Vazyme

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VAHTS Universal Plus DNA Library Prep Kit for MGI V2

NDM627

Vazyme

Instruction for Use

Version 23.1

Contents

01/Product Description

VAHTS Universal Plus DNA Library Prep Kit for MGI V2 is a fragment enzyme DNA library preparation kit developed specifically for the MGI sequencing platform. This kit was optimized and upgraded for the original version, which significantly reduced the proportion of Artificial Invert Chimera Reads in the FFPE sample DNA library and improved the reliability of SNV detection while maintaining the original high performance. This kit combines DNA fragmentation, end repair and dA tailing into one step. The product does not require purification. The adapter ligation, library enrichment and sorting can be performed directly. The 100 pg - 1 μg Input DNA can be converted into a dedicated library for MGI sequencing platforms. This kit is fully compatible with DNA from different sources and different amount of Input DNA. The required library can be achieved simply by 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

▲ Control DNA is salmon genome DNA.

03/Storage

Store at -30 ~ -15℃ and transport at ≤0℃.

04/Applications

This kit is suitable for preparing 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 various species such as animals, plants and microorganisms, as well as genomic DNA or paraffin-embedded DNA (FFPE DNA), etc. It is recommend to use this kit for:

◇ Whole genome sequencing
◇ Whole exome sequencing or other target capture sequencing
◇ Metagenome 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);
VAHTS Library Quantification Kit for MGI (Vazyme #NQ101-106);

DDNA adapter:

VAHTS DNA Adapters for MGI (Vazyme #NM108).

VAHTS PCR-Free DNA Adapters for MGI (Vazyme #NM10901- NM10904).

#NM108 contains 96 types of single-end 10 bp indexed adapters.

#NM10901- NM10904 contain 96 types of single-end 10 bp PCR-free indexed adapters in total.

Other materials: Absolute ethanol, ddH$_2$O; Low binding EP tube, PCR tube, Magnetic stand, PCR instruments, etc.

06/Notes

For research use only. Not for use in diagnostic procedures.

Due to a number of factors such as the sample type, plan, equipment and operations, it may be necessary to adjust the parameters for the library preparation according to practical application. In order to obtain a high-quality library, it is important to read the following precautions carefully. If having any questions during using of this kit, please contact Vazyme at info.biotech@vazyme.com.

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 Input DNA amounts for conventional applications

Application	Sample type	Recommended amount of Input DNA
Whole Genome Sequencing	Complex gDNA	50 ng - 1 μg
Target Capture Sequencing	Complex gDNA	10 ng - 1 μg
Whole Genome/Target 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 (no size selection) ≥200 ng (with size selection)

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

2. It is recommended to use ddH$_2$O to dissolve DNA samples. Since FEA Enzyme Mix V2 is sensitive to EDTA, EDTA concentration must be checked. 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(Page.10)**.

06-2/DNA Adapter

1. For the MGI sequencing platform, Vazyme offers four sets of Indexed Adapters, which can be selected according to the different usage and the number of Pooling samples:
   #NM108 contains 96 types of single-end 10 bp indexed adapters.
   #NM10901- NM10904 contain 96 types of single-end 10 bp PCR-free indexed adapters in total.
2. The quality and amount of adapters directly affect the preparation efficiency and library quality. If the adapter input is excessive, it may lead to residual adapters or adapter dimers. If the adapter input is too little, it may affect ligation efficiency and reduce library yield. Table 2 lists the recommended adapter usage for different Input DNA amounts.

Table 2. Recommended adapter concentration for 100 pg - 1 μg Input DNA

Input DNA	Adapter: Input DNA molar ratio	Concentration of adapter from other source	Vazyme adapter dilution ratio
500 ng - 1 μg	10:1 - 20:1	10 μM	Undiluted
100 - 500 ng	20:1 - 100:1	10 μM	Undiluted
25 - 100 ng	40:1 - 200:1	5 μM	1:2
5 - 25 ng	50:1 - 200:1	1 μM	1:30
100 pg - 5 ng	60:1 - 3,000:1	0.1 - 0.2 μM	1:400 - 1:60

▲ The mole number of Input DNA can be roughly calculated according to the following formula:
Input DNA mole number (pmol) ≈ Input DNA mass (ng)/[0.66 × average length of Input DNA (bp)]

▲ It is recommended to use 0.1 × TE to dilute adapters according to the above table. This ensures that the adapters are used with a fixed volume (5 μl) during the library preparation process, avoiding incorrect loading volume.

