DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V3.0(TaiM 4)

Instructions for Use

Version: 2.0

For Research Use Only.
Not for use in diagnostic procedures.

Qingdao MGI Tech Co., Ltd.

About the instructions for use

This instructions for use is applicable to DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V3.0 (hereinafter referred to as preparation set). The instructions for use version is 2.0.

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Figures in this instructions for use are for illustrative purpose only. The content may be slightly different from the preparation set. For the most up-to-date details, refer to the preparation set purchased.

Revision history

	Date	Version	Revision summary
Revision	December 25, 2024	2.0	• Added the requirements for nucleus samples. • Modified the procedures for preparing the sample phase suspension. • Added instructions for preparing the sample phase and beads phase suspensions, as well as for sample loading onto flow cells. • Added a figure for the aqueous phase after demulsification • Deleted descriptions related to the DNBSEQ-T7RS high-throughput sequencing kit V2.0.
Initial release	May 30, 2024	1.0	/

Tips

- Please download the latest user manual, and use it with the corresponding version of the preparation set.
- Download the user manual through search according to the catalog number or product name from the website:
  https://en.mgi-tech.com/download/files/type_id/1/p/4.

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Chapter 1 Product overview

This chapter describes basic information of the product, including the intended use, compatible sequencing platforms, and component information.

1.1 About the product

Based on the DNBelab C series single-cell library preparation and DNBSEQ sequencing technologies, combined with self-developed single-cell analysis software, the DNBelab C Series Single Cell Omics Solution can realize a one-stop single-cell omics research workflow.

Based on droplet microfluidic technology, the DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V3.0 (TaiM 4) can rapidly prepare single-cell (nucleus) suspensions into dedicated libraries applicable to MGI's DNBSEQ series sequencing platforms, by utilizing MGI independently designed DNBelab C-TaiM 4 Single Cell Droplet Generator, magnetic beads for mRNA capture, and droplets recognition microbeads. This product employs a droplet-based reverse transcription strategy, along with efficient mRNA capture magnetic beads and droplet-identifying microbeads, which can significantly increase the quantity of captured mRNA, and can decrease a contamination rate, and increase a gene detection capability of the single-cell RNA library. All reagents, flow cells, and consumables included in the preparation set have undergone strict quality control and function verification, to ensure single-cell RNA library preparation stability and reproducibility.

1.2 Intended use

This preparation set is applicable to preparation of high-throughput single-cell 3'RNA libraries for eukaryote. Before use, it is necessary to prepare single-cell (nucleus) suspensions from the samples.

⚠ **WARNING** This preparation set is for scientific research use only, and cannot be used for clinical diagnosis.

1.3 Compatible sequencing platform

Sequencing platform	DNBSEQ-G400RS
	DNBSEQ-T7RS
Sequencing recipe for the cDNA library	47 (Read1) + 100 (Read2) + 10
Sequencing recipe for the Oligo library	32 (Read1) + 42 (Read2) + 10

1.4 Component information

The reagent kit has two packaging specifications: 16RXN and 4RXN, both contain 5 modules. For detailed information, see the tables below:

Table 1 DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V3.0 (TaiM 4) (Cat. No.: 940-001818-00, 16RXN)

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 1 Droplet Generation) (Cat. No.: 940-001820-00)	Cell Beads-V3	Natural	560 μL/tube×2
	Index Carrier	Natural	280 μL/tube×2
	Lysis Buffer-V3	Natural	72 μL/tube×2
	Breakage Reagent	Brown	800 μL/tube×2
	P100 Oil	Natural	7.6 mL/bottle×2
	Cover Oil	Natural	3.2 mL/bottle×2
	DNA Clean Beads	Natural	8.352 mL/bottle×2
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 2 Droplet Generation) (Cat. No.: 940-001819-00)	Beads Buffer	Natural	728 μL /tube×2
	Cell Solution-V3	Natural	142 μL/tube×2
	RT Primer-V3	Natural	33 μL/tube×2
	DIR Regent-V3	Natural	13 μL/tube×2
	RT Enzyme-V3	Natural	64 μL/tube×2
	RNase Inhibitor	Natural	132 μL/tube×2
	cDNA Amp Enzyme	Natural	400 μL/tube×2
	cDNA Amp Primer-V3	Natural	33 μL/tube×2
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 3 Library Preparation) (Cat. No.: 940-001821-00)	Frag Enzyme-V3	Natural	80 μL/tube×2
	Frag Buffer-V3	Natural	40 μL/tube×2
	DNA Ligase-V3	Natural	80 μL/tube×2
	Ligation Buffer-V3	Natural	160 μL/tube×2
	scRNA Adapter-V3	Natural	40 μL/tube×2
	PCR Amp Enzyme	Natural	600 μL/tube×2
DNBelab C Series Flow Cell (TaiM 4) (Cat. No.: 940-001822-00)	Flow Cell	/	16 EA/box×1
	Sealing Gasket	/	5 EA/bag×1

Name	Component	Cap color	Specification
DNBelab C Series Single-cell Library Preparation Sample Barcode Kit S (Cat. No.: 940-001920-00)	Barcode Primer-1 to 8	Natural	8-strip tube×2
	Barcode Primer-9 to 16	Natural	8-strip tube×2
	Barcode Primer-17 to 24	Natural	8-strip tube×2
	Barcode Primer-25 to 32	Natural	8-strip tube×2

Table 2 DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V3.0 (TaiM 4) (Cat. No.: 940-001924-00, 4RXN)

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 1 Droplet Generation) (Cat. No.: 940-001927-00)	Cell Beads-V3	Natural	280 μL/tube×1
	Index Carrier	Natural	140 μL/tube×1
	Lysis Buffer-V3	Natural	36 μL/tube×1
	Breakage Reagent	Brown	400 μL/tube×1
	P100 Oil	Natural	3.8 mL/bottle×1
	Cover Oil	Natural	1.6 mL/bottle×1
	DNA Clean Beads	Natural	4.176 mL/bottle×1
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 2 Droplet Generation) (Cat. No.: 940-001929-00)	Beads Buffer	Natural	364 μL /tube×1
	Cell Solution-V3	Natural	71 μL/tube×1
	RT Primer-V3	Natural	17 μL/tube×1
	DIR Regent-V3	Natural	7 μL/tube×1
	RT Enzyme-V3	Natural	32 μL/tube×1
	RNase Inhibitor	Natural	66 μL/tube×1
	cDNA Amp Enzyme	Natural	200 μL/tube×1
	cDNA Amp Primer-V3	Natural	17 μL/tube×1
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 3 Library Preparation) (Cat. No.: 940-001925-00)	Frag Enzyme-V3	Natural	40 μL/tube×1
	Frag Buffer-V3	Natural	20 μL/tube×1
	DNA Ligase-V3	Natural	40 μL/tube×1
	Ligation Buffer-V3	Natural	80 μL/tube×1
	scRNA Adapter-V3	Natural	20 μL/tube×1
	PCR Amp Enzyme	Natural	300 μL/tube×1

Name	Component	Cap color	Specification
DNBelab C Series Flow Cell (TaiM 4) (Cat. No.: 940-001822-00)	Flow Cell	/	4 EA/box×1
	Sealing Gasket	/	2 EA/bag×1
DNBelab C Series Single-cell Library Preparation Sample Barcode Kit S (Cat. No.: 940-001926-00)	Barcode Primer-1 to 8	Natural	8-strip tube×1
	Barcode Primer-9 to 16	Natural	8-strip tube×1

1.5 Storage and transportation condition

Table 3 Transportation and storage condition

Name	Storage temperature	Transportation temperature	Validity period
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 1 Droplet Generation) (Cat. No.: 940-001820-00, 16RXN) (Cat. No.: 940-001927-00, 4RXN)	2 ℃ to 8 ℃ (36 ℉ to 46 ℉)	2 ℃ to 8 ℃ (36 ℉ to 46 ℉)	Refer to the label on the box.
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 2 Droplet Generation) (Cat. No.: 940-001819-00, 16RXN) (Cat. No.: 940-001929-00, 4RXN)	-25 ℃ to -15 ℃ (-13 ℉ to 5 ℉)	-80 ℃ to -15 ℃ (-112 ℉ to 5 ℉)	
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 3 Library Preparation) (Cat. No.: 940-001821-00, 16RXN) (Cat. No.: 940-001925-00, 4RXN)	-25 ℃ to -15 ℃ (-13 ℉ to 5 ℉)	-80 ℃ to -15 ℃ (-112 ℉ to 5 ℉)	
DNBelab C Series Flow Cell (TaiM 4) (Cat. No.: 940-001822-00, 16RXN) (Cat. No.: 940-001928-00, 4RXN)	10 ℃ to 30 ℃ (50 ℉ to 86 ℉)	0 ℃ to 30 ℃ (32 ℉ to 86 ℉)	
DNBelab C Series Single-cell Library Preparation Sample Barcode Kit S (Cat. No.: 940-001920-00, 16RXN) (Cat. No.: 940-001926-00, 4RXN)	-25 ℃ to -15 ℃ (-13 ℉ to 5 ℉)	-80 ℃ to -15 ℃ (-112 ℉ to 5 ℉)	

Tips

- When the product is transported by using dry ice, please check whether any dry ice remains upon receipt.
- When the product is transported, stored, and used appropriately, all of the components retain full activity within the validity period.

1.6 Self-provided materials

Table 4 Self-provided equipment

Name	Recommended brand	Cat. No.
DNBelab C-TaiM 4 Single Cell Droplet Generator	MGI	900-000637-00 (China) 900-000780-00 (CE) 900-000781-00 (EAC) 900-000782-00 (UKCA)
Clean bench	/	/
Microscope (For nucleus, counting should be perf ormed using a fluorescence microscope)	/	/
Electronic balance	/	/
Vortex mixer	/	/
Mini centrifuge	/	/
Manual single-channel pipette, with a measurement range as follows: • 0.1 μL to 2.5 μL • 0.5 μL to 10 μL • 2 μL to 20 μL • 10 μL to 100 μL • 20 μL to 200 μL • 100 μL to 1000 μL	/	/
Manual 8-channel pipette, with a measurement range as follows: • 1 μL to 10 μL • 2 μL to 10 μL • 5 μL to 50 μL • 20 μL to 200 μL	/	/
Deep-well PCR device (for 100 μL mixture, with heated lid)	/	/
Centrifuge or equivalents	Eppendorf	5810R
Magnetic separation rack for 1.5 mL tubes	Thermo Fisher	12321D
Magnetic separation rack for 0.2 mL tubes	New England Biolabs	S1515S
Qubit 3.0 fluorometer or equivalents	Thermo Fisher	Q33216

Name	Recommended brand	Cat. No.
Fragment analyzer	/	/

Table 5 Self-provided reagents

Name	Recommended brand	Cat. No.
DNA-OFF SOLUTION	TAKARA	9036
RNase Zap	AMBION	AM9782
75% ethanol	/	/
PBS, pH 7.4	Gibco	10010031
BSA (Bovine Serum Albumin)	Sangon Biotech	A600332-0005
Trypan Blue Solution, 0.4%, or equivalents	Gibco	15250061
DAPI (for nuclear staining)	Sigma-Aldrich	D9542
Nuclease-free water (NF Water)	Ambion	AM9937
TE buffer, pH 8.0	Ambion	AM9858
Absolute ethanol (analytical grade)	/	/
MGIEasy Circularization Kit	MGI	1000005259
HotMPS High-throughput Sequencing Set (G400 HM FCL PE100)	MGI	940-000489-00
HotMPS High-throughput Sequencing Set (G400 HM FCL PE150)	MGI	940-000244-00
DNBSEQ-G400RS High-throughput Sequencing Set (FCL PE100)	MGI	1000016950
DNBSEQ-G400RS High-throughput Sequencing Set (FCL PE150)	MGI	1000016952
DNBSEQ-T7RS High-throughput Sequencing Set (FCL PE100) V3.0	MGI	940-000269-00
DNBSEQ-T7RS High-throughput Sequencing Set (FCL PE150) V3.0	MGI	940-000268-00
Qubit ssDNA Assay Kit	Invitrogen	Q10212
Qubit dsDNA HS Assay Kit	Invitrogen	Q32854
Analytical reagents applicable to the fragment analyzer	/	/

Table 6 Self-provided consumables

Name	Recommended brand	Cat. No.
1 mL syringe	/	/
Cell strainer with appropriate specification	/	/
0.22 μm filter membrane	PALL	4612
C-chip disposable hemocytometer or universal hemocytometer	INCYTO	DHC-N01
Low-binding sterile filter tips, boxed, with a capacity of 10 μL, 20 μL, 100 μL, 200 μL, or 1000 μL	Axygen	/
Universal low-binding tips, with a capacity of 10 μL, 20 μL, 100 μL, 200 μL, or 1000 μL	Axygen	/
200 μL wide-bore tips	Axygen	T-205-WB-C
0.2 mL low-binding PCR tube	Axygen	PCR-02-L-C
1.5 mL low-binding centrifuge tube	Eppendorf	0030108051
0.2 mL PCR tube	Axygen	PCR-02-C
0.2 mL 8-strip tube	Axygen	PCR-0208-CP-C
1.5 mL centrifuge tube	Axygen	MCT-150-C
15 mL centrifuge tube	CORNING	430791
50 mL centrifuge tube	CORNING	430291
Qubit Assay tube, or 0.5 mL thin wall PCR tube	Invitrogen; Axygen	Q32856; PCR-05-C

