Part No.:H-940-001530-00-01

User Manual

Version:3.0

MGIEasy Whole Genome Methylation Sequencing Library Prep Kit

Cat. No.: 940-001530-00 (16 RXN)
940-001527-00 (96 RXN)
Kit Version: V3.0

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About the user manual

Manufacturer information

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Revision history

Manual version	Kit version	Date	Description
3.0	V3.0	Mar. 2025	• Change the Control DNA to fragmentation only, without purification. • Add application of DNBSEQ-G400RS StandardMPS 2.0 sequencing. • Add application of DNBSEQ-T7RS StandardMPS methylation sequencing.
2.0	V3.0	Sep. 2024	• Update sequencing platform version. • Add the recommended table of sample input and end repair input in the precaution. • Add the precautions for using adapter. • Update the workflow. • Update sample fragment and size selection. • Cleanup of end repair product merged with "A" tailing. • Update DNB loading method. • Add shearing condition and barcode using guide in appendix. • Update TE Buffer specification for 70 mL DNA Clean Beads.
1.0	V3.0	Aug. 2023	Trial version 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 Whole Genome Methylation Sequencing Library Prep Kit is specifically designed for MGI high-throughput sequencing platforms. This kit prepares the selected DNA through end repair, "A"-tailing, adapter ligation, enzymatic conversion, PCR, and circularization to construct methylation library specifically for the MGI high-throughput sequencing platform. All reagents provided in the kit have passed strict quality control and functional verification procedures, ensuring high stability and reproducibility.

1.2 Intended use

This kit can be used to prepare libraries for whole genome methylation sequencing. It is applicable to genomic DNA extracted from human (blood, fresh tissues, cells, and FFPE samples), animals (mouse, etc.), plants (*A. thaliana*, etc.), as well as cell-free DNA from plasma.

1.3 Applicable sequencing platform

The prepared libraries are applicable to the following sequencing platforms and types:

Table 1 Sequencing platform and sequencing type recommendation

Sequencing platform	Sequencing type	ISW version	Basecall version
DNBSEQ-G400RS	PE100 / PE150 (StandardMPS)	1.8.0.2158 and above	1.7.1.613 and above
DNBSEQ-G400RS	PE100 / PE150 (StandardMPS 2.0)	1.8.2.2293 and above	1.7.1.658 and above
DNBSEQ-T7RS	PE150 (StandardMPS)	1.5.0.887 and above	BCS_1.5.0.316 and above

1.4 Components

This library preparation kit comes in two specifications: 16 RXN and 96 RXN. Three or four separated modules are included for each specification. For component details, refer to the following table.

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

Table 2
MGIEasy Whole Genome Methylation Sequencing Library Prep Kit (16 RXN) (Cat. No.: 940-001530-00)

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy Whole Genome Methylation Sequencing Library Prep Module Cat. No.: 940-001529-00	ER Buffer	Orange	144 µL/tube × 1
	ER Enzyme	Orange	16 µL/tube × 1
	Purif Suppl	Colorless	18 µL/tube × 1
	AT Buffer	Green	152 µL/tube × 1
	AT Enzyme	Green	8 µL/tube × 1
	WGMS Adapter	Red	80 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
	U-PCR Enzyme	Blue	400 µL/tube × 1
	X Enhancer	Purple	64 µL/tube × 1
	PCR BC Primer 1-3, 18, 5-8	8-tube strips	5 µL/well × 8
	PCR BC Primer 9-16	8-tube strips	5 µL/well × 8
MGIEasy Circularization Module Cat. No.: 1000005260	Splint Buffer	Purple	186 µL/tube × 1
	DNA Rapid Ligase	Purple	8 µL/tube × 1
	Digestion Buffer	White	23 µL/tube × 1
	Digestion Enzyme	White	42 µL/tube × 1
	Digestion Stop Buffer	White	120 µL/tube × 1

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy DNA Clean Beads Cat. No.: 940-001174-00	DNA Clean Beads	White	15 mL/tube ×1
	TE Buffer	White	17 mL/tube ×1

Table 3
MGIEasy Whole Genome Methylation Sequencing Library Prep Kit (96 RXN) (Cat. No.: 940-001527-00)

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy Whole Genome Methylation Sequencing Library Prep Module Cat. No.: 940-001528-00	ER Buffer	Orange	864 µL/tube × 1
	ER Enzyme	Orange	96 µL/tube × 1
	Purif Suppl	Colorless	106 µL/tube × 1
	AT Buffer	Green	912 µL/tube × 1
	AT Enzyme	Green	48 µL/tube × 1
	Ad-Lig Buffer	Red	864 µL/tube × 2
	Ad Ligase	Red	480 µL/tube × 1
	Ligation Enhancer	Brown	192 µL/tube × 1
	U-PCR Enzyme	Blue	1200 µL/tube × 2
	X Enhancer	Purple	384 µL/tube × 1
MGIEasy Methylation Adapter and Single Barcode Primer Module Cat. No.: 940-001531-00	WGMS Adapter	Red	480 µL/tube × 1
	PCR BC Primer-96	/	5 µL/well × 96
MGIEasy Circularization Module Cat. No.: 1000017573	Splint Buffer	Purple	1144 µL/tube × 1
	DNA Rapid Ligase	Purple	48 µL/tube × 1
	Digestion Buffer	White	135 µL/tube × 1
	Digestion Enzyme	White	250 µL/tube × 1
	Digestion Stop Buffer	White	720 µL/tube × 1

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy DNA Clean Beads Cat. No.: 940-001526-00	DNA Clean Beads	White	70 mL/tube × 1
	TE Buffer	White	35 mL/tube × 1

1.5 Storage and transportation

Table 4 Storage and transportation conditions

Item	Storage temperature	Transportation temperature
MGIEasy Whole Genome Methylation Sequencing Library Prep Module	-25℃ to -15℃	-80℃ to -15℃
MGIEasy Methylation Adapter and Single Barcode Primer Module		
MGIEasy Circularization Module		
MGIEasy DNA Clean Beads	2℃ to 8℃	

Tips

- For ice packs or dry ice shipments, ensure that there is enough ice or dry ice remaining after transportation.
- Check the expiration date on the kit label. With proper transport, storage, and use, all components can maintain complete activity within their shelf life.
- Ligation Enhancer in the MGIEasy Whole Genome Methylation Sequencing Library Prep Module should be stored away from light at room temperature and avoid repeated freezing and thawing.

1.6 User-supplied materials

Table 5 Order information for MGI products

Catalog number	Model	Name
1000016950	FCL PE100	DNBSEQ-G400RS High-throughput Sequencing Set (StandardMPS)
1000016952	FCL PE150	DNBSEQ-G400RS High-throughput Sequencing Set (StandardMPS)
940-001344-00	G400 FCL PE100	DNBSEQ-G400RS High-throughput Sequencing Set (StandardMPS 2.0)
940-001356-00	G400 FCL PE150	DNBSEQ-G400RS High-throughput Sequencing Set (StandardMPS 2.0)
940-002625-00	T7 Methylation FCL PE150	DNBSEQ-T7RS High-throughput Methylation Sequencing Set

Catalog number	Model	Name
1000005033	V3.0	Standard Library Reagent*

Tips * It is necessary to order V3.0 Standard Library Reagent, when sequencing genomic DNA methylation libraries at DNBSEQ-T7RS platform with V1.0 camera. The following situations do not need V3.0 Standard Library Reagent: sequencing cell-free DNA at DNBSEQ-T7RS platform, sequencing at DNBSEQ-T7RS platform with V2.0 camera, or sequencing at DNBSEQ-G400RS platform.

Table 6 User-supplied equipment list

Equipment	Recommended brand
Covaris focused ultrasonicator	Covaris
Vortex mixer	Kylin Bell (Cat. No.: VORTEX-6) or equivalent
Mini centrifuge	yooning (Cat. No.: Mini-7KS) or equivalent
Pipettes	Eppendorf Research (Cat. No.: 3120000216, 3120000224, 3120000232, 3120000240, 3120000259 and 3120000267) or equivalent
Magnetic rack for 96-well plate	ALPAQUA (Cat. No.: A00400) or equivalent
Magnetic rack for 1.5 mL tubes	Thermo Fisher (Cat. No.: 12321D) or equivalent
Qubit 3.0 Fluorometer	Thermo Fisher (Cat. No.: Q33216) or equivalent
Agilent 2100 Bioanalyze	Agilent Technologies (Cat. No.: G2939AA) or equivalent

Table 7 Recommended reagent/consumable list

Reagent/consumable	Recommended brand
Unmethylated Lambda DNA*	Promega (Cat. No.: D1521) or equivalent
NEBNext Enzymatic Methyl-seq Conversion Module	NEB (Cat. No.: E7125S / E7125SL)
Formamide*	Diamond (Cat. No.: A100314-0100) or equivalent
2 M NaOH*	Aladdin (Cat. No.: S128511-1L) or equivalent
Nuclease Free water (NF water)	Ambion (Cat. No.: AM9937)
1 x TE Buffer, pH 8.0	Ambion (Cat. No.: AM9858)
Absolute ethanol (for analysis)	XiLONG SCIENTIFIC(Cat. No.: 72188-01)
Qubit ssDNA Assay Kit	Invitrogen (Cat. No.: Q10212) or YEASEN (Cat. No.: 12645ES60/12645ES76)
Qubit dsDNA HS Assay Kit	Invitrogen (Cat. No.: Q32854) or YEASEN (Cat. No.: 12640ES60/12640ES76)
Agilent High Sensitivity DNA Kit	Agilent (Cat. No.: 5067-4626)
Agilent DNA 1000 Kit	Agilent (Cat. No.: 5067-1504) or equivalent

Reagent/consumable	Recommended brand
Covaris AFA Tubes for use with Ultrasonicator	Covaris
Pipette tips	Axygen (Cat. No.: T-300 10 μL, T-200-Y 200 μL, T-1000-B 1000 μL)
1.5 mL tube	Ambion (Cat. No.: AM12450)
0.2 mL PCR tube or 96-well plate	Axygen (Cat. No.: PCR-02-C) or Axygen (Cat. No.: PCR-96M2-HS-C)
Qubit Assay Tubes or 0.5 mL Thin Wall PCR Tubes	Invitrogen (Cat. No.: Q32856) or Axygen (Cat. No.: PCR-05-C)

Tips * Unmethylated Lambda DNA does not need to be sheared, and is used to protect ligation product during oxidation reaction. It can be pre-diluted to 200 ng/μL.

