Part No.: H-020-000552-00

DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0

User Manual

Version: 1.0

Leading Life Science Innovation

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About the user manual

This user manual is applicable to DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (hereinafter called preparation set). The manual version is 1.0, and the set version is V1.0.

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Figures in this user manual are all illustrations. The content might be slightly different from the set, please refer to the set purchased.

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Revision history

	Date	Version
Initial release	November 11, 2022	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.

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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 proprietary 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 microfluidics, the preparation set, together with the Cell Beads, Index Carrier, and single-cell RNA library preparation kit, cooperates with a self-designed DNBelab C Series C4 Station, to quickly prepare the dedicated libraries applicable to DNBSEQ sequencing platforms of MGI by using single-cell or single-cell nucleus suspension. This product is used together with a demulsification recovery system, the Cell Beads, and the Index Carrier, to improve beads recovery efficiency, increase the amount of captured mRNA, decrease a contamination rate, and increase a gene detection capability of the single-cell RNA library. All reagents, chips, 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 human or mouse samples. Before use, it is necessary to prepare single-cell or 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 5 boxes.

Table 1 DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (16 RXN) (Cat. No.: 940-000519-00)

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 1 Droplet Formation kit) (Cat. No.: 940-000508-00)	Cell Beads-V2	White	1.6 mL/tube × 1
	Lysis Buffer	Black	1.584 mL/tube × 1
	Cell Solution	Blue	528 μL/tube×1
	Index Carrier	Green	608 μL/tube×1
	Suspension Reagent-V2	Brown	320 μL/tube × 1
	P50 Oil	Natural	12.8 mL/bottle × 1
	Stop Buffer	Natural	12.8 mL/tube × 1
	Denaturation Buffer (10×)	Natural	6.4 mL/bottle × 1
	DNA Clean Beads	Natural	7.19 mL/bottle × 2
	Collection Buffer	Brown	22.4 mL/bottle × 1
	Wash Buffer	Brown	32 mL/bottle × 1
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 2 Droplet Formation kit) (Cat. No.: 940-000509-00)	DIR Reagent-V2	Black	176 μL/tube × 1
	RNase Inhibitor	Brown	176 μL/tube × 1
	RT Buffer	Blue	1.54 mL/tube × 2
	RT Primer-V2	Blue	160 μL/tube × 1
	RT Enzyme	Blue	160 μL/tube × 1
	D Buffer	Orange	320 μL/tube × 1
	D Enzyme	Orange	160 μL/tube × 1
	Second Strand Buffer-V2	Green	1.08 mL/tube × 2
	Second Strand Primer-V2	Green	240 μL/tube × 1
	Second Strand Enzyme-V2	Green	800 μL/tube × 1
	cDNA Amp Enzyme	White	800 μL/tube × 2
	cDNA Amp Primer-V2	White	128 μL/tube × 1

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 3 Library Preparation kit) (Cat. No.: 940-000510-00)	Frag Enzyme-V2	Purple	80 μL/tube × 1
	Frag Buffer-V2	Purple	160 μL/tube × 1
	DNA Ligase-V2	Orange	80 μL/tube × 1
	Ligation Buffer-V2	Orange	320 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
DNBelab C Series C4 Station (Cat. No.: 940-000507-00)	C4 station	/	1
DNBelab C Series C4 Chip V2.0 (Cat. No.: 940-000506-00)	C4 scRNA chip	/	16/box × 1
	C4 filter connection hose	/	1/case × 1
	C4 filter	/	16/case × 1
	C4 chip sleeve	/	16/box × 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 Set V2.0 (Box 1 Droplet Formation kit) (Cat. No.: 940-000508-00)	2 ℃ to 8 ℃	2 ℃ to 8 ℃	Refer to the label on the box.
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 2 Droplet Formation kit) (Cat. No.: 940-000509-00)	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃	
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 3 Library Preparation kit) (Cat. No.: 940-000510-00)	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃	
DNBelab C Series C4 Station (Cat. No.: 940-000507-00)	10 ℃ to 30 ℃	0 ℃ to 30 ℃	
DNBelab C Series C4 Chip V2.0 (Cat. No.: 940-000506-00)	10 ℃ to 30 ℃	0 ℃ to 30 ℃	

Tips

- The kits with the transportation temperature of -80 ℃ to -15 ℃ should be 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	Clean bench	/	/
	Microscope	/	/
	Tips A fluorescence microscope is required for counting the number of nuclei.		
	Electronic balance	/	/
	Mini desktop vacuum pump or equivalents	Kylin-Bell	GL-802A
	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	/	/
	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	A600903-0010
	SSC (20×)	Invitrogen	AM9763
	DAPI	Sigma-Aldrich	D9542
	Trypan Blue Solution, 0.4%	Gibco	15250061
	Nuclease-free water (NF Water)	Ambion	AM9937
	TE buffer, pH 8.0	Ambion	AM9858
	Absolute ethanol (analytical grade)	/	/
	MGIEasy Circularization Kit	MGI	1000005259
	DNBSEQ-G400RS High-throughput Sequencing Set (FCL PE100)	MGI	1000016950
	DNBSEQ-T7RS High-throughput Sequencing Set (FCL PE100)	MGI	1000028455
	Qubit ssDNA Assay Kit	Invitrogen	Q10212
	Qubit dsDNA HS Assay Kit	Invitrogen	Q32854
	Analytical reagents applicable to the fragment analyzer	/	/
Consumables	1 mL syringe	/	/
	40 μm cell strainer, blue, individually packaged	CORNING	352340
	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	/

Type	Name	Recommended brand	Cat. No.
Consumables	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 formation, 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 (nucleus) 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/nucleus 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: 10000 to 30000 cells • PBMC, nucleus, 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	208 < N < 2000
10000	416 < N < 2000
20000	832 < N < 2000
30000	1250 < 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.
- 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:

- BSA

Table 6 10% BSA

Component	Volume
BSA powder	1 g
PBS (without Ca2+ and Mg2+)	10 mL

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

Tips The 10% BSA can be stored at -20 ℃ to -15 ℃ for up to 6 months.

- PBS

Table 7 PBS (containing 0.04% BSA)

Component	Volume
PBS	49.8 mL
10% BSA	200 μL

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

Tips The PBS can be stored at 2 ℃ to 8 ℃ for up to 1 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 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.

- For a nucleus 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 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 cell (nucleus) sample thoroughly by pipetting with a wide-bore tip.

  CAUTION When cells (nuclei) 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 formation

This chapter describes how to form droplets from the prepared cell (nucleus) suspension by using the preparation set, and how to perform cytolysis and capture mRNA with magnetic beads. The whole procedure takes about 50 minutes.

Tips
- It is recommended to perform the operations at normal atmospheric pressure. Do not carry out the droplet formation experiment in a positive or negative pressure laboratory.
- It is recommended to use low-binding filter tips and low-binding centrifuge tubes in the experiment.

3.1 Pre-experiment preparation

3.1.1 Preparing reagents and device

Table 8 Required reagent kit and device

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 1 Droplet Formation kit) (Cat. No.: 940-000508-00)	Cell Beads-V2	White	1.6 mL/tube × 1
	Lysis Buffer	Black	1.584 mL/tube × 1
	Cell Solution	Blue	528 μL/tube×1
	Index Carrier	Green	608 μL/tube×1
	P50 Oil	Natural	12.8 mL/bottle × 1
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 2 Droplet Formation kit) (Cat. No.: 940-000509-00)	DIR Reagent-V2	Black	176 μL/tube × 1
	RNase Inhibitor	Brown	176 μL/tube × 1
DNBelab C Series C4 Station (Cat. No.: 940-000507-00)	C4 station	/	1
DNBelab C Series C4 Chip V2.0 (Cat. No.: 940-000506-00)	C4 scRNA chip	/	16/box × 1
	C4 chip sleeve	/	16/box × 1

Tips Take out the P50 Oil for at least 30 minutes in advance to equilibrate to room temperature.