1.7 Library preparation workflow

The entire library preparation workflow takes approximately 12 hours by using DNBelab C Series High-Throughput Single-Cell RNA Library Preparation Kit V3.0. The time required for each operation and the stopping points are shown in the table below:

Table 7 Overview of the library preparation workflow

Operation	Time	Stop point and storage
1. Sample processing	**~ 60 min**	/
Preparing cell (nucleus) suspension and counting cells	~ 60 min	/

Operation	Time	Stop point and storage
2. Droplet generation	**~ 30 min**	/
Pre-experiment preparation	~ 15 min	/
Performing droplet generation	~ 15 min	/
3. RT reaction in droplet	**~ 160 min**	/
Collecting droplets	~ 10 min	/
RT reaction	~ 150 min	Stop point The collected droplets can be stored at 2 ℃ to 8 ℃ (36 ℉ to 46 ℉) for up to 24 hours.
4.Performing demulsification and size selection of RT product	**~ 60 min**	/
Performing demulsification	~ 20 min	/
Performing size selection of RT product	~ 40 min	Stop point The “cDNA Intermediate Product” can be stored at -25 ℃ to -15 ℃ (-13 ℉ to 5 ℉) for up to one week. The“Oligo Product 2” can be stored at -25 ℃ to -15 ℃ (-13 ℉ to 5 ℉) for up to six months.
5. Amplifying and purifying cDNA Intermediate Products	**~ 140 min**	/
Amplifying cDNA Intermediate Products	~ 100 min	Stop point The cDNA amplification product can be stored at 2 ℃ to 8 ℃ (36 ℉ to 46 ℉) for up to 24 hours or at -25 ℃ to -15 ℃ (-13 ℉ to 5 ℉) for up to one week.
Purifying cDNA products	~ 20 min	Stop point The purification product can be stored at -25 ℃ to -15 ℃ (-13 ℉ to 5 ℉) for up to six months.
Performing cDNA product quantification and fragment QC	~ 20 min	/
6. Oligo library preparation	**~ 90 min**	/

Operation	Time	Stop point and storage
Preparing the Oligo library	~ 40 min	/
Performing size selection on Oligo library	~ 30 min	Stop point The Oligo library can be stored at -25 °C to -15 °C (-13 °F to 5 °F) for up to six months.
Oligo library quantification and fragment QC	~ 20 min	/
7. cDNA library preparation	**~ 195 min**	/
Performing fragmentation and end repair	~ 35 min	/
Performing adapter ligation	~ 20 min	/
Performing purification and size selection on adapter ligation product	~ 50 min	Stop point The purified adapter ligation product can be stored at -25 °C to -15 °C (-13 °F to 5 °F) for up to 24 hours.
Performing PCR amplification	~ 40 min	/
Performing size selection on PCR amplification product	~ 30 min	Stop point The cDNA library can be stored at -25 °C to -15 °C (-13 °F to 5 °F) for up to six months.
Performing cDNA library quantification and fragment QC	~ 20 min	/

1.8 Precautions

- This product is for research use only. Not for use in diagnostic procedures. Please read this user manual carefully before use.
- Before the experiment, be sure to be familiar with and master the operation methods and precautions of various reagents and devices to be used.
- Adjust and optimize library preparation procedures according to specific experiment design, sample characteristics, sequencing applications, and devices. The experiment procedures provided in this user manual are universal, and the reaction parameters can be adjusted as needed to achieve high performance and efficiency.

- Take out all components of the preparation set in advance, and briefly centrifuge the Enzymes and keep them on ice for further use. Thaw other components on ice, invert them up and down several times after thawing to thoroughly mix them, briefly centrifuge, and place them on ice for further use.
- To avoid an experiment failure caused by cross contamination, it is recommended to perform experiment operations such as sample processing, droplet generation, reverse transcription, demulsification, and cDNA amplification in a clean laboratory, use low-binding filter tips, and change tips for aspirating different samples.
- It is recommended to proceed with reaction steps in a PCR device with a heated lid mode. The PCR device should be preheated to a required reaction temperature before use.
- Avoid aerosol contamination caused by improper operations on PCR products, which may reduce the accuracy of experimental results. It is recommended to physically separate the PCR reaction solution preparation area from the PCR product purification and detection area. Use special pipettes or other devices, and regularly clean experimental areas (wipe by using 0.5% sodium hypochlorite or 10% bleach) to ensure the cleanliness of the experimental environment.
- Direct contact with skin and eyes should be avoided for all samples and reagents. Do not swallow. Once this happens, immediately rinse with a large amount of water and go to the hospital in time.
- All samples and wastes should be disposed of in accordance with relevant regulations.
- Any other questions, contact the technical support at *MGI-service@mgi-tech. com.*

Chapter 2 Sample requirements and processing

This chapter describes sample processing and requirements, including precautions and preparation before experiment, and preparation of reagents and cell suspensions.

2.1 Precautions before experiment

- It is recommended to operate single-cell experiments in a ISO Class 8 or 9 clean laboratory, or in a clean bench.
- Avoid exogenous nucleic acid contamination when performing single-cell RNA experiment in the clean bench.

- Wear protective equipment such as mask and gloves when performing experiments. During the operation, do not expose the skin of wrist. If the gloves touch the area outside the clean bench, carefully wipe the surface of the gloves with RNase-Zap before continuing the experiment.
- Place all samples on ice during the experiment.
- Consumables such as pipette tips, centrifuge tubes, and sterile water should be sterile, nucleic acid-free, and nuclease-free, and cannot be used for other purposes. Tips should be low-binding filter tips.

2.2 Pre-experiment preparation

2.2.1 Sample requirements

Table 8 Sample requirements

Cell (nucleus) size	Recommended diameter: < 60 μm
Recommended total cell (nucleus) input	A total of 5000 to 30000 cells (nucleus), with a sample load as follows: • Cell-line samples: 5000 to 30000 cells (nucleus) • PBMC and other tissue dissociation samples: 10000 to 30000 cells (nucleus)
Cell requirements	• Cytoactivity: > 80% • Clumping rate: < 5% • Impurity rate: < 5%
Nucleus requirements	• Cytoactivity: < 5% • Clumping rate: < 5% • Impurity rate: < 5%

Table 9 Recommended cell (nucleus) input

Target cell (nucleus) number	Recommended cell (nucleus) concentration (cells/μL)
5000	145 < N < 1000
10000	275 < N < 2000
20000	550 < N < 2000
30000	825 < N < 2000

Table 10 Reference for captured cell and multi-cell rate (taking human-mouse cell lines as an example)

Input cells	Captured cells	Multi-cell rate
5000	>3000	1.19%
10000	>6000	2.61%
20000	>12000	4.06%
30000	>18000	6.37%

Tips
- N represents the cell (nucleus) concentration.
- It is recommended to calculate the concentration of viable cells when you calculate cell concentration.

2.2.2 Experiment requirements

- Before experiment, carefully wipe the gloves, pipettes, bench, and devices with RNase-Zap. Pay special attention to wipe the pipettes and bench.
- If a clean bench is used, turn on the light of the clean bench in advance and perform the following steps:

  1) Wipe the device and operating deck of the clean bench with DNA-OFF, especially the metal and plastic surfaces.
  2) Wait for 10 minutes for degrading the DNA.
  3) Turn off the light, and turn on the UV lamp for sterilization for at least 15 minutes.
  4) Turn on the light and ventilator after sterilization.

2.2.3 Preparing reagents

Prepare the following reagents:

- PBS (containing 10% BSA)

Table 11 PBS (containing 10% BSA) preparation

Component	Volume
BSA powder	1 g
PBS (without Ca^{2+} and Mg^{2+})	Fixed volume to 10 mL

After the BSA powder fully dissolves, use a syringe and 0.22 μm filtermembrane to filter the solution.

Tips The PBS (containing 10% BSA) can be stored at -25 ℃ to -15 ℃ (-13 ℉ to 5 ℉)for up to 6 months.

- PBS (containing 0.04% BSA)

Table 12 PBS (containing 0.04% BSA) preparation

Component	Volume
PBS (without Ca^{2+} and Mg^{2+})	49.8 mL
PBS(containing 10% BSA)	200 μL

Add the components with the required volumes in proportion and mix them thoroughly.

Tips The PBS (containing 0.04% BSA) can be stored at 2 ℃ to 8 ℃ (36 ℉ to 46 ℉) for up to one month.

2.3 Preparing cell (nucleus) suspension

Perform the following steps:

1. Prepare single-cell (nucleus) suspension in an appropriate way, and wash the single-cell suspension twice with the PBS (containing 0.04% BSA).
2. Resuspend cells (nucleus) with an appropriate volume of the PBS (containing 0.04% BSA) to obtain cell suspension.
3. After filtering the cell suspension with the cell strainer with a appropriate specification of less than 60 μm, quantify the cell (nucleus) suspension, and record the concentration.

Tips
- It is recommended to mix the sample thoroughly by pipetting with a wide-bore tip.
- When cells are counted by using the C-chip disposable hemocytometer or the universal hemocytometer, a counting result must be correct, or it might affect a final yield. It is recommended to repeat the counting step at least 3 times.

Chapter 3 Droplet generation

This chapter describes how to generate droplets from the prepared cell (nucleus) suspension by using the DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V3.0 (TaiM 4). The whole procedure takes about 30 minutes.

3.1 Pre-experiment preparation

3.1.1 Preparing reagents and equipment

Table 13 Required reagent kit

Name	Component	Cap color	Specification	
			16RXN	4RXN
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 1 Droplet Generation) (Cat. No.: 940-001820-00, 16RXN) (Cat. No.: 940-001927-00, 4RXN)	Cell Beads-V3	Natural	560 μL/tube×2	280 μL/tube×1
	Index Carrier	Natural	280 μL/tube×2	140 μL/tube×1
	Lysis Buffer-V3	Natural	72 μL/tube×2	36 μL/tube×1
	P100 Oil	Natural	7.6 mL/bottle×2	3.8 mL/bottle×1
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 2 Droplet Generation) (Cat. No.: 940-001819-00, 16RXN) (Cat. No.: 940-001929-00, 4RXN)	Beads Buffer	Natural	728 μL/tube×2	364 μL/tube×1
	Cell Solution-V3	Natural	142 μL/tube×2	71 μL/tube×1
	RT Primer-V3	Natural	33 μL/tube×2	17 μL/tube×1
	DIR Reagent-V3	Natural	13 μL/tube×2	7 μL/tube×1
	RNase Inhibitor	Natural	132 μL/tube×2	66 μL/tube×1
	RT Enzyme-V3	Natural	64 μL/tube×2	32 μL/tube×1
DNBelab C Series Flow Cell (TaiM 4) (Cat. No.: 940-001822-00, 16RXN) (Cat. No.: 940-001928-00, 4RXN)	Flow Cell	/	16 EA/box×1	4 EA/box×1
	Sealing Gasket	/	5 EA/bag×1	2 EA/bag×1

Table 14 Required equipment

Name	Component	Cap color	Specification	
			16RXN	4RXN
DNBelab C-TaiM 4 Single Cell Droplet Generator Cat. No.: 900-000637-00 (China) 900-000780-00 (CE) 900-000781-00 (EAC) 900-000782-00 (UKCA)	/	/	1	

Tips
- Take out the P100 Oil for at least 30 minutes in advance to equilibrate to room temperature.
- Take out the Lysis Buffer-V3 in advance to equilibrate to room temperature until all crystals in the solution dissolves.
- Do not vortex to mix the following components: Cell Beads-V3, Index Carrier, RT Enzyme-V3, RNase Inhibitor, and the prepared cell phase suspension and beads phase suspension.
- It is recommended to use low-binding filter tips and low-binding centrifuge tubes for the experimental steps in this chapter.

3.1.2 Preparing sample phase suspension

Tips Appropriately increase the volume when preparing the sample phase suspension, to avoid a volume insufficiency (less than 80 µL) of the sample phase suspension in subsequent droplet generation.

Perform the following steps:

Gently pipette the cell (nucleus) suspension prepared in *"2.3 Preparing cell (nucleus) suspension" on page 14* by using a pipette to mix it thoroughly, and prepare the sample phase suspension according to the following table. After preparation, place it on ice for later use.

Table 15 Sample phase suspension

Component	Volume (µL) required for each reaction
Cell Solution-V3	17.7
RT Primer-V3	4
DIR Reagent-V3	1.6
RNase Inhibitor	16.5
RT Enzyme-V3	8
PBS (containing 0.04% BSA)	32.2-X
Cell suspension	X
Total	80

Tips
- "X" indicates the volume of the cell (nucleus) suspension.
- The prepared sample phase suspension can be mixed by pipetting just before being added to the droplet generator. After mixing, it can be directly added for droplet generation, no need to pipette again to reduce loss and avoid not meeting the sample input volume requirements.

