Part No.:H-940-000190-00-01

User Manual

Version:4.0

MGIEasy Cell-free DNA Library Prep Kit

Cat. No.: 940-000190-00 (48 RXN)
940-000191-00 (96 RXN)
Kit Version: V1.0

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About the user manual

Manufacturer information

Company	Shenzhen MGI Biological Electronic Technology Co., Ltd.
Address	2/F, Building 11, Beishan Industrial Zone, No.146, Beishan Road, Yantian Street, Yantian District, Shenzhen, 518083, P.R. China
Service hotline	(+86) 4000-688-114
Email	MGI-service@mgi-tech.com
Website	http://en.mgi-tech.com

Revision history

Manual version	Kit version	Date	Description
4.0	V1.0	Jun. 2024	Correct the adapter ligation reaction time
3.0	V1.0	May. 2024	Update the manufacturer information
2.0	V1.0	Sep. 2023	• Update the components specification • Update the service hotline • Update the manual style
1.0	V1.0	Dec. 2021	Update Cat. No.

Tips Please download the latest version of the manual, and use it with the corresponding kit. Search for the manual by Cat. No. or product name from the following website.

https://en.mgi-tech.com/download/files.html

Contents

1 Product overview

1.1 Introduction

The MGIEasy Cell-freeDNA Library Prep Kit is specifically designed for MGI high-throughput sequencing platforms. This library prep kit is optimized to convert cell free DNA or 150 - 250 bp fragmented DNA into a custom library. All reagents provided in this kit have passed strict quality control and functional verification procedures, ensuring stability and reproducibility.

1.2 Intended use

This library prep kit is used for cell-free DNA and 150 to 250 bp fragmented DNA. The kit can be used for researching cell-free DNA and pathogen detection.

1.3 Applicable sequencing platforms

The prepared libraries are applicable to the following MGI sequencing platforms.

- BGISEQ-500RS (SE50)
- MGISEQ-200RS (SE50), DNBSEQ-G50RS (SE50)
- MGISEQ-2000RS (SE50), DNBSEQ-G400RS (SE50)

1.4 Components

This library prep kit comes in two specifications: 48 RXN and 96 RXN. Two or three separate boxes are included for each specification. For component details, refer to the following table.

Each kit contains an information card. Relevant manuals and SDS files can be downloaded from the MGI website provided on the information card.

Table 1 MGIEasy Cell-free DNA Library Prep Kit (48 RXN) (Cat. No.: 940-000190-00)

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy Cell-free DNA Library Perp Kit (Box 1) Cat. No.: 940-000163-00	ERAT Buffer Mix	Colorless	155 μL/tube × 3
	ERAT Enzyme Mix	Colorless	10 μL/tube × 3
	Ligation Buffer Mix	Red	384 μL/tube × 3
	Ligation Enzyme	Red	16 μL/tube × 3
	PCR Enzyme Mix	Blue	400 μL/tube × 3
	PCR Primer Mix	Blue	64 μL/tube × 3
	DNA Control	Yellow	10 μL/tube × 1
	Adapter Mix (Barcode 01-48)	Colorless	10 μL/well × 48
MGIEasy Cell-free DNA Library Perp Kit (Box 2) Cat. No.:940-000164-00	Elution Buffer	White	1500 μL/tube × 3
	Purification Beads	White	1500 μL/tube × 3

Table 2 MGIEasy Cell-free DNA Library Prep Kit (96 RXN) (Cat. No.:940-000191-00)

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy Cell-free DNA Library Perp Kit (Box 1) Cat. No.:940-000165-00	ERAT Buffer Mix	Colorless	903 μL/tube × 1
	ERAT Enzyme Mix	Colorless	58 μL/tube × 1
	Ligation Buffer Mix	Red	1152 μL/tube × 2
	Ligation Enzyme	Red	96 μL/tube × 1
	PCR Enzyme Mix	Blue	1200 μL/tube × 2
	PCR Primer Mix	Blue	384 μL/tube × 1
	DNA Control	Yellow	10 μL/tube × 1
MGIEasy Cell-free DNA Library Perp Kit (Box 2) Cat. No.:940-000166-00	DNA Adapters-96 (1 pmol/μL)	/	10 μL/well × 96
MGIEasy Cell-free DNA Library Perp Kit (Box 3) Cat. No.:940-000167-00	Elution Buffer	White	6100 μL/tube × 2
	Purification Beads	White	5500 μL/tube × 2

1.5 Storage and transportation

Table 3 Kit storage and transportation temperatures

Item	Cat. No.	Storage temperature	Transportation temperature
MGIEasy Cell-free DNA Library Perp Kit (Box 1), 48 RXN	940-000163-00	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃
MGIEasy Cell-free DNA Library Perp Kit (Box 1), 96 RXN	940-000165-00		
MGIEasy Cell-free DNA Library Perp Kit (Box 2), 96 RXN	940-000166-00		
MGIEasy Cell-free DNA Library Perp Kit (Box 2), 48 RXN	940-000164-00	2 ℃ to 8 ℃	2 ℃ to 8 ℃
MGIEasy Cell-free DNA Library Perp Kit (Box 3), 96 RXN	940-000167-00		

Tips
- Production date and expiration date: refer to the label.
- For ice packs or dry ice shipments, ensure that there is enough ice or dry ice remaining after transportation.
- With proper transport, storage, and use, all components can maintain complete activity within their shelf life.

