STOmics

Stereo-CITE PROTEO-TRANSCRIPTOMICS LIBRARY PREPARATION

USER MANUAL

With Stereo-seq 16 Barcode Library Preparation Kit V1.0

Cat. No.: 111KL160 (16 RXNs)
Kit Version: V1.0
Manual Version: A

STUM-LP003

REVISION HISTORY

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Note: Please download the latest version of the manual and use it with the corresponding Stereo-seq Library Preparation Kit.

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NOTE: Additional operation tips and guidance.

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

QUALITY CHECK POINT

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

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

CHAPTER 1

INTRODUCTION

1.1. Intended Use

This user manual provides experimental instructions specifically for Stereo-seq 16 Barcode Library Preparation Kit.

Stereo-CITE Proteo-Transcriptomics Library Preparation requires the use of Stereo-seq 16 Barcode Library Preparation Kit, which is designed for samples using Stereo-seq technology, including fresh frozen (FF) transcriptome cDNA samples, multi-protein antibody-derived tags (ADT) samples, and Formalin-Fixed Paraffin-Embedded (FFPE) transcriptome cDNA samples. It enables the addition of sample barcodes and library construction, suitable for multi-sample mixed sequencing, supporting up to 16 samples for mixed sequencing.

All reagents provided in this kit have passed stringent quality control and functional verification, ensuring performance stability and reproducibility of library construction, as well as the uniformity and accuracy of sequencing data splitting.

1.2. Sequencing Guidelines

Sequencing libraries produced using the Stereo-seq 16 Barcode Library Preparation Kit require the DNBSEQ sequencing platform. For details, refer to Chapter 3: Library Structure and Sequencing of Stereo-CITE Proteo-Transcriptome Library.

1.3. List of Kit Components

Table 1-1 Stereo-seq 16 Barcode Library Preparation Kit Components

Stereo-seq 16 Barcode Library Preparation Kit V1.0		Cat. No.: 111KL160	
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (tube)**
KMB	1000047709	○	160 μL × 1
KME	1000047770	○	80 μL × 1
PCR Barcode Primer Mix 1	1000043201	●	25 μL × 1
PCR Barcode Primer Mix 2	1000043202	●	25 μL × 1
PCR Barcode Primer Mix 3	1000043203	●	25 μL × 1
PCR Barcode Primer Mix 4	1000043204	●	25 μL × 1
PCR Barcode Primer Mix 5	1000043205	●	25 μL × 1
PCR Barcode Primer Mix 6	1000043206	●	25 μL × 1
PCR Barcode Primer Mix 7	1000043207	●	25 μL × 1
PCR Barcode Primer Mix 8	1000043208	●	25 μL × 1
PCR Barcode Primer Mix 9	1000043209	●	25 μL × 1
PCR Barcode Primer Mix 10	1000043210	●	25 μL × 1

Stereo-seq 16 Barcode Library Preparation Kit V1.0		Cat. No.: 111KL160	
Component	**Reagent Cat. No.**	**Cap Color**	**Quantity (tube)**
PCR Barcode Primer Mix 11	1000043211	●	25 μL × 1
PCR Barcode Primer Mix 12	1000043212	●	25 μL × 1
PCR Barcode Primer Mix 13	1000043213	●	25 μL × 1
PCR Barcode Primer Mix 14	1000043214	●	25 μL × 1
PCR Barcode Primer Mix 15	1000043215	●	25 μL × 1
PCR Barcode Primer Mix 16	1000043216	●	25 μL × 1
PCR Amplification Mix	1000043217	●	800 μL × 1
Storage Temperature: -25°C~-18°C	Transported by cold chain		Expiration Date: refer to label

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.

Refer to Appendix I: PCR Barcode Primer Mix Use Rules for information on barcode combinations.

1.4. Additional Equipment and Materials

Table 1-2 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).