3.1.2 Preparing cell (nucleus) reaction solution

Tips Appropriately increase the volume of the cell (nucleus) reaction solution when preparing the reaction solution, to avoid a volume insufficiency (less than 80 μL) of the reaction solution in subsequent droplet formation.

Perform the following steps:

1. Gently pipette the cell (nucleus) suspension prepared in *Preparing cell (nucleus) suspension on Page 10* by using a pipette to mix it thoroughly, and prepare the cell (nucleus) reaction solution according to the following table.

Table 9 Cell (nucleus) reaction solution

Component	Volume (μL) required for each tube
Cell Solution	24
Index Carrier	28
RNase Inhibitor	4
PBS (containing 0.04% BSA)	24-X
Cell suspension	X
Total	80

Tips
- X indicates the volume of the cell suspension.
- The total number of input cells (nuclei) ranges from 5000 to 30000, and the volume of the cell (nucleus) suspension should be 24 μL and might vary according to the cell (nucleus) concentration. If the volume is less than 24 μL, the PBS (containing 0.04% BSA) is added as a complement.

2. After the reaction solution is prepared, gently pipette it to mix thoroughly by using a pipette with the measurement range of 70 μL.
3. Briefly centrifuge the reaction solution, and place it on ice for further use.

Tips
- 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 (nuclei) 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.

3.1.3 Preparing beads suspension

Tips
- You should prepare the beads suspension in the clean bench.
- Appropriately increase the volume of the beads suspension when preparing the beads reaction solution, to avoid a volume insufficiency (less than 80 μL) of the beads suspension in subsequent droplet formation.

Perform the following steps:

1. Take out the Cell Beads-V2, and gently invert or pipette it to mix it thoroughly.
2. Aspirate 100 μL of the Cell Beads-V2 (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.

4. Gently remove and dispose of the supernatant, to avoid loss of the beads.
5. Remove the PCR tube from the magnetic separation rack, and add 72 μL of the Lysis Buffer.
6. Add 8 μL of the DIR Reagent-V2 to the beads suspension.
7. Gently pipette the mixture to mix it thoroughly by using the pipette with the measurement range of 70 μL.

   Tips The DIR Reagent-V2 should be added to the beads suspension before droplet formation, and the mixture should be gently pipetted and mixed thoroughly, to avoid formation of bubbles.

8. Briefly centrifuge the PCR tube, and place it on ice for subsequent loading.

   Tips
   - A capacity of a PCR tube varies with the number of samples.
   - If cells (nuclei) 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.

3.2 Performing droplet formation

Perform the following steps:

1. Take out the C4 scRNA chip and C4 station, and place the C4 station on a horizontal table.

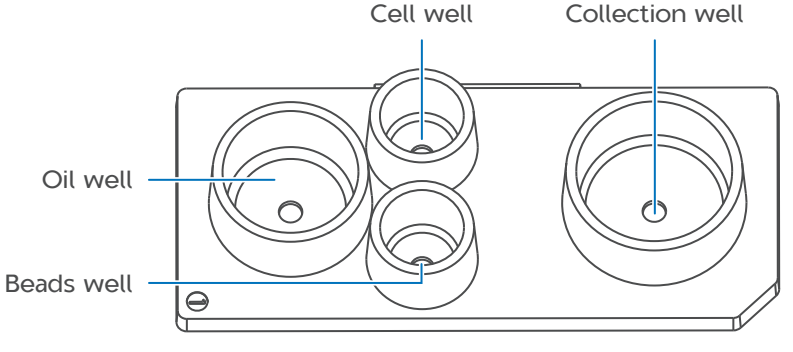

Figure 1 C4 scRNA chip

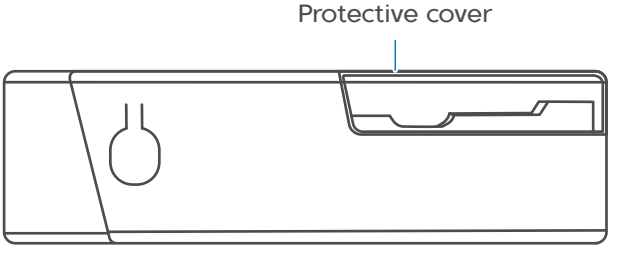

Figure 2 C4 station

2. Perform the following steps:

   1) Gently and evenly press the buckle on the protective cover with the right thumb, and hold the other end with the other fingers.

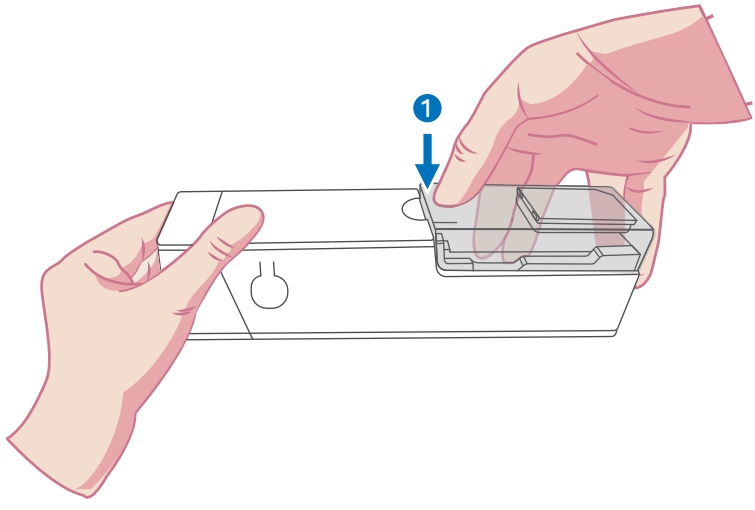

Figure 3 Pressing the front end

   2) Gently lift up the protective cover to open it.

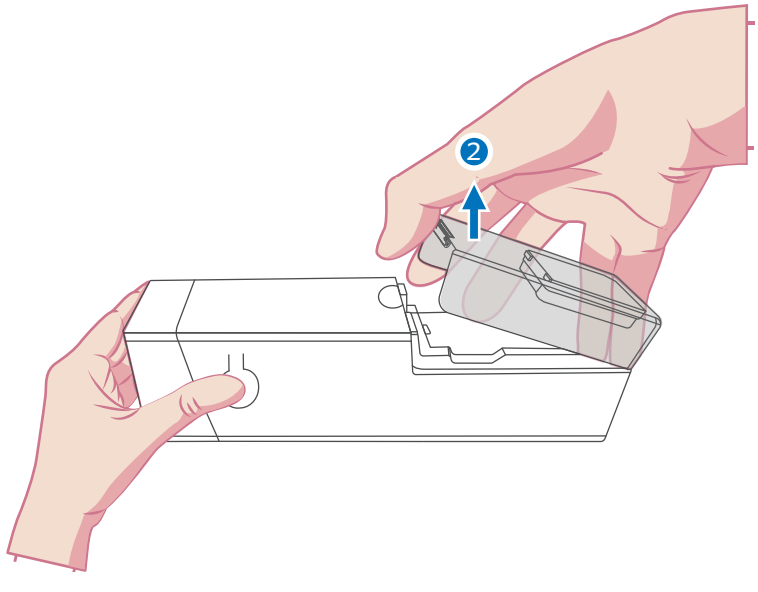

Figure 4 Opening the protective cover

3. Clean the inner pad of the protective cover with the 75% ethanol before the experiment.
4. Install the chip by pushing it into the chip slot from the side.

