DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V3.0

Instructions for use

Version: 1.0

Research Use Only

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 1.0.

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Revision history

	Date	Version
Initial release	January 31, 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:

  www.mgi-tech.com/download/files.

Contents

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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 Cell Omics Solution can realize a portable, instant, and one-stop single-cell omics research workflow.

Based on droplet microfluidic technology, the DNBelab C-Series High-throughput Single-cell RNA Library Preparation Kit V3.0 can rapidly prepare single-cell (nucleus) suspensions into dedicated libraries applicable to MGI's DNBSEQ series sequencing platforms, by utilizing MGI independently designed 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 RNA libraries from eukaryote such as human or mouse samples. 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 preparation set includes 4 boxes.

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

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (TaiM 4) (Box 1 Droplet Generation) (Cat. No.: 940-001820-00)	Cell Beads-V3	White	560 μL/tube × 1
	Index Carrier	White	280 μL/tube×1
	Lysis Buffer-V3	White	72 μL/tube × 1
	Breakage Reagent	Brown	800 μL/tube × 1
	P100 Oil	Natural	7.6 mL/bottle × 1
	Cover Oil	Natural	3.2 mL/bottle × 1
	DNA Clean Beads	Natural	4.32 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)	Beads Buffer	White	728 μL/tube × 1
	Cell Solution-V3	White	142 μL/tube × 1
	RT Primer-V3	White	32 μL/tube × 1
	DIR Regent-V3	White	13 μL/tube × 1
	RT Enzyme-V3	White	64 μL/tube × 1
	RNase Inhibitor	White	32 mL/tube × 1
	cDNA Amp Enzyme	White	400 μL/tube × 1
	cDNA Amp Primer-V3	White	32 μL/tube × 1
DNBelab C Series Flow Cell (TaiM 4) (Cat. No.: 940-001822-00)	scRNA Flow Cell	/	8 EA/box × 1
	Sealing Gasket	/	5 EA/bag × 1

Name	Component	Cap color	Specification
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	White	80 μL/tube × 1
	Frag Buffer-V3	White	40 μL/tube × 1
	DNA Ligase-V3	White	80 μL/tube × 1
	Ligation Buffer-V3	White	160 μL/tube × 1
	scRNA Adapter-V2	Orange	80 μL/tube × 1
	PCR Amp Enzyme	White	1.2 mL/tube × 1
	scRNA Barcode Primer II-1	Red	32 μL/tube × 1
	scRNA Barcode Primer II-2	Red	32 μL/tube × 1
	scRNA Barcode Primer II-3	Red	32 μL/tube × 1
	scRNA Barcode Primer II-4	Red	32 μL/tube × 1
	scRNA Barcode Primer II-5	Red	32 μL/tube × 1
	scRNA Barcode Primer II-6	Red	32 μL/tube × 1
	scRNA Barcode Primer II-7	Red	32 μL/tube × 1
	scRNA Barcode Primer II-8	Red	32 μL/tube × 1
	scRNA Barcode Primer II-9	Red	32 μL/tube × 1
	scRNA Barcode Primer II-10	Red	32 μL/tube × 1
	scRNA Barcode Primer II-11	Red	32 μL/tube × 1
	scRNA Barcode Primer II-12	Red	32 μL/tube × 1
	scRNA Barcode Primer II-13	Red	32 μL/tube × 1
	scRNA Barcode Primer II-14	Red	32 μL/tube × 1
	scRNA Barcode Primer II-15	Red	32 μL/tube × 1
	scRNA Barcode Primer II-16	Red	32 μL/tube × 1

1.5 Storage and transportation condition

Table 2 Transportation and storage condition

Name	Storage temperature	Transportation temperature	Validity period
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (TaiM 4) (Box 1 Droplet Generation) (Cat. No.: 940-001820-00)	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)	-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)	-25 ℃ to -15 ℃ (-13 ℉ to 5 ℉)	-80 ℃ to -15 ℃ (-112 ℉ to 5 ℉)	
DNBelab C Series Flow Cell (TaiM 4) (Cat. No.: 940-001822-00)	10 ℃ to 30 ℃ (50 ℉ to 86 ℉)	0 ℃ to 30 ℃ (32 ℉ to 86 ℉)	

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 3 Self-provided materials

Type	Name	Recommended brand	Cat. No.
Equipment	Single Cell Droplet Generator (Optional)	MGI	900-000637-00
	Clean bench	/	/
	Microscope (For nucleus, counting should be performed 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
	Fragment analyzer	/	/

Type	Name	Recommended brand	Cat. No.
Reagents	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) V2.0	MGI	1000028455
	DNBSEQ-T7RS High-throughput Sequencing Set (FCL PE150) V2.0	MGI	1000028454
	Qubit ssDNA Assay Kit	Invitrogen	Q10212
	Qubit dsDNA HS Assay Kit	Invitrogen	Q32854
	Analytical reagents applicable to the fragment analyzer	/	/