Table 1-2 Additional Equipment and Materials

Equipment		
Brand	**Description**	**Cat. No.**
Thermo Fisher Scientific	PCR Thermal Cycler	4483636 (or similar)
NEBNext®*	Magnetic Separation Rack for <200 μL Tubes	S1515S
Invitrogen*	Magnet Separation Rack: DynaMag™-PCR	492025
Thermo Fisher Scientific	Magnetic Separation Rack DynaMag™-2 for 1.5-2mL Tubes	12321D
	Qubit™3 Fluorometer	Q33216 (or similar)
-	Benchtop Centrifuge	-
-	Vortex Mixer	-
Agilent Technologies™	Agilent 2100 Bioanalyzer	G2939AA (or similar)

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

Reagent

Brand	Description	Cat. No.
Invitrogen*	Nuclease-free Water	2186768
Ambion	Nuclease-free Water	AM9937
	1X TE Buffer, pH 8.0	AM9858
-	100% Ethanol (Analytical grade)	-
Beckman Coulter^	AMPure® XP	A63882
	SPRIselect	B23317/B23318/B23319
VAZYME^	VAHTS™ DNA Clean Beads	N411-02
Invitrogen	Qubit dsDNA HS Assay Kit	Q32854
	Qubit ssDNA Assay Kit	Q10212
Agilent Technologies™	High Sensitivity DNA Kit	5067-4626/5067-1506

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

Consumables

Brand	Description	Cat. No.
Axygen	1.5 mL Centrifuge Tubes	MCT-150-A
	0.2 mL PCR Tubes*	PCR-02-C
	0.2 mL Thin-wall 8 Strip PCR Tubes*	PCR-0208-CP-C
	1,000 μL Filtered Tips	TF-1000-L-R-S
	200 μL Filtered Tips	TF-200-L-R-S
	100 μL Filtered Tips	TF-100-R-S
	10 μL Filtered Tips	TXLF-10-L-R-S
	0.5 mL Thin Wall PCR Tubes	PCR-05-C
Invitrogen	Qubit Assay Tubes	Q32856

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

1.5. Precautions and Warnings

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

CHAPTER 2

LIBRARY PREPARATION FOR Stereo-CITE FF cDNA/ADT SAMPLES

2.1. Stereo-CITE cDNA Library Preparation

2.1.1. Experiment Preparation

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

Table 2-1 Experiment Preparation Steps

Reagent	Preparation Steps	Maintenance
KMB	Thaw the reagent 5 min in advance.	On ice until use
80% Ethanol	Dilute 100% ethanol to 80%.	Room temperature up to 1 day
Magnetic Beads	Take the beads out and equilibrate to room temperature at least 30 min prior to use.	Room temperature up to 6 hr

2.1.2. cDNA Multiple Displacement Amplification and ssDNA Purification

a. Use **100 ng** cDNA sample for the amplification reaction. If the cDNA yield is less than 100 ng, add up to a maximum volume of **35 μL**.

b. Prepare the Amplification Reaction Mix on ice according to Table 2-2, gently vortex the mix, and briefly centrifuge.

Table 2-2 Multiple Displacement Amplification Mix

Component	1X (μL)
KMB	10
cDNA Product (100ng)	X
Nuclease-free water	35-X
Total	45

cDNA Input: X (μL) = 100 ng/Concentration of cDNA (ng/μL)

c. Program a PCR Thermal Cycler according to Table 2-3. When the module starts to heat up, place the reaction tube into the PCR Thermal Cycler and run the following protocol.

Table 2-3 Reaction Program

Temperature	Time
(Heated lid) 105 °C	on
95°C	5 min
40°C	3 min
4°C	∞

d. After the reaction program has finished, take out the reaction tube. Centrifuge the tube for **5 sec** in a mini centrifuge, and then add **5 μL** of KME.

e. Vortex and briefly spin down the reaction mix prepared above. Incubate the mix in a PCR Thermal Cycler using the following incubation protocol (Table 2-4).

Table 2-4 Multiple Displacement Amplification Reaction Program

Temperature	Time
(Heated lid) 105 °C	on
37°C	10 min
12°C	∞

f. For ssDNA purification:

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

1) Mix the PCR product obtained above with the magnetic beads in a volume ratio of **1:0.8 (PCR product: Beads = 50 μL : 40 μL)** in a PCR tube.

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

3) Spin down and place the tube on a magnetic separation rack for **3-5 min** until the liquid is clear. Carefully discard the supernatant with a pipette.

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

5) Repeat **step 4)**.

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

7) Mix the dried beads with **27 μL** of TE Buffer, vortex to mix, and incubate at room temperature for **5 min**. Spin down briefly and place the tube on the magnetic separation rack for **3-5 min** until the liquid is clear. Transfer the supernatant to a new 1.5 mL tube.

