcF

a. Take the Chip T Slide out of the vacuum sealed aluminum bag and record Chip ID (SN number) that is on the back side of the slide. Make sure not to touch the front side chip surface.

Once opened, please check if all the Stereo-seq Chip Slides in the slide container are well orientated with the front-side facing upward. The front-side of a chip has a shiny surface which contains DNB-probes for mRNA capture. DO NOT scratch the surface.

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

c. Equilibrate Stereo-seq Chip Slide to room temperature for **1 min** on the benchtop, then rinse with **100 µL** nuclease-free water **twice** with a pipette or rinse the slide up and down **twice** in a **50 mL** corning tube with enough nuclease-free water.

Store unused slides in original packaging (first in the slide container and then the sealable aluminum bag) and keep sealed at -25°C ~ 8°C for up to two weeks. KEEP the desiccant in the aluminum bag.

d. Remove excess water on the chip by blowing gently with a power dust remover (MATIN, M-6318) from one side of the chip at a 30~45-degree angle horizontal to the plane of the chip. Wipe excess water around the chip and on the slide with dust-free paper.

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

f. Prepare enough methanol in a **50 mL** corning tube or an empty slide container at a volume that could submerge all the chips on the slide. Immerse a regular glass slide in the methanol containing tube to check if the volume is enough. Close the lid and pre-cool methanol for **5-30 min** at -20°C.

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

h. Tissue mounting could be achieved via either cold method (option A) or warm method (option B). We recommend practicing tissue mounting and section placement on plain glass slides first.

A. Cold Method

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

Prolonged cooling for 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 chip center carefully with forceps and brushes. Make sure the tissue section is complete and without wrinkles.

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

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

5) Once complete all tissue mounting, immediately dry the Stereo-seq Chip Slide at 37°C on a PCR thermal cycler with PCR adaptor at 37°C 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 obtain two or four consecutive tissue sections (depending on the number of chips on the Stereo-seq Chip Slide), carefully flatten the tissue sections out by gently touching the surrounding OCT with cryostat brushes.

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

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

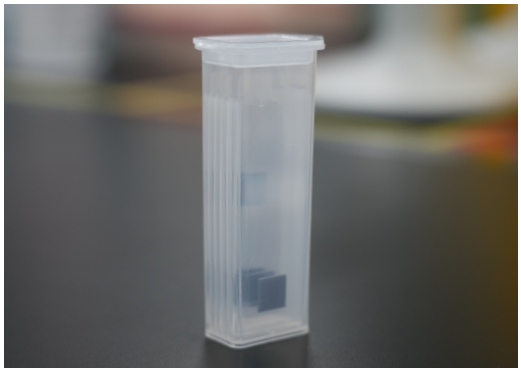
4) Repeat step 3) until all the tissue sections have been mounted on to the chips of the Stereo-seq Chip Slide.

5) Turn the Stereo-seq Chip Slide over, and immediately dry it on a PCR thermal cycler with PCR adaptor front-side up for **5 min**.

If two different tissue blocks need to be cryosectioned and mounted on to the same Stereo-seq Chip Slide, it is recommended to first trim both tissue blocks beforehand. Perform tissue sectioning and mounting for one tissue block first with the warm method, and then place the tissue mounted Stereo-seq Chip Slide on the PCR thermal cycler for no longer than 5 min while preparing for the second tissue block. Perform tissue section and mount the second tissue block using the warm method, then place the tissue mounted Stereo-seq Chip Slide on the PCR thermal cycler to dry for 5 min.

Stop Point:

- **After drying the tissue containing Stereo-seq Chip Slides on a PCR Thermal Cycler, they can be stored in a slide container then transferred to −80°C freezer on dry ice.**
- **Store the sealed slide container containing Stereo-seq Chip Slides with tissue at −80°C for up to four weeks.**
- **When retrieving Stereo-seq Chip Slides with tissue from the freezer, transfer out the slide container on dry ice, and take out the tissue containing Stereo-seq Chip Slides then immediately incubate at 37°C with PCR Adaptor for 5 min.**



5.4. Tissue Fixation

a. After drying, immediately immerse the tissue-mounted Stereo-seq Chip Slide in pre-cooled methanol prepared in section 5.3 f. for a **30-min** fixation at -20°C. When immersing Stereo-seq Chip Slide in methanol, ensure that all the tissue sections are completely submerged.

b. While waiting for the fixation to be done, prepare the reagents required for tissue blocking and antibody incubation according to Table 5-1 and prepare the blocking solution according to Table 5-2 then leave it on ice until use.

