MGIEasy

Whole Genome Bisulfite Sequencing Library Prep Kit User Manual

Cat. No：1000005251 (16RXN)

Kit Version：V2.0

Manual Version：B2

Revision History

Manual Version	Kit Version	Date	Description
B2	V2.0	Jan. 2021	◆ Update contact information.
B1	V2.0	Jul.2020	◆ Update the manual style
B0	V2.0	Nov.2019	◆ Kit Version update to V2.0, Manual version update to B0. ◆ Change ERAT step to ER and AT. Add Quench step in between. ◆ delete all the Single Strand DNA Circularization steps and change to Double Strand DNA Circularization steps ◆ Change DNB pooling recommendation and WGBS make DNB instruction.
A1	V1.0	Sep. 2019	◆ 1.3 Add DNBSEQ series sequencing platform
A0	V1.0	Oct. 2018	◆ Initial release.

Note: Please download the latest version of the manual and use it with the corresponding kit.

Search manual by Cat. No. or product name from website:

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

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Chapter 1 Product Description

1.1 Introduction

MGIEasy Whole Genome Bisulfite Sequencing Library Prep Kit is specifically designed for preparing WGBS libraries for MGI High-throughput sequencing platforms. This library prep kit is optimized to convert 10-100ng of fragmented DNA into a sequencing library for MGI High-throughput sequencing platforms. This kit incorporates improved Adapter Ligation and High-fidelity PCR Enzymes which significantly increases library yield and amplification efficiency. All reagents provided within this kit have passed stringent quality control and functional verification, ensuring high performance stability and repeatability.

1.2 Applications

This library prep kit is universal kit which is applicable for samples from all common animals, plants, fungus, bacteria, including Humans, mice, rice, Arabidopsis, and yeast. Stable performance across all such sample types is expected.

1.3 Platform Compatibility

Constructed libraries are compatible with:

BGISEQ-500RS (PE100)

MGISEQ-2000RS (PE100), DNBSEQ-G400RS (PE100)

1.4 Contents

MGIEasy Whole Genome Bisulfite Sequencing Library Prep Kit is 16RXN. Further information about cat. no. and components are listed in Table 1:

Table 1 MGIEasy Whole Genome Bisulfite Sequencing Library Prep Kit (16 RXN)

Modules & Cat. No.	Components	Color Coded Screw Caps	Spec & Quantity
MGIEasy Whole Genome Bisulfite Sequencing Library Prep Kit Cat. No.: 1000005251	Lambda DNA	Yellow	18 μL/tube× 1tube
	ER Buffer Mix	Orange	117 μL/tube× 1tube
	ER Enzyme Mix	Orange	44 μL/tube× 1tube
	ER Stop Buffer	White	100 μL/tube× 1tube
	AT Buffer Mix	Orange	65 μL/tube× 1tube
	AT Enzyme Mix	Orange	4 μL/tube× 1tube
	Ligation Buffer Mix	Red	375 μL/tube× 1tube
	DNA Ligase	Red	26 μL/tube× 1tube
	WGBS Adapter (16 barcodes)	Colorless	5 μL/tube× 16tubes
	WGBS PCR Enzyme Mix	Blue	400 μL/tube× 1tube
	PCR Primer Mix	Blue	80 μL/tube× 1tube
	DS Digestion Buffer	Purple	150 μL/tube× 1tube
	DS Digestion Enzyme Mix	Purple	16 μL/tube× 1tube
	DS Ligation Buffer	Purple	800 μL/tube× 1tube
	DNA Rapid Ligase	Purple	8 μL/tube× 1tube
	WGBS Make DNB Buffer	White	320 μL/tube× 1tube

⚠ **Note：Lambda DNA concentration is 200 ng/μL.**

1.5 Storage Conditions and Shelf Life

- Storage temperature：-25°C--15°C.
- Expiration date：9 months.
- Transport conditions：transported on dry ice. Please ensure sufficient dry ice remains after transportation.
- Performance of kit components are guaranteed until the expiration date under appropriate transport, storage, and usage conditions.

1.6 Equipment and Materials Required but not Provided

Table 2 Equipment and Materials Required but not Provided

Equipment	Covaris™ Focused-ultrasonicator (Thermo Fisher Scientific™ Vortex Mixer High speed centrifuge (Eppendorf) Desktop centrifuge Pipettes Thermocycler Magnetic rack DynaMagTM-2 (Thermo Fisher Scientific™， Cat. No. 12321D)or equivalent Qubit® 3.0 Fluorometer (Thermo Fisher Scientific™, Cat. No. Q33216) Electrophoresis equipment or Agilent 2100 Bioanalyzer (Agilent Technologies™, Cat. No. G2939AA)
Reagents	Nuclease free water (NF water) (Ambion™, Cat. No. AM9937) TE Buffer，pH 8.0 (Ambion™, Cat. No. AM9858) 100% Ethanol (Analytical Grade) AMPure XP beads (Agencourt™, Cat. No. A63882) QIAquick PCR Purification Kit (QIAGEN™, Cat. No. 28104/28106) EZ DNA Methylation-Gold Kit (Zymo Research™, Cat. No. D5005/D5006) Qubit® ssDNA Assay Kit (Thermo Fisher Scientific™, Cat. No. Q10212) Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific™, Cat. No. Q32854) High Sensitivity DNA Analysis Kits (Agilent Technologies™, Cat. No. 5067-4626) DNA Analysis Kits (Agilent Technologies™, Cat. No. 5067-1504)
Consumables	Covaris AFA tubes for use with ultrasonicator (Thermo Fisher Scientific™) Pipette tips 1.5 mL tube, Non-stick RNase-Free, 1.5 mL Microfuge Tubes (Axygen™ ,Cat. No. MCT-150-C) 0.2 mL PCR tube (Axygen™, Cat. No. PCR-02-C) or 96-well plate (Axygen™, Cat. No. PCR-96M2-HS-C) Qubit® Assay Tubes (Thermo Fisher Scientific™， Cat. No. Q32856) or 0.5mL thin wall PCR tubes (Axygen™, Cat. No. PCR-05-C)

1.7 Precautions and Warnings

1.7.1 General Recommendations

- Instructions provided in this manual are intended for general use only, and may require further adjustments to optimize performance. We recommend making adjustments while taking into consideration the experimental design, sample characteristics, sequencing application, and other equipment.
- Retrieve the reagents from storage before starting experiment and prepare them before use. For Enzymes, centrifuge briefly and place on ice during the experiment. For other reagents, first thaw at room temperature, invert several times to mix completely, centrifuge briefly, and place on ice for further use.
- When preparing mixtures and working solutions, we recommend pipetting up and down at least 10 times to mix thoroughly. Note that vigorous shaking may decrease library yield.
- To prevent cross-contamination, we recommend using filtered Pipette Tips. Use a new tip each time for pipetting different solutions.
- We recommend using Thermocyclers with heated lids for reactions. Preheat to reaction temperature before use.
- Improper handling of samples and reagents may contribute to aerosol contamination of PCR Products and may negatively affect experimental accuracy. Therefore, we recommend physically separating two working areas in the laboratory for PCR reaction preparation and PCR product purification. Use designated equipment for each area and perform regular cleaning to ensure a sterile working environment. (Use 0.5% Sodium Hypochlorite or 10% Bleach to clean working environment).