Keep the supernatant.

8) Take **2 μL** of purified PCR product and measure the concentration using Qubit ssDNA HS Kit.

Normally, the concentration of ssDNA is greater than 5 ng/μL.

Refer to Appendix II: Instructions for Using Qubit to Quantify the ssDNA for instructions on using Qubit ssDNA Assay Kit to quantify the purified PCR product.

Stop Point: Purified products can be stored at -20°C for 1 month.

g. Take 25 μL of purified ssDNA product and prepare PCR Library Mix according to Table 2-5 to perform amplification.

Table 2-5 PCR Library Mix

Component	1X (μL)
Purified ssDNA Product	25
PCR Amplification Mix	50
PCR Barcode Primer Mix	25
Total	100

Refer to Appendix I: PCR Barcode Primer Mix Use Rules for instructions on using PCR Barcode Primer Mix.

h. Vortex and briefly spin down the reaction mix prepared above. Incubate it in a PCR Thermal Cycler using the following incubation protocol (Table 2-6) and start the program.

Table 2-6 PCR Amplification Program (for 100 μL)

Temperature	Time	Number of Cycles
(Heated lid) 105 °C	on	-
95°C	5 min	1
98°C	20 sec	
58°C	20 sec	13
72°C	30 sec	
72°C	5 min	1
12°C	∞	-

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

Normally, the concentration of the PCR product is greater than 5 ng/μL.

2.1.3. PCR Product Size Selection

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

b. Briefly spin down the reaction mix and place the tube on a magnetic separation rack for **3 min** until the reaction mix is clear. Then, carefully transfer the supernatant to a new PCR tube.

Keep the supernatant and discard the beads.

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

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

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

f. Repeat **step e**.

g. Spin down the tube and place it on the magnetic separation rack. Use a smaller pipette tip to remove and discard the remaining liquid.

h. Air-dry the beads for **3-5 min** until the bead surface is not reflective or cracked.

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

Keep the supernatant.

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

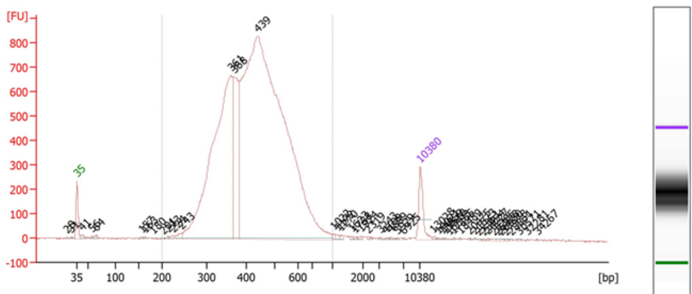
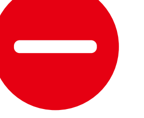


Figure 1 Agilent 2100 Bioanalyzer fragment size distribution of the purified transcriptome library



Stop Point: Purified products can be stored at -20°C for 1 month.

2.2. Stereo-CITE ADT Library Preparation

2.2.1. Experiment Preparation

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

Table 2-7 Experiment Preparation Steps

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

2.2.2. ADT Product PCR Amplification

a. Prepare 20 ng ADT product for the following reaction.

b. Prepare PCR Library Mix according to Table 2-8. Vortex and briefly spin down the reaction mix.

Table 2-8 ADT Product PCR Library Mix

Component	1X (μL)
ADT Product (20 ng)	X
Nuclease-free Water	25-X
PCR Barcode Primer Mix	25
PCR Amplification Mix	50
Total	100

Different sample barcodes are required when sequencing ADT libraries and transcriptome libraries together.

Refer to Appendix I: PCR Barcode Primer Mix Use Rules for instructions on using PCR Barcode Primer Mix.

c. Incubate the ADT product in a PCR Thermal Cycler using the following incubation protocol (Table 2-9).

Table 2-9 ADT PCR Program

Temperature	Time	Number of Cycles
(Heated lid) 105 °C	on	-
95°C	5 min	1
98°C	20 sec	8
58°C	20 sec	
72°C	1 min	
72°C	5 min	1
12°C	∞	-

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

Normally, the concentration of the ADT PCR product is greater than 10 ng/μL.

