VAHTS Universal Plus DNA Library Prep Kit for MGI V2

NDM627

Vazyme

Instruction for Use

Version 25.1

Contents

For Research Use Only. Not for use in diagnostic procedures.

01/Product Description

VAHTS Universal Plus DNA Library Prep Kit for MGI V2 is an enzyme-based fragmented DNA library preparation kit specifically developed for the MGI sequencing platform. This kit has been optimized and upgraded from the original version, significantly reducing the proportion of Artificial Invert Chimera Reads in the FFPE DNA library and improving the reliability of SNV detection, while maintaining the original high performance. The kit combines DNA fragmentation, end repair, and dA tailing into one step. The resulting product does not require purification, allowing direct adapter ligation, library enrichment, and size selection. 100 pg - 1 μg DNA can be converted into a dedicated library for MGI sequencing platforms. The kit is fully compatible with DNA from various sources and accommodates different input amounts. The desired library can be obtained by simply adjusting the fragmentation time according to the size of the target insert fragment. All the reagents provided in the kit have undergone rigorous quality control and functional testing to ensure the optimal stability and repeatability of library preparation.

02/Components

Components	NDM627-01 (24 rxns)	NDM627-02 (96 rxns)
FEA Buffer V2	120 μl	480 μl
FEA Enzyme Mix V2	240 μl	960 μl
Rapid Ligation Buffer V2	600 μl	4 × 600 μl
Rapid DNA Ligase V2	120 μl	480 μl
VAHTS HiFi Amplification Mix	600 μl	4 × 600 μl
PCR Primer Mix for MGI	120 μl	480 μl
Neutralization Buffer	120 μl	480 μl
Control DNA (100 ng/μl)	10 μl	10 μl

▲ The Control DNA is derived from the salmon genome and should be re-quantified before use.

03/Storage

Store at -30 ~ -15°C and ship at ≤0°C.

04/Applications

It is applicable to the preparation of a dedicated library for the MGI sequencing platform. It can be used with an initial input of 100 pg - 1 μg, and is suitable for genomic DNA derived from various species, including animals, plants, and microorganisms, as well as FFPE DNA. This kit is recommended for:

◇ Whole genome sequencing

◇ Whole exome sequencing or other targeted capture sequencing

05/Self-prepared Materials

Magnetic beads: VAHTS DNA Clean Beads (Vazyme #N411);

DNA quality control: Agilent Technologies 2100 Bioanalyzer or equivalent; Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121);

DNA Adapter for MGI: VAHTS Maxi Unique Dual Index Primers Set 1 - Set 4 for MGI (Vazyme #NM34401/NM34402/NM34403/NM34404); #NM34401/NM34402/NM34403/NM34404 are stubby adapters with 10 bp Unique Indexes at two ends, providing 384 different indexes across 4 sets.

Other materials: Absolute ethanol, ddH_2O; low-adsorption EP tube, PCR tube, magnetic rack, PCR instrument, etc.

06/Notes

Due to various factors such as the sample type, experiment protocol, equipment, and operations, it may be necessary to adjust the parameters for the library preparation in practical applications. To obtain a high-quality library, please read the following precautions carefully.

06-1/Input DNA and Fragmentation

1. Starting material: 100 pg - 1 μg Input DNA. High-quality Input DNA (A260/A280 = 1.8 - 2.0) should be used. Table 1 lists the recommended amount of Input DNA for conventional applications.

Table 1. Recommended amount of Input DNA for conventional applications

Application	Sample type	Recommended amount of Input DNA
Whole Genome Sequencing	Complex gDNA	50 ng - 1 μg
Targeted Capture Sequencing	Complex gDNA	10 ng - 1 μg
Whole Genome/Targeted Capture Sequencing	FFPE DNA	≥50 ng
Whole Genome Sequencing	Microbial genome	1 ng - 1 μg
Whole Genome Sequencing (PCR-free)	Complex/Simple genome	≥50 ng (without size selection) ≥200 ng (with size selection)

▲ The table above shows the recommended amount of high-quality Input DNA. When the Input DNA is of poor quality, the usage amount should be increased appropriately.

2. It is recommended to use ddH_2O to dissolve DNA samples. Verify EDTA concentration as FEA Enzyme Mix V2 is sensitive to EDTA. For example, if the final EDTA concentration in the end repair reaction solution is higher than 0.1 mM, pretreat DNA samples according to step **08/Experiment Process/Step 1: Fragmentation, End Repair & dA-tailing/Page 10**.

06-2/DNA Adapter

1. The quality and amount of adapters directly affect the preparation efficiency and library quality. Excess adapter input may lead to residual adapters or adapter dimers, while insufficient adapter input may affect ligation efficiency and reduce library yield. Table 2 lists the recommended usage amount of the adapter for different amounts of Input DNA.