1.7.2 Insert Size Requirements

- This protocol is used for constructing libraries from 250 bp DNA fragments. See the size selection conditions in Table 3 for purifying other fragment sizes. Sequencing quality may decrease slightly as the insert size increases. To achieve high quality sequencing, we recommend using uniform DNA fragment sizes for library construction.

⚠ **Warning: Pooling DNA libraries of different insert sizes for sequencing is not recommended.**

- If you have any question, please contact MGI technical support MGI-service@mgi-tech.com

Chapter 2 Sample Requirements and preparation

2.1 Sample Requirements

This library kit supports library preparation from gDNA of all common animals, plants, fungus, etc, including Humans, Mice, Rice, Arabidopsis, and Yeast. It is also compatible with human FFPE samples. To meet the Step 3.1 Library Requirements, it is strongly recommended to use fragmented DNA samples with an input of 10-100ng and volume less than 30 μL.

2.1.1 gDNA

- Sample DNA refers to DNA fragments after physical fragmentation and size selection. This kit is compatible with sample DNA between 10-100ng. The gDNA sample volume for fragmentation cannot exceed the maximum sample volume for Covaris sonication (Appendix A).
- Nanodrop measurements: High quality gDNA with A260/A280=1.8 ~ 2.0, A260/A230>2.0. Electrophoresis measurement: Main DNA bands >23 Kb, intact or slightly degraded DNA. DNA quality significantly influences the quality of library and sequencing.
- DNA sample should be free of RNA contamination. Contamination during DNA extraction caused by high concentrations of metal ion chelators or other salts may have negative effects on end repair and A-tailing efficiency.

2.1.2 FFPE sample

- Sample DNA refers to FFPE DNA fragments after physical fragmentation and size selection. This kit is compatible with the sample DNA between 10-100 ng. The gDNA sample volume for fragmentation cannot exceed the maximum sample volume of Covaris sonication (Appendix A).
- Electrophoresis measurement: intact DNA with main bands>23 Kb or slightly degraded FFPE DNA. Using highly degraded FFPE DNA for library construction might decrease the library quality.
- DNA sample should be free of RNA contamination. Contamination during DNA extraction caused by high concentrations of metal ion chelators or other salts may have negative effects on end repair and A-tailing efficiency.

2.2 DNA Fragmentation and Size Selection

2.2.1 Fragmentation

- Fragment gDNA into sizes between 100-700 bp. The optimal main size of gDNA/ FFPE DNA is 250 bp. Refer to Appendix A.
- For additional requirements of Covaris Focused-ultrasonication, please visit Covaris' official website.
- If other fragmentation methods are used, we recommend doing trial runs to determine optimal parameters for obtaining recommended fragment sizes before getting started.
- To evaluate the efficiency of C to T conversion in library, you must perform fragmentation and size selection on Lambda DNA using the same conditions as the sample. Add selected Lambda DNA at 1/50 amount to the End Repair and A-tailing input. For example, add 1ng selected Lambda DNA to 50ng End repair and A-tailing sample input or add 0.4 ng selected Lambda DNA to 20ng End repair and A-tailing sample input.

⚠ **Note: The concentration of Lambda DNA in the kit is 200 ng/μL.**

2.2.2 Size Selection

- DNA fragmentation results in a wide fragment size distribution. Therefore, it is necessary to perform fragment size selection to ensure that the final library has uniform insert size. For size selection, we recommend using magnetic beads (see Table 3).
- Fragment size selection is achieved through controlling the volume ratio of suspended beads. Bead Volume / Sample Volume = Bead Ratio (×). By increasing the ratio, the selection can purify shorter fragment sizes. On the contrary, using a lower ratio will purify longer DNA fragments.1st Bead Selection: Beads bind to fragments longer than the desired upper limit. Discard beads to remove the bead-binding fragments; 2nd Bead Selection: Beads bind to fragments longer than the desired lower limit. Remove the supernatant to remove smaller unwanted fragments. Finally, collect DNA fragments in main size.

Table 3 Bead: DNA Fragment Ratio

Main Fragment size(bp)	180	230	250	300	350
1st Bead Selection (×)	1.0	0.9	0.8	0.7	0.6
2nd Bead Selection (×)	0.5	0.2	0.2	0.2	0.2

- The DNA loss during bead selection is approximately 60%-95%. For important samples you may

collect the beads from the 1st Bead Selection, wash twice with 80% Ethanol, air dry, then elute with TE Buffer and store as a backup sample.

- To purify 250bp fragments, we recommend using 0.8×+0.2× bead selection on fragmented gDNA/FFPE DNA. Use 10-100ng of selection product to begin library construction following Step 3.1.

2.3 Sample DNA Quantitation and Quality Control

- Sample DNA refers to DNA fragments that have not yet undergone the End Repair process. Quantitate sample DNA after physical fragmentation and size selection using Qubit® dsDNA HS Assay Kit or Quant-iT™ PicoGreen® dsDNA Assay Kit. This kit is compatible with sample DNA amounts between 10-100 ng.
- Ensure a narrow distribution of main fragment size (Table 3). A wide distribution will decrease sequencing quality. Please use optimal library insert sizes based on your sequencing options. The ideal main size is 250bp for PE100 sequencing. Warning: pooling DNA libraries of different insert sizes is not recommended.
- Contamination of sample DNA during preparation by metal chelates or other salts may have a negative effect on the efficiency of the End Repair process.

Chapter 3 Library Construction Protocol

3.1 End Repair and A-tailing

⚠ **Warning: Please read Precautions and Warnings and PART 2.1 Sample Requirements carefully before starting library construction. To evaluate the efficiency of C-to-T conversion, follow Step 3.1.1 to add 20ng sample together with 0.4ng Lambda DNA of the same fragment size to a new 0.2mL PCR tube and add TE Buffer to 30 μL total volume. Then continue to Step 3.1.2 and the following steps.**

This protocol describes the method for constructing a library from 20ng DNA fragments with main peak at 250 bp.

3.1.1 Add the appropriate amount of sample (20 ng recommended) to a new 0.2 mL PCR tube and then add TE Buffer to a final volume of 30 μL.

3.1.2 Prepare the End Repair Reaction Mixture on ice (see Table 4).

Table 4 The End Repair Reaction Mixture

Components	Volume
ER Buffer Mix	7.3 μL
ER Enzyme Mix	2.7 μL
Total	10 μL

3.1.3 Transfer 10 μL of the End Repair reaction mixture to the PCR tube from Step 3.1.1. Vortex 3 times (3 s each) and briefly centrifuge (5 s) to collect the solution to the bottom of the tube.