2.2.3. ADT PCR Product Purification

a. Mix the PCR product obtained above with the magnetic beads in a volume ratio of **1:2 (PCR product: Beads = 100 μL : 200 μL)** in a new 1.5 mL tube. Vortex the mixture, then incubate it at room temperature for **10 min**.

b. Briefly spin down the reaction mix and place the tube on a magnetic separation rack for **3-5 min** until the reaction mix is clear. Then discard the supernantant.

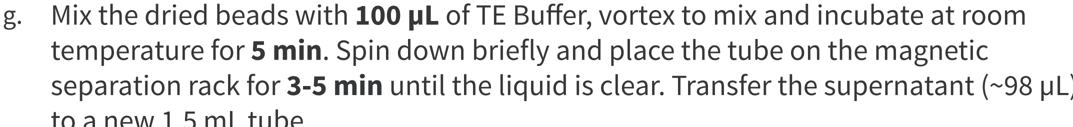
Keep the beads and discard the supernantant.

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

d. Repeat **step c**.

e. Spin down the tube and place it on the magnetic separation rack. Use a smaller pipette tip to remove and discard the remaining liquid.

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



g. Mix the dried beads with **100 μL** of TE Buffer, vortex to mix and incubate at room temperature for **5 min**. Spin down briefly and place the tube on the magnetic separation rack for **3-5 min** until the liquid is clear. Transfer the supernatant (~98 μL) to a new 1.5 mL tube.

Keep the supernatant.

h. Take **1 μL** of purified PCR product and measure the concentration using Qubit dsDNA HS Kit. Use Bioanalyzer, Tapestation (Agilent Technologies), LabChip® GX, GXII, GX Touch (PerkinElmer), Fragment Analyzer™ (Advanced Analytical), or other equipment based on the principle of electrophoretic separation to detect the fragment distribution of the purified PCR products.

The main peak in the fragment distribution must be 200-260 bp (Figure 2). Normally, the PCR yield is greater than 100 ng.

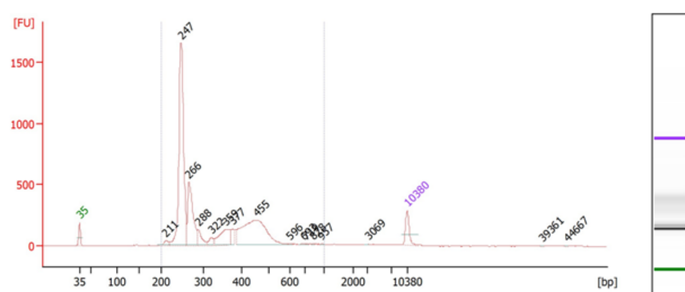
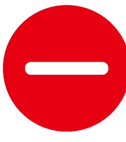


Figure 2 Agilent 2100 Bioanalyzer fragment size distribution of the purified ADT library



Stop Point: Purified products can be stored at -20°C for 1 month.

CHAPTER 3

LIBRARY STRUCTURE AND SEQUENCING OF Stereo-CITE PROTEO-TRANSCRIPTOME LIBRARY

The Stereo-CITE transcriptome and ADT library can be sequenced together or separately. This chapter introduces the sequencing instruments, reagents, and strategies for the Stereo-CITE transcriptome and ADT libraries.

3.1. Stereo-CITE Transcriptome and ADT Library Pooling

When sequencing on **DNBSEQ-T7RS**:

- Pooling both Stereo-CITE transcriptome and ADT libraries on the same flowcell is recommended.
- It is recommended that you measure the DNB concentration after preparing DNB, and calculate the DNB pooling volume for each sample based on the required data volume and the DNB concentration. Refer to **DNBSEQ-T7RS Stereo-seq Visualization Reagent Set User Manual, 940-001895-00 (MGI)/940-001889-00 (CG)** for more information.
- For base balancing, it is recommended that you mix the transcriptome and ADT libraries with different sample barcodes at an input mass ratio of at least 1:1.

3.1.1. Sequencing Strategy for Stereo-CITE Transcriptome and ADT Libraries

Use the following parameters to perform the sequencing run (Figure 3 and Figure 4).

Choose paired-ended mode with 50 cycles of Read 1 and 100 cycles of Read 2 and an additional 10 cycles of sample barcode. **Use dark cycles on Read 1 from cycle 26 to 40**.