Table 2. Recommended adapter concentration for 1 ng - 1 μg Input DNA

Input DNA	Concentration of adapter from other sources	Vazyme Adapter: ddH_2O
1 ng	0.1 μM	1:99
10 ng	1 μM	1:9
50 ng	5 μM	1:1
100 ng - 1 μg	10 μM	Undiluted

▲ It is recommended to dilute the adapter according to the concentration provided in the table or based on the dilution ratio of the Vazyme Adapter, ensuring that a fixed volume of 5 μl is used during library preparation to avoid pipetting errors.

▲ The adapter quality directly affects ligation efficiency and library yield. Avoid repeated freeze-thaw cycles.

▲ Increasing the use of adapters can improve the library yield to some extent; however, it is important to note that elevated adapter concentrations may increase adapter residues in the library, resulting in wasted sequencing data.

06-3/Adapter Ligation Product Purification

1. Excess adapters must be removed before library amplification (for PCR amplification library) or sequencing (for PCR-free library). The recommended purification condition of 0.6 × (100 μl of products, 60 μl of beads) is suitable for most cases. To obtain libraries with larger insert sizes, the amount of beads can be reduced to lower the content of small DNA fragments. However, this adjustment only changes the position of the main peak of the library. If you need to accurately control the library distribution, you can carry out a size selection after purification.
2. If library size selection is to be performed, the recommended elution volume is 105 μl. Otherwise, the recommended elution volume is 25 μl.
3. If the data indicate that the purified products are heavily contaminated with adapters or adapter dimers, an additional bead purification step can be performed. Adjust the volume of the initial purified product to 50 μl with ddH_2O, then perform a second purification using 50 μl of beads (1 ×). This process significantly reduces residual adapter dimers, particularly during the preparation of PCR-free libraries. It may also be necessary to reduce the amount of adapter used to eliminate adapter dimer residues.

06-4/Magnetic Beads

1. It is recommended to use VAHTS DNA Clean Beads (Vazyme #N411) for purification.
   ▲The purification conditions may need to be changed if beads from other sources are used!
2. General precautions for the use of magnetic beads:
   a. The amount of beads used is indicated by the multiplier "×", which represents the proportion of bead volume relative to the original sample volume. For example, if the original sample volume is 100 μl, using 1 × beads for purification means adding 1 × 100 μl = 100 μl of beads. A 0.6 ×/0.2 × size selection means 0.6 × 100 μl = 60 μl of beads are used in the first round, followed by 0.2 × 100 μl = 20 μl in the second round.
   b. The amount of magnetic beads used directly affects the minimum size of DNA fragments that can be efficiently purified. A higher bead-to-sample ratio allows for the recovery of shorter DNA fragments, whereas a lower ratio favors the purification of longer fragments. For example, at a 1 × bead ratio, only DNA fragments longer than 250 bp can be efficiently purified, with shorter fragments largely lost during purification. When the ratio is increased to 1.8 ×, fragments as short as 150 bp can be effectively recovered.
   c. Equilibrate the beads at room temperature for 30 min before use, or the yield and selection effect may be affected.
   d. Mix the beads well by vortexing or pipetting before use.
   e. Mix the sample well with the beads, and place the tube on the magnetic rack for separation. Leave about 2 - 3 μl of supernatant to avoid disturbing the beads, or the yield, selection effect, and the subsequent enzyme reaction may be affected. If disturbance occurs, remix the beads and place the tube on the magnetic rack for another separation. Due to the different performances of the magnetic rack, the default separation time may need to be extended for complete separation between beads and liquid.
   f. Rinse the beads with freshly prepared 80% ethanol. Keep the EP tube on the magnetic rack during the rinse. Do not disturb the beads.
   g. Air-dry the beads at room temperature before product elution. Insufficient drying may leave residual absolute ethanol, which can interfere with the subsequent reactions, while excessive drying may cause the beads to crack, reducing recovery efficiency. The beads usually can dry sufficiently when left at room temperature for 5 - 10 min. Do not heat the beads (e.g., drying at 37°C in an oven).
   h. Eluent (10 mM Tris-HCl, pH 8.0 - 8.5) is generally recommended to ensure stable product storage. However, if the library will undergo targeted capture, elution with ddH_2O is preferred. This facilitates drying and concentration of the library before capture and helps avoid interference with subsequent capture reactions.

06-5/Size Selection

1. If the Input DNA distribution range is wide, size selection is usually required to control the final library size distribution. Two Rounds Size Selection is recommended; gel extraction is also an option.
2. Size selection can be carried out after Adapter Ligation or Library Amplification. The size selection steps are not included in the standard experimental protocol. Refer to **Appendix I: Two Rounds Size Selection (Page 15)** for more information.
3. Size selection often results in substantial DNA loss. Sometimes, a trade-off must be made between achieving a defined library size distribution (with size selection) and preserving library complexity (without size selection). When the Input DNA amount is low, size selection should be performed only once, as two rounds or above can significantly reduce library complexity and yield.
4. Over-amplification typically results in a trailing band or a tailing peak in the high-molecular-weight region. These products are primarily caused by non-complementary strand cross-annealing (see **06-6/Library Amplification/Page 06**). It is recommended to adjust the number of amplification cycles to avoid over-amplification, while size selection is not recommended for eliminating the trailing band or tailing peak.
5. The high concentration of PEG in Rapid Ligation Buffer V2 significantly impacts Two Rounds Size Selection and gel extraction. Therefore, if size selection is to be performed after Adapter Ligation, the purification of Adapter Ligation product (**08/Experiment Process/Step 2/6. Purify the reaction product using VAHTS DNA Clean Beads as follows/Page 12**) must not be omitted. The purified product must be eluted in a suitable volume of eluent, followed by Two Rounds Size Selection or gel extraction. If size selection must be performed after Adapter Ligation, the selection conditions should be explored separately. If size selection takes place after Library Amplification, the original purification step can be replaced with Two Rounds Size Selection.