3.1.4 Place the 0.2 mL PCR tube from Step 3.1.3 into the Thermocycler. Set up the reaction conditions listed in Table 5 and start the reaction.

Table 5 The Reaction Conditions of the End Repair

Temperature	Time
Heated lid	On
25°C	30 minutes
4°C	Hold

3.1.5 Briefly centrifuge to collect the solution to the bottom of the tube.

3.1.6 Transfer 6 μL of the ER stop Buffer to the PCR tube from Step 3.1.5. Vortex 3 times (3 s each) and briefly centrifuge (5 s) to collect the solution to the bottom of the tube, incubate for 2 minutes.

3.1.7 Place 0.2 mL PCR tube from Step 3.1.3 in the preheated Thermocycler(75°C). Set up the reaction conditions listed in Table 6 and start the reaction.

Table 6 The Reaction Conditions of the End Repair Quenching

Temperature	Time
Heated lid	On
75°C	20 minutes
4°C	Hold

3.1.8 Briefly centrifuge to collect the solution to the bottom of the tube.

3.1.9 Prepare the A tailing Reaction Mixture on ice (see Table 7).

Table 7 The A Tailing Reaction Mixture

Components	Volume
AT Buffer Mix	3.8 μL
AT Enzyme Mix	0.2 μL
Total	4 μL

3.1.10 Transfer 4 μL of A Tailing Reaction Mixture to the PCR tube from Step 3.1.8. Vortex 3 times (3 s each) and briefly centrifuge (5 s) to collect the solution to the bottom of the tube.

3.1.11 Place 0.2 mL PCR tube from Step 3.1.10 in the Thermocycler. Set up the reaction conditions listed in Table 8 and start the reaction.

Table 8 The Reaction Conditions of the A tailing

Temperature	Time
Heated lid	On
65°C	15 minutes
4°C	Hold

3.1.12 Briefly centrifuge to collect the solution to the bottom of the tube.

⚠ **Note: We do not recommend stopping at this step. Please continue to Step 3.2. If the operation must be put on hold, the End Repair product can be stored at -20°C overnight with a risk of 20% decrease in yield.**

3.2 Adapter Ligation

Note: Please read Appendix D and E carefully before you begin.

3.2.1 Please refer to the instructions for WGBS Adapters (16 barcode) (see Appendix D). Dilute WGBS Adapter with TE buffer at a ratio of 1:4. The dilution ratio depends on the amount of sample DNA used in library construction. See Table 24 in Appendix D for detailed information. Add 5 μL of diluted WGBS Adapters to the 0.2 mL PCR tube from Step 3.1.5. Vortex 3 times (3 s each) and centrifuge briefly to collect solution.

3.2.2 Prepare the following Adapter Ligation reaction mixture on ice (see Table 9).

Table 9 The Adapter Ligation Reaction Mixture

Components	Volume
Ligation Buffer Mix	23.4 μL
DNA Ligase	1.6 μL
Total	25 μL

3.2.3 Use pipette to slowly transfer 25 μL of Adapter Ligation reaction mixture to the 0.2 mL PCR tube from Step 3.2.1. Vortex 6 times (3 s each) and centrifuge briefly to collect the solution.

3.2.4 Place 0.2 mL PCR tube from Step 3.2.3 in the Thermocycler. Set up the reaction conditions listed in Table 10 and start the reaction.

Table 10 The Reaction Conditions of Adapter Ligation

Temperature	Time
Heated lid	40°C
23°C	30 minutes
4°C	Hold

3.2.5 Centrifuge briefly to collect solution to the bottom of the tube.

3.2.6 Transfer the entire product to a new 1.5 mL tube.

Stopping Point: Adapter-ligation DNA can be stored at -20°C for a maximum of 16 hours.

3.3 Cleanup of Adapter-ligated DNA

Note: Please read Appendix B carefully before you begin. For cleanup of adapter-ligated DNA, we recommend using QIAquick PCR Purification Kit. See the manual on the official website for detailed instruction (https://www.qiagen.com/cn/shop/sample-technologies/dna/dna-clean-up/qiaquick-pcr-purification-kit/#resources). Add the correct amount of 96-100%

ethanol according to the label on the Buffer PE and mix thoroughly before use. Perform cleanup at room temperature (20°C ~25°C).

3.3.1 Transfer the ligation product from Step 3.2.6 to a 1.5mL centrifuge tube and add 400 µL (5 times the volume) Buffer PB. Vortex 6 times (3 seconds each). Centrifuge briefly to collect the solution to the bottom of the tube.

3.3.2 Place QIAquick column on the 2 mL collection tube. Transfer the mixture to QIAquick column. Centrifuge at 13000 rpm for 1 minute. Remove and discard the supernatant and place QIAquick column back on the collection tube.

3.3.3 Add 750 µL freshly prepared Buffer PE to QIAquick column. Centrifuge at 13000 rpm for 1 minute. Remove and discard the supernatant and place QIAquick column back on the collection tube.

3.3.4 Centrifuge again at 13000 rpm for 1 minute. Remove and discard the supernatant.

3.3.5 Transfer QIAquick column to a new 1.5 mL tube. Open the cap of QIAquick column and air dry for 2 minutes until no droplet is seen on the inner and outer wall of QIAquick column.

3.3.6 Slowly add 21 µL Buffer EB to the center of QIAquick column membrane and incubate for 1 minute. Centrifuge at 13000 rpm for 1 minute. Purified ligated products are collected in 1.5 mL EP.

⚠ **Note 1：Do not replace QIAquick PCR Purification Kit (purification of 100 bp-10 kb DNA in size) with magnetic beads or MinElute PCR Purification Kit (purification of 70 bp-4 kb DNA in size).**

⚠ **Note 2：We do not recommend adding pH Indicator I to Buffer PB. If added, please use pH Indicator I in strict accordance with the QIAquick PCR Purification Kit instructions.**

✔ **Stopping Point: After cleanup, PCR Products can be stored at -20°C.**

3.4 Bisulfite Treatment and Cleanup

⚠ **Note：Please read Precautions and Warning carefully before you begin. For bisulfite treatment and cleanup of adapter-ligated products, we recommend using EZ DNA Methylation-Gold Kit. See the manual on the website for detailed instruction (https://www.zymoresearch.eu/ez-dna-methylation-gold-kit).**

3.4.1 Prepare CT Conversion Reagent as follows: Add 900 µL nuclease-free (NF) water, 300 µL M-Dilution Buffer and 50 µL M-Dissolving Buffer to a tube of CT Conversion Reagent powder (centrifuge briefly before opening the cap). Frequently vortex for 10 minutes to mix at room temperature. **The CT Conversion Reagent is light sensitive, so minimize its exposure to light**. For best results, the CT Conversion Reagent should be used immediately following preparation. If

not used immediately, the CT Conversion Reagent solution can be stored overnight at room temperature, one week at 4°C, or up to one month at -20°C. CT Conversion Reagent solution must be warmed to 37°C, then vortexed for 10 minutes prior to use.