* Formamide and 2M NaOH are used for denaturation in enzymatic conversion, and are only needed to purchase one of them.

1.7 Precautions

1.7.1 Sample requirements

1.7.1.1 Sample type

This kit is applicable to genomic DNA extracted from human (blood, fresh tissues, cells, and FFPE samples), animals (mouse, etc.), plants (*A. thaliana*, etc.), as well as cell-free DNA from plasma.

1.7.1.2 Sample integrity

For genomic DNA, it is recommended to use samples with good integrity, with the main band ≥ 23 Kb on agarose gel electrophoresis and no significant smearing for library construction. For cell-free DNA, it is recommended to use samples with a main peak distribution between 160 ~ 180 bp for library construction.

1.7.1.3 Sample purity

It is strongly recommended to use high quality DNA (absorbance detection: 1.8 ≤ OD_{260}/OD_{280} ≤ 2.0, OD_{260}/OD_{230} ≥ 1.7, and no significant protein and RNA bands on agarose gel electrophoresis) for library preparation. If there are high concentrations of proteins or polysaccharide impurities, it is recommended to purify with 2x volume of beads and elute with TE Buffer.

1.7.1.4 Genomic DNA input

100 ng to 1000 ng gDNA can be used for library preparation. If the amount of genomic DNA is sufficient, it recommend using a high input of genomic DNA for library preparation. Qubit or FLUOstar Omega is recommended for quantification of sample concentrations.

Table 8 Recommended sample input range

Minimum input (ng)	Input range (ng)	Size selection method
100	100 ~ 500	single size selection
	500 ~ 1000	single size selection or double size selection

Tips The recovery efficiency for single size selection should be ≥ 30%, and ≥ 10% for double size selection. Otherwise, it is necessary to appropriately adjust the input and/or the number of PCR cycles.

- Single size selection: The smear size is 300 bp ~ 700 bp with the peak size between 350 bp ~ 480 bp.
- Double size selection: The smear size is 300 bp ~ 500 bp with the peak size between 350 bp ~ 450 bp.
- For specific experimental procedures, please refer to "Cleanup of fragmentation product" on page 11.

1.7.1.5 Cell-free DNA input

This kit can be used for library preparation of 5 ~ 20 ng of cell-free DNA (cfDNA) without shearing and size selection, allowing direct end repair. It is recommended to elute cell-free DNA with TE Buffer. If high concentrations of metal ion chelators or other salts are introduced during the extraction process, it may affect the efficiency of the end repair step.

1.7.1.6 End repair (ER) input

Table 9 ER input

Sample type	ER input (ng)
size-selected gDNA	10 ~ 50
cell-free DNA	5 ~ 20

1.7.2 Use of Adapters

Due to differences in design processes, **only the WGMS Adapter from this kit can be used**. Using adapters from other kits will result in library construction failure.

1.7.3 Other precautions and warnings

- This product is for research use only, not for clinical diagnosis. Read this manual carefully before use.
- Familiarize yourself with the precautions and operation methods of various instruments before performing the experiment.
- This manual aims to provide a standard protocol. Changes can be made for different applications, but changes must be tested prior to starting the protocol.
- It is recommended to use pipette tips with filters to prevent cross-contamination. Use a new tip each time for pipetting different solutions or samples.
- It is recommended to 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.
- To prevent yield loss, try to avoid transferring reaction product to a new tube for bead purification.
- Avoid skin and eye contact with samples and reagents. In case of contact with skin and eyes, rinse immediately with plenty of water and seek medical advice.
- Conform to the law and regulations when disposing of all samples and reagents.
- If you have questions, contact Technical Support: **MGI-service@mgi-tech.com**.

1.8 Workflow

Table 10 Workflow

Section	Workflow	Hands-on time (1 RXN)	Total time (1 RXN)
2.1	Sample shearing ⏸	2 min	10min
2.2	Cleanup of fragmentation product ⏸	5 min	20 min
2.3	QC of size selection product ⏸	2 min	5 min
3.1	End repair	2 min	32 min
3.2	Cleanup of adapter-ligated product and A-tailing	5 min	25 min
3.3	Adapter ligation	3 min	18 min
3.4	Cleanup of adapter-ligated product ⏸	5 min	18 min
3.5.1	Oxidation ⏸	5 min	1 h 35 min
3.5.2	Cleanup of oxidation product ⏸	5 min	18 min

Section	Workflow	Hands-on time (1 RXN)	Total time (1 RXN)
3.5.3	Denaturation	2 min	17 min
3.5.4	Deamination ⏸	2 min	3 h 2 min
3.5.5	Cleanup of deamination product ⏸	5 min	28 min
3.6	PCR	2 min	30 min
3.7	Cleanup of PCR product ⏸	5 min	18 min
3.8	QC of PCR product ⏸	2 min	5 min
4.1	Denaturation and single strand circularization	5 min	45 min
4.2	Digestion	2 min	12 min
4.3	Cleanup of digestion product ⏸	5 min	28 min
4.4	QC of digestion product ⏸	2 min	5 min
total		~ 66 min	~ 10 h 11 min

Tips

- Total time: The time that it takes to perform 1 reaction. The time will increase as the number of reactions increases.
- Hands-on time: The total required hands-on time in the workflow. Due to varying levels of operator proficiency, the time may fluctuate.
- ⏸ Stop point.

2 Sample fragmentation and selection

2.1 Fragmentation

2.1.1 Preparation

Table 11 Reagent preparation

Reagent	Requirement
TE Buffer	Place at RT (room temperature)

Table 12 Sample preparation

Source	Component	Application	Requirement
User-supplied	DNA	Samples for Methylation Library Preparation	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
User-supplied, NEBNext Enzymatic Methyl-seq Conversion Module, or equivalent	Control DNA Unmethylated Lambda (2 ng/μL)	Evaluate the Conversion Rate of Unmethylated C in the Library	
	Control DNA CpG methylated pUC19 (0.1 ng/μL)	Evaluate the Oxidation Efficiency of TET2 Enzyme	

Tips
- Unmethylated Lambda DNA FASTA website: https://www.ncbi.nlm.nih.gov/nuccore/J02459
- CpG methylated pUC19 FASTA file website: https://www.ncbi.nlm.nih.gov/nuccore/L09137

2.1.2 DNA shearing

1. This kit is only compatible with mechanical DNA shearing and not compatible with enzymatic fragmentation.
2. Shear genomic DNA into smears ranging from 100 bp to 1000 bp, with a peak size between 300 bp and 700 bp.
3. Appendix "Shearing condition" on page 48 lists the shearing parameters for different Covaris Focused-ultrasonicators with a volume of 55 μL. For DNA shearing conditions with other volumes (15 μL, 130 μL, or 200 μL, etc.), please refer to the Quick Guide: DNA Shearing with E220 Focused-ultrasonicator (Part Number 01308, Rev O, Date 12/2020) .
4. If using other consumables for DNA shearing, it is recommended to design a gradient experiment to determine the optimal DNA shearing condition.
5. The shearing conditions for Control DNA can be consistent with those for genomic DNA.
6. Shear Control DNA CpG unmethylated Lambda and Control DNA CpG methylated pUC19 together in the same tube. Taking a 55 μL shearing volume as an example: use 27.5 μL (55 ng) of Control DNA CpG unmethylated Lambda and 27.5 μL (2.75 ng) of Control DNA CpG methylated pUC19. Control DNA from other brands with equivalent functionality can also be used as a substitute.

CAUTION
- Purification of the Control DNA is not required after shearing.
- The sheared product can be stored at -20°C.

2.2 Cleanup of fragmentation product

Tips
- Please carefully read "Table 8 Recommended sample input range" on page 7 before proceeding. Choose the appropriate size selection method (single or double size selection) based on different experimental protocols.
- Size selection should be performed after increasing the volume of sheared genomic DNA to 100 μL with TE Buffer.

2.2.1 Single size selection

Tips
- The following steps use 80 μL (0.8 x) beads to perform the size selection on 100 μL of sheared product.
- 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.

2.2.1.1 Preparation

Table 13 Reagent preparation

Reagent	Requirement
80% Ethanol	User-supplied. Freshly prepared.
TE Buffer	Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

2.2.1.2 Single size selection

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

1. Check the sample volume after shearing. Add TE buffer to make the total volume to 100 µL if the sheared DNA volume is not enough.
2. Mix the DNA Clean Beads well. Add **80 µL DNA Clean Beads** to each sample tube, mix with a vortexer to suspend the beads.
3. Incubate the sample(s) at room temperature for 5 min.
4. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
5. Keep the PCR tube(s) on the magnetic rack, add **150 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 cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the surface of beads. There should be no visible cracking on the surface of beads.

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

8. Remove the PCR tube(s) from the magnetic rack. Add **32 µL TE Buffer** to elute the DNA. Vortex to ensure beads are suspended.

   Tips The elution volume can be adjusted according to requirements.

9. Incubate the sample(s) at room temperature for 5 min.
10. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully transfer **30 µL** supernatant to a new 0.2 mL PCR tube.

    Stop point After cleanup, products can be stored at -20℃.

2.2.2 Double size selection

Tips
- The following steps use 55 μL (0.55 ×) beads + 20 μL (0.2 ×) beads for size selection.
- 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.

2.2.2.1 Preparation

Table 14 Reagent preparation

Reagent	Requirement
80% Ethanol	User-supplied. Freshly prepared.
TE Buffer	Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

2.2.2.2 Double size selection

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

1. Check the sample volume after shearing. Add TE buffer to make the total volume to 100 μL if the sheared DNA volume is insufficient.
2. Mix the DNA Clean Beads well. Add **55 μL DNA Clean Beads** to each sample tube, mix with a vortexer to suspend the beads.
3. Incubate the sample(s) at room temperature for 5 min.
4. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully remove 150 μL supernatant to a new 0.2 mL PCR tube.