06-6/Library Amplification

1. PCR Primer Mix for MGI is designed to amplify libraries containing full-length adapters on MGI high-throughput sequencing platforms. For stubby adapters or libraries intended for other sequencing platforms, replace the amplification primers accordingly. The recommended amplification concentration for each primer is 5 - 20 μM.
2. In the later cycles of PCR, primers are typically depleted before dNTPs. At this stage, excessive cycling can lead to nonspecific annealing following denaturation of the amplification products, resulting in cross-annealing between non-complementary strands. These products migrate slowly during electrophoresis-based detection and appear as diffuse bands in the high molecular weight region. They are composed of single-stranded libraries of correct length and, after denaturation, can hybridize normally to the flow cell and be sequenced. Therefore, their presence has no significant impact on sequencing performance. However, the presence of such products has a critical impact on the choice of library quantification method. Since these

products are not fully double-stranded, quantification using fluorescence dyes that specifically bind to double-stranded DNA (such as Equalbit 1 × dsDNA HS Assay Kit, Vazyme #EQ121) may underestimate the actual library concentration. In contrast, qPCR-based quantification methods, which include a denaturation step during quantification, can still accurately measure the concentration of such over-amplified libraries.

3. Library Amplification requires strict control of the number of amplification cycles. Insufficient cycles may lead to an insufficient library yield, while excessive cycles will lead to various adverse effects such as over-amplification, increased amplification bias, PCR duplicates and chimeric products, and amplification mutations. Table 3 provides the recommended number of amplification cycles to obtain a library of 100 ng or 1 μg when 100 pg - 1 μg of high-quality Input DNA is used.

Table 3. Recommended amplification cycles for 100 pg - 1 μg Input DNA

Input DNA	Number of cycles required for expected yield	
	100 ng	1 μg
100 pg	13 - 15	16 - 18
1 ng	9 - 11	13 - 15
10 ng	4 - 6	8 - 10
50 ng	2 - 4	5 - 7
100 ng	0 - 2	4 - 5
250 ng	/	3 - 4
500 ng	/	2 - 3
1 μg	/	0 - 2

▲ The table above shows the number of cycles used for library preparation with high-quality mouse gDNA fragmented for 15 min at 37°C. When DNA quality is poor, the number of cycles must be adjusted to obtain sufficient library yield.

▲ If size selection is performed during library preparation, a higher number of amplification cycles should be used; otherwise, a lower number of cycles is sufficient.

4. When performing adapter ligation with full-length adapters (VAHTS PCR-Free DNA Adapters Set 1 - Set 4 for MGI, Vazyme #NM10901 - #NM10904), and the library yield meets application requirements, the library amplification step can be omitted to obtain a PCR-free library.

5. When using stubby adapters in the adapter ligation step, at least 2 additional PCR cycles are required to complete the adapter sequences at the ends of the library.

06-7/Library Quality Control

Generally, a prepared library can be evaluated through size distribution and concentration analysis.

1. Library size distribution analysis:
   Library size distribution can be analyzed using electrophoresis-based instruments, such as LabChip GX, GXII, GX Touch (PerkinElmer); Bioanalyzer, Tapestation (Agilent Technologies); and Fragment Analyzer (Advanced Analytical).

2. Library concentration analysis:
   Common methods of library quantification: methods based on dsDNA fluorescent dyes, e.g., Qubit, PicoGreen, or Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121).

06-8/Further Precautions

1. The size and distribution range of DNA fragments are determined by a time-dependent enzyme-based reaction, thus the fragmentation reaction should be carried out on ice.
2. Since this kit will be transported on dry ice, both buffer and enzyme will be frozen. Thaw all the components at room temperature before use. After thawing, mix well and centrifuge briefly before putting them on ice.
3. To avoid cross-contamination of samples, it is recommended to use tips with a filter and replace them between samples.
4. It is recommended to use a PCR instrument with a heated lid for the reaction at each step. Preheat the PCR instrument to approximately the reaction temperature before use.
5. PCR products are highly susceptible to aerosol contamination caused by improper handling, which can affect the accuracy of the experiment results. Therefore, we recommend physically isolating the PCR reaction preparation area from the PCR product purification and testing area, using equipment such as specialized pipettes, and regularly cleaning each laboratory area (wipe down with 0.5% sodium hypochlorite or 10% bleach) to ensure proper cleanliness of the laboratory environment.