▲ The quality of the adapters directly affects the molar ratio of the adapter and the Input DNA, which in turn affects ligation efficiency and the library yield. High-quality adapters should be used. Use 0.1 × TE to dilute and store adapters. Avoid repeated freezing and thawing.

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

06-3/Adapter Ligation Product Purification

1. Excessed 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 this purification process.
2. If library size selection is being performed later, the recommended elution amount is 105 μl. Otherwise, the recommended elution amount is 22.5 μl.
3. If the data shows that the purification products are heavily contaminated with the adapters or adapter dimers, they can be further purified with beads: the first purified product volume is made up to 50 μl with ddH$_2$O and the second purification is done with 50 μl beads (1 ×). This significantly reduces the residue level of the adapters or adapter dimers, especially when building PCR-free libraries. It may also be necessary to reduce the amount of adapter in order to completely eliminate the adapters or adapter dimers residue.

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 magnetic beads usage:
   a. The amount of beads used is indicated by the usual multiplier "×", which indicates the proportion of the amount of beads used in relation to the original sample volume. For example, if the original volume of the sample is 100 μl, 1 × beads used for purification is 1 × 100 μl = 100 μl. 0.6 ×/0.2 × size selection means the beads amount is 0.6 × 100 μl = 60 μl in the first round and 0.2 × 100 μl = 20 μl in the second round.
   b. The amount of beads used directly affects the lower limit of DNA length that can be purified.
   c. Beads should be balanced to room temperature (30 min at room temperature) before use; Otherwise, it could result in poor yield or poor selection effect.
   d. Thoroughly vortex or mix the beads before use.
   e. When a sample is fully mixed with the beads and placed on the magnetic stand to separate, the solution must be completely clear before the supernatant is aspirated. About 2 - 3 μl supernatant should be left. If the beads were accidentally removed, reduced yields or poor selection effect may be caused, and this may even affect subsequent enzyme reactions. To solve this problem, the beads can be fully mixed again and placed back on the magnetic stand for further separation. Due to the different performance of the magnetic stand, the default separation time may sometimes need to be extended to completely separate the beads and liquid.
   f. Rinse the beads with freshly prepared 80% ethanol. The EP tube should always be placed on the magnetic stand during the rinse process. Make sure not to disturb the beads.
   g. The beads should be allowed to dry at room temperature before elution. Insufficient drying can lead to absolute ethanol residue which affects the subsequent reaction. Excessive drying can cause the beads to crack, which reduces the purification rate. The beads will usually dry sufficiently when left at room temperature for 5 - 10 min. Do not heat to dry the beads (e.g. drying out at 37℃ in an oven).
   h. Generally, it is recommended to use an eluent (10 mM Tris-HCl, pH 8.0 - 8.5) to perform product elution, as this is better for the stable storage of the product. If targeted capture of the library is required in the future, in order to facilitate the drying and concentration of the library before capture and to prevent any impact on the subsequent capture reaction, the product elution should be carried out with ddH_2O.

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. It is recommended to use Two Rounds Beads Selection, or selection can also be performed using gel extraction.
2. Size selection can be carried out after Adapter Ligation, or after Library Amplification. The size selection steps are not included in the standard experimental protocol. Refer to **Appendix I: Two Rounds Beads Selection (Page.14)** for more information.
3. There is a large amount of DNA loss involved in size selection. Sometimes it is necessary to choose between the library size distribution (with size selection) and the library complexity (no size selection). When the amount of Input DNA is low, it must be guaranteed that the size selection stage occurs only once. Two or more size selections can lead to a significant reduction in library complexity and yield.
4. Over-amplification typically results in the trailing band or tail peak appearing at the high molecular weight position. The corresponding products are mostly non-complementary chain cross-annealing products (refer to **06-6/Library Amplification/Page.06**). The recommended solution is to adjust the number of amplification cycles to avoid over-amplification. It is not recommended to resolve over-amplification by the mean of size selection.
5. The high concentration of PEG in Rapid Ligation Buffer V2 has a significant impact on Two Rounds Beads Selection and gel extraction. Therefore, if size selection is performed after Adapter Ligation, the Adapter Ligation product purification steps (**08/Experiment Process/Step 2/6. Use VAHTS DNA Clean Beads to purify the reaction products/Page.12**) must not be omitted and the purified product must be eluted in a suitable volume of eluent, followed by Two Rounds Beads Selection or gel extraction. If selection must be performed after Adapter Ligation, the selection condition should be explored separately. If size selection takes place after Library Amplification, the original purification step can be replaced with Two Rounds Beads Selection or gel extraction.