3.4.2 Add the correct volume of 100% ethanol to M-Wash Buffer as the label guided and mix before use.

3.4.3 Prepare bisulfite treatment reaction mixture to a new 0.2 mL PCR tube at room temperature (see Table 11). No need for Lambda DNA fragmentation. Note: the Lambda DNA concentration is 200 ng/μL:

Table 11 Bisulfite treatment reaction mixture

Components	Volume
CT Conversion Reagent	130 μL
Purified adapter-ligated DNA	19 μL
Lambda DNA	1 μL
Total	150 μL

3.4.4 Place 0.2 mL PCR tube from Step 3.4.3 in a Thermocycler. Set up the reaction conditions listed in Table 12 and start the reaction.

Table 12 Bisulfite treatment reaction conditions

Temperature	Time
Heated lid	On
98°C	10 minutes
64°C	2.5 h
4°C	Hold

✔ **Stopping Point: Bisulfite-treated DNA products can be hold overnight in Thermocycler at 4°C or stored 24 hours at -20°C.**

3.4.5 Transfer bisulfite-treated products from Step 3.4.4 to a new 1.5 mL tube. Add 600 μL M-Binding Buffer and vortex 6 times (3 seconds each). Centrifuge briefly to collect the solution.

3.4.6 Place Zymo-Spin™ IC Column on a 2 mL Collection Tube. Transfer the mixture to Zymo-Spin™ IC Column. Centrifuge at 13000 rpm for 30 seconds. Remove and discard the supernatant. Place Zymo-Spin™ IC Column back to Collection Tube.

3.4.7 Add 100 μL M-Wash Buffer to Zymo-Spin™ IC Column and centrifuge at 13000 rpm for 30 seconds.

3.4.8 Add 200 μL M-Desulphonation Buffer to Zymo-Spin™ IC Column. Screw the cap on the tube tightly and incubate for 15-20 minutes at room temperature. Centrifuge at 13000 rpm for 30 seconds. Remove and discard the supernatant and place Zymo-Spin™ IC Column back to Collection Tube.

3.4.9 Add 200 μL M-Wash Buffer to Zymo-Spin™ IC Column. Centrifuge at 13000 rpm for 30 seconds. Remove and discard the supernatant. Then Place Zymo-Spin™ IC Column back to Collection Tube.

3.4.10 Add 200 μL M-Wash Buffer to Zymo-Spin™ IC Column. Centrifuge at 13000 rpm for 30 seconds. Remove and discard the supernatant. Then Place Zymo-Spin™ IC Column back to Collection Tube. Centrifuge at 13000 rpm for 30 seconds. Discard Collection Tube. Remove the liquid on outer surface of Zymo-Spin™ IC Column with pipette and place Column to a new 1.5 mL tube.

3.4.11 Open the cap of Zymo-Spin™ IC Column and air dry for 2 minutes at room temperature. Place Zymo-Spin™ IC Column on another new 1.5 mL tube.

3.4.12 Slowly add 10 μL M-Elution Buffer to the center of Zymo-Spin™ IC Column membrane. Let stand for 1 minute at room temperature. Centrifuge at 13000 rpm for 30 seconds and collect the purified bisulfite-treated products in 1.5 mL tube.

⚠ **Note 1: An unbalanced centrifuge from Step 3.4.6-3.4.12 or incomplete drying from Step 3.4.10-3.4.11 can cause failed library construction.**

⚠ **Note 2: Collected products from Step 3.4.12 should be less than 10 μL. Otherwise, it may indicate that library construction failed.**

✔ Stopping Point: Purified bisulfite-treated DNA products can be stored for 24 hours at -20°C.

3.5 PCR Amplification

⚠ **Note: Please read Appendix E carefully before you start.**

3.5.1 Transfer all purified bisulfite-treated products into a new 0.2 mL PCR Tube and add NF water to a final volume of 20 μL

3.5.2 Prepare the PCR Amplification mixture on ice (see table 13):

Table 13 PCR Amplification Mixture

Components	Volume
WGBS PCR Enzyme Mix	25 μL
PCR Primer Mix	5 μL
Total	30 μL

3.5.3 Transfer 30 μL of PCR Amplification mixture to the 0.2 mL PCR tube from Step 3.5.1. Vortex 3 times (3 seconds each) and centrifuge briefly to collect the solution.

3.5.4 Place the 0.2 mL PCR tube from Step 3.5.3 into the Thermocycler. Set up the reaction conditions (see Table 14) and start the reaction.

Table 14 The Reaction Conditions of PCR Amplification

Temperature	Time	Cycles
Heated lid	on	
95°C	2 min	1 cycle
98°C	20 s	
62°C	20 s	13 cycles
72°C	30 s	
72°C	3 min	1 cycle
4°C	Hold	

3.5.5 Centrifuge briefly to collect solution to the bottom of the tube.

3.5.6 Transfer all of the solution into a new 1.5 mL tube.

3.6 Cleanup of PCR

⚠ **Note：Please read Appendix B carefully before you start.**

3.6.1 Remove AMPure® XP Beads from the refrigerator and let stand at room temperature for 30 min beforehand. Vortex and mix thoroughly before use.

3.6.2 Transfer 50 μL (1.0×) AMPure® XP Beads to the 50 μL PCR products from Step 3.5.6. Pipette up and down 10 times to mix thoroughly. Ensure that all liquid and beads are expelled into the 1.5 mL tube before you continue to the next step.

3.6.3 Incubate for 5 minutes at room temperature.

3.6.4 Centrifuge briefly and place the 1.5 mL tube on the Magnetic Separation Rack for 5 minutes until the liquid is clear. Carefully remove and discard the supernatant with a pipette (Do not disturb

the beads).

3.6.5 Keep the 1.5 mL tube on the Magnetic Separation Rack and add 200 µL freshly prepared 80% Ethanol to wash the beads and walls of the tube. Incubate for 30 seconds, then carefully remove and discard the supernatant.

3.6.6 Repeat Step 3.6.5 once. Remove all liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid to the bottom of the tube, separate beads with a Magnetic Separation Rack and then remove any remaining liquid using a small volume pipette.

3.6.7 Keep the 1.5 mL tube on the Magnetic Separation Rack with the lid open, and allow beads to air dry until they no longer appear shiny but before they start to crack.

3.6.8 Remove the 1.5 mL tube from the Magnetic Separation Rack and add 32 µL TE Buffer to elute the DNA. Pipette up and down at least 10 times to mix thoroughly.