07/Workflow

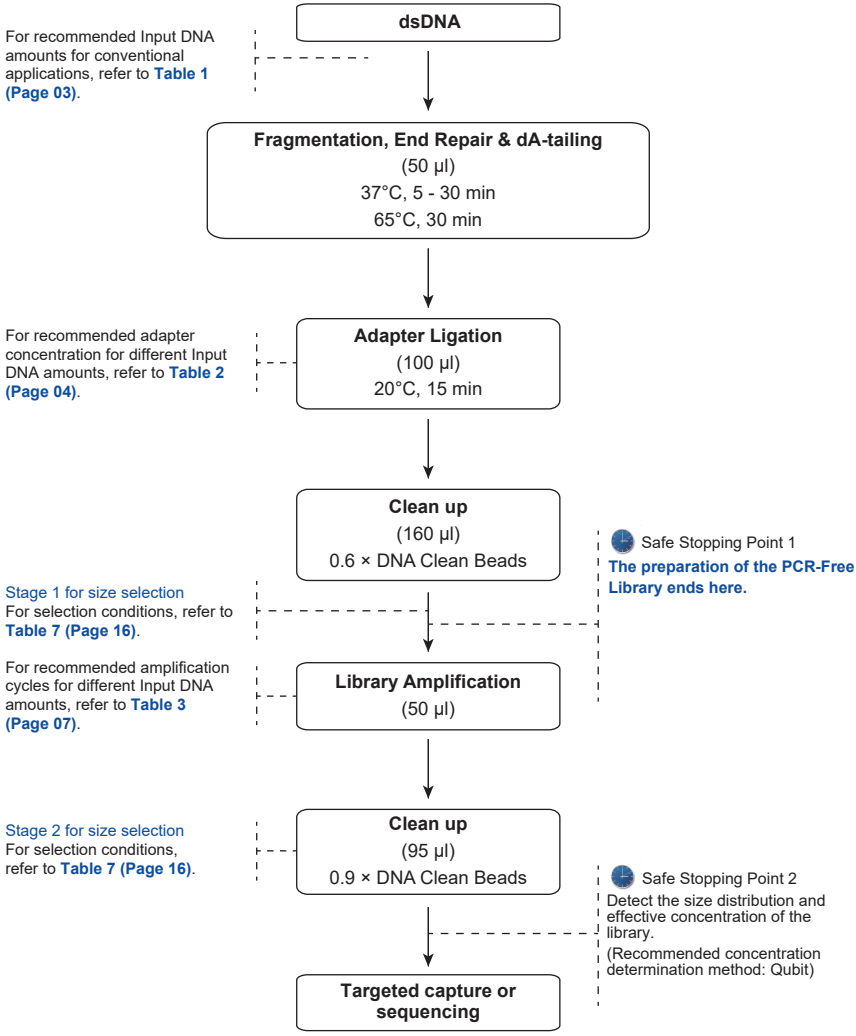


Fig 1. Workflow of VAHTS Universal Plus DNA Library Prep Kit for MGI V2

08/Experiment Process

▲ Do not mix components from different kit lots!

▲ To ensure reagent performance, please ensure the sealing integrity of the tubes during transportation and storage.

Step 1: Fragmentation, End Repair & dA Tailing

This step is performed to fragment the Input DNA while simultaneously repairing the fragmented ends, phosphorylating the 5' ends, and adding a dA tail to the 3' ends.

1. Before starting the experiment, confirm which solvent the template DNA is dissolved in (ddH_2O is recommended) and whether the solvent contains EDTA. If it does not contain EDTA, proceed directly to step 3. If it contains EDTA, pre-treat the sample according to step 2.
2. If the solvent contains EDTA, the template DNA can be purified using 2.2 × beads and eluted with ddH_2O. Alternatively, the corresponding amount of neutralization buffer can be added according to the final concentration of EDTA in the fragmentation solution to neutralize the EDTA.

   ▲ Final concentration of EDTA in fragmentation solution = EDTA concentration in DNA solution × amount of DNA used/50 μl. For example, if the DNA is dissolved in TE containing 1 mM EDTA and 10 μl is used for library preparation, the EDTA final concentration will be 1 mM × 10 μl/50 μl = 0.2 mM.

Final concentration of EDTA in fragmentation solution	Volume of Neutralization Buffer
1 mM	5 μl
0.8 mM	4 μl
0.6 mM	3 μl
0.5 mM	2.5 μl
0.4 mM	2 μl
0.2 mM	1 μl
0.1 mM	0.5 μl
<0.1 mM	0 μl

3. Thaw the FEA Buffer V2 and FEA Enzyme Mix V2. Mix well, centrifuge briefly, and put them on ice before use. All of the following steps are performed on ice.
4. Prepare the reaction solution in a sterile PCR tube as follows:

Components	Volume	
Input DNA	x μl	
Neutralization Buffer	y μl	☐
FEA Buffer V2	5 μl	■
ddH_2O	To 40 μl	

▲ If the solvent does not contain EDTA, there is no need to add Neutralization Buffer. Too much Neutralization Buffer can cause overreaction during fragmentation.