1.6 User-supplied materials

Table 4 Order information for MGI products

Catalog number	Model	Name
1000002072	SE50	BGISEQ-500RS High-throughput Sequencing Set
1000004635	SE50	MGISEQ-200RS High-throughput Sequencing Set
1000016959	FCL SE50	MGISEQ-G50RS High-throughput Sequencing Set
1000012551	SE50	MGISEQ-2000RS High-throughput Sequencing Set
1000016941	FCL SE50	DNBSEQ-G400RS High-throughput Sequencing Set

Table 5 User-supplied equipment list

Equipment	Recommended brand
Vortex mixer	/
Desktop centrifuge	/
Pipettes	/

Equipment	Recommended brand
Thermocycler	/
Magnetic rack DynaMag -2, or equivalent	Thermo Fisher Scientific, Cat. No. 12321D
Qubit Fluorometer, or equivalent	Thermo Fisher, Cat. No. Q33216
Agilent 2100 Bioanalyzer, or equivalent	Agilent Technologies , Cat. No. G2939AA

Table 6 Recommended reagent/consumable list

Reagent/consumable	Recommended brand
Nuclease Free (NF) water or Molecular Grade Water	Ambion, Cat. No. AM9937, or equivalent
TE Buffer, pH 8.0	Ambion, Cat. No. AM9858, or equivalent
100% Ethanol (Analytical Grade)	/
Qubit dsDNA HS Assay Kit	Invitrogen, Cat. No. Q32854), or equivalent
Pipette tips	/
1.5 mL tube	/
0.2 mL PCR tube or 96-well plate	/
Qubit Assay Tubes or 0.5mL Thin Wall PCR Tubes	Invitrogen or Axygen, or equivalent

1.7 Precautions and warnings

- This product is for research use only, not for in vitro diagnosis. Please read this manual carefully before use.
- Familiarize yourself with the precautions and operation methods of various instruments before performing the experiment.
- This manual aims to provide a standard protocol. Changes can be made for different applications, but changes must be tested prior to starting the protocol.
- It is recommended that you use pipette tips with filters to prevent cross-contamination. Use a new tip each time for pipetting different solutions or samples.
- It is recommended that you use the thermocyclers with heated lids for reactions. Preheat the thermocyclers to reaction temperature before use. If the thermocycler does not allow for lid temperature adjustments, the preset lid temperature of 105 ℃ is sufficient.
- Aerosol contamination may cause inaccurate results. It is recommended that you prepare separate working areas in the laboratory for PCR reaction preparation, PCR reaction, and PCR product cleanup. Use designated equipment for each area and clean the area regularly to ensure a sterile working environment (use 0.5% Sodium Hypochlorite or 10% bleach to clean the working area).
- Avoid skin and eyes contact with samples and reagents. Do not eat or drink the samples and reagents. In case of contact with skin and eyes, rinse immediately with plenty of water and seek medical advice.

- Conform to the law and regulations when disposing of all samples and reagents.
- If you have questions, contact Technical Support: **MGI-service@mgi-tech.com**.

1.8 Workflow

Section	Workflow	Total time	Hands-on time
3.1	End repair	35 - 40 min	5 - 10 min
3.2	Adapter ligation	25 - 30 min	5 - 10 min
3.3	Cleanup of adapter-ligated ⏸	30 - 40 min	20 - 30 min
3.4	PCR	35 - 50 min	5 - 8 min
3.5	Cleanup of PCR product ⏸	30 - 40 min	20 - 30 min
3.6	QC of PCR product ⏸	15 - 60 min	10 - 20 min

Tips

- Total time: The theoretical use time of 8 reactions. The time will be extended if the number of reactions increases.
- Hands-on time: The total required hands-on time in the process.
- ⏸ : The stop point.

2 Sample requirements

- Peripheral blood used for extracting cell-free DNA should be stored in EDTA anticoagulant tubes and is not suitable for plasma stored in heparin sodium blood collection tubes. It is recommended to start DNA extraction from 200 μL of plasma.
- If the sample is fragmented DNA, the peak size of the DNA fragment is concentrated in the range of 150 bp to 250 bp.
- The input DNA is between 2 ng and 6 ng.

  dsDNA quantitation kits such as Qubit dsDNA HS Assay Kit or Quant- iT PicoGreen dsDNA Assay Kit are recommended to quantify the sample in accordance with the instructions of the relevant kit user manuals.
- DNA samples are recommended to be eluted with **TE Buffer**. Any residual impurities in the DNA sample, such as metal chelators or other salts, may adversely affect the efficiency of the end repair step.