06-6/Library Amplification

1. PCR Primer Mix for MGI is designed to be used for amplification of the MGI high-performance sequencing platform library with full length adapters. Replace the amplification primers for short adapters or other platform libraries, and the recommended amplification concentration for each primer is 5 - 20 μM.
2. During the late stage of PCR, primers are usually depleted before dNTP. At this point, too many cycles can cause non-specific annealing after the amplification products unwind, resulting in non-complementary chain cross-annealing products. These products migrate slower and are diffused in higher molecular weight region in electrophoresis-based analysis. They are made up of single-strand libraries that have the correct length, which can be normally combined with Flow Cell and sequenced after denaturation. The existence of these products has no significant impact on sequencing. Because the product is not a complete double-stranded

structure, when library quantification is performed by using fluorescent dyes (Equalbit 1 × dsDNA HS Assay Kit, Vazyme #EQ121) that recognize double-stranded DNA, the quantification results are lower than the actual values. But when library quantification is performed by using the qPCR-based library quantification methods (such as VAHTS Library Quantification Kit for MGI, Vazyme #NQ101-NQ106), which includs the denaturation process in the quantification process, it can still accurately quantify this over-amplified library.

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

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

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

▲ The above table shows the number of cycles measured for high-quality mouse gDNA which was fragmented for 15 min at 37℃. When DNA quality is poor, the number of cycles must be adjusted to obtain sufficient library.

▲ If the size selection is performed during library preparation, Library Amplification should be carried out with a higher number of cycles; otherwise, the lower number of cycles is sufficient.

06-7/Library Quality Control

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

1. Library size distribution analysis:
   Library size distribution analysis can be performed using equipment based on electrophoretic separation, such as LabChip GX、GXII、GX Touch (PerkinElmer); Bioanalyzer、Tapestation (Agilent Technologies); Fragment (Advanced Analytical), etc.
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/Other Notes

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 thoroughly 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 to replace the tips between samples.
4. It is recommended to use a PCR instrument with a heat lid function when carrying out the reaction in each step. Preheat the PCR instrument close to 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 and the PCR product purification testing area, using equipment such as specialist pipettes, and periodically cleaning each laboratory area (wipe down with 0.5% sodium hypochlorite or 10% bleach) to ensure proper cleanliness of the laboratory environment.