3.6.9 Incubate for 5 minutes at room temperature.

3.6.10 Centrifuge briefly then place the 1.5 mL tube back onto the Magnetic Separation Rack for 5 minutes until the liquid is clear. Transfer 30 µL supernatant to a new 1.5 mL tube.

✔ **Stopping Point: After cleanup, purified PCR Products can be stored at -20°C.**

3.7 Quality Control of PCR Product

3.7.1 Quantitate the purified PCR products with dsDNA Fluorescence Assay Kits such as: Qubit® dsDNA HS Assay Kit or Quant-iT™ PicoGreen® dsDNA Assay Kit. The required yield for PCR products is ≥ 1 pmol. See Table 15 for the corresponding yield for different fragment sizes (detailed calculations in Appendix F). For pooled sequencing, please follow instructions provided for MGIEasy DNA Adapters. See Appendix D for detailed information about how to design your sample pooling. Quantitate your Adapter-ligated samples before pooling. The total yield after pooling should be 1 pmol, with a total volume ≤ 48 µL.

Table15 The Corresponding Yield in 1 pmol for PCR Products with Different Fragment Sizes

Insert Size (bp)	PCR Product Size(bp)	Corresponding Yield in 1 pmol (ng)
180	264	170
230	314	210
250	334	220
300	384	250
350	434	280

3.7.2 Assess the fragment size distribution of purified PCR products with electrophoresis-based equipment such as Bioanalyzer, Tapestation (Agilent Technologies), LabChip® GX, GXII, GX Touch (PerkinElmer) or Fragment Analyzer™ (Advanced Analytical). See the Agilent 2100 Bioanalyzer Results of purified PCR Product in Figure 1.

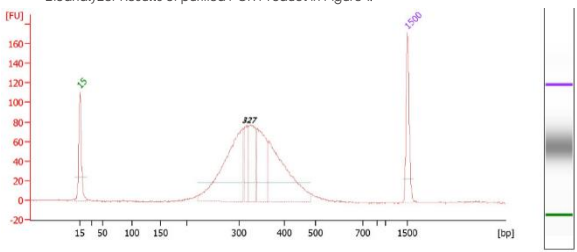

Figure 1 The Agilent 2100 Bioanalyzer Results of purified PCR Product

3.8 Double Strand DNA Digestion

Note: Please read Appendix F carefully before you begin.

3.8.1 According to the targeted fragment size of purified PCR product, transfer 1 pmol of PCR Product to a new 0.2 mL PCR tube. Add TE Buffer to final volume of 40 μL.

3.8.2 Set up the reaction conditions for Double Strand Digestion (see Table 16) and start the reaction.

Table 16 Double Strand DNA Digestion Mixture

Components	Volume
DS Digestion Buffer	9 μL
DS Digestion Enzyme Mix	1 μL
Total	10 μL

3.8.3 Transfer 10 μL of Double Strand DNA Digestion Mixture to the PCR tube from Step 3.8.2. Vortex 3 times (3 s each) and briefly centrifuge (5 s) to collect the solution to the bottom of the tube.

3.8.4 Place 0.2 mL PCR tube in the Thermocycler. Set up the reaction conditions for digestion (see Table 17) and start the reaction.

Table 17 The Reaction Conditions of Double Strand DNA Digestion

Temperature	Time
Heated lid	On
37°C	20 min
75°C	10 min
4°C	Hold

3.8.5 After the reaction is complete, centrifuge briefly and place the 0.2 mL PCR tube on ice. Continue to the next step immediately.

3.9 Double Strand DNA Circularization

3.9.1 Prepare the Double Stranded DNA Circularization mixture on ice (see Table 18):

Table 18 Double Strand DNA Circularization mixture

Components	Volume
DS Ligation Buffer	49.5 μL
DNA Rapid Ligase	0.5 μL
Total	50 μL

3.9.2 Transfer 50 μL Double Strand Circularization mixture to the 0.2 mL PCR Tube from Step 3.8.5. Vortex 3 times (3 seconds each) and centrifuge briefly to collect the solution.

3.9.3 Place the 0.2 mL PCR tube into the Thermocycler. Set up the reaction conditions for Double Strand Circularization (see Table 19) and start the reaction.

Table 19 The Reaction Conditions of Double Strand DNA Circularization

Temperature	Time
Heated lid	On
37°C	60 min
4°C	Hold

3.9.4 Briefly centrifuge and place the 0.2 mL PCR tube on ice.

3.10 Cleanup of Double Strand DNA Product

Note：Please read Appendix B carefully before you begin.

3.10.1 Remove AMPure® XP Beads from the refrigerator and let stand for 30 minutes at room temperature. Vortex and mix thoroughly before use.

3.10.2 Transfer 250 μL (2.5×) AMPure® XP Beads to the 100 μL of Double Strand DNA Circularization Products from Step 3.9.4. Pipette up and down at least 10 times to mix thoroughly. Ensure that all liquid is expelled into the 1.5 mL tube before you continue to the next step.

3.10.3 Incubate for 10 minutes at room temperature.

3.10.4 Centrifuge briefly and place the 1.5 mL tube onto a Magnetic Separation Rack for 5 minutes until the liquid clears. Carefully remove and discard the supernatant with a pipette.

3.10.5 Keep the tube on the Magnetic Separation Rack and add 500 μL freshly prepared 80% Ethanol to wash the beads and the sides of the tube. Incubate for 30 seconds and then carefully remove and discard the supernatant (Do not disturb the beads).

3.10.6 Repeat Step 3.10.5 once. Remove all liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid to the bottom of the tube, separate beads with a Magnetic Separation Rack, and then remove any remaining liquid using a small volume pipette.

3.10.7 Keep the 1.5 mL tube on the Magnetic Separation Rack with the lid open and allow beads to air dry until they no longer appear shiny but before they start to crack.

3.10.8 Remove the 1.5 mL tube from the Magnetic Separation Rack and add 32 μL TE Buffer to elute the DNA. Pipette up and down at least 10 times to mix thoroughly.

3.10.9 Incubate for 10 minutes at room temperature.

3.10.10 Centrifuge briefly, then place the tube back onto the Magnetic Separation Rack for 5 minutes until the liquid clears. Transfer 30 μL supernatant to a new 1.5 mL tube.

Stopping Point: After cleanup, Double strand DNA Products can be stored at -20°C.

