Part No.:SOP-013-B02-129

User Manual

Version:8.0

MGIEasy FS PCR-Free DNA Library Prep Set

Cat. No.: 1000013454 (16 RXN, V1.2)
1000013455 (96 RXN, V1.3)

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About the user manual

Manufacturer information

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Revision history

Manual version	Kit version	Date	Description
8.0	V1.2 (16 RXN) V1.3 (96 RXN)	Mar. 2024	• Update the manufacture information • Update Cat. No. of MGIEasy DNA Clean Beads in section 1.4 • Update the manual style • Delete the appendix "Magnetic beads and cleanup" and "About Adapter ligation"
A6	V1.2 (16 RXN) V1.3 (96 RXN)	Feb. 2022	• Updated the protocol of step 3.2. • Optimized the description
A5	V1.2 (16 RXN) V1.3 (96 RXN)	Jul. 2021	Corrected typos
A4	V1.2 (16 RXN) V1.3 (96 RXN)	Apr. 2021	• Changed the application scope, deleted WGA samples and human tissue • Added the compatible sequence platform DNBSEQ-T10X4 RS(PE100) • Updated the sample requirement to $2.0 \geq OD_{260/280} \geq 1.8$, $OD_{260/230} \geq 1.7$ • Changed the name of the fragmentation enzyme to FS Buffer II and FS Enzyme Mix II • Updated the kit version of 16 RXN to V1.2. • Updated the kit version of 96 RXN to V1.3. • Changed the normalization method of gDNA, fragmentation reaction condition, the two-step beads purification condition in step 3.2. • Changed the table number after table 8
A3	V1.1 (16 RXN) V1.2 (96 RXN)	Jan. 2021	Updated contact information
A2	V1.1 (16 RXN) V1.2 (96 RXN)	May. 2020	• Updated kit version of 96 RXN to V1.2. • Updated the specifications of each component from MGIEasy FS PCR-Free DNA Library Prep Kit in Table 2

Manual version	Kit version	Date	Description
A1	V1.1	Dec. 2019	• Updated kit version to V1.1. • Reduced the minimum gDNA input to 50 ng • Added WGA DNA to input DNA type • Added beads ratio for single beads purification in step 3.3.2. • Added DNBSEQTM series sequencing platform • Changed the reaction time in steps 3.2.2, 3.5.5, 3.8.3. • Changed the prep volume of En-TE buffer in step 3.1.2 (Table 6). • Changed the elute volume for low gDNA input in step 3.10.8. • Changed the QC criterion for quantification of the ligated product
A0	V1.0	Mar. 2019	Initial release

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 FS PCR-Free DNA Library Prep Set is specifically designed for construct WGS libraries without PCR for MGI High-throughput Sequencing Platforms.

The library prep set is optimized to convert 50 ng to 1000 ng genomic DNA into a customized library. This set incorporates a high-quality fragmentation enzyme and improved Adapter Ligation technology, which significantly increases library conversion rate. All reagents provided in this set have passed strict quality control and functional verification procedures, ensuring stability and reproducibility.

1.2 Intended use

This library prep set is applicable to samples from human (including but not limited to blood, saliva), animals (including but not limited to mouse), plants (including but not limited to rice), bacteria (including but not limited to *E. coli*), fungi (including but not limited to *Candida gabrata*), and other microbial species. The fragmentation time should be titrated before library construction for optimal fragment size.

1.3 Applicable sequencing platforms

The prepared libraries are applicable to the following sequencing platforms.

- MGISEQ-200RS/DNBSEQ-G50RS (PE100)
- MGISEQ-2000RS/DNBSEQ-G400RS (PE100/PE150)
- DNBSEQ-T7RS (PE100/PE150)
- DNBSEQ-$T10\times4$ RS(PE100)

1.4 Components

This library prep set comes in two specifications: 16 RXN and 96 RXN. Three separate boxes are included for each specification. For component details, refer to the following table.

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

Table 1 MGIEasy FS PCR-Free DNA Library Prep Set V1.2 (16 RXN) (Cat. No.: 1000013454)

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy FS PCR-Free DNA Library Prep Kit V1.2 Cat. No.: 1000013458 Configuration: 16 RXN	20x Elute Enhancer	Black	3 µL/tube × 1
	FS Buffer II	Green	160 µL/tube × 1
	FS Enzyme Mix II	Green	80 µL/tube × 1
	ER Buffer	Orange	112 µL/tube × 1
	ER Enzyme Mix	Orange	48 µL/tube × 1
	Ad-Lig Buffer	Red	288 µL/tube × 1
	Ad Ligase	Red	80 µL/tube × 1
	Ligation Enhancer	Brown	32 µL/tube × 1
	Cir Buffer	Purple	184 µL/tube × 1
	Cir Enzyme Mix	Purple	8 µL/tube × 1
	Exo Buffer	White	23 µL/tube × 1
	Exo Enzyme Mix	White	42 µL/tube × 1
	Exo Stop Buffer	White	48 µL/tube × 1
MGIEasy PF Adapters-16 (Tube) Kit Cat. No.: 1000013460	DNA Adapters	Colorless	5 µL/tube × 16
MGIEasy DNA Clean Beads Cat. No.: 940-001596-00	DNA Clean Beads	White	8 mL/tube × 1
	TE Buffer	White	4 mL/tube × 1

Table 2 MGIEasy FS PCR-Free DNA Library Prep Set V1.3 (96 RXN) (Cat. No.: 1000013455)