   Tips In this step, keep the supernatant and discard the beads. Recycle the DNA on the beads if necessary.
5. Add **20 μL DNA Clean Beads** to the supernatant sample tube. Gently pippete at least 10 times until all beads are suspended. Or, vortex to mix.
6. Incubate the sample(s) at room temperature for 5 min.
7. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
8. Keep the PCR tube(s) on the magnetic rack, add **150 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 cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the surface of beads. There should be no visible cracking on the surface of beads.

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

11. Remove the PCR tube(s) from the magnetic rack. Add **32 μL TE Buffer** to elute the DNA. Vortex to make all beads are suspended.

    Tips The elution volume can be adjusted appropriately according to the actual situation.

12. Incubate the sample(s) at room temperature for 5 min.
13. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully transfer **30 μL** supernatant to a new 0.2 mL PCR tube.

    Stop point After cleanup, products can be stored at -20℃.

2.3 QC of size selection product

Tips If the experimental process is stable, this quality control step can be skiped.

Take 1 μL of supernatant for size distribution detection by Bioanalyzer. Use double-stranded DNA fluorescence quantification kit such as Qubit dsDNA HS Assay Kit or Quant-iT PicoGreen dsDNA Assay Kit. Follow the instructions of the kit to quantify the size selection product.

Below figure is the Agilent 2100 bioanalyzer results of 0.8 x single size selection.

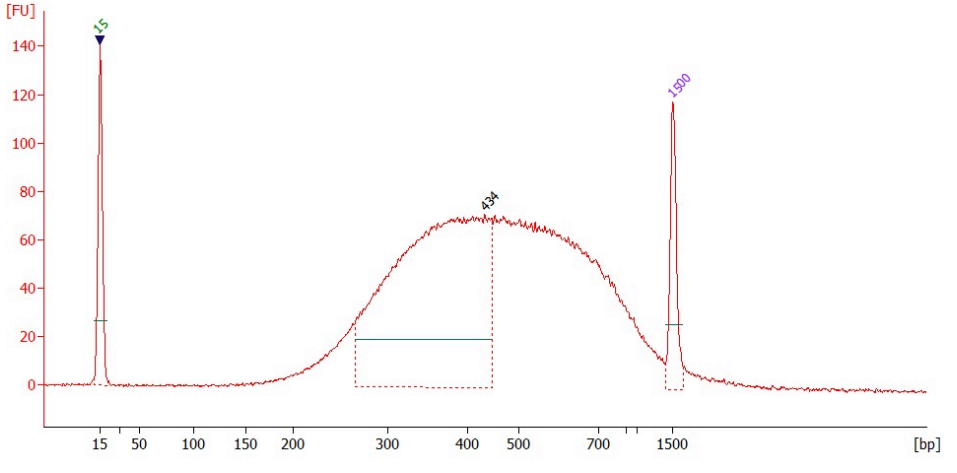


Figure 1 Agilent 2100 bioanalyzer results of 0.8 x single size selection

Below is the Agilent 2100 bioanalyzer results of 0.55 x + 0.2 x double size selection.

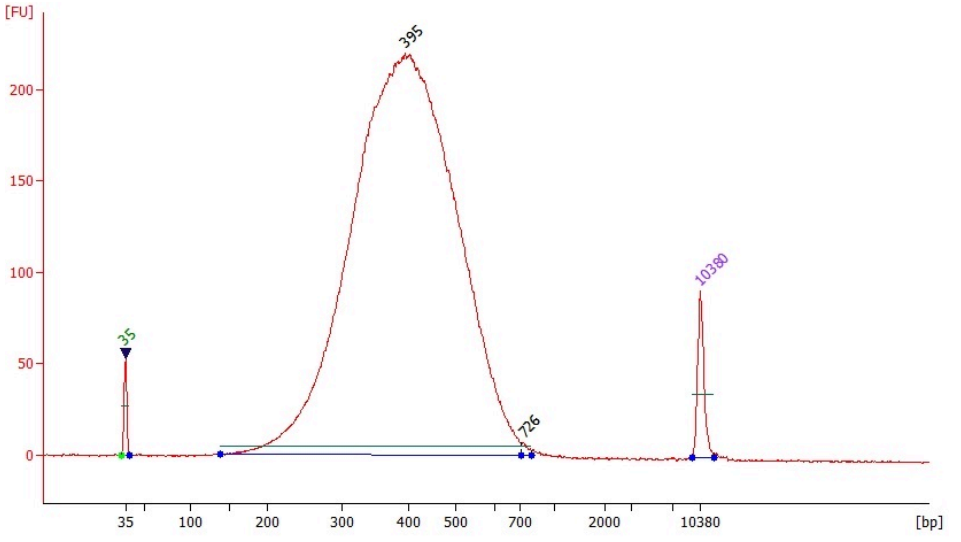


Figure 2 Agilent 2100 bioanalyzer results of 0.55 x+0.2 x double size selection

3 Library preparation protocol

3.1 End repair

Tips
- Please carefully read "Precautions" on page 6 before proceeding.
- It is recommended to use 50 ng of selection product (concentration ≥ 1.75 ng/μL) for end repair, with a minimum of 10 ng of selected product (concentration ≥ 0.35 ng/μL) as a trial.
- For cell-free DNA, it is recommended to use 20 ng (concentration ≥ 0.69 ng/μL) directly for end repair, with a minimum of 5 ng of cell-free DNA (concentration ≥ 0.18 ng/μL) as a trial.
- When the sample quality is sufficient, please use the recommended input for end repair. The lower input (less than recommended) for end repair might be attempted, but it will increase duplication rate and decrease coverage.
- Preheat the thermocycler to 20 ℃.
- End Repair is abbreviated as ER.

3.1.1 Preparation

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

Table 15 Reagent preparation

Reagent	Requirement
Sheared Control DNA	User-supplied. Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
TE Buffer	Place at RT.
ER Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
ER Enzyme	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.
Purif Suppl	

Reagent	Requirement
DNA Clean Beads	Take out 30 min in advance to equilibrate to RT. Mix thoroughly by vortexing before each use.

3.1.2 End repair

1. According to the sample concentration, take an appropriate input of sample (50 ng of selected DNA or 20 ng of cell-free DNA is recommended) into a new 0.2 mL PCR tube. The volume should be ≤ 29 μL. Add TE Buffer to make the total volume to 29 μL.
2. Prepare the end-repair mixture on ice according to the reaction number. Vortex to mix well, centrifuge briefly, and place on ice.

Table 16 ER mixture

Reagent	Volume per reaction
Sheared Control DNA	1 μL
ER Buffer	9 μL
ER Enzyme	1 μL
Total	11 μL

3. Add **11 μL of ER mixture** to each sample tube (from step 1 in section 3.1.2). Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.
4. Place the PCR tube(s) into the thermocycler and skip the first step (20 ℃ Hold) to start the reaction.

CAUTION
- **Do not set 4℃ Hold.**
- **Do not put the sample(s) on ice after the reaction.**

Table 17 ER reaction conditions (Volume: 40 μL)

Temperature	Time
35 ℃ Heated lid	On
20 ℃	Hold
20 ℃	30 min
20 ℃	Hold

5. When there are 5 min remaining in the ER reaction, prepare the ER Clean Beads Mixture according to the table below. Vortex to mix well and leave at **room temperature**.

Table 18 ER Clean Beads Mixture (Prepare fresh and use immediately)

Reagent	Volume per reaction
DNA Clean Beads	60 μL
Purif Suppl	0.1 μL
Total	60.1 μL

Tips If the number of reactions is <= 4, it is recommended to prepare the "ER Clean Beads Mixture" for 5 reactions.

6. Centrifuge the PCR tube(s) briefly to collect the liquid in the bottom of the tube after the reaction and purify **immediately**.

CAUTION Do not stop at this step. Proceed the cleanup of end repair product **immediately**.

3.2 Cleanup of end repair product and A-tailing

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.
- Preheat the thermocycler to 65 ℃.
- A-tailing is abbreviated as AT.

3.2.1 Preparation

Table 19 Reagent preparation

Reagent	Requirement
80% Ethanol	User-supplied. Freshly prepared.
TE Buffer	Place at RT.
AT Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
AT Enzyme	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

3.2.2 Cleanup of end repair product

1. Mix the ER Clean Beads Mixture well. Add **60.1 μL ER Clean Beads Mixture** to the sample tube(s) (from step 6 in section 3.1.2). Vortex to mix until all the beads are suspended.

2. Incubate the sample(s) at room temperature for 5 min. Simultaneously, prepare the AT reaction mixture on ice according to the number of reactions needed. Vortex to mix well, briefly centrifuge, and then place on ice.

Table 20 AT mixture

Reagent	Volume per reaction
AT Buffer	9.5 μL
AT Enzyme	0.5 μL
Total	10 μL

3. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
4. Keep the PCR tube(s) on the magnetic rack, add **150 μL of 80% Ethanol** to each tube to wash the beads and tube wall. Wait for 30 sec and carefully remove and discard the supernatant. 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.
5. Keep the tube(s) on the magnetic rack. Open the cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the surface of beads. There should be no visible cracking on the surface of beads.

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

6. Remove the PCR tube(s) from the magnetic rack. Add **40 μL TE Buffer** to elute the DNA.
7. Add **10 μL AT mixture** atroom temperature. Vortex it 3 times (3 sec each) and centrifuge **briefly**. **Immediately** place the sample tube on the preheated thermocycler, skip the first step (65 ℃ Hold), and proceed with the reaction according to the conditions in the table below.

CAUTION
- Ensure that the beads are fully resuspended after mixing and centrifugation.
- The sample tube with the added AT reaction mixture should **not be placed on ice** and should be **immediately placed on thermocycler** for the reaction.

Table 21 AT reaction conditions (Volume: 50 μL)

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

8. After reaction, centrifuge the tube(s) briefly and **immediately proceed next step.**

CAUTION Do not stop at this step. Proceed adapter ligation **immediately**.

3.3 Adapter ligation

Tips
- Preheat the thermocycler to 25 ℃.
- The adapter is double-stranded. Do not expose it to temperatures above 30 ℃, as it may denature and affect performance.

