

**VAHTS® Universal Plus DNA
Library Prep Kit for Illumina V2**

ND627



Instruction for Use

Version 21.1

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01/Product Description

VAHTS Universal Plus DNA Library Prep Kit for Illumina V2 is a fragment enzyme DNA library preparation kit developed specifically for the Illumina high-performance 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 while maintaining the original high performance, and improved the reliability of SNV detection. This kit combines DNA fragmentation, end repair and dA tailing into one step. The product does not require purification, and adapter ligation, library enrichment and sorting can be performed directly. The 100 pg - 1 µg template DNA can be converted into a dedicated library for Illumina high-performance sequencing platforms. Mechanical disruption of the genome is eliminated. Library preparation is simplified and the operating time is reduced. It is applicable to PCR library construction. This kit is fully compatible with DNA from different sources and different input amounts. 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 | ND627-01 (24 rxns) | ND627-02 (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 3 for Illumina | 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°C and transport at ≤0°C.

04/Applications

This kit is suitable for preparing a dedicated library for the Illumina high-performance 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. Suitable for:

- ◇ Whole genome sequencing
- ◇ Whole exome sequencing or other target capture sequencing
- ◇ Metagenome sequencing
- ◇ Methylation sequencing (in combination with Phanta UC Super-Fidelity DNA Polymerase for Library Amplification, Vazyme #P507)

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 Illumina (Vazyme #NQ101-106);

DNA adapter:

VAHTS Dual Index UMI DNA Adapters for Illumina (Vazyme #N351/352/353/354);

#N351-N354 are adapters with an 8 bp Unique Dual Index at two ends and a 5 - 6 bp UMI

VAHTS Multiplex Oligoes Set 4 - Set 5 for Illumina (Vazyme #N321/N322);

#N321/N322 are adapters with an 8 bp Unique Dual Index at two ends, allowing the construction of up to 384 different libraries by adapter combination.

VAHTS DNA Adapters Set 1 - Set 6 for Illumina (Vazyme #N801/N802 or #N805/N806/N807/N808);

#N801/N802 are 6 bp single-Index adapters, each containing 12 kinds.

#N805/N806/N807/N808 are 8 bp-single-Index adapters, each containing 24 kinds.

Other materials: Absolute ethanol, ddH₂O; Low adsorption EP tube, PCR tube, Magnetic stand, PCR instruments, etc.

06/Notes

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 the actual situation. In order to obtain a high-quality library, it is important to read the following precautions carefully. In case of any queries during the process, please contact Vazyme at global@vazyme.com.

06-1/About 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 input DNA amounts 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 ddH₂O to dissolve DNA samples. Since FEA Enzyme Mix is sensitive to EDTA, thus EDTA concentration must be examined. For example, if the final EDTA concentration in the end repair reaction solution is greater than 0.1 mM, pre-treat DNA samples according to step **08/Experiment Process/Step 01(Page.11)**.

06-2/About DNA Adapter

- For the Illumina sequencing platform, Vazyme offers four sets of Indexed Adapters, which can be selected according to different usage and the number of Pooling samples:
N351/N352/N353/N354: up to 96 kinds of non-repeating dual-ended 8 bp Indexed Adapters with 5 - 6 bp UMI sequences, 24 kinds/each set.
#N321/N322: up to 384 kinds of dual-ended 8 bp Indexed Adapters.
#N801/N802: up to 24 kinds of single-ended 6 bp Indexed Adapters, 12 kinds/each set;
#N805/N806/N807/N808: up to 96 kinds of single-ended 8 bp Indexed Adapters, 24 kinds/each.
- VAHTS Universal Plus DNA Library Prep Kit for Illumina V2 is also compatible with a variety of non-index, single-Index and dual-Index Adapters used in conventional systems such as Illumina TruSeq.
- The quality and amount of adapters directly affects the preparation efficiency and library quality. If the adapter input is too high, this may lead to residual adapter or adapter dimer. If the adapter input is too low, this 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) \approx input DNA mass (ng)/[0.66 \times average length of input DNA (bp)]
- ▲ It is recommended to use 0.1 \times 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 \times 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/About Adapter Ligation Product Purification