Type	Name	Recommended brand	Cat. No.
Consumables	1 mL syringe	/	/
	40 μm cell strainer, individually packaged	/	/
	0.22 μm filter membrane	PALL	4612
	C-flow cell 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
	1.5 mL centrifuge tube	Axygen	MCT-150-C
	15 mL centrifuge tube	CORNING	430791
	50 mL centrifuge tube	CORNING	430291
	Qubit Assay tube	Invitrogen	Q32856
	0.5 mL thin wall PCR tube	Axygen	PCR-05-C

1.7 Precautions

- This product is for research use only. 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 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, demulsification, reverse transcription, 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 class 100,000 or 300,000 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 in 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 4 Sample requirements

Cell size	Recommended diameter: < 40 μm
Recommended total cell input	A total of 5000 to 30000 cells, with a sample load as follows: • Cell-line samples: 5000 to 30000 cells • PBMC and other tissue dissociation samples: 10000 to 30000 cells
Cell requirements	• Cytoactivity: > 80% • Clumping rate: < 5% • Impurity rate: < 5%

Table 5 Recommended cell input

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

Tips
- N represents the cell concentration.
- It is recommended to calculate the concentration of viable cells when 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 6 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 filter membrane 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 7 PBS (containing 0.04% BSA) preparation

Component	Volume
PBS (without Ca^{2+} and Mg^{2+})	49.8 mL
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

For different samples such as a cell-line or live tissue sample, perform the following steps:

1. Prepare single-cell 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 40 μm cell strainer, quantify the cell 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-flow cell 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 Kit V3.0 (TaiM4). The whole procedure takes about 30 minutes.

3.1 Pre-experiment preparation

3.1.1 Preparing reagents and equipment

Table 8 Required reagent kit and equipment

Type	Name	Component	Cap color	Specification
Reagent	DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (TaiM 4) (Box 1 Droplet Generation) (Cat. No.: 940-001820-00)	Cell Beads-V3	White	560 μL/tube × 1
		Index Carrier	White	280 μL/tube×1
		Lysis Buffer-V3	White	72 μL/tube × 1
		P100 Oil	Natural	7.6 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)	Beads Buffer	White	728 μL/tube × 1
		Cell Solution-V3	White	142 μL/tube × 1
		RT Primer-V3	White	32 μL/tube×1
		DIR Reagent-V3	White	13 μL/tube×1
		RNase Inhibitor	White	32 μL/tube × 1
		RT Enzyme-V3	White	64 μL/tube×1
	DNBelab C Series Flow Cell (TaiM 4) (Cat. No.: 940-001822-00)	scRNA Flow Cell	/	8/box × 1
		Sealing Gasket	/	5/bag × 1
Equipment	DNBelab C-TaiM 4 Droplet Generator (Cat. No.: 900-000637-00)	/	/	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 crystallization in the solution dissolves.
- 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:

1. Gently pipette the cell (nucleus) suspension prepared in *2.3 Preparing cell (nucleus) suspension on Page 11* by using a pipette to mix it thoroughly, and prepare the sample phase suspension according to the following table.

Table 9 Sample phase suspension

Component	Volume (μL) required for each tube
Cell Solution-V3	17.7
RT Primer-V3	4
DIR Reagent-V3	1.6
RNase Inhibitor	4
RT Enzyme-V3	8
PBS (containing 0.04% BSA)	44.7-X
Cell suspension	X
Total	80

Tips
- 'X' indicates the volume of the cell (nucleus) suspension.
- The total number of input cells (nucleus) ranges from 5000 to 30000, and the maximum volume of the cell (nucleus) suspension should be 44.7 μL and might vary according to the cell (nucleus) concentration. If the volume is less than 44.7 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.

2. After the sample phase suspension is prepared, adjust the pipette measurement range to 70 μL and gently pipette it to mix thoroughly.

3. Briefly centrifuge the sample phase suspension, and place it on ice for further use.

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.
5. Adjust the pipette measurement range to 90 µL and gently pipette the mixture to mix it thoroughly. Briefly centrifuge the PCR tube, and place it on ice for subsequent loading.

   Tips
   - 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 scRNA 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.