3.3.1 Preparation

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

Table 22 Reagent preparation

Reagent	Requirement
TE Buffer	Place at RT.
Ligation Enhancer	Mix by vortexing, centrifuge briefly, and place at RT.
WGMS Adapter	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Ad-Lig Buffer	
Ad Ligase	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

Tips
- Mix the adapter(s) well before use. Do not directly add the adapters into the adapter ligation mixture.
- The Ad-Lig Buffer is highly viscous. Mix it by vortexing 6 times (3 sec each), and centrifuge briefly before use. Slowly aspirate and pipette when pipetting the ligation buffer to ensure that the volume is accurate.
- After Ligation Enhancer is used for the first time, store it at 10 ℃ - 30 ℃ away from light.

3.3.2 Adapter ligation

1. Dilute the adapters based on the DNA input used. Refer to the table below. Use TE Buffer in the kit to dilute the adapter.

Tips The quality and quantity of the adapter will directly affect the quality of library. Please select the appropriate Adapter dilution ratio.

Table 23 Relationship between WGMS Adapter dilution ratio and size-selected DNA input

Size-selected DNA input (X, ng)	Dilution ratio of WGMS Adapter	WGMS Adapter volume (μL)	TE Buffer volume (μL)	Total volume after dilution (μL)
25 < X ≤ 50	No dilution	5	-	-
10 < X ≤ 25	2	5	5	10
X = 10	5	2	8	10

Table 24 Relationship between WGMS Adapter dilution ratio and cell-free DNA input

Cell-free DNA input (X, ng)	Dilution ratio of WGMS Adapter	WGMS Adapter volume (μL)	TE Buffer volume (μL)	Total volume after dilution (μL)
10 < X ≤ 20	No dilution	5	-	-
5 ≤ X ≤ 10	2	5	5	10

CAUTION After briefly centrifuging the AT product, add the WGMS Adapter and vortex to suspend all beads. Centrifuge briefly, and then add the ligation reaction mixture.

2. **Add 5 μL of WGMS Adapter to corresponding sample tube** (from step 8 in section 3.2.2). Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.
3. According to the reaction number, prepare the adapter ligation mixture on ice. Vortex 6 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.

Table 25 Adapter ligation mixture

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

Tips Prepare the adapter ligation mixture when there are 5 min remaining in the A-tailing reaction. Vortex to mix well, place it on ice, and use it within 30 min.

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

CAUTION The adapter ligation mixture is highly viscous. Slowly aspirate and pipette when pipetting the ligation buffer to ensure that the volume is accurate.

Ensure that the magnetic beads are completely suspended after mixing and briefly centrifugation.

5. Place the PCR tube(s) into the thermocycler preheated to 25℃ and skip the first step (25℃ Hold) to start the reaction.

Table 26 Adapter ligation reaction conditions (Volume: 80 μL)

Temperature	Time
35 ℃ Heated lid	On
25 ℃	Hold
25 ℃	15 min
4 ℃	Hold

6. When the program is completed, centrifuge the PCR tube(s) briefly.
7. Add **20 μL of TE Buffer** to each tube to make a total volume of 100 μL.

CAUTION Do not stop at this step. Proceed the cleanup of adapter-ligated product **immediately**.

3.4 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.
- The elution buffer in this cleanup step is **NF Water**.

3.4.1 Preparation

Table 27 Reagent preparation

Reagent	Requirement
80% Ethanol	User-supplied. Freshly prepared.
NF Water	User-supplied. Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.4.2 Cleanup of adapter-ligated product

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

1. Mix the DNA Clean Beads thoroughly. Add **40 μL of DNA Clean Beads** to each sample tube (from step 7 in section 3.3.2). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
2. Incubate at room temperature for 5 min.

3. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
4. While keeping the tube(s) on the magnetic rack, gently add **150 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 centrifuge 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 **30 µL of NF Water** to elute the DNA. Gently pipette at least 10 times until all beads are suspended. Or, mix with a vortexer.
8. Incubate at room temperature for 5 min.
9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully transfer **28 µL** of supernatant to a new 0.2 mL PCR tube.

   Stop point After cleanup, the adapter-ligated product can be stored at - 20 ℃.

3.5 Enzymatic conversion

Tips
- Before oxidation, make sure that the adapter-ligated DNA is eluted in **NF Water**.
- Refer to the following NEB website for detailed instructions: NEBNext Enzymatic Methyl-seq Conversion Module Instruction Manual v4.0 (E7125S/L).

3.5.1 Oxidation

Tips Preheat the thermocycler to the reaction temperature.

3.5.1.1 Preparation

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

Table 28 Reagent preparation

Source	Reagent	Requirement
User-supplied. Non-fragmented unmethylated Lambda DNA (Promega, Cat. No.:D1521) can protect ligation product and can be pre-diluted to 200 ng/μL.	Unmethylated Lambda DNA (**non-fragmented**)	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
User-supplied.	NF Water	Place at RT.
User-supplied: NEBNext Enzymatic Methyl-seq Conversion Module	TET2 Reaction Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
	Oxidation Supplement	
	TET2 Reaction Buffer Supplement (**powder**)	Centrifuge breifly. Add TET2 Reaction Buffer according to the insruction on the lable. Mix by vortexing, centrifuge briefly, and place on ice.
	DTT	Thaw at RT, mix by vortexing, centrifuge briefly, and place at RT.
	Fe (II)	
	Oxidation Enhancer	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.
	TET2	
	Stop Reagent	

3.5.1.2 Oxidation

1. According to the reaction number, prepare the oxidation mixture on ice. Mix by vortexing, centrifuge briefly, and place on ice.

Table 29 Oxidation mixture

Reagent	Volume per reaction
Unmethylated Lambda DNA (**non-fragmented**, 200 ng/μL)	0.75 μL
TET2 Reaction Buffer Supplement	10 μL
Oxidation Supplement	1 μL
DTT	1 μL
Oxidation Enhancer	1 μL
TET2	4 μL
Total	17.75 μL

2. Add **17.75 μL of oxidation mixture** to each sample tube (from step 9 in section 3.4.2). Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.
3. Dilute the Fe (II) Solution: add **1 μL Fe (II)** to **1249 μL NF Water**. Vortex 6 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place at room temperature.

Table 30 Dilution of Fe(II) Solution

Reagent	Volume
Fe (II)	1 μL
NF water	1249 μL
Total	1250 μL

CAUTION Use the freshly-prepared Fe (II) solution. Discard after use.

4. Add **5 μL Diluted Fe(II) Solution** to each sample tube (from step 2). Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.
5. Place the PCR tube(s) into the preheated thermocycler at 37°C, skip the first step (37°C Hold), and run the program with the following conditions.

Table 31 Oxidation reaction conditions (Volume: 50.75 μL)

Temperature	Time
45 ℃ Heated lid	On
37 ℃	Hold
37 ℃	60 min
4 ℃	Hold

6. Centrifuge the tube(s) briefly after reaction. Add **1 μL Stop Reagent** to each sample tube. Vortex the tube(s) 3 times (3 sec each), centrifuge briefly, and place on ice.
7. Place the PCR tube(s) into the preheated thermocycler at 37℃, skip the first step (37℃ Hold), and run the program with the following conditions.

Table 32 Stop reaction conditions (Volume: 51.75 μL)

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

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

Stop point Oxidation product(s) can be stored at -20℃ for no more than 24 hrs.

3.5.2 Cleanup of oxidation 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.
- The elution buffer in this cleanup step is **NF Water**.

3.5.2.1 Preparation

Table 33 Reagent preparation

Reagent	Requirement
80% Ethanol	User-supplied. Freshly prepared.
NF Water	User-supplied. Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.5.2.2 Cleanup of oxidation product

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

1. Mix the DNA Clean Beads thoroughly. Add **90 µL DNA Clean Beads** to each sample tube (from step 8 in section 3.5.1.2). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
2. Incubate at room temperature for 5 min.
3. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
4. While keeping the tube(s) on the magnetic rack, gently add **150 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. Add **28 µL NF Water** to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
8. Incubate at room temperature for 5 min.
9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2min to 5 min until the liquid is clear. Carefully transfer **26 µL** supernatant to new 0.2 mL PCR tube(s).

   Stop point Product(s) can be stored at -20 ℃ for no more than 24 hrs.

3.5.3 Denaturation

Tips
- Preheat the thermocycler to the reaction temperature.
- Place the denatured DNA on ice to keep single-stranded. Otherwise, the deamination efficiency will be affected, resulting in a conversion rate lower than 99%
- Use either formamide or 0.15 M NaOH for DNA denaturation.

3.5.3.1 Preparation

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

Table 34 Reagent preparation

Source	Reagent	Requirement
User-supplied. Diamond (Cat. No.:A100314-0100), or equivalent	Formamide	Store at 2℃ - 8℃ and place at RT before use.
User-supplied.	0.15 M NaOH	Freshly-prepared.
User-supplied.	NF Water	Place at RT.

Tips
- Dilute the 2 M NaOH solution to 1.5 M with NF Water, for example, add 100 µL 2 M NaOH solution and 33.3 µL NF Water to a 1.5 mL tube, mix well, aliquot and store at -20℃.
- Take out an aliquoted tube for each experiment. Make a 10-time dilution to 0.15 M for denaturation. Discard after use.

3.5.3.2 Method 1: Formamide denaturation (recommended)

1. Add 4 µl Formamide to the 26 µl oxidized product (from step 9 in section 3.5.2.2). Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.
2. Place the PCR tube(s) into the preheated thermocycler, skip the first step (85℃ Hold), and run the program with the following conditions.

Table 35 Denaturation reaction conditions (Volume: 30 µL)

Temperature	Time
105 ℃ Heated lid	On
85 ℃	Hold
85 ℃	10 min
4 ℃	5 min
4 ℃	Hold

CAUTION Alternative program: 85℃ 10 min (heated lid 105℃), **immediately** place on ice for 2 min after the reaction.

3. **Immediately** place the PCR tube(s) on ice after reaction.

3.5.3.3 Method 2: 0.15 M NaOH denaturation (optional)

1. Add 4 µl 0.15 M NaOH to the 26 µl oxidized product (from step 9 in section 3.5.2.2). Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.
2. Place the PCR tube(s) into the preheated thermocycler, skip the first step (50℃ Hold), and run the program with the following conditions.

Table 36 Denaturation reaction conditions (Volume: 30 μL)

Temperature	Time
75 ℃ Heated lid	On
50 ℃	Hold
50 ℃	10 min
4 ℃	5 min
4 ℃	Hold

CAUTION Alternative program: 50℃ 10 min (heated lid 75℃), **immediately** place on ice for 2 min after the reaction.