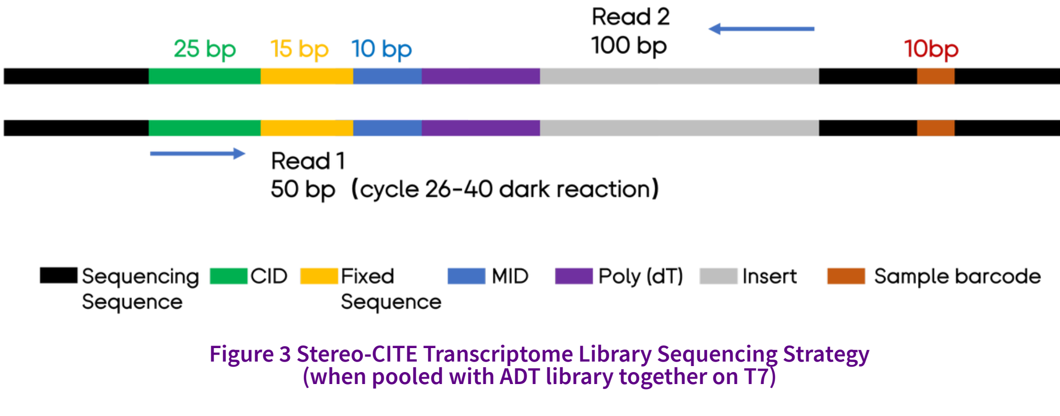


Figure 3 Stereo-CITE Transcriptome Library Sequencing Strategy (when pooled with ADT library together on T7)

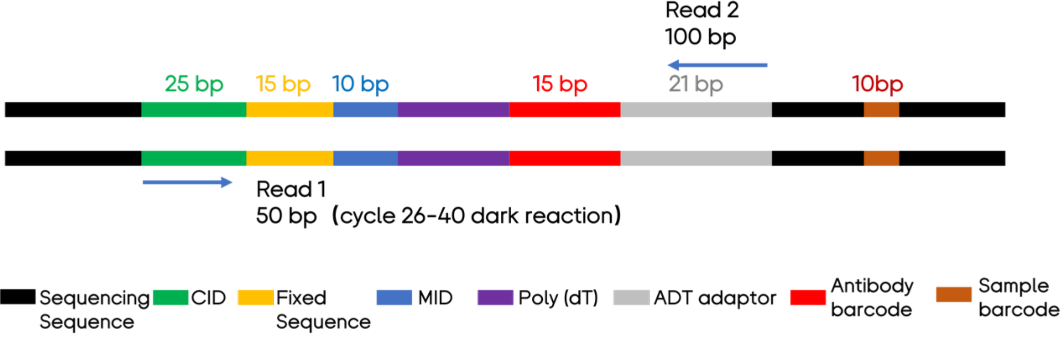


Figure 4 Stereo-CITE ADT Library Sequencing Strategy (when pooled with cDNA library together on T7)

Necessary input parameters for Stereo-CITE proteo-transcriptome libraries when pooled together for the Stereo-seq Analysis Workflow (SAW) bioinformatics pipelines are:

--kit-version="Stereo-CITE T FF V1.1"

--sequencing-type="PE75_50+100"

For DNB preparation instructions, refer to **the DNBSEQ-T7RS Stereo-seq Visualization Reagent Set User Manual, 940-001895-00 (MGI)/940-001889-00 (CG)**.

Transcriptome library recommends **400 fmol** input, and ADT recommends **400 fmol** input to make DNB. If you have any questions about sequencing, contact your local technical support service for DNBSEQ platforms.

3.2. Stereo-CITE Transcriptome and ADT Library Sequencing

When sequencing on **DNBSEQ-G400**:

It is recommended that you sequence Stereo-CITE transcriptome and ADT libraries separately on different flowcells.

Pooling both Stereo-CITE transcriptome and ADT libraries on the same flowcell is **not** recommended.

3.2.1. Sequencing Strategy for Stereo-CITE Transcriptome and ADT Libraries

- cDNA library (Figure 5):

Choose paired-ended mode with 50 cycles of Read 1 and 100 cycles of Read 2 and an additional 10 cycles of sample barcode (if multiple samples are pooled). **Use dark cycles on Read 1 from cycle 26 to 40**.

