STOmics

Stereo-seq TRANSCRIPTOMICS FRESH FROZEN LIBRARY PREPARATION

USER MANUAL

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

Cat. No.: 111KL160 (16 RXNs) / 111KL160-CG (16 RXNs)
Kit Version: V1.0
Manual Version: B

STUM-LP002

REVISION HISTORY

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

Manual Version: A_1
Kit Version: V1.0
Date: Dec. 2024
Description:
- Modification of the ssDNA beads purification ratio and elution volume in chapter 2.2.
- Modification of the purified product input in chapter 2.2.
- Modification of the make DNB library input in chapter 3.

Manual Version: B
Kit Version: V1.0
Date: May. 2025
Description:
- Deleted the make DNB library input recommendation in this manual.
- Adjust the PCR reaction system to 50μL in chapter 2.2.
- Add a secondary PCR reaction step for the double size selection in chapter 2.4.
- Add Precautions and Warnings in chapter 1.5.

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

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 / 111KL160-CG			
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 / 111KL160-CG			
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 ~ -15°C	Transportation Temperature: -25°C ~ -15°C	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

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	-
-	Benchtop Centrifuge	-

Equipment		
Agilent Technologies™	Agilent 2100 Bioanalyzer	G2939AA (or similar)

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

Reagents		
Brand	**Description**	**Cat. No.**
-	100% Ethanol (Analytical grade)	-
Ambion	Nuclease-free Water	AM9937
	1X TE Buffer, pH 8.0	AM9858
*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

* 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 *).

^ Choose either one of the listed brands (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.
- 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 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 cDNA Multiple Displacement Amplification and ssDNA Purification

a. Use **100 ng** 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.

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 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	∞

Once the PCR thermal cycler has reached 12°C, immediately take out the reaction tube and proceed to the next step.

f. Use the magnetic beads to purify the MDA (Multiple Displacement Amplification) product in a volume ratio of 1:0.8 (DNA:Beads) as follows:

1) Mix the MDA product (**50 μL**) 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 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.

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 usually take approximately **5-8 min**.

7) Add **15 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 (**~ 15 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 20 ng/μL.

Refer to Appendix II 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 product can be stored at -20°C for up to 1 month.

h. Amplification of the purified product: Take **12.5 μL** of the purified product. Prepare the PCR Mix according to Table 2-5.

If using a mixed PCR Barcode Primer Mix, prepare at least 25 μL in advance. Note that only 12.5 μL is required for each amplification reaction.

Table 2-5 PCR Mix

Component	1X (μL)
Purified Product	12.5
PCR Amplification Mix	25
PCR Barcode Primer Mix	12.5
Total	**50**

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

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 (for 50μL)

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 = 50 μL:27.5 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 **7.5 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 **15 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 (**~15 μL**) to a new **1.5 mL** tube.

b. Take 1 μL of purified PCR product and measure the concentration using the Qubit dsDNA HS Kit.

2.4 Secondary Amplification of Selection products

a. Secondary Amplification: Take **100 ng** of the selection PCR product and prepare the PCR Mix according to Table 2-7. If the selection product yield is less than 100 ng, add up to a maximum volume of 12.5 μL.

For the same sample, Table 2-5 and Table 2-7 should use the same PCR barcode primer mix.

For mixed PCR Barcode Primer Mix, use the remaining PCR Barcode Primer Mix prepared in step f. of section 2.2 cDNA Multiple Displacement Amplification and ssDNA Purification.

Table 2-7 PCR Mix

Component	1X (μL)
Selection Product (100ng)	Y
PCR Amplification Mix	25
PCR Barcode Primer Mix	12.5
Nuclease-free Water	12.5 - Y
Total	**50**

Selection Product Input: Y (μL) = 100 ng/Concentration of the selection product (ng/μL)

b. Vortex and briefly spin down the reaction mix prepared above. Incubate it in a PCR thermal cycler using the following incubation protocol (Table 2-8), and then start the program.

If the selection product is less than 50 ng, it is recommended to increase the number of PCR cycles to 8.

