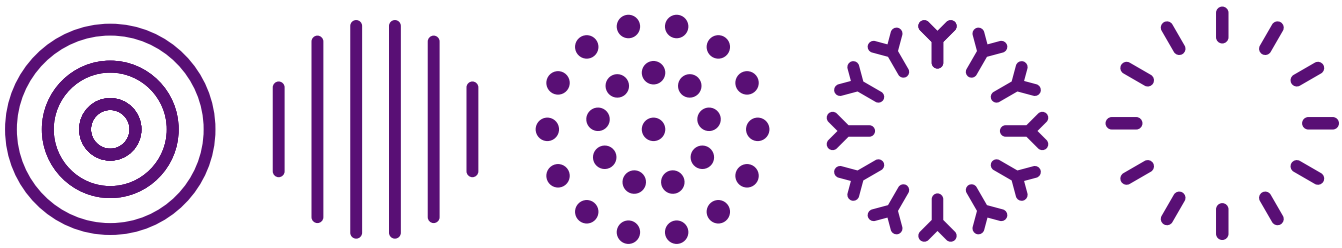
STOmics

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

USER MANUAL



Cat. No.: 211ST13114 (4 RXNs)

Kit Version: V1.3

Manual Version: A

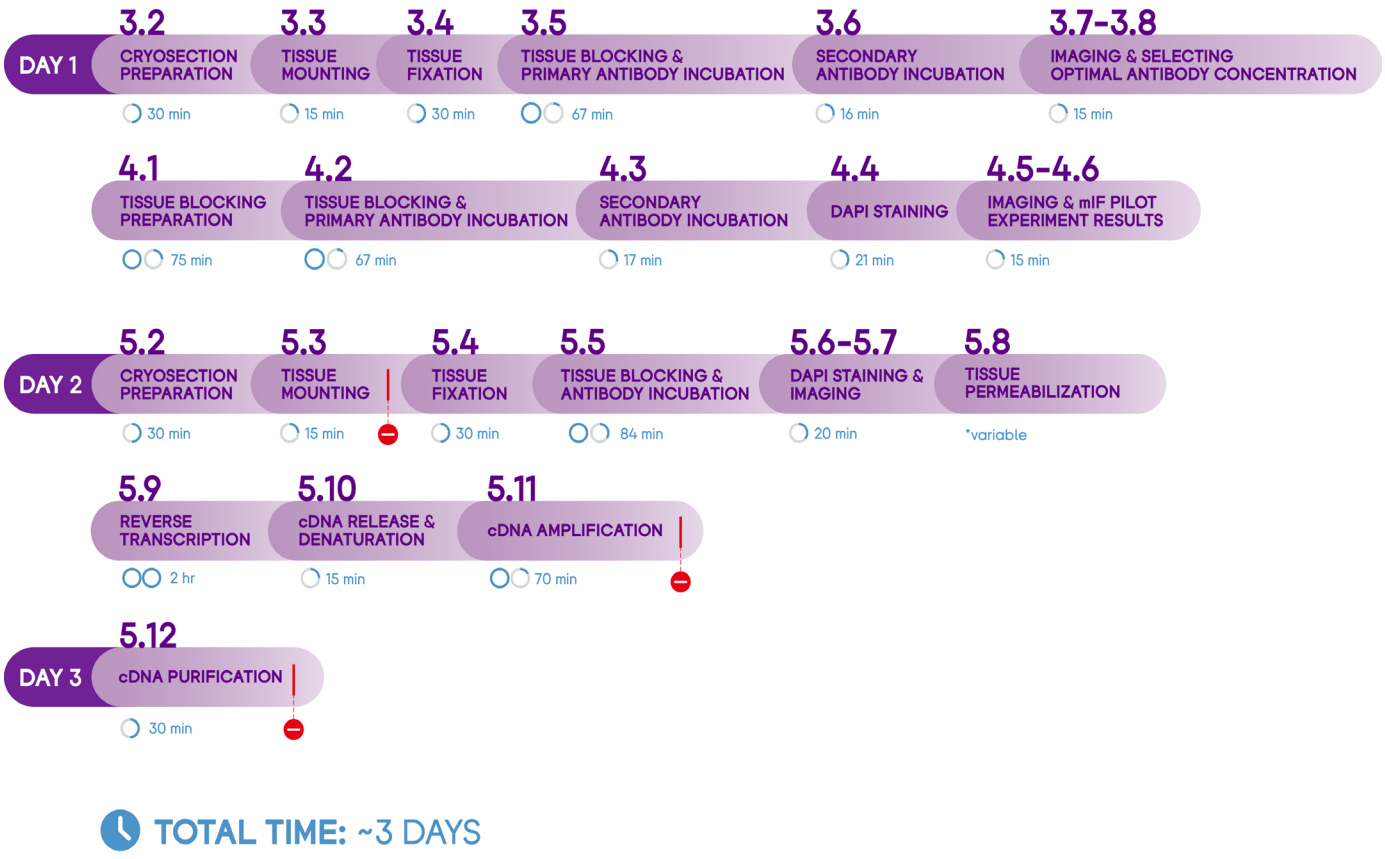
STUM-TT003

# REVISION HISTORY

| | |
|---|---|
| **Manual Version:** | A |
| **Kit Version:** | V1.3 |
| **Date:** | Jan. 2025 |
| **Description:** | Initial release |

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

# WORKFLOW




**TOTAL TIME:** ~3 DAYS

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**NOTE:** Additional operation tips and guidance.

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

**QUALITY CHECK POINT**

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

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

# CHAPTER 1
# INTRODUCTION

## 1.1. Intended Use

STOmics Stereo-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 (FOV). This kit uses DNB patterned array chips loaded with spatially-barcoded probes that capture and prime poly-adenylated mRNA from tissue sections *in situ*. Each cDNA synthesized from mRNA captured on a particular spot is linked to its spatially-barcoded probe, allowing subsequent gene expression mapping of a tissue section following sequencing and visualization analysis using the StereoMap visualization platform.

By integrating mIF staining method into the process of Stereo-seq Transcriptomics 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 evaluate valuable samples in depth, and to parse complex pathological and physiological processes. The number of proteins that can be detected depends on the user's antibody selection and imaging configuration. In this user manual, DAPI with the staining of three antibodies is used as an example.

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

## 1.2. Sequencing Guideline

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

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

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

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

## 1.3. List of Kit Components

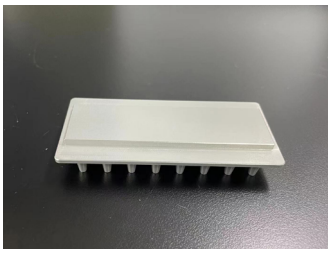
Each Stereo-seq Transcriptomics Set for Chip-on-a-slide consists of:

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

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


Compatible auxiliary but not included:

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

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

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

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

Table 1-1 Stereo-seq Transcriptomics T Kit Components

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

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

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

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

Table 1-3 STOmics Accessory Kit Components

| STOmics Accessory Kit Cat. No.: 1100033700 | | |
|---|---|---|
| **Component** | **Reagent Cat. No.** | **Quantity (per kit)** |
| Cassette | 1000033699 | 1 EA |
| Gasket | 1000033698 | 4 EA |
| Sealing Tape | 1000042970 | 6 EA |
| Storage Temperature: 18°C ~ 25°C | Transported at 10°C ~ 30°C | Expiration Date: refer to label |

Table 1-4 Stereo-seq PCR Adaptor Kit Components

| Stereo-seq PCR Adaptor Cat. No.: 301AUX001-02 | |
|---|---|
| **Component** | **Quantity (per kit)** |
| Stereo-seq PCR Adaptor | 2 EA |
| Storage Temperature: 18°C ~ 25°C | Transported at 10°C ~ 30°C / Expiration Date: refer to label |

## 1.4. Additional Equipment and Materials

Table 1-5 lists the equipment and materials needed for this protocol. The user is expected to have access to common laboratory equipment not named in the document (equipment such as an ice maker, biological safety cabinet, freezers, and so on). For specific microscope requirements, refer to STOmics Microscope Assessment Guideline.

**Table 1-5 Additional Equipment and Materials**

| Equipment | | |
|---|---|---|
| **Brand** | **Description** | **Catalog Number** |
| - | Cryostat | - |
| - | Microcentrifuge | - |
| Eppendorf | Refrigerated Centrifuge (for Stereo-seq mIF application) | 5418R |
| - | Pipettes | - |
| - | Fluorescence Microscope | - |
| - | Vortex Mixer | - |
| - | Metal Bath (or equivalent instrument) | - |
| **Bio-Rad*** | T100™ Thermal Cycler | 1861096 |
| **ABI*** | ProFlex™ 3 x 32-well PCR System | 4483636 |
| Labnet | Slide Spinner (optional) | C1303-T |
| NEB | NEBNext® Magnetic Separation Rack for <200 µL tubes | S1515S |
| Thermo Fisher Scientific | Magnetic Rack: DynaMag™-2 Magnet for 1.5-2 mL Tubes | 12321D |
| | Qubit™ 3 Fluorometer | Q33216 (or similar) |
| Agilent Technologies™ | Agilent 2100 Bioanalyzer | G2939AA (or similar) |

**Choose either one of the listed brands (marked with *). 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 |
| Sigma Aldrich | Hydrochloric Acid, HCl | 2104-50ML |
| | Methanol | 34860-1L-R |
| Thermo Fisher Scientific | RNase Inhibitor (RI) | EO0382 |
| SigmaAldrich | Triton X-100 Solution, 10% | 93443-100ML |
| Invitrogen | Gibco ™ Horse Serum | 26050070 |
| SAKURA | SAKURA Tissue-Tek® O.C.T. Compound | 4583 |
| **Beckman Coulter*** | SPRIselect | B23317/B23318/ B23319 |
| | AMPure® XP DNA Cleanup Beads | A63882 |
| **VAZYME*** | VAHTS™ DNA Clean Beads | N411-02 |
| Thermo Fisher Scientific | DAPI Solution (1 mg/mL) | 62248 |
| Biolegend | TruStain FcX ™ PLUS (anti-mouse CD16/32) Antibody | 156604 |
| | Human TruStainFcX ™ (Fc Receptor Blocking Solution) | 422301 |
| FcR Blocking Reagent is used for blocking Fc receptors on the cell membrane. Select either one according to the host species of your sample. Choose Human TruStain FcX™ (Fc Receptor Blocking Solution) for human tissues and choose TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody for mouse tissues. 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 Series Secondary Antibodies | - |
| Millipore Sigma | Potassium Hydroxide Solution, 8M KOH | P4494-50ML |
| Invitrogen | Qubit dsDNA HS Assay Kit | Q32854 |
| Agilent Technologies™ | High Sensitivity DNA Kit | 5067-4626 |
| | High Sensitivity RNA Kit | 5067-1513 |