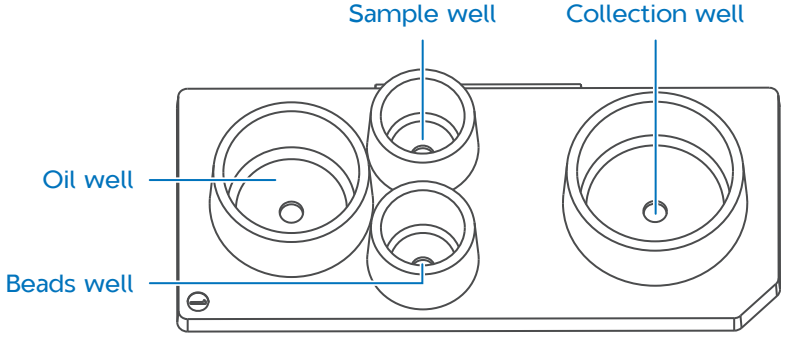

Figure 1 scRNA Flow Cell

2. Take out the Single Cell Droplet Generator.

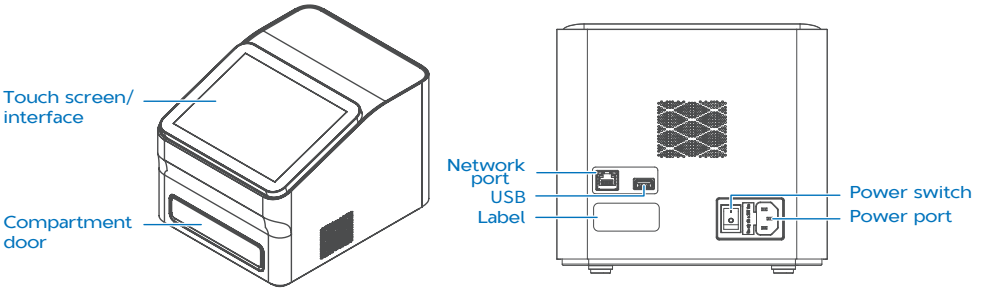

Figure 2 Front view and back view of the single cell droplet generator

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

4. Open the latch on the flow cell holder.

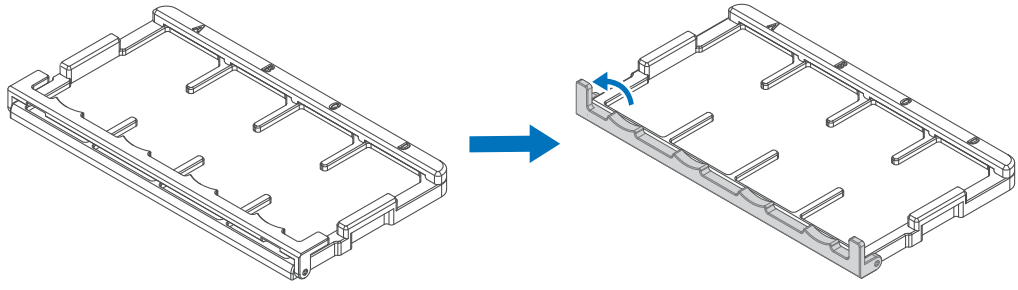

Figure 3 Opening the latch

5. 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.

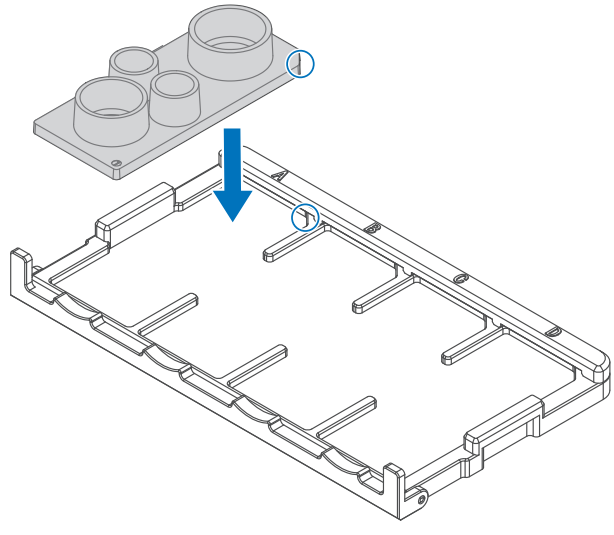

Figure 4 Loading the flow cell

6. 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.

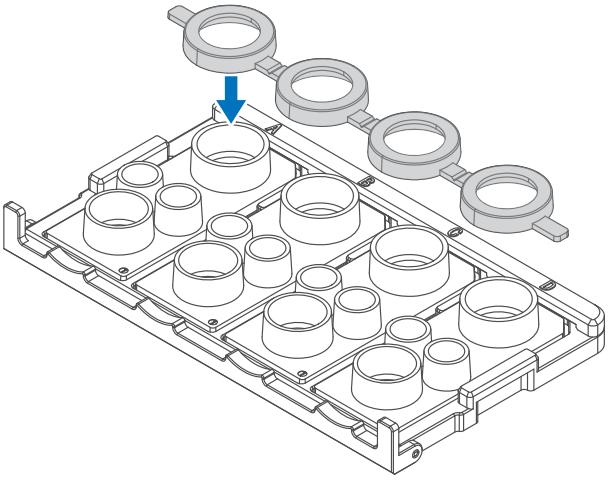

Figure 5 Installing the sealing gasket

Tips

Each flow cell should be equipped with a sealing gasket.

7. After installing the sealing gasket, close the latch until you hear a click to install the flow cells in place.
8. 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.

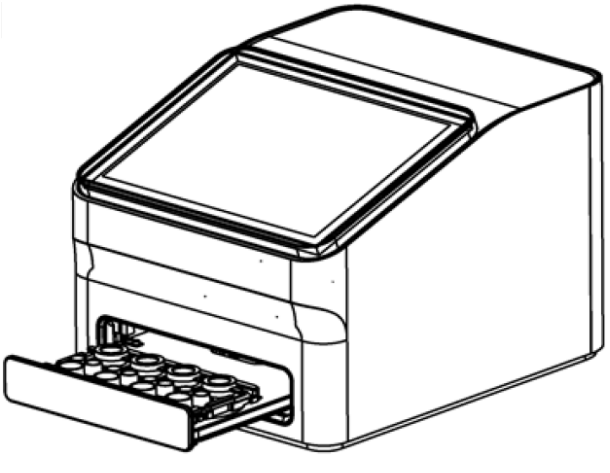

Figure 6 Placing flow cell holder into the flow cell compartment

9. 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.

