STOmics

Stereo-seq TRANSCRIPTOME (FRESH FROZEN) 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-LP002

REVISION HISTORY

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

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.

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

CHAPTER 1
INTRODUCTION

1.1. Intended Use

Stereo-seq Transcriptome (Fresh Frozen, FF) 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 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 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-seq Transcriptome FF Library**.

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 16 Barcode Library Preparation Kit consists of:

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 Mix1	1000043201	●	25 μL × 1
PCR Barcode Primer Mix2	1000043202	●	25 μL × 1
PCR Barcode Primer Mix3	1000043203	●	25 μL × 1
PCR Barcode Primer Mix4	1000043204	●	25 μL × 1
PCR Barcode Primer Mix5	1000043205	●	25 μL × 1
PCR Barcode Primer Mix6	1000043206	●	25 μL × 1
PCR Barcode Primer Mix7	1000043207	●	25 μL × 1
PCR Barcode Primer Mix8	1000043208	●	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 Mix9	1000043209	●	25 μL × 1
PCR Barcode Primer Mix10	1000043210	●	25 μL × 1
PCR Barcode Primer Mix11	1000043211	●	25 μL × 1
PCR Barcode Primer Mix12	1000043212	●	25 μL × 1
PCR Barcode Primer Mix13	1000043213	●	25 μL × 1
PCR Barcode Primer Mix14	1000043214	●	25 μL × 1
PCR Barcode Primer Mix15	1000043215	●	25 μL × 1
PCR Barcode Primer Mix16	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 A: PCR Barcode Primer Mix Use Rules for information on barcode combinations.

1.4. Additional Equipment and Materials

The table below 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.**
Bio-Rad*	T100 Thermal Cycler	1861096
Thermo Fisher Scientific *	ProFlex 3 x 32-well PCR System	4483636
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)
-	Vortex mixer	-
Agilent Technologies™	Agilent 2100 Bioanalyzer	G2939AA (or similar)

Reagents		
Brand	**Description**	**Cat. No.**
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

Consumables		
Brand	**Description**	**Cat. No.**
Axygen	1.5 mL Centrifuge Tubes	MCT-150-A
	0.2 mL PCR Tubes*	PCR-02-C
	96-well PCR Plate*	PCR-96M2-HS-C
	1,000 µL Filtered Tips	TF-1000-L-R-S
	200 µL Filtered Tips	TF-200-L-R-S
	100 µL Filtered Tips	TF-100-R-S
	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 *).**

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

CHAPTER 2
LIBRARY PREPARATION FOR FF SAMPLES

2.1. 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	Take it out of -20°C, and thaw on ice prior to use.	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

2.2 ccDNA Multiple Displacement Amplification and ssDNA Purification

a. Use 100ng cDNA sample for the following 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 Amplification Reaction Mix

Components	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.

Table 2-3 Reaction program (100μL)

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. Gently vortex the reaction mix and centrifuge it in the mini centrifuge for 5 sec. 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. Use the magnetic beads to purify the MDA (Multiple Displacement Amplification) product (in a volume ratio of 1:1 (DNA:Beads) as follows:

1) Mix the MDA product (100 μL) with the magnetic beads in a ratio of 1:1.

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

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

4) Keep the tube on the magnetic rack and add **200 μL** of freshly prepared 80% ethanol that has been equilibrated to room temperature. Wash the beads by rotating the tube on the magnetic rack. Incubate for **30 sec** and then carefully remove and discard the supernatant with a pipette.

Always position the pipette tips on the tube wall 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).

5) Repeat **step 4**.

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

7) Add 5**0 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 (**~ 50 μL**) into a new PCR tube.

g. Take **2 μL** of purified PCR product and measure the concentration using the **Qubit ssDNA Assay Kit**.

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

Refer to Appendix B for instructions on using Qubit ssDNA Assay Kit to quantify the purified PCR product. This step is crucial for obtaining optimal experiment results.