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

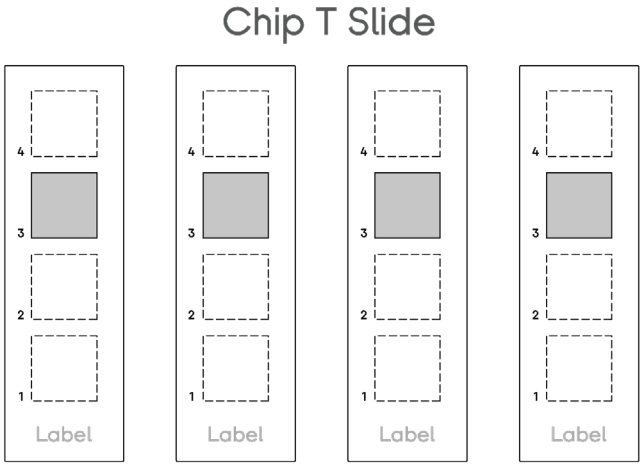
| Consumbales | | |
|---|---|---|
| **Brand** | **Description** | **Catalog Number** |
| - | Aluminum Foil | - |
| - | Forceps | - |
| - | Microscope Slides or Adhesion Microscope Slides | - |
| - | Microscope Glass Coverslip (size: 18 mm x 18 mm, thickness: 0.13 - 0.16 mm) | - |
| - | Super PAP Pen (hydrophobic barrier pen) | - |
| - | Microscope Slide Storage Box | - |
| - | Slide Container | - |
| - | Sterilized Syringe | - |
| - | Slide Staining Rack | - |
| Millipore (or other brands) | Millex Syringe Filter, Durapore PVDF, 0.22 μm pore size | SLGV033N |
| BBI | 5.0 mL Centrifuge Tubes | F611888-0001 |
| Corning | Corning® 100 mm TC-treated Culture Dish | 353003 |
| | 50 mL Centrifuge Tubes | 430829 |
| | 15 mL Centrifuge Tubes | 430791 |
| Kimtech | Kimwipes™ Delicate Task Wipes | 34155 |
| MATIN | Power Dust Remover | M-6318 |
| Axygen | 1.5 mL Centrifuge Tubes | MCT-150-A |
| | 0.2 mL Centrifuge Tubes* | PCR-02-C |
| | 0.2 mL Thin-wall 8 Strip PCR Tubes* | PCR-0208-CP-C |
| | 1000 μL Filtered Tips | TF-1000-R-S |
| | 200 μL Filtered Tips | TF-200-L-R-S |
| | 100 μL Filtered Tips | TF-100-R-S |
| | 10 μL Filtered Tips | TXLF-10-L-R-S |
| | 0.5 mL Thin Wall PCR Tubes^ | PCR-05-C |
| Invitrogen | Qubit Assay Tubes^ | Q32856 |

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

## 1.5. Stereo-seq Chip Slide Information

### Stereo-seq Chip T Slide

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

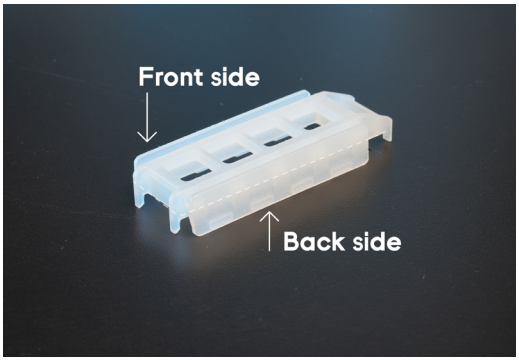


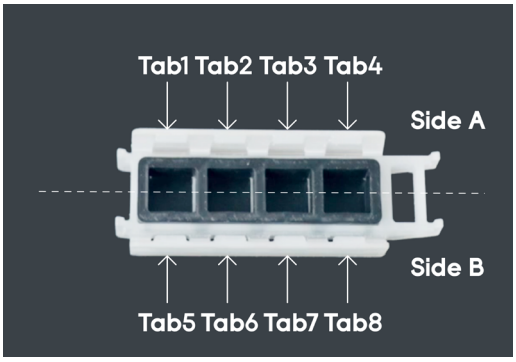

### Stereo-seq Chip T Slide Storage

Always store unused slides in their original slide container and keep them sealed in a sealable aluminum bag at 4°C. Keep sealed with tape or another re-sealable bag. **Always KEEP the desiccant in the bag.**

### Stereo-seq Slide Cassette

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



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

**Refer to Appendix I: Stereo-seq Slide Cassette Assembly**

## 1.6. Precautions and Warning

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

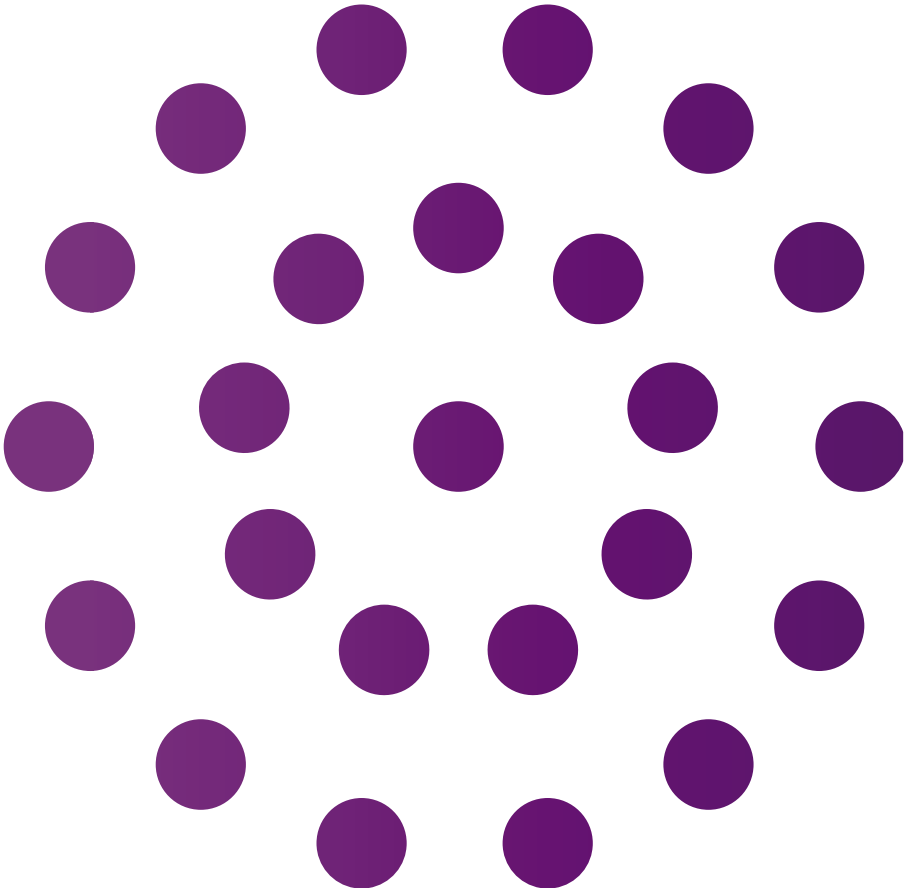
# CHAPTER 2
# SAMPLE AND EXPERIMENT PREPARATION

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

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

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

# 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 is 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 that you 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 the Stereo-seq Transcriptomics - mIF co-assay.

## 3.1. Experiment Preparation

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

**Table 3-1 Experiment Preparation Steps**

| Reagent | Preparation Steps | Maintenance |
|---|---|---|
| 0.1X SSC | Dilute 350 µL of 20X SSC to 70 mL. | Room Temperature |
| 5X SSC | Dilute 1 mL of 20X SSC to 4 mL. | Room Temperature |
| Filtered Serum Aliquot | Thaw the horse serum, then filter it with a 0.22 µm pore-sized filter and a sterilized syringe. Aliquot the filtered serum in 200 µL/ tube and store at -20°C. Thaw the aliquoted serum on ice and centrifuge at 14,000 g for 10 min at 4°C. Place on ice until use. | On ice until use |
| **Do not freeze and thaw the aliquot more than 3 times. Keep the aliquots at -20°C for long-term storage.** | | |
| 10% Triton X-100 | Use 10% Triton X-100 or DILUTE 100% Triton X-100 with Nuclease-free Water. | Room temperature |
| Primary Antibody | Take them out of -20°C or 4°C (depending on the manufacturer's instructions) and centrifuge at 14,000g, 4°C for 10 min. | On ice until use |
| Secondary Antibody | Take them out of -20°C or 4°C (depending on the manufacturer's instructions) and centrifuge at 14,000g, 4°C for 10 min. | On ice until use |

| Reagent | Preparation Steps | Maintenance |
|---|---|---|
| Diluted Primary Antibody and Secondary Antibody (Optional) | Primary antibodies can be diluted with blocking solution to a desired concentration if needed. | On ice until use |
| FcR Blocking Reagent | Choose Human TruStain FcX™ (Fc Receptor Blocking Solution) for human tissues, and choose TruStain FcX™ PLUS (antimouse CD16/32) Antibody for mouse tissue. | On ice until use |
| FcR Blocking Reagent is used for blocking Fc receptors on the cell membrane. 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 experiment, an alternative solution is to skip adding FcR Blocking Reagent and replenish with nuclease-free water to the required volume. | | |
| Glycerol | Equilibrate to room temperature 5 min in advance.<br>Prepare 5 µL per chip. | Room temperature |

| Other Preparation | | |
|---|---|---|
| **Equipments** | **Set up** | **Note** |
| Cryostat | Set the cryostat chamber temperature to -20°C and specimen disc temperature (object temperature) to -10°C~-15°C. | The specimen disc temperature depends on the tissue type. |
| PCR Thermal Cycler | Set the temperature to 37°C for slide drying. | Check the PCR Thermal Cycler for any abnormalities and replace it if necessary. |
| Refrigerated Centrifuge | Adjust the temperature to 4°C in advance. | Centrifuge the filtered serum, primary antibody and secondary antibody. |
| Fluorescence Microscope | DAPI/FITC/TRITC/CY5 | Check the microscope for any abnormalities and replace it if necessary. |

## 3.2. Cryosection Preparation

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

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

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

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

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

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

f. Trim again if necessary to ensure a good fit for mounting. Now, the specimen is ready for cryosection.