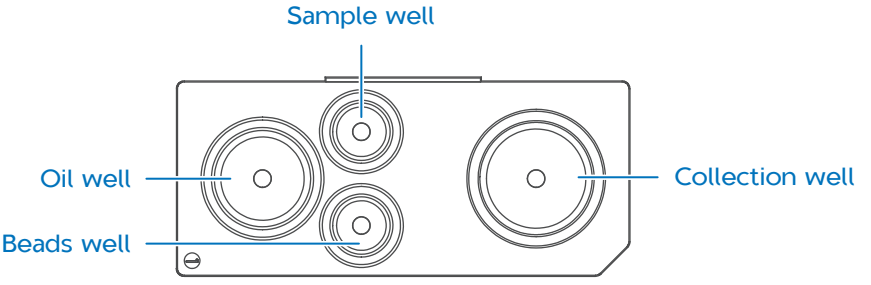

Figure 7 scRNA Flow Cell

Table 10 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

- When adding the solutions, do not suspend the tip, but gently pipette down the sample near the edge of the well.
- 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 formation.

10. Tap **Close** to close the compartment door.
11. Select **mRNA** for the reaction type.
12. 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.

13. Tap ▶ to start the reaction.

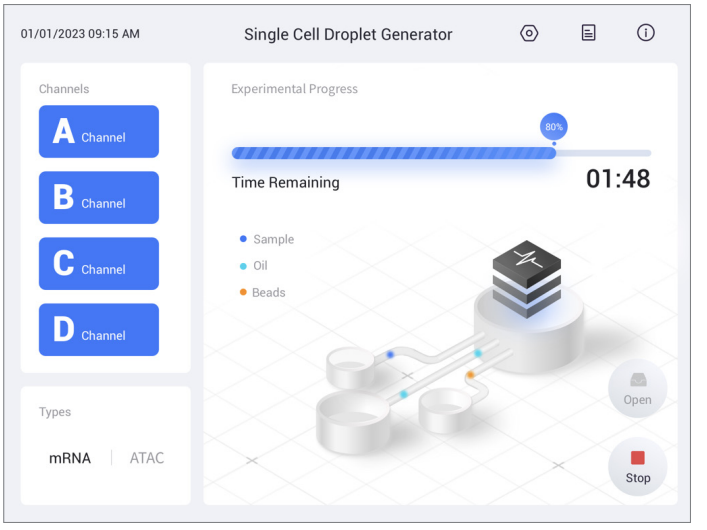

Figure 8 Reaction started

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

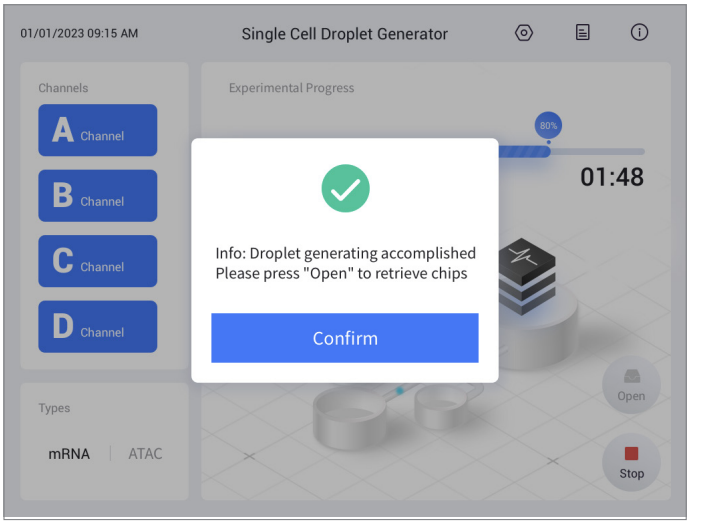

Figure 9 Droplet generating accomplished

15. 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 11 Required reagent kit

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

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

4.2 Collecting droplets

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 tips. 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 PCR tubes, 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.

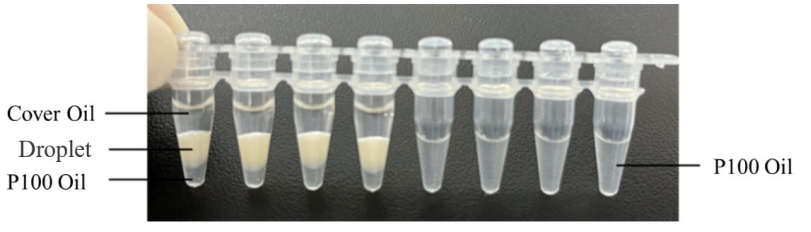

Figure 10 Droplet collection

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

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

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

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 13 Required reagent kit

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (TaiM 4) (Box 1 Droplet Generation) (Cat. No.: 940-001820-00)	Breakage Reagent	Brown	800 mL/tube × 1
	DNA Clean Beads	Natural	4.32 mL/bottle × 1

5.2 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.

   Tips Do not shake or vortex the tube vigorously.

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 110 µL to 120 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 120 µL, add the NF Water to the tube to complement the volume to 120 µL.