07/Workflow

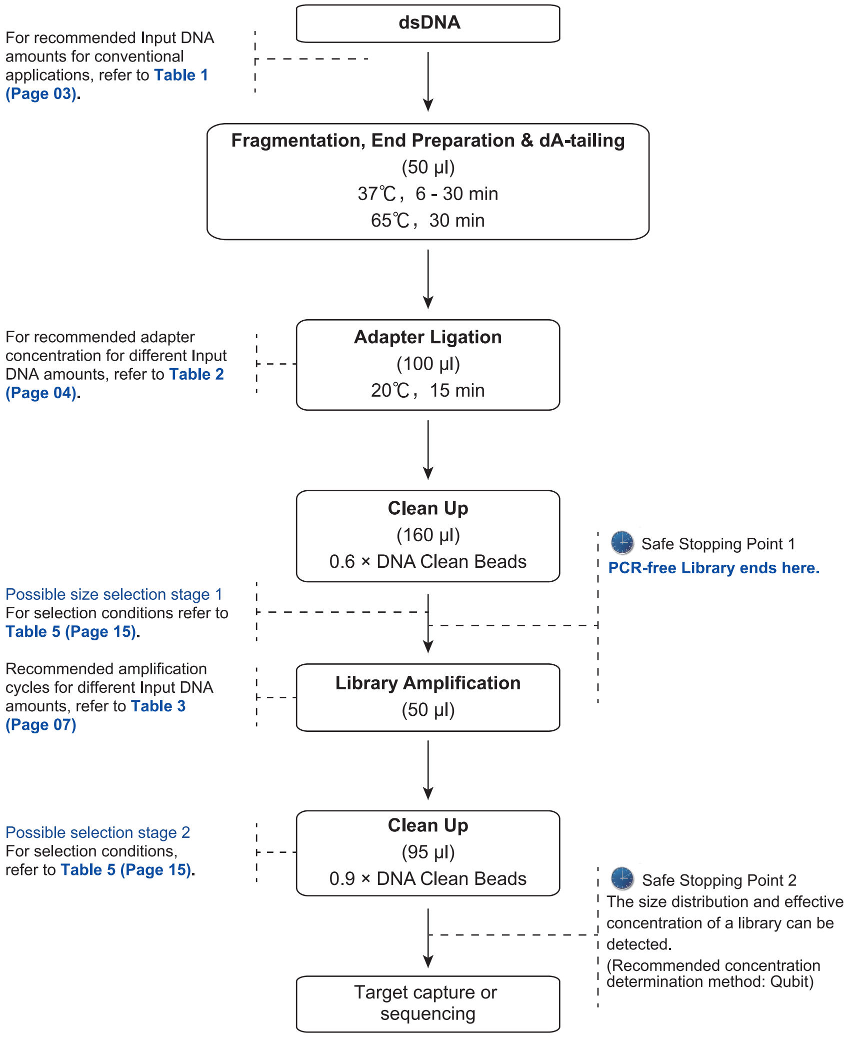


Fig 1. Workflow of VAHTS Universal Pro DNA Library Prep Kit for MGI V2

08/Experiment Process

Step 1: Fragmentation, End Preparation and dA Tailing

This step is for fragmenting the Input DNA while simultaneously repairing the fragmented DNA end, as well as the 5' end phosphorylation and the 3' end dA tailing.

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 does contain 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, in order to neutralize the EDTA.
   ▲ EDTA final concentration of 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.

EDTA final concentration of fragmentation solution	The 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 gently and 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.

▲ When there are a large number of samples and the samples contain EDTA, different amounts of neutralization buffer must be calculated and added, which is relatively complicated during actual operation. Refer to **Appendix III: Solution for Fragmentation of Multiple Samples (Page.17).**

5. Add 10 μl FEA Enzyme Mix V2 to each sample, pipetting up and down or vortexing to mix, and centrifuge to collect the reaction solution to the bottom of the tube and **place it in the PCR instrument immediately for reaction! ! !**
   - ▲ Fragmentation reaction is a time-dependent enzyme-based reaction, 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, so the FEA Buffer V2 and FEA Enzyme Mix V2 caps should be tightly screwed on as soon as possible after use, and then store them at -20℃.
6. Place the PCR tube into the PCR instrument and perform the following program:

Temperature	Time
Hot lid 105℃	On
37℃	Refer to the table below*
65℃	30 min
4℃	Hold

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

Expected insert size	Fragmentation time
150 bp	20 - 30 min
250 bp	15 - 20 min
350 bp	10 - 15 min
550 bp	6 - 10 min

▲ The recommended time given above was measured by using high-quality human placenta gDNA as a template. When using high-quality human placenta gDNA for library preparation, different inputs within the recommended input range (100 pg - 1 μg) with the same reaction time resulted 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 its integrity if there is no strict requirement for the size of the inserted fragments in the library. The FFPE DNA samples with different integrity were fragmented for 15 - 20 min, and the inserted fragments of 150 - 250 bp were obtained. For different segmented time experiments, please refer to **Appendix III: Solution for Fragmentation of Multiple Samples (Page.17).**

Step 2: Adapter Ligation

This step is to add adapters to the products in the previous step.