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy FS PCR-Free DNA Library Prep Kit V1.3 Cat. No.: 1000013459 Configuration: 96 RXN	20x Elute Enhancer	Black	20 μL/tube × 1
	FS Buffer II	Green	1120 μL/tube × 1
	FS Enzyme Mix II	Green	640 μL/tube × 1
	ER Buffer	Orange	896 μL/tube × 1
	ER Enzyme Mix	Orange	352 μL/tube × 1
	Ad-Lig Buffer	Red	1108 μL/tube × 1
	Ad Ligase	Red	560 μL/tube × 1
	Ligation Enhancer	Brown	304 μL/tube × 1
	Cir Buffer	Purple	1456 μL/tube × 1
	Cir Enzyme Mix	Purple	60 μL/tube × 1
	Exo Buffer	White	282 μL/tube × 1
	Exo Enzyme Mix	White	374 μL/tube × 1
	Exo Stop Buffer	White	512 μL/tube × 1
MGIEasy PF Adapters-96 (Tube) Kit Cat. No.: 1000013461	DNA Adapters-96 plate	-	5 μL/well × 96
MGIEasy DNA Clean Beads Cat. No.: 940-001594-00	DNA Clean Beads	White	50 mL/tube × 1
	TE Buffer	White	25 mL/tube × 1

1.5 Storage and transportation

Table 3 Kit storage and transportation temperatures

Item		Storage temperature	Transportation temperature
MGIEasy FS PCR-Free DNA Library Prep Kit	20 × Elute Enhancer	2 ℃ to 30 ℃	-80 ℃ to -15 ℃
	Exo Stop Buffer		
	Ligation Enhancer	2 ℃ to 30 ℃ Away from light	
	Other components	-25 ℃ to -15 ℃	
MGIEasy PF Adapters-16 (Tube) Kit			
MGIEasy PF Adapters-96 (Plate) Kit			
MGIEasy DNA Clean Beads		2 ℃ to 8 ℃	

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.
- In MGIEasy FS PCR-Free DNA Library Prep Kit, after the first use of 20× Elute Enhancer, Exo Stop Buffer, and Ligation Enhancer, store them at room temperature. Avoid repeated freeze-thawing. The Ligation Enhancer should be stored away from light.

1.6 User-supplied materials

Table 4 User-supplied equipment list

Equipment	Recommended brand
Vortex mixer	/
Desktop centrifuge	/
Pipettes	/
Thermocycler	/
96M MagnumTM Plate	ALPAQUA, Cat. No. A000400 recommended
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
Horizontal electrophoresis tank	/

Equipment	Recommended brand
Gel Imager	/
Gel Electrophoresis apparatus	/

Table 5 Recommended reagent/consumable list

Reagent/consumable	Recommended brand
Nuclease free (NF) water	Ambion, Cat. No. AM9937 or equivalent
TE Buffer, pH 8.0	Ambion, Cat. No. AM9858 or equivalent
100% Ethanol (Analytical Grade)	/
Qubit ssDNA Assay Kit	Invitrogen, Cat. No. Q10212, or equivalent
Qubit dsDNA HS Assay Kit	Invitrogen, Cat. No. Q32854) or equivalent
Agilent High Sensitivity DNA Kit	Agilent, Cat. No. 5067-4626, or equivalent
Agilent DNA 1000 Kit	Agilent, Cat. No. 5067-1504, or equivalent
REGULAR AGAROSE G-10	BIOWEST, CB005-100G or equivalent
GelStain (10000x)	TRANSGEN, Cat. No. GS101-01 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.
- To prevent yield loss, try to avoid transferring the reaction product to a new tube for beads purification especially in Exo Digestion Product purification step. Adding the En-Beads directly to the reaction tube for product purification is recommended.
- 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, and 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 the MGI technical support: **MGI-service@mgi-tech.com**

1.8 Workflow

Section	Workflow	Total time	Hands-on time
3.1	Reagent prep	10 min	10 min
3.2	Fragmention	35 - 45 min	10 min
3.3	Magnetic beads size selection ⏸	30 - 45 min	10 - 15 min
3.4	End repair	65 min	10 min
3.5	Adapter ligation	20 min	10 min
3.6	Cleanup of adapter-ligated ⏸	30 min	10 - 15 min
4.1	Denaturation and single strand circularization	30 min	15 min
4.2	Digestion	40 min	10 min
4.3	Cleanup of digestion product ⏸	50 min	10 - 15 min
4.4	QC of digestion product ⏸	15 - 20 min	10 - 15 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 preparation

2.1 Sample requirements

- This library prep set is suitable for samples from common animals, plants, fungi, bacteria etc. This includes Humans (blood, saliva), rice, *Candida glabrata*, *E. coli*. It is strongly recommended to use high quality genomic DNA ($2.0 \geq OD_{260/280} \geq 1.8$, $OD_{260/230} \geq 1.7$) for fragmentation.
- Since FS Enzyme Mix II is sensitive to the pH and components of the DNA storage buffer, we recommend using 1x TE buffer (pH 8.0) or H_2O for dissolution of DNA. If other buffers, such as 10 mM Tris (pH 6.8-8.0), AE Buffer(pH 8.5), 0.1x TE (pH 8.0) or other special buffers are present, refer to "Fragmentation" on page 10 to do a demo test before you start the real test. If the demo test result is unsatisfactory, please re-purify the gDNA and elute it in 1x TE buffer (pH 8.0) or H_2O.
- Any residual impurities (e.g. metal chelators or other salts) in the gDNA sample may adversely affect the efficiency of the fragmentation step and the fragment size.