Chapter 4 DNB Making and Sequencing

4.1 WGBS and WGS library Pooled Sequencing

⚠ **Note: Note: WGBS library has an unbalanced base composition and needs to be pooled with WGS library as a balance library for sequencing. We recommend that insert sizes difference of the WGBS library and a balance library should be within 20 bp. DNA libraries with big difference in insert sizes are not recommended to be pooled for sequencing. We recommend DNB pooling and it is recommended that WGBS DNB and WGS DNB be pooled at a ratio of 2:1. Follow Step 4.2 to make DNB before pooling.**

4.2 WGBS DNB Making

⚠ **Note：Please read Precautions and Warnings carefully before you begin. For Double strand WGBS Circularization library（dsDNA library）, you should use the WGBS Make DNB Buffer in our kit to replace the Make DNB Buffer in the MGISEQ-2000RS High-throughput Rapid Sequencing Set for Making DNB**

4.2.1 Take 0.2 mL PCR 8-tube strip or PCR tubes. Prepare reaction mix following Table 20 below on ice.

Table 20 WGBS Make DNB reaction mix

Component	volume（μL）
ds DNA library	4 μL
Low TE Buffer	16 μL
WGBS Make DNB Buffer	20 μL
Make DNB Enzyme Mix I	40 μL
Make DNB Enzyme Mix II	4 μL
Total	84 μL

⚠ **Note: Do not place Make DNB Enzyme Mix II（LC）at room temperature and avoid holding the tube in such a way as to heat the contents.**

4.2.2 Mix gently by vortexing and spin down for 5 seconds using the mini centrifuge. Place the mix into a PCR machine and start the reaction. PCR machine settings are described in Table 21:

Table 21 WGBS DNB reaction condition

Temperature	Time
Heated lid（35°C）	On
30°C	25 min
4°C	Hold

⚠ **Note：We recommend setting the temperature of the heated lid to 35°C or to the temperature closest to 35°C.**

4.2.3 Add 20 μL Stop DNB Reaction Buffer immediately after the reaction enters the cold hold step at 4°C. Mix Gently by wide bore pipetting 5-8 times. Do not vortex or shake the tube. Store DNB at 4°C and perform sequencing within 48 hours.

⚠ **Note：It is very important to mix DNB gently by wide bore pipetting. Do not centrifuge, vortex, or shake the tube.**

4.2.4 If the DNB concentration is higher than 5 ng/μL, continue to DNB Loading in Section 4.3.

4.3 DNB Loading

4.3.1 See Table 22 for the recommended amount of DNB to be loaded. Add DNB Loading Buffer I to 50μL of the DNB volume required for sequencing.

Table22 The Recommended Amount of DNB Loaded for Sequencing

	DNB pooling	DNB pooling	
	DNB amount for pooled library in each lane	The amount of WGBS DNB in each lane	The amount of WGS DNB in each lane
MGISEQ-2000	450 ng	300 ng	150 ng
BGISEQ-500	660 ng	440ng	220 ng

⚠ **Note: Refer to MGI Sequencing Kit User Manual for detailed instructions.**

⚠ **Note: After DNB pooling，if the concentrations of WGBS DNB and WGS DNB are higher than 5 ng/μL and the volume of DNB mixture exceeds the required volume, you can decrease the amount of WGBS DNB and WGS DNB loaded while maintaining a 2:1 mass ratio. If you use other DNB volume required for sequencing, please change the DNB amount for pooled library in each lane proportionally。**

⚠ **Note: To ensure the accuracy of the mixing ratio, it is recommended to use normal pipette for taking samples and wide bore pipette for mixing.**

Appendix

Appendix A DNA Fragmentation Conditions

Below are the Fragmentation parameters for Covaris series models supporting 55 μL sample volumes. For reference only.

Please follow the parameters below to fragment gDNA into sizes between 100-700 bp. The main fragment length of sample DNA should be between 250-300 bp for PE100 sequencing.

Table 23 The Fragmentation Parameters of Covaris series (55 μL Sample Volume)

	Vessel	microTUBE-50 AFA Fiber-Screw-Cap (PN 520166) 						
	Sample Volume	55 μL						
S220	Holder	S-Series Holder microTUBE-50 Screw-Cap (PN 500492)						
	Water Level	10						
	Temperature (°C)	7						
	Target BP (Peak)	150	200	250	300	350	400	550
	Peak Incident Power (W)	100	75	75	75	75	75	50
	Duty Factor	30%	25%	20%	20%	15%	10%	10%
	Cycles per Burst	1000	1000	1000	1000	1000	1000	1000
	Treatment Time (s)	150	95	65	45	45	55	50

	Vessel	MicroTUBE-50 Screw-Cap (PN 520166)	8 microTUBE-50 AFA Fiber Strip V2 (PN 520174)	96 microTUBE-50 AFA Fiber Plate (PN 520168)
			8 microTUBE-50 AFA Fiber H Slit Strip V2 (PN 520240)	96 microTUBE-50 AFA Fiber Plate Thin Foil (PN 520232)

				
	Sample Volume	55 µL		
E220	Racks	Rack 24 Place microTUBE Screw-Cap (PN 500308)	Rack 12 Place 8 microTUBE Strip (PN 500444)	No Rack needed
	Plate Definitions	"E220_500308 Rack 24 Place microTUBE- 50 Screw-Cap +6.5mm offset"	"E220_500444 Rack 12 Place 8 microTUBE-50 Strip V2 -10mm offset"	"E220_520168 96 microTUBE-50 Plate -10.5mm offset" "E220_520232 96 microTUBE-50 Plate Thin Foil -10.5mm offset"
E220 evolution	Racks	Rack E220e 4 Place microTUBE Screw Cap (PN 500432) Rack E220e 8 microTUBE Strip V2 (PN 500437) Non Compatible	Rack E220e 4 Place microTUBE Screw Cap (PN 500432) Rack E220e 8 microTUBE Strip V2 (PN 500437) Non Compatible	Rack E220e 4 Place microTUBE Screw Cap (PN 500432) Rack E220e 8 microTUBE Strip V2 (PN 500437) Non Compatible
	Plate Definitions	"500432 E220e 4 microTUBE-50 Screw Cap - 8.32mm offset" "500437 E220e 8 microTUBE-50 Strip V2 -	"500432 E220e 4 microTUBE-50 Screw Cap - 8.32mm offset" "500437 E220e 8 microTUBE-50 Strip V2 -	"500432 E220e 4 microTUBE-50 Screw Cap - 8.32mm offset" "500437 E220e 8 microTUBE-50 Strip V2 -

		10mm offset" N/A		10mm offset" N/A		10mm offset" N/A		
All	Temperature (°C)	7						
	Water Level	6		-2		0		
	Intensifier (PN 500141)	Yes		Yes		Yes		
	Y-dithering	No		No		Yes (0.5 mm Y-dither at 10 mm/s)		
	Target BP (Peak)	150	200	250	300	350	400	550
Screw-Cap	Peak Incident Power (W)	100	75	75	75	75	75	30
	Duty Factor	30%	20%	20%	20%	20%	10%	10%
	Cycles per Burst	1000	1000	1000	1000	1000	1000	1000
	Treatment Time (s)	130	95	62	40	30	50	70
8-Strip	Peak Incident Power (W)	75	75	75	75	75	75	50
	Duty Factor	15%	15%	20%	20%	20%	10%	10%
	Cycles per Burst	500	500	1000	1000	1000	1000	1000
	Treatment Time (s)	360	155	75	45	35	52	50
Plate	Peak Incident Power (W)	100	100	75	75	75	75	75
	Duty Factor	30%	30%	20%	20%	20%	10%	10%
	Cycles per Burst	1000	1000	1000	1000	1000	1000	1000
	Treatment Time (s)	145	90	70	49	34	50	32

Appendix B Magnetic Beads and Cleanup Procedures

For magnetic beads-based cleanup, we recommend using the MGIEasy DNA Clean Beads（MGI, Cat. No.940-200073-00 or 940-200074-00)or AMPure® XP (Agencourt, Cat. No. A63882). For Magnetic Beads from other sources, cleanup conditions may differ.