Table 5-1 Reagent Preparation for Tissue Blocking and Primary Antibody Incubation

Reagent	Preparation Steps	Maintenance
Primary Antibodies	Take them out of -20°C or 4°C (depending on the manufacturer's instruction) and centrifuge at 14,000g, 4°C for 10 min then leave them on ice.	On ice until use
Secondary Antibodies	Take them out of -20°C or 4°C (depending on the manufacturer's instruction) and centrifuge at 14,000g, 4°C for 10 min then leave them on ice.	On ice until use
Post-centrifuged serum	Take aliquoted serum out of the -20°C freezer and thaw. Then centrifuge at 14,000g, 4°C for 10 min, then leave it on ice.	On ice until use
Each antibody dilution requires about 200 μL of serum. The rest of the aliquoted serum can be reused.		
Wash Buffer	For one chip: add 35 μL of RI to 665 μL of 0.1X SSC	On ice until use
10% Triton X-100	If without readily available 10% Triton X-100, prepare by diluting 100% Triton X-100 with nuclease-free water	Room Temperature
FcR Blocking Reagent	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.	On ice until use
FcR Blocking Reagent is for blocking of Fc receptors on the cell membrane. Please select either one according to the host species of your sample. Store at 4°C. TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody is not recommended if the host species of the primary antibody is rat. If a primary antibody of rat host species is required for the experimental design, an alternative solution is to skip adding FcR Blocking Reagent and replenish with nuclease-free water to a total volume of 200 μL.		
Glycerol	Take it out in advance and equilibrate to room temperature at least 5 min prior to use.	Room Temperature
Diluted primary antibody (optional)	Primary antibodies can be diluted with blocking solution to a desired concentration if needed.	On ice until use
Diluted secondary antibody (optional)	Secondary antibodies can be diluted with blocking solution to a desired concentration if needed.	On ice in the dark until use

Table 5-2 Blocking solution

Components	1X (μL)	2X + 10% (μL)	3X + 10% (μL)	4X + 10% (μL)
5X SSC	120	240	360	480
Post-centrifuged serum (use the supernatant and pipette away from the bottom)	20	40	60	80
10% Triton X-100	2	4	6	8
*FcR Blocking Reagent	10	20	30	40
RI	10	20	30	40
Nuclease-free water	38	76	114	152
Total	200	400	600	800

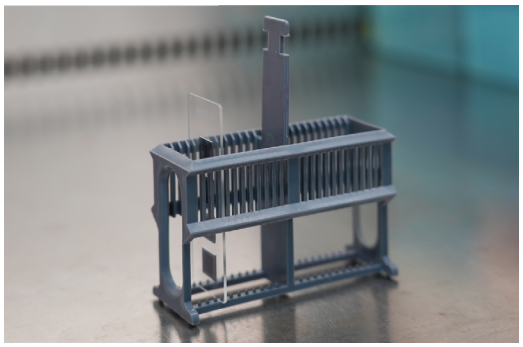
*** TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody is not recommended if the host species of the primary antibody is rat. If a primary antibody of rat host species is required for the experimental design, an alternative solution is to skip adding FcR Blocking Reagent and replenish with nuclease-free water to a total volume of 330 μL.**

The blocking buffer prepared for one chip (1X) is enough for three usages: tissue blocking, primary antibody solution preparation and secondary antibody solution preparation.

c. After fixation, move the 50 mL corning tube or slide container to a sterile fume hood.

d. Take out the Stereo-seq Chip Slide and wipe off excess methanol from around and the back of the slide with dust-free paper without touching the chips. Make sure there is no methanol residue between chips.

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



f. Once methanol is fully evaporated, transfer the Stereo-seq Chip Slide on to a flat and clean bench top surface.

5.5. Tissue Blocking & Primary Antibody Incubation

a. Vortex the blocking solution that was prepared in Table 5-2 and add **60 μL per chip** of blocking solution drop wise on the tissue surface then incubate at room temperature for **15 min**.

b. While waiting for the incubation to be done, prepare primary antibody solution according to Table 5-3. Prepare the volume for all antibodies according to the optimal dilution ratio selected for each primary antibody during antibody titration. After vortex mixing and brief centrifugation, leave the primary antibody solution on ice until use.