- The total number of input cells (nucleus) ranges from 5000 to 30000, and the maximum volume of the cell (nucleus) suspension should be 32.2 µL and might vary according to the cell (nucleus) concentration. If the volume is less than 32.2 µL, the PBS (containing 0.04% BSA) is added as a complement.
- For a resuscitated or fragile cell sample, it is recommended to use a wide-bore tip to pipette and mix the cell reaction solution thoroughly.
- If cells are divided and prepared as multiple tubes of samples, it is recommended to prepare the total amount of reaction solution required for all tubes of samples in a 1.5 mL low-binding centrifuge tube.
- Add RT Enzyme-V3 and cells before operations on the droplet generator.

3.1.3 Preparing beads phase suspension

Tips
- Prepare the Beads phase suspension in the clean bench.
- Appropriately increase the volume when preparing the beads phase suspension, to avoid a volume insufficiency (less than 100 µL) of the beads phase suspension in subsequent droplet generation.

Perform the following steps:

1. Take out the Cell Beads-V3 and Index Carrier, and gently invert or pipette it to mix it thoroughly.
2. Aspirate 70 µL of the Cell Beads-V3 and 35 µL of the Index Carrier (for each sample), and transfer it to a 0.2 mL low-binding PCR tube (the volume varies according to the number of samples).
3. Place and keep the PCR tube on the magnetic separation rack for 3 to 5 minutes. Gently remove and dispose of the supernatant, to avoid loss of the beads.
4. Remove the PCR tube from the magnetic separation rack and add 91 µL of the Beads Buffer and 9 µL of the Lysis Buffer-V3 in order into the PCR tube. Prepare the beads phase suspension according to the following table. After preparation, place it on ice for later use.

Table 16 Beads phase suspension

Component	Volume (µL) required for each tube	Remarks
Cell Beads-V3	70	Remove and dispose of the supernatant
Index Carrier	35	
Beads Buffer	91	/
Lysis Buffer-V3	9	/

Component	Volume (μL) required for each tube	Remarks
Total	100	

Tips
- The prepared beads phase suspension can be mixed by pipetting just before being added to the droplet generator. After mixing, it can be directly added for droplet generation, no need to pipette again to reduce loss and avoid not meeting the sample input volume requirements.
- If cells are divided and prepared as multiple tubes of samples, it is recommended to prepare the total amount of reaction solution required for all tubes of samples in a 1.5 mL low-binding centrifuge tube.
- The beads phase suspension should be transferred to the droplet generator within 20 minutes for droplet generation.

3.2 Performing droplet generation

Perform the following steps:

1. Prepare the Flow Cell.

WARNING
- Use the flow cell immediately after unwrapping the outer plastic package, to prevent dust from falling into the flow cell wells and result in blockage.
- If the flow cell accidentally drops to the floor and breaks, handle with care in case of personal injury.

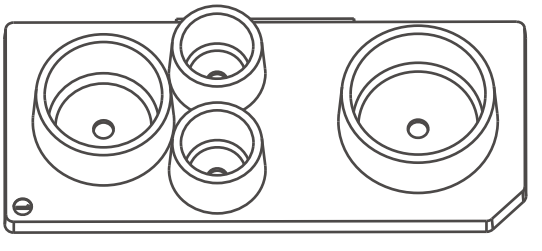

Figure 1 Flow Cell

2. Power on the droplet generator. Tap **Open** in the main interface to open the flow cell compartment door.

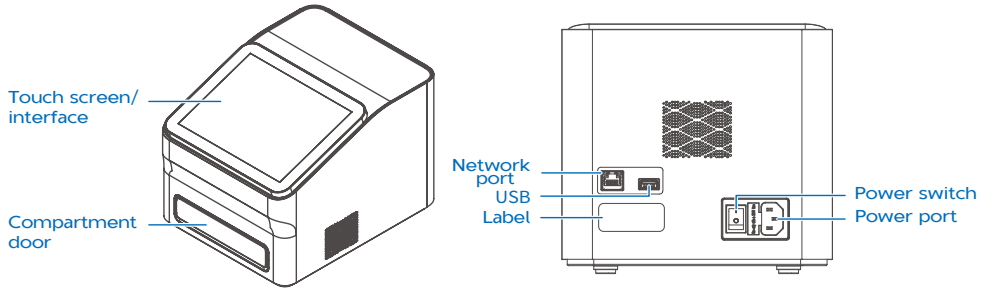

Figure 2 Front view and back view of the single cell droplet generator

3. Open the latch on the flow cell holder.

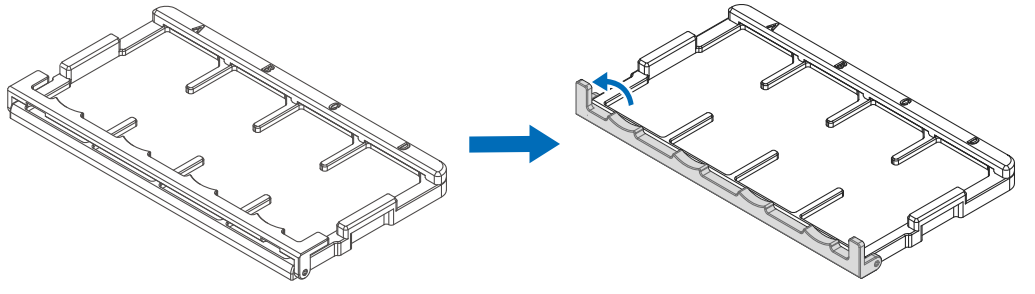

Figure 3 Opening the latch

4. Place the flow cell onto the holder. Ensure that the notch on the upper right of the flow cell is aligned with the notch on the channel of the holder. Place flow cells onto the other channels according to your requirement.

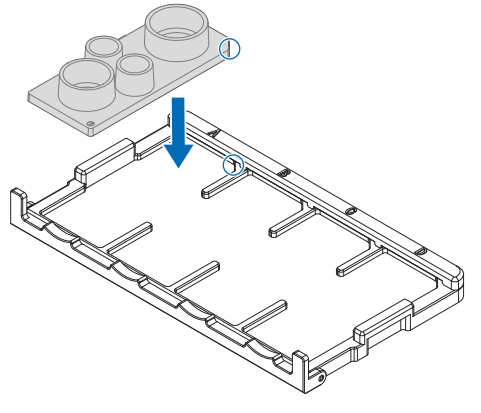

Figure 4 Loading the flow cell

5. Gently install the sealing gasket onto each collection well. You can cut the required number of sealing gaskets according to the number of loaded flow cells.

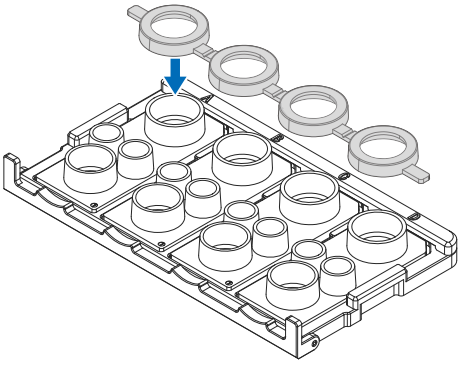

Figure 5 Installing the sealing gasket

Tips Each flow cell should be equipped with a sealing gasket.

6. After installing the sealing gasket, close the latch to install the flow cells in place.
7. Place the flow cell holder that is loaded with flow cells onto the flow cell stage of flow cell compartment, as shown in the following figure.

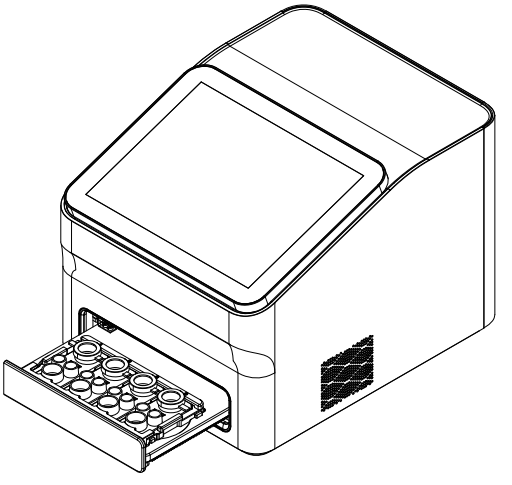

Figure 6 Placing flow cell holder into the flow cell compartment

8. According to the table below, add solutions to the flow cell wells in the following order: the sample phase suspension, P100 Oil, and beads phase suspension.

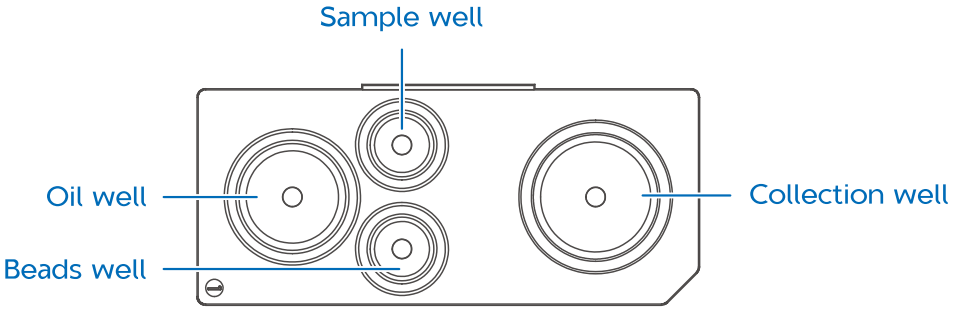

Figure 7 Wells on flow cell

Table 17 Adding solutions to the flow cell

Order	Name	Volume	Well Name
1	Sample phase suspension	80 µL	Sample well
2	P100 Oil	950 µL	Oil well
3	Beads phase suspension	100 µL	Beads well

Tips
- Before adding the sample phase and beads phase suspension, ensure that the suspension is mixed thoroughly by pipetting. Avoid creating air bubbles while pipetting. When adding the samples, do not hover the pipette tip over the liquid inlet. Keep it in contact with the bottom of the inlet, and add the liquid slowly. Fill the inlet completely with the liquid, so as to ensure the smooth progress of droplet generation.
- The total time for adding the three solutions should be within 1 minute.
- Strictly add solutions in the following order: sample phase suspension, P100 Oil, and beads phase suspension. Otherwise, it may result in failure of droplet generation.
- When generating droplets for multiple samples (for example, 4 samples), you can sequentially add the sample suspensions of the 4 samples, then add the P100 Oil for each of the 4 samples in sequence, and finally add the beads suspensions for the 4 samples in sequence, before proceeding to droplet generation for all of them.

9. Tap **Close** to close the compartment door.
10. Select **RNAV3** for the reaction type.
11. Select the channel corresponding to the loaded channel on the stage.

    Tips The channels **A**, **B**, **C** and **D** on the screen corresponds to the channels **A**, **B**, **C** and **D** on the stage.

12. Tap ▶ to start the reaction.

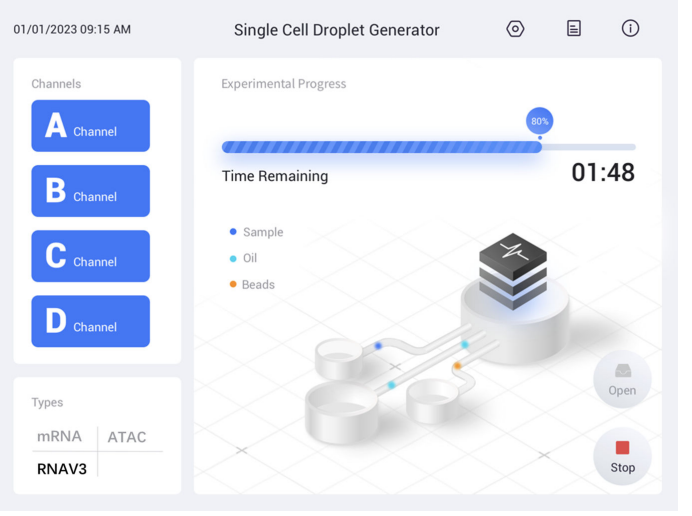

Figure 8 Reaction started

13. After the reaction, tap **Confirm** > **Open** .

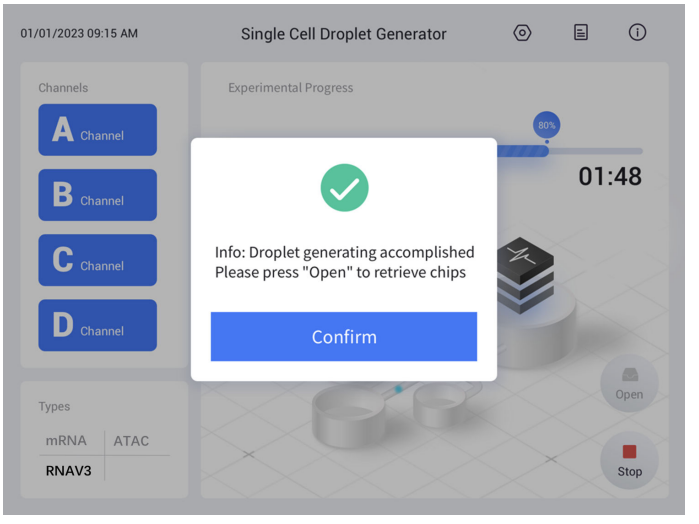

Figure 9 Droplet generating accomplished

14. Remove the sealing gaskets and open the latch. Take out the flow cell. Proceed the droplet generation procedures.

    For details about droplet generation, refer to the relevant reagent kit user manual.