- Unused adapters must be removed before library amplification (for PCR amplification library) or sequencing (for PCR-free library). The default purification condition of 0.6 \times (100 µl of products, 60 µl of beads) is suitable for most cases. To obtain libraries with larger insert size, 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.
- 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 adapter or adapter dimer, it can be further purified with beads: the first purified product volume is made up to 50 μl with ddH₂O and the second purification is done with 50 μl beads (1 \times). This significantly reduces the residue level of the adapter or adapter dimer, especially when building pCR-free libraries. It may also be necessary to reduce the amount of adapter in order to completely eliminate the adapter or adapter dimer residue.

06-4/About 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 "x", 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 \times beads used for purification is 1 \times 100 μl = 100 μl . 0.6 \times /0.2 \times size selection means the beads amount is 0.6 \times 100 μl = 60 μl in the first round and 0.2 \times 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. The higher the multiplier is, the shorter the purified DNA length is, and vice versa. For example: 1 \times magnetic beads can only be used to efficiently purify DNA longer than 250 bp, shorter DNA will be lost in large quantities during purification and 150 bp DNA can also be efficiently purified after the multiplier has been increased to 1.8 \times .
 - 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 by 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°C 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 ddH₂O.

06-5/About 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 of beads selection, or selection can also be performed using gel extraction.
2. Size selection can be carried out before DNA Damage Repair & End Preparation, after Adapter Ligation, or after Library Amplification. The size selection steps are not included in the standard experiment protocol. See [Appendix 1 \(Page.15\)](#). Two Rounds Beads selection 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 appeared at the high molecular weight position. The corresponding products are mostly non-complementary chain cross-annealing products (see [06-6/About Library Amplification/Page.07](#)). The recommended solution is to adjust the number of amplification cycles to avoid over-amplification. It is not recommended to resolve over-amplification by means of size selection.
5. The high concentration of PEG in Rapid Ligation Buffer 3 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.13](#)) 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/About Library Amplification

1. PCR Primer Mix for Illumina is designed to be used for amplification of the Illumina high-performance sequencing platform library with full length adapter. 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 unwinding, 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 cyclized and sequenced after denaturation. The existence of these products has a decisive impact on the library's quantitative methods. When library quantification is performed by using fluorescent dyes (Equalbit 1 \times 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 Illumina, Vazyme #NQ101-NQ106), the quantification results are lower than the actual values.
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 8 min at 37°C. 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 higher number of cycles; otherwise, the lower number of cycles is sufficient.
4. If the adapters are complete (e.g. Vazyme #N801/N802 or #N805/N806/N807/N808) and a sufficient amount of library is available, it may be possible to skip library amplification to obtain PCR-Free libraries.
 5. When using incomplete adapters (e.g. Vazyme #N321/N322), a minimum number of amplification cycles (at least 2) is required to complete adapter sequences.

06-7/About Library Quality Control

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

1. Library size distribution analysis:

- a. 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.
- b. Conventional Adapters suitable for Illumina platform are "Y" type adapters with single chain bifurcation area at the end. Therefore, there were single-strand bifurcation regions at both ends of the unamplified PCR-free library after Adapter Ligation. When the length distribution of library is detected, the bifurcation structure of single chain will slow down the migration rate of library, and lead to some problems such as longer detection results, wider distribution range and abnormal peak type. In order to accurately detect the size distribution of the library, a small number of PCR-free libraries should be used for moderate PCR amplification, and the amplification products should be taken for detection to reflect the size distribution of the PCR-free library.