3 Library preparation protocol

3.1 End repair

Tips
- Preheat the thermocycler to reaction temperature in advance if the thermocycler heat up slowly.
- A DNA Control is recommended to be added as a quality control for library construction in each batch, which is used for quality control of reagents, library construction, and sequencing operations. The DNA Control is human genomic DNA with a fragment size of 150 bp to 250 bp. Sequence the DNA Control library together with the libraries constructed in the same batch. The library concentration and sequencing results of DNA Control should meet the quality control standards.

3.1.1 Preparation

Mix the reagents before using and store the remaining reagents immediately after use.

Table 7 Preparing the reagents

Reagent	Requirement
NF water or Molecular Grade Water	User-supplied; place at RT.
DNA Control	Thaw at RT, mix well, centrifuge briefly, and place on ice.
ERAT Buffer Mix	Thaw at RT, mix well, centrifuge briefly, and place on ice.
ERAT Enzyme Mix	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

3.1.2 End repair

1. Transfer the DNA extracted from 200 µL plasma or 2 ng - 6 ng fragmented DNA (150 - 200 bp) into a new 0.2 mL PCR tube. Add NF water or Molecular Grade Water to make a total volume of 40 µL. Mix it well and centrifuge briefly.

2. A DNA Control is recommended to be added as a quality control for library construction in each batch. Transfer 1.5 μL of DNA Control into a new 0.2 mL PCR tube. Add 38.5 μL of NF water or Molecular Grade Water for a total volume of 40 μL. Mix it well and centrifuge briefly.
3. According to the desired reaction number, prepare the end repair mixture in a 0.2 mL PCR tube on ice. Mix it well by vortexing, centrifuge briefly, and place on ice.

Table 8 End repair mixture

Reagent	Volume per reaction
ERAT Buffer Mix	9.4 μL
ERAT Enzyme Mix	0.6 μL
Total	10 μL

4. Add 10 μL of end repair mixture to each sample tube (from step 1 and 2). Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.
5. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 9 End repair reaction conditions (Volume: 50 μL)

Temperature	Time
85 ℃ Heated lid	On
37 ℃	10 min
65 ℃	15 min
4 ℃	Hold

6. When the program is completed, centrifuge the PCR tube(s) briefly to collect the liquid to the bottom of the tube.

WARNING
- Do not stop at this step. Please proceed to next reaction.
- If the operation stops here, store the end repair product(s) at –20 ℃ overnight with a risk of 20% decrease in yield.

3.2 Adapter ligation

Tips Before operation, carefully read “Using adapters” on page 16.

3.2.1 Preparation

Mix the reagents before using and store the remaining reagents immediately after use.

Table 10 Preparing the reagents

Reagent	Requirement
Ligation Buffer Mix	Thaw at RT, mix well, centrifuge briefly, and place on ice.
Ligation Enzyme	Flick or invert the tube gently, centrifuge briefly, and place on ice.
Adapters Mix (Barcode 01-48) or DNA Adapters-96	Mix thoroughly, centrifuge briefly, and place on ice.

Tips
- Mix the adapter(s) well before using. Adapters should not be mixed directly with the adapter ligation mixture.
- The Ligation Buffer is highly viscous. Mix it by vortexing 6 times (3 sec each) and centrifuge briefly. When pipetting the Ligation Buffer, slowly aspirate to ensure that the volume is accurate.

3.2.2 Adapter ligation

1. **Add 5 µL of Adapters Mix (Barcode 01-48) or DNA Adapters-96** to the corresponding sample tube (from step 6 in section 3.1.2). Vortex the tube(s) 3 times (3 sec each), centrifuge briefly, and place on ice.
2. According to the desired reaction number, prepare the adapter ligation mixture in a 0.2 mL PCR tube on ice. Mix it well by vortexing 3 times (3 sec each), centrifuge briefly, and place on ice.

Table 11 Adapter ligation mixture

Reagent	Volume per reaction
Ligation Buffer Mix	24 µL
Ligation Enzyme	1 µL
Total	25 µL

3. Slowly pipette 25 µL of adapter ligation mixture to each sample tube and vortex 6 times (3 sec each). Centrifuge briefly to collect the liquid to the bottom of the tube and place on ice.

   Tips The adapter ligation mixture is highly viscous. Slowly aspirate to ensure the volume is accurate.

4. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 12 Adapter ligation reaction conditions (Volume: 80 µL)

Temperature	Time
30 ℃ Heated lid	On
23 ℃	20 min
4 ℃	Hold

5. When the program is completed, centrifuge the PCR tube(s) briefly and place on ice.

WARNING Do not stop at this step. Please proceed to next reaction. Otherwise, the yield may decrease.