5. Add 10 μl of FEA Enzyme Mix V2 to each sample and mix well by pipetting or vortexing. Centrifuge to collect the reaction solution to the tube bottom and **place it in the PCR instrument immediately for reaction! ! !**

▲ Fragmentation reaction is time-dependent and enzyme-based, and the size of the fragment product depends on the reaction time. It is therefore recommended to add the FEA Enzyme Mix V2 to the reaction solution separately at the end. Mix immediately, and then carry out the follow-up reaction.

▲ Fragmentation reactions are sensitive to oxidation. Therefore, the FEA Buffer V2 and FEA Enzyme Mix V2 should be tightly capped immediately after use and stored at -20°C.

6. Place the PCR tube into the PCR instrument and perform the following program:

Temperature	Time
Heated lid 105°C	On
37°C	Refer to the table below*
65°C	30 min
4°C	Hold

* Fragmentation time depends on the size of Input DNA and target fragment:

Expected insert size	Fragmentation time
150 bp	25 - 30 min
250 bp	17 - 22 min
350 bp	10 - 15 min
550 bp	5 - 10 min

▲ The recommended fragmentation time is based on tests using 100 ng of high-quality human genomic DNA dissolved in ddH_2O. Please note that the fragmentation efficiency may vary slightly depending on the type and quality of the DNA template.

▲ When high-quality human gDNA is used for library preparation, different inputs within the recommended input range (100 pg - 1 μg) with the same reaction time result in little variation in the distribution of fragmentation product (the distribution range is essentially consistent, but the main peak position may vary slightly).

▲ If the Input DNA quality is poor or the fragment size is not within the expected range, it is recommended to increase or decrease the fragmentation time by 2 - 5 min. For FFPE DNA samples, there is no need to adjust the fragmentation time according to their integrity if there is no strict requirement for the size of the inserted fragments in the library.

Step 2: Adapter Ligation

Adapter is added to the products from the previous step.

1. Dilute the adapters to the appropriate concentration according to **Table 4 (same as Table 2)**.

Table 4. Recommended adapter concentration for 1 ng - 1 μg Input DNA

Input DNA	Concentration of adapter from other sources	Vazyme Adapter: ddH_2O
1 ng	0.1 μM	1:99
10 ng	1 μM	1:9
50 ng	5 μM	1:1
100 ng - 1 μg	10 μM	Undiluted

2. Thaw the Rapid Ligation Buffer V2 and Rapid DNA Ligase V2. Mix well, centrifuge briefly, and put them on ice before use.
3. Prepare the reaction solution according to the table below:

Components	Volume
Products from the previous step	50 μl
Rapid Ligation Buffer V2	25 μl
Rapid DNA Ligase V2	5 μl
ddH_2O	15 μl
DNA Adapter X*	5 μl

* When using different types of adapters, the usage of DNA Adapter X also varies.

When full-length adapters are used, different samples are distinguished by using different DNA Adapter X during the ligation step.

When stubby adapters are used, all samples are ligated with universal adapters, and the samples are distinguished during the final library amplification using different index primers.

Therefore, the corresponding component name of DNA Adapter X in the actual adapter kit is listed in the table below:

Product Form	Cat. No. of Adapter	Type of Adapter	Name of DNA Adapter X*
Tube-based	NM108	Full-length	DNA Adapter 96-XX
	NM10901 - NM10904		DNA Adapter 96-XX
	NM35101 - NM35108	Stubby	VAHTS Dual UMI Adapters for MGI UDB
	NM34401 - NM34404		VAHTS Universal Adapter for MGI
Plate-based	NMB108	Full-length	DNA Adapter 96-XX
	NMB34401 - NMB34404	Stubby	VAHTS Universal Adapter for MGI

▲ White precipitates may form in the Rapid Ligation Buffer V2 during storage, which is a normal phenomenon. This does not affect product performance after proper thawing and complete dissolution.

4. Mix well and centrifuge briefly.
5. Place the PCR tube into the PCR instrument and perform the following program:

Temperature	Time
Heated lid 105°C	On
20°C	15 min
4°C	Hold

▲ If the Input DNA amount is too low, consider doubling the ligation time. However, longer reaction time may lead to increased adapter dimers. If necessary, the adapter concentration may also need to be optimized.

6. Purify the reaction product using VAHTS DNA Clean Beads as follows:
   a. Equilibrate VAHTS DNA Clean Beads to room temperature, and mix well by vortexing.
   b. Add 60 μl of VAHTS DNA Clean Beads to 100 μl of Adapter Ligation solution. Mix well by vortexing or pipetting.
   c. Incubate for 5 min at room temperature.

d. Briefly centrifuge the PCR tube and place it on the magnetic rack to separate the beads from the liquid. Carefully **discard the supernatant** after the solution is clear (about 5 min).

e. Keep the PCR tube on the magnetic rack, and add 200 µl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature, then carefully **discard the supernatant**.

f. Repeat step e (twice in total).

g. Keep the PCR tube on the magnetic rack, and air-dry the beads.