Before You Use

1) To ensure capture efficiency of the Magnetic Beads, remove beads from 4°C refrigerator storage and let sit at room temperature (RT) for 30 min. Vortex and mix thoroughly before use.

2) Vortex or pipet up and down to ensure that the beads are thoroughly mixed before use.

3) The volume of the beads is denoted as a multiplier "×" which represents the ratio between the volume of beads and the volume of the original sample. For example: The volume if using 1×Beads in conjunction with 50 μL of original sample is 1×50 μL = 50 μL; For a procedure of 0.8×+ 0.2×Bead Selection, the volume for use for the 1st Bead Selection is 0.8×50 μL = 40 μL, and the volume for use for the 2nd Bead Selection is 0.2×50 μL = 10 μL.

4) The volume of the beads determines the lower limit of fragment size that can be purified. The higher the multiplier, the smaller the fragments can be purified. For example: 1×Beads can effectively purify DNA fragments ≥ 200 bp; 2×Beads can effectively purify DNA fragments ≥ 100 bp.

Operation Notes

1) If the sample volume decreases due to evaporation during incubation, add TE buffer to designated volume before using beads to purify. This is to ensure that the correct multiplier for the beads is used.

2) During the magnetic separation step, please allow the solution to become completely clear before removing the supernatant. This process takes approximately 2-3 minutes. Take into consideration the different magnetic strength of your specific Separation Plate / Rack, and allow enough time for the solution to turn completely clear.

3) Avoid touching the beads with the pipette tip. 2-3 μL of fluids can be left in the tube to avoid contact. If the tip touches the beads, dispense all beads and solution into the tube and restart the separation process.

4) Use freshly prepared 80% Ethanol (at room temperature) to wash the beads. The 1.5 mL tube should remain on the Magnetic Separation Rack while washing. Do not shake or disturb the beads in any way.

5) After the 2nd wash of the beads with Ethanol, try to remove all liquid within the tube. You may centrifuge briefly to collect any remaining liquid to the bottom, separate beads magnetically, and remove remaining liquid by using a small volume pipette.

6) After washing twice with Ethanol, allow the beads to air dry at room temperature. Insufficient drying (the bead surface is shiny) will allow Anhydrous Ethanol to deposit, affecting subsequent reactions. Over-drying (bead pellet appears cracked) may cause a reduction in yield. Drying takes approximately 5-10 minutes depending on your specific lab environment. Observe closely until the pellet appears sufficiently dry with a matte appearance, then continue to elution with TE Buffer.

7) During the elution step, do not disturb the beads with tips when removing the supernatant. Contamination of DNA by the beads may affect subsequent purification. Therefore, the total volume of TE buffer and beads should be 2 μL more than the volume of the supernatant.

8) Pay attention when opening / closing the lids of 1.5 mL tubes on the Separation Rack. Strong vibrations may cause sample loss through spilling the liquid or bead. Secure the tubes well before opening the lids.

Appendix C Magnetic Beads Size Selection

The following example uses 0.8×+ 0.2×Beads to select fragments of 250 bp as sample DNA. To select other fragment sizes, please refer to PART 2 Table 3 for details.

Protocol

1) Remove AMPure® XP Beads from refrigerator and let stand at RT for 30 min beforehand. Vortex and mix thoroughly before use.
2) Transfer all Fragmentation Products to a new 1.5 mL tube. Add TE Buffer to final volume of 100 μL.
3) Transfer 80 μL (0.8×) AMPure® XP Beads to the 1.5 mL tube. Pipette up and down at least 10 times to mix thoroughly. Ensure that all liquid is dispensed into the tube before proceeding.
4) Incubate for 5 minutes at room temperature.
5) Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid is clear, then carefully transfer the supernatant to a new 1.5 mL tube.

⚠ **Note: Retain the Supernatant and discard the Beads.**

6) Transfer 20 μL (0.2×) AMPure® XP Beads to the tube with supernatant. Pipette at least 10 times to mix thoroughly.
7) Incubate for 5 minutes at room temperature.
8) Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid clears, then carefully remove and discard the supernatant with a pipette.
9) Keep the tube on the Magnetic Separation Rack, add 200 μL freshly prepared 80% Ethanol to wash the beads and the walls of the tube. Then, remove and discard the supernatant.
10) Repeat Step 9 and try to remove all liquid from the tube.
11) Keep the tube on the Magnetic Separation Rack with the lid open and allow beads to air dry until they no longer appear shiny, but before bead pellets start to crack.
12) Remove the 1.5 mL tube from the Magnetic Separation Rack, add the specified volume of TE Buffer to elute the DNA. Pipette up and down at least 10 times to mix thoroughly.
13) Incubate for 5 minutes at room temperature.
14) Centrifuge briefly then place the 1.5 mL tube back onto the Magnetic Separation Rack for 2-5 minutes until the liquid clears. Transfer 20 μL supernatant to a new 1.5 mL tube.

Appendix D Using WGBS Adapters

- We currently offer 16 tubes of WGBS Adapters (8-tube strip). WGBS Adapter is developed to meet requirements for batch processing library construction and Multiplex Sequencing. We selected the best WGBS adapter combination based on the principle of balancing base composition. However, the tube numbers of WGBS Adapters are not continuous. For optimal performance, please refer to the instructions in Appendix D-1.
- WGBS adapters are double stranded. Please do not place above room temperature. Structural changes such as denaturation may affect performance.
- Before you use, please centrifuge to collect liquid to the bottom of the tube. Gently remove the cap to prevent liquid from spilling and cross-contamination. Mix the adapters by pipetting before you use and remember to reseal the adapters immediately after use.
- Do not use any Adapters from other kits, including but not limited to MGIEasy DNA Adapters-16 (Tube) Kit and the MGIEasy DNA Adapters-96 (Plate) Kit and Adapters from other MGI library kits Using these incompatible kits and adapter can cause failed library construction.
- WGBS Adapter quality as well as quantity affects the efficiency and quality of library construction. We recommend the WGBS Adapter to sample DNA ratio be 10:1. An excessive input of adapters may lead to Adapter Dimers; whereas insufficient input may lower library yield and construction efficiency.