2. Library concentration analysis:

There are two methods of library quantification: one is based on dsDNA fluorescent dyes, e.g. Qubit, PicoGreen, or Equalbit dsDNA HS Assay Kit (Vazyme #EQ111); the other is qPCR-based quantification, such as VAHTS Library Quantification Kit (Vazyme #NQ101 - NQ106). The first one is easy to proceed, however, qPCR-based method is recommended due to the following reasons:

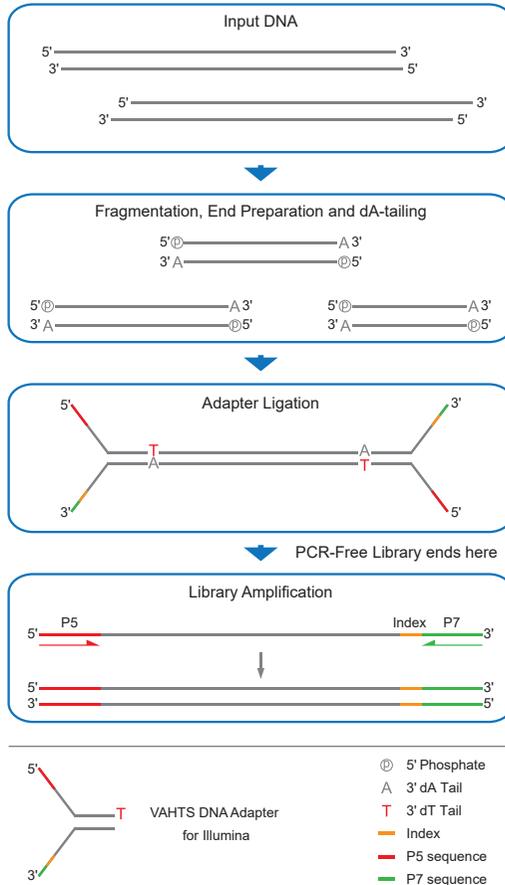
- a. When using full-length adapters, and once ligation has been completed, qPCR-based quantification kit can quantify Library concentration at different stages of the workflow. Thus the efficiency of End Preparation, purification/size selection, and Library Amplification can be assessed, to provide useful data for optimization or troubleshooting.
- b. Since PCR-free Library lacks Library Amplification, the constructed Library contain some fragments with single-end adapters or without adapters. When using the method of double-stranded DNA dye, such as Qubit and PicoGreen, these products cannot be effectively distinguished. But qPCR quantification only quantifies those molecules with double-end adapters in the correct orientation for sequencing. Therefore, PCR-Free libraries can only be quantified by qPCR-based method.
- c. Over-amplified libraries contain non-complete fragment and can't be measured by Qubit or PicoGreen methods. Measurements with qPCR-based method are not affected by library over amplification ([refer to 06-6/About Library Amplification/Page.07](#)).