2.2 Library insert size requirement

A narrow size distribution of fragmented DNA is preferable. Better sequencing quality can be obtained with a narrow size distribution, while a wide distribution results in lower quality.

- The recommended peak size of double size selection library is 450 bp to 600 bp.
- The recommended peak size of single size selection library is 600 bp to 750 bp.

Tips Do not pool double size selection library and single size selection library together for sequencing.

2.3 gDNA amount and size selection

The brief scheme of Library Construction Protocol using this kit is as following:

50 ng - 1000 ng of gDNA is fragmented by Enzyme Fragmentation, and purified DNA fragments is obtained and ready for further library construction. The recommend instructions for different gDNA amount are listed in table below.

Table 6 Recommend instructions for different gDNA amounts

gDNA amount (A)	gDNA input	Size selection method for fragment DNA	purified DNA fragments input
1000 ng < N	1000 ng	Double size selection	80 - 200 ng
800 ng ≤ N ≤ 1000 ng	800 ng - 1000 ng (fully used)	Double size selection	80 - 200 ng
200 ng < N < 800 ng	200 ng	Single size selection	fully used
50 ng ≤ N ≤ 200 ng	50 ng - 200 ng (fully used)	Single size selection	fully used

Tips

- The single size selection method will result in lower sequence reads, because its range of insert size is wider than that of the double size selection method.
- 500 ng - 800 ng of gDNA also can be used as shearing input followed by the double size selection for size selection. However, it may cause low yield.
- Library construction with 50 ng - 200 ng gDNA input results in low yield of ssCir. Usually, it is insufficient for a sequencing run, which can be pooled and sequenced with other PCR-free libraries.

3 Library preparation protocol

3.1 Reagent prep

3.1.1 Preparation

Table 7 Preparing the reagents

Reagent	Requirement
Nuclease-Free Water	User-supplied; place at room temperature (RT), mix well.
TE Buffer	
20 × Elute Enhancer	Thaw at RT, mix by vortexing, centrifuge briefly, and place at RT. Store at RT after first using.
DNA Clean Beads	Mix by vortexing and place at RT.

3.1.2 Operation

CAUTION The preparation volume of reagents listed blow is enough for 6 samples. Increase the preparation reagent volumes in proportion if there are more samples.

1. Prepare the 1x Elute Enhancer in a 1.5 mL sterile centrifuge tube. Mix the tube by vortexing and centrifuge briefly. Store at room temperature before using. The shelf life of the 1x Elute Enhancer is 7 days.

Table 8 1x Elute Enhancer

Reagent	Volume per reaction
20 × Elute Enhancer	1 μL
Nuclease-Free Water	19 μL
Total	20 μL

2. Prepare the En-TE in a 1.5 mL sterile centrifuge tube. Mix the tube by vortexing. Store at 4 ℃ before using. The shelf life of the En-TE is 7 days.

Table 9 En-TE

Reagent	Volume per reaction
1 × Elute Enhancer	2.4 μL
TE Buffer	1197.6 μL
Total	1200 μL

3. Prepare the En-Beads in a 1.5 mL sterile centrifuge tube. Mix the tube by vortexing. Store at 4 ℃ before using. The shelf life of the En-Beads is 7 days.

Table 10 En-Beads

Reagent	Volume per reaction
1 × Elute Enhancer	15 μL
DNA Clean Beads	1485 μL
Total	1500 μL

3.2 Fragmentation

Tips The following fragmentation conditions are suitable for human blood, saliva, animal, plant, or bacterial gDNA. Fragment size should be 100 bp to 2000 bp, with a peak size of 450 bp to 600 bp. If the sample is not listed above, refer to the following conditions to shorten or extend the 30 ℃ incubation time to achieve the optimum results.

3.2.1 Preparation

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

Table 11 Preparing the reagents

Reagent	Requirement
Dilution Buffer	User-supplied; place at RT.
FS Buffer II	Thaw at RT, vortex, centrifuge briefly, and place on ice.
FS Enzyme Mix II	Keep on ice.
En-TE	Refer to 3.1.2, place at RT.

CAUTION • DO NOT vortex FS Enzyme Mix II.

- Please strictly follow the instructions on the manual. Insufficient mixing would affect the fragmentation process.

3.2.2 Fragmentation

1. Add 50 ng to 1000 ng gDNA to a new 0.2 mL PCR tube. Normalize the gDNA to a total volume of 45 μL. Vortex 3 times (3 sec each), centrifuge briefly, and place on ice.

Table 12 Normalization of gDNA Dissolved in 1xTE (pH 8.0)

Components	Volume
1 × TE (pH 8.0)	45-X μL
gDNA (50 ng - 1000 ng)	X μL
Total	45 μL

Tips
- This enzyme is sensitive to the pH. The lower the pH is, the smaller the peak size of fragmentation. In principle, the normalization buffer should be the same as the DNA elution buffer.
- If the same sequencing batch of genomic DNAs are dissolved in different types of DNA dissolving solution (pH range: 6.8-8.5):
  1) when using double size selection method for fragment DNA purification, it is recommended to use 1 x TE buffer (pH 8.0) or H_2O as the DNA normalization buffer.
  2) when using single size selection for fragment DNA purification, it is recommended to re-purify the genomic DNAs and dissolve in 1 x TE buffer (pH 8.0) or H_2O.