3.3 Cleanup of adapter-ligated product

Tips Do not disturb or pipette the beads when adding reagents or transferring supernatant. If you accidentally disturb or pipette the beads, pipette the solution and beads back into the tube and restart the separation process.

3.3.1 Preparation

Table 13 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied. Freshly prepared.
Elution Buffer	Place at RT.
Purification Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.3.2 Cleanup of adapter-ligated product

Tips Transfer all liquid to a new 1.5 mL centrifuge tube (one tube per reaction) in advance if using a 1.5 mL centrifuge tube and a corresponding magnetic rack for purification.

1. Mix the Purification Beads thoroughly. Add 40 µL of Purification Beads to each sample tube (from step 5 in section 3.2.2). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
2. Incubate the sample(s) at room temperature for 5 min.
3. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
4. While keeping the tube(s) on the magnetic rack, add 200 µL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
5. Repeat step 4. Try to remove all liquid from the tube. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.

6. Keep the tube(s) on the magnetic rack. Open the tube cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the beads' surface. There should be no visible cracking on the surface of the beads.

   Tips Over-drying the beads will result in reduced yield.

7. Remove the tube(s) from the magnetic rack and add 23 µL of Elution Buffer to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
8. Incubate the sample(s) at room temperature for 5 min.
9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 21 µL of supernatant to a new 0.2 mL PCR tube.

   Stop point After cleanup, the adapter-ligated product(s) can be stored at -20 °C.

3.4 PCR

3.4.1 Preparation

Mix the reagents before using and store the remaining reagents immediately after use.

Table 14 Preparing the reagents

Reagent	Requirement
PCR Enzyme Mix	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
PCR Primer Mix	Thaw at RT, mix by vortexing, centrifuge briefly, and place at RT.

3.4.2 PCR

1. According to the desired reaction number, prepare the PCR mixture in a 0.2 mL PCR tube on ice. Mix it well by vortexing 3 times (3 sec each), centrifuge briefly, and place on ice.

Table 15 PCR mixture

Reagent	Volume per reaction
PCR Enzyme Mix	25 µL
PCR Primer Mix	4 µL
Total	29 µL

2. Add 29 µL of PCR mixture to each sample tube (from step 9 in section 3.3.2). Vortex the tube(s) 3 times (3 sec each) and centrifuge briefly to collect the liquid to the bottom of the tube.
3. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 16 PCR reaction conditions (Volume: 50 μL)

Temperature	Time	Cycles
105 ℃ Heated lid	On	-
98 ℃	2 min	1
98 ℃	15 sec	12
56 ℃	15 sec	
72 ℃	30 sec	
72 ℃	5 min	1
4 ℃	Hold	-

4. When the program is completed, centrifuge the tube(s) briefly.

3.5 Cleanup of PCR product

Tips Do not disturb or pipette the beads when adding reagents or transferring supernatant. If you accidentally disturb or pipette the beads, pipette the solution and beads back into the tube and restart the separation process.

3.5.1 Preparation

Table 17 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied. Freshly prepared.
Elution Buffer	Place at RT.
Purification Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.5.2 Cleanup of PCR product

Tips Transfer all liquid to a new 1.5 mL centrifuge tube (one tube per reaction) in advance if using a 1.5 mL centrifuge tube and a corresponding magnetic rack for purification.

1. Mix the Purification Beads thoroughly. Add 50 μL of Purification Beads to each sample tube (from step 4 in section 3.4.2). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
2. Incubate the sample(s) at room temperature for 5 min.

3. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
4. While keeping the tube(s) on the magnetic rack, add 200 µL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
5. Repeat step 4. Try to remove all liquid from the tube. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.
6. Keep the tube(s) on the magnetic rack. Open the tube cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the beads' surface. There should be no visible cracking on the surface of the beads.

   Tips Over-drying the beads will result in reduced yield.

7. Remove the tube(s) from the magnetic rack and add 32 µL of Elution Buffer to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
8. Incubate the sample(s) at room temperature for 5 min.
9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 30 µL of supernatant to a new 1.5 mL centrifuge tube or PCR tube.

   Stop point After cleanup, PCR product(s) can be stored at -20 ℃.

3.6 QC of PCR product

- **dsDNA fluorescence quantification method**: Quantify the purified PCR products with dsDNA fluorescence assay kits and instructions.