Tips

- After droplet generation, the droplets should be collected immediately to prevent evaporation of droplets in the collection wells or loss of samples due to droplets hanging on the walls of the collection wells during prolonged exposure to the air.
- For details about the maintenance of the Single Cell Droplet Generator, refer to *DNBelab C-TaiM 4RS Single Cell Droplet Generator User Manual*.

Chapter 4 Droplet-based reverse transcription

This chapter describes the process of collecting droplets after droplet generation and performing droplet-based RT. The whole procedure takes approximately 2 hours and 40 minutes.

4.1 Pre-experiment preparation

Table 18 Required reagent kit

Name	Component	Cap color	Specifications	
			16RXN	4RXN
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 1 Droplet Generation) (Cat. No.:940-001820-00, 16RXN) (Cat. No.:940-001927-00, 4RXN)	Cover Oil	Natural	3.2 mL/bottle × 2	1.6 mL/bottle × 1

Tips Take out the Cover Oil at least 30 minutes in advance to equilibrate to room temperature.

4.2 Collecting droplets and performing RT reaction

Perform the following steps:

1. Take a clean PCR 8-strip tube and mark it with a marker pen.

   Tips One sample corresponds to one PCR 8-strip tube.

2. Gently aspirate all droplets from the collection well of the flow cell by using a 200 μL low-binding tip. You can slightly tilt the flow cell to facilitate droplet collection. Hang the pipette tip vertically a few seconds, and wait for the droplets to float to the upper layer of the oil phase. Gently dispense the bottom oil phase into the last four tubes of the PCR 8-strip tube, then transfer the droplets to the first four tubes of the PCR 8-strip tube. Repeat this step until all droplets have been transferred.

   Tips
   - The first four tubes of the PCR 8-strip tube should contain all the droplets from one sample, while the last four tubes should contain P100 Oil. When transferring droplets, move gently, as vigorous pipetting can cause the droplets to break.

- Distribute the droplets from one sample evenly into the first four tubes of the PCR 8-strip tube, with each tube containing approximately 50 µL to 100 µL.
- Use the oil phase in the last four tubes of the PCR 8-strip tube to wash any residual droplets in the collection well (avoid aspirating and dispensing), and transfer them to the first four tubes of the PCR 8-strip tube to collect as many droplets as possible.
- Do not place the droplets for more than 30 minutes after droplet generation, Otherwise, the data quality may be influenced.

3. Add 100 µL of Cover Oil to the surface of the droplets in the first four tubes of the PCR 8-strip tube.

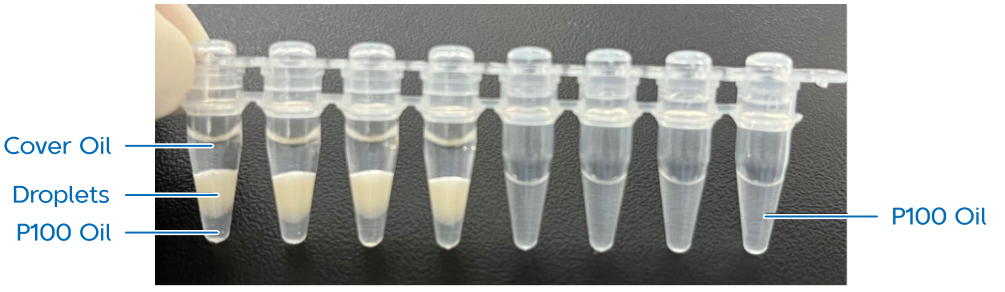

Figure 10 Droplet collection

Tips The liquid in the first four tubes of the 8-strip tube is divided into three layers: the upper layer is Cover Oil, the middle layer is droplets, and the bottom layer is P100 Oil (little or none).

4. Proceed with droplet-based reverse transcription reaction by using the PCR device according to the condition shown in the following table:

Table 19 Droplet-based reverse transcription reaction condition (100 µL reaction solution)

Temperature	Time	Cycles
70 °C (158 °F) (heated lid)	On	/
42 °C (108 °F)	90 min	1
50 °C (122 °F)	2 min	10
42 °C (108 °F)	2 min	
85 °C (185 °F)	5 min	1
4 °C (39 °F)	Hold	/

Stop point After the RT reaction, the collected droplets can be stored for up to 24 hours at 2 °C to 8 °C (36 °F to 46 °F).

Chapter 5 Performing demulsification and size selection of RT product

This chapter describes how to perform demulsification and purify RT products. The whole procedure takes about one hour.

Tips
- Take out the DNA Clean Beads at least 30 minutes in advance to equilibrate to room temperature, and vortex it to mix thoroughly before use.
- Breakage Reagent should be used in a fume hood.

5.1 Pre-experiment preparation

Table 20 Required reagent kit

Name	Component	Cap color	Specifications	
			16RXN	4RXN
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 1 Droplet Generation) (Cat. No.: 940-001820-00, 16RXN) (Cat. No.: 940-001927-00, 4RXN)	Breakage Reagent	Brown	800 μL/tube ×2	400 μL/tube ×1
	DNA Clean Beads	Natural	8.352 mL/bottle ×2	4.176 mL/bottle ×1

5.2 Performing demulsification and size selection of magnetic beads

Tips This section provides two methods for the process of the demulsification and size selection of magnetic beads, and you can choose either one to proceed with.

5.2.1 Method 1

5.2.1.1 Performing demulsification

Perform the following steps:

1. After the RT reaction, transfer the droplets in the middle layer of the first 4 PCR tubes of the PCR 8-strip tube to a new 1.5 mL low-binding centrifuge tube.

   Tips Avoid aspirating the Cover Oil in the upper layer while transferring droplets; aspirating the P100 Oil in the bottom layer will not cause any effects.

2. Add 100 µL of Breakage Reagent into the centrifuge tube, then add 200 µL of NF Water. Invert the tube 15 to 20 times, and let it stand at room temperature for 3 minutes.
3. Centrifuge the tube at 1000 × g at room temperature for 2 minutes.
4. Place and keep the centrifuge tube on the magnetic separation rack for 3 to 5 minutes.
5. Slowly aspirate 300 µL of the aqueous phase into a new 1.5 mL low-binding centrifuge tube. If the volume of the aqueous phase is less than 300 µL, add the NF Water to the tube to complement the volume to 300 µL.

   Tips When aspirating the aqueous phase, gently insert the pipette tip into the aqueous layer and keep it above the water-oil interface, and avoid aspirating the P100 Oil in the bottom layer and the water-oil interface in the middle layer.

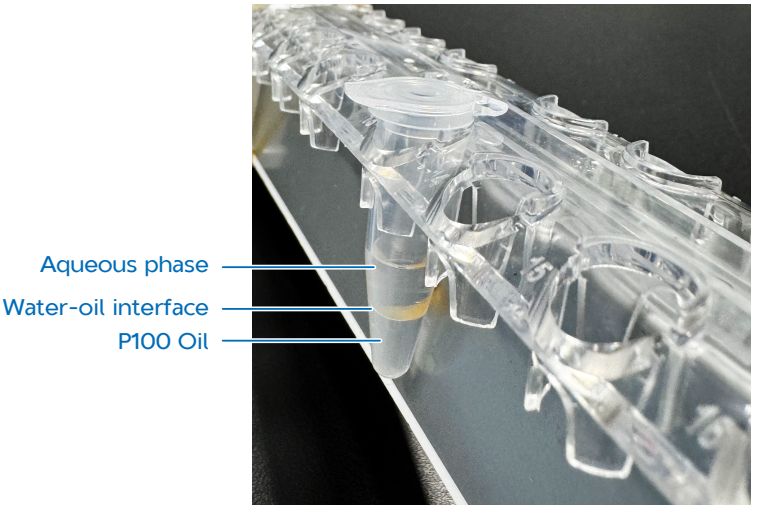

Figure 11 Aqueous phase after demulsification

5.2.1.2 Performing size selection of magnetic beads

Tips
- Take out the DNA Clean Beads at least 30 minutes in advance to equilibrate to room temperature, and vortex it to mix thoroughly before use.
- Before starting the magnetic bead purification, carefully read *"Appendix 1 About the DNA Clean Beads and purification" on page 54*.

Perform the following steps:

1. Aspirate 180 µL (0.6×) of DNA Clean Beads into the aqueous phase from step 5 of *"5.2.1.1 Performing demulsification" on page 26*. Gently pipette the liquid to mix it thoroughly, and ensure that all liquid and beads in the pipette tip are pipetted down into the centrifuge tube in the last pipetting.
2. Incubate the centrifuge tube at room temperature for 5 minutes.
3. After briefly centrifuging the tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear.
4. Transfer the supernatant of step 3 to a new 1.5 mL centrifuge tube, and mark it as "Oligo Product 1". Purify magnetic beads adsorbed in step 3.

   Tips
   - Do not dispose of but reserve the supernatant in this step.
   - Do not aspirate the magnetic beads.
5. Keep the centrifuge tube on the magnetic separation rack, and add 700 µL of freshly prepared 80% ethanol to rinse the beads and tube wall.
6. Keep the tube on the magnetic separation rack for 30 seconds, and then remove and dispose of the supernatant.
7. Repeat steps 5 and 6, and try to remove all liquid from the tube. If some residuals remain on the tube wall, briefly centrifuge the tube, place it on the magnetic separation rack for separation, and remove all liquid at the bottom of the tube by using a pipette with a small measurement range.
8. Keep the centrifuge tube on the magnetic separation rack, and open the lid of the tube for drying the beads at room temperature until no wetness or glossiness is visible on the beads surface and no beads crack.

   Tips Do not overdry the beads as the beads may crack.
9. Remove the centrifuge tube from the magnetic separation rack, and add 48 µL of the NF Water by using a pipette.
10. Gently pipette the liquid to mix it thoroughly.
11. Incubate the centrifuge tube at room temperature for 5 minutes.
12. After briefly centrifuging the tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and transfer 46 µL of the supernatant to a new 1.5 mL centrifuge tube, and mark it as"cDNA Intermediate Product", and proceed to amplifying and purifying the cDNA Intermediate Products. For details, refer to *"Chapter 6 Amplifying and purifying cDNA Intermediate Products" on page 31*.

Stop point The "cDNA Intermediate Product" can be stored at -25 °C to -15 °C (-13 °F to 5 °F) for up to one week.

13. Add 240 µL (0.8×) of DNA Clean Beads to the "Oligo Product 1" retained in the step 4. Gently pipette the liquid to mix it thoroughly, and ensure that all liquid and beads in the pipette tip are pipetted into the centrifuge tube in the last pipetting.
14. Incubate the centrifuge tube at room temperature for 5 minutes.
15. After briefly centrifuging the tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, remove and dispose of the supernatant.
16. Keep the centrifuge tube on the magnetic separation rack, and add 700 µL of freshly prepared 80% ethanol to rinse the beads and tube wall.
17. Keep the tube on the magnetic separation rack for 30 seconds, and then remove and dispose of the supernatant.
18. Repeat steps 16 and 17, and try to remove all liquid from the tube. If some residuals remain on the tube wall, briefly centrifuge the tube, place it on the magnetic separation rack for separation, and remove all liquid at the bottom of the tube by using a pipette with a small measurement range.
19. Keep the centrifuge tube on the magnetic separation rack, and open the lid of the tube for drying the beads at room temperature until no wetness or glossiness is visible on the beads surface and no beads crack.

    Tips Do not overdry the beads as the beads may crack.

20. Remove the centrifuge tube from the magnetic separation rack, and add 65 µL of the NF Water by using a pipette.
21. Gently pipette the liquid to mix it thoroughly.
22. Incubate the centrifuge tube at room temperature for 5 minutes.
23. After briefly centrifuging the tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and transfer 63 µL of the supernatant to a new 1.5 mL centrifuge tube, and mark it as "Oligo Product 2".

    Stop point The "Oligo Product 2" can be stored at -25 °C to -15 °C (-13 °F to 5 °F) for up to six months.

5.2.2 Method 2

5.2.2.1 Performing demulsification

Perform the following steps:

1. After the RT reaction, transfer the droplets in the middle layer of the first 4 PCR tubes of the PCR 8-strip tube to a new 1.5 mL low-binding centrifuge tube.