Table 5-3 Primary Antibody Solution

Components	1X (μL)
Blocking Solution	60-(V1+V2+...+Vn)
Primary Antibody #1	V1
Primary Antibody #2	V2
...	...
Primary Antibody #N	Vn
Total	60

Before mixing multiple primary antibodies, antibody titration should be performed for each antibody. To avoid cross-reactivity of secondary antibodies, please choose primary antibodies of different host species.

c. Discard the blocking solution with a pipette. Slowly add **55 μL per chip** of primary antibody solution from the non-tissue area until the solution covers the tissue section. 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 in Table 5-4 according to the recommended dilution or manufacturer's instruction for each secondary antibody. After vortex mixing and brief centrifugation, leave the secondary antibody solution on ice **in the dark** until use.

Table 5-4 Secondary Antibody Solution

Components	1X (μL)
Blocking Solution	60-(V1+V2+...+Vn)
Secondary Antibody #1	V1
Secondary Antibody #2	V2
...	...
Secondary Antibody #N	Vn
Total	60

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.

5.6. Secondary Antibody Incubation

a. Discard the primary antibody solution with a pipette.

b. Wash by adding **100 µL per chip** of Wash Buffer. Incubate for **3 min** then discard.

c. Repeat **b.** twice for a total three-time wash.

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. Slowly add **55 µL per chip** of secondary antibody solution to the tissue. Incubate for **25 min** at room temperature **in the dark**.

5.7. DAPI Staining

a. While waiting for the secondary antibody incubation to be done, prepare DAPI staining solution according to Table 5-5. Mix with pipette then leave it on ice **in the dark** until use.

Table 5-5 DAPI staining solution

Components	1X (µL)
5X SSC	60
50X-diluted DAPI solution	1
RI	5
Nuclease-free water	34
Total	100

b. Discard the secondary antibody solution with a pipette.

c. Wash by adding 100 µL per chip of Wash Buffer. Incubate for **3 min** then discard.

d. Repeat **c.** twice for a total three-time wash.

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.

e. Slowly add 100 µL per chip of DAPI staining solution to the tissue. Incubate for **2 min** at room temperature **in the dark**.

f. Discard the DAPI staining solution with a pipette. Wash by gently adding 100 µL per chip of Wash Buffer.

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

h. Transfer the Stereo-seq Chip Slide onto dust-free paper. Hold on to the slide with one hand and completely dry the chips further with a power dust remover in the other hand at a distance 2-3 cm away from the chip surface by blowing gently from one side of the chip at a 30-degree angle horizontal to the plane of the chip.

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.

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 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.

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.

5.8. Imaging

a. Take fluorescent images with microscopes that have stitching functions and both brightfield and fluorescence capacity. Select epifluorescence mode for scanning.

b. Required fluorescence channels will depend on antibody selection.

Recommended fluorescence configuration:

- Light source with a wavelength range of 380 - 680 nm
- Monochrome camera (≥ 12 bit)
- DAPI filter cube (Excitation 375/28nm, Emission 460/50nm)
- FITC filter cube (Excitation 480/30nm, Emission 525/50nm)
- TRITC filter cube (Excitation 545/25nm, Emission 605/70nm)
- CY5 filter cube (Excitation 620/50nm, Emission 690/50nm)
- Maximum pixel size of 5 µm
- Exposure time 1 milli sec - 2 sec

Exposure times will depend on antibody sensitivity and fluorophores.

c. Create a new folder in a fluorescent microscope-connected PC.

- For antibody IF image, name the folder as: <chip ID number>_<antibody name>_IF (i.e. Y00035N1_NeuN_IF).
- For DAPI image, name the folder with only the chip ID (i.e. Y00035N1)

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

d. Add a few drops of water (~2 μL) 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 depending on different microscopes. As long as the tissue can be imaged clearly, the light intensity should be kept in the lower range to prevent fluorescence quenching.

For multi-channel imaging, adjust the imaging parameters for each channel individually before imaging.

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 brain tissue as an example, the red box in the middle picture is the selected tissue area, and the small blue boxes are 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.