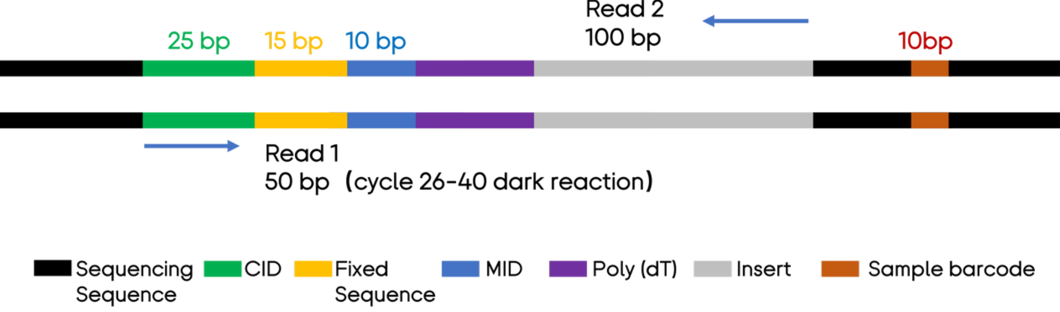


Figure 5 Stereo-CITE Transcriptome Library Sequencing Strategy (when sequenced individually on G400)

- Protein ADT library (Figure 6):

Choose paired-ended mode with 50 cycles of Read 1 and 36 cycles of Read 2 and an additional 10 cycles of sample barcode (if multiple samples are pooled).

Use dark cycles on Read 1 from cycle 26 to 40 and on Read 2 from cycle 1 to 21.

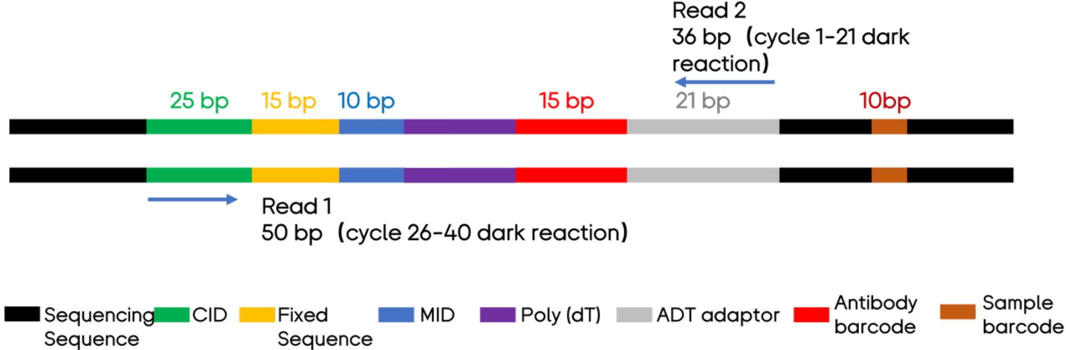


Figure 6 Stereo-CITE ADT Library Sequencing Strategy (when sequenced individually on G400)

Necessary input parameters for Stereo-CITE proteo-transcriptome libraries when sequenced separately for the Stereo-seq Analysis Workflow (SAW) bioinformatics pipelines are:

--kit-version="Stereo-CITE T FF V1.1"

--sequencing-type="PE75_50+100", "PE75_50+36"

For DNB preparation instructions, refer to **the DNBSEQ-G400RS Stereo-seq Visualization Reagent Set User Manual, 940-001886-00 (MGI)/940-001885-00 (CG)**. If you have any questions about sequencing, contact your local technical support service for DNBSEQ platforms.

Appendix I: PCR Barcode Primer Mix Usage Rules

Stereo-seq 16 Barcode Library Preparation Kit V1.0 provides 16 types of PCR Barcode Primer Mix, which are designed to meet the needs of high-throughput library construction for a large number of samples and multiplex sequencing of multiple samples. This kit is based on the design principle of base balancing and has been tested in repeated experiments. To ensure the best results, read the following usage rules carefully.

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

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

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

Be sure to centrifuge the PCR Barcode Primer Mix thoroughly before use. Gently open the lid during use to prevent the liquid from splashing and to prevent cross-contamination. While using the PCR Barcode Primer Mix, pipette to mix well, and briefly centrifuge..

N types of PCR Barcode Primer Mix method: Take the same volume of each, mix them, and then add the mixtures to the sample.

When the data amount requirements for each sample are the same

For different sample sizes, refer to the recommended barcode combinations in Table A-1 below.