   Tips Avoid aspirating the Cover Oil in the upper layer while transferring droplets; aspirating the P100 Oil in the bottom layer will not cause any effects.

2. Add 100 µL of Breakage Reagent to the centrifuge tube. Invert the tube 15 to 20 times, and let it stand at room temperature for 3 minutes.
3. Centrifuge the tube at 1000 × g at room temperature for 2 minutes.
4. Aspirate 300 µL of DNA Clean Beads into the centrifuge tube, gently invert it several times to mix it thoroughly.
5. Incubate the centrifuge tube at room temperature for 5 minutes.
6. Briefly centrifuge the tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and then remove and dispose of the supernatant.
7. Keep the centrifuge tube on the magnetic separation rack, and add 700 µL of freshly prepared 80% ethanol to rinse the beads and tube wall. Keep the tube on the magnetic separation rack for 30 seconds, and then remove and dispose of the supernatant.
8. Repeat step 7, and try to remove all liquid from the tube. If some residuals remain on the tube wall, briefly centrifuge the tube, place it on the magnetic separation rack for separation, and remove all liquid at the bottom of the tube by using a pipette with a small measurement range.
9. Keep the centrifuge tube on the magnetic separation rack, and open the lid of the tube for drying the beads at room temperature until no wetness or glossiness is visible on the beads surface and no beads crack.

   Tips Do not overdry the beads as the beads may crack.

10. Remove the centrifuge tube from the magnetic separation rack, and add 102 µL of the NF Water by using a pipette. Gently pipette the liquid to mix it thoroughly.
11. Incubate the centrifuge tube at room temperature for 5 minutes.
12. Briefly centrifuge the tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and transfer 100 µL of the supernatant to a new 1.5 mL centrifuge tube.

5.2.2.2 Performing size selection of magnetic beads

Tips
- Take out the DNA Clean Beads at least 30 minutes in advance to equilibrate to room temperature, and vortex it to mix thoroughly before use.
- Before starting the magnetic bead purification, carefully read *"Appendix 1 About the DNA Clean Beads and purification" on page 54*.

Perform the following steps:

1. Aspirate 60 µL (0.6×) of DNA Clean Beads into the centrifuge tube in step 12 of *"5.2.2.1 Performing demulsification" on page 29* Gently pipette the liquid to mix it thoroughly, and ensure that all liquid and beads in the pipette tip are pipetted down into the centrifuge tube in the last pipetting.
2. Incubate the centrifuge tube at room temperature for 5 minutes.
3. After briefly centrifuging the tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear.
4. Transfer the supernatant of step 3 to a new 1.5 mL centrifuge tube, and mark it as "Oligo Product 1". Purify magnetic beads adsorbed in step 3.

   Tips
   - Do not dispose of but reserve the supernatant in this step.
   - Do not aspirate the magnetic beads.
5. Keep the centrifuge tube on the magnetic separation rack, and add 700 µL of freshly prepared 80% ethanol to rinse the beads and tube wall.
6. Keep the tube on the magnetic separation rack for 30 seconds, and then remove and dispose of the supernatant.
7. Repeat steps 5 and 6, and try to remove all liquid from the tube. If some residuals remain on the tube wall, briefly centrifuge the tube, place it on the magnetic separation rack for separation, and remove all liquid at the bottom of the tube by using a pipette with a small measurement range.
8. Keep the centrifuge tube on the magnetic separation rack, and open the lid of the tube for drying the beads at room temperature until no wetness or glossiness is visible on the beads surface and no beads crack.

   Tips Do not overdry the beads as the beads may crack.
9. Remove the centrifuge tube from the magnetic separation rack, and add 48 µL of the NF Water by using a pipette.
10. Gently pipette the liquid to mix it thoroughly.
11. Incubate the centrifuge tube at room temperature for 5 minutes.
12. After briefly centrifuging the tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and transfer 46 µL of the supernatant to a new 1.5 mL centrifuge tube, and mark it as"cDNA Intermediate Product", and proceed to amplifying and purifying the cDNA Intermediate Products. For details, refer to *"Chapter 6 Amplifying and purifying cDNA Intermediate Products" on page 31*.

Stop point The "cDNA Intermediate Product" can be stored at -25 °C to -15 °C (-13 °F to 5 °F) for up to one week.

13. Add 80 µL (0.8×) of DNA Clean Beads to the "Oligo Product 1" retained in the step 4. Gently pipette the liquid to mix it thoroughly, and ensure that all liquid and beads in the pipette tip are pipetted into the centrifuge tube in the last pipetting.
14. Incubate the centrifuge tube at room temperature for 5 minutes.
15. After briefly centrifuging the tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, remove and dispose of the supernatant.
16. Keep the centrifuge tube on the magnetic separation rack, and add 700 µL of freshly prepared 80% ethanol to rinse the beads and tube wall.
17. Keep the tube on the magnetic separation rack for 30 seconds, and then remove and dispose of the supernatant.
18. Repeat steps 16 and 17, and try to remove all liquid from the tube. If some residuals remain on the tube wall, briefly centrifuge the tube, place it on the magnetic separation rack for separation, and remove all liquid at the bottom of the tube by using a pipette with a small measurement range.
19. Keep the centrifuge tube on the magnetic separation rack, and open the lid of the tube for drying the beads at room temperature until no wetness or glossiness is visible on the beads surface and no beads crack.

    Tips Do not overdry the beads as the beads may crack.

20. Remove the centrifuge tube from the magnetic separation rack, and add 65 µL of the NF Water by using a pipette.
21. Gently pipette the liquid to mix it thoroughly.
22. Incubate the centrifuge tube at room temperature for 5 minutes.
23. After briefly centrifuging the tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and transfer 63 µL of the supernatant to a new 1.5 mL centrifuge tube, and mark it as "Oligo Product 2".

    Stop point The "Oligo Product 2" can be stored at -25 °C to -15 °C (-13 °F to 5 °F) for up to six months.

Chapter 6 Amplifying and purifying cDNA Intermediate Products

This chapter describes how to amplify cDNA Intermediate Products and how to purify the amplified cDNA products. The whole procedure takes about 2 hours and 20 minutes.

6.1 Pre-experiment preparation

Table 21 Required reagent kit

Name	Component	Cap color	Specification	
			16RXN	4RXN
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 1 Droplet Generation) (Cat. No.: 940-001820-00, 16RXN) (Cat. No.: 940-001927-00, 4RXN)	DNA Clean Beads	Natural	8.352 mL/bottle×2	4.176 mL/bottle×1
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 2 Droplet Generation) (Cat. No.: 940-001819-00, 16RXN) (Cat. No.: 940-001929-00, 4RXN)	cDNA Amp Enzyme	Natural	400 μL/tube×2	200 μL/tube×1
	cDNA Amp Primer-V3	Natural	33 μL/tube×2	17 μL/tube×1

Tips Take out the DNA Clean Beads at least 30 minutes in advance to equilibrate to room temperature, and vortex it to mix thoroughly before use.

6.2 Amplifying cDNA Intermediate Products

Perform the following steps:

1. Prepare the cDNA amplification reaction solution on ice according to the following table.

Table 22 cDNA amplification reaction solution

Component	Volume (μL) required for each reaction
cDNA Amp Enzyme	50
cDNA Amp Primer-V3	4
cDNA Intermediate Product	46
Total	100

2. Vortex the prepared reaction solution to mix it thoroughly, and briefly centrifuge the tube.
3. Proceed with cDNA amplification reaction by using the PCR device according to the condition shown in the following table:

Table 23 cDNA amplification reaction condition (100 µL reaction solution)

Temperature	Time	Cycles
105 °C (221 °F) (heated lid)	On	/
95 °C (203 °F)	3 min	1
98 °C (208 °F)	20 s	X
65 °C (149 °F)	30 s	
72 °C (162 °F)	3 min	
72 °C (162 °F)	5 min	1
4 °C (39 °F)	Hold	/

Tips The PCR cycle varies with the sample and single-cell input:

- For cell-line samples (with an input of 10000 to 20000), 11 to 13 cycles are recommended.
- For PBMC samples (with an input of 10000 to 20000), 13 to 15 cycles are recommended.
- For cells or nucleus (with an input of 10000 to 20000) from live tissue, 15 to 20 cycles are recommended based on sample conditions.

Stop point The cDNA amplification product can be stored at 2 °C to 8 °C (36 °F to 46 °F) for up to 24 hours or at -25 °C to -15 °C (-13 °F to 5 °F) for up to one week.

6.3 Purifying cDNA products

Tips

- Take out the DNA Clean Beads at least 30 minutes in advance to equilibrate to room temperature, and vortex it to mix thoroughly before use.
- Before starting size selection, carefully read *"Appendix 1 About the DNA Clean Beads and purification" on page 54*.

Perform the following steps:

1. Aspirate 60 µL (0.6×) of the DNA Clean Beads to the cDNA amplification products from step 3 in *"6.2 Amplifying cDNA Intermediate Products" on page 32* by using a pipette.
2. Gently pipette the liquid to mix it thoroughly, and ensure that all liquid and beads in the pipette tip are pipetted down into the PCR tube in the last pipetting.
3. Incubate the PCR tube at room temperature for 5 minutes.

4. After briefly centrifuging the PCR tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear. Remove and dispose of the supernatant.
5. Keep the PCR tube on the magnetic separation rack, and add 200 µL of freshly prepared 80% ethanol to rinse the beads and tube wall.
6. Keep the tube on the magnetic separation rack for 30 seconds, and then remove and dispose of the supernatant.
7. Repeat steps 5 and 6, and try to remove all liquid from the tube.
8. If some residuals remain on the tube wall, briefly centrifuge the tube, place it on the magnetic separation rack for separation, and remove all liquid at the bottom of the tube by using a pipette with a small measurement range.
9. Keep the PCR tube on the magnetic separation rack, and open the lid of the tube for drying the beads at room temperature until no wetness or glossiness is visible on the beads surface and no beads crack.

   Tips Do not overdry the beads as the beads may crack.

10. Remove the centrifuge tube from the magnetic separation rack, and add 32 µL of the NF Water for cDNA elution by using a pipette.
11. Gently pipette the liquid to mix it thoroughly.
12. Incubate the PCR tube at room temperature for 5 minutes.
13. After briefly centrifuging the tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and transfer 30 µL of the supernatant to a new 1.5 mL centrifuge tube, and mark it as “cDNA Product”.
14. Take 1 µL of the “cDNA Product” and quantify it by using the Qubit dsDNA HS Assay Kit, and take a proper amount of the cDNA Product to assess fragment size distribution.

    Reference value: The concentration of the cDNA Product is greater than 10 ng/µL, and a peak for the fragment size distribution ranges between 600 bp to 2000 bp.

    Tips This reference value is obtained by testing with standard human PBMC. The concentration of the cDNA Product may vary with sample types.

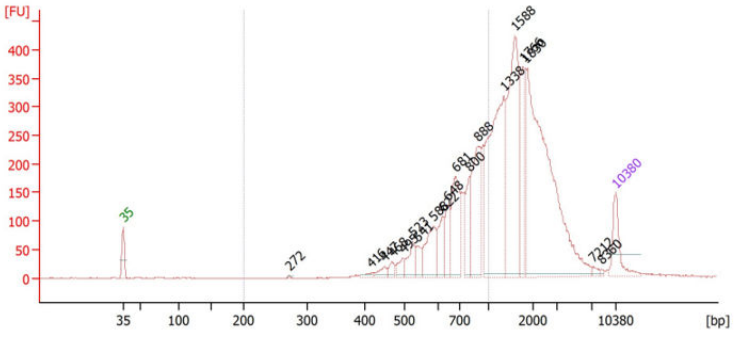

Figure 12 Fragment size distribution of the cDNA Product (a reference graph obtained through assessment by using the Agilent 2100 Bioanalyzer)

Stop point The purification product can be stored at -25 ℃ to -15 ℃ (-13 ℉ to 5 ℉) for up to six months.

Chapter 7 Oligo library preparation

This chapter describes how to perform sample barcode marking on the Oligo product through PCR. The whole procedure takes about 1 hour and 30 minutes.

7.1 Pre-experiment preparation

Table 24 Required reagent kit

Name	Component	Cap color	Specification	
			16RXN	4RXN
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 1 Droplet Generation) (Cat. No.: 940-001820-00,16RXN) (Cat. No.: 940-001927-00, 4RXN)	DNA Clean Beads	Natural	8.352 mL/ bottle×2	4.176 mL/ bottle×1
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 3 Library Preparation) (Cat. No.: 940-001821-00, 16RXN) (Cat. No.: 940-001925-00, 4RXN)	PCR Amp Enzyme	Natural	600 μL/tube×2	300 μL/ tube×1

Name	Component	Cap color	Specification	
			16RXN	4RXN
DNBelab C Series Single-cell Library Preparation Sample Barcode Kit S (Cat. No.: 940-001920-00, 16RXN)	Barcode Primer-1 to 8	Natural	8-strip tube×2	/
	Barcode Primer-9 to 16	Natural	8-strip tube×2	/
	Barcode Primer-17 to 24	Natural	8-strip tube×2	/
	Barcode Primer-25 to 32	Natural	8-strip tube×2	/
DNBelab C Series Single-cell Library Preparation Sample Barcode Kit S (Cat. No.: 940-001926-00, 4RXN)	Barcode Primer-1 to 8	Natural	/	8-strip tube×1
	Barcode Primer-9 to 16	Natural	/	8-strip tube×1

Tips Take out the DNA Clean Beads at least 30 minutes in advance to equilibrate to room temperature, and vortex it to mix thoroughly before use.