Stop Point: The purified cDNA product can be stored at -20°C for up to 1 month.

h. Amplification of the purified product: Take 100 ng of the purified product. If the amount of purified product is less than 100 ng, add up to a maximum volume of 25 μL. Prepare the PCR Mix according to Table 2-5.

Table 2-5 PCR Mix

Component	1X (μL)
cDNA Product	X
PCR Amplification Mix	50
PCR Barcode Primer Mix	25
Nuclease-free water	25-X
Total	**100**

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

i. 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 then start the program.

Table 2-6 Reaction program (100μL)

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	30 sec	
72°C	5 min	1
12°C	∞	-

j. Take 1 μL of the PCR product and use the Qubit dsDNA HS Assay Kit to measure the concentration. Normally, the concentration of the PCR product is greater than 5 ng/μL.

2.3 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.

1) Vortex the mixture then incubate it at room temperature for **5 min**.

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

Keep the supernatant and discard the beads.

3) Add **15 μL** of beads to the new PCR tube with the supernatant from step 2). Mix thoroughly by vortexing. Incubate at room temperature for **5 min**.

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

5) Keep the tube on the magnetic 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 then carefully remove the supernatant with a pipette.

6) Repeat **step 5**.

7) Spin down and place the tube on the magnetic rack and allow the beads to collect on the tube wall. Use a smaller pipette tip to remove the remaining liquid and discard it.

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

9) Mix the dried beads with **20 μL** of TE Buffer by vortexing, and incubate at room temperature for **5 min**. Spin down briefly and place the centrifuge tube onto a magnetic separation rack for **3 min** until the liquid is clear. Transfer the supernatant (**~20 μL**) to a new **1.5 mL** tube.

Keep the supernatant.

b. Take 1 μL of purified PCR product and measure the concentration using the Qubit dsDNA HS Kit. Use Bioanalyzer, Tapestation (Agilent Technologies), LabChip® GX, GXII, GX Touch (PerkinElmer), Fragment Analyzer™ (Advanced Analytical), or other equipment based on the principle of electrophoretic separation to detect the fragment distribution of the purified PCR products. The main peak of fragment distribution must be 200-600 bp (Figure 1). Normally, the PCR yield is greater than 60 ng.

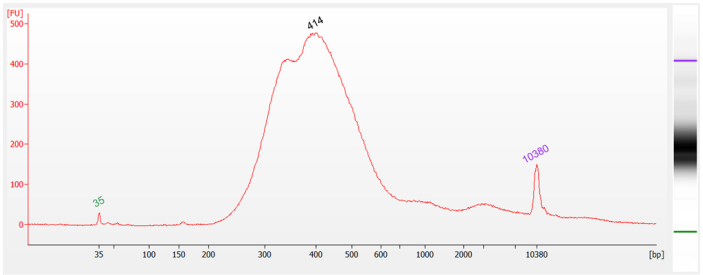


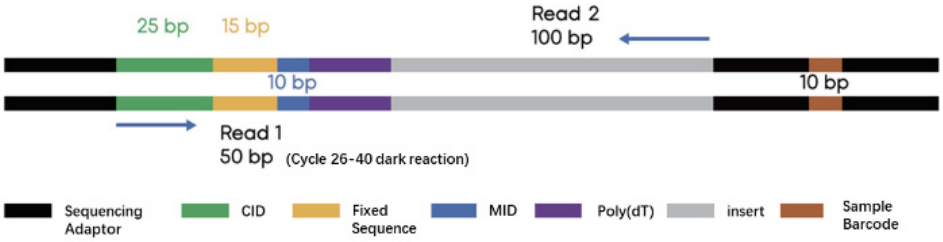
Figure 1. Agilent 2100 Bioanalyzer fragment size distribution of the FF library

Stop Point: The PCR product can be stored at -20°C for long-term storage.

CHAPTER 3

LIBRARY STRUCTURE AND SEQUENCING OF Stereo-seq TRANSCRIPTOME FF LIBRARY

The Stereo-seq Transcriptome FF Library structure is shown in Figure 2.