▲ Elute the beads when their surface changes from shiny brown to matte brown. Avoid over-drying as it can reduce the DNA recovery efficiency.

h. Remove the PCR tube from the magnetic rack for elution:

▲ If the purified products do not undergo Two Rounds Size Selection: Add 25 µl of eluent (10 mM Tris-HCl, pH 8.0 - 8.5) or ddH_2O for elution. Mix well by vortexing or pipetting, and incubate at room temperature for 2 min. Briefly centrifuge the PCR tube and place it on the magnetic rack. Carefully transfer 20 µl of supernatant to a new EP tube after the solution is clear (about 5 min). Do not disturb the magnetic beads.

▲ If the purified products undergo Two Rounds Size Selection: Add 105 µl of eluent (10 mM Tris-HCl, pH 8.0 - 8.5) or ddH_2O for elution. Mix well by vortexing or pipetting and incubate at room temperature for 2 min. Briefly centrifuge the PCR tube and place it on the magnetic rack. Carefully transfer 100 µl of supernatant to a new EP tube after the solution is clear (about 5 min). Do not disturb the magnetic beads. Perform size selection according to the criteria for Two Rounds Size Selection in **Table 7 (Page 16)**.

Store the samples at -20°C and avoid repeated freeze-thaw cycles.

Step 3: Library Amplification

This step involves PCR amplification of the Adapter Ligation products following purification or size selection. Whether this step is necessary depends on factors such as the Input DNA amount, the completeness of the adapters, and the downstream application. If the adapters are not full-length, PCR amplification is required. When full-length adapters are used, library amplification is recommended if the Input DNA is <50 ng. If the Input DNA is ≥50 ng or library amplification is not needed, skip this step and proceed directly to **Step 4: Library Quality Control (Page 15)**.

1. Thaw the PCR Primer Mix for MGI and the VAHTS HiFi Amplification Mix. Mix well by inversion and centrifuge briefly. Prepare the reaction solution in a sterile PCR tube as follows:

Components	Volume	
Purified or selected Adapter Ligation products	20 µl	
PCR Primer Mix for MGI*	5 µl	■
VAHTS HiFi Amplification Mix	25 µl	■
Total	50 µl	

* The amplification primers should be selected based on the adapter type. Please follow the instructions listed in the table below.

Product Form	Cat. No. of Adapter	Type of Adapter	Name of PCR Primer	Kit for Component Sourcing
Tube-based	NM108 NM10901 - NM10904	Full-length	PCR Primer Mix for MGI PCR Primer Mix for MGI	Library prep kit
	NM35101 - NM35108	Stubby	VAHTS Unique Dual Barcode Primer for MGI (UDB XXX)	Adapter kit
	NM34401 - NM34404		VAHTS Unique Dual Barcode Primer for MGI (UDB XXX)	
Plate-based	NMB108	Full-length	PCR Primer Mix for MGI	Library prep kit
	NMB34401 - NMB34404	Stubby	VAHTS Unique Dual Barcode Primer for MGI (UDB XXX)	Adapter kit

▲ If stubby adapters or adapters from other platforms are used, amplification primers must be replaced accordingly.

2. Mix well and centrifuge briefly.

3. Place the PCR tube in the PCR instrument and perform the reaction below:

Temperature	Time	Volume
95°C	3 min	1
98°C 60°C 72°C	20 sec 15 sec 30 sec	For the number of cycles, see Table 5 (same as Table 3)
72°C	5 min	1
4°C	Hold	

Table 5. Recommended amplification cycles for 100 pg - 1 μg Input DNA

Input DNA	Number of cycles required for expected yield	
	100 ng	1 μg
100 pg	13 - 15	16 - 18
1 ng	9 - 11	13 - 15
10 ng	4 - 6	8 - 10
50 ng	2 - 4	5 - 7
100 ng	0 - 2	4 - 5
250 ng	/	3 - 4
500 ng	/	2 - 3
1 μg	/	0 - 2

4. For size selection, refer to **Appendix I: Two Rounds Size Selection (Page 15)**. Use VAHTS DNA Clean Beads to purify the reaction products if size selection is not required:
   a. Equilibrate VAHTS DNA Clean Beads to room temperature, and mix well by vortexing.
   b. Add 45 μl of VAHTS DNA Clean Beads to 50 μl of Library Amplification solution. Mix well by vortexing or pipetting.
   c. Incubate for 5 min at room temperature.
   d. Briefly centrifuge the PCR tube and place it on the magnetic rack to separate the beads from the liquid. Carefully **discard the supernatant** after the solution is clear (about 5 min).
   e. Keep the PCR tube on the magnetic rack and add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate at room temperature for 30 sec, then carefully **discard the supernatant**.

f. Repeat step e (twice in total).

g. Keep the PCR tube on the magnetic rack. Air-dry the beads for 5 - 10 min until there is no ethanol residue.

h. Remove the PCR tube from the magnetic rack for elution:

▲ Add 25 µl of eluent (10 mM Tris-HCl, pH 8.0 - 8.5) or ddH_2O (ddH_2O elution is necessary if targeted capture is required later) for elution. Mix well by vortexing or pipetting and incubate at room temperature for 2 min. Briefly centrifuge the PCR tube and place it on the magnetic rack. Carefully transfer 20 µl of supernatant to a new EP tube after the solution is clear (about 5 min). Do not disturb the magnetic beads.