## 3.3. Tissue Mounting on Microscope Slides

a. Pre-cool the methanol: Add more than 2/3 volume of the methanol to the slide container, close the lid, and pre-cool the methanol for **5-30 min** at -20°C.

b. Tissue mounting can be achieved using either the cold method (option A) or the warm method (option B). We recommend placing one tissue slice on one microscope slide. Perform cryosectioning and obtain 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 instructions for each antibody. Take the primary antibody of NeuN (Abcam, ab104224) as an example. If the instruction recommended 1:1000, then set the experiment groups 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 the microscope slide inside the cryostat chamber with the front facing up and pre-cool the slide inside the cryostat chamber for **no less than 1 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. Carefully place the tissue section onto the microscope slide center using forceps and brushes. Make sure the tissue section is complete and without wrinkles.

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

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

**B. Warm Method**

1) Perform cryosection, then 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) Verify that the tissue section has been mounted on to the microscope slide.

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

## 3.4. Tissue Fixation

a. After drying the tissue-mounted microscope slide, immediately immerse it in the precooled methanol prepared in section 3.3-a for a **30-min** fixation at -20°C. When immersing the microscope slide in methanol, ensure that the tissue section on the slide is completely submerged.

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

**Table 3-2 Blocking Solution (for Antibody Titration)**

| Components | 1X (μL) | 6X (μL) + 10% |
|---|---|---|
| 5X SSC | 120 | 732 |
| 10% Triton X-100 | 2 | 12.2 |
| FcR Blocking Reagent | 10 | 61 |
| Filtered Serum | 20 | 122 |
| Nuclease-Free Water | 48 | 292.8 |
| **Total** | 200 | 1220 |

c. After fixation is completed, 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 the edges 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 for **4-6 min** to allow the methanol to evaporate completely.

f. Using a Super PAP Pen (hydrophobic barrier pen), draw a circle around the tissue on the microscope slide to create a hydrophobic exclusion zone that will 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 you prepared in Table 3-2 and add **no more than 100 μL/slide** of blocking solution drop-by-drop on the tissue surface, then incubate at room temperature for **20 min**.

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

b. While waiting for the incubation to be completed, 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). There will be a total of 6 groups for antibody titration. Vortex to mix, centrifuge briefly, then 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 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, dilute the primary antibody 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 experiment 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**. 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**.

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

d. While waiting for the primary antibody incubation to be completed, prepare secondary antibody solution according to Table 3-4. Vortex to mix, centrifuge briefly, then leave the secondary antibody solution on ice in the dark until use.

**Prepare the necessary number of secondary antibody solutions based on the number of primary antibodies used for the titration.**

**Table 3-4 Secondary antibody solution (for Antibody Titration)**

| Components | 1X (µL) |
|---|---|
| 5X SSC | 60 |
| Secondary Antibody* | 0.2 |
| Nuclease-Free Water | 39.8 |
| **Total** | 100 |

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

## 3.6. Secondary Antibody Incubation

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

b. Wash by adding **100 µL/slide** of 0.1X SSC. Incubate for **1 min** then discard.

c. Repeat **step b.**

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

d. Slowly add **100 µL/slide** of secondary antibody solution to the tissue. Incubate for **15 min** at room temperature in the dark.

e. Discard the secondary antibody solution with a pipette.

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

g. Repeat **step f.**

**Do not 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 per slide gently onto the center of the tissue without introducing air bubbles. Using 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 3.7 Imaging immediately.

## 3.7. Imaging

a. Take images with fluorescent microscopes that have stitching functions.

b. Required fluorescence channels will depend on antibody selection. Scan using 10X objective lens.

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

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

## 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.

An example of the serial dilution of Anti-NeuN antibody (Abcam, ab104224) shown in Figure 1 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. Determine the optimal concentrations for all antibodies before proceeding to subsequent mIF pilot experiment and Stereo-seq Transcriptomics experiment on the Stereo-seq Chip.

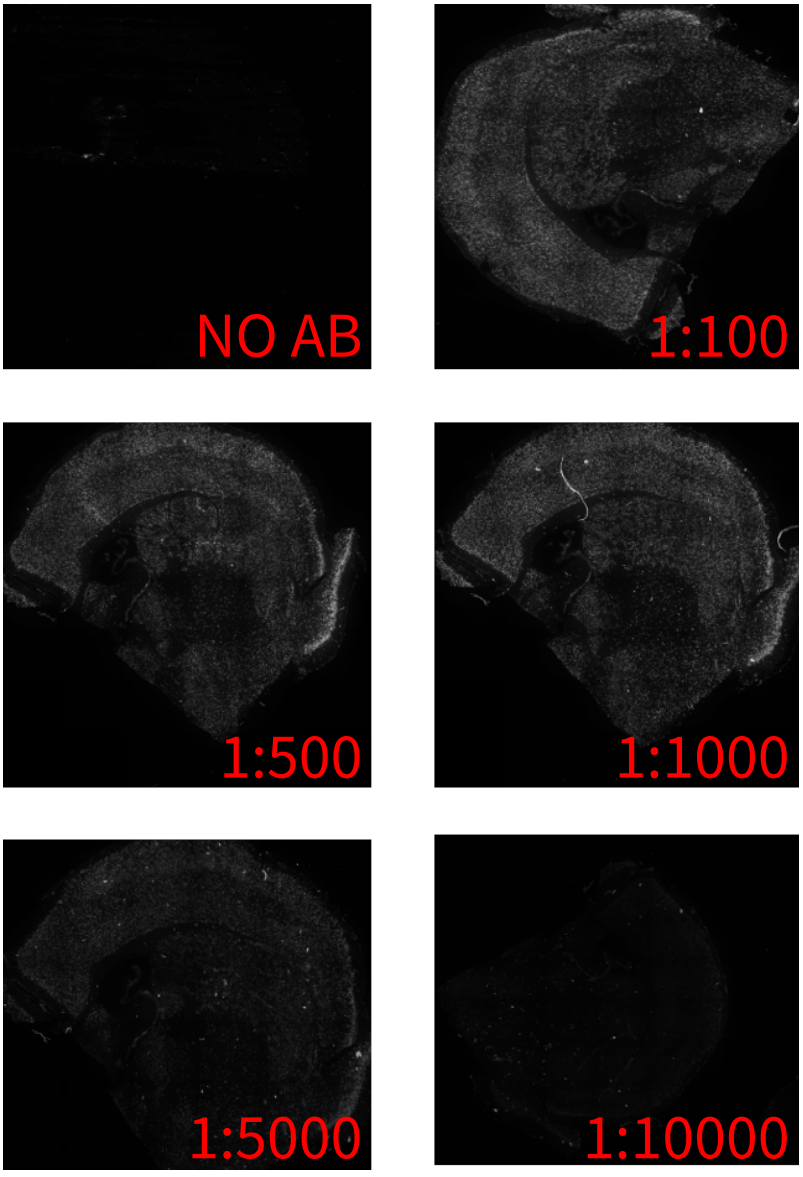
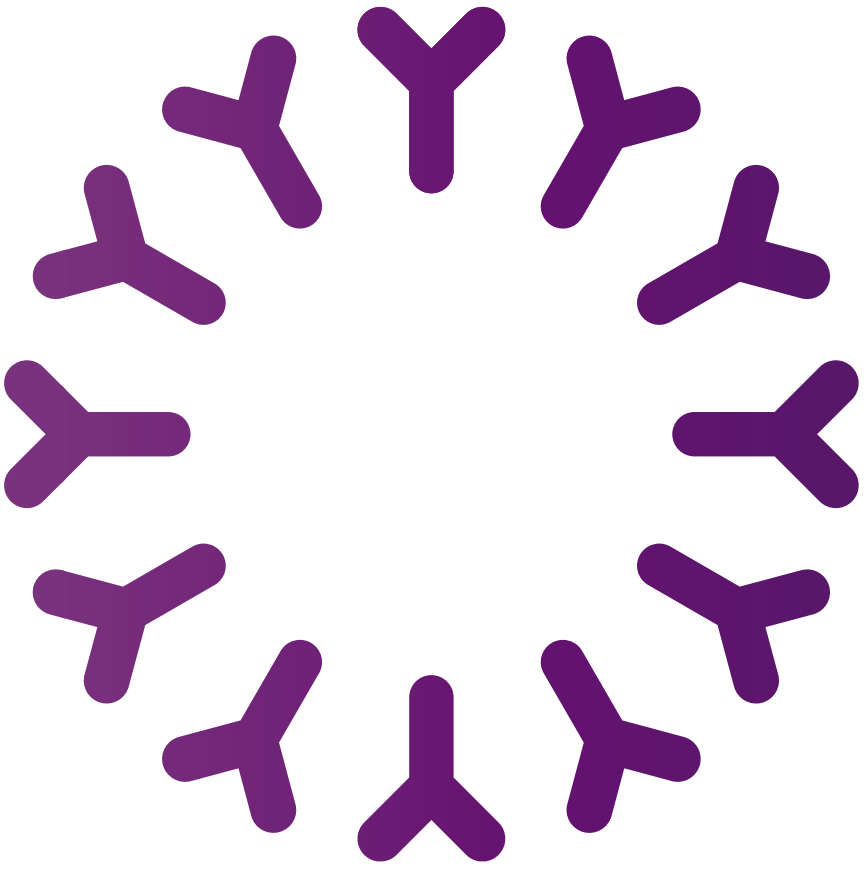



**Figure 1. Serial Anti-NeuN antibody titration results of mouse brain (hemisphere)**

# CHAPTER 4
# mIF PILOT EXPERIMENT



When the optimal dilution ratio for each antibody has been determined, it is recommended that you 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 you proceed to the formal STOmics multiple immunofluorescence (mIF) and Stereo-seq Transcriptomics co-detection experiment on the Stereo-seq Chip.

## 4.1. Tissue Blocking Preparation

Refer to **sections 3.1 - 3.4 in Chapter 3** for the procedures for experiment preparations, cryosection preparation, tissue mounting on microscope glass slides and tissue fixation and blocking solution preparation.