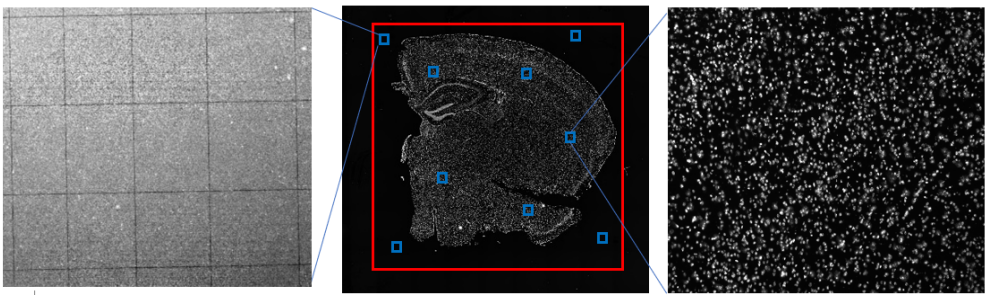


Figure 4. DAPI image of mouse brain tissue

e. Once you complete the first scan of the DAPI channel, switch to the next channel directly **WITHOUT** moving the Stereo-seq Chip Slide, re-scanning the map, re-adding the focal points, or modifying the red box of the selected tissue area. Save original tile (FOV) image files and stitched images. Simply create a new folder, name it with the next antibody and save the folder. Then adjust the focus and exposure until the stained tissue is clearly displayed. Finally complete the full scan on the capturing area with 10X objective lens.

f. After the scanning is completed, continue imaging with the next channel until all the antibody IF images have been acquired.

g. Save original tile (FOV) image files and stitched images.

Glycerol mounted chips could be stored for a maximum of 4 hr after imaging.

h. Open the ImageStudio software and the Image Quality Control functional module within the software. Upload your nuclei-stained (DAPI) image and IF images, 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.

i. After imaging, gently push the coverslip with a pair of forceps until it is slightly beyond the edge of Stereo-seq Chip Slide.

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

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

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

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

l. 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.

m. Grip along the Stereo-seq Cassette to make sure the Stereo-seq Chip Slide has been locked in place.

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

5.9. Tissue Permeabilization

a. Set aside the 2 mL of 0.01N HCl and 1X Permeabilization Reagent Solution you prepared in **5.1. Experimental Preparation**.

b. Make sure 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. Warm up the 1X permeabilization Reagent Solution inside the 37°C PCR thermal cycler for **>3 min**.

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

e. Place the Stereo-seq Slide Cassette in the 37°C PCR Thermal Cycler. Add **150 µL** of 1X permeabilization Reagent Solution onto the chip by first pipetting one droplet at each corner of the chip and then adding the rest of the solution to the middle to merge all the droplets.

Make sure the chip is completely covered with 1X Permeabilization Reagent Solution.

f. Place unpeeled sealing tape on the Stereo-seq Slide Cassette 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 chip-on-a-slide.

g. While waiting for permeabilization to be done, prepare RT mix according to Table 5-6 and leave it on ice until use. **【PREPARED AHEAD】**

Table 5-6 RT Mix

Components	1X (µL)	2X + 10% (µL)	3X + 10% (µL)	4X + 10% (µL)
RT Reagent	80	176	246	352
RT Additive	5	11	16.5	22
RI	5	11	16.5	22
RT Oligo	5	11	16.5	22
ReverseT Enzyme	5	11	16.5	22
Total	100	220	330	440

h. Place the PCR Adaptor in another PCR Thermal Cycler in advance and set the temperature to 42°C with heated lid set to 47°C.

i. Once complete, remove the Stereo-seq Slide Cassette from the PCR Adaptor (37°C).

j. Slightly tilt the Stereo-seq Slide Cassette, remove 1X Permeabilization Reagent Solution with a pipette from the corner of each well without touching the chip surface.

k. Add 100 µL of PR Rinse Buffer (with 5% RI) per chip from the corner of each well.

l. Slightly tilt the Stereo-seq Slide Cassette, remove PR Rinse Buffer with a pipette from the corner of each well without touching the chip surface. Keep the chip surface moisturized.

Make sure not to dry the chip completely.

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

5.10. Reverse Transcription

a. Make sure the temperature of the PCR Thermal Cycler with PCR Adaptor has been set to 42°C in advance.

b. Pipette up and down, then short spin the prepared RT Mix. Gently add **100 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 make sure 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	1
42°C	Hold	-

5.11. 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 make sure 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 **10 min** with the following incubation protocol.

Temperature	Time	Cycle
(Heated lid) 60°C	on	-
55°C	10 min	1
55°C	Hold	-

e. While waiting, prepare cDNA Release Mix according to Table 5-7.【**PREPARED AHEAD**】

Table 5-7 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.