Store the samples at -20°C and avoid repeated freeze-thaw cycles.

Step 4: Library Quality Control

Refer to **06-7/Library Quality Control (Page 07).**

Appendix I: Two Rounds Size Selection

1. To meet the needs of different applications, Two Rounds Size Selection is often required during library preparation to control the distribution range of the library insert size. See **Table 6** for information on choosing when to perform selection, as well as the advantages and disadvantages of performing size selection at different stages. **It must be guaranteed that the selection process is performed only once. Two or more selections can lead to a significant reduction in library complexity and yield!**

Table 6. Stage options for size selection

Stage for size selection	Applicable conditions	Advantages	Disadvantages	Examples of applicable samples
After adapter ligation	Input DNA distribution is suitable and adequate[a]	The final library exhibits a relatively sharp peak profile	Cannot accurately evaluate the library distribution[a]	Proper fragmentation of genomic DNA or FFPE DNA with wider distribution range
After library amplification	Low Input DNA amount[b]	Reducing the loss of Input DNA during library preparation and increasing the library complexity	Library size distribution range is slightly broad	
No size selection during library preparation	Input DNA distribution range meets the library preparation requirements; Input DNA amount is low	Reducing the loss of Input DNA during library preparation and increasing the library complexity	Library insert size cannot be controlled	Proper fragmentation of genomic DNA

a. The effectiveness of Two Rounds Size Selection is influenced by the state of the DNA ends. Single-stranded overhangs at the Input DNA ends and non-complementary regions of the adapters can result in a broader distribution of the selected product sizes.

b. If the Input DNA amount is ≥100 ng, it is recommended to perform the size selection after Adapter Ligation. If the Input DNA amount is <100 ng or the sample copy number is limited, perform size selection after Library Amplification.

2. Two Rounds Size Selection is achieved by controlling the amount of beads used for DNA size selection. The basic principle is as follows: in the first round the beads bind to DNA with a larger molecular weight, and this kind of DNA is removed when the beads are discarded; in the second round the beads bind to DNA with a larger molecular weight in the remaining products, and the smaller-sized DNA is removed by discarding the supernatant. Many components in the initial sample interfere with the effectiveness of Two Rounds Size Selection. Therefore, when the stage for size selection is different, the amount of beads used for Two Rounds Size Selection will be different. Select the most appropriate selection parameter according to **Table 7** based on the expected library insert size and the stage for size selection.

   ▲ The efficiency of library size selection is directly influenced by the main peak size and the distribution concentration of DNA fragments after shearing. If the size selection result does not meet expectations, the selection parameters can be adjusted accordingly to achieve the desired library profile.

   ▲ Size selection performance may vary slightly across magnetic bead brands. Please optimize the selection conditions based on experimental results.

Table 7. Library size selection

Stage and conditions for performing size selection	Purification rounds	Expected insert size (bp)							
		150	200	250	300	350	400	450	500
After adapter ligation	1st round **X** (μl)	78	68	65	59	56	53	51	50
(100 μl sample volume)	2nd round **Y** (μl)	20	20	15	15	12	12	10	10
After library amplification	1st round **X** (μl)	78	70	63	55	50	46	45	44
(fill sample volume up to 100 μl)	2nd round **Y** (μl)	20	20	20	20	20	20	20	15

3. If stubby adapters are used, please refer to the following table to choose the volume of beads according to the expected insert size and the stage for size selection.

Stage and conditions for performing size selection	Purification rounds	Expected insert size (bp)							
		150	200	250	300	350	400	450	500
After adapter ligation	1st round **X** (μl)	100	90	75	65	60	55	53	50
(100 μl sample volume)	2nd round **Y** (μl)	20	20	20	20	20	20	20	18

   ▲ The size selection conditions for stubby adapters are the same as those in Table 7 after library amplification.

   ▲ When magnetic beads are used for size selection, the larger insert size, the broader size distribution. However, magnetic beads are ineffective for selecting DNA fragments with insert sizes greater than 700 bp, and two rounds of bead-based purification have minimal size selection effect in this range. In such cases, gel extraction is recommended for size selection.

   ▲ The volume ratio of samples and beads is important for size selection. Please ensure the accuracy of the initial sample volume and pipetting volume.

4. Sample pretreatment (IMPORTANT!)

   ▲ If size selection takes place after the purification of Adapter Ligation products, the sample volume should be 100 μl. If not, the sample should be filled up to 100 μl with ddH_2O.