Table 2-8 PCR Amplification Program (for 50 μL)

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

c. 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.

d. Use the magnetic beads to purify the PCR product in a volume ratio of 1:0.8 (DNA:Beads) as follows:

1) Mix the PCR product (**50 μL**) 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 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.

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 usually take approximately **5-8 min**.

7) Add **50 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.

e. 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 200 ng.

If the purified PCR products yield is less than 200ng, contact your local Field Application Scientist for troubleshooting.

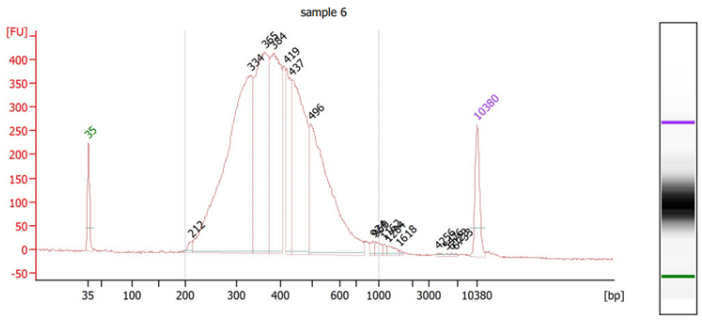


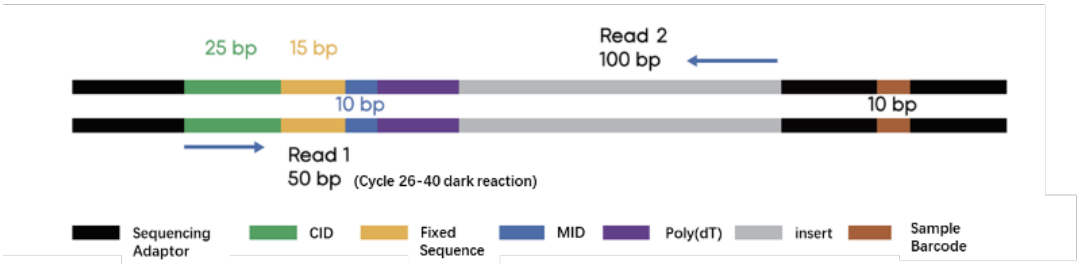
Figure 1. Agilent 2100 Bioanalyzer fragment size distribution of the mouse eyeball library

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)**.

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 I: 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 read counts for each sample are equal

For different sample sizes, refer to the recommended barcode combinations shown in the table (Table I-1) below:

Table I-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. Mix equal volumes of PCR Barcode Primer Mix 1 and PCR Barcode Primer Mix 2 (prepare at least 25 μL of the mixed PCR Barcode Primer Mix). Add the mixed primer to Sample 1 following the instructions.

2. Mix equal volumes of PCR Barcode Primer Mix 3 and PCR Barcode Primer Mix 4 (prepare at least 25 μL of the mixed PCR Barcode Primer Mix). Add the mixed primer to Sample 2 following the instructions.

Example 2: 13 samples/lane

1. Add PCR Barcode Primer Mix 1 to Sample 1, Mix 2 to Sample 2, ..., and Mix 12 to Sample 12, following the operation instructions.

2. Mix equal volumes of PCR Barcode Primer Mixes 13, 14, 15, and 16 to prepare at least 25 μL of the mixed primer. Add the mixed primer to Sample 13 following the instructions.

When the read counts for each sample are different

Libraries that require more than 20% read counts in a lane are required to use grouped PCR Barcode Primer Mixes.

For example:

Nine samples pooled into one sequencing lane

Reads Proportion:

Samples 1- 8: 8.75% reads each, 70% reads in total
Sample 9: 30% reads

Barcode Application:

Samples 1-8: Individual PCR Barcode Primer Mixes 1-8.
Sample 9: Grouped 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.

Avoid touching the wall of detection tubes.

Ensure that there are no bubbles in detection tubes.

This instruction is intended to provide reference information. For detailed operating procedures, consult the user manuals of your selected quantification instrument and reagent kit.

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. Avoid light before use.

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 requires 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.