VAHTS Universal Plus DNA Library Prep Kit for Illumina®

Catalog# ND617

Version 9.1

Vazyme biotech co., ltd.

01/ Introduction

VAHTS Universal Plus DNA Library Prep Kit for Illumina® is specially designed for DNA library preparation for next generation sequencing (NGS) on Illumina® platforms. This kit combines DNA fragmentation, end repair and dA tailing into one step. The products can be directly used to adapter Ligation without purification, then for library enrichment and size selection. This kit is suitable for library preparation from 100 pg - 1 μg of input DNA. No mechanical fragmentation of the genome simplifies the process of library construction and shortens the operation time, suitable for the construction of PCR and PCR-Free libraries. This kit is perfectly compatible with DNA from different sources and different inputs, and the required fragment size library can be obtained by adjusting the fragmentation time according to the size of the target insert. All kit components are subjected to stringent functional quality control, ensuring the consistency and reproducibility of library preparation.

02/ Components

Components	ND617-01 (24 rxn)	ND617-02 (96 rxn)
FEA Buffer	120 μl	480 μl
FEA Enzyme Mix	240 μl	960 μl
Rapid Ligation Buffer 3	600 μl	4 × 600 μl
Rapid DNA Ligase	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 genomic DNA.

03/ Storage

All components should be stored at - 30℃ ~ - 15℃, and transported at - 20℃ ~ 0℃.

04/ Applications

Applicable to DNA library preparation for NGS on Illumina® platforms and compatible with 100 pg - 1 μg of input DNA and various kinds of DNA template including genomic DNA (from different species such as animals, plants, and microorganisms), paraffin sections DNA (FFPE DNA), etc.

It is recommended to use this kit for:

◇ Whole genome sequencing.

◇ Whole exome or targeted sequencing (using IDT xGen™ Lockdown™ Probes, Roche® NimbleGen™ SeqCap™ EZ or other hybridization capture systems).

◇ Metagenome sequencing.

◇ Methylation Sequencing (in combination with Phanta UC Super-Fidelity DNA Polymerase for Library Amplification, Vazyme #P507).

05/ Additional Materials Required

Purification Beads: VAHTS DNA Clean Beads (Vazyme #N411);

DNA quality control:

Agilent Technologies 2100 Bioanalyzer or other equivalent product;

Equalbit dsDNA HS Assay Kit (Vazyme #EQ111);

VAHTS Library Quantification Kit for Illumina (Vazyme #NQ101-106).

DNA Adapters:

VAHTS Dual Index UMI DNA Adapters for Illumina® (Vazyme #N331/332/333/334);

#N331-N334 are adapters with an 8 bp Unique Dual Index at two ends and a 10 bp UMI (Unique Molecular Identifier)

VAHTS Multiplex Oligoes Set 4/5 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 1-6 for Illumina® (Vazyme #N801/N802 or #N805/N806/N807/N808);

#N801/N802 are 6 bp single-Index adaptors, each containing 12 kinds. #N805/N806/N807/N808 are 8 bp-single-Index adaptors, each containing 24 kinds.

Other materials:

Absolute ethanol, Ultrapure sterile water (ddH2O), 0.1 × TE, Elution Buffer (10 mM Tris-HCl, pH 8.0 - pH 8.5), Low adsorption EP tubes, PCR tubes, Magnetic stand, PCR instrument.

06/ Notes

The parameters of library preparation procedures may be adjusted according to sample types, experimental designs, instruments, and operations. To obtain libraries of high quality, please read the following notes carefully. For any questions during procedures, please contact Vazyme for help at global@vazyme.com.

06-1/ Input DNA & Fragmentation

◇ The recommended input DNA range is 100 pg - 1 μg. If possible, please use high quality DNA (with A260/A280 ratio of between 1.8 and 2.0) for library preparation. The recommended input DNA amounts are listed in **Table 1**.

Table 1. Recommended Amount of Input DNA

Application	Sample Type	Recommended Amount of Input DNA
Whole Genome Sequencing	Complex gDNA	50 ng - 1μg
Targeted Sequencing	Complex gDNA	10 ng - 1μg
Whole Genome/Targeted Sequencing	FFPE DNA	≥ 50 ng
Whole Genome Sequencing	Microbial genome	1 ng - 1μg
Whole Genome Sequencing (PCR-Free)	Complex/Simple genome	≥ 50 ng (no size selection)
		≥ 200 ng (with size selection)

▲ The amount recommended above is for DNA with high quality. For DNA with low quality, however, the input amount should be increased.