5.12. cDNA Release and Collection

a. Add 400 µL of cDNA Release Mix per chip prepared in 5.11 e. into each well of the Stereo-seq Slide Cassette.

b. Apply sealing tape to Stereo-seq Slide Cassette and make sure 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	1
55°C	Hold	-

Stop Point:

DNA collection step may be left overnight. If it is left overnight, make sure the Stereo-seq Slide Cassette has been sealed tightly with the sealing tape and the cassette is tightly clipped in place.

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.

Make sure to collect as complete 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.

The Stereo-seq Chip Slide may be discarded. Ensure all the chip ID numbers on the Slide have been recorded, as it is required for downstream analysis.

5.13. 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 VAHTSTM DNA Clean Beads (VAZYME, Cat. No. N411-02). *If magnetic beads from other sources are used, please optimize the cleanup conditions before getting started.*

Before 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 amount of magnetic beads directly affects the distribution of purified DNA fragments.

Operation Notes

- 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.
- 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 direct contacting the beads. If the beads are mistakenly taken up, dispense everything and redo the magnetic separation.
- Use freshly prepared 80% ethanol (at room temperature) to wash the beads. Keep the sample tube on the magnetic separation rack during the washing step. Do not shake or disturb the beads.
- After the 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.
- 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.
- 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 direct contacting the beads, always collect the eluate in 2 µL less than the initial volume of TE buffer used for the elution.
- **Pay attention when opening/closing the lid of a sample tube on a separation rack. Strong vibrations may cause samples or beads to spill from the tubes. Hold the body of the tube while opening the lid.**

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

b. If white precipitate is observed in the collected cDNA, dissolve it by heating at 55°C and equilibrate to room temperature before the puritification step.

c. cDNA Purification Procedures with 0.8X Magnetic Bead

1) Mix the collected cDNA (450-490 μL) with the beads in a ratio of 1 : 0.8. 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 remove and discard the supernatant with a pipette (If foams are seen on the cap, discard them with a pipette).

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

8) Obtain eluted cDNA (~42 μL) by transferring the supernatant to a new 0.2 mL PCR tube.

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

Store the beads with 42 μL of nuclease-free water at 4°C after collecting the eluted cDNA till your cDNA final product has passed QC.

e. Prepare PCR Mix by referring to Table 5-8. The total volume for the PCR reaction is 100 µL.

Table 5-8 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

f. 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 5-9.

Table 5-9 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	-

g. Prepare Qubit dsDNA Mix and record the concentration of PCR product according to Table 5-10.

Table 5-10 Qubit dsDNA Mix

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

h. 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.

i. Use magnetic beads to purify the PCR product in a volume ratio of 1 : 0.6 (DNA : beads).

1) Mix the cDNA PCR product (100 µL) with beads in a ratio of 1 : 0.6. 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 42 µL of nuclease-free water at 4°C after purification till your cDNA final product has passed QC.

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

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 1,000- 1,500 bp (Figure 2), and a yield higher than 20 ng.