Table 18 Different QC methods and standards for library

Method	Equipment/Reagent	Standard
dsDNA fluorescence quantification method	Qubit dsDNA HS Assay Kit, Quant-iT PicoGreen dsDNA Assay Kit	concentration for PCR products: ≥ 2 ng /µL

- Refer to Formula 1 in "About samples pooling" on page 23 to calculate the mass (in ng) that corresponds to 1 pmol of dsDNA sample with varying fragment sizes.
- For multiple samples pooled sequencing, refer to "Using adapters" on page 16 and "About samples pooling" on page 23. The mass of PCR products is recommended to be 168 ng with a total volume ≤ 48 µL.
- The pooled library can be stored at -20 ℃ or continue to circularization. If the kit is applied to other high depth application study, we recommend using the MGIEasy Circularization Kit (Cat. No.: 1000005259). PCR products are converted into circularized single strand DNA (ssCir DNA) which can be sequenced on BGISEQ/MGISEQ/DNBSEQ sequencers.

4 Sequencing

Please follow the protocol described in "BGISEQ/MGISEQ/DNBSEQ High-throughput Sequencing Set Instruction Manual" for DNB making and sequencing. The available sequencing kits including:.

- BGISEQ-500RS sequencing platform: SE50, PE50, PE100;
- MGISEQ-200RS\DNBSEQ-G50RS sequencing platform: SE50, PE100;
- MGISEQ-2000RS\DNBSEQ-G400RS sequencing platform: SE50, PE100.

5 Appendix

5.1 Using adapters

MGI currently offers the Adapter Reagent Kits with two specifications based on the number of reactions.

Both kits were developed to meet requirements for batch processing of library construction and multiplex sequencing. We selected the best adapter combination based on the principle of balanced base composition. However, the numbers of barcode adapter are not continuous. For optimal performance, read the instructions of Adapter Reagent Kits carefully before use.

- Adapters from the two kits contain overlapping barcodes and cannot be sequenced in the same lane.
- All adapters are double stranded. To prevent structural changes, such as denaturation, which might affect performance, do not place the adapters above 30 ℃.
- Before use, mix the adapter(s) well and centrifuge to collect the liquid at the bottom of tubes or plates.
- Change tips when pipetting different adapters to prevent cross-contamination.
- For tube reagent, carefully open the tube cap to prevent spills or to prevent cross-contamination. Close the cap immediately after use.
- For plate reagent, spray 75% alcohol and wipe the surface of the aluminum film of the plate with absorbent paper. The aluminum film is penetrable and do not touch the surface of the aluminum film with sharp objects. Pierce the aluminum film to pipette solutions for first-time use. After use, separately transfer the remaining reagents to 1.5 mL centrifuge tube(s) or 0.2 mL PCR tube(s), label the tubes clearly, and store them at -20 ℃.
- Adapters from other MGI Library Prep Kits are designed differently and cannot be mixed with the adapters described here. Otherwise, errors will occur during barcode demultiplexing procedures on DNBSEQ platforms.

5.1.1 Sample barcode pooling strategies

- For pooled sequencing, sample barcode pooling should follow the principle of base balance.
- Using an 8 bp barcode as an example, the ratio of ATGC at 1-8 bp bases should be 25%, as shown in the table below.

Table 19 Example of sample barcode pooling strategies

Barcode	Sequence	1	2	3	4	5	6	7	8
Example 1	TAGGTCCG	T	A	G	G	T	C	C	G
Example 2	GGACGGAA	G	G	A	C	G	G	A	A
Example 3	CTTACTGC	C	T	T	A	C	T	G	C
Example 4	ACCTAATT	A	C	C	T	A	A	T	T
Barcode 1-8 bp A%		25%	25%	25%	25%	25%	25%	25%	25%
Barcode 1-8 bp T%		25%	25%	25%	25%	25%	25%	25%	25%
Barcode 1-8 bp G%		25%	25%	25%	25%	25%	25%	25%	25%
Barcode 1-8 bp C%		25%	25%	25%	25%	25%	25%	25%	25%

- If the proportion cannot reach 25%, then ATGC should appear in each cycle. The minimum base proportion should not be less than 12.5% and the maximum base proportion should not be greater than 62.5%.
- If the proportion is not between 12.5% and 62.5%, then sequencing quality could be reduced. In this case, it is possible that the sample barcodes might not be properly split.

5.1.2 Instructions for Adapters Mix (Barcode 01-48)

Based on the principles of balanced base composition, adapters must be used in specific groups. Please follow the instructions below to use the adapters in the proper combinations.

- 4 sets of 4 adapters: 01-04, 05-08, 09-12, 13-16.
- 4 sets of 8 adapters: 17-24, 25-32, 33-40, 41-48.

If the sequencing data output requirement is the same for all samples in one lane, choose the barcode adapter combinations in the table below.

⚠ CAUTION The number of the adapter should not be repeated between samples in one lane.

Table 20 Instructions for Adapters Mix (Barcode 01-48)

Sample/lane	Instruction (Example)
1	Requires at least 1 set of adapters: • For a set of 4 adapters, add 4 adapters to each sample. For example: 01-04. Mix 4 adapters with equal volume and add the mixture to the sample. • Or, for a set of 8 adapters, add 8 adapters to each sample. For example: 41-48. Mix 8 adapters with equal volume and add the mixture to the sample.