06-8/Further Precautions

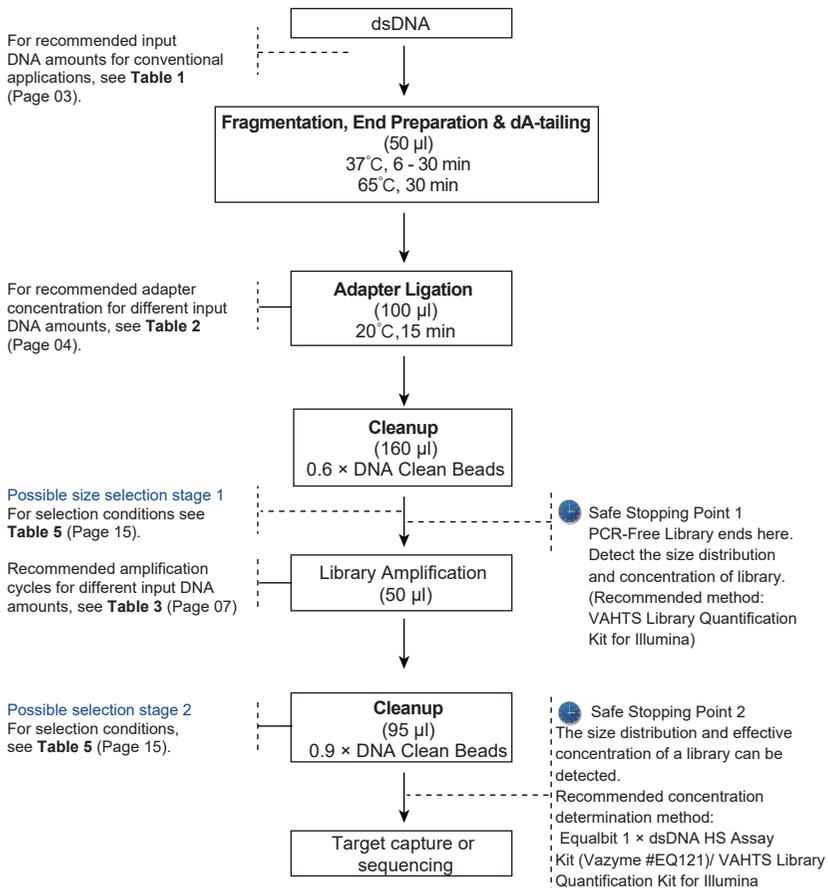
1. The size and distribution range of DNA fragments are determined by a time-dependent enzyme-based reaction, thus the fragmentation reaction should be carried out on ice.
2. Since this kit will be transported on dry ice, both Buffer and enzyme will be frozen. Thaw all the components at room temperature before use. After thawing, mix thoroughly and centrifuge briefly before putting them on ice.

- To avoid cross-contamination of samples, it is recommended to use tips with a filter and to replace the tips between samples.
- It is recommended to use a PCR instrument with heat lid function when carrying out the reaction in each step. Preheat the PCR instrument close to the reaction temperature before use.
- 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/Mechanism & Workflow



Construction Principle for VAHTS Universal Plus DNA Library Prep Kit for Illumina V2



Process Outline for Library Construction Using
VAHTS Universal Pro DNA Library Prep Kit for Illumina 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 (ddH₂O 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 ddH₂O. 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 of 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 and FEA Enzyme Mix. 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 <input type="checkbox"/> |
| FEA Buffer V2 | 5 μl <input checked="" type="checkbox"/> |
| ddH ₂ O | 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. See **Appendix 3**. Solution for Fragmentation of Multiple Samples (Page.19).

5. Add 10 µl of 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°C.
6. Place the PCR tube into the PCR instrument and perform the following program:

| Temperature | Time |
|---------------|---------------------------|
| Hot 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 | 20 - 30 min |
| 250 bp | 15 - 20 min |
| 350 bp | 10 - 15 min |
| 550 bp | 6 - 10 min |

▲ The recommended time given above 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 II Experiments Example (Page.18).

Step 2: Adapter Ligation

This step is for ligating adapter to the products in previous step.

1. Dilute the adapter 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 previous step | 50 μ l |
| Rapid Ligation Buffer V2 | 25 μ l ■ |
| Rapid DNA Ligase V2 | 5 μ l ■ |
| ddH ₂ O | 15 μ l |
| DNA Adapter X | 5 μ l |

▲ If VAHTS Multiplex Oligos Set 4 - Set 5 for Illumina (Vazyme #N321/N322) is used, the adapter should be of the DNA adapter-S for Illumina in the kit, and still be used in an amount of 5 μ l.