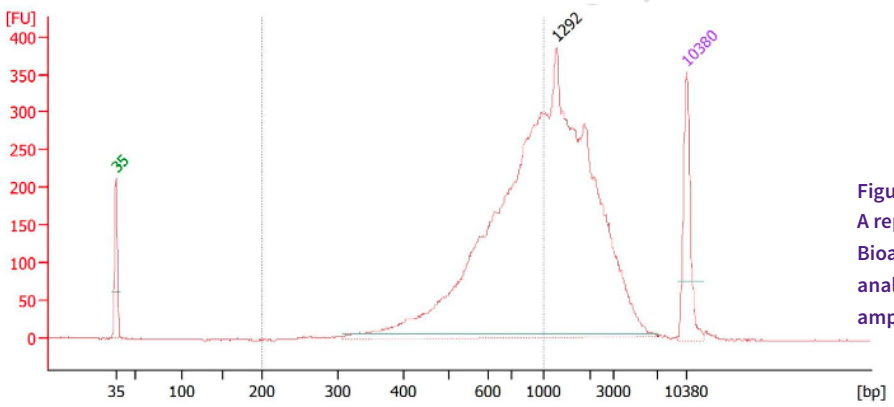
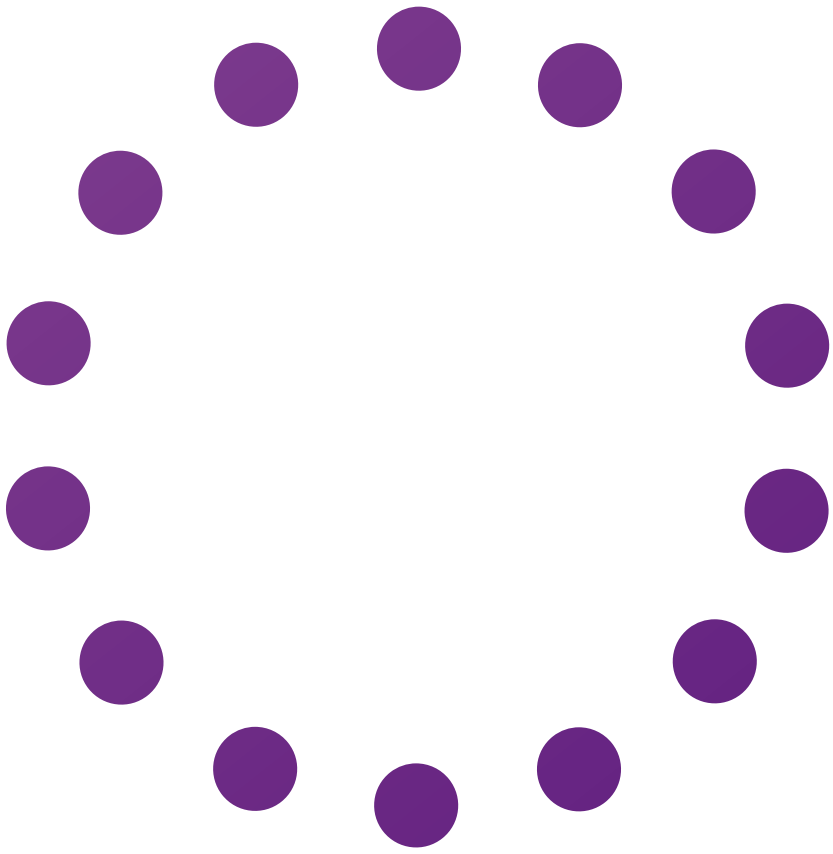


Figure 5.
A representative Agilent Bioanalyzer 2100 analysis result of a PCR amplified cDNA sample

CHAPTER 6
LIBRARY PREPARATION



The later steps will require the usage of Stereo-seq Library Preparation kit. Please make sure you have purchased them separately and prepare them in advance before constructing your library.

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: on dry ice | 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.

6.1. 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

6.2. cDNA Fragmentation and Amplification

a. Use 20 ng cDNA sample prepared in section 5.13. for the following fragmentation reaction.

b. Prepare the Fragmentation Reaction Mix on ice through gentle pipetting according to Table 6-1. 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 6-1 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 6-2. 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 6-2 Fragmentation reaction program

Temperature	Time
(Heated lid) 60°C	on
55°C	10 min
4°C	Hold

d. After the fragmentation reaction program is done, take out the reaction tube and 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 6-3 to start the amplification process of fragmented cDNA.

Table 6-3 PCR Library Mix

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

Please refer to Appendix A 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 6-4) and start the program.

Table 6-4 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.

6.3. 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 incubates 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 extract the beads out of the liquid. 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 or cracked.