   Tips Move gently when aspirating the aqueous phase, to avoid aspirating the oil phase in the bottom layer, the interface layer between the aqueous phase and the oil phase, and the remaining Cover Oil floating at the top layer.

5.3 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 48*.

Perform the following steps:

1. Aspirate 72 µL (0.6×) of DNA Clean Beads into the aqueous phase from step 5 of *5.2 Performing demulsification on Page 23*. 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 500 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”.

    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 96 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 down 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 500 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 32 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 30 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 ℃ to -15 ℃ (-13 ℉ to 5 ℉) for up to six months.

Chapter 6 cDNA product amplification and purification

This chapter describes how to amplify cDNA 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 14 Required reagent kit

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (TaiM 4) (Box 1 Droplet Generation) (Cat. No.: 940-001820-00)	DNA Clean Beads	Natural	4.32 mL/bottle × 1

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 2 Droplet Generation) (Cat. No.: 940-001819-00)	cDNA Amp Enzyme	White	400 μL/tube × 1
	cDNA Amp Primer-V3	White	32 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 products

Perform the following steps:

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

Table 15 cDNA amplification reaction solution

Component	Volume (μL) required for each tube
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 16 cDNA amplification reaction condition (100 μL reaction solution)

Temperature	Time	Cycles
105 ℃ (221 ℉) (heated lid)	On	/
95 ℃ (203 ℉)	3 min	1
98 ℃ (208 ℉)	20 s	X
65 ℃ (149 ℉)	30 s	
72 ℃ (162 ℉)	3 min	
72 ℃ (162 ℉)	5 min	1
4 ℃ (39 ℉)	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), 15 to 17 cycles are recommended.
- For cells or nucleus (with an input of 10000 to 20000) from live tissue, 18 to 20 cycles are recommended based on sample conditions.

Stop point The cDNA 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 48*.

Perform the following steps:

1. Aspirate 60 µL (0.6×) of the DNA Clean Beads to the cDNA products from step 3 in *6.2 Amplifying cDNA products on Page 26* 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 centrifuge 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.

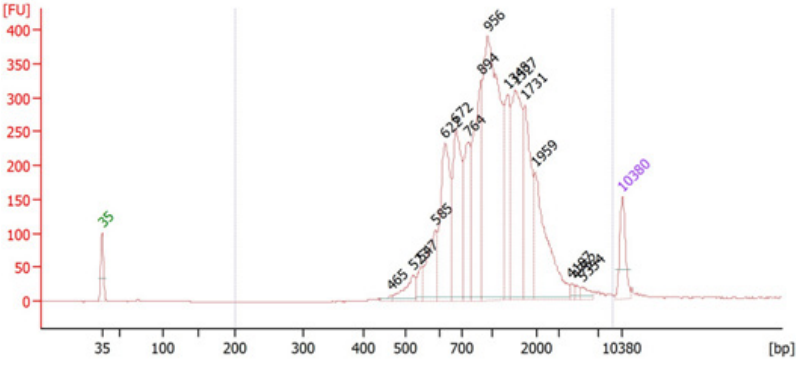

Figure 11 Fragment size distribution of the cDNA product (a reference graph obtained through assessment by using the Agilent 2100 Bioanalyzer)

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 barcode marking on the Oligo product through PCR for subsequent preparation of an Oligo circularization library. The whole procedure takes about 1 hour and 30 minutes.

7.1 Pre-experiment preparation

Table 17 Required reagent kit

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (TaiM 4) (Box 1 Droplet Generation) (Cat. No.: 940-001820-00)	DNA Clean Beads	Natural	4.32 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)	PCR Amp Enzyme	White	1.2 mL/tube × 1
	scRNA Barcode Primer II-1	Red	32 μL/tube × 1
	scRNA Barcode Primer II-2	Red	32 μL/tube × 1
	scRNA Barcode Primer II-3	Red	32 μL/tube × 1
	scRNA Barcode Primer II-4	Red	32 μL/tube × 1
	scRNA Barcode Primer II-5	Red	32 μL/tube × 1
	scRNA Barcode Primer II-6	Red	32 μL/tube × 1
	scRNA Barcode Primer II-7	Red	32 μL/tube × 1
	scRNA Barcode Primer II-8	Red	32 μL/tube × 1
	scRNA Barcode Primer II-9	Red	32 μL/tube × 1
	scRNA Barcode Primer II-10	Red	32 μL/tube × 1
	scRNA Barcode Primer II-11	Red	32 μL/tube × 1
	scRNA Barcode Primer II-12	Red	32 μL/tube × 1
	scRNA Barcode Primer II-13	Red	32 μL/tube × 1
	scRNA Barcode Primer II-14	Red	32 μL/tube × 1
	scRNA Barcode Primer II-15	Red	32 μL/tube × 1
	scRNA Barcode Primer II-16	Red	32 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.