7.2 Preparing the Oligo library

Perform the following steps:

1. Take out a new 0.2 mL PCR tube, aspirate 21 μL of the"Oligo Product 2" from step 23 in *"5.2.1.2 Performing size selection of magnetic beads" on page 27* or *"5.2.2.2 Performing size selection of magnetic beads" on page 30* to the PCR tube. Prepare Oligo library preparation reaction solution according to the following table:

Table 25 Oligo library preparation reaction solution

Component	Volume (μL) required for each reaction
Oligo Product 2	21
Barcode Primer	4
PCR Amp Enzyme	25
Total	50

Tips
- Before starting Oligo library preparation, carefully read *"Appendix 1 About the DNA Clean Beads and purification" on page 54*.
- Record the number of the Barcode Primer added to each sample.

2. Vortex the prepared reaction solution to mix it thoroughly, and briefly centrifuge it.
3. Prepare the Oligo library according to the condition shown in the following table:

Table 26 Oligo library preparation condition (50 µL reaction solution)

Temperature	Time	Cycles
105 °C (221 °F) (heated lid)	On	/
95 °C (203 °F)	3 min	1
98 °C (208 °F)	15 s	9
60 °C (140 °F)	30 s	
72 °C (162 °F)	10 s	
72 °C (162 °F)	5 min	1
4 °C (39 °F)	Hold	/

7.3 Performing size selection on Oligo library

Tips
- Take out the DNA Clean Beads at least 30 minutes in advance to equilibrate to room temperature, and vortex it to mix thoroughly before use.
- Before starting size selection, carefully read *"Appendix 1 About the DNA Clean Beads and purification" on page 54*.

Perform the following steps:

1. Aspirate 30 µL (0.6×) of the DNA Clean Beads to the PCR tube from step 3 in *"7.2 Preparing the Oligo library" on page 36* by using a pipette.
2. Gently pipette the liquid to mix it thoroughly, and ensure that all liquid and beads in the pipette tip are pipetted down into the PCR tube in the last pipetting.
3. Incubate the PCR tube at room temperature for 5 minutes.
4. After briefly centrifuging the PCR tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear.
5. Carefully remove the supernatant by using a pipette, and transfer it to a new PCR tube.

   Tips Do not dispose of but reserve the supernatant in this step.
6. Add 40 µL (0.8×) of the DNA Clean Beads to the PCR tube with the supernatant by using a pipette.
7. Gently pipette the liquid to mix it thoroughly, and ensure that all liquid and beads in the pipette tip are pipetted down into the PCR tube in the last pipetting.
8. Incubate the PCR tube at room temperature for 5 minutes.

9. After briefly centrifuging the PCR tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and remove and dispose of the supernatant.
10. Keep the PCR tube on the magnetic separation rack, and add 200 µL of freshly prepared 80% ethanol to rinse the beads and tube wall.
11. Keep the PCR tube on the magnetic separation rack for 30 seconds, and then remove and dispose of the supernatant.
12. Repeat steps 10 and 11, and try to remove all liquid from the PCR tube.

    If some residuals remain on the tube wall, after briefly centrifuging the tube and placing it on the magnetic separation rack for separation, remove all liquid at the bottom of the tube by using a pipette with a small measurement range.
13. Keep the PCR tube on the magnetic separation rack, and open the lid of the tube for drying the beads at room temperature until no wetness or glossiness is visible on the beads surface and no beads crack.

    Tips Do not overdry the beads as the beads may crack.
14. Remove the PCR tube from the magnetic separation rack, and add 32 µL of the TE Buffer by using a pipette.
15. Gently pipette the liquid to mix it thoroughly.
16. Incubate the PCR tube at room temperature for 5 minutes.
17. After briefly centrifuging the PCR tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and transfer 30 µL of the supernatant to a new centrifuge tube, and mark it as “Oligo library”.
18. Take 1 µL of the size-selection product and quantify it by using the Qubit dsDNA HS Assay Kit, and take a proper amount of the size-selection product to assess fragment size distribution.

    Reference value: The concentration of the Oligo library is greater than 10 ng/µL, and a peak for the fragment size distribution is located at 180±10 bp.

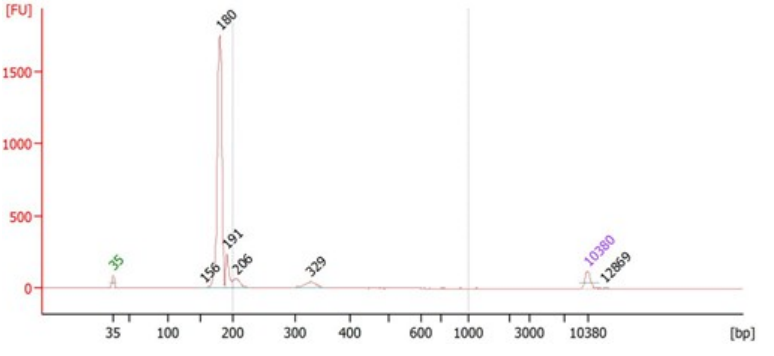

Figure 13 Fragment size distribution of the Oligo library (a reference graph obtained through assessment by using the Agilent 2100 Bioanalyzer)

19. Use the Oligo library to prepare circularization libraries.
    For details, refer to *"8.7 Preparing circularization libraries (cDNA library and Oligo library)" on page 47*.

Stop point The Oligo library can be stored at -25 ℃ to -15 ℃ (-13 ℉ to 5 ℉) for up to six months.

Chapter 8 cDNA library preparation

This chapter describes how to prepare a cDNA library by using the cDNA Product. This library preparation procedure mainly includes fragmentation and end repair, adapter ligation, and PCR, and takes about 3 hours and 15 minutes.

Tips It is unnecessary to prepare the cDNA library in the clean bench.

8.1 Pre-experiment preparation

Table 27 Required reagent kit

Name	Component	Cap color	Specification	
			16RXN	4RXN
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 1 Droplet Generation) (Cat. No.: 940-001820-00, 16RXN) (Cat. No.: 940-001927-00, 4RXN)	DNA Clean Beads	Natural	8.352 mL/ bottle×2	4.176 mL/ bottle ×1
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 3 Library Preparation) (Cat. No.: 940-001821-00, 16RXN) (Cat. No.: 940-001925-00, 4RXN)	Frag Enzyme-V3	Natural	80 μL/tube×2	40 μL/tube×1
	Frag Buffer-V3	Natural	40 μL/tube×2	20 μL/tube×1
	DNA Ligase-V3	Natural	80 μL/tube×2	40 μL/tube×1
	Ligation Buffer-V3	Natural	160 μL/tube×2	80 μL/tube×1
	scRNA Adapter-V3	Natural	40 μL/tube×2	20 μL/tube×1
	PCR Amp Enzyme	Natural	600 μL/tube×2	300 μL/ tube×1
DNBelab C Series Single-cell Library Preparation Sample Barcode Kit S (Cat. No.: 940-001920-00, 16RXN)	Barcode Primer-1 to 8	Natural	8-strip tube×2	/
	Barcode Primer-9 to 16	Natural	8-strip tube×2	/
	Barcode Primer-17 to 24	Natural	8-strip tube×2	/
	Barcode Primer-25 to 32	Natural	8-strip tube×2	/

Name	Component	Cap color	Specification	
			16RXN	4RXN
DNBelab C Series Single-cell Library Preparation Sample Barcode Kit S (Cat. No.: 940-001926-00, 4RXN)	Barcode Primer-1 to 8	Natural	/	8-strip tube×1
	Barcode Primer-9 to 16	Natural	/	8-strip tube×1

Tips Take out the DNA Clean Beads at least 30 minutes in advance to equilibrate to room temperature, and vortex it to mix thoroughly before use.

8.2 Performing fragmentation and end repair

Perform the following steps:

1. Take out the Frag Enzyme-V3, gently tap and invert to mix, briefly centrifuge and then place on ice for later use. Do not vortex to mix the Frag Enzyme-V3.
2. Prepare a fragmentation and end repair reaction solution on ice according to the following table:

Table 28 Fragmentation and end repair reaction mixture

Adding order	Component	Volume (μL) required for each reaction
1	NF Water	25
2	Frag Buffer-V3	5
3	cDNA product	10
4	Frag Enzyme-V3	10
Total		50

Tips
- The maximum input of cDNA Product is 800 ng. If the concentration of the cDNA Product is greater than 80 ng/μL, input 800 ng of the product. Add the NF Water to the PCR tube to complement to 40 μL.
- The Frag Enzyme-V3 is the final component to be added into the reaction mixture.

3. Vortex the PCR tube to mix the liquid thoroughly. Briefly centrifuge the liquid to collect the reaction solution to the bottom of the tube.
4. After the temperature of the PCR device decreases to 4 ℃ (39 ℉), place the PCR tube in the PCR device and proceed with the reaction according to the condition shown in the following table:

Table 29 Fragmentation and end repair reaction condition

(50 µL reaction solution)

Temperature	Time
70 °C (158 °F) (heated lid)	On
4 °C (39 °F)	1 min
32 °C (90 °F)	10 min
65 °C (149 °F)	30 min
4 °C (39 °F)	Hold

8.3 Performing adapter ligation

Perform the following steps:

1. Prepare an adapter ligation reaction solution on ice according to the following table:

Table 30 Adapter ligation reaction solution

Component	Volume (µL) required for each reaction
Ligation Buffer-V3	20
DNA Ligase-V3	10
scRNA Adapter-V3	5
NF Water	15
Total	50

Tips
- The adapter ligation reaction solution is viscous. Gently pipette it up and down to ensure that the added amount is correct.
- Vortex the adapter ligation reaction solution multiple times to ensure that the reaction solution is mixed thoroughly.

2. Gently add 50 µL of the adapter ligation reaction solution to the PCR tube from step 4 in *"8.2 Performing fragmentation and end repair" on page 40* by using a pipette.
3. Vortex the PCR tube to mix the liquid thoroughly.
4. Briefly centrifuge the liquid to collect the reaction solution to the bottom of the tube.
5. Place the PCR tube in the PCR device to proceed with the reaction according to the condition shown in the following table.

Tips The heated-lid mode is disabled in this step. If the heated lid temperature is higher than 25 °C (77 °F), open the cover of the PCR device for the reaction.

Table 31 Adapter ligation reaction condition (100 µL reaction solution)

Temperature	Time
Heated lid	Off
20 °C (68 °F)	15 min
4 °C (39 °F)	Hold

6. After the reaction, briefly centrifuge the PCR tube to collect the reaction solution to the bottom of the tube.

8.4 Performing purification and size selection on adapter ligation product

Tips
- Take out the DNA Clean Beads at least 30 minutes in advance to equilibrate to room temperature, and vortex it to mix thoroughly before use.
- Before starting size selection, carefully read *"Appendix 1 About the DNA Clean Beads and purification" on page 54*.

Perform the following steps:

1. Add 100 µL (1×) of the DNA Clean Beads to the PCR tube with the adapter ligation product from step 6 in *"8.3 Performing adapter ligation" on page 41* by using a pipette.
2. Gently pipette the liquid to mix it thoroughly, and ensure that all liquid and beads in the pipette tip are pipetted down into the PCR tube in the last pipetting.
3. Incubate the PCR tube at room temperature for 5 minutes.
4. After briefly centrifuging the PCR tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and remove and dispose of the supernatant.
5. Keep the PCR tube on the magnetic separation rack, and add 200 µL of freshly prepared 80% ethanol to rinse the beads and tube wall.
6. Keep the PCR tube on the magnetic separation rack for 30 seconds, and then remove and dispose of the supernatant.
7. Repeat steps 5 and 6, and try to remove all liquid from the PCR tube.
   If some residuals remain on the tube wall, after briefly centrifuging the tube and placing it on the magnetic separation rack for separation, remove all liquid at the bottom of the tube by using a pipette with a small measurement range.

8. Keep the PCR tube on the magnetic separation rack, and open the lid of the tube for drying the beads at room temperature until no wetness or glossiness is visible on the beads surface and no beads crack.

   Tips Do not overdry the beads as the beads may crack.