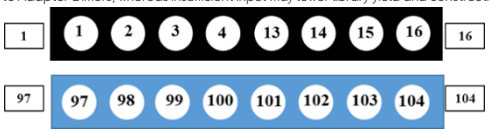

Figure 2 WGBS Adapters-16 (Tube) Kit (8-tube strip).

Table 24 Recommended Adapter input according to the amount of Sample DNA (250 bp)

Sample DNA (ng)	WGBS Adapter Dilution Ratio	WGBS Adapter Input after dilution (μL)
100	No Dilution	5
75	1.33	5
50	2	5
20	5	5
10	10	5

Formula 1: The number of moles of sample DNA can be roughly calculated by the formula below:

sample DNA (pmol) ≈ sample DNA mass (ng) / [0.66× DNA average length (bp)]

- Increasing Adapter input may increase the library yield to a certain extent, especially when sample DNA ≤ 25 ng. If you need to optimize library construction efficiency, you may follow Table 24 to increase Adapter input (within the range of 2-10 times). If the yield is limited by the original Adapter concentration, you may also try increasing Adapter volume input.

D-1 WGBS Adapters-16 (8-tube strip) Kit Instructions

Based on the principles of balancing base composition, WGBS Adapters must be used in specific groups. Please follow the instructions bellow to use Adapters in proper combination:

2 sets of 4 Adapters: (01-04) and (13-16)

1 set of 8 Adapters: (97-104)

Assuming data output requirement is the same for all samples in a lane, please refer to the table 25 below to choose your Barcode Adapter combinations:

Table 25 WGBS Adapters-16 (8-tube strip) Kit Instruction

Sample /lane	Instructions (Example)
1	Requires at least 1 set of Adapter 1. Take a set of 4 Adapters (01-04) and mix equal volumes and then add to the sample. Or 2. Take a set of 8 Adapters (97-104) and mix equal volumes and then add to the sample.
2	Requires at least 1 set of Adapter: 1. Take a set of 4 Adapters (01-04) and mix equal volumes in pairs to obtain 2 mixtures of equal volume. Add 1 mixture to each sample. (e.g. Mix 01 & 02, then add to sample 1; Mix 03 & 04, then add to sample 2) Or 2. Take a set of 8 Adapters (97-104), mix equal volumes in groups of 4 to obtain 2 mixtures of equal volume. Add 1 mixture to each sample. (e.g. Mix 97-100, then add to sample 1; Mix 101-104, then add to sample 2)
3	Requires at least 2 sets of Adapters: For sample 1&2, use the method for (2 samples/lane) above. For sample 3, use the method

	for (1 sample/lane) above. Note that you should use different Adapter sets for sample 1,2 and 3.
4	Requires at least 1 set of Adapter: (1.) Take a set of 4 Adapters (01-04) and add 1 Adapter for each sample in equal volumes. (e.g. Respectively add Adapters 01, 02, 03, 04 to samples 1, 2, 3, 4.) Or (2.) Take a set of 8 Adapters (97-104) and mix equal volumes in pairs to obtain 4 mixtures of equal volume. Add 1 mixture to each sample. (e.g. Mix 97-98, 99-100, 101-102, 103-104, then add respectively to samples 1, 2, 3, 4.)
5	Requires at least 2 Adapter sets: For samples 1-4, use the method for (4 samples/lane) above. For sample 5, use the method for (1 sample/lane) above. Note that you should use different Adapter sets for sample 1-4 and 5.
6	Requires at least 2 Adapter sets: For samples 1-4, use the method for (4 samples/lane) above. For sample 5-6, use the method for (2 sample/lane) above. Note that you should use different Adapter sets for sample 1-4 and 5-6.
7	Requires all 3 Adapter sets, follow these 3 steps: (1) For samples 1-4, use the method for (4 samples/lane) above. (Use 1st Adapter set) (2) For samples 5-6, use the method for (2 samples/lane) above. (Use 2nd Adapter set) (3) For sample 7, use the method for (1 sample/lane) above. (Use 3rd Adapter set). Or take all Adapter sets in the same volume and mix to obtain Adapter mix. Note that you should use different Adapter sets for sample 1-4, sample 5-6 and sample 7.
8	Requires at least 1 set of Adapter: 1. Take a set of 8 Adapters (97-104), add Adapter to each sample in equal volumes. Or 2. Take 2 sets of 4 Adapters (01-04 and 13-16), add 1 Adapter to each sample in equal volumes.

In cases where data output requirements are different between samples, any sample with a data output of more than 20% for each lane must use a separate set of Adapters. (e.g. 9 samples are pooled into 1 lane and one of which requires 30% of the total data output. In this case, the other 8 samples may use Adapters (97-104), whereas the final sample must use a full Adapter set of 01-04 or 13-16 instead of using only a single Adapter.)

Appendix E Adapter Ligation and PCR

- The Adapter Reaction mixture contains a high concentration of PEG which increases the viscosity of the mixture. Please pipette slowly and ensure that the correct amount has been added.
- The amount of PCR cycles must be kept in strict control. Insufficient cycles may reduce yield. Excessive cycles may also lead to adverse effects such as over amplification, an increase in bias, PCR duplicates, chimeric sequences, and accumulated mutation. Table 26 below shows the number of PCR cycles required to generate 300 ng and 1 μg library, respectively, when using 10-100 ng high quality DNA sample (250 bp). When sample DNA is of lower quality and consists of a longer fragment, PCR cycles should be increased accordingly to generate an appropriate yield.

Table 26 PCR Cycles required to yield 300 ng and 1 μg Libraries

Sample DNA (ng)	PCR Cycles required for corresponding yield	
	300 ng	1 μg
10	13-15	15-17
25	12-14	14-16
50	11-13	13-15
75	10-12	12-14
100	10-12	12-14

Appendix F Conversion between DNA Molecular Mass and number of Moles

Formula 2 shows the calculation of the Mass in (ng) that corresponds to 1 pmol of dsDNA sample with varying fragment sizes. Please refer to the formula to calculate the amount of DNA needed.

Formula 2 dsDNA sample pmol and ng Conversion

$$\text{The Mass (ng) corresponding to 1 pmol PCR Products} = \frac{\text{DNA Fragement Size (bp)}}{\text{1000 bp}} \times 660 \text{ ng}$$

The yield for circularized ssDNA after cleanup must be at least 108 fmol or above for sequencing twice. Please refer to Formula 3 below to calculate the number of mols needed:

Formula 3 Circular ssDNA fmol and ng Conversion:

$$\text{The Mass (ng) corresponding to 108 fmol circular ssDNA} = 0.108 \times \frac{\text{DNA Fragment Size (bp)}}{\text{1000 bp}} \times 330 \text{ ng}$$

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