Sample/lane	Instruction (Example)
2	Requires at least 1 set of adapters: • For a set of 4 adapters, add 2 adapters to each sample. For example: 01-04. Mix 01 and 02 with equal volume and add the mixture to sample 1; Mix 03 and 04 with equal volume and add the mixture to sample 2. • Or, for a set of 8 adapters, add 4 adapters to each sample. For example: 41-48. Mix 41-44 with equal volume and add the mixture to sample 1; Mix 45-48 with equal volume and add the mixture to sample 2.
3	Requires at least 2 sets of Adapters: 1. For samples 1 and 2, use the method for (2 samples/lane) above. 2. For sample 3, use the method for (1 sample/lane) above. Tips Use different adapter sets for samples 1, 2, and 3.
4	Requires at least 1 set of adapters: • For a set of 4 adapters, add 1 adapter to each sample. For example: 01-04. Add adapter 01, 02, 03, 04 to sample 1, 2, 3, 4, respectively. • Or, for a set of 8 adapters, add 2 adapters to each sample. For example: 41-48. Mix 41-42, 43-44, 45-46, and 47-48 with equal volume. Add the mixture to sample 1, 2, 3, 4, respectively.
5	Requires at least 2 sets of Adapters: • For samples 1-4, use the method for (4 samples/lane) above. • For sample 5, use the method for (1 sample/lane) above. Tips Use different adapter sets for samples 1-4 and for sample 5.
6	Requires at least 2 sets of adapters: 1. For samples 1-4, use the method for (4 samples/lane) above. 2. For samples 5-6, use the method for (2 sample/lane) above. Tips Use different adapter sets for samples 1-4 and for samples 5-6.

Sample/lane	Instruction (Example)
7	Requires all 3 Adapter sets and follow 3 steps: 1. For samples 1-4, use the method for (4 samples/lane) above (use the first adapter set). 2. For samples 5-6, use the method for (2 samples/lane) above (use the second adapter set). 3. For sample 7, use the method for (1 sample/lane) above (use the third adapter set). ▪ Add a single adapter within the adapter set. ▪ Or, add the adapter mix which is mixed from all adapters within the adapter set with an equal volume. Tips Use different adapter sets for samples 1-4, for samples 5-6 and for sample 7.
8	Requires at least 1 set of adapters: • For a set of 8 adapters, add 1 adapter to each sample. For example: 41-48. Add adapters 41-48 to samples 1-8, respectively. • Or, for 2 sets of 4 adapters, add 1 adapter to each sample. For example: 01-04 and 5-8. Add 1 adapter to each sample.
8n+x (x=1-8, Total 9-48)	Perform the following 3 steps: 1. For samples 1 to 8n, use the method for (8 samples/lane) above. 2. For samples X, according to the value of X, use the methods above for 1-8 sample/lane accordingly. Tips Use different adapter sets for steps 1 and 2.

For situations in which sequencing data output requirements are different among samples, any sample with a data output of more than 20% for each lane should use a separate set of adapters.

For example, 9 samples are pooled into one lane, one sample of which requires 30% of the total data output.

1. 8 samples may use adapter set (41-48).
2. The final sample should use a full adapter set instead of using only a single adapter. (For example: adapter set (01-04) or (13-16)).

5.1.3 Instructions for DNA Adapters-96 (Plate)

Based on the principles of balanced base composition, adapters should be used in specific groups. Follow the instructions below to use the adapters in the proper combinations.