9. Remove the PCR tube from the magnetic separation rack, and add 102 µL of the NF Water by using a pipette.
10. Gently pipette the liquid to mix it thoroughly.
11. Incubate the PCR tube at room temperature for 5 minutes.
12. After briefly centrifuging the PCR tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and transfer 100 µL of the supernatant to a new 0.2 mL PCR tube.
13. Add 55 µL (0.55×) of the DNA Clean Beads to the PCR tube with the supernatant by using a pipette.
14. Gently pipette the liquid to mix it thoroughly, and ensure that all liquid and beads in the pipette tip are pipetted down into the PCR tube in the last pipetting.
15. Incubate the PCR tube at room temperature for 5 minutes.
16. After briefly centrifuging the PCR tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear.
17. Carefully remove the supernatant by using a pipette, and avoid removing the beads, and transfer the supernatant to a new PCR tube.

    Tips Do not dispose of but reserve the supernatant.

18. Add 15 µL (0.15×) of the DNA Clean Beads to the PCR tube with the supernatant by using a pipette.
19. Gently pipette the liquid to mix it thoroughly.
20. Incubate the PCR tube at room temperature for 5 minutes.
21. After briefly centrifuging the PCR tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and remove and dispose of the supernatant.
22. Keep the PCR tube on the magnetic separation rack, and add 200 µL of freshly prepared 80% ethanol to rinse the beads and tube wall.
23. Keep the PCR tube on the magnetic separation rack for 30 seconds, and then remove and dispose of the supernatant.
24. Repeat steps 22 and 23, and try to remove all liquid from the PCR tube.

    If some residuals remain on the tube wall, after briefly centrifuging the tube and placing it on the magnetic separation rack for separation, remove all liquid at the bottom of the tube by using a pipette with a small measurement range.

25. Keep the PCR tube on the magnetic separation rack, and open the lid of the tube for drying the beads at room temperature until no wetness or glossiness is visible on the beads surface and no beads crack.

    Tips Do not overdry the beads as the beads may crack.

26. Remove the PCR tube from the magnetic separation rack, and add 48 µL of the NF Water by using a pipette.
27. Gently pipette the liquid to mix it thoroughly.
28. Incubate the PCR tube at room temperature for 5 minutes.
29. After briefly centrifuging the PCR tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and transfer 46 µL of the supernatant to a new 0.2 mL PCR tube.

    Stop point The purified adapter ligation product can be stored at -25 °C to -15 °C (-13 °F to 5 °F) for up to 24 hours.

8.5 Performing PCR amplification

Tips
- Before starting PCR amplification, carefully read *"Appendix 2 Using Barcode Primer" on page 56*
- If pooling cDNA libraries and Oligo libraries for sequencing, attention should be paid to the following:
  - The barcode primers added to the two types of libraries must be different, to avoid issues with separating the barcodes during bioinformatics analysis.
  - Ensure that the barcode primers added to each type of library are balanced in terms of their nucleotide composition.

Perform the following steps:

1. Prepare the PCR amplification reaction mixture according to the following table:

Table 32 PCR amplification reaction mixture

Component	Volume (µL) required for each reaction
Supernatant in Step 29 in *"8.4 Performing purification and size selection on adapter ligation product" on page 42*	46
Barcode Primer	4
PCR Amp Enzyme	50

Component	Volume (μL) required for each reaction
Total	100

2. Place the PCR tube in the PCR device to proceed with the PCR reaction according to the condition shown in the following table:

Table 33 PCR reaction condition (100 μL reaction solution)

Temperature	Time	Cycles
105 °C (221 °F) (heated lid)	On	/
95 °C (203 °F)	3 min	1
98 °C (208 °F)	20 s	12
58 °C (136 °F)	20 s	
72 °C (162 °F)	30 s	
72 °C (162 °F)	5 min	1
4 °C (39 °F)	Hold	/

8.6 Performing size selection on PCR amplification product

Tips
- Take out the DNA Clean Beads at least 30 minutes in advance to equilibrate to room temperature, and vortex it to mix thoroughly before use.
- Before starting size selection, carefully read *"Appendix 1 About the DNA Clean Beads and purification" on page 54*.

Perform the following steps:

1. Add 55 μL (0.55×) of the DNA Clean Beads to the PCR amplification product by using a pipette.
2. Gently pipette the liquid to mix it thoroughly, and ensure that all liquid and beads in the pipette tip are pipetted down into the PCR tube in the last pipetting.
3. Incubate the PCR tube at room temperature for 5 minutes.
4. After briefly centrifuging the PCR tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear.
5. Carefully remove the supernatant by using a pipette, to avoid removing the beads, and transfer the supernatant to a new PCR tube.

   Tips Do not dispose of but reserve the supernatant.

6. Add 15 μL (0.15×) of the DNA Clean Beads to the PCR tube with the supernatant by using a pipette.
7. Gently pipette the liquid to mix it thoroughly.
8. Incubate the PCR tube at room temperature for 5 minutes.
9. After briefly centrifuging the PCR tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and remove and dispose of the supernatant.
10. Keep the PCR tube on the magnetic separation rack, and add 200 μL of freshly prepared 80% ethanol to rinse the beads and tube wall.
11. Keep the PCR tube on the magnetic separation rack for 30 seconds, and remove and dispose of the supernatant.
12. Repeat steps 10 and 11, and try to remove all liquid from the PCR tube.

    If some residuals remain on the tube wall, after briefly centrifuging the tube and placing it on the magnetic separation rack for separation, remove all liquid at the bottom of the tube by using a pipette with a small measurement range.
13. Keep the PCR tube on the magnetic separation rack, and open the lid of the tube for drying the beads at room temperature until no wetness or glossiness is visible on the beads surface and no beads crack.

    Tips Do not overdry the beads as the beads may crack.
14. Remove the PCR tube from the magnetic separation rack, and add 32 μL of the TE Buffer by using a pipette.
15. Gently pipette the liquid to mix it thoroughly.
16. Incubate the PCR tube at room temperature for 5 minutes.
17. After briefly centrifuging the PCR tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear, and transfer 30 μL of the supernatant to a new 1.5 mL centrifuge tube, and mark it as “cDNA library”.
18. Take 1 μL of the “cDNA library” and quantify it by using the Qubit dsDNA HS Assay Kit, and take a proper amount of the size-selection product to assess fragment size distribution.

    Reference value: The concentration of the cDNA library is greater than 10 ng/μL, and a peak for the fragment size distribution ranges between 350 bp to 550 bp.

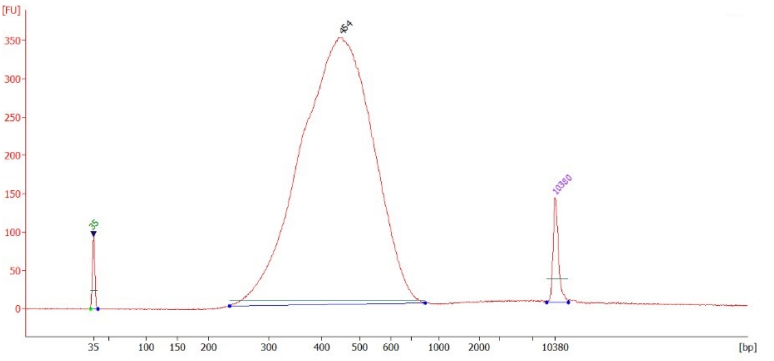

Figure 14 Fragment size distribution of the cDNA library (a reference graph obtained through assessment by using the Agilent 2100 Bioanalyzer)

Stop point The cDNA library can be stored at -25 ℃ to -15 ℃ (-13 ℉ to 5 ℉) for up to six months.

8.7 Preparing circularization libraries (cDNA library and Oligo library)

Perform the following steps:

1. Prepare the following reagents kit: MGIEasy Circularization Kit (Cat. No.: 1000005259)

   Tips Carefully read *MGIEasy Circularization Reagent Kit User Manual* (downloaded from the website: *https://en.mgi-tech.com/download/files?q=1000005259*) before starting circularization, and strictly perform all operations according to the instructions in the user manual.

2. Input the cDNA and Oligo libraries for circularization according to the following table. When the input of the cDNA or Oligo library is insufficient, pool multiple samples (up to 4 libraries are recommended) for circularization or re-prepare the cDNA or Oligo library.

Table 34 Circularization library preparation requirements

Type	Input for circularization
cDNA library	400 ng
Oligo library	400 ng

Chapter 9 Sequencing

This chapter describes genetic sequencers, sequencing reagent kits, read lengths, and library structures.

Tips The single-cell scATAC libraries of MGI cannot be pooled with libraries converted by MGI's APP series adaptor conversion kits on the same flow cell or in the same lane for sequencing.

9.1 cDNA and Oligo library structures

The cDNA and Oligo library structures are shown as follows:

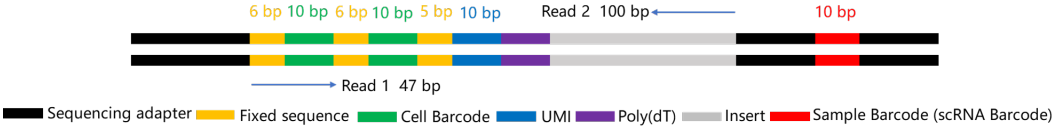

Figure 15 cDNA library structure

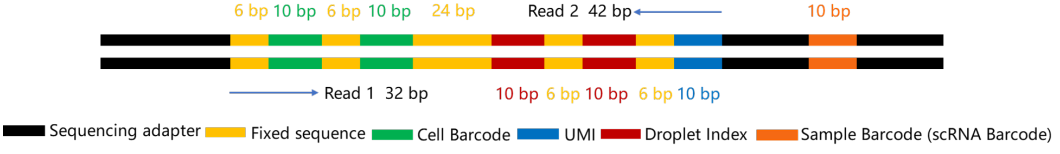

Figure 16 Oligo library structure

Tips Sequencing read length:

- For the cDNA library:

  Read1=30 bp (the fixed sequence used for Read1 dark reaction is 6+6+5=17 bp),

  Read2=100 bp.

- For the Oligo library:

  Read1=20 bp (the fixed sequence used for Read1 dark reaction is 6+6=2 bp),

  Read2=30 bp (the fixed sequence used for Read2 dark reaction is 6+6=12 bp).

9.2 Sequencing requirements of DNBSEQ-G400RS

9.2.1 Pre-experiment preparation

Table 35 Material list

Type	Model No.	Cat. No.
Genetic sequencer	DNBSEQ-G400RS	/
Sequencing set	DNBSEQ-G400RS High-throughput Sequencing Set (FCL PE100)	1000016950
	DNBSEQ-G400RS High-throughput Sequencing Set (FCL PE150)	1000016952

Tips According to the sequencing set, carefully read *DNBSEQ-G400RS High-throughput (Rapid) Sequencing Set User Manual* before starting sequencing, and strictly perform all operations according to the operations in the user manual.

9.2.2 Making DNB

Make DNB according to the following table:

Table 36 DNBSEQ-G400RS making DNB requirements

Sequencing set	PE100/PE150	
Type	cDNA library	Oligo library
Make DNB input	10 ng	6 ng
RCA time	20 min	20 min

Tips If the quality of the sscDNA library is no greater than 10 ng, you may adjust the input of the sscDNA library to 6 ng and the RCA time to 26 minutes when making DNB.

9.2.3 Pooling libraries

- If pooling is required, ensure that different samples are pooled before preparation of DNBs.
- For details about protocols for pooling different libraries based on the Barcode Primer, refer to *"Appendix 2 Using Barcode Primer" on page 56*.

9.2.4 Sequencing parameter

Table 37 DNBSEQ-G400RS (StandardMPS) sequencing software version and read length (in the case of pooling samples and sequencing the barcode)

	cDNA library				Oligo library			
Software version	ECR 3.0	ECR 4.0	ECR 6.0	ECR7.0	ECR 3.0	ECR 4.0	ECR 6.0	ECR7.0
Control software version	DNBSEQ-G400_1.0.0.34 or later version				DNBSEQ-G400_1.0.0.34 or later version			
Basecall version	Basecall_1.0.8.208 and above				Basecall_1.0.8.208 and above			
Sequencing script	C4_scRNA_BC_PE47+100+10	C4_scRNA_BC_PE47+100+10-ECR4.0	Z_C4_scRNA_BC-ECR6.0	Customize	C4_Oligo_BC_PE32+42+10	C4_Oligo_BC_PE32+42+10-ECR4.0	Z_C4_Oligo_BC-ECR6.0	Customize
Read1	47 cycles (1 bp to 6 bp, 17 bp to 22 bp, and 33 to 37 bp are set for dark reaction)				32 cycles (1 bp to 6 bp and 17 bp to 22 bp are set for dark reaction)			
Read2	100 cycles				42 cycles (11 bp to 16 bp and 27 bp to 32 bp are set for dark reaction)			
Sample barcode	10 cycles				10 cycles			
Depth of sequencing	> 50 k Reads/cell				> 50 M reads / library			

Table 38 DNBSEQ-G400RS (StandardMPS) sequencing software version and read length (in the case of neither pooling samples nor sequencing the barcode)

	cDNA library				Oligo library			
Software version	ECR 3.0	ECR 4.0	ECR 6.0	ECR7.0	ECR 3.0	ECR 4.0	ECR 6.0	ECR7.0
Control software version	DNBSEQ-G400_1.0.0.34 or later version				DNBSEQ-G400_1.0.0.34 or later version			
Basecall version	Basecall_1.0.8.208 and above				Basecall_1.0.8.208 and above			
Sequencing script	C4_scRNA_noBC_PE47+100	C4_scRNA_noBC_PE47+100-ECR4.0	Z_C4_scRNA_noBC-ECR6.0	Customize	C4_Oligo_noBC_PE32+42	C4_Oligo_noBC_PE32+42-ECR4.0	Z_C4_Oligo_noBC-ECR6.0	Customize
Read1	47 cycles (1 bp to 6 bp, 17 bp to 22 bp, and 33 to 37 bp are set for dark reaction)				32 cycles (1 bp to 6 bp and 17 bp to 22 bp are set for dark reaction)			
Read2	100 cycles				42 cycles (11 bp to 16 bp and 27 bp to 32 bp are set for dark reaction)			
Sample barcode	/				/			
Depth of sequencing	> 50 k Reads/cell				> 50 M reads / library			

9.3 Sequencing requirements of DNBSEQ-T7RS

9.3.1 Pre-experiment preparation

Table 39 Material list

Type	Model No.	Cat. No.
Genetic sequencer	DNBSEQ-T7RS	/
Sequencing set	DNBSEQ-T7RS High-throughput Sequencing Set (FCL PE100) V3.0	940-000269-00
	DNBSEQ-T7RS High-throughput Sequencing Set (FCL PE150) V3.0	940-000268-00

Tips According to the sequencing set, carefully read *DNBSEQ-T7RS High-throughput Sequencing Set User Manual* before starting sequencing, and strictly perform all operations according to the operations in the user manual.