DAPI solution (Thermo Fisher Scientific 62248) is required in this chapter. Prepare:

| Reagent | Preparation Steps | Maintenance |
|---|---|---|
| 50X-diluted DAPI solution | Dilute DAPI Solution (1 mg/mL) with 5X SSC then keep it on ice in the dark. | On ice in the dark until use, up to 6 hr |

## 4.2. Tissue Blocking & Primary Antibody Incubation

a. Prepare the blocking solution according to Table 4-1, vortex it, and add no more than **100 μL/slide** of blocking solution drop-by-drop on the tissue surface, then incubate at room temperature for **20 min**.

**The amount of blocking solution used per slide is dependent on the size of the hydrophobic area. For a hydrophobic area 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)**

| Reagent | 1X (μL) | 1X (μL) + 10% |
|---|---|---|
| 5X SSC | 120 | 132 |
| 10% Triton X-100 | 2 | 2.2 |
| FcR Blocking Reagent | 10 | 11 |
| Filtered Serum | 20 | 22 |
| Nuclease-Free Water | 48 | 52.8 |
| **Total** | 200 | 220 |

**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 experiment, an alternative solution is to skip adding FcR Blocking Reagent and replenish with nuclease-free water to the required volume.**

**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 completed, 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. Vortex to mix, centrifuge briefly, then leave the primary antibody solution on ice until use.

**Table 4-2 Primary Antibody Solution (for mIF Pilot Experiment)**

| Reagent | 1X (μL) |
|---|---|
| Blocking Solution | 100-(V1+V2+...+Vn) |
| Primary Antibody 1 | V1 |
| Primary Antibody 2 | V2 |
| Primary Antibody 3 | V3 |
| ... | ... |
| Primary Antibody #N | Vn |
| **Total** | 100 |

**Before mixing multiple primary antibodies, perform antibody titration for each antibody. To avoid cross-reactivity of secondary antibodies, 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**.

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

d. While waiting for the primary antibody incubation to be completed, prepare secondary antibody solution in Table 4-3 according to the recommended dilution or manufacturer's instruction for each secondary antibody. Vortex to mix, centrifuge briefly, then leave the secondary antibody solution on ice in the dark until use.

**Table 4-3 Secondary antibody solution (for mIF Pilot Experiment)**

| Reagent | 1X (μL) |
|---|---|
| 5X SSC | 60 |
| Secondary Antibody 1 | V1 |
| Secondary Antibody 2 | V2 |
| Secondary Antibody 3 | V3 |
| … | …… |
| Secondary Antibody n | Vn |
| Nuclease Free Water | 40-(V1+V2+V3+....+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 are used, 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 **1 min** then discard.

c. Repeat step b. twice (for a total of three washes).

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

d. Slowly add 100 μL/slide of secondary antibody solution to the tissue. Incubate for **15 min** at room temperature in the dark.

## 4.4. DAPI Staining

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

**Table 4-4 DAPI Staining Solution (for mIF Pilot Experiment)**

| Reagent | 1X (μL) | 1X (μL) + 10% |
|---|---|---|
| 5X SSC | 60 | 66 |
| 50X Diluted DAPI | 1 | 1.1 |
| Nuclease Free Water | 39 | 42.9 |
| **Total** | 100 | 110 |

b. Discard the secondary antibody solution with a pipette.

c. Wash by adding **100 μL/slide** of 0.1X SSC. Incubate for **1 min** then discard.

d. Repeat **step c.**

**Do not 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 in the dark.

f. Discard the DAPI staining solution with a pipette. Wash the slide by immersing it in a 50 mL centrifuge tube or a slide container with enough 0.1X SSC for **10 sec**, ensure that all tissue sections are completely submerged. Discard the solution.

g. Repeat **step f.**

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 per slide gently onto the center of the tissue without introducing air bubbles. Using 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 **4.5 Imaging** immediately.

## 4.5. Imaging

a. Take images with fluorescent microscopes that have stitching functions.

b. Required fluorescence channels will depend on antibody selection. Scan using the 10X objective lens.

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

**If there is any region with extremely high signal in the field of view, wash the slide by immersing it in a 50 mL centrifuge tube or a slide container with enough 0.1X SSC for 10 sec, ensure that all tissue sections are completely submerged. Discard the solution. Repeat the washing one more time.**

## 4.6. mIF Pilot Experiment Results

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

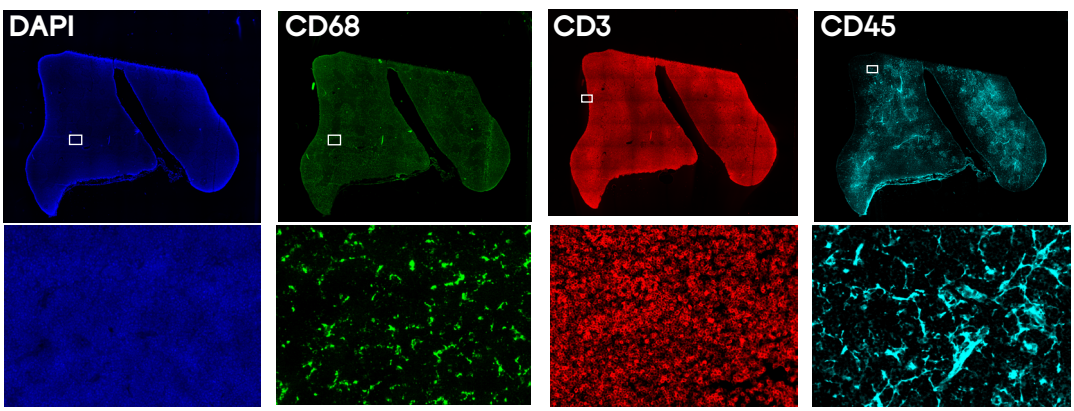
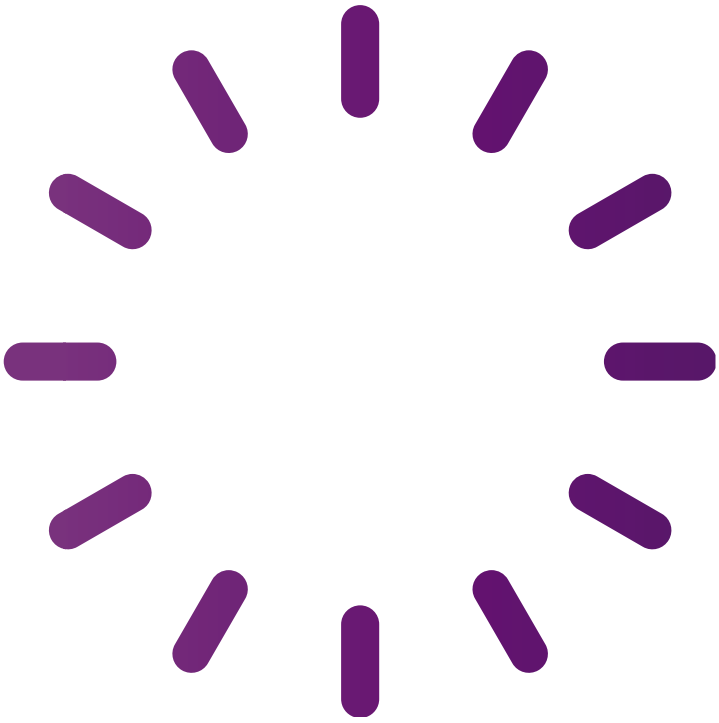



Figure 2. mIF pilot experiment results on mouse thymus with co-staining of Anti-CD68, Anti-CD3, and Anti CD45 Antibodies

# CHAPTER 5

# Stereo-seq TRANSCRIPTOMICS SET FOR CHIP-ON-A-SLIDE FOR mIF STANDARD OPERATING PROCEDURE



## 5.1. Experiment Preparation

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

| Reagent | Preparation Steps | Maintenance |
|---|---|---|
| Methanol | Pre-cool at -20°C for no longer than 30 min before use. | -20°C |
| 0.1X SSC | Dilute 100 µL of 20x SSC to 20 mL | Room temperature |
| Wash Buffer | Prepare at least 2200 µL per chip (Add 110µL RI into 2090 µL 0.1X SSC ) | On ice until use |
| 5X SSC | Dilute 1 mL of 20x SSC to 4 mL | Room temperature |
| Filtered Serum Aliquot | Thaw the horse serum, then filter it with a 0.22 µm pore-sized filter and a sterilized syringe. Aliquot 200 µL of the filtered serum per tube and store at -20°C. Thaw the aliquoted serum on ice and centrifuge at 14,000 g for 10 min at 4°C . Place on ice until use. | On ice until use |
| **Do not freeze and thaw the aliquot more than 3 times. Keep the aliquots at -20°C for long term storage.** | | |
| RI | Take the RI out of -20°C and place it on ice until use. | On ice until use |
| 10% Triton X-100 | Use 10%Triton X-100 or DILUTE 100% Triton X-100 with Nuclease-free water. | Room temperature |
| Primary Antibody | Take them out of -20°C or 4°C (depending on the manufacturer's instructions) and centrifuge at 14,000g, 4°C for 10 min, then place them on ice. | On ice until use |
| Secondary Antibody | Take them out of -20°C or 4°C (depending on the manufacturer's instructions) and centrifuge at 14,000g, 4°C for 10 min, then place them on ice. | On ice until use |
| Diluted Primary Antibody and Secondary Antibody (Optional) | Primary antibodies can be diluted with blocking solution to a desired concentration if needed. | On ice until use |