2. Set and run the program with the following conditions. The thermocycler will perform the first step reaction described in table below and be kept at 4 ℃.

Table 13 Fragmentation reaction conditions (Volume: 60 μL)

Temperature	Time
70 ℃ Heated lid	On
4 ℃	Hold
30 ℃	11 to 18 min
65 ℃	15 min
4 ℃	Hold

3. Mix the FS Enzyme Mix II by inverting 10 times and flicking the bottom gently, ensure that no residual reagent is left at the bottom. Centrifuge briefly and place on ice.

Tips DO NOT vortex the FS Enzyme Mix II. Insufficient mixing would affect the fragmentation process.

4. According to the desired reaction number, prepare the fragmentation mixture in a 0.2 mL PCR tube on ice. Mix it well by vortexing, centrifuge briefly, and place on ice.

Table 14 Fragmentation mixture

Reagent	Volume per reaction
FS Buffer II	10 μL
FS Enzyme Mix II	5 μL
Total	15 μL

5. Add 15 μL of fragmentation mixture to each sample tube (from step 1). Pipette 10 times or vortex 3 times (3 sec each), centrifuge briefly, and place on ice.
6. Make sure the thermocycler has cooled to 4 ℃ (see step 2). Place the PCR tube(s) into the thermocycler and skip the 4 ℃ hold step to start the reaction at 30 ℃.
7. After reaction, centrifuge the tube(s) briefly. Add 20 μL of En-TE to each tube to make a total volume of 80 μL. Vortex 3 times (3 sec each), centrifuge briefly, and place on ice.

Tips
- For the first fragmentation test, it is recommended to take 40 μL of product from the 80 μL mixture in Step 6 for purification with 1.8X beads and elute in 25 μL of En-TE. Take 1 μL of elute product for Agilent 2100 High Sensitivity test and make sure the smear size is 100 to 2000 bp with the peak size between 300 bp - 800 bp (Figure 1).
- If the size is too large or too small, titrate the 30 ℃ incubation time from "Table 13 Fragmentation reaction conditions (Volume: 60 μL)" on page 11. For samples in which an ideal fragmentation size cannot be attained by incubation time titration, we recommend re-purifying the sample DNA with 1.8x magnetic beads and eluting into 1x TE buffer (pH 8.0) or Nuclease-Free Water. After re-purification, re-titrate the incubation time (10 - 20 min is recommended).

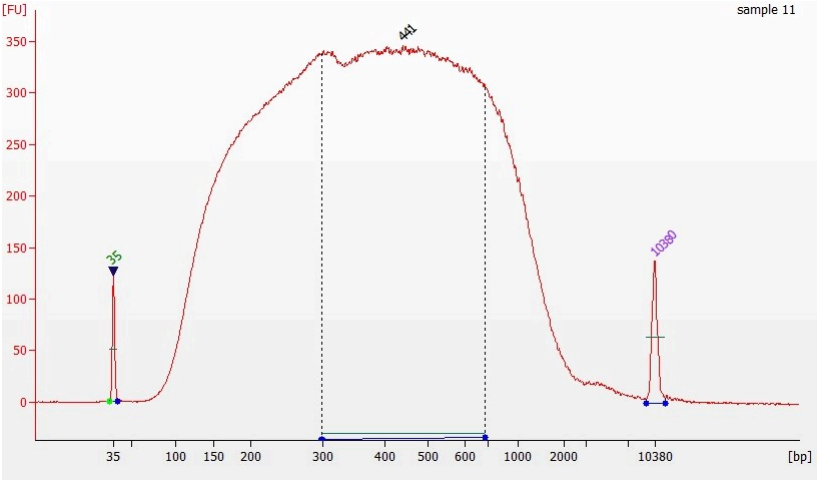

Figure 1 1000 ng gDNA (dissolved in pH8.0 1xTE buffer) 1.8x beads purification fragmentation product (30 ℃,11 min)

3.3 Magnetic beads size selection

Tips Select one of the two methods based on your needs.

- After fragmentation, the DNA has a wide size distribution and it is usually necessary to perform a fragment screen to control the concentration of the final library fragments.

- Select the double size selection if the input DNA is higher than 800 ng.
- Select the single size selection if the input sample is small than 800 ng.
- Less than 200 ng of purified fragmented DNA should be used for End Repair. If the fragmented DNA is less than 40 ng, library preparation may fail.

Table 15 Double size selection process (75 μL sample of the theoretical majority of DNA fragments using magnetic beads selection)

Target fragment peak size (bp)	450 - 600
1st Bead selection (μL)	40
2nd Bead selection (μL)	12
Sequencing strategy	PE100/PE150

Tips The selection condition in table above is used for reference. For different samples, the target fragment peak size may have a ±100 bp deviation. The ideal recovery rate of double size selection is 15% to 20%.

3.3.1 Double size selection

Tips
- The DNA sample may loss approximately 75% to 90% in double size selection process. If the sample is rare, recycle the DNA which adsorb on the 1st beads. Process Step 8 to 13 to recycle the DNA and store the eluted DNA as a backup.
- 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.