9.3.2 Making DNB

Make DNB according to the following table:

Table 40 DNBSEQ-T7RS making DNB requirements

Sequencing set	PE100		PE150	
Type	cDNA library	Oligo library	cDNA library	Oligo library
Make DNB input	10 ng	6 ng	10 ng	6 ng
RCA time	20 min	20 min	10 min	10 min

9.3.3 Pooling libraries

For details about protocols for pooling different libraries based on the Barcode Primer, refer to *"Appendix 2 Using Barcode Primer" on page 56*.

9.3.4 Sequencing parameters

Table 41 DNBSEQ-T7RS sequencing software version and read length

	cDNA library	Oligo library
Software version	ECR 4.0 and above	
Control software version	1.4.1.812 and above	
Basecall version	1.4.21.72_Ubuntu and above	
Sequencing script	Customized	
Custom Primers	No	
Read1	47 cycles (1 bp to 6 bp, 17 bp to 22 bp, and 33 to 37 bp are set for dark reaction)	32 cycles (1 bp to 6 bp and 17 bp to 22 bp are set for dark reaction)
Read2	100 cycles	42 cycles (11 bp to 16 bp and 27 bp to 32 bp are set for dark reaction)

	cDNA library	Oligo library
Sample barcode	10 cycles	10 cycles
Depth of sequencing	> 50 k Reads/cell	> 50 M reads / library

Appendix 1 About the DNA Clean Beads and purification

Precautions before use of the DNA Clean Beads

- To ensure recovery efficiency of the DNA Clean Beads (hereinafter called the beads), take it out from the 4 °C refrigerator 30 minutes in advance, vortex it to mix thoroughly and equilibrate to room temperature.
- Vortex or invert the beads before use each time, to ensure that the beads are mixed thoroughly.
- A volume of the beads directly affects a lower limit length of the purified DNA fragments. The higher volume of the beads leads to the smaller lower limit length of the purified DNA fragments.

Precautions during purification

- If the volume of to be purified samples decreases due to evaporation caused by incubation, the TE Buffer should be added to complement the liquid to the required volume, and then the recommended volume of the beads is added to perform purification.
- After mixing the sample and the beads thoroughly and placing the centrifuge tube on the magnetic separation rack for separation, remove the supernatant after the solution becomes completely clear. This process usually takes 2 to 5 minutes. Because magnetism of magnetic separation racks might be different, the separation time may be longer, depending on the time in which the solution becomes completely clear.
- When separating the beads from the solution, avoid contact between the pipette tip and the beads. Reserve 2 or 3 µL of the solution, to avoid aspirating the beads. If the beads are aspirated accidentally, pipette down all the beads and the liquid into the centrifuge tube, and re-aspirate the supernatant after separation.
- Rinse the beads by using 80% ethanol that is freshly prepared and equilibrated to room temperature. During rinsing, keep the centrifuge tube on the magnetic separation rack, and operate the pipette tip on the side that is away from the magnetic separation rack. Do not pipette and stir the beads.
- Try to remove all liquid from the tube in second rinsing with the ethanol.

- If some residuals remain on the tube wall, briefly centrifuge the tube, place it on the magnetic separation rack for separation, and remove all liquid at the bottom of the tube by using a pipette with a small measurement range.
- After rinsing the beads with the ethanol two times, completely dry the beads at room temperature. Incomplete drying of the beads (with a reflective surface) easily causes absolute ethanol residuals and affects subsequent reactions, and over-drying of the beads (with cracks) reduces a purification yield. Drying at room temperature usually takes 5 to 10 minutes. The drying time varies with the room temperature and humidity. Proceed with the elution process after observing that the beads have a matte appearance.
- Avoid contact between the pipette tip and the beads when removing the supernatant after elution, as removal of the beads might affect subsequent purification reactions. Therefore, the elution volume should be 2 µL greater than the volume of the aspirated supernatant.
- Carefully open or close the lid of the 1.5 mL centrifuge tube on the magnetic separation rack, to avoid spill of the beads or liquid caused by strong shaking. It is recommended to hold the tube at the middle and lower part with your fingers when opening the lid.

Appendix 2 Using Barcode Primer

Based on different reaction specifications, this set provides either 16 or 32 Barcode Primers: DNBelab C Series Single-cell Library Preparation Sample Barcode Kit S (Cat. No.: 940-001920-00, 32 types; Cat. No.: 940-001926-00, 16 types). The Barcode Primer in the preparation set is designed based on the base balancing principle. To ensure high performance, carefully read rules for using the Barcode Primer.

Tips

- Avoid placing the Barcode Primer at a temperature higher than the room temperature. Otherwise, melting occurs, which affects the performance.
- Mix each tube of scRNA Barcode Primer II thoroughly and centrifuge it before use, and wipe the lid of the tube with the lint-free paper. Gently open the lid during use, to prevent the liquid from splashing and avoid cross-contamination. Close the lid in time after use.

The rules are described as follows:

Based on the base balancing principle, the Barcode Primers should be used individually or in groups.

- First group: The Barcode Primer-1 to Barcode Primer-4 are used as a base balancing barcode group.
- Second group: The Barcode Primer-5 to Barcode Primer-8 are used as a base balancing barcode group.
- Third group: The Barcode Primer-9 to Barcode Primer-12 are used as a base balancing barcode group.
- Fourth group: The Barcode Primer-13 to Barcode Primer-16 are used as a base balancing barcode group.
- Fifth group: The Barcode Primer-17 to Barcode Primer-24 are used as a base balancing barcode group.
- Sixth group: The Barcode Primer-25 to Barcode Primer-32 are used as a base balancing barcode group.

Totally six groups exist. When all samples have the same data amount requirement, the group of Barcode Primer varies according to the number of samples. For details of recommended groups of Barcode Primer, refer to the following table.

Table 42 Recommended method for using Barcode Primer

Sample/ lane	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
1	1 to 4	5 to 8	9 to 12	13 to 16	/	/
2	Sample 1: 1 to 2 Sample 2: 3 to 4	Sample 1: 5 to 6 Sample 2: 7 to 8	Sample 1: 9 to 10 Sample 2: 11 to 12	Sample 1: 13 to 14 Sample 2: 15 to 16	Sample 1: 17 to 20 Sample 2: 21 to 24	Sample 1: 25 to 28 Sample 2: 29 to 32
3	Sample 1: 1 Sample 2: 2 Sample 3: 3 to 4	Sample 1: 5 Sample 2: 6 Sample 3: 7 to 8	Sample 1: 9 Sample 2: 10 Sample 3: 11 to 12	Sample 1: 13 Sample 2: 14 Sample 3: 15 to 16	Sample 1: 17 to 18 Sample 2: 19 to 20 Sample 3: 21 to 24	Sample 1: 25 to 26 Sample 2: 27 to 28 Sample 3: 29 to 32
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	Sample 1: 17 to 18 Sample 2: 19 to 20 Sample 3: 21 to 22 Sample 4: 23 to 24	Sample 1: 25 to 26 Sample 2: 27 to 28 Sample 3: 29 to 30 Sample 4: 31 to 32
5	Sample 1: 1 Sample 2: 2 Sample 3: 3 Sample 4: 4 Sample 5: select any group from the remaining 3 groups	Sample 1: 5 Sample 2: 6 Sample 3: 7 Sample 4: 8 Sample 5: select any group from the remaining 3 groups	Sample 1: 9 Sample 2: 10 Sample 3: 11 Sample 4: 12 Sample 5: select any group from the remaining 3 groups	Sample 1: 13 Sample 2: 14 Sample 3: 15 Sample 4: 16 Sample 5: select any group from the remaining 3 groups	Sample 1: 17 Sample 2: 18 Sample 3: 19 Sample 4: 20 Sample 5: 21 to 24	Sample 1: 25 Sample 2: 26 Sample 3: 27 Sample 4: 28 Sample 5: 29 to 32

Sample/ lane	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
6	Sample 1: 1 Sample 2: 2 Sample 3: 3 Sample 4: 4 Sample 5 and Sample 6: select any two groups from the remaining 3 groups	Sample 1: 5 Sample 2: 6 Sample 3: 7 Sample 4: 8 Sample 5 and Sample 6: select any two groups from the remaining 3 groups	Sample 1: 9 Sample 2: 10 Sample 3: 11 Sample 4: 12 Sample 5 and Sample 6: select any two groups from the remaining 3 groups	Sample 1: 13 Sample 2: 14 Sample 3: 15 Sample 4: 16 Sample 5 and Sample 6: select any two groups from the remaining 3 groups	Sample 1: 17 Sample 2: 18 Sample 3: 19 Sample 4: 20 Sample 5: 21 to 22 Sample 6: 23 to 24	Sample 1: 25 Sample 2: 26 Sample 3: 27 Sample 4: 28 Sample 5: 29 to 30 Sample 6: 31 to 32
7	Sample 1: 1 Sample 2: 2 Sample 3: 3 Sample 4: 4 Sample 5 to 7: select groups by referencing the methods used for 3 samples/lane	Sample 1: 5 Sample 2: 6 Sample 3: 7 Sample 4: 8 Sample 5 to 7: select groups by referencing the methods used for 3 samples/ lane	Sample 1: 9 Sample 2: 10 Sample 3: 11 Sample 4: 12 Sample 5 to 7: select groups by referencing the methods used for 3 samples/lane	Sample 1: 13 Sample 2: 14 Sample 3: 15 Sample 4: 16 Sample 5 to 7: select groups by referencing the methods used for 3 samples/lane	Sample 1: 17 Sample 2: 18 Sample 3: 19 Sample 4: 20 Sample 5: 21 Sample 6: 22 Sample 7: 23 to 24	Sample 1: 25 Sample 2: 26 Sample 3: 27 Sample 4: 28 Sample 5: 29 Sample 6: 30 Sample 7: 31 to 32
8	Select any two groups from the four groups.		17 to 24		25 to 32	
8+x (x= 1 to 8, totally 9 to 16 samples)	Perform the following steps: 1. Classify samples 1 to 8 as a group, and add the Barcode Primer by referencing the methods used for 8 samples/lane. 2. Classify the remaining samples as a group, and correspondingly add different groups of Barcode Primer based on a value of x by referencing the methods used for 1 to 8 samples/lane.					

Tips
- It is required to add a mixture of the scRNA Barcode Primer II-1 to 16 to a sample. Specifically, take the same volume of the N types of scRNA Barcode Primer II, mix them, and add the mixture to the sample.

- Barcode Primer (1 to 16) are used as a base balancing barcode group. 1 to 4 are used as a base balancing barcode group, 5 to 8 are used as a base balancing barcode group, and so on, Barcode Primer (17 to 32) are used as a base balancing barcode group, 17 to 24 are used as a base balancing barcode group, 25 to 32 are used as a base balancing barcode group.

Appendix 3 Manufacturer information

Manufacturer	Qingdao MGI Tech Co., Ltd.
Address	Building 4, No.2, Hengyunshan Road, Qingdao Area, Pilot Free Trade Zone, Shandong, China
Technical support	Qingdao MGI Tech Co., Ltd.
Technical support E-mail	MGI-service@mgi-tech.com

Part No.: H-020-000897-00

Leading Life Science Innovation

Address: Building 4, No.2, Hengyunshan Road, Qingdao Area, Pilot Free Trade Zone, Shandong, China
E-mail: MGI-service@mgi-tech.com
Website: www.mgi-tech.com