| | | |
|---|---|---|
| FcR Blocking Reagent | Choose Human TruStain FcX™ (Fc Receptor Blocking Solution) for human tissues and choose TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody for mouse tissue. | On ice until use |
| FcR Blocking Reagent is used for blocking Fc receptors on the cell membrane. 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 experiment, an alternative solution is to skip adding FcR Blocking Reagent and replenish with nuclease-free water to the required volume. | | |
| 50X-diluted DAPI Solution | Dilute DAPI Solution (1mg/mL) with 5X SSC, then place it on ice in the dark. | On ice in the dark until use, up to 6 hr |
| **50X-diluted DAPI solution can be stored at 4°C for up to 1 day in the dark.** | | |
| Glycerol | Equilibrate to room temperature 5 min in advance.<br>Prepare 5 µL per chip. | Room temperature |
| 0.01N HCl | Prepare at least 2 mL of 0.01N HCl per sample. Configure HCl to 0.01N. Measure and make sure the pH = 2. | Room temperature for 48 hr **(Storing longer than 48 hr will affect the desired pH. Use within 48 hr of preparation)** |
| **Always use freshly prepared 0.01N HCl (pH = 2.0 ± 0.1). For pre-made 0.1N HCl and newly purchased HCl, check the pH prior to the experiments.** | | |
| 10X Permeabilization Reagent Stock Solution | Add 1 mL of freshly prepared 0.01N HCl to dissolve PR Enzyme (red cap, in powder form), and thoroughly mix the reagent through pipetting. | On ice until use, up to 1 hr |
| **Do not vortex the permeabilization enzyme.** Mix by pipette before using. **Aliquot 10X stock solution to avoid freeze-thaw cycles. Keep the aliquots at -20°C for long-term storage.** | | |
| 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 |
| 0.1M KOH | Dilute 10 µL 8M KOH with Nuclease free water to 800 µL | Room temperature |
| **Always use freshly prepared 0.1M KOH.** For newly purchased KOH, check the pH prior to the experiments (diluted to 1M, the pH value should be 14 ± 0.3). **Do not prepare until you are ready to use it.** | | |
| Elute Additive | Take it out of -20°C, and thaw on ice in advance; use 5 µL per chip. | On ice until use |

| | | |
|---|---|---|
| Neutralization Solution | Take it out of -20°C, and thaw at room temperature; use 23 μL per chip. | Room temperature |
| Magnetic Beads | Take it out in advance and equilibrate to room temperature at least 30 min prior to use. | Room temperature |
| 80% Ethanol | Dilute 100% ethanol to 80% | Room temperature up to 1 day |
| **Other Preparation** | | |
| **Equipment** | **Set up** | **Notes** |
| 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:<br>1. 37°C for slide drying and permeabilization (heating lid at 60°C);<br>2. 45°C for reverse transcription (heating lid at 60°C);<br>3. 55°C for cDNA release (heating lid at 60°C).<br>4. 95°C for denaturation (heated lid at 105°C). | Check the PCR Thermal Cycler for any abnormalities and replace it if necessary. |
| Metal Bath (or the equipment with same function) | 37°C for pre-heating of 1X Permeabilization Reagent Solution. | Check the instrument for any abnormalities and replace it if necessary. |
| Refrigerated Centrifuge | Adjust the temperature to 4°C in advance. | Centrifuge the thawed filtered serum, primary antibody and secondary antibody. |
| Fluorescence Microscope | DAPI/FITC/TRITC/CY5 | Choose the fluorescence channel according to the secondary antibody. |

## 5.2. Cryosection Preparation

a. Set the PCR thermal cycler to 37°C in advance, and pre-heat the PCR Adaptor in the PCR thermal cycler to the desired temperatures.

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

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

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

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

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

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

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

## 5.3. Tissue Mounting

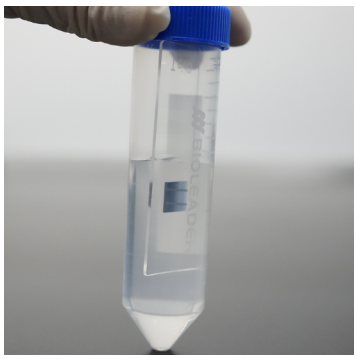
For a demonstration video of tissue mounting onto the Stereo-seq Chip Slide, refer to the link below or scan the QR code:
**https://en.stomics.tech/resources/videos/list.html**

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

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

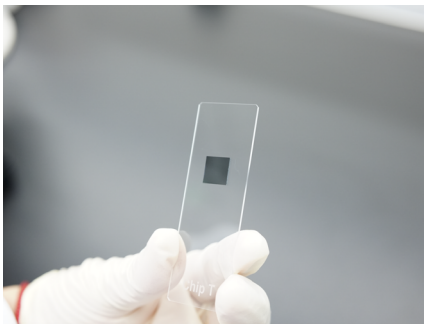
b. Make sure the PCR thermal cycler has been turned on and set to **37°C**.

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



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

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

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

f. Prepare enough methanol in a 50 mL centrifuge tube or an empty slide container with sufficient volume for submerging all the chips on the slide. Immerse a regular glass slide in the methanol-containing tube to confirm that there is sufficient volume. Close the lid and pre-cool the 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 can be achieved using either the cold method (option A) or the warm method (option B). We recommend practicing tissue mounting and section placement on plain glass slides first.

**A. Cold Method**

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

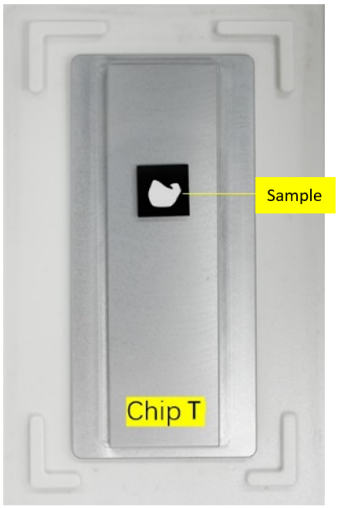
**Prolonged cooling for over 6 min may cause mist formation on the chip surface.**

2) Perform cryosection, then carefully flatten the tissue section out by gently touching the surrounding OCT with cryostat brushes. Carefully place a tissue section onto the chip center using forceps and brushes. Make sure the tissue section is complete and without wrinkles.

3) Immediately pick up the Stereo-seq Chip Slide and place a finger on the back of the Stereo-seq Chip Slide directly under the chip for a few 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 the remaining chips.

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




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

**B. Warm Method**

1) Perform cryosection and obtain two to 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 such that the space between each is greater than the chip spacing on the Stereo-seq Chip Slide.

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

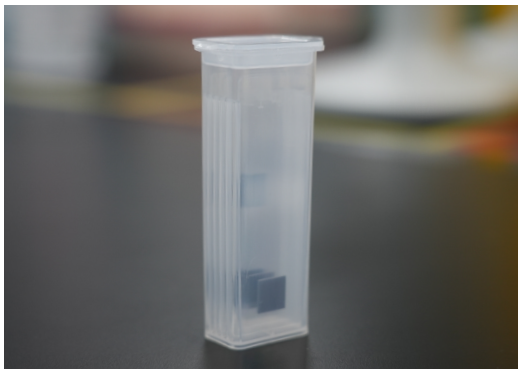
4) Repeat **step 3)** until all the tissue sections have been mounted onto the chips of the Stereo-seq Chip Slide.

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

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

**Stop Point:**

- **After drying the tissue containing Stereo-seq Chip Slides in the PCR thermal cycler, transfer the Stereo-seq Chip Slide into a slide container (or 50 mL tube), then place the slide container in a sealable plastic bag. Place one desiccant pack per container into a sealable bag, push out as much air as possible and seal the bag tightly. Transfer sealed container to a -80°C freezer on dry ice.**
- **Store the sealed bag containing the Stereo-seq Chip Slides with tissue at -80°C for up to 21 days.**
- **When retrieving Stereo-seq Chip Slides with tissue from the freezer, transfer out the slide container on dry ice, take out the tissue containing Stereo-seq Chip Slides then immediately incubate at 37°C with PCR Adaptor for 5 min.**



## 5.4. Tissue Fixation

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

b. While waiting for the fixation to be completed, prepare the reagents required for tissue blocking and antibody incubation according to Table 5-2.

**Table 5-2 Blocking Solution**

| Components | 1X (μL) | 1X + 10% (μL) | 2X + 10% (μL) | 3X + 10% (μL) | 4X + 10% (μL) |
|---|---|---|---|---|---|
| 5X SSC | 180 | 198 | 396 | 594 | 792 |
| 10% Triton X-100 | 3 | 3.3 | 6.6 | 9.9 | 13.2 |
| FcR Blocking Reagent | 15 | 16.5 | 33 | 49.5 | 66 |
| RI | 15 | 16.5 | 33 | 49.5 | 66 |
| Filtered Serum | 30 | 33 | 66 | 99 | 132 |
| Nuclease-Free Water | 57 | 62.7 | 125.4 | 188.1 | 250.8 |
| **Total** | 300 | 330 | 660 | 990 | 1320 |

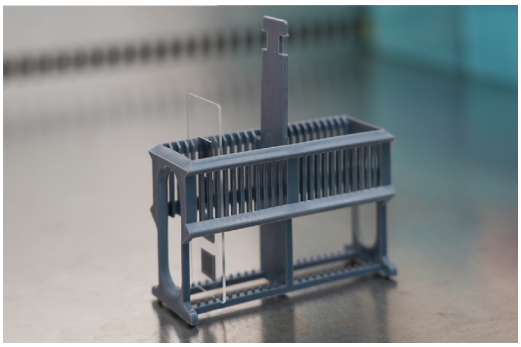
**TruStain FcX™ PLUS (anti-mouse CD16/32) Antibody is not recommended if the host species of the primary antibody is rat. If a primary antibody of rat host species is required for the experiment, an alternative solution is to skip adding FcR Blocking Reagent and replenish with nuclease-free water to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

**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 is completed, move the 50 mL centrifuge tube or slide container to a sterile fume hood.

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

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



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

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

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

## 5.5. Tissue Blocking & Antibody Incubation

a. Vortex the blocking solution that you prepared in Table 5-2, add **150 µL** per chip of blocking solution slowly on the tissue surface. Apply an **unpeeled** sealing tape on top of the Stereo-seq Slide Cassette assembly and incubate at room temperature for **20 min**.

b. While waiting for the incubation to be completed, 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. Vortex to mix, centrifuge briefly, then leave the primary antibody solution on ice until use.

**Table 5-3 Primary Antibody Solution**

| Components | 1X (μL) |
|---|---|
| Blocking Solution | 150 - (V1+V2+V3+....+Vn) |
| Primary Antibody 1 | V1 |
| Primary Antibody 2 | V2 |
| Primary Antibody 3 | V3 |
| ... | … |
| Primary Antibody n | Vn |
| **Total** | 150 |

**Before mixing multiple primary antibodies, perform antibody titration for each antibody. Centrifuge the primary antibodies before use. While pipetting, do not allow the pipette tip to directly contact the bottom of the tube. To avoid cross-reactivity of secondary antibodies, choose primary antibodies of different host species.**

c. Discard the blocking solution with a pipette. Slowly add **150 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**.