The left edge of the chip should fit exactly with the left edge of the chip slot without any gap.

5. Add the solutions to the wells according to the following table:

Order	Name	Volume	Well name
1	Cell (nucleus) suspension	80 μL	Cell well
2	P50 Oil	800 μL	Oil well
3	Beads suspension	80 μL	Beads well

The chip with the solutions added is shown as follows:

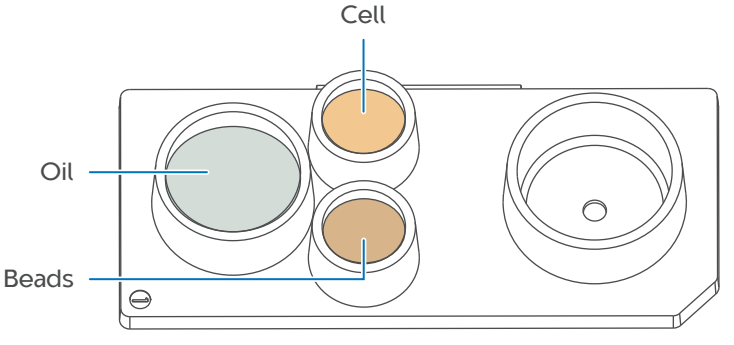

Figure 5 Chip with the solutions added

Tips
- Before adding the cell (nucleus) suspension and the beads suspension, separately pipette them to mix thoroughly. Ensure that no bubble forms during pipetting.
- 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.

6. Close the protective cover by performing the following steps:
   1) Insert the tail end of the protective cover into the groove of the outer housing of the C4 station.
   2) Press and hold the other end of the C4 station with the left thumb.

3) Press the front end of the protective cover into the groove with the right thumb until a click is heard.

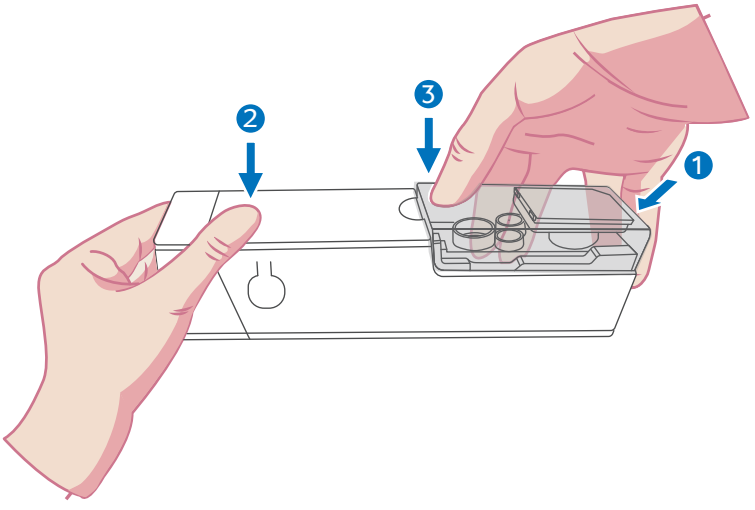

Figure 6 Closing the protective cover

7. Hold the front of the C4 station and pull out the tail until a click is heard.

   Then, droplet formation starts. In the first 1 or 2 minutes during droplet formation, carefully observe whether droplets are normally formed in the collection well.

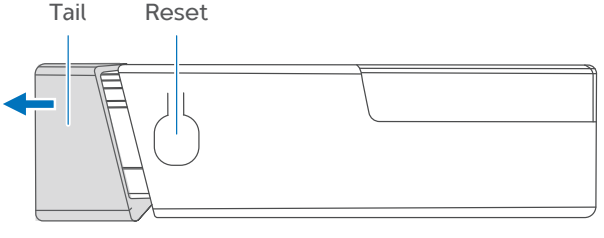

Figure 7 Starting droplet formation

Tips Keep the C4 station stable when pulling out the tail. Otherwise, the liquid might spill, affecting droplet formation.

8. After droplet formation is performed (about 9 minutes later), press the buckle to loosen the protection cover, so as to stop droplet collection.

   CAUTION Strictly keep droplet formation time within 9 minutes.

9. Incubate the collected solution at room temperature for 20 minutes.

CAUTION

- Incubation at room temperature aims to fully hybridize primers on the beads with mRNA. Short incubation time might reduce mRNA capturing efficiency. Long incubation time (which should not exceed 30 minutes) might cause mRNA degradation.
- During incubation, do not move the chip to prevent droplets in the collection well from splashing.
- During incubation, keep the protective cover closed but not buckled, to prevent foreign matters from falling into the station.

10. Take down the protection cover, mount the chip sleeve on the chip by correctly aligning the sleeve with the well to prevent the droplets from splashing, and remove the chip for subsequent demulsification.

Tips The droplets should be used for demulsification immediately.

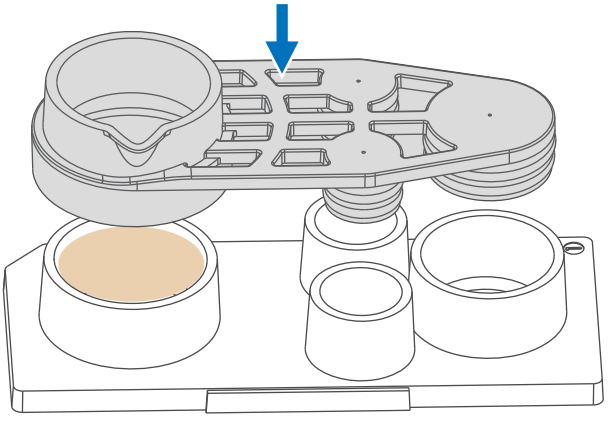

Figure 8 Aligning the chip sleeve

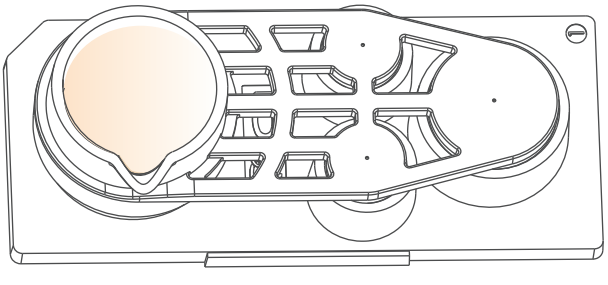

Figure 9 Chip with the sleeve mounted

11. Press and hold the reset button on both sides of the C4 station, and push the tail to its original position.
12. Clean the inner pad of the protective cover with the 75% ethanol.

Chapter 4 Demulsification

This chapter describes how to recover single-cell beads by using a demulsification recovery system (with self-provided vacuum pump). The whole procedure takes about 20 minutes.

Tips It is recommended to use low-binding filter tips and low-binding centrifuge tubes in the demulsification experiment.

4.1 Pre-experiment preparation

4.1.1 Preparing reagents and device

Table 10 Required reagent kit and device

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 1 Droplet Formation kit) (Cat. No.: 940-000508-00)	Collection Buffer	Brown	22.4 mL/bottle × 1
DNBelab C Series C4 Chip V2.0 (Cat. No.: 940-000506-00)	C4 filter connection hose	/	1/case × 1
	C4 filter	/	16/case × 1

4.1.2 Preparing self-provided reagents

Perform the following steps:

1. Prepare 6×SSC according to the following table:

Table 11 6×SSC

Name	Volume
20×SSC	15 mL
NF Water	35 mL

2. Vortex the reagents to mix thoroughly for further use.

4.1.3 Preparing consumables

Prepare the following consumables:

- 50 mL centrifuge tubes
- C4 filter
- Vacuum pump
- C4 filter connection hose

4.2 Performing demulsification

Perform the following steps:

1. Connect the C4 filter and the tubes B and A of the vacuum pump (not shown in the following figure), and turn on the vacuum pump.