3. **Immediately** place the PCR tube(s) on ice after reaction and proceed next step.

3.5.4 Deamination

Tips Preheat the thermocycler to the reaction temperature.

3.5.4.1 Preparation

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

Table 37 Reagent preparation

Source	Reagent	Requirement
User-supplied.	NF Water	Place at RT.
User-supplied: NEBNext Enzymatic Methyl-seq Conversion Module	APOBEC Reaction Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
	BSA	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.
	APOBEC	

3.5.4.2 Deamination

1. According to the reaction number, prepare the deamination mixture on ice. Mix it well by vortexing, centrifuge briefly, and place on ice.

Table 38 Deamination mixture

Reagent	Volume per reaction
NF Water	58 μL
APOBEC Reaction Buffer	10 μL
BSA	1 μL
APOBEC	1 μL
Total	70 μL

2. Add **70 μL deamination mixture** to each sample tube (from step 3 in section 3.5.3). Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.
3. Place the PCR tube(s) into the preheated thermocycler, skip the first step (37 ℃ Hold), and run the program with the following conditions.

Table 39 Deamination reaction conditions (Volume: 100 μL)

Temperature	Time
45 ℃ Heated lid	On
37 ℃	Hold
37 ℃	3 hrs
4 ℃	Hold

4. Centrifuge the PCR tube(s) briefly to collect the liquid in the bottom of the tube.

Stop point Deamination product(s) can be stored at -20 ℃ for no more than 24 hrs.

3.5.5 Cleanup of deamination 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.
- The elution buffer in this cleanup step is **NF Water**.

3.5.5.1 Preparation

Table 40 Reagent preparation

Reagent	Requirement
80% Ethanol	User-supplied. Freshly prepared.
NF Water	User-supplied. Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.5.5.2 Cleanup of deamination product

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

1. Mix the DNA Clean Beads thoroughly. Add **100 µL DNA Clean Beads** to each sample tube (from step 4 in section 3.5.4.2). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
2. Incubate at room temperature for **10 min**.
3. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
4. While keeping the tube(s) on the magnetic rack, gently add **200 µL of 80% Ethanol** to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
5. Repeat step 4. Try to **remove all liquid** from the tube. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. **Remove all liquid** by using a low-volume pipette.

CAUTION Ethanol must be thoroughly removed; otherwise, it will result in reduced PCR yield.

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. Add **22 µL NF Water** to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
8. Incubate at room temperature for **10 min**.
9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully transfer **20 µL** supernatant to new 0.2 mL PCR tube(s).

   Stop point Product(s) can be stored at -20℃ for no more than 24 hrs.

3.6 PCR

Tips Before operation, carefully read "PCR BC Primer using guide" on page 51. Follow the principle of base balance to choose the appropriate Barcode.

3.6.1 Preparation

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

Table 41 Reagent preparation

Reagent	Requirement
U-PCR Enzyme	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
PCR BC Primer 1-3, 18, 5-8	
PCR BC Primer 9-16	
PCR BC Primer-96	

3.6.2 PCR

1. Add U-PCR Enzyme and PCR BC Primer to the tube(s) (from step 9 in section 3.5.5.2). Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly.

   CAUTION U-PCR Enzyme and PCR BC Primer need to be added separately. Avoid mixing before use.

Table 42 PCR reaction mixture

Reagent	Volume per reaction
Purified deamination product	20 μL
U-PCR Enzyme	25 μL
PCR BC Primer	5 μL
Total	50 μL

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

Table 43 PCR reaction conditions (Volume: 50 μL)

Temperature	Time	Cycles
105 ℃ Heated lid	on	-
95 ℃	2 min	1
98 ℃	20 s	N (refer to the table below)
62 ℃	20 s	
72 ℃	30 s	
72 ℃	3 min	1
4 ℃	Hold	-

Table 44 Number of PCR cycles with different ER input

Size-selected DNA (X, ng)	PCR cycle number
X = 50	10
25 ≤ X < 50	11
10 ≤ X < 25	12
cell-free DNA (X, ng)	**PCR cycle number**
X = 20	11
10 ≤ X < 20	12
5 ≤ X < 10	13

Tips The PCR amplification requires strict control of amplification cycles. Insufficient cycles will result in inadequate library yield, while excessive cycles can negatively impact data performance. It is necessary to increase the number of cycles appropriately to obtain sufficient product when the genomic DNA has poor quality and longer peak size.

3. Centrifuge the tube(s) briefly after reaction.

3.7 Cleanup of PCR product

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

3.7.1 Preparation

Table 45 Reagent preparation

Reagent	Requirement
80% Ethanol	User-supplied. Freshly prepared.
TE Buffer	Place at RT.
Purif Suppl	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.7.2 Cleanup of PCR product

1. According to the reaction number, prepare the PCR clean beads mixture. Mix it well by vortexing, centrifuge briefly and place at **room temperature**.

Table 46 PCR clean beads mixture

Reagent	Volume per reaction
DNA Clean Beads	50 μL
Purif Suppl	1 μL
Total	51 μL

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

2. Mix the DNA Clean Beads thoroughly. Add **51 μL of DNA Clean Beads** to each sample tube (from step 3 in section 3.6.2). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
3. Incubate at room temperature for 5 min.
4. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
5. While keeping the tube(s) on the magnetic rack, gently add **150 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. Add **32 μL TE Buffer** to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
9. Incubate at room temperature for 5 min.
10. Centrifuge the tube(s) briefly and place on the magnetic rack for 2min to 5 min until the liquid is clear. Carefully transfer **30 μL supernatant** to new 1.5 mL PCR tube(s).

    Stop point Product(s) can be stored at - 20 ℃.

3.8 QC of PCR product

WARNING Do not perform multi-sample pooling with PCR product. It is recommended to perform pooling after DNB preparation.

- dsDNA fluorescence quantification method: Quantify the purified PCR product with dsDNA fluorescence assay kits and instructions.
- Electrophoresis method: Assess the size range of purified PCR product with electrophoresis based equipment and instructions.

Table 47 Different QC methods and standards for library

Method	Equipment/Reagent	Standard
dsDNA fluorescence quantification method	Qubit dsDNA HS Assay Kit Quant-iT PicoGreen dsDNA Assay Kit, etc	Yield of PCR product ≥ 1 pmol
Electrophoresis method	Tapestation (Agilent Technologies) Bioanalyzer, LabChip GX, GXII, GX Touch (PerkinElmer), Fragment Analyzer (Advanced Analytical), etc	/

Refer to the formula below to calculate the mass (in ng) that corresponds to 1 pmol of PCR product with different sizes. For example, when the size of PCR product is 425 bp, the yield should reach 280.5 ng.

Formula 1 Conversion between 1 pmol of PCR product and mass in ng

$$\text{Mass corresponding to 1 pmol PCR product (ng)} = \text{PCR product peak size (bp)} \times 0.66$$

Formula 2 Sample mass calculation

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

Formula 3 Sample volume calculation

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

The following image shows the Agilent 2100 Bioanalyzer results for the PCR purification product.

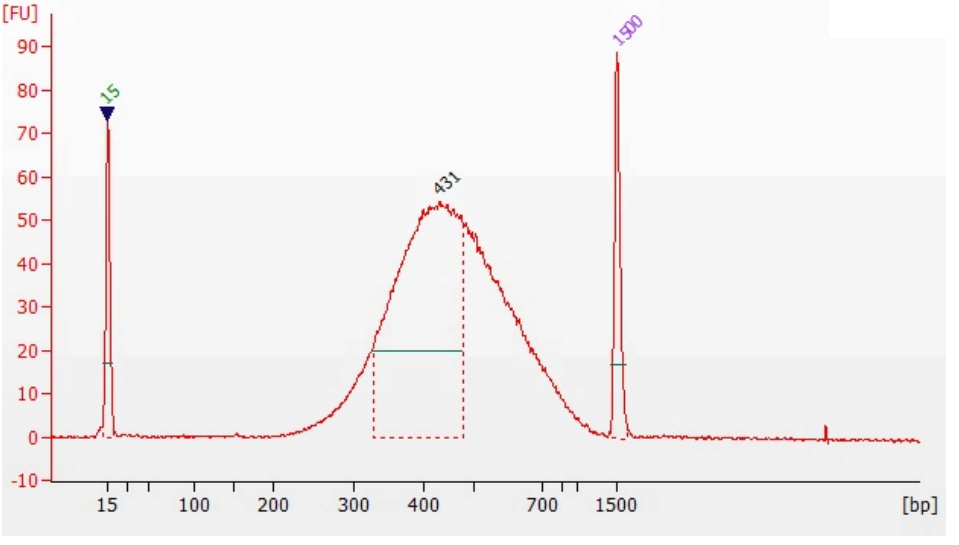


Figure 3 Agilent 2100 Bioanalyzer results for the PCR purification product of single size selection.

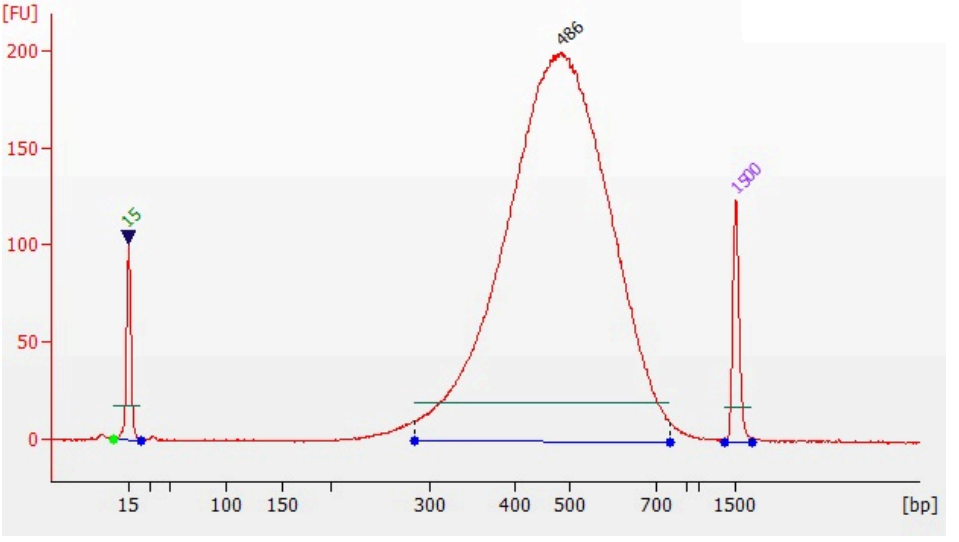


Figure 4 Agilent 2100 Bioanalyzer results for the PCR purification product of double size selection.