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

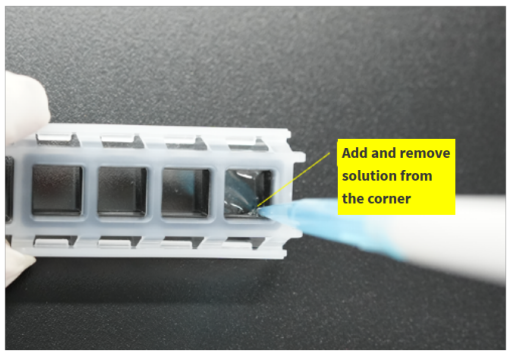
d. While waiting for the primary antibody incubation to be completed, prepare secondary antibody solution in Table 5-4 according to the recommended dilution or manufacturer's instruction for each secondary antibody. Vortex to mix, centrifuge briefly, then leave the secondary antibody solution on ice in the dark until use.

**Table 5-4 Secondary Antibody Solution**

| Components | 1X (μL) |
|---|---|
| 5X SSC | 90 |
| RI | 7.5 |
| Secondary Antibody | 0.3 |
| Nuclease-Free Water | 52.2 |
| **Total** | 150 |

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

e. Pipette to remove the blocking solution from the corner of the well; do not touch the chip surface. Keep it moist.




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

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

h. Repeat **steps f.** and **g**.

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

i. Slowly add **150 µL per chip** of secondary antibody solution to the tissue. Apply **unpeeled** sealing tape on top of the Stereo-seq Slide Cassette and incubate for **15 min** at room temperature in the dark.

## 5.6. DAPI Staining

| Reagent | Preparation Steps | Maintenance |
|---|---|---|
| 50X Diluted DAPI solution | Dilute DAPI stock solution with 5X SSC then keep it on ice in the dark. Mix, centrifuge shortly and avoid pipetting the precipitation at the bottom. | On ice in the dark until use, up to 6 hr |

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

**Table 5-5 DAPI Staining Solution**

| Components | 1X (μL) | 1X + 10% (μL) | 2X + 10% (μL) | 3X + 10% (μL) | 4X + 10% (μL) |
|---|---|---|---|---|---|
| 5X SSC | 90 | 99 | 198 | 297 | 396 |
| 50X-diluted DAPI | 1.5 | 1.65 | 3.3 | 5 | 6.6 |
| RI | 7.5 | 8.25 | 15.8 | 24.8 | 33 |
| Nuclease-Free Water | 51 | 56.1 | 119.9 | 168.2 | 224.4 |
| **Total** | 150 | 165 | 330 | 495 | 660 |

b. Pipette to remove the Secondary Antibody Solution from the corner of the well; do not touch the chip surface. Keep it moist.

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

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

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

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

f. Slowly add **150μL per chip** of DAPI staining solution from the non-tissue area until the solution covers the tissue section. Apply an **unpeeled** sealing tape on top of the Stereo-seq Slide Cassette assembly and incubate for 2 min at room temperature in the dark.

g. Remove the sealing tape for later use and leave it on the bench. Slightly tilt the Stereo-seq Cassette and, using a pipette, remove the DAPI staining buffer from the corner of each well without touching the chip surface. Keep it moist.

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

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

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

k. Disassemble the Cassette and Gasket according to the instructions in Appendix I: Stereo-seq Slide Cassette Assembly and place the cassette and gasket on the bench for later use.

**Do not touch the front-side of the chip while disassembling the Stereo-seq Slide Cassette.**

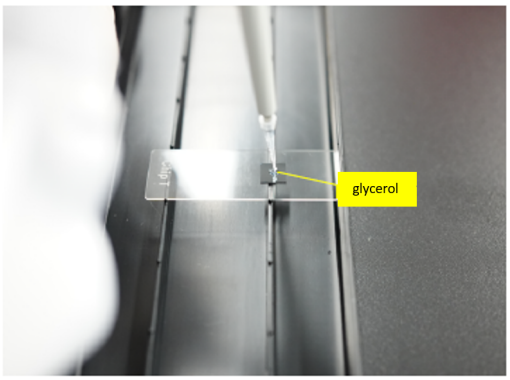
l. Transfer the Stereo-seq Chip Slide onto dust-free paper. Hold the slide with one hand and completely dry the chips using a power dust remover held in the other hand at an approximate distance of 2-3 cm from the chip surface. Blow gently from one side of the chip at a 30-degree angle horizontal to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

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

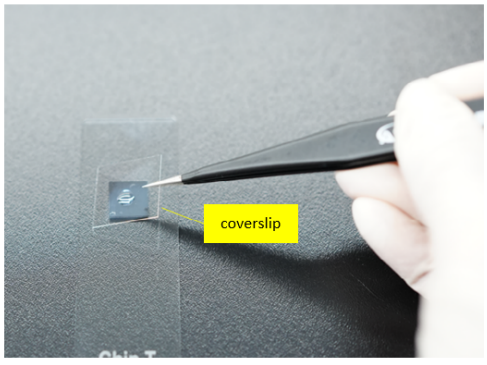
**Equilibrate the glycerol at room temperature for 5 min in advance.**

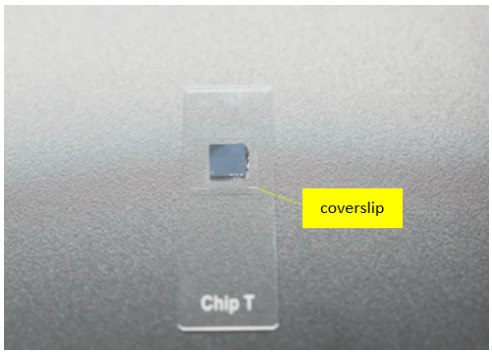
**Ensure that the coverslip is clean and free of any dust or debris. Wipe with an alcohol swab or blow the debris off with a power dust remover.**

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




n. Using clean forceps, place one end of the coverslip onto the chip while holding the other end and then gradually lower the coverslip onto the chips. Ensure that the tissue is completely covered with glycerol and the coverslip. To avoid fluorescent bleaching, IMMEDIATELY proceed to **5.7 Imaging**.



## 5.7. Imaging

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

This section uses a general-purpose microscope as an example to describe the imaging operation. For specific requirements for microscopes, refer to **STOmics Microscope Assessment Guideline**.

Required fluorescence channels will depend on antibody selection. Recommended fluorescence configuration:

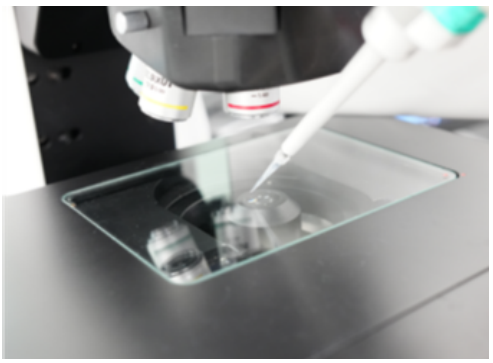
- Light source with a wavelength range of 380 - 680 nm
- Monochrome camera (≥ 8 bit)
- DAPI filter cube (Excitation 375/28nm, Emission 460/50nm)
- FITC filter cube (Excitation 480/30nm, Emission 525/50nm)
- TRITC filter cube (Excitation 545/25nm, Emission 605/70nm)
- CY5 filter cube (Excitation 620/50nm,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

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

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

**Example chip ID number: B00249A1**

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



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

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

d. Turn on the fluorescence microscope and select the epifluorescence mode (DAPI channel).

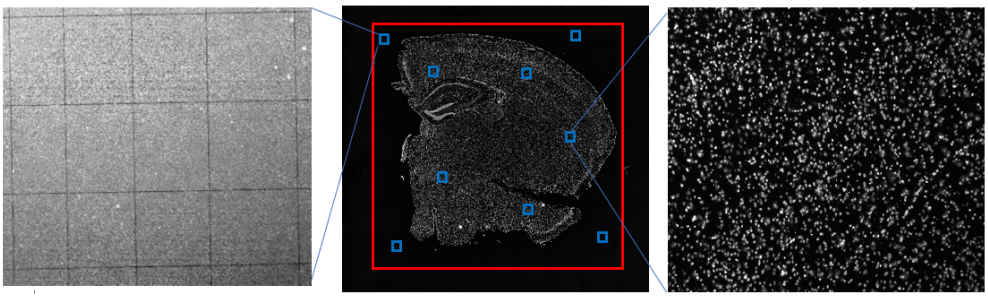
e. Determine the tissue location: Select the 4X objective lens, move the Field of View (FOV) to the tissue area on the chip, adjust Brightness, Gain, and Exposure, then use the focusing knob to adjust the focus until the tissue and cell boundaries are clear and within focus (the light intensity should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

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

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

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

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



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

k. When 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. Create a new folder, name it, and save it in the format of chip number_protein name_IF, and select the appropriate fluorescence channel (FITC channel, TRITC channel or CY5 channel) according to the fluorescent dye coupled to the secondary antibody to take the IF image. Then adjust the focus and exposure until the stained tissue is clearly displayed. Finally complete the full scan on the capture area with 10X objective lens. Save the original tile (FOV) image files and stitched images.

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

**Example folder name: B00249A1_NeuN_IF**

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

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

**The captured images need to pass Image QC before you can proceed to further image analysis (register).**

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

**Glycerol-mounted chips can not be stored longer than 1 hr after imaging at room temperature. For tissues prone to RNA degradation, such as pancreas, proceed to the next step immediately to avoid RNA degradation.**

m. After imaging, take the slide from microscope and keep it mounting. Prepare the 1X Permeabilization Reagent Solution according to Table 5-6.