3.3.1.1 Preparation

Table 16 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied. Freshly prepared.
En-TE	Refer to section 3.1.2. Place at RT.
En-Beads	Refer to section 3.1.2. Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.3.1.2 Double size selection

1. Transfer 75 μL of Fragmentation Products to a new 0.2 mL PCR tube. Add En-TE to make a final volume of 75 μL if the volume is less than 75 μL.
2. Mix the En-Beads thoroughly. Add 40 μL of En-Beads to each sample tube (from step 7 in section 3.2.2). Gently pipette at least 10 times until all beads are suspended. Ensure all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.

3. Incubate the sample(s) at room temperature for 10 min.

   Tips In the next step, keep the supernatant and discard the beads. If necessary, recycle the DNA on that beads.

4. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer all of supernatant to a new 0.2 mL PCR tube.
5. Add 12 µL of En-Beads to each sample tube (contain supernatant). Gently pipette at least 10 times until all beads are suspended. Or, mix with a vortexer.
6. Incubate the sample(s) at room temperature for 10 min.
7. Centrifuge the sample tube(s) briefly and place on the magnetic rack for at least 5 min until the liquid is clear. Carefully remove and discard the supernatant.
8. While keeping the tube(s) on the magnetic rack, add 160 µL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
9. Repeat step 8. 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.
10. 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.

11. Remove the tube(s) from the magnetic rack and add 45 µL of En-TE to elute the DNA. Gently pipette the liquid at least 10 times. Or, mix with a vortexer.
12. Incubate the sample(s) at room temperature for 5 min.
13. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 44 µL of supernatant to a new 0.2 mL PCR tube.
14. Quantify the Size selection products with dsDNA Fluorescence Assay Kits such as Qubit dsDNA HS Assay Kit or Quant-iT PicoGreen dsDNA Assay Kit.

    Stop point Products can be stored at -20 ℃.

    Tips For the demo test, it is recommended to take 1 µL of eluted product for Agilent 2100 High Sensitivity test (Figure 2), to ensure that the peak size of the selected fragments is approximately 450 bp to 600 bp. It should be noted that the peak size of the sequenced library is normally smaller than the peak size shown in the Agilent 2100 Bioanalyzer.

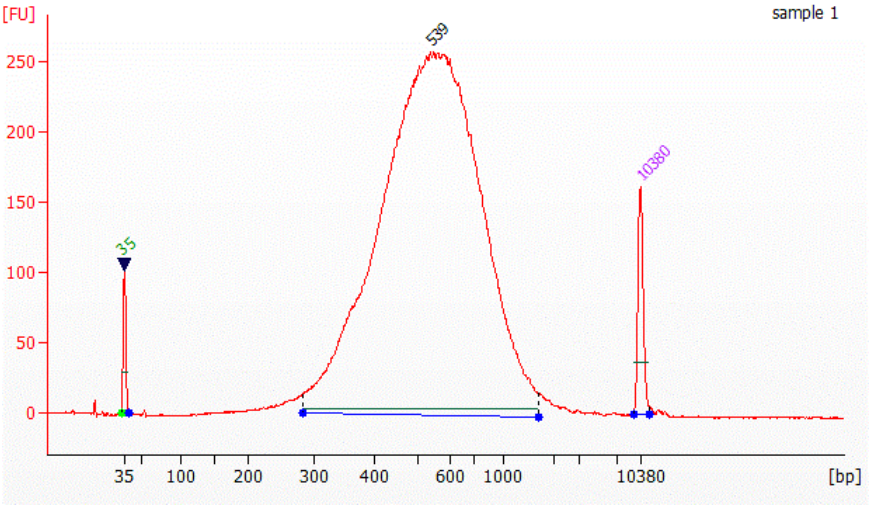

Figure 2 Agilent 2100 Bioanalyzer results of 1000 ng gDNA#dissolved in pH8.0 1X TE buffer#double size selection fragmentation product#30 °C,11 min#

3.3.2 Single size selection

Tips

- The DNA sample may loss approximately 30% to 60% in single size selection process.
- Do not touch or pipette the beads when adding reagents or transferring supernatant. If you accidentally pipette the beads, pipette all of the solution and beads back into the tube and restart the separation process.

3.3.2.1 Preparation

Table 17 Preparing the reagents

Reagent	Requirement
80% ethanol	User supplied. Freshly prepared.
En-TE	Refer to section 3.1.2. Place at RT.
En-Beads	Refer to section 3.1.2. Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.3.2.2 Single size selection

1. Transfer 75 μL of Fragmentation Products to a new 0.2 mL PCR tube. Add En-TE to make a total volume of 75 μL if the volume is less than 75 μL.
2. Mix the En-Beads thoroughly. Add 60 μL of En-Beads to each sample tube (from step 7 in section 3.2.2). Gently pipette at least 10 times until all beads are suspended. Or, mix with a vortexer.
3. Incubate the sample(s) at room temperature for 10 min.
4. 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.
5. While keeping the tube(s) on the magnetic rack, add 160 μL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
6. Repeat step 5. 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.
7. 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.

8. Remove the tube(s) from the magnetic rack and add 45 μL of En-TE to elute the DNA. Gently pipette the liquid at least 10 times. Or, mix with a vortexer.
9. Incubate the sample(s) at room temperature for 5 min.
10. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 44 μL of supernatant to a new 0.2 mL PCR tube.
11. Quantify the purified fragmentation products with dsDNA Fluorescence Assay Kits such as Qubit dsDNA HS Assay Kit or Quant-iT PicoGreen dsDNA Assay Kit.

    Stop point Products can be stored at -20 ℃.