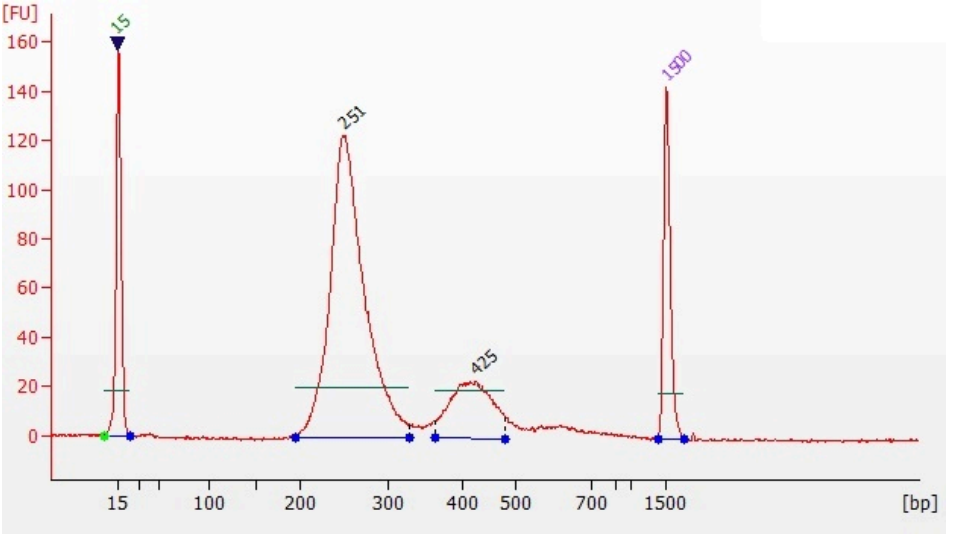


Figure 5 Agilent 2100 Bioanalyzer results for the PCR purification product of cell-free DNA.

4 Circularization and digestion

4.1 Denaturation and single strand circularization

Tips Calculate the required purified PCR product volume based on Formula 1 and 3 in "QC of PCR product" on page 34.

4.1.1 Preparation

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

Table 48 Reagent preparation

Reagent	Requirement
TE Buffer	Place at RT.
Splint Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
DNA Rapid Ligase	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.
X Enhancer	

4.1.2 Denaturation

1. Add **1 pmol PCR product** into a new 0.2 mL PCR tube. Add TE Buffer to make a total volume to **44.5 µL**.
2. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 49 Denaturation reaction conditions (Volume: 44.5 μL)

Temperature	Time
105 ℃ Heated lid	On
95 ℃	3 min
4 ℃	5 min
4 ℃	Hold

CAUTION Alternative program: 95℃ 3 min (heated lid 105 ℃), **immediately** place on ice for 2 min after the reaction.

3. **Immediately** place the PCR tube(s) on ice after reaction and proceed next step.

4.1.3 Single strand circularization

1. According to the reaction number, prepare the single strand circularization mixture on ice. Mix it well by vortexing, centrifuge briefly, and place on ice.

Table 50 Single strand circularization mixture for size-selected DNA

Reagent	Volume per reaction
Splint Buffer	11.6 μL
DNA Rapid Ligase	0.5 μL
X Enhancer	3.5 μL
Total	15.6 μL

Table 51 Single strand circularization mixture for cell-free DNA

Reagent	Volume per reaction
Splint Buffer	11.6 μL
DNA Rapid Ligase	0.5 μL
X Enhancer	1 μL
TE Buffer	2.5 μL
Total	15.6 μL

2. Add **15.6 μL single strand circularization mixture** to each sample tube (from step 3 in section 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 52 Single strand circularization reaction conditions (Volume: 60.1 μL)

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

Tips Prepare the digestion mixture in advance at this step.

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

4.2 Digestion

4.2.1 Preparation

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

Table 53 Reagent preparation

Reagent	Requirement
Digestion Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Digestion Enzyme	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.
Digestion Stop Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place at RT.

4.2.2 Digestion

1. According to the reaction number, prepare the digestion mixture on ice. Mix it well by vortexing, centrifuge briefly, and place on ice.

Table 54 Digestion mixture

Component	Volume per reaction
Digestion Buffer	1.4 μL
Digestion Enzyme	2.6 μL
Total	4.0 μL

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

Table 55 Digestion reaction conditions (Volume: 64.1 μL)

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

4. Centrifuge the PCR tube briefly. Immediately add **7.5 μL Digestion Stop Buffer**. Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and transfer all liquid into new 1.5 mL tube(s).

CAUTION Do not stop at this step. Continue to the cleanup of digestion product.

When having to stop, store the sample at -20 ℃ for no longer than 16 hrs and the yield will drop by around 20%.

4.3 Cleanup of 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 56 Reagent preparation

Reagent	Requirement
80% Ethanol	User-supplied. Freshly prepared.
TE Buffer	Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

4.3.2 Cleanup of digestion product

1. Mix the DNA Clean Beads thoroughly. Add **170 μL DNA Clean Beads** to each sample tube (from step 4 in section 4.2.2). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
2. Incubate at room temperature for **10 min**.
3. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully remove and discard the supernatant.

4. While keeping the tube(s) on the magnetic rack, gently add **500 µL 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. Add **32 µL TE Buffer** to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
8. Incubate at room temperature for **10 min**.
9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 min to 5 min until the liquid is clear. Carefully transfer **30 µL** supernatant to new 1.5 mL PCR tube(s).

   Stop point The digestion product(s) after cleanup can be stored at - 20 ℃.

4.4 QC of digestion product

WARNING Do not perform multi-sample pooling with the ssCir DNA. It is recommended perform pooling after DNB preparation.

Pipette 2 µL of single-stranded circular DNA (ssCir DNA) and use the Qubit ssDNA Assay Kit for quantification according to the kit's instructions.

The single-stranded circular DNA (ssCir DNA) should be ≥ 160 fmol, which can be calculated using Formula 4. For example, if the peak size of the PCR product is 425 bp, the ssCir DNA should reach 22.44 ng.

Formula 4 Conversion between fmol and ng of ssCir DNA

$$160\ \text{fmol ssCir DNA (ng)} = 0.16 \times \text{PCR product peak size (bp)} \times 0.33$$

The yield of ssCir DNA must reach at least 160 fmol to be sufficient for two sequencing runs.

5 Sequencing

5.1 DNBSEQ-G400RS WGMS DNB making

Tips
- The sequencing quality may decrease slightly as the insert size increases. Better sequencing quality can be obtained with narrow size distribution, while wide distribution results in lower quality.
- DNA libraries with different insert size are not recommended pooling. When pooling libraries with different insert size for sequencing (such as pooling single size selection product with double size selection product), there might be a decrease in sequencing quality.
- DNA libraries with similar insert size and size range(± 50 bp) can be pooled with DNB.
- 80 fmol ssCir DNA (ng) = 0.08 × PCR product peak size (bp) × 0.33

CAUTION The DNB making reagents of StandardMPS and StandardMPS 2.0 sequencing are different. Corresponding DNB making reagents must be used when preparing DNB.

5.1.1 Preparation

DNB preparation reagents come from DNBSEQ-G400RS High-throughput Sequencing Set. Mix the reagents before using and store the remaining reagents immediately after use.

Table 57 Reagent preparation

Reagent	Catalog number of sequencing set	Requirement
Low TE Buffer	1000016950 or 1000016952 (StandardMPS)	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Make DNB Buffer		
Make DNB Enzyme Mix I		
Stop DNB Reaction Buffer		
Make DNB Enzyme Mix II (LC)		Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

Reagent	Catalog number of sequencing set	Requirement
Low TE Buffer	940-001344-00 or 940-001356-00 (StandardMPS 2.0)	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Make DNB Buffer		
Make DNB **High-efficiency** Enzyme Mix I		
Stop DNB Reaction Buffer		
Make DNB Enzyme Mix II (LC)		Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

5.1.2 Annealing

1. Add **80 fmol ssCir DNA** to new 0.2 mL PCR tube, and make the total volume to 10 μL using Low TE Buffer. Add 10 μL Make DNB Buffer. Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly.

Table 58 Annealing reaction mix

Component	Volume per reaction
ssCir DNA	X μL
Low TE Buffer	10-X μL
Make DNB Buffer	10 μL
Total	20 μL

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

Table 59 Annealing reaction conditions (Volume: 20 μL)

Temperature	Time
105℃ Heated lid	On
95℃	1 min
65℃	1 min
40℃	1 min
4℃	Hold

3. After the reaction, centrifuge briefly to collect the reaction solution to the bottom of the tube.

5.1.3 DNB making

1. According to the reaction number, prepare the DNB mixture on ice. Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.

Table 60 DNB making mixture (StandardMPS)

Component	Volume per reaction
Make DNB Enzyme Mix I	20 μL
Make DNB Enzyme Mix II (LC)	2 μL
Total	22 μL

Table 61 DNB making mixture (StandardMPS 2.0)

Component	Volume per reaction
Make DNB **High-efficiency** Enzyme Mix I	20 μL
Make DNB Enzyme Mix II (LC)	2 μL
Total	22 μL

2. Add **22 μL DNB making mixture** to each sample tube (from step 3 in section 5.1.2). Vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.
3. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 62 DNB making reaction conditions (Volume: 42 μL)

Temperature	Time
35℃ Heated lid	On
30℃	**15 min**
4℃	Hold

CAUTION

- The reaction time for DNB preparation is different from conventional sequencing, requiring only **15 min**.
- The heating and cooling speed of the heated lid of some thermocyclers is slow. During the heating and cooling process, the heating module is at room temperature and the program is not running. For this type of thermal cycler, it is necessary to preheat the heated lid to ensure that the heated lid is at working temperature when performing DNB reactions.