For DNB preparation, refer to the **DNBSEQ-G400RS Stereo-seq Visualization Reagent Set User Manual, 940-001886-00 (MGI)/940-001885-00 (CG)** or the **DNBSEQ-T7RS Stereo-seq Visualization Reagent Set User Manual, 940-001895-00 (MGI)/940-001889-00 (CG)**. Recommended library input is **60 ng**.

Read the corresponding user manual carefully before performing sequencing and strictly follow the instructions. If you have any questions about sequencing, contact your local Technical Support representative for DNBSEQ platforms.

Use the following parameters to perform the sequencing run:

• Without sample barcode sequenced (for only one sample): choose paired-ended mode with 50 cycles of Read 1 and 100 cycles of Read 2. Use dark cycles on Read 1 from 26 to 40 cycles.

• With sample barcode sequenced (for two or more samples): choose paired-ended mode with 50 cycles of Read 1 and 100 cycles of Read 2 and an additional 10 cycles of sample barcode. Use dark cycles on Read 1 from 26 to 40 cycles.

Appendix A: PCR Barcode Primer Mix Use Rules

The Stereo-seq 16 Barcode Library Preparation Kit V1.0 provides 16 types of PCR Barcode Primer Mix, 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 shown in the table (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 to 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 to 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 to 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 to the methods used for 3 samples/lane
8	Select any two groups from the four groups.			
N= 9~16 **(N, number of mixed samples)**	Perform the following steps: 1. Classify samples 1 to 8 as a group, and add the PCR Barcode Primer Mix by referring to the methods used for 8 samples/lane. 2. Classify the remaining samples as a group, and correspondingly add different groups of PCR Barcode Primer Mix based on a value of (N-8) by referring to the methods used for 1 to 8 samples/lane.			

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

Example 1: 2 samples /lane (refer to Method 1 in the above table)

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 of each of the PCR Barcode Primer Mixes 13, 14, 15, and 16, mix them in equal volumes and 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 are required to 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 B: Instructions for using Qubit to quantify the ssDNA

Working solution should be used within 30 min following preparation.

Avoid touching the wall of tapered detection tubes.

Ensure that there are no bubbles in detection tubes.

Perform the following steps:

a. Prepare the Qubit working solution by diluting the Qubit ssDNA Reagent 1:200 in Qubit ssDNA Buffer. Use a clean plastic tube each time you prepare Qubit working solution. Do not mix the working solution in a glass container.

Each standard tube requires 190 µL of Qubit working solution, and each sample tube requires 180 - 199 µL of Qubit working solution.

b. Prepare sufficient Qubit working solution to accommodate all standards and samples. For example, for 8 samples, prepare enough working solution for the samples and 2 standards: ~ 200 µL per tube in 10 tubes yields 2 mL of working solution (10 µL of Qubit reagent plus 1990 µL of Qubit Buffer).

c. Add 190 µL of Qubit working solution to each tube used for standards.

d. Add 10 µL of each Qubit standard to the appropriate tube, then mix by vortexing for 3 to 5 sec. Be careful not to introduce bubbles.

e. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit ssDNA Assay requires 2 standards.

- Use only thin-wall, clear, 0.5-mL PCR tubes. Acceptable tubes include either Qubit assay tubes (Cat. No.: Q32856) or Axygen PCR-05-C tubes (Cat No.: 10011-830).
- The number of Qubit test tubes needed are the number of samples plus 2 standard tubes. For example, if you have 3 samples, you will need 5 tubes.

f. Label the tube lids. Do not label the side of the tube.

g. Prepare the solutions used for standards and sample tests according to the table below.

	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(μL)	/	/	2	2	2
Total volume	200	200	200	200	200

h. Mix the tubes by using a vortex mixer, centrifuge briefly for 5 sec, then incubate at room temperature for 2 min.

i. Refer to the Qubit user manual for instructions on reading standards and samples. Follow the appropriate procedure for your instrument.