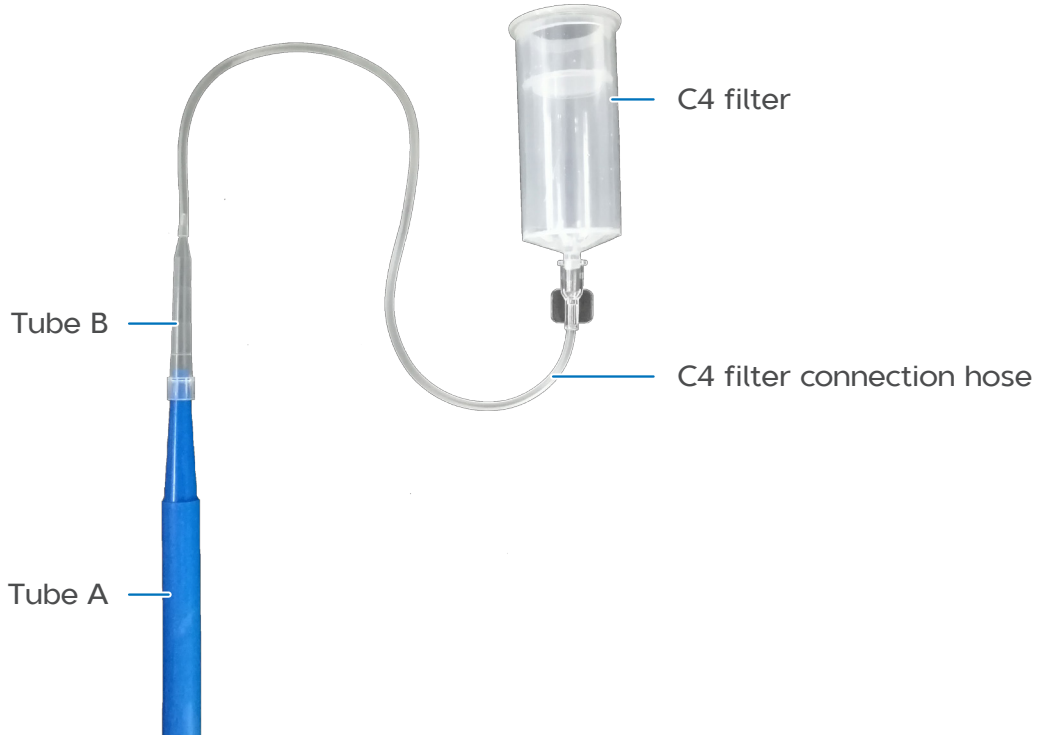

Figure 10 Connecting the C4 filter

Tips
- The vacuum pump is equipped with the tubes A and B.
- The tubes of the vacuum pump are connected to the C4 filter with the C4 filter connection hose.
- The pressure parameter of the vacuum pump should be adjusted to 0.08 MPa or 800 mbar.
- During beads recovery, keep the vacuum pump in the on state, and ensure that the beads are not excessively dry.

2. Pre-rinse the C4 filter with approximately 4.5 mL of the 6×SSC.
3. After observing that no liquid remains on the filter membrane, evenly pour all droplets in the collection well of the chip onto the filter membrane.
4. Add 700 µL of the 6×SSC to the collection well with a pipette.
5. Pipette the liquid to mix it thoroughly to collect residual droplets.
6. Transfer the liquid to the C4 filter, and reserve the tip for continued use in the next step.
7. Repeat steps 4 to 6 twice with the reserved tip. That is, pipette the liquid in the collection well several times with the tip, to collect beads on the bottom and wall, and transfer it to the C4 filter.
8. After observing that no liquid remains on the filter membrane, quickly pour approximately 4.5 mL of the 6×SSC to wash the beads.
9. Repeat step 8 twice.
10. After observing that no liquid remains on the filter membrane, turn off the vacuum pump, and disconnect the vacuum pump from the C4 filter.
11. Connect the C4 filter to a 50 mL centrifuge tube, add 700 µL of the Collection Buffer, and gently pipette the surface of the filter membrane several times until the beads completely suspend.

    Tips
    - Carefully perform the pipetting operation to prevent the liquid from splashing.
    - Avoid contact between the filter membrane and the tip, to prevent the tip from puncturing the filter membrane.
    - Do not touch the surrounding of the filter membrane during beads recovery, to avoid RNA degradation caused by the heat conducted from the hand.

12. Transfer the collection solution containing the beads to a 1.5 mL low-binding centrifuge tube, and reserve the tip for continued use in steps 13 and 14.
13. Gently pipette the surface of the filter membrane several times by using 700 µL of the Collection Buffer, to suspend all remaining beads.
14. Transfer the collection solution containing the beads to the 1.5 mL low-binding centrifuge tube.

15. Place and keep the centrifuge tube on the magnetic separation rack for 3 to 5 minutes until the liquid becomes clear.
16. Gently remove and dispose of the supernatant, to avoid removing the beads.

    Tips If residual beads exist on the filter membrane, aspirate the supernatant to rinse the filter membrane again.
17. Remove the centrifuge tube from the magnetic separation rack, and add 200 μL of the 6×SSC with the pipette.
18. Pipette and rinse the beads on the tube wall, to suspend and mix the beads thoroughly.
19. Evenly transfer the suspension into two 0.2 mL PCR tubes, approximately 100 μL per tube, and reserve the tip for continued use in the next step.
20. Add another 200 μL of the 6×SSC to the 1.5 mL centrifuge tube with the pipette.
21. Pipette and rinse the remaining beads to mix them thoroughly.
22. Evenly transfer the suspension into the two 0.2 mL PCR tubes.

    Tips Use the reserved tip to perform steps 20 to 22, but do not use the tip for a different sample.
23. Place and keep the 0.2 mL PCR tubes on the magnetic separation rack for 3 to 5 minutes until the liquid becomes clear.

    Tips Prepare a reverse transcription reaction solution in advance, to prevent the beads from being overdried.

Chapter 5 Reverse transcription and digestion

This chapter mainly describes how to transcribe mRNA captured by the beads into cDNA. The whole procedure takes about 3 hours.

5.1 Pre-experiment preparation

Table 12 Required reagent kit

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 1 Droplet Formation kit) (Cat. No.: 940-000508-00)	Stop Buffer	Natural	12.8 mL/tube × 1
	Wash Buffer	Brown	32 mL/bottle × 1

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 2 Droplet Formation kit) (Cat. No.: 940-000509-00)	RNase Inhibitor	Brown	176 μL/tube × 1
	RT Buffer	Blue	1.54 mL/tube × 2
	RT Primer-V2	Blue	160 μL/tube × 1
	RT Enzyme	Blue	160 μL/tube × 1
	D Buffer	Orange	320 μL/tube × 1
	D Enzyme	Orange	160 μL/tube × 1

Tips Take out the Stop Buffer in advance to equilibrate to room temperature until all crystallization in the solution dissolves.