- It is recommended to set the temperature of the heated lid to 35℃, or as close to 35℃ as possible.

4. Immediately add **10 μL Stop DNB Reaction Buffer** when the temperature of the PCR instrument reaches 4℃. Gently pipette DNB with **wide bore pipette tips** for 5-8 times. After mixing, the DNB can be stored at 4℃ (use within 24 hrs).

   CAUTION Do not centrifuge, vortex, or pipette DNB vigorously.

5. Take 2 μL DNB and quantify it with ssDNA Fluorescence Assay Kits. If DNB concentration is more than 8 ng/μL, the DNB is qualified. If DNB concentration is less than 8 ng/μL, prepare DNB again.

5.2 DNBSEQ-G400RS DNB loading

Tips
- DNB pooling is specifically recommended for methylation libraries, whereas sequencing pooling may be determined based on the data quantity of the library.
- It is recommended to use normal pipptte tip(s) for sampling and wide bore pipette tip(s) for mixing.

CAUTION The DNB loading mixture of StandardMPS and StandardMPS 2.0 sequencing are different. Corresponding loading mixture must be used according to different sequencing reagents.

5.2.1 DNB loading (StandardMPS)

5.2.1.1 Preparation

DNB loading reagents come from DNBSEQ-G400RS High-throughput Sequencing Set. Mix the reagents before using and store the remaining reagents immediately after use.

Table 63 Reagent preparation

Reagent	Requirement
DNB Load Buffer II	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Make DNB Enzyme Mix II (LC)	lick and/or invert the tube gently, centrifuge briefly, and place on ice.

5.2.1.2 Preparing DNB loading mixture

1. Prepare the DNB loading mixture on ice:

Table 64 DNB loading mixture (StandardMPS)

Component	Volume per reaction
DNB	25 μL
DNB Load Buffer II	8 μL
Make DNB Enzyme Mix II (LC)	0.25 μL
Total	33.25 μL

2. After mixing with wide-bore pipette tips, please refer to Chapter 6 DNB loading in the DNBSEQ-G400RS High-throughput (Rapid) Sequencing Set User Manual for detail operations.

5.2.2 DNB loading (StandardMPS 2.0)

5.2.2.1 Preparation

DNB loading reagents come from DNBSEQ-G400RS High-throughput Sequencing Set. Mix the reagents before using and store the remaining reagents immediately after use.

Table 65 Reagent preparation

Reagent	Requirement
DNB Load Buffer II	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Make DNB Enzyme Mix II (LC)	lick and/or invert the tube gently, centrifuge briefly, and place on ice.

5.2.2.2 Preparing DNB loading mixture

1. Prepare the DNB loading mixture on ice:

Table 66 DNB loading mixture (StandardMPS 2.0)

Component	Volume per reaction
DNB	16.5 μL
DNB Load Buffer II	16.5 μL
Make DNB Enzyme Mix II (LC)	0.25 μL
Total	33.25 μL

2. After mixing with wide-bore pipette tips, please refer to the DNB loading section in DNBSEQ-G400RS System Quick Start Guide (StandardMPS 2.0) for detail operations.

5.3 DNBSEQ-T7RS Sequencing

Tips The methylation library constructed with this kit needs to be sequenced using the DNBSEQ-T7RS High-throughput Methylation Sequencing Set (Cat.No. 940-002625-00) at DNBSEQ-T7RS platform.

1. Please select the appropriate sequencing strategy according to the table below:

Table 67 Methylation library sequencing strategy

Camera of DNBSEQ-T7RS	Sample type	Size selection	Balanced library	Ratio of balanced library
V1.0	Cell free DNA	No need	No need	0%
V1.0	Genomics DNA	Single size selection or double size selection	V3.0 Standard Library	30%
V2.0	Cell free DNA	No need	No need	0%
V2.0	Genomics DNA	Single size selection or double size selection	No need	0%

2. Detail operations for DNB making and DNB pooling can refer to chapter 6 in DNBSEQ-T7RS High-throughput Methylation Sequencing Set Instructions.
3. Detail operations for DNB loading can refer to chapter 7 in DNBSEQ-T7RS High-throughput Methylation Sequencing Set Instructions.
4. Detail operations for sequencing can refer to chapter 8 and chapter 9 in DNBSEQ-T7RS High-throughput Methylation Sequencing Set Instructions.

6 Appendix

6.1 Shearing condition

The below table shows the shearing conditions for different Covaris Focused-ultrasonicators with 55 μL volume, as provided on the Covaris official website, for reference only. Please adjust the specific parameters to shear the genomic DNA to the smear size between 100 bp and 1000 bp, with the peak size between 300 bp and 700 bp.

Table 68 Conditions for shearing genomic DNA (55 μL) to 150-550 bp using Covaris S220

S220	Vessel	microTUBE-50 AFA Fiber-Screw-Cap (PN 520166)						
								
	Sample Volume	55 μL						
	Holder	S-Series Holder microTUBE-50 Screw-Cap (PN 500492)						
	Water Level	10						
	Temperature (℃)	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

Table 69 Conditions for shearing genomic DNA (55 μL) to 150-550 bp using different Covaris Focused-ultrasonicators

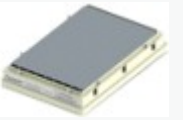
E220	Vessel	MicroTUBE-50 Screw-Cap (PN 520 166)	8 microTUBE-50 AFA Fiber Strip V2 (PN 520174) 8 microTUBE-50 AFA Fiber H Slit Strip V2 (PN 520240)	96 microTUBE-50 AFA Fiber Plate (PN 520168) 96 microTUBE-50 AFA Fiber Plate Thin Foil (PN 520232)
				
	Sample Volume	55 μL		
	Racks	Rack 24 Place micro TUBE 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 +6.5 mm 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
	Plate Definitions	"500432 E220e 4 microTUBE-50 Screw Cap -8.32mm offset"	"500437 E220e 8 microTUBE-50 Strip V2 -10mm offset"	N/A
All	Temperature (℃)	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)

Table 70 Conditions for shearing genomic DNA (55 μL) to 150-550 bp using different Covaris Focused-ultrasonicators (continued from the previous table)

All	Target BP (Peak)	150	200	250	300	350	400	550
Screw-Cap	Peak Incident Power (W)	75	75	75	75	75	75	30
	Duty Factor (%)	15	15	20	20	20	10	10
	Cycles per Burst	1000	1000	1000	1000	1000	1000	1000
	Treatment Time (s)	340	145	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)	75	75	75	75	75	75	75
	Duty Factor (%)	15	15	20	20	20	10	10
	Cycles per Burst	1000	1000	1000	1000	1000	1000	1000
	Treatment Time (s)	360	155	70	49	34	50	32

6.2 PCR BC Primer using guide

1. This kit is designed for the construction of single barcode libraries only, and the barcode sequence is located on the primer.
2. There are two specifications of the PCR BC Primer: 16 RXN and 96 RXN.

- The 16 RXN primers consist of 2 strips of 8-tube strips.
- The 96 RXN primers consist of a 96-well plate.

3. For detailed barcode information for each specification, refer to "Figure 6 Barcode layout (16 RXN)" on page 51 and "Figure 7 PCR BC Primer-96 layout" on page 53.

6.2.1 Note for PCR BC Primer

1. Barcode with same ID number share same sequence, thus cannot be sequenced in the same lane.
2. To prevent cross contamination, change tips when pipetting different barcode primer.
3. Before use, centrifuge to collect liquid at the bottom of tubes or plates. Gently remove the cap/sealing film to prevent liquid from spilling and cross-contamination. Mix BC Primer with a pipette before use. Remember to reseal the BC Primer immediately after use.
4. For PCR BC Primer-96 (Plate), if the seal film is contaminated, discard the old seal film and use a new one to reseal the 96-well plate.
5. Due to different design, it is prohibited to use barcode primer from other MGI library preparation kits. Otherwise, it may result in library preparation failure.

6.2.2 Barcode using guide (16RXN)

1. Vortex PCR BC Primer before using. Centrifuge to collect the liquid at the bottom of tubes. For the PCR BC Primer in 8-tube strips, gently remove the lid of the tube to prevent liquid from spilling and cross-contamination. Close the lid in time after use.

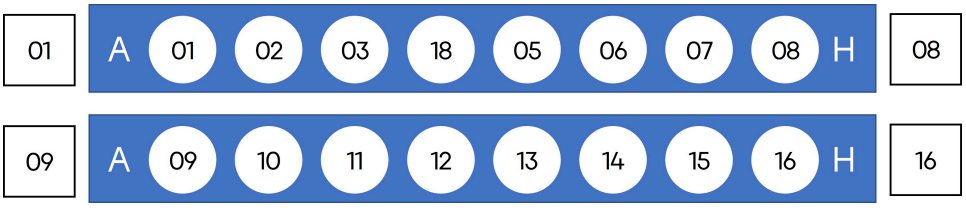


Figure 6 Barcode layout (16 RXN)

- Set of 4 barcodes: 05-08, 09-12, 13-16, 3 sets in total;
- Set of 8 barcodes: 01-03, 18, 05-08, 1 set in total.

2. The PCR BC Primer must be used based on the principle of balanced base compostion. Follow the instructions below for using.
3. When each sample requires same amount of data, refer to the table below to determine the recommended barcodes set for different sample numbers.