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°C | On |
| 20°C | 15 min |
| 4°C | Hold |

- ▲ If the input DNA amount is 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:
- When the beads are balanced to room temperature, vortex to mix the VAHTS DNA Clean Beads.
 - Add 60 μ l of VAHTS DNA Clean Beads to 100 μ l of Adapter Ligation solution. Mix thoroughly by vortexing or pipetting.
 - Incubate for 5 min at room temperature.
 - 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.
 - Always keep the PCR tube on the magnetic stand, add 200 μ l of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature, then carefully **remove the supernatant**.
 - Repeat [step e](#); rinse twice in total.
 - 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.
 - Remove the PCR tube from the magnetic stand for elution:

▲ If the purification products do not undergo two rounds beads selection: Add 22.5 μ l of eluent (10 mM Tris-HCl, pH 8.0 - 8.5) or ddH₂O 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 μ l of supernatant to a new EP tube. Do not disturb the magnetic beads.

▲ If the purification products undergo two rounds beads selection: Add 105 µl of eluent (10 mM Tris-HCl, pH 8.0 - 8.5) or ddH₂O 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 of supernatant to a new EP tube. Do not disturb the magnetic beads. Perform size selection according to the two rounds beads selection criteria in **Table 5** (Page 16).

● Samples can remain stable for one week at 4°C. Store at -20°C 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. Whether to proceed with this step depends on the amount of input DNA, whether adapters are in complete length, and downstream application. If adapters are not in complete length (e.g. Vazyme #N321/N322), this step is necessary. If adapters are in complete length, for input DNA <50 ng, library amplification is recommended. Skip this step and proceed directly to Step 4: Library Quality Control (Page.15) , if input DNA is ≥50 ng or there is no need for library amplification.

1. Thaw the PCR Primer Mix 3 for Illumina 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 3 for Illumina | 5 µl ■ |
| VAHTS HiFi Amplification Mix | 25 µl ■ |
| Total | 50 µl |

▲ If using Vazyme #N321/N322, the i5 PCR Primer (RM5XX) and i7 PCR Primer (RM7XX) should be used in an amount of 2.5 µl, respectively.

2. Mix gently and centrifuge briefly.

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

| Temperature | Time | Volume |
|-------------|--------|---|
| 95°C | 3 min | 1 |
| 98°C | 20 sec | For number of cycles, see Table 3 (Page 07) |
| 60°C | 15 sec | |
| 72°C | 30 sec | |
| 72°C | 5 min | |
| 4°C | Hold | 1 |

4. For size selection, refer to **Appendix 1: Two Rounds Beads Selection (Page.15)**. Use VAHTS DNA Clean Beads to purify the reaction products if size selection is not required:

- When the beads are balanced to room temperature, vortex to mix the VAHTS DNA Clean Beads.
- Add 45 µl of VAHTS DNA Clean Beads to 50 µl of 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 μ l of 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 μ l of eluent (10 mM Tris-HCl, pH 8.0 - 8.5) or ddH₂O (ddH₂O 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 μ l of supernatant to a new EP tube. Do not disturb the magnetic beads.
 - Samples can remain stable for one week at 4°C. Store at -20°C for long-term storage. Avoid repeated freezing and thawing.

Step 4: Library Quality Control

See [06-7/About Library Quality Control \(Page.08\)](#).

Appendix 1. 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. See **Table 4** for information on choosing when to perform selection and the advantages and disadvantages of the different stage. **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.

- 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 bind 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 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 (sample volume 100 μ l) | One round X (μ l) | 78 | 68 | 65 | 59 | 56 | 53 | 51 | 50 |
| | Two round Y (μ l) | 20 | 20 | 15 | 15 | 12 | 12 | 10 | 10 |
| After library amplification (fill sample volume up to 100 μ l) | One round X (μ l) | 78 | 70 | 63 | 55 | 50 | 46 | 45 | 44 |
| | Two round Y (μ l) | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 15 |

3. If adapters are not in complete length (e.g., Vazyme #N321/N322), 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 (Sample volume is 100 μ l) | 1st-Round X (μ l) | 100 | 90 | 75 | 65 | 60 | 55 | 53 | 50 |
| | 2nd-Round Y (μ l) | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 18 |