5.2 Performing reverse transcription

Perform the following steps:

1. Prepare the reverse transcription reaction solution on ice for each PCR tube according to the following table.

Table 13 Reverse transcription reaction solution

Component	Volume (μL) required for each tube
RT Buffer	87.5
RT Primer-V2	5
RT Enzyme	5
RNase Inhibitor	2.5
Total	100

Tips This table shows the volume of the reverse transcription reaction solution required for reverse transcription for each tube. Each sample tube requires two tubes of the reverse transcription reaction solution.

2. Vortex the PCR tube to mix the reaction solution thoroughly, and place it on ice for further use.
3. Slowly remove and dispose of the supernatant in each of the 0.2 mL PCR tubes from step 23 in *Performing demulsification on Page 20*.

Tips

- If the supernatant is not completely removed, remove remaining supernatant by using a pipette with the measurement range of 10 µL, and avoid removing the beads.
- After removing the supernatant, add the reverse transcription reaction solution, to prevent the beads from being overdried.

4. Pipette 100 µL of the reverse transcription reaction solution, and add it to the 0.2 mL PCR tube with the beads.
5. Mix the liquid by gently shaking or inverting the tube, to resuspend the beads until the liquid is mixed thoroughly.

   Tips Do not mix the liquid by pipetting or vortexing, to avoid loss of the beads due to attachment to the tip.

6. Briefly centrifuge the liquid to collect the reaction solution to the bottom of the tube.
7. Proceed with reverse transcription reaction by using the PCR device according to the condition shown in the following table.

Table 14 Reverse transcription reaction condition (reaction solution: 100 µL)

Temperature	Time	Cycles
70 ℃ (heated lid)	On	/
42 ℃	90 min	1
50 ℃	2 min	10
42 ℃	2 min	
65 ℃	10 min	1
4 ℃	Hold	/

Stop point The beads can suspend in the reaction solution, and the solution can be stored for up to 24 hours at 4 ℃.

8. After the reaction is completed, briefly centrifuge the PCR tube, and keep it on the magnetic separation rack for 3 to 5 minutes until the liquid becomes clear. Remove and dispose of the supernatant, and reserve about 10 µL of the supernatant to avoid loss of the beads.
9. Add 200 µL of the Wash Buffer to the tube for proceeding with digestion.

   Stop point The beads can suspend in the Wash Buffer, and the solution can be stored for up to 24 hours at 4 ℃.

5.3 Performing digestion

Perform the following steps:

1. Prepare the digestion reaction solution according to the following table.

Table 15 Digestion reaction solution

Component	Volume (μL) required for each tube
D Buffer	10
D Enzyme	5
NF Water	85
Total	100

Tips This table shows the volume of the digestion reaction solution required for digestion reaction for each tube. Each sample tube requires two tubes of the digestion reaction solution.

2. Remove and dispose of all the supernatant in the PCR tube from step 9 in *Performing reverse transcription on Page 23*.
3. Add 100 μL of the digestion reaction solution to the tube.
4. Thoroughly mix the solution by gently shaking or inverting the tube.
5. Briefly centrifuge the tube to collect the solution to the bottom of the tube.
6. Proceed with digestion by using the PCR device according to the following table.

Table 16 Digestion condition (reaction solution: 100 μL)

Temperature	Time
50 ℃ (heated lid)	On
37 ℃	15 min
4 ℃	Hold

7. After the reaction is completed, briefly centrifuge the tube, place and keep it on the magnetic separation rack for 3 to 5 minutes until the liquid becomes clear. Remove and dispose of the supernatant, and reserve about 10 μL of the supernatant to avoid loss of the beads.
8. Remove the PCR tube from the magnetic separation rack, and add 200 μL of the Stop Buffer to the tube.
9. Thoroughly mix the liquid by gently shaking or inverting the tube to stop reaction.
10. After briefly centrifuging the tube, place and keep it on the magnetic separation rack for 3 to 5 minutes.

11. Remove and dispose of the supernatant, and reserve about 10 μL of the supernatant to avoid loss of the beads.
12. Keep the tube on the magnetic separation rack, and add 200 μL of the Wash Buffer to the tube.

Stop point The beads can suspend in the Wash Buffer, and the solution can be stored for up to 24 hours at 4 ℃.

Chapter 6 Second-strand synthesis

This chapter mainly describes cDNA second-strand synthesis. The whole procedure takes about 50 minutes.

6.1 Pre-experiment preparation

Table 17 Required reagent kit

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 1 Droplet Formation kit) (Cat. No.: 940-000508-00)	Stop Buffer	Natural	12.8 mL/tube × 1
	Denaturation Buffer (10×)	Natural	6.4 mL/bottle × 1
	Wash Buffer	Brown	32 mL/bottle × 1
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 2 Droplet Formation kit) (Cat. No.: 940-000509-00)	Second Strand Buffer-V2	Green	1.08 mL/tube × 2
	Second Strand Primer-V2	Green	240 μL/tube × 1
	Second Strand Enzyme-V2	Green	800 μL/tube × 1

Tips

- Take out the Stop Buffer in advance to equilibrate to room temperature until all crystallization in the solution dissolves.
- Dilute the Denaturation Buffer (10×) in advance until its concentration becomes one tenth of the original concentration. Specifically, add 900 μL of the NF Water to 100 μL of the Denaturation Buffer (10×), vortex the liquid to mix it thoroughly.
- The Denaturation Buffer should be used immediately after dilution.

6.2 Performing second-strand synthesis

Perform the following steps:

1. Place and keep the PCR tube from step 12 in *Performing digestion on Page 25* on the magnetic separation rack for 3 to 5 minutes until the liquid becomes clear, and remove and dispose of the supernatant.

   Tips Reserve about 10 µL of the supernatant to avoid loss of the beads.

2. Add 200 µL of the diluted Denaturation Buffer to the tube.
3. Remove the tube from the magnetic separation rack, and thoroughly mix the liquid by gently shaking or inverting the tube.
4. Briefly centrifuge the tube to collect the reaction solution to the bottom of the tube.
5. Incubate the tube at room temperature for 5 minutes, and mix the liquid during incubation.
6. Briefly centrifuge the tube, place and keep it on the magnetic separation rack for 3 to 5 minutes until the liquid becomes clear, and remove and dispose of the supernatant.

   Tips Reserve about 10 µL of the supernatant to avoid loss of the beads.

7. Keep the tube on the magnetic separation rack, and add 200 µL of the Wash Buffer to the tube.
8. Keep the tube on the magnetic separation rack for 2 minutes.

   Tips Prepare a second-strand synthesis reaction solution in advance, to prevent the beads from being overdried. It is recommended to prepare the second-strand synthesis reaction solution when keeping the tube on the magnetic separation rack.

9. Prepare the second-strand synthesis reaction solution on ice according to the following table.

Table 18 Second-strand synthesis reaction solution

Component	Volume (µL) required for each tube
Second Strand Buffer-V2	67.5
Second Strand Primer-V2	7.5
Second Strand Enzyme-V2	25
Total	100

Tips This table shows the volume of the second-strand synthesis reaction solution required for second-strand synthesis reaction for each tube. Each sample tube requires two tubes of the second-strand synthesis reaction solution.

10. Remove and dispose of all the supernatant in the tube from step 8.
11. Add 100 µL of the second-strand synthesis reaction solution to the tube, and thoroughly mix the liquid by gently shaking or inverting the tube.
12. Briefly centrifuge the tube to collect the reaction solution to the bottom of the tube.
13. Proceed with the second-strand synthesis reaction by using the PCR device according to the condition shown in the following table.