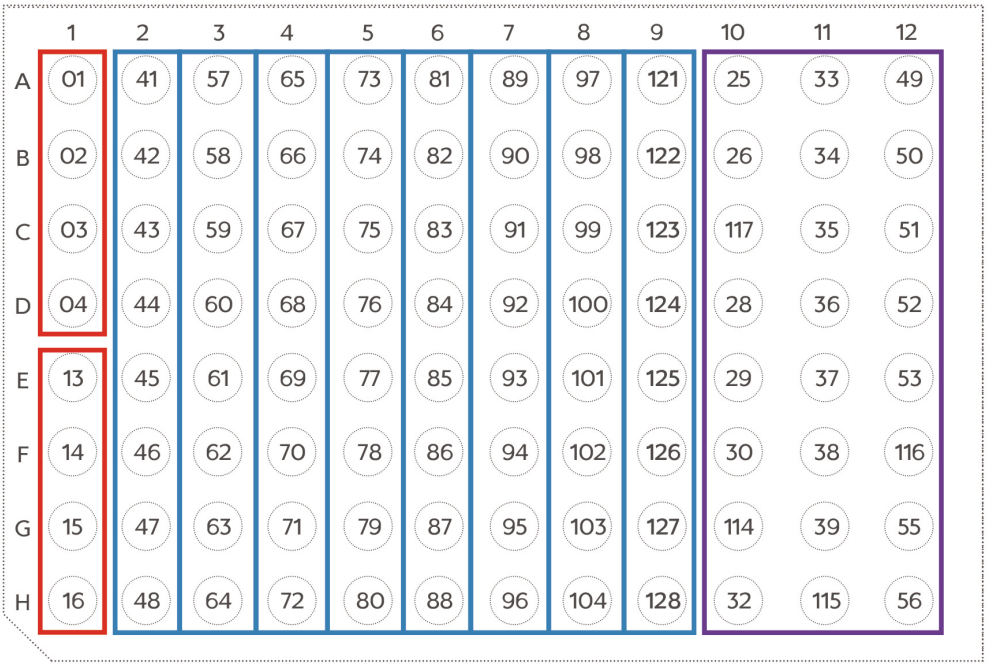

Figure 1 DNA adapters-96 (plate) adapters layout and combination instructions

- 2 sets of 4 adapters: Column 1 (01-04, 13-16) (see the red box in the figure above)
- 8 sets of 8 adapters: Columns 2-9 (41-48, 57-64, 65-72, 73-80, 81-88, 89-96, 97-104, and 121-128) (see the blue box in the figure above)
- 1 set of 24 adapters: Columns 10-12 (see the purple box in the figure above)

If the sequencing data output requirement is the same for all samples in a lane, please refer to the table below to organize your barcode adapter combinations.

CAUTION The number of the adapter should not be repeated between samples in one lane.

Table 21 Instructions for DNA Adapters-96 (Plate)

Sample/lane	Instruction (Example)
1	• For a set of 4 adapters, add 4 adapters to each sample. For example: 01-04. Mix 4 adapters with equal volume and add the mixture to the sample. • Or, for a set of 8 adapters, add 8 adapters to each sample. For example: 41-48. Mix 8 adapters with equal volume and add the mixture to the sample.

Sample/lane	Instruction (Example)
2	• For a set of 4 adapters, add 2 adapters to each sample. For example: 01-04. Mix 01 and 02 with equal volume and add the mixture to sample 1; Mix 03 and 04 with equal volume and add the mixture to sample 2. • Or, for a set of 8 adapters, add 4 adapters to each sample. For example: 41-48. Mix 41-44 with equal volume and add the mixture to sample 1; Mix 45-48 with equal volume and add the mixture to sample 2.
3	1. For samples 1 and 2, use the method for (2 samples/lane) above. 2. For sample 3, use the method for (1 sample/lane) above. Tips Use different adapter sets for samples 1, 2, and 3.
4	• For a set of 4 adapters, add 1 adapter to each sample. For example: 01-04. Add adapters 01, 02, 03, 04 to samples 1, 2, 3, 4, in that order. • Or, for a set of 8 adapters, add 2 adapters to each sample. For example: 41-48. Mix 41-42, 43-44, 45-46, and 47-48 with equal volume. Add the mixture to sample 1, 2, 3, 4, in that order.
5	1. For samples 1-4, use the method for (4 samples/lane) above. 2. For sample 5, use the method for (1 sample/lane) above. Tips Use different adapter sets for samples 1-4 and 5.
6	1. For samples 1-4, use the method for (4 samples/lane) above. 2. For samples 5-6, use the method for (2 sample/lane) above. Tips Use different adapter sets for samples 1-4 and 5-6.

Sample/lane	Instruction (Example)
7	1. For samples 1-4, use the method for (4 samples/lane) above (use the first adapter set). 2. For samples 5-6, use the method for (2 samples/lane) above (use the second adapter set). 3. For sample 7, use the method for (1 sample/lane) above (use the third adapter set). Tips Use different adapter sets for samples 1-4, samples 5-6, and sample 7.
8	• For a set of 8 adapters, add 1 adapter to each sample. For example: 41-48. Add adapters 41-48 to samples 1 - 8, in that order.
8n+x (n=1 or 2, x=1-8, total 9-24)	Perform the following 3 steps: 1. For samples 1-8, ▪ Use the method for (8 samples/lane) above. ▪ Or, separate into 2 groups of 4 and use the method for (4 samples/lane) above for each group. 2. For samples 9-8n, separate samples into groups of 8, and use the method for (8 samples/lane) above. 3. For samples 8n+1 - 8n+X, according to the value of X, use the methods above for 1-8 sample/lane accordingly. Tips Use different adapter sets for steps 1, 2, and 3.
8n+x (3≤n<11, x=1-8, total 25-96)	Perform the following 3 steps: 1. For samples 1-24, use a set of 24 adapters and add 1 adapter to each sample. 2. For samples 25-8n, separate the samples into groups of 8, and use the method for (8 samples/lane) above. 3. For samples 8n+1 - 8n+X, according to the value of X, use the methods above for 1-8 sample/lane accordingly. Tips Use different adapter sets for steps 1, 2, and 3.