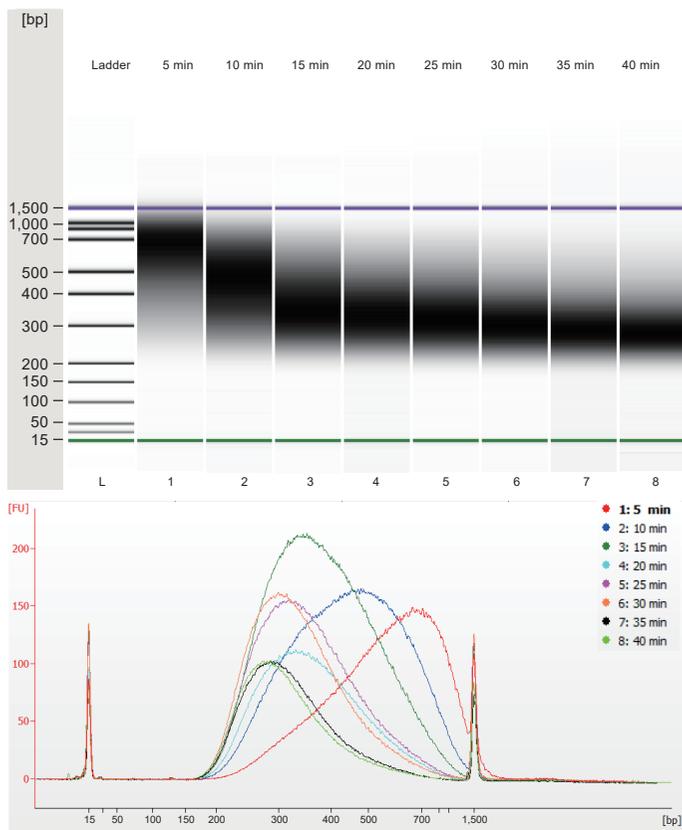
- ▲ When magnetic beads are used for size selection, the larger Insert Size, the broader size distribution. However, beads cannot select DNA with insert size is greater than 700 bp, two rounds purification with magnetic beads has almost no size-selection effect. In this case, it is recommended to carry out size selection through gel extraction.
 - ▲ The volume ratio of samples and beads is important for size selection. Please ensure the accuracy of 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 μ l. If not, the sample should be filled up to 100 μ l with ddH₂O.
 - ▲ If size selection takes place after Library Amplification, the sample volume should be 100 μ l. If not, the sample should be filled up to 100 μ l with ddH₂O.
 - ▲ 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 μ l is not recommended.
5. Protocol for selection (see **Table 5 (Page 16)** 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** μ l of VAHTS DNA Clean Beads to the above 100 μ l solution. Mix thoroughly by vortexing or pipetting.
 - ▲ If the solution is less than 100 μ l. Fill up to 100 μ l with ddH₂O.
 - 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** μ l of 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 μ l of 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 μ l of eluent (10 mM Tris-HCl, pH 8.0 - 8.5) or ddH₂O (ddH₂O 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 μ l of supernatant to a new EP tube. Do not disturb the magnetic beads.

Appendix 2. Experiments Example

Experiments example with different fragmentation time

Using 100 ng human genome DNA as template, the kit was used to construct the library. The fragmentation conditions were 37°C 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 3. 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 difference of fragmentation between different samples due to the long loading time.

1. For example, for each 10 μl of DNA sample, 2.5 μl of Neutralization Buffer must be added by means of calculation and dilution. Dilute the DNA samples according to the calculated results and arrange sequentially in 8-pipe or 96-well plate.
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 <input type="checkbox"/> |
| FEA Buffer V2 | 5 μl | 500 μl <input checked="" type="checkbox"/> |
| FEA Enzyme Mix V2 | 10 μl | 1,000 μl <input checked="" type="checkbox"/> |
| 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 of 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.



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