Table 19 Second-strand synthesis reaction condition (reaction solution: 100 µL)

Temperature	Time	Cycles
50 ℃ (heated lid)	On	/
25 ℃	10 min	1
37 ℃	30 min	
4 ℃	Hold	/

14. After the reaction is completed, briefly centrifuge the tube, place and keep it on the magnetic separation rack for 3 to 5 minutes. Remove and dispose of the supernatant, and reserve about 10 µL of the supernatant to avoid loss of the beads.
15. Remove the tube from the magnetic separation rack, add 200 µL of the Stop Buffer to the tube, and thoroughly mix the liquid by gently shaking the tube to stop the reaction.
16. After briefly centrifuging the tube, place and keep it on the magnetic separation rack for 3 to 5 minutes. Remove and dispose of the supernatant, and reserve about 10 µL of the supernatant to avoid loss of the beads.
17. Keep the tube on the magnetic separation rack, and add 200 µL of the Wash Buffer to the tube.

Tips Prepare a cDNA and Oligo amplification reaction solution in advance, to prevent the beads from being overdried. It is recommended to prepare the cDNA amplification reaction solution when keeping the tube on the magnetic separation rack.

Chapter 7 cDNA and Oligo amplification and size selection

This chapter describes how to amplify cDNA and Oligo products and how to perform size selection on amplified cDNA and Oligo products. The whole procedure takes about 1.5 hours.

7.1 Pre-experiment preparation

Table 20 Required reagent kit

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 1 Droplet Formation kit) (Cat. No.: 940-000508-00)	Suspension Reagent-V2	Brown	320 μL/tube × 1
	DNA Clean Beads	Natural	7.19 mL/bottle × 2
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 2 Droplet Formation kit) (Cat. No.: 940-000509-00)	cDNA Amp Enzyme	White	800 μL/tube × 2
	cDNA Amp Primer-V2	White	128 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 Amplifying cDNA and Oligo products

Perform the following steps:

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

Table 21 cDNA amplification reaction solution

Component	Volume (μL) required for each tube
cDNA Amp Enzyme	50
cDNA Amp Primer-V2	4
Suspension Reagent-V2	10
NF Water	36
Total	100

Tips This table shows the volume of the cDNA amplification reaction solution required for cDNA amplification for each tube. Each sample tube requires two tubes of the cDNA amplification reaction solution.

2. Place and keep the PCR tube from step 17 in *Performing second-strand synthesis on Page 27* on the magnetic separation rack for 3 to 5 minutes until the liquid becomes clear, and remove and dispose of the supernatant.
3. Add 100 µL of the cDNA amplification reaction solution to the tube.
4. Gently shake the tube to mix the liquid thoroughly, and briefly centrifuge the tube.
5. Proceed with cDNA amplification reaction by using the PCR device according to the condition shown in the following table.

Table 22 cDNA amplification reaction condition (required reaction solution: 100 µL)

Temperature	Time	Cycles
105 ℃ (heated lid)	On	/
95 ℃	3 min	1
98 ℃	20 s	13 to 18
60 ℃	30 s	
72 ℃	3 min	
72 ℃	5 min	1
4 ℃	Hold	/

Tips

- The PCR cycle varies with the sample and single-cell input volume. For details about how to set PCR cycles, refer to *cDNA amplification cycles for common sample types on Page 50*.
- For cell-line samples (with an input volume of 20000), 13 or 14 cycles are recommended.
- For PBMC samples (with an input volume of 20000), 17 cycles are recommended.
- For cells and nuclei (with an input volume of 20000) from live tissue, 16 to 18 cycles are recommended based on sample conditions.

Stop point The cDNA product can be stored at 4 ℃ for up to 24 hours or at -20 ℃ for up to one week.

7.3 Performing size selection on cDNA and Oligo products

Tips Before starting size selection, carefully read *About the DNA Clean Beads and purification on Page 51*.

Perform the following steps:

1. Briefly centrifuge two tubes of PCR products, and place them on the magnetic separation rack for 3 to 5 minutes.
2. Remove 95 µL of the supernatant from each tube, and transfer a total of 190 µL of the supernatant to a new 1.5 mL centrifuge tube.
3. Add 114 µL (= 190 µL × 0.6) of the DNA Clean Beads to the 1.5 mL centrifuge tube by using a pipette.
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.
5. Incubate the centrifuge tube at room temperature for 10 minutes.
6. After briefly centrifuging the centrifuge tube, place and keep it on the magnetic separation rack for 2 to 5 minutes until the liquid becomes clear.
7. Transfer the supernatant to a new 1.5 mL centrifuge tube, which is marked as Oligo product 1.

   Tips Do not dispose of but reserve the supernatant in this step.

8. Purify the adsorbed beads in the centrifuge tube from step 6 by performing the following steps:

   1) 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.
   2) Keep the tube on the magnetic separation rack for 30 seconds, and then remove and dispose of the supernatant.
   3) Repeat steps 1) and 2), 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.

   4) 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.

      CAUTION Do not over dry the beads as the beads might crack.

   5) Remove the centrifuge tube from the magnetic separation rack, and add 32 µL of the NF Water for cDNA elution by using a pipette.

6) Gently pipette the liquid to mix it thoroughly.
7) Incubate the centrifuge tube at room temperature for 5 minutes.
8) 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, which is marked as cDNA product.

9. 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.

Stop point The purification product can be stored at -20 ℃ for up to 6 months.

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.

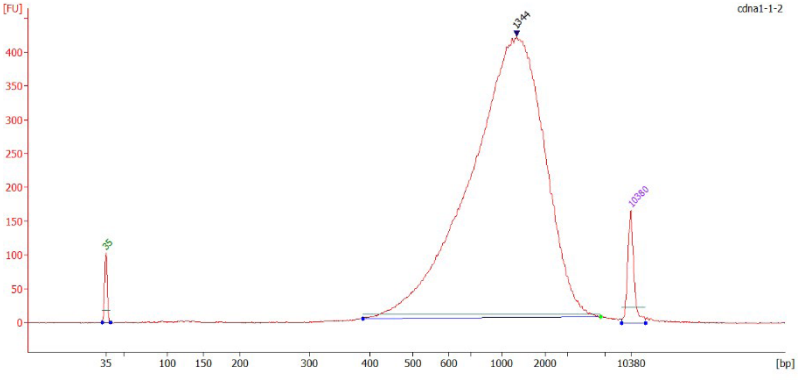

Figure 11 Fragment size distribution of the cDNA product (a reference graph obtained through assessment by using the Agilent 2100 Bioanalyzer)

10. Add 152 μL (= 190 μL × 0.8) of the DNA Clean Beads to the centrifuge tube with the Oligo product 1.
11. 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.
12. Incubate the centrifuge 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 remove and dispose of the supernatant.
14. 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.
15. Keep the tube on the magnetic separation rack for 30 seconds, and then carefully remove and dispose of the supernatant.
16. Repeat steps 14 and 15, 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.

17. 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.

    CAUTION Do not over dry the beads as the beads might crack.

18. Remove the centrifuge tube from the magnetic separation rack, and add 32 µL of the NF Water for Oligo elution by using a pipette.
19. Gently pipette the liquid to mix it thoroughly.
20. Incubate the centrifuge tube at room temperature for 5 minutes.
21. 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, which is marked as Oligo product 2.
22. Take 1 µL of the Oligo product 2 and quantify it by using the Qubit dsDNA HS Assay Kit.

    Reference value: The concentration of the Oligo product is greater than 5 ng/µL.

    Stop point The purification product can be stored at -20 ℃ for up to 6 months.

Chapter 8 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.

8.1 Pre-experiment preparation

Table 23 Required reagent kit

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 1 Droplet Formation kit) (Cat. No.: 940-000508-00)	DNA Clean Beads	Natural	7.19 mL/bottle × 2

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 3 Library Preparation kit) (Cat. No.: 940-000510-00)	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.