1. Dilute the adapters to the appropriate concentration according to **Table 2 (Page. 04)**.
2. Thaw the Rapid Ligation Buffer V2 and Rapid DNA Ligase V2. Mix thoroughly and 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$_2$O	15 μl	
DNA Adapter X	5 μl	

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

Temperature	Time
Hot lid 105℃	On
20℃	15 min
4℃	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. When the beads are balanced to room temperature, vortex to mix the VAHTS DNA Clean Beads.
   b. Add 60 μl VAHTS DNA Clean Beads to 100 μl Adapter Ligation solution. Mix thoroughly by vortexing or pipetting.
   c. Incubate for 5 min at room temperature.
   d. Briefly centrifuge the PCR tube and place it on the magnetic stand to separate the beads and liquid. After the solution is clear (approximately 5 min), **carefully remove the supernatant**.
   e. Always keep the PCR tube on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature, then carefully **remove the supernatant**.
   f. Repeat step e; rinse twice in total.
   g. Always keep the PCR tube on the magnetic stand. Open the lid to air dry the beads for 3 - 5 min until there is no ethanol residue.
      ▲ The surface of the magnetic beads changes from dark brown to brown, and the surface can be eluted without reflection. If the magnetic beads are excessively dry, the elution experience and efficiency will be affected.
   h. Remove the PCR tube from the magnetic stand for elution:
      ▲ If the purification products do not undergo Two Rounds Beads Selection: Add 22.5 μl eluent (10 mM Tris-HCl, pH 8.0 - 8.5) or ddH$_2$O for elution, vortex or gently pipette up and down to mix thoroughly and place it at room temperature for 2 min. Briefly centrifuge the PCR tube and place it on the magnetic stand. After the solution is clear (approximately 5 min), carefully transfer 20 μl supernatant to a new EP tube. Do not disturb the magnetic beads.

- ▲ If the purification products undergo Two Rounds Beads Selection: Add 105 μl eluent (10 mM Tris-HCl, pH 8.0 - 8.5) or ddH_2O for elution, vortex or gently pipette up and down to mix thoroughly and place it at room temperature for 2 min. Briefly centrifuge the PCR tube and place it on the magnetic stand. After the solution is clear (approximately 5 min), carefully transfer 100 μl supernatant to a new EP tube. Do not disturb the magnetic beads. Perform size selection according to **Two Rounds Beads Selection criteria in Table 5 (Page. 15).**

Samples can remain stable for one week at 4℃. Store at -20℃ for long-term storage. Avoid repeated freezing and thawing.

Step 3: Library Amplification

This step is for PCR amplification of the Adapter Ligation products after purification or size selection.

1. Thaw the PCR Primer Mix for MGI and the VAHTS HiFi Amplification Mix. Once thawed, mix thoroughly 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	

2. Mix gently and centrifuge briefly.
3. Place the PCR tube in the PCR instrument and perform the below reaction:

Temperature	Time	Volume
95℃	3 min	1
98℃	20 sec	For the number of cycles, refer to **Table 3** (Page. 07)
60℃	15 sec	
72℃	30 sec	
72℃	5 min	1
4℃	Hold	

4. For size selection, refer to **Appendix I: Two Rounds Beads Selection (Page.14)**. Use VAHTS DNA Clean Beads to purify the reaction products if size selection is not required:
   a. When the beads are balanced to room temperature, vortex to mix the VAHTS DNA Clean Beads.
   b. Add 45 μl VAHTS DNA Clean Beads to 50 μl Library Amplification solution. Mix thoroughly by vortexing or pipetting.
   c. Incubate for 5 min at room temperature.
   d. Briefly centrifuge the PCR tube and place it on the magnetic stand to separate the beads and liquid. After the solution is clear (approximately 5 min), carefully **remove the supernatant**.
   e. Always keep the PCR tube on the magnetic stand, add 200 μl freshly prepared 80% ethanol to rinse the beads. Incubate at room temperature for 30 sec, then carefully **remove the supernatant**.
   f. Repeat step e; rinse twice in total.
   g. Always keep the PCR tube on the magnetic stand. Open the lid to air dry the beads for 5 - 10 min until there is no ethanol residue.
   h. Remove the PCR tube from the magnetic stand for elution:
      - ▲ Add 22.5 μl eluent (10 mM Tris-HCl, pH 8.0 - 8.5) or ddH_2O (ddH_2O elution must be used if targeted capture is required later) for elution, vortex or gently pipette up and down to mix thoroughly and place it at room temperature for 2 min. Briefly centrifuge the PCR tube and place it on the magnetic stand. Once the solution is clear (approximately 5 min), carefully transfer 20 μl supernatant to a new EP tube. Do not disturb the magnetic beads.

   Samples can remain stable for one week at 4℃. Store at -20℃ for long-term storage. Avoid repeated freezing and thawing.