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. QC

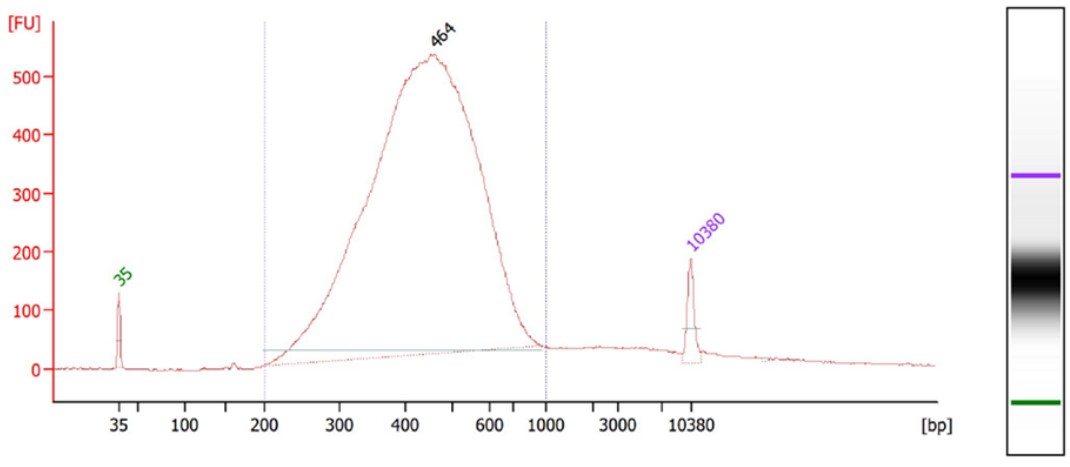
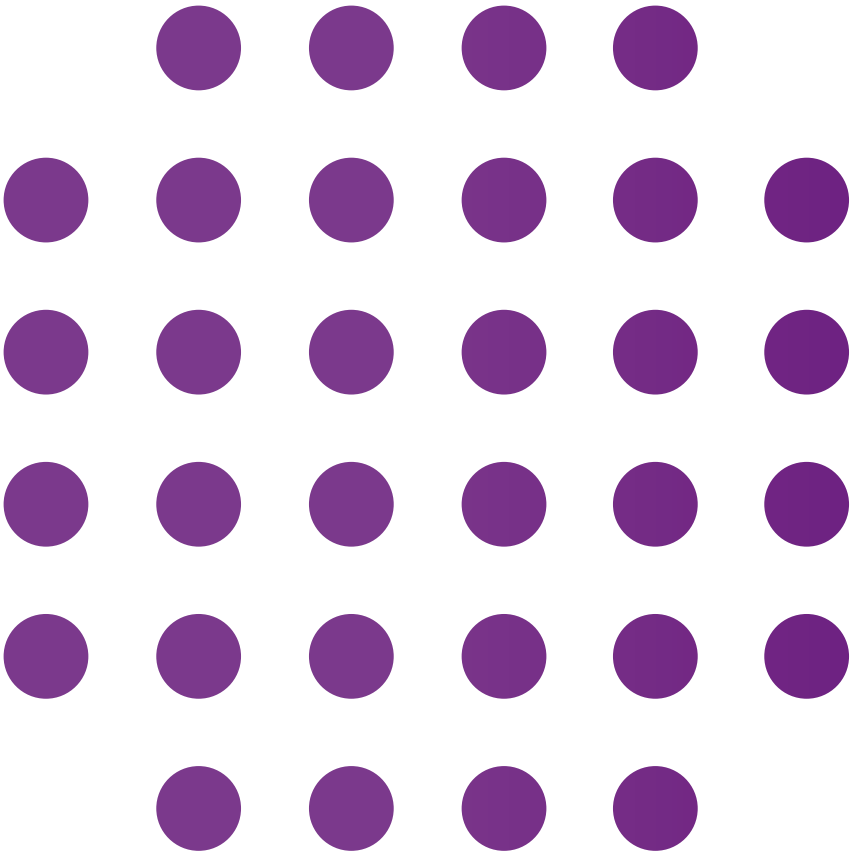


Figure 6. Agilent 2100 Bioanalyzer fragment size distribution of the purified PCR product

CHAPTER 7
LIBRARY CONSTRUCT & SEQUENCING



This chapter introduces the compatible sequencing instruments and sequencing reagents for the Stereo-seq system. The library construct is illustrated in Figure 7.

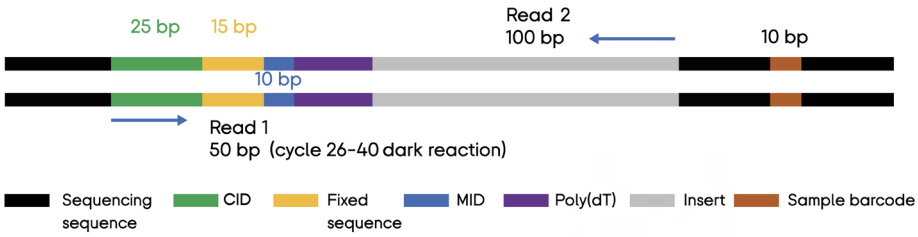


Figure 7. Stereo-seq Transcriptome Library Construct

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: Stereo-seq Transcriptomics Set for Chip-on-a-slide Experimental Record

For recording and self-checking experimental procedures, users can access and download the Stereo-seq Transcriptomics Set Experimental Record we provided here: **https://drive.google.com/drive/folders/18Od8IPO-H1805iycXlZtdTjTtGXIVGex?usp=sharing**