Table A-1

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

Sample/Lane	Method 1	Method 2	Method 3	Method 4
6	Sample 1: 1 Sample 2: 2 Sample 3: 3 Sample 4: 4 Sample 5 and 6: select any two groups from the remaining three groups	Sample 1: 5 Sample 2: 6 Sample 3: 7 Sample 4: 8 Sample 5 and 6: select any two groups from the remaining three groups	Sample 1: 9 Sample 2: 10 Sample 3: 11 Sample 4: 12 Sample 5 and 6: select any two groups from the remaining three groups	Sample 1: 13 Sample 2: 14 Sample 3: 15 Sample 4: 16 Sample 5 and 6: select any two groups from the remaining three groups
7	Sample 1: 1 Sample 2: 2 Sample 3: 3 Sample 4: 4 Sample 5 and 7: select groups by referring the methods used for 3 samples/lane	Sample 1: 5 Sample 2: 6 Sample 3: 7 Sample 4: 8 Sample 5 and 7: select groups by referring the methods used for 3 samples/lane	Sample 1: 9 Sample 2: 10 Sample 3: 11 Sample 4: 12 Sample 5 and 7: select groups by referring the methods used for 3 samples/lane	Sample 1: 13 Sample 2: 14 Sample 3: 15 Sample 4: 16 Sample 5 and 7: select groups by referring the methods used for 3 samples/lane
8	Select any two groups from the four groups.			
N= 9~16 **(N, number of mixed samples)**	Perform the following steps: 1. Classify samples 1 to 8 as a group and add PCR Barcode Primer Mix by referring to the methods used for 8 samples/lane 2. Classify the remaining samples as a group, based on the number of the remaining samples (N-8), and follow the described methods above to add sample PCR Barcode Primer Mix accordingly. Do not use the same barcode mix for different samples.			

The following are examples of the mixture with different PCR Barcode Primer Mixes.

Example 1: 2 samples/lane (refer to Method 1 in Table A-1)

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

Example 2: 13 samples/lane

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

When the library data amount requirements for each sample are different

Libraries that require more than 20% data in a lane must use grouped PCR Barcode Primer Mixes.

For example:

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

Appendix II: Instructions for Using Qubit to Quantify the ssDNA

Working solution should be used within 30 min following preparation.

Do not touch the walls of the tapered detection tubes.

Ensure that there are no air bubbles in the detection tubes.

Perform the following steps:

a. Prepare the Qubit working solution:

1) Mix Qubit ssDNA Buffer with Qubit ssDNA Reagent in a 199:1 ratio in a dark tube.

2) Vortex and briefly spin down the mixture prepared above, then set it aside at room temperature.

- To quantify ssDNA, Qubit Working solutions (200 μL/sample) are required.
- To establish a standard curve, two Qubit working solutions (200 μL) are required.

b. Prepare (2+N) Qubit test tubes and label them as follows: S1 (Qubit ssDNA standard#1 0 ng/μL), S2 (Qubit ssDNA standard#2 20 ng/μL), D1 (sample ssDNA), D2 (sample ssDNA), D3 (sample ssDNA), and so on.

c. Prepare the standard tubes and the sample tubes to be tested according to the table below.

Temperatur	S1 (μL)	S2 (μL)	D1 (μL)	D2 (μL)	D3 (μL)
Working Solution	190	190	198	198	198
S1 (0ng/μL)	10	/	/	/	/
S2 (20ng/μL)	/	10	/	/	/
Sample ssDNA	/	/	2	2	2
Total	200	200	200	200	299

d. Vortex and mix the prepared sample tube and the standard liquid tube, centrifuge for 5 sec, and incubate for 2 min in the dark.

e. According to the instructions in the Qubit ssDNA HS Kit user manual, select the ssDNA detection channel, use the standard solution S1 and the standard solution S2 respectively to establish the standard curve, and then after the curve is generated, use S2 as a sample for measurement. The sample input amount is 10 μL, and the unit of measure is ng/μL.

  - When the measured value is 19.9-20 ng/μL, the standard curve is acceptable and can be used to test the sample. Otherwise, mix it again and re-establish the standard curve.
  - The standard curve can only be used if S2 is within the acceptable range; otherwise, reformulate the standard.

f. Place it in the sample tube to be measured, select 2 μL and the unit of measure is ng/μL for sample input, measure it, and then record the measured value.