    Tips For the demo test, it is recommended to take 1 μL of step 3.3.1.12 eluted product for Agilent 2100 High Sensitivity test (Figure 3), to ensure that the peak size of the selected fragments is approximately 600 bp to 750 bp. It should be noted that the peak size of the sequenced library is normally smaller than the peak size shown in the Agilent 2100 Bioanalyzer.

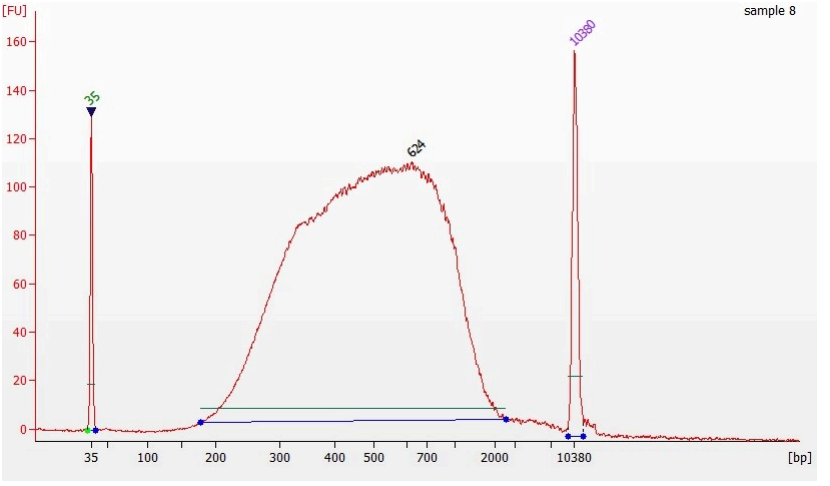

Figure 3 Agilent 2100 Bioanalyzer results of 200 ng gDNA#dissolved in pH8.0 1xTE buffer#single size selection fragmentation product#30 #C, 11 min#

3.4 End repair

Tips Preheat the thermocycler to reaction temperature in advance if the thermocycler heat up slowly.

3.4.1 Preparation

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

Table 18 Preparing the reagents

Reagent	Requirement
En-TE	Refer to 3.1.2, place at RT.
ER Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
ER Enzyme Mix	Mix by flicking with a finger, centrifuge briefly, and place on ice.

3.4.2 End repair

1. Transfer an appropriate amount of sample (80 ng - 200 ng is recommended) to a new 0.2 mL PCR tube. Add En-TE to make a total volume of 40 μL.
2. 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 19 End repair reaction mixture

Reagent	Volume per reaction
ER Buffer	7 μL
ER Enzyme Mix	3 μL
Total	10 μL

3. Add 10 μL of end repair reaction mixture to each sample tube (from step 13 in 3.3.1.2 or step 10 in 3.3.2.3). Vortex the tube(s) 3 times (3 sec each), centrifuge briefly, and place on ice.
4. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Tips Preheat the thermocycler to reaction temperature before use.

Table 20 End repair reaction conditions (Volume: 50 μL)

Temperature	Time
70 ℃ Heated lid	On
14 ℃	15min
37 ℃	25 min
65 ℃	15 min
4 ℃	Hold

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

CAUTION Do not stop at this step. Please proceed to next reaction.

3.5 Adapter ligation

Tips Before operation, carefully read “Using adapters” on page 27.

3.5.1 Preparation

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

Table 21 Preparing the reagents

Reagent	Requirement
Adapters	Thaw on ice, mix by vortexing, centrifuge briefly, and place on ice.
Ad-Lig Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Ad Ligase	Mix by flicking with a finger, centrifuge briefly, and place on ice.

Reagent	Requirement
Ligation Enhance	Thaw at RT, mix by vortexing, centrifuge briefly, and place at RT. Store at RT and away from light after first using.
En-TE	Refer to 3.1.2, and place at RT.

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

3.5.2 Adapter ligation

1. Add **5 μL of adapter(s)** to the corresponding sample tube (from step 5 in 3.4.2). Vortex it 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 6 times (3 sec each), centrifuge briefly, and place on ice.

Table 22 Adapter ligation mixture

Reagent	Volume per reaction
Ad-Lig Buffer	18 μL
Ad Ligase	5 μL
Ligation Enhancer	2 μL
Total	25 μL

4. 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.

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

Table 23 Adapter ligation reaction conditions (Volume: 80 μL)

Temperature	Time
30 ℃ Heated lid	On
25 ℃	10 min
4 ℃	Hold

Tips The ligation incubation time for 25 ℃ can extend into 30 min for improving ssCir output as needed.

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

8. Add 20 μL of En-TE to make a total volume of 100 μL. Mix it well, centrifuge briefly, and place on ice.

CAUTION Do not stop at this step. Please proceed to next reaction.

3.6 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.

3.6.1 Preparation

Table 24 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied; freshly prepared.
En-TE	Refer to section 3.1.2, and place at RT.
En-Beads	Refer to section 3.1.2. Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.6.2 Cleanup of adapter-ligated product

1. Mix the En-Beads thoroughly. Add 50 μL of En-Beads to each sample tube (from step 7 in 3.5.2). Gently pipette at least 10 times until all beads are suspended. Or, mix with a vortexer.
2. Incubate the sample(s) at room temperature for 10 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 160 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 50 μL of En-TE buffer to elute the DNA. Gently pipette the liquid at least 10 times. 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 48 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.