7.2 Preparing the Oligo library

Perform the following steps:

1. Take out a new 0.2 mL PCR tube, aspirate 10 µL of the "Oligo Product 2" from step 23 in *5.3 Performing size selection of magnetic beads on Page 23* to the PCR tube. Prepare Oligo library preparation reaction solution according to the following table:

Table 18 Oligo library preparation reaction solution

Component	Volume (µL) required for each tube
Oligo Product 2	10
NF Water	11
scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16 (16 optional components)	4
PCR Amp Enzyme	25
Total	50

Tips
- Before starting Oligo library preparation, carefully read *Appendix 2 Using scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16 on Page 49*.
- Record the number of the scRNA Barcode Primer II 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 19 Oligo library preparation condition (50 µL reaction solution)

Temperature	Time	Cycles
105 ℃ (221 ℉) (heated lid)	On	/
95 ℃ (203 ℉)	3 min	1
98 ℃ (208 ℉)	15 s	9
60 ℃ (140 ℉)	30 s	
72 ℃ (162 ℉)	10 s	
72 ℃ (162 ℉)	5 min	1
4 ℃ (39 ℉)	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 48*.

Perform the following steps:

1. Aspirate 30 µL of the DNA Clean Beads to the PCR tube from step 3 in *7.2 Preparing the Oligo library on Page 30* 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 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.

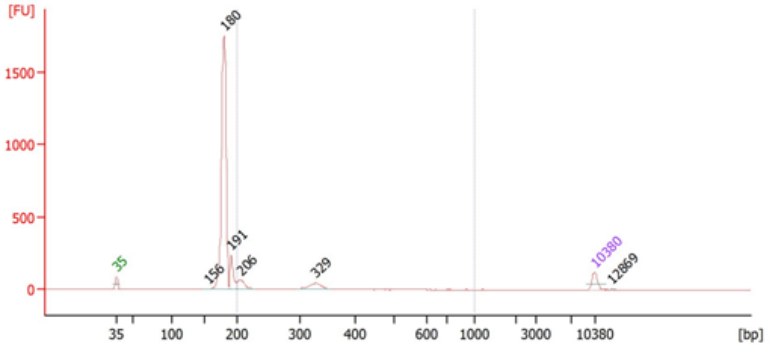

Figure 12 Fragment size distribution of the Oligo library (a reference graph obtained through assessment by using the Agilent 2100 Bioanalyzer)

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 40*.

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 20 Required reagent kit

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (TaiM 4) (Box 1 Droplet Generation) (Cat. No.: 940-001820-00)	DNA Clean Beads	Natural	4.32 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)	Frag Enzyme-V3	White	80 μL/tube × 1
	Frag Buffer-V3	White	40 μL/tube × 1
	DNA Ligase-V3	White	80 μL/tube × 1
	Ligation Buffer-V3	White	160 μL/tube × 1
	scRNA Adapter-V2	Orange	80 μL/tube × 1
	PCR Amp Enzyme	White	1.2 mL/tube × 1
	scRNA Barcode Primer II-1	Red	32 μL/tube × 1
	scRNA Barcode Primer II-2	Red	32 μL/tube × 1
	scRNA Barcode Primer II-3	Red	32 μL/tube × 1
	scRNA Barcode Primer II-4	Red	32 μL/tube × 1
	scRNA Barcode Primer II-5	Red	32 μL/tube × 1
	scRNA Barcode Primer II-6	Red	32 μL/tube × 1
	scRNA Barcode Primer II-7	Red	32 μL/tube × 1
	scRNA Barcode Primer II-8	Red	32 μL/tube × 1
	scRNA Barcode Primer II-9	Red	32 μL/tube × 1
	scRNA Barcode Primer II-10	Red	32 μL/tube × 1
	scRNA Barcode Primer II-11	Red	32 μL/tube × 1
	scRNA Barcode Primer II-12	Red	32 μL/tube × 1
	scRNA Barcode Primer II-13	Red	32 μL/tube × 1
	scRNA Barcode Primer II-14	Red	32 μL/tube × 1
	scRNA Barcode Primer II-15	Red	32 μL/tube × 1
	scRNA Barcode Primer II-16	Red	32 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.

8.2 Performing fragmentation and end repair

Perform the following steps:

1. Take out the Frag Enzyme-V3, mix and briefly centrifuge it, and place it on ice for further use.
2. Prepare a fragmentation and end repair reaction solution on ice according to the following table:

Table 21 Fragmentation and end repair reaction solution

Component	Volume (μL) required for each tube
Frag Buffer-V3	5
NF Water	25
cDNA Product	10
Total	40

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.

3. Aspirate 10 μL of the Frag Enzyme-V3 to the PCR tube of step 2 by using a pipette.
4. Vortex the PCR tube to mix the liquid thoroughly.
5. Briefly centrifuge the liquid to collect the reaction solution to the bottom of the tube.
6. After the temperature of the PCR device decreases to 4 °C (39 °F), place the PCR tube in the PCR device and proceed with the reaction according to the condition shown in the following table.

Table 22 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 23 Adapter ligation reaction solution

Component	Volume (μL) required for each tube
Ligation Buffer-V3	20
DNA Ligase-V3	10
scRNA Adapter-V2	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 6 in *8.2 Performing fragmentation and end repair on Page 34* 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 ℃ (77 ℉), open the cover of the PCR device for the reaction.

Table 24 Adapter ligation reaction condition (100 μL reaction solution)

Temperature	Time
Heated lid	Off
20 ℃ (68 ℉)	15 min
4 ℃ (39 ℉)	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 48*.