◇It is recommended to use ultrapure sterile water (ddH2O) to dissolve DNA samples. FEA Enzyme Mix is sensitive to EDTA. Please confirm the concentration of EDTA in the DNA sample. If the final concentration of EDTA in the fragmentation and end repair reaction system is greater than 0.1 mM, please pretreat the DNA sample according to the instructions in **08/ Standard Protocol for Library Preparation, Step 01. Fragmentation, End Preparation & dA-tailing, Step 1.**

06-2/ Adapters

◇ For the Illumina sequencing platform, Vazyme offers four sets of Indexed Adapters, which can be selected according to different usage needs and the number of Pooling samples:

▲ Vazyme, # N331/N332/N333/N334: up to 96 kinds of non-repeating dual-ended 8 bp Indexed adapters with 10 bp UMI sequences, 24 kinds/each set.

▲ Vazyme, #N321/N322: up to 384 kinds of dual-ended 8 bp Indexed Adapters.

▲ Vazyme, #N801/N802: up to 24 kinds of single-ended 6 bp Indexed Adapters, 12 kinds/each set;

▲ Vazyme, #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® 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 affect the efficiency of library construction and the library quality. High Adapter input may lead to residual Adapter/Adapter Dimer. Low Adapter input may affect ligation efficiency and reduce library yields. Please refer to **Table 2** for the recommended adapter concentrations for different DNA inputs.

Table 2. Recommended adapter concentrations for libraries prepared from 100 pg - 1 μg input DNA

Input DNA	Adapter: Input DNA Molar Ratio	Adapter concentration from other source (Working concentration)	Vazyme Adapter Dilution Ratio
500 ng - 1 μg	10:1 - 20:1	10 μM	Undiluted
100 ng - 500 ng	20:1 - 100:1	10 μM	Undiluted
25 ng - 100 ng	50:1 - 200:1	5 μM	1:2
5 ng - 25 ng	40:1 - 200:1	1 μM	1:10
100 pg - 5 ng	60:1 - 3000:1	0.1 μM - 0.2 μM	1:30 - 1:200

▲Calculate the moles of Input DNA:

moles of Input DNA (pmol) ≈ mass of Input DNA (ng) / [0.66 × average length of Input DNA (bp)]

▲According to the concentration or dilution ratio, dilute Adapter with 0.1× TE. Make the volume of Adapter fixed (5 μl) to avoid pipetting error.

▲The quality of adapters will affect the molar ratio of Adapter and Input DNA and further affect ligation rate and library yields. Please see the adapter with high quality for library preparation. Dilute and store the Adapter solution with 0.1× TE. Minimize the number of freeze-thaw cycles.

▲Increasing adapter inputs can increase library yields, especially when the amount of Input DNA is ≤ 25 ng. When optimizing workflows to increase the efficiency of library construction, several higher adapter concentrations should be evaluated: based on the recommended adapter concentration (Table 2), try several additional concentrations in a range that is 2-10 times higher than the recommended concentration. If the adapter concentration is limited, try using more volume to increase adapter amount. For example, if Input DNA is 500 ng-1 μg while default volume of adapter is 5 μl, please increase to 10 μl to enhance 5% - 15% library output. However, it should be noted that increasing the concentration of adapter may increase the residue of adapter in the library, resulting in wasted sequencing data.

06/ Notes

06-3/ Cleanup of Adapter Ligation Products

◇ Unused Adapters should be removed before library amplification (for PCR amplification library) or sequencing (for PCR-free library). The default purification condition 0.6× (60 μl beads/100 μl products) 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. Please note this is just a rough adjust. To control the library distribution accurately, please process size selection after cleanup.

◇ If proceeding with size selection, an elution volume of 105 μl is recommended. If proceeding directly to other subsequent steps, the recommended elution volume is 22.5 μl.