8.2 Preparing the Oligo library

Perform the following steps:

1. Take out a new 0.2 mL PCR tube, and take 8 μL of the Oligo product 2 to prepare the Oligo library.

   The Oligo library preparation reaction solution is shown in the following table:

Table 24 Oligo library preparation reaction solution

Component	Volume (μL) required for each tube
Oligo product 2	8
NF Water	13
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 *Using scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16 on Page 52*.
- 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 25 Oligo library preparation condition (reaction solution: 50 μL)

Temperature	Time	Cycles
105 ℃ (heated lid)	On	/
95 ℃	3 min	1
98 ℃	20 s	10
62 ℃	30 s	
72 ℃	10 s	
72 ℃	1 min	1
4 ℃	Hold	/

8.3 Performing size selection on Oligo library

Tips Before starting size selection, carefully read *About the DNA Clean Beads and purification on Page 51*.

Perform the following steps:

1. Add 35 µL of the DNA Clean Beads to the PCR tube from step 3 in *Preparing the Oligo library on Page 34* 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 35 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.

    CAUTION Do not over dry the beads as the beads might crack.
14. Remove the PCR tube from the magnetic separation rack, and add 32 µL of the TE Water 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.
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.

    Stop point The Oligo library can be stored at -20 ℃ for up to 1 month.

    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.

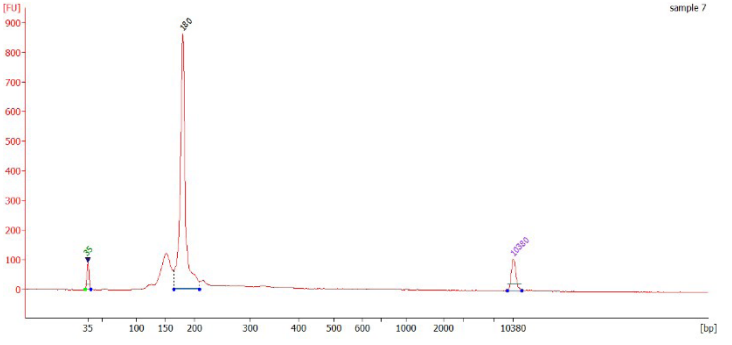

Figure 12 Fragment size distribution of the Oligo library (a reference graph obtained through assessment by using the Agilent 2100 Bioanalyzer)

19. Use the Oligo library to prepare circularization libraries.

    For details, refer to *Preparing circularization libraries (cDNA library and Oligo library) on Page 45*.

Chapter 9 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.

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

9.1 Pre-experiment preparation

Table 26 Required reagent kit

Name	Component	Cap color	Specification
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 1 Droplet Formation kit) (Cat. No.: 940-000508-00)	DNA Clean Beads	Natural	7.19 mL/bottle × 2
DNBelab C Series High-throughput Single-cell RNA Library Preparation Set V2.0 (Box 3 Library Preparation kit) (Cat. No.: 940-000510-00)	Frag Enzyme-V2	Purple	80 μL/tube × 1
	Frag Buffer-V2	Purple	160 μL/tube × 1
	DNA Ligase-V2	Orange	80 μL/tube × 1
	Ligation Buffer-V2	Orange	320 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.

9.2 Performing fragmentation and end repair

Perform the following steps:

1. Take out the Frag Enzyme-V2, mix and briefly centrifuge it, and place it on ice for further use.
2. Prepare a fragmentation and end repair reaction solution according to the following table.

Table 27 Fragmentation and end repair reaction solution

Component	Volume (μL) required for each tube
Frag Buffer-V2	10
Frag Enzyme-V2	5
Total	15

3. According to the concentration of the cDNA product, take out 150 ng of the cDNA product (or take out all 30 μL of the cDNA product when it is less than 150 ng), and add it to a 0.2 mL PCR tube.
4. Add the NF Water to the PCR tube to complement the volume to 45 μL, and place the PCR tube on ice.
5. Add 15 μL of the prepared fragmentation and end repair reaction solution to the PCR tube by using a pipette.
6. Vortex the PCR tube to mix the liquid thoroughly.
7. Briefly centrifuge the liquid to collect the reaction solution to the bottom of the tube.
8. After the temperature of the PCR device decreases to 4 ℃, place the PCR tube in the PCR device. Then, tap **Next** in the interface of the PCR device, to proceed with the reaction at 30 ℃.

Table 28 Fragmentation and end repair reaction condition (reaction solution: 60 μL)

Temperature	Time
105 ℃ (heated lid)	On
4 ℃	Hold
30 ℃	9 min
65 ℃	20 min
4 ℃	Hold

9.3 Performing adapter ligation

Perform the following steps:

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

Table 29 Adapter ligation reaction solution

Component	Volume (μL) required for each tube
Ligation Buffer-V2	20
DNA Ligase-V2	5
scRNA Adapter-V2	5
NF Water	10
Total	40

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 40 μL of the adapter ligation reaction solution to the PCR tube from step 8 in *Performing fragmentation and end repair on Page 39* 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.

Table 30 Adapter ligation reaction condition (reaction solution: 100 μL)

Temperature	Time
Heated lid	Off
20 ℃	15 min
4 ℃	Hold

Tips The heated-lid mode is disabled in this step. If the heated lid temperature is higher than 25 ℃, open the cover of the PCR device for the reaction.

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

9.4 Performing purification and size selection on adapter ligation product

Tips Before starting purification and size selection, carefully read *About the DNA Clean Beads and purification on Page 51*.

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 *Performing adapter ligation on Page 40* 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.

   CAUTION Do not over dry the beads as the beads might 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, to 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.

    CAUTION Do not over dry the beads as the beads might 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 -20 ℃ refrigerator for 24 hours.

9.5 Performing PCR amplification

Tips Before starting PCR amplification, carefully read *Using scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16 on Page 52*.

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 *Performing purification and size selection on adapter ligation product on Page 41*, 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 31 PCR reaction condition (reaction solution: 100 µL)

Temperature	Time	Cycles
105 ℃ (heated lid)	On	/
95 ℃	3 min	1
98 ℃	20 s	12
58 ℃	20 s	
72 ℃	30 s	
72 ℃	5 min	1
4 ℃	Hold	/

9.6 Performing size selection on PCR amplification product

Tips Before starting size selection, carefully read *About the DNA Clean Beads and purification on Page 51*.

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.

    CAUTION Do not over dry the beads as the beads might crack.

14. Remove the PCR tube from the magnetic separation rack, and add 32 µL of the TE Water 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, which is marked 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.

    Stop point The size selection product can be stored at -20 ℃ for up to 6 months.

    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.

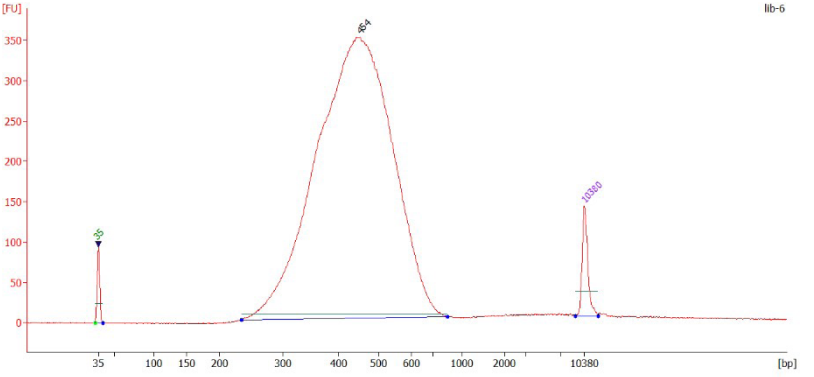

Figure 13 Fragment size distribution of the cDNA library (a reference graph obtained through assessment by using the Agilent 2100 Bioanalyzer)

9.7 Preparing circularization libraries (cDNA library and Oligo library)

Perform the following steps:

1. Prepare the following reagents and consumables:

Name	Recommended brand	Cat. No.
MGIEasy Circularization Kit	MGI	1000005259

Tips Carefully read *MGIEasy Circularization Reagent Kit User Manual* (downloaded from the website: *https://www.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.