Perform the following steps:

1. Add 100 µL 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 35* 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 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 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 in a -25 °C to -15 °C (-13 °F to 5 °F) refrigerator for up to 24 hours.

8.5 Performing PCR amplification

Tips Before starting PCR amplification, carefully read *Appendix 2 Using scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16 on Page 49*.

Perform the following steps:

1. Add 4 µL of the scRNA Barcode Primer II to the PCR tube with the supernatant from step 29 in *8.4 Performing purification and size selection on adapter ligation product on Page 36*, and record the number of the scRNA Barcode Primer II added to each sample.
2. Add 50 µL of the PCR Amp Enzyme to the reaction solution in the previous step.
3. Vortex the liquid to mix it thoroughly, and briefly centrifuge the liquid to collect the reaction solution to the bottom of the tube.
4. Place the PCR tube in the PCR device to proceed with the PCR reaction according to the condition shown in the following table:

Table 25 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 48*.

Perform the following steps:

1. Add 55 μL 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 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.

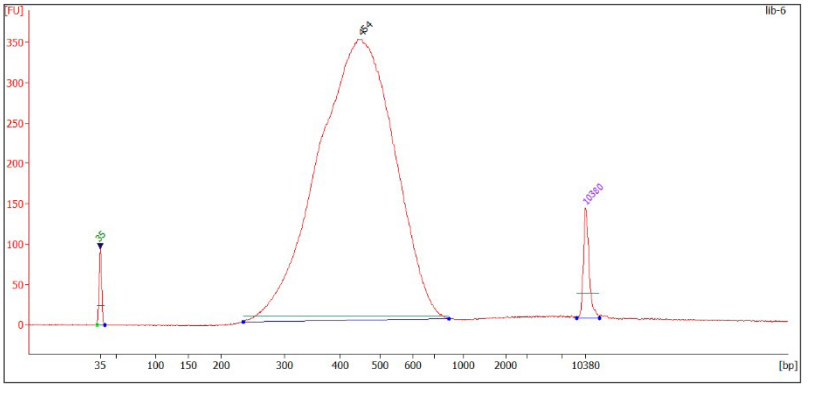

Figure 13 Fragment size distribution of the cDNA library (a reference graph obtained through assessment by using the Agilent 2100 Bioanalyzer)

Stop point The size selection product 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 26 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.

9.1 cDNA and Oligo library structures

The cDNA and Oligo library structures are shown as follows:

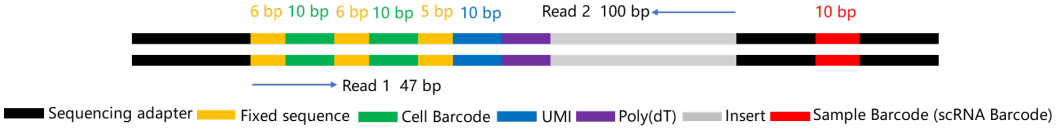

Figure 14 cDNA library structure

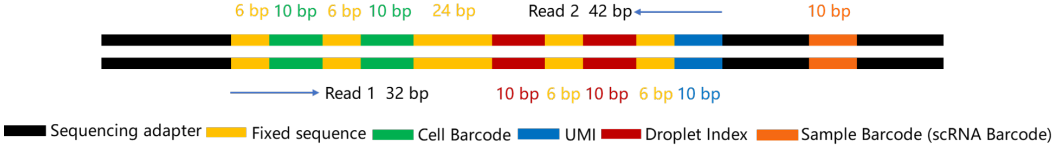

Figure 15 Oligo library structure

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 = 12 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 27 Material list

Type	Model No.	Cat. No.
Genetic sequencer	DNBSEQ-G400RS	/
Sequencing set	HotMPS High-throughput Sequencing Set (G400 HM FCL PE100)	940-000489-00
	HotMPS High-throughput Sequencing Set (G400 HM FCL PE150)	940-000244-00
	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 HotMPS High-throughput Sequencing Set User Manual* or *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 28 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 scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16, refer to *Appendix 2 Using scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16 on Page 49*.

9.2.4 Sequencing parameter

Table 29 DNBSEQ-G400RS (HotMPS) sequencing software version and read length (in the case of pooling samples and sequencing the barcode)

Type	cDNA library	Oligo library
Software version	ECR 1.0	ECR 1.0
Control software version	1.7.1.1799	1.7.1.1799
Basecall version	1.5.0.323	1.5.0.323
Sequencing script	C4_scRNA_BC	C4_Oligo_BC
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	> 60 k reads/cell	> 50 M reads/library

Table 30 DNBSEQ-G400RS (HotMPS) sequencing software version and read length (in the case of neither pooling samples not sequencing the barcode)

Type	cDNA library	Oligo library
Software version	ECR 1.0	ECR 1.0
Control software version	1.7.1.1799	1.7.1.1799
Basecall version	1.5.0.323	1.5.0.323
Sequencing script	C4_scRNA_noBC	C4_Oligo_noBC

Type	cDNA library	Oligo library
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	> 60 k reads/cell	> 50 M reads/library

Table 31 DNBSEQ-G400RS (StandardMPS) sequencing software version and read length (in the case of pooling samples and sequencing the barcode)