Step 4: Library Quality Control

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

Appendix I: Two Rounds Beads Selection

1. To meet the needs of different applications, Two Rounds Beads Selection are often required during library preparation to control the distribution range of the library insert size. Refer to **Table 4** for information on choosing when to perform selection and the advantages and disadvantages of the 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 4: Choosing the stage of the size selection

Stage of size selection	Applicable conditions	Advantages	Disadvantages	Examples of applicable samples
After adapter ligation	Input DNA distribution is suitable and adequate[a]	Reduce the loss of short Input DNA;	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]	Reduce the loss of input DNA during library preparation and increase the complexity of the library	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	Reduce the loss of input DNA during library preparation and increase the complexity of the library	Library insert size cannot be controlled	Proper fragmentation of genomic DNA

a. The effect of Two Rounds Beads Selection is affected by the state of the DNA end. The single-stranded part of the Input DNA end and the adapter non-complementary region will lead to a wider distribution of selected product length.

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 Beads Selection is made by controlling the amount of beads used to perform DNA size selection. The basic principle is: the first round of beads binds to DNA with a larger molecular weight and this kind of DNA is removed when the beads are discarded; while the second round of beads binds to DNA with a larger molecular weight in the remainder of the products and the smaller-sized DNA is removed by discarding the supernatant. Many components in the initial sample interfere with the Two Rounds Beads Selection effect. Therefore, when the stage of the size selection is different, the amount of beads used for two rounds of selection would be different. Select the most appropriate selection parameter according to **Table 5** based on the expected library insert size and the stage of the size selection.

Table 5: Library size selection

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

3. If adapters are not in complete length (e.g., Vazyme #NM35101-NM35108), please refer to the following table to choose the volume of beads according to expected insert size and selection points.

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
(Sample volume is 100 μl)	2nd-Round Y (μl)	20	20	20	20	20	20	20	18

▲ When magnetic beads are used for size selection, the larger Insert Size, the broader size distribution. However, beads cannot select DNA with insert size greater than 700 bp, two rounds of purification with magnetic beads has almost no size-selection effect. In this case, it is recommended to carry out size selection through gel extraction.

▲ 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 Adapter Ligation products purification, 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 is not pretreated by volume, the beads amount can also be adjusted in proportion to the actual volume of the sample. However, if the sample volume is too small, this will increase pipetting errors, which in turn affect the accuracy of size selection. Therefore, direct size selection of samples <50 μl is not recommended.

5. Protocol for selection (refer to **Table 5 (Page. 15)** to confirm values of **X** and **Y**)

a. When the beads are balanced to room temperature, vortex to mix the VAHTS DNA Clean Beads.

b. Add **X** μl VAHTS DNA Clean Beads to the above 100 μl solution. Mix thoroughly 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. Centrifuge the PCR tube briefly and place it on the magnetic stand to separate the beads and the solution. After the solution is clear (approximately 5 min), carefully transfer the supernatant to the new PCR tube and **discard the beads**.

e. Add **Y** μl VAHTS DNA Clean Beads to the supernatant. Mix thoroughly by vortexing or pipetting.

f. Incubate for 5 min at room temperature.

g. Centrifuge the PCR tube briefly and place it on the magnetic stand to separate the beads and the solution. After the solution is clear (approximately 5 min), carefully **remove the supernatant**.

h. Always keep the PCR tube on the magnetic stand, add 200 μl freshly prepared 80% ethanol to rinse the beads. Incubate at room temperature for 30 seconds, then carefully **remove the supernatant**.

i. Repeat step h; rinse twice in total.

j. Always keep the PCR tube in the magnetic stand. Open the lid to air dry the beads for 3 - 5 min until there is no ethanol residue.