◇ A second cleanup may be performed using a 1× bead to DNA ratio, if post-ligation analysis reveals unacceptable levels of adapter and/or adapter-dimer carry-over after the first cleanup. Make the volume of the purification products from the first round up to 50 μl with ddH2O, and then add 50 μl of beads for second round purification. A second cleanup may be particularly beneficial when libraries are prepared in PCR-free workflows for direct sequencing on Illumina platforms. Sometimes it may be necessary to reduce the amount of adapter to completely eliminate the residual adapter and/or adapter-dimer.

06-4/ Beads

◇ This protocol has been validated for use with VAHTS DNA Clean Beads (Vazyme, #N411).

Note: If you use beads from other vendors, the purification conditions may need to be changed.

◇General notes on beads manipulations:

▲The amount of beads is calculated by "×" (multiple), indicating the multiple of beads volume compared to sample volume. For example, if sample volume is 100 μl, 1× beads means the volume of beads is 1 × 100 μl = 100 μl; 0.6×/0.2× size selection means the first round of bead volume is 0.6 × 100 μl = 60 μl and the second round is 0.2 × 100 μl = 20 μl.

▲The volume of beads directly affects the purified DNA size of lower limit. The higher multiple, the smaller insert of lower limit, and vise-versa. For example, 1× beads can only purify DNA longer than 250 bp. The smaller fragments will be discarded during cleanup. While 1.8× beads can purify DNA of 150 bp.

▲ Equilibration to room temperature (place in room temperature for 30 min) before use is essential to achieve specified size distribution and high yield of libraries.

▲ Beads will settle gradually. Always ensure that they are fully resuspended before use by vortexing or up-and-down pipetting several times.

▲The time required for complete capture of beads varies according to the reaction vessel and magnet used. It is important not to discard or transfer any beads with the removal or transfer of supernatant. Please transfer the supernatant after the solution is completely clarified, and leave 2 μl - 3 μl of supernatant behind to avoid disturbing the beads. If the magnetic beads are accidentally drawn out with the supernatant, the yield will decrease, the effect of size selection will be poor, and even the subsequent enzymatic reaction will be affected. In this case, the magnetic beads can be mixed and placed on magnetic stand again to completely separate the beads.

▲ Always use freshly prepared 80% ethanol. Keeping tubes on magnet stand without disturbing the beads during elution.

▲It is important to remove all the ethanol before proceeding with subsequent reactions. However, over-drying of beads may make them difficult to resuspend, resulting in a dramatic loss of DNA. Normally, drying of beads for 5 min–10 min at room temperature should be sufficient. Do not heat and dry (such as oven drying at 37°C).

▲DNA should be eluted from beads with elution buffer (10 mM Tris-HCl, pH 8.0 - pH 8.5) for stable preservation. However, DNA must be eluted and stored in PCR-grade water (ultrapure sterile water) for subsequent target capture reaction, to facilitate concentrating of DNA libraries before probe hybridization.

06-5/ Size Selection

◇If the distribution range of input DNA is broad, size selection will be necessary to control the final library size. It is recommended to use a dual bead-based size selection, while gel-based size selection technique is also usable.

◇Size selection may be carried out at several time points in the overall workflow, for example:

▲ prior to End Preparation;

▲ after the cleanup of adapter ligation products;

▲ after library amplification.

◇The standard protocol of this manual (Refer to **08/ Standard Protocol for Library Preparation**) does not include size-selection. Please refer to **Appendix 1** for detailed protocols for size-selection.

◇Size selection inevitably leads to a loss of sample material. The potential advantages of size selection in a library construction workflow should be weighed against the potential loss of library complexity, especially when input DNA is limited. The position of size selection steps should be consistent. Two or more size selection steps will result in dramatic decrease in library complexity and yields.

◇Over-amplification typically results in the observation of secondary, higher molecular weight peaks in the electrophoretic profiles of amplified libraries. These higher molecular weight peaks are artifacts (Refer to **06-6/ Library Amplification**) of the analysis, and typically contain authentic library molecules of the appropriate length. To eliminate these artifacts, optimization of library amplification reaction parameters (cycle number and primer concentration), rather than post-amplification size selection, is recommended.

06/ Notes

◇Rapid Ligation Buffer 3 contains a high concentration of PEG, which, if