Table 71 Barcode using guide (16 RXN)

Number of sampe/lane	Using guide
1	Use at least 1 barcode set: • Use 4 barcodes from one barcode set. Add 4 barcodes into each sample. E.g., barcode 13-16: mix equal volume of the 4 barcodes and add to the sample.
2	Use at least 1 barcode set: • Use 4 barcodes from one barcode set. Add 2 barcodes into each sample. E.g., barcode 13-16: mix equal volume of barcodes 13 and 14 and add to sample 1; mix equal volume of barcodes 15 and 16 and add to sample 2
3	Use at least 2 barcode set: 1. Add barcodes into Sample 1 and 2 using the above (2 samples/lane) method. 2. Add barcodes into Sample 3 using the above (1 sample/lane) method. Tips Use different barcode sets in sample 1-2 and sample 3.
4	Use at least 1 barcode set: • Use 4 barcodes from one barcode set. Add 1 barcode into each sample. E.g., barcode 13-16: Add barcode 13, 14, 15,16 to sample 1, 2, 3, 4.
5	Use at least 2 barcode set: 1. Add barcodes into Sample 1-4 using the above (4 samples/lane) method. 2. Add barcodes into Sample 5 using the above (1 sample/lane) method. Tips Use different barcode sets in sample 1-4 and sample 5.
6	Use at least 2 barcode set: 1. Add barcodes into Sample 1-4 using the above (4 samples/lane) method. 2. Add barcodes into Sample 5-6 using the above (2 samples/lane) method. Tips Use different barcode sets in sample 1-4 and sample 5-6.
7	Use all of the 3 barcode sets: 1. Add barcodes into Sample 1-4 using the above (4 samples/lane) method. 2. Add barcodes into Sample 5-6 using the above (2 samples/lane) method. 3. For Sample 7, use any one of the remaining barcode set or take equal volumes of all barcodes in the remainng set, mix well and add them to the sample. Tips Use different barcode sets in sample 1-4, sample 5-6 and sample 7.
8	• Use 8 barcodes in two barcode sets (e.g., Barcode 09-12, Barcode 13-16). Add 1 barcode in each sample.

Number of sampe/lane	Using guide
8+X (X=1-8, 9-16 in total)	2-step operation: 1. Add barcodes into Sample 1-8 using the above (8 samples/lane) method. 2. Add barcodes into sampel 9-X using the above (X samples/lane) method. Tips Use different barcode sets in sample 1-8 and sample 9-X.

4. 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 barcode. For example, 9 samples are pooled into one lane, one sample of which requires 30% of the total data output. It is recommended that eight samples may use barcode set (09-16) and the the final sample should use a full barcode set (05-08) instead of using only a single barcode.

6.2.3 Barcode using guide (96 RXN)

1. For the PCR BC Primer in 96-well plate, clean the aluminum film surface with 75% ethanol and dry with absorbent paper. The film is penetrable and and the surface should not be exposed to sharp objects. For the first use, it is recommended to use a pipette tip to pierce the film and draw up liquid directly. After use, transfer the remaining reagents in the used-wells to new tubes one by one, labeled, and stored at -20 ℃.
2. The PCR BC Primer must be used in sets based on the principle of balanced base. Follow the instructions below for proper use.

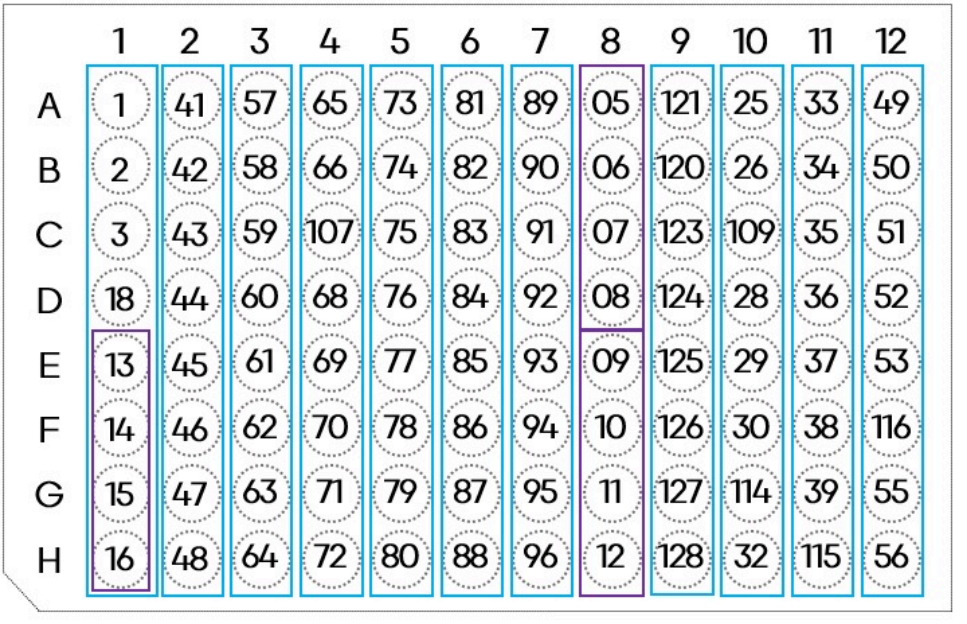


Figure 7 PCR BC Primer-96 layout

3. PCR BC Primer-96 consists of barcode combinations with preset balanced bases, grouped by columns A to H in sets of 8 (13-16, 05-08, 09-12 form a group of 4 barcodes).

4. For sample numbers < 8 with the same data volume, refer to Appendix "Barcode using guide (16RXN)" on page 51 to select barcodes.
5. For sample numbers ≥ 8 with the same data volume, you can select barcodes according to the table below.

Tips X means positive integer. For example: 8X = 8 multiplied by X, which means there are 8X samples.

Table 72 PCR BC Primer-96 using guide

Number of Sample	Using guide
8X	Use X Barcode sets
8X+1	Use X Barcode sets, and 1 barcode from one of the unused barcode sets
8X+2	Use X Barcode sets, and 2 barcodes from one of the unused barcode sets
8X+3	Use X Barcode sets, and 3 barcodes from one of the unused barcode sets
8X+4	Use X Barcode sets, and 4 barcodes from one of the unused barcode sets
8X+5	Use X Barcode sets, and 5 barcodes from one of the unused barcode sets
8X+6	Use X Barcode sets, and 6 barcodes from one of the unused barcode sets
8X+7	Use X Barcode sets, and 7 barcodes from one of the unused barcode sets

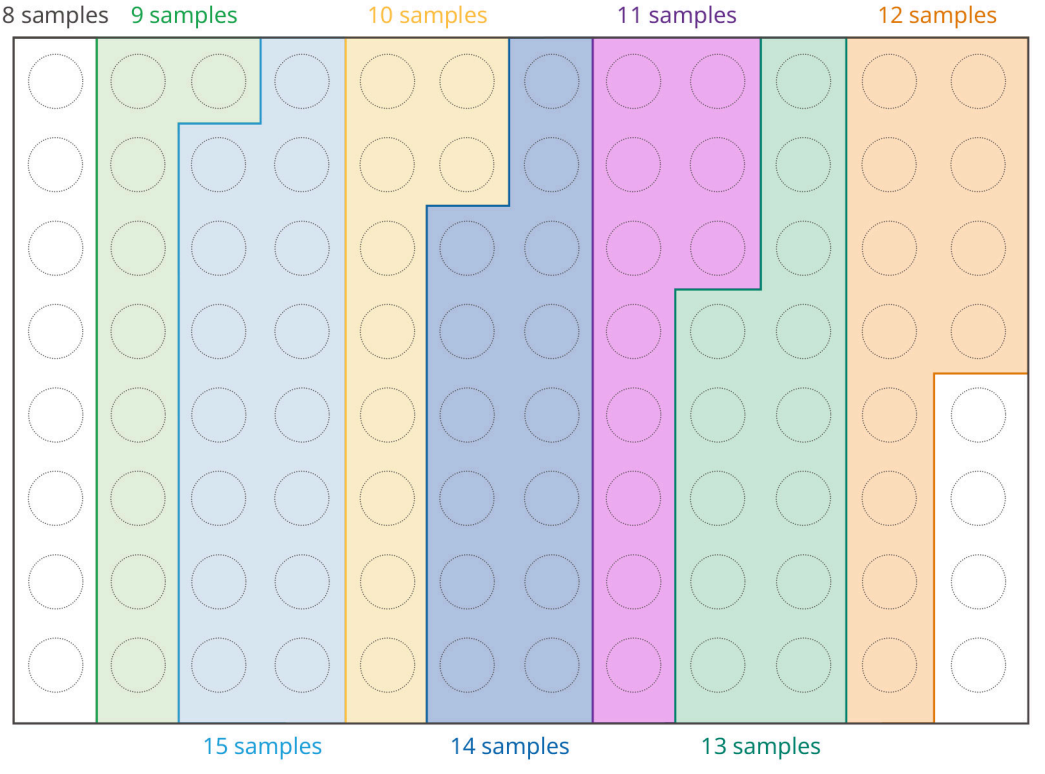


Figure 8 8 to 15 samples pooling example

6. Under exceptional circumstances (for example, insufficient reagents for a well), when the requirement of at least one barcode primer combination for standard pooling cannot be met, or if the required data amount of each library pooled is not equal, make sure to determine the pooling strategy by calculating the content of each base in each sequencing cycle. It is necessary to ensure that each base content is **not less than 12.5% and is not greater than 62.5%** in single sequencing position in the same lane.

Table 73 Perfect balanced 8 barcode Pooling strategy (8 barcode from one entire column)

Sample 1	A	G	G	A	C	G	T	A	G	A
Sample 2	C	T	G	A	A	C	C	G	A	A
Sample 3	G	A	A	C	G	T	G	T	C	G
Sample 4	T	C	C	G	T	G	A	C	T	C
Sample 5	A	A	T	T	C	A	C	T	G	T
Sample 6	C	C	T	G	A	A	G	G	A	T
Sample 7	T	T	C	C	T	T	A	C	T	G
Sample 8	G	G	A	T	G	C	T	A	C	C
Signal %	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0

Table 74 Unbalanced 9 barcode Pooling strategy (barcode from different columns)

Sample 1	A	G	G	A	C	G	T	A	G	T
Sample 2	A	C	G	A	A	G	G	T	C	C
Sample 3	G	A	A	C	G	T	G	T	C	G
Sample 4	T	C	C	G	T	G	A	C	T	C
Sample 5	A	A	T	T	C	A	C	T	G	T
Sample 6	G	C	T	G	A	A	G	G	A	T
Sample 7	T	G	C	C	T	T	A	C	T	G
Sample 8	G	G	A	T	G	A	T	A	C	C
Sample 9	G	A	C	G	G	T	C	G	A	G
A signal %	33.3	33.3	22.2	22.2	22.2	33.3	22.2	22.2	22.2	0
T signal %	22.2	0	22.2	22.2	22.2	33.3	22.2	33.3	22.2	33.3
C signal %	0	33.3	33.3	22.2	22.2	0	22.2	22.2	33.3	33.3
G signal %	44.4	33.3	22.2	33.3	33.3	33.3	33.3	22.2	22.2	33.3