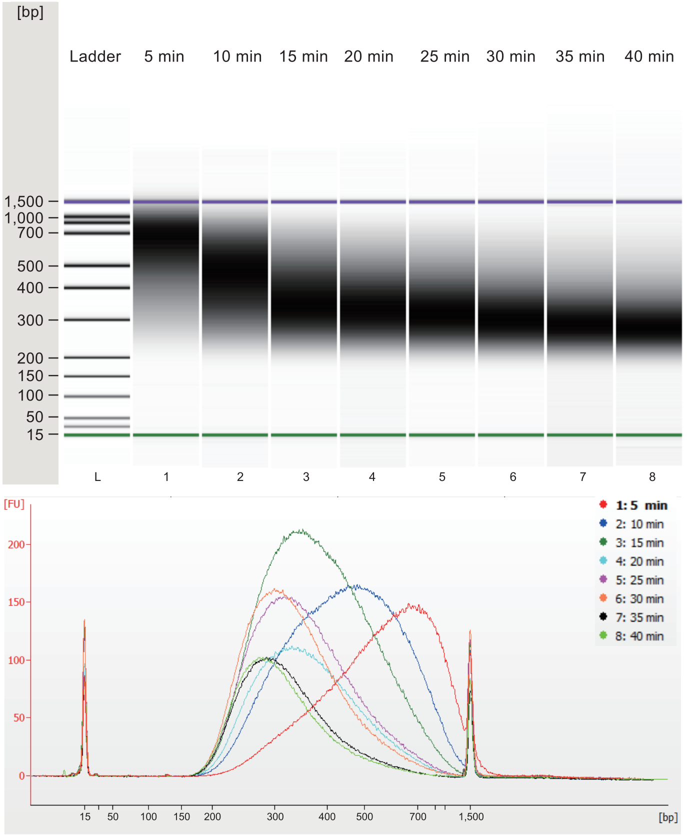
k. Remove the PCR tube from the magnetic stand for elution:

▲ Add 22.5 μl eluent (10 mM Tris-HCl, pH 8.0 - 8.5) or ddH_2O (ddH_2O elution must be used if targeted capture is required later). Vortex or gently pipette up and down to mix thoroughly and place it at room temperature for 2 min. Briefly centrifuge the PCR tube and place it on the magnetic stand. After the solution is clear (approximately 5 min), carefully transfer 20 μl supernatant to a new EP tube. Do not disturb the magnetic beads.

Appendix II: Experiments Example

Experiments example with different fragmentation time

Using 100 ng human genome DNA as a template, the kit was used to construct the library. The fragmentation conditions were 37℃ for 5/10/15/20/25/30/35/40 min, respectively. PCR amplification lasted for 4 cycles, and the final library distribution was shown as follows:



Appendix III: Solution for Fragmentation of Multiple Samples

When there are a large number of samples and the samples contain EDTA, different amounts of Neutralization Buffer must be calculated and added, which is relatively complicated during actual operation. At this point, you can dilute the sample to the same concentration using the same solvent, ensuring that multiple samples are added in the same volume and that an equal volume of Neutralization Buffer is added. As shown in the following table, the reaction solution mixture is prepared, mixed and divided into appropriate volumes in each tube. Quickly proceed to PCR to prevent great differences of fragmentation between different samples due to the long loading time.

1. For example, for each 10 μl DNA sample, 2.5 μl Neutralization Buffer must be added by means of calculation and dilution. Dilute the DNA samples according to the calculated results and arrange them sequentially in an 8-pipe or 96-well plates.
2. Thaw the FEA Buffer V2, FEA Enzyme Mix V2 and Neutralization Buffer, Mix gently and centrifuge briefly. Put them on ice before use. All of the following steps are performed on ice.

Components	Single reaction volume	96-reaction mixture volume	
Neutralization buffer	2.5 μl	250 μl	☐
FEA Buffer V2	5 μl	500 μl	■
FEA Enzyme Mix V2	10 μl	1,000 μl	■
ddH$_2$O	22.5 μl	2,250 μl	

▲ When preparing multiple reaction mixtures, it is recommended to prepare them with 1.1 times the volume of the actual number required in order to ensure there is a sufficient amount for dispensing.

▲ Fragmentation reaction mixture should be freshly prepared and used and should not be stored for a long time.

3. Mix gently and centrifuge briefly.
4. Distribute the reaction mixture into reaction tubes or 96-well plates, with 40 μl in each well.
5. Using a pipette or an automated workstation, add 10 μl the DNA sample to each reaction well as quickly as possible, Mix thoroughly and centrifuge briefly. **Then immediately proceed to PCR**.

▲ Fragmentation reaction is a time-dependent enzyme-based reaction, and the size of the fragment products depends on time. Therefore, operate quickly to minimize the difference between different samples. Mix immediately after adding and perform the follow-up reaction.