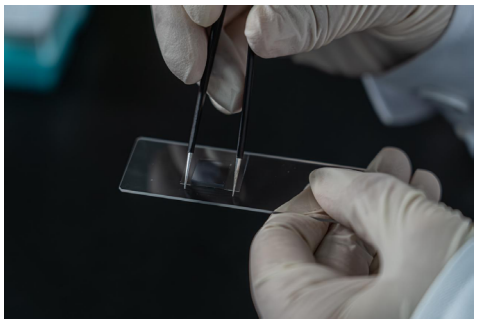
**Table 5-6 1X Permeabilization Reagent Solution**

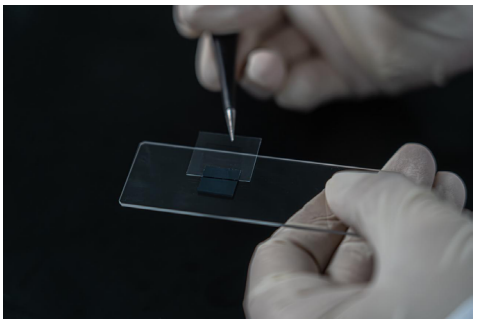
| Components | 1X (μL) | 1X + 10% (μL) | 2X + 10% (μL) | 3X + 10% (μL) | 4X + 10% (μL) |
|---|---|---|---|---|---|
| 0.01N HCl | 135 | 148.5 | 297 | 445.5 | 594 |
| 10X Permeabilization Stock | 15 | 16.5 | 33 | 49.5 | 66 |
| **Total** | 150 | 165 | 330 | 495 | 660 |

n. Set the temperature of the metal bath (or equivalent instrument) to 37°C, and the PCR thermal cycler at 37°C Hold.

o. Place the 1X Permeabilization Reagent Solution in the metal bath (or equivalent instrument) and incubate for **10 min (no longer than 30 min)** at 37°C before use.

p. After incubating the solution for **8 min**, use clean forceps to grip the coverslip, and then slowly pull and slide the coverslip over the Stereo-seq Chip Slide edge until the chips and the coverslip are completely separated.





q. Wipe off excess solution from around the edges and the back of the slide with dust-free paper without touching the chips. Ensure that there is no liquid residue around the chips.

r. Change the gasket and assemble the Cassette and Gasket according to the instructions in Appendix I: Stereo-seq Slide Cassette Assembly.

**Do not touch the surface of chips.**

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

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

u. Repeat **steps s**. and **t**.

## 5.8. Tissue Permeabilization

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

b. Ensure that the PCR thermal cycler has been set to 37°C **(highlighted in bold)**.

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

c. Place the Stereo-seq Slide Cassette in the 37°C PCR thermal cycler, 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 droplets.

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

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

Optimal permeabilization time is determined by the Stereo-seq Permeabilization Kit (211KP11118). Refer to the **Stereo-seq Permeabilization Set for Chip-on-a-slide for mIF User Manual (Document No.: STUM-PR003)** for more information.

e. While waiting for permeabilization to be completed, prepare RT Mix according to Table 5-7 then leave it on ice until use. **【PREPARED AHEAD】**

**Table 5-7 RT Mix**

| Components | 1X (μL) | 1X + 10% (μL) | 2X + 10% (μL) | 3X + 10% (μL) | 4X + 10% (μL) |
|---|---|---|---|---|---|
| RT Buffer Mix | 166 | 182.6 | 365.2 | 547.8 | 730.4 |
| RT Plus | 4 | 4.4 | 8.8 | 13.2 | 17.6 |
| RT Oligo | 10 | 11 | 22 | 33 | 44 |
| RT Enzyme Mix | 20 | 22 | 44 | 66 | 88 |
| **Total** | 200 | 220 | 440 | 660 | 880 |

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

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

h. Add **200 μL** of Wash Buffer per chip and then slightly tilt the Stereo-seq Slide Cassette to remove the solution from the corner of each well. Keep the chip surface moist.


**CAUTION! Do not dry the chip completely.**

**To prevent RNA degradation, proceed immediately to 5.9 Reverse Transcription.**

## 5.9. Reverse Transcription

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

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

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

c. Apply sealing tape to the Stereo-seq Slide Cassette and seal it tightly. Incubate the Stereo-seq Slide Cassette at 45°C for **2 hr** or longer (no longer than **5 hr**).

## 5.10. cDNA Release and Denaturation

a. Five (5) min before the Reverse Transcription Program is completed, prepare the cDNA Release Mix according to Table 5-8, and then place the mix at room temperature.

**Table 5-8 cDNA Release Mix**

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

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

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

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

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

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

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

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

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

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

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

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

**CAUTION! Be careful not to pipette cDNA Release Mix onto the gasket.**

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

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

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

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

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

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

## 5.11. cDNA Amplification

a. Prepare PCR Mix according to Table 5-9. Pipette up and down to mix, then centrifuge briefly. Store it on ice until use.

**Table 5-9 PCR Mix**

| Component | 1X (μL) | 1X + 10% (μL) | 2X + 10% (μL) | 3X + 10% (μL) | 4X + 10% (μL) |
|---|---|---|---|---|---|
| 4X cDNA PCR Mix | 76.5 | 84.15 | 168.3 | 252.5 | 336.6 |
| cDNA Primer | 12 | 13.2 | 26.4 | 39.6 | 52.8 |
| Nuclease-free water | 13.5 | 14.85 | 29.7 | 44.5 | 59.4 |
| **Total** | 102 | 112.2 | 224.4 | 336.6 | 448.8 |

b. Aliquot **34 μL** PCR Mix into each PCR tube with 66 μL cDNA (from step m. in 5.10 cDNA Release and Denaturation). Vortex to mix well.

c. Briefly spin the three PCR tubes then proceed to Amplification based on the following incubation protocol.

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

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

d. Prepare Qubit dsDNA Mix in a PCR tube according to Table 5-10.

**Table 5-10 Qubit dsDNA Mix**

| Component | 1X (μL) | 2X (μL) | 3X (μL) | 4X (μL) |
|---|---|---|---|---|
| Invitrogen™ Qubit dsDNA HS Buffer | 199 | 398 | 597 | 796 |
| Qubit dsDNA HS Reagent 200X | 1 | 2 | 3 | 4 |
| **Total** | 200 | 400 | 600 | 800 |

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

f. Take **1 µL** PCR product and add into the detection tube with **199 µL** Qubit dsDNA Mix. Vortex, mix and briefly centrifuge the detection tube, then measure the concentration of the PCR product using the Qubit fluorometer. The DNA concentration is usually greater than **5 ng/µL**.

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

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

a. Proceed to 5.12 cDNA Purification and perform the **1X** magnetic bead purification steps from **steps b.1) to b.5)**.

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

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

d. Add **25.5 µL** of 4X cDNA PCR Mix and **4 µL** cDNA Primer, mix well and centrifuge, then place the PCR tube back into the PCR thermal cycler for **8 cycles** (follow the incubation protocol in 5.11 cDNA Amplification, step c., but for 8 cycles only).

e. Continue to perform **0.8X** magnetic bead purification according to the instructions in 5.12 cDNA Purification, starting from **step a**.

## 5.12. cDNA Purification

### Recommended Magnetic Beads

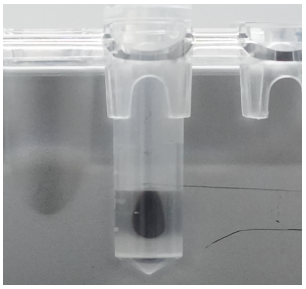
For bead-based purification, we recommend using DNA Cleanup Beads AMPure® XP (Beckman Coulter, Cat. No.: A63882), SPRIselect (Beckman Coulter, Cat. No.: B23317/B23318/B23319), or VAHTS™ DNA Clean Beads (VAZYME, Cat. No.: N411-02). *If magnetic beads from other sources are used, please optimize the cleanup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

### Before Using the Beads

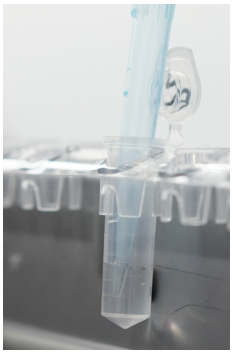
- To ensure the DNA capture efficiency of the magnetic beads, equilibrate the beads to room temperature **30 min before use**.
- Vortex or pipette up and down to ensure that the beads are thoroughly mixed every time before use.
- The number of magnetic beads directly affects the distribution of purified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

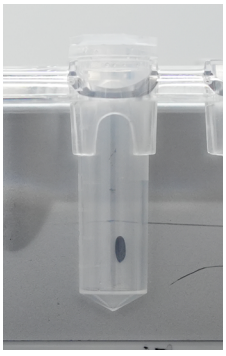
### Operation Notes

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

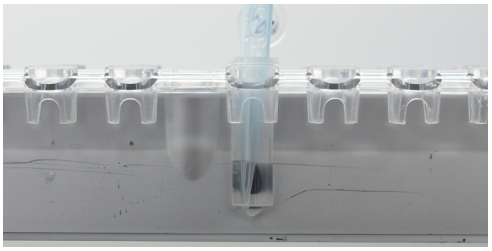


2. When collecting the supernatant after magnetic separation, avoid taking up the beads in the pipette. Instead of collecting the entire supernatant fraction, leave 2-3 µL in the tube to prevent the pipette from directly contacting the beads. If the beads are accidentally taken up, dispense everything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

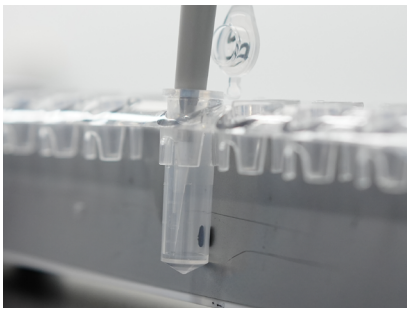




3. Use freshly prepared 80% ethanol (at room temperature) to wash the beads. Keep the sample tube on the magnetic separation rack during the washing step. Do not shake or disturb the beads.



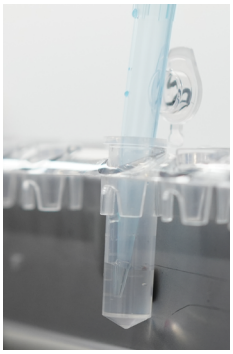
4. After the second washing of beads with ethanol, try to remove all of the liquid in the tube. You may centrifuge briefly to accumulate any remaining liquid at the bottom of the tube, then separate the beads magnetically, and remove the remaining liquid by using a small-volume pipette.

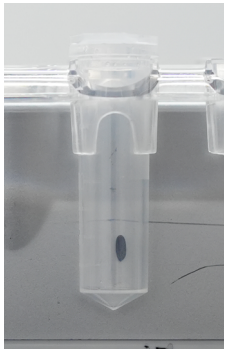


5. After washing twice with ethanol, air-dry the beads at room temperature. Drying usually takes approximately **5-10 min**, depending on the lab temperature and humidity level. Watch closely until the pellet appears sufficiently dry with a matte appearance, then continue to the elution step with TE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

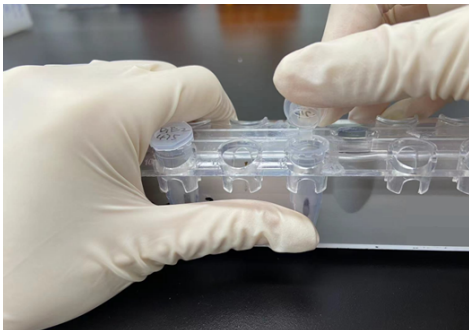


6. During the elution step, do not touch the beads with the pipette tip when removing the supernatant. Contamination of a DNA sample with beads may affect subsequent purification steps. Therefore, to prevent the pipette tip from directly contacting the beads, always collect the eluate in 2 μL less than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





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



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

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

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

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

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

4) Carefully remove and discard the supernatant with a pipette (if foam is visible on the cap, discard it with a pipette).