For situations in which sequencing data output requirements are different among samples, any sample with a data output of more than 20% for each lane should use a separate set of adapters.

For example, 9 samples are pooled into one lane, one sample of which requires 30% of the total data output.

1. 8 samples may use adapters (41-48).
2. The final sample should use a full adapter set instead of using only a single adapter. For example: adapter set (01-04) or (13-16).

5.2 About samples pooling

CAUTION Do not pool samples with different insert size distributions in the same lane.

Tips Before pooling, carefully read Appendix Using adapters.

Purified PCR products pooling

Quantify the purified PCR products before pooling. The total yield after pooling should be 1 pmol, with a total volume ≤ 48 μL.

Calculate the percentage of the required amount of sequencing data for samples on the same lane. Refer to Formula 1 and 2 to calculate the required mass of each sample. Formula 3 shows the calculation of sample volume.

Formula 1 Conversion between 1 pmol of dsDNA sample and mass in ng

Mass corresponding to 1 pmol PCR product (ng) = PCR product peak size (bp) × 0.66

Formula 2 Calculation of each sample mass before pooling

Sample mass (ng) = Mass corresponding to 1 pmol PCR product (ng) × Ratio of sample data (%)

Formula 3 Calculation of sample volume

$$\text{Sample volume (μL)} = \frac{\text{Sample mass (ng)}}{\text{Sample concentration (ng/μL)}}$$

For example: For 4 samples (belong to 150 to 200 bp insert size libraries, 84 bp adapter) pooled sequencing. The mass of PCR products is recommended to be 168 ng and be equal to a total yield of 1 pmol.

1. Calculate the mass for each sample.

- The expected amount of sequencing data for each sample is the same. The ratio of each sample sequencing data is 25%. Referring to Formula 2, the required mass of each PCR sample is 168 ng × 25% = 42 ng.
- The expected amount of sequencing data for each sample is different. The ratios of sequencing data for samples 1-4 are 20%, 20%, 30%, and 30%. Referring to Formula 2, the required mass of sample 1 is 33.6 ng. Calculate the mass of samples 2 to 4 in the same way.

2. The concentration of sample 1 is 10 ng/μL. Refer to Formula 3 and the required volume is "A μL". Calculate the volume of samples 2 to 4 in the same way.
3. Transfer A μL of sample 1 into a new 0.2 mL PCR tube.
4. Add other samples into the same PCR tube.
5. Add TE Buffer to make a total volume of 48 μL.

Table 22 Multiple samples pooling (each sample volume should be at least 1 μL)

Name	Volume
Sample 1	A μL
Sample 2	B μL

Name	Volume
Sample 3	C μL
Sample 4	D μL
TE Buffer	48 - (A+B+C+D) μL
Total	48 μL

Tips The volumes of A, B, C, and D should each be ≥ 1 μL.

Follow one of the two methods below to pool the samples when the required volume of a sample is less than 1 μL. Method 1 is recommended.

Method 1: Increase the volume of all samples by Z (Z > 1) times. After mixing the samples, take 1/Z of the total volume W μL. Add TE Buffer to make a total volume of 48 μL.

Table 23 Samples mixture: All samples volume increases by Z times

Name	Volume
Sample 1	A × Z μL
Sample 2	B × Z μL
Sample 3	C × Z μL
Sample 4	D × Z μL
Total	W μL

Table 24 Method 1: Multiple samples pooling

Name	Volume
Samples mixture	(W ÷ Z) μL
TE Buffer	48 - (W ÷ Z) μL
Total	48 μL

Tips If necessary, quantify the samples mixture and calculate a new volume X μL (1 pmol). Replace "(W ÷ Z) μL" with "X μL".

Method 2: Dilutes a high concentration sample by Y (Y > 1) times if the required volume is less than 1 μL. Quantify the diluted sample and calculate a new volume. Pool the diluted sample with other samples.

For example: The required volume of sample 3 is < 1 μL. It needs to be diluted by Y times.

Table 25 Diluted sample: Dilute the high concentration sample by Y times

Name	Volume
Sample 3	5 μL*
TE Buffer	5Y - 5 μL
Total	5Y μL

Tips *: The volume of high concentration sample is recommended to be more than 5 μL.

Quantify the diluted sample. Refer to Formula 3 to calculate a new volume "E μL". Pool the diluted sample with other samples. Add TE Buffer to make a total volume of 48 μL.

Table 26 Method 2: Multiple samples pooling

Name	Volume
Sample 1	A μL
Sample 2	B μL
Sample 4	D μL
Diluted sample 3	E μL
TE Buffer	48 - (A+B+D) - E μL
Total	48 μL