   ▲ If size selection takes place after Library Amplification, the sample volume should be 100 μl. If not, the sample should be filled up to 100 μl with ddH_2O.

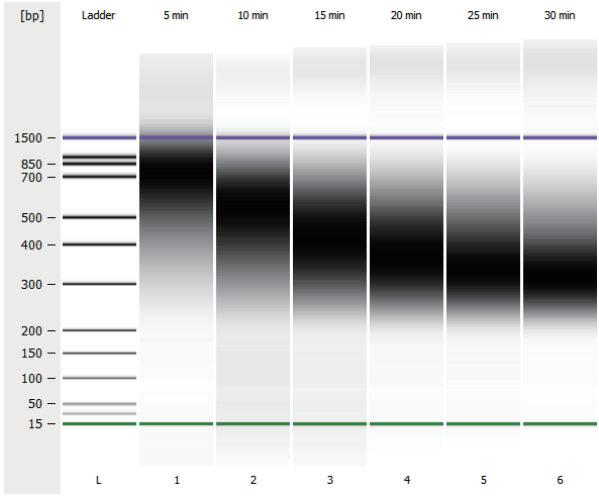
▲ If the sample volume is not adjusted in advance, the bead amount can be adjusted in proportion to the actual volume of the sample. However, a small sample volume can increase pipetting errors, which may affect the accuracy of size selection. Therefore, direct size selection of samples <50 μl is not recommended.

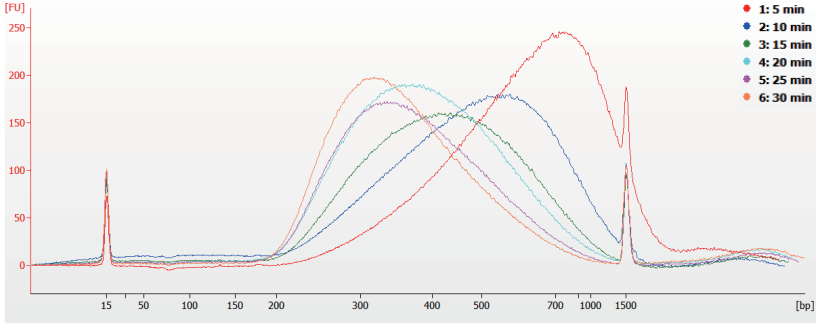
5. Protocol for selection (refer to **Table 7 (Page 16)** to confirm values of **X** and **Y**)
   a. Equilibrate VAHTS DNA Clean Beads to room temperature and mix well by vortexing.
   b. Add **X** μl of VAHTS DNA Clean Beads to 100 μl of the solution above. Mix well by vortexing or pipetting.
      ▲ If the solution is less than 100 μl, fill up to 100 μl with ddH_2O.
   c. Incubate for 5 min at room temperature.
   d. Briefly centrifuge the PCR tube and place it on the magnetic rack to separate the beads from the liquid. Carefully **transfer the supernatant to a new PCR tube** after the solution is clear (about 5 min) and **discard the beads**.
   e. Add **Y** μl of VAHTS DNA Clean Beads to the supernatant. Mix well by vortexing or pipetting.
   f. Incubate for 5 min at room temperature.
   g. Briefly centrifuge the PCR tube and place it on the magnetic rack to separate the beads from the solution. Carefully **transfer the supernatant** after the solution is clear (about 5 min).
   h. Keep the PCR tube on the magnetic rack and add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate at room temperature for 30 sec, then carefully **discard the supernatant**.
   i. Repeat step h (twice in total).
   j. Keep the PCR tube in the magnetic rack. Air-dry the beads for 3 - 5 min until there is no ethanol residue.
   k. Remove the PCR tube from the magnetic rack for elution:
      ▲ Add 25 μl of eluent (10 mM Tris-HCl, pH 8.0 - 8.5) or ddH_2O (ddH_2O elution is necessary if targeted capture is required later). Mix well by vortexing or pipetting and incubate at room temperature for 2 min. Briefly centrifuge the PCR tube and place it on the magnetic rack. Carefully transfer 20 μl of supernatant to a new EP tube after the solution is clear (about 5 min). Do not disturb the magnetic beads.

Appendix II: Experimental Example

Experimental example involving different fragmentation times:

With 100 ng of human genome DNA as a template, the kit was used to prepare the library. The fragmentation conditions were 37°C for 5/10/15/20/25/30 min, respectively. PCR amplification lasted for 4 cycles. The final library distribution is shown as follows:


[bp]
Ladder
5 min
10 min
15 min
20 min
25 min
30 min
1500
850
700
500
400
300
200
150
100
50
15
L
1
2
3
4
5
6


[FU]
250
200
150
100
50
0
15
50
100
150
200
300
400
500
700
1000
1500
[bp]
1: 5 min
2: 10 min
3: 15 min
4: 20 min
5: 25 min
6: 30 min

Vazyme Biotech Co.,Ltd.
www.vazyme.com
400-600-9335 (China) +86 400-168-5000 (Global)
support@vazyme.com