CAUTION Do not mix the samples for multiple samples sequencing after cleanup of adapter-ligated product. The residual adapter dimer may cause the sample cross-contamination.

4 Circularization and digestion

4.1 Denaturation and single strand circularization

4.1.1 Preparation

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

Table 25 Preparing the reagents

Reagent	Requirement
Cir Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Cir Enzyme Mix	Mix by flicking with a finger, centrifuge briefly, and place on ice.

4.1.2 Denaturation

1. Place the PCR tube(s) (from step 9 in 3.6.2) into the thermocycler and run the program with the following conditions.

Table 26 Denaturation reaction conditions (Volume: 48 μL)

Temperature	Time
100 ℃ Heated lid	On
95 ℃	3 min
4 ℃	10 min

Tips There is another alternative to Denaturation Reaction Conditions: 95 ℃ 3 min(Heated Lid 100 ℃). After reaction, quickly place on ice 2 min.

2. After reaction, centrifuge the tube(s) briefly and place on ice.

4.1.3 Single strand circularization

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

Table 27 Circularization reaction mixture

Reagent	Volume per reaction
Cir Buffer	11.5 μL
Cir Enzyme Mix	0.5 μL
Total	12 μL

2. Add 12 μL of circularization reaction mixture to each sample tube (from step 2 in 4.1.2). Vortex the tube(s) 3 times (3 sec each), centrifuge briefly, and place on ice.
3. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 28 Single strand DNA circularization reaction conditions (Volume: 60 μL)

Temperature	Time
42 ℃ Heated lid	On
37 ℃	10 min
4 ℃	Hold

4. When the program is completed, place the PCR tube(s) on ice, centrifuge briefly, and proceed to the next step.

4.2 Exo digestion

4.2.1 Preparation

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

Table 29 Preparing the reagents

Reagent	Requirement
Exo Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Exo Enzyme Mix	Mix by flicking with a finger, centrifuge briefly, and place on ice.
Exo Stop Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place at RT.

4.2.2 Exo digestion

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

Table 30 Exo digestion mixture

Reagent	Volume per reaction
Exo Buffer	1.4 μL
Exo Enzyme Mix	2.6 μL
Total	4.0 μL

2. Add 4 μL of exo digestion mixture to each sample tube (from step 4 in section 4.1.3). Vortex the tube(s) 3 times (3 sec each), centrifuge briefly, and place on ice.
3. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 31 Exo digestion reaction conditions (Volume: 64 μL)

Temperature	Time
42 ℃ Heated lid	On
37 ℃	30 min
4 ℃	Hold

4. When the program is completed, centrifuge the tube(s) briefly. Immediately add **3 μL of Exo Stop Buffer** to each sample tube. Vortex the tube(s) 3 times (3 sec each) and centrifuge briefly.

CAUTION The yield may loss if transfer the liquid into 1.5 mL tube to cleanup. It is not recommended to transfer the liquid.

4.3 Cleanup of exo digestion 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.

4.3.1 Preparation

Table 32 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied; freshly prepared.
En-TE	Refer to section 3.1.2, and place at RT.

Reagent	Requirement
En-Beads	Refer to section 3.1.2. Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

4.3.2 Cleanup of exo digestion product

1. Mix the En-Beads thoroughly. Add 120 µL of En-Beads to each sample tube (from step 4 in 4.2.2). Gently pipette at least 10 times until all beads are suspended. Or, mix with a vortexer.
2. Incubate the sample(s) at room temperature for 10 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 160 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 25 µL of En-TE to elute the DNA. Gently pipette the liquid at least 10 times. Or, mix with a vortexer.

   Tips If the gDNA input is 50 to 100 ng, it is recommended to use 12 µL of En-TE for DNA elution and to collect 11 µL of the supernatant in step 9.

8. Incubate the sample(s) at room temperature for 10 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 24 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 ℃.

4.4 QC of digestion product

- Quantify the purified Exo Digestion Products with Qubit ssDNA Assay Kit.
- The final yields should be ≥75, 60 and 30 fmol when using 200 ng - 1000 ng, 100 ng - 200 ng, and 50 ng - 100 ng gDNA as input respectively. Please refer to table below or Formula 1.

Formula 1: Conversion between circular ssDNA fmol and mass in ng

$$75 \text{ fmol ssDNA (ng)} = 0.075 \times \text{DNA fragment peak size (bp)} \times 0.33$$

Table 33 The corresponding molecular weight equal to 75 fmol circularized ssDNA for different selected fragment size

Peak size of selected fragment (bp)	Corresponding yield in 75 fmol (ng)
360	9
400	10
490	12.2
530	13.2

- Sequencing requires a single strand circle input is 75 fmol/lane.
- If you plan to pool multiple samples in one lane for sequencing, you can pool the single strand circles of different samples by certain mole ratio at this step. The barcodes used in the pooled samples should strictly adhere to the instructions for MGIEasy PF Adapters (see "Using adapters" on page 27). And the mole ratio is based on your required sequencing data of each sample being pooled.

Tips The insert size and the size range affect sequencing quality and amount of effective sequencing reads. Therefore, pooling libraries with different insert sizes or using different purification methods (for example, pooling single size selection products with double size selection products for sequencing) may lead to decreased sequencing quality and fewer effective sequencing reads. If you have to pool the libraries, it is recommended to pool PCR-free libraries with similar insert sizes and size ranges.

5 Appendix

5.1 Using adapters

MGI currently offers the Adapter Reagent Kits with two specifications based on the number of reactions: the MGIEasy PF Adapters-16 (Tube) Kit and MGIEasy PF Adapters-96 (Plate) Kit.