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

**Always place the pipette tips on the tube wall and away from the magnetic beads. Do not disturb the beads while transferring the supernatant (if foam is visible on the cap, clean the cap with 80% ethanol).**

6) Repeat **step 5**.

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

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

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

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

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

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

**A qualified cDNA sample should have fragment distribution appearing at approximately 200-2,000 bp (Figure 3) and a yield that is greater than 100 ng.**

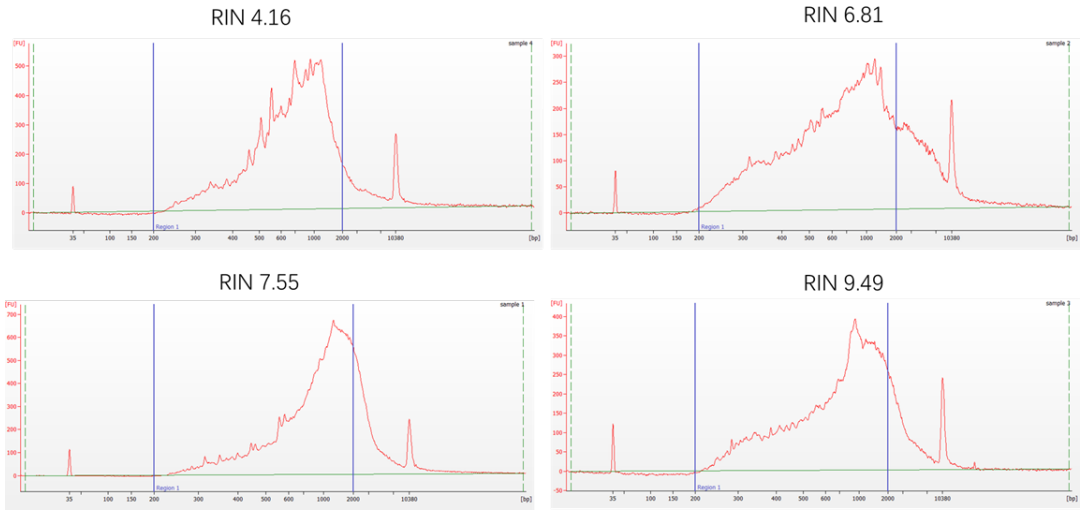


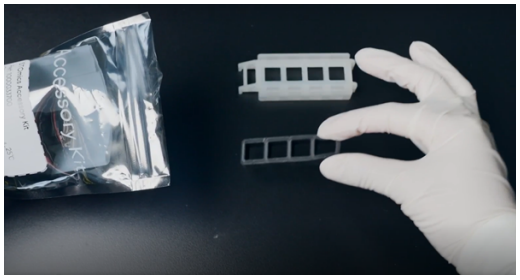

**Figure 3. Representative Agilent Bioanalyzer 2100 analysis results of PCR-amplified cDNA samples with different RIN values**

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

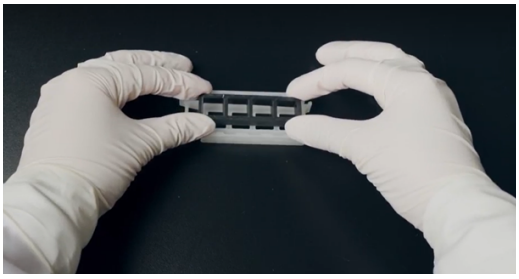
# Appendix I: Stereo-seq Slide Cassette Assembly

## Stereo-seq Slide Cassette Assembly

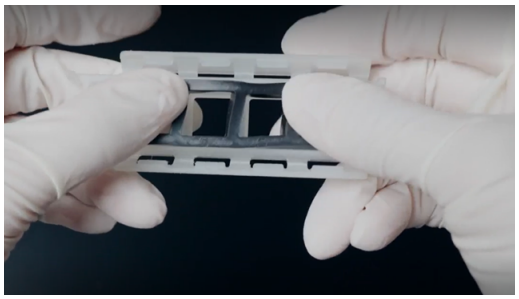
a. Take the Stereo-seq Slide Cassette and Gasket out of the STOmics Stereo-seq Accessory Kit.



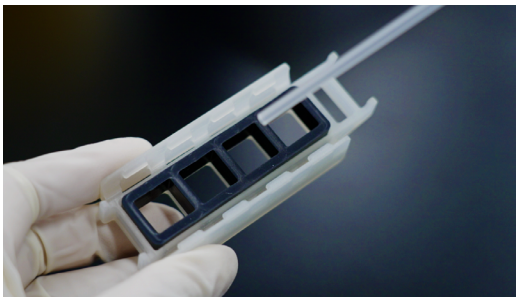
b. Pick up the Stereo-seq Slide Cassette and flip it over. Insert the gasket into the Stereo-seq Slide Cassette, ensuring that the cutouts are aligned.



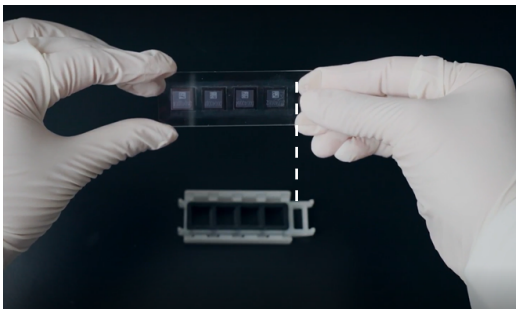
c. Press the gasket down to secure it in the cassette.



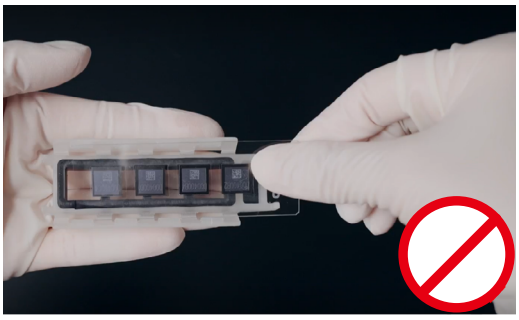
d. If necessary, use a power dust remover to blow any debris off the gasket.



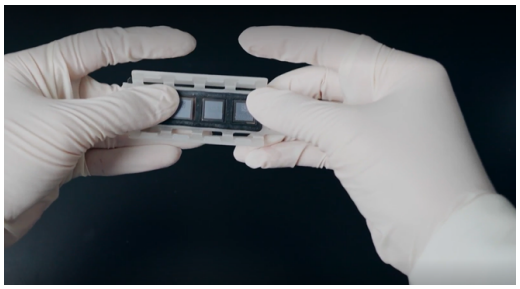
e. Pick up the Stereo-seq Chip Slide and flip it over with the chip surface facing down. Align the engraved label with the long edge of the Stereo-seq Slide Cassette.



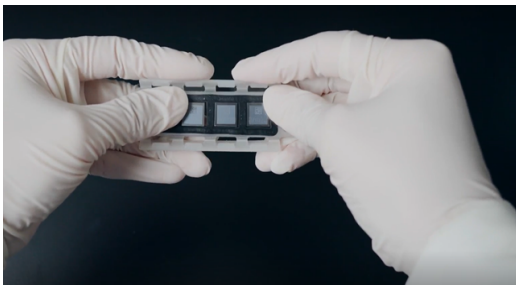
f. Ensure that the chips are aligned within the empty space of the gasket and avoid touching the chip surface with the gasket or cassette during slide placement. Insert the Stereo-seq Chip Slide under the bottom 4 tabs.



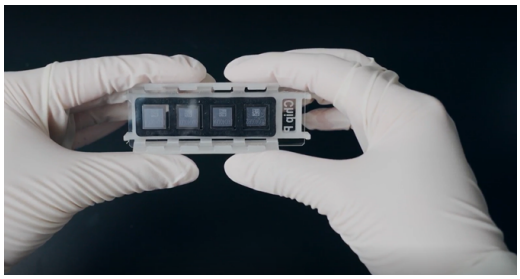
g. Support the back of the cassette with both middle fingers. Place your left thumb between tab 1 and tab 2 and place your right thumb between tab 3 and tab 4.



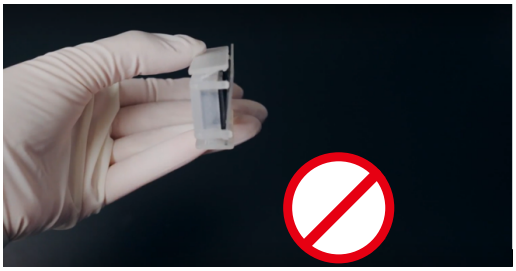
h. Press the upper side (A side) of the slide (near the edge) evenly and then simultaneously press the top edge down firmly with both index fingers to clip the slide in place until you hear it click.



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

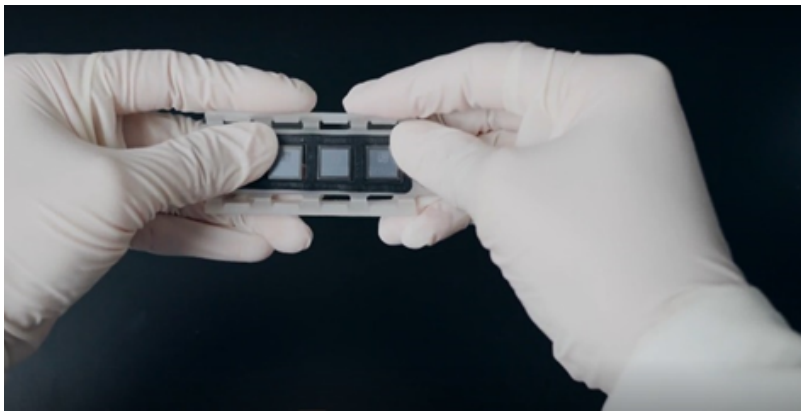


j. Recheck the Stereo-seq Slide Cassette and verify that the slide is clipped in place.



## Stereo-seq Slide Cassette Removal

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



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