Type	cDNA library			Oligo library		
Software version	ECR 3.0	ECR 4.0	ECR 6.0	ECR 3.0	ECR 4.0	ECR 6.0
Control software version	DNBSEQ-G400_1.0.0.34 or later version					
Basecall version	Basecall_1.0.8.208 or later version					
Sequencing script	C4_scRNA_BC_PE47+100+10	C4_scRNA_BC_PE47+100+10-ECR4.0	Z_C4_scRNA_BC-ECR6.0	C4_Oligo_BC_PE32+42+10	C4_Oligo_BC_PE32+42+10-ECR4.0	Z_C4_Oligo_BC-ECR6.0
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	> 60 k reads/cell			> 50 M reads/library		

Table 32 DNBSEQ-G400RS (StandardMPS) sequencing software version and read length (in the case of neither pooling samples not sequencing the barcode)

Type	cDNA library			Oligo library		
Software version	ECR 3.0	ECR 4.0	ECR 6.0	ECR 3.0	ECR 4.0	ECR 6.0
Control software version	DNBSEQ-G400_1.0.0.34 or later version					
Basecall version	Basecall_1.0.8.208 or later version					
Sequencing script	C4_scRNA_noBC_PE47+100	C4_scRNA_noBC_PE47+100-ECR4.0	Z_C4_scRNA_noBC-ECR6.0	C4_Oligo_noBC_PE32+42	C4_Oligo_noBC_PE32+42-ECR4.0	Z_C4_Oligo_noBC-ECR6.0
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	> 60 k reads/cell			> 50 M reads/library		

9.3 Sequencing requirements of DNBSEQ-T7RS

9.3.1 Pre-experiment preparation

Table 33 Material list

Type	Model No.	Cat. No.
Genetic sequencer	DNBSEQ-T7RS	/
Sequencing set	DNBSEQ-T7RS High-throughput Sequencing Set (FCL PE100) V2.0	1000028455
	DNBSEQ-T7RS High-throughput Sequencing Set (FCL PE150) V2.0	1000028454

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 34 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

Tips
- When using the PE100 sequencing set, 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.
- When using the PE150 sequencing set, 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 20 minutes when making DNB.

9.3.3 Pooling libraries

For details about protocols for pooling different libraries based on the scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16, refer to *Appendix 2 Using scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16 on Page 49*.

9.3.4 Sequencing parameters

Table 35 DNBSEQ-T7RS sequencing software version and read length

Type	cDNA library	Oligo library
Software version	ECR 3.0 and above	
Control software version	1.3.3.553 and above	
Basecall version	1.4.2.47_Ubuntu and above	
Sequencing script	Customized	

Type	cDNA library	Oligo library
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)
Sample barcode	10 cycles	10 cycles
Depth of sequencing	> 60 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 ℃ 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 scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16

This preparation set includes 16 tubes of scRNA Barcode Primer II, that is, DNBelab C Series High-throughput Single-cell RNA Library Preparation Kit V3.0 (Box 3 Library Preparation). The scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16 in the preparation set are designed based on the base balancing principle. To ensure high performance, carefully read rules for using the scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16.

Tips
- Avoid placing the 16 tubes of scRNA Barcode Primer II 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 scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16 are used individually or in groups.

- First group: The scRNA Barcode Primer II-1 to scRNA Barcode Primer II-4 are used as a base balancing barcode group.

- Second group: The scRNA Barcode Primer II-5 to scRNA Barcode Primer II-8 are used as a base balancing barcode group.
- Third group: The scRNA Barcode Primer II-9 to scRNA Barcode Primer II-12 are used as a base balancing barcode group.
- Fourth group: The scRNA Barcode Primer II-13 to scRNA Barcode Primer II-16 are used as a base balancing barcode group.

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

Table 36 Recommended method for using scRNA Barcode Primer II

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

Samples/lane	Method 1	Method 2	Method 3	Method 4
6	• Sample 1: 1 • Sample 2: 2 • Sample 3: 3 • Sample 4: 4 • Samples 5 and 6: select any two groups from the remaining 3 groups	• Sample 1: 5 • Sample 2: 6 • Sample 3: 7 • Sample 4: 8 • Samples 5 and 6: select any two groups from the remaining 3 groups	• Sample 1: 9 • Sample 2: 10 • Sample 3: 11 • Sample 4: 12 • Samples 5 and 6: select any two groups from the remaining 3 groups	• Sample 1: 13 • Sample 2: 14 • Sample 3: 15 • Sample 4: 16 • Samples 5 and 6: select any two groups from the remaining 3 groups
7	• Sample 1: 1 • Sample 2: 2 • Sample 3: 3 • Sample 4: 4 • Samples 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 • Samples 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 • Samples 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 • Samples 5 to 7: select groups according to the methods used for 3 samples/lane
8	Select any two groups from the four groups.			
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 scRNA Barcode Primer II by referencing the methods used for 8 samples/lane. 2. Classify the remaining samples as a group, and correspondingly add different groups of scRNA Barcode Primer II 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.

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

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E-mail: MGI-service@mgi-tech.com
Website: www.mgi-tech.com