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 adapters 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.
- Do not place the adapters above 30 ℃ to avoid structural changes such as denaturation, which might affect performance.
- Before use, mix the adapter(s) well and centrifuge to collect liquid at the bottom of tubes or plates. Wipe the surface of the tube cap or aluminum film with absorbent paper.
- For MGIEasy PF Adapters-16 (Tube) Kit, carefully open the tube cap to prevent spills or to prevent cross-contamination. Close the cap immediately after use.
- For MGIEasy PF Adapters-96 (Plate) Kit, pierce the aluminum film to pipette solutions for first use. During the process, remember to replace the tip to avoid contamination. After use, transfer the remaining reagents to individual 1.5 mL tube(s) or 0.2 mL PCR tube(s) , label them and store at -20 ℃.
- Adapters from other MGI library Kits are designed for library construction with amplification strategies and are incompatible with PCR-Free Kits.

5.1.1 Instructions for PF Adapters-16 (Tube)

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 proper combinations.

- 2 sets of 4 adapters: (01-04) and (13-16)
- 1 set of 8 adapters: (97-104)

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 34 Instructions for PF Adapters-16 (Tube)

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: 97-104. Mix 8 adapters with equal volume and add the mixture to the sample. • Or, add one adapter to the sample, if you don't need to sequence the barcode. This method should not be used for samples pooling sequencing.
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: 97-104. Mix 97-100 with equal volume and add the mixture to sample 1; Mix 101-104 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 adapter 01, 02, 03, 04 to sample 1, 2, 3, 4, in that order. • Or, for a set of 8 adapters, add 2 adapters to each sample. For example: 97-104. Mix 97-98, 99-100, 101-102, and 103-104 with equal volume. Add the mixture to sample 1, 2, 3, 4, in that order.

Sample/lane	Instruction (Example)
5	• 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	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.
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, for samples 5-6 and for sample 7.
8	• For a set of 8 adapters, add 1 adapter to each sample. For example: 97-104. Add adapters 97-104 to samples 1-8, in that order. • Or, for 2 sets of 4 adapters, add 1 adapter to each sample. For example: 01-04 and 13-16. Add 1 adapter to each sample.
8+x (x=1-8, Total 9-16)	Perform the following 3 steps: 1. For samples 1 to 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 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 (97-104).
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)).

5.1.2 Instructions for PF 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 proper combinations.

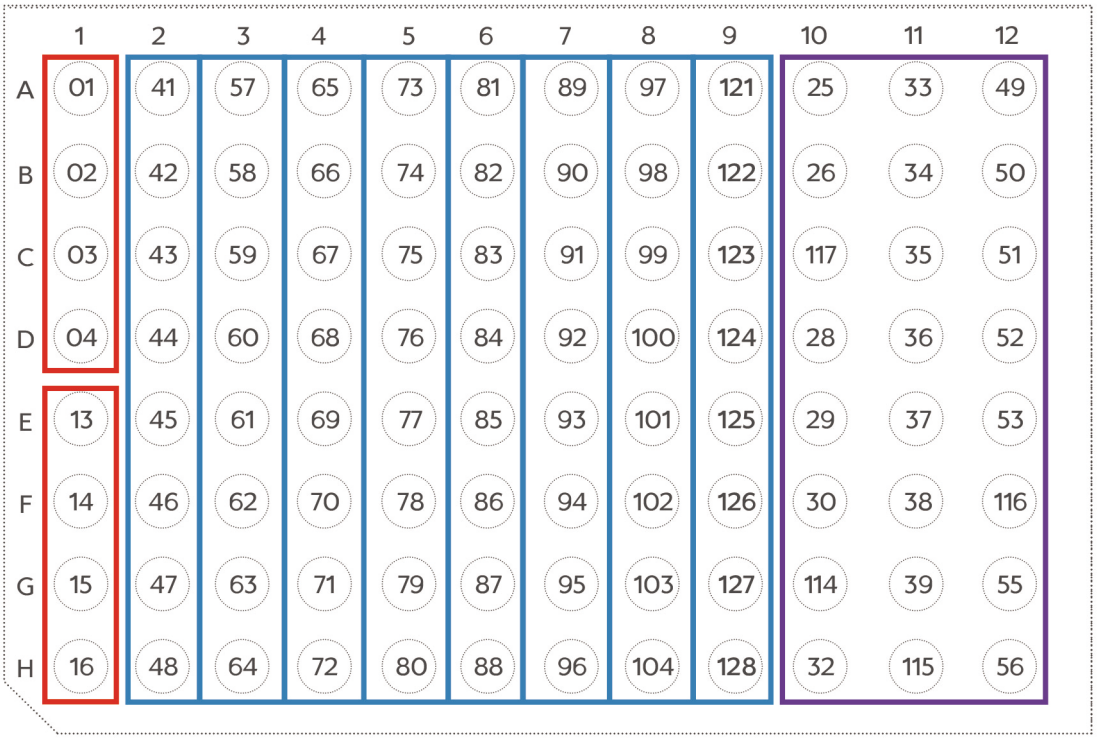

Figure 4 PF adapters-96 (plate) adapters layout and combination instructions

- 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 35 Instructions for PF 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. • Or, add one adapter to the sample, if you don't need to sequence the barcode. This method should not be used for samples pooling sequencing.

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)).