Table 32 Circularization library preparation requirements

Type	Input volume for circularization
cDNA library	400 ng
Oligo library	300 ng

Tips When the volume of the cDNA or Oligo library is insufficient, re-prepare the cDNA or Oligo library for subsequent circularization library preparation.

Chapter 10 Sequencing

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

10.1 cDNA and Oligo library structures

The cDNA and Oligo library structures are shown as follows:

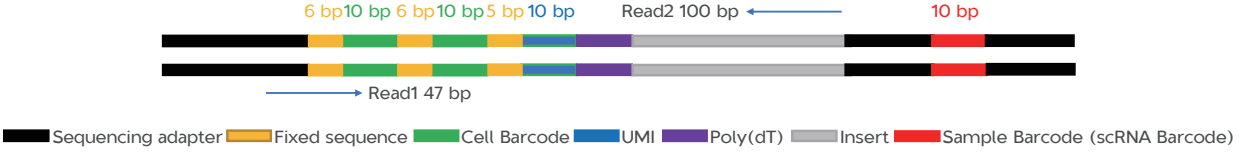

Figure 14 cDNA library structure

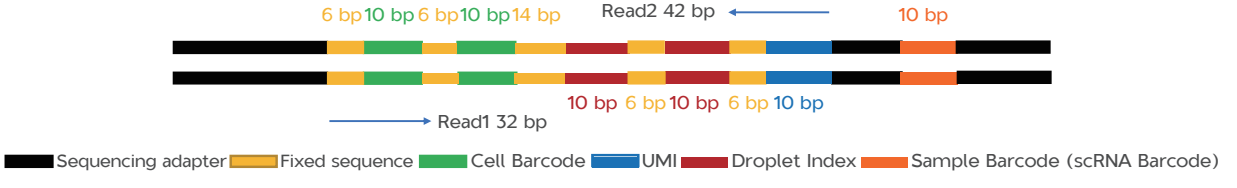

Figure 15 Oligo library structure

Tips After the script is run:

- 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 part 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).

10.2 Sequencing requirements of DNBSEQ-G400RS

- Preparation of the DNBSEQ-G400RS and reagent

Genetic sequencer	DNBSEQ-G400RS
Reagent	DNBSEQ-G400RS High-throughput Sequencing Set (FCL PE100) (Cat. No.: 1000016950)

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

- Preparation of DNBs

Table 33 DNB preparation requirements

Type	Make DNB input volume	RCA time
cDNA library	10 ng	30 min
Oligo library	10 ng	20 min

Tips
- 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 *Using scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16 on Page 52*.

- Sequencing parameters

Table 34 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 or later version	ECR 3.0 or later version
Control software version	ZebraV2Seq_1.4.0.184 or later version	ZebraV2Seq_1.4.0.184 or later version
Basecall version	Basecall_1.0.8.208 or later version	Basecall_1.0.8.208 or later version
Sequencing script	C4_scRNA_BC_PE47+100+10 (for ECR 4.0 and control software of a later version, C4_scRNA_BC_PE47+100+10-ECR4.0 is used)	C4_Oligo_BC_PE32+42+10 (for ECR 4.0 and control software of a later version, C4_Oligo_BC_PE32+42+10-ECR4.0 is used)

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	10 cycles	10 cycles
Depth of sequencing	> 60 k reads/cell	> 50 M reads/library

Table 35 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 or later version	ECR 3.0 or later version
Control software version	ZebraV2Seq_1.4.0.184 or later version	ZebraV2Seq_1.4.0.184 or later version
Basecall version	Basecall_1.0.8.208 or later version	Basecall_1.0.8.208 or later version
Sequencing script	C4_scRNA_noBC_PE47+100 (for ECR 4.0 and control software of a later version, C4_scRNA_noBC_PE47+100-ECR4.0 is used)	C4_Oligo_noBC_PE32+42 (for ECR 4.0 and control software of a later version, C4_Oligo_noBC_PE32+42-ECR4.0 is used)
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

10.3 Sequencing requirements of DNBSEQ-T7RS

- Preparation of the DNBSEQ-T7RS and reagent

Genetic sequencer	DNBSEQ-T7RS
Reagent	DNBSEQ-T7RS High-throughput Sequencing Set (FCL PE100) (Cat. No.: 1000028455)

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

- Preparation of DNBs

Table 36 DNB preparation requirements

Type	Make DNB input volume	RCA time
cDNA library	10 ng	30 min
Oligo library	10 ng	20 min

Tips For details about protocols for pooling different libraries based on the scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16, refer to *Using scRNA Barcode Primer II-1 to scRNA Barcode Primer II-16 on Page 52*.

- Sequencing parameters

Table 37 Sequencing software version and read length

Type	cDNA library	Oligo library
Software version	ECR 3.0 or later version	ECR 3.0 or later version
Control software version	1.3.3.553 or later version	1.3.3.553 or later version
Basecall version	1.4.2.47_Ubuntu or later version	1.4.2.47_Ubuntu or later version
Sequencing script	Custom	Custom
Custom Primers	No	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)

Type	cDNA library	Oligo library
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 cDNA amplification cycles for common sample types

Table 1 Recommended cDNA amplification cycles for dissociated cell/nuclei or cell-line samples from organs and tissues of human and rat

Species	Sample type	Cell input volume	Recommended cycles
Human	PBMC	20000	18
	Gastric cancer cell	20000	18
	Lung cancer cell	20000	18
	Renal cancer cell	20000	18
	Mesenchymal stem cell	20000	13
	K562 cell	20000	13
	293T cell	20000	13
Rat	Brain cell	20000	18
	Splenocyte	20000	18
	Renal cell	20000	18
	Brain nucleus	20000	18
	Spleen nucleus	20000	18
	Renal nucleus	20000	18
	Cardiac nucleus	20000	18
	NIH3T3 cell	20000	13

Appendix 2 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 3 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 Set V2.0 (Box 3 Library Preparation Kit). 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 1 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 three groups	• Sample 1: 5 • Sample 2: 6 • Sample 3: 7 • Sample 4: 8 • Sample 5: select any group from the remaining three groups	• Sample 1: 9 • Sample 2: 10 • Sample 3: 11 • Sample 4: 12 • Sample 5: select any group from the remaining three groups	• Sample 1: 13 • Sample 2: 14 • Sample 3: 15 • Sample 4: 16 • Sample 5: select any group from the remaining three groups

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 three groups	• Sample 1: 5 • Sample 2: 6 • Sample 3: 7 • Sample 4: 8 • Samples 5 and 6: select any two groups from the remaining three groups	• Sample 1: 9 • Sample 2: 10 • Sample 3: 11 • Sample 4: 12 • Samples 5 and 6: select any two groups from the remaining three groups	• Sample 1: 13 • Sample 2: 14 • Sample 3: 15 • Sample 4: 16 • Samples 5 and 6: select any two groups from the remaining three groups
7	• Sample 1: 1 • Sample 2: 2 • Sample 3: 3 • Sample 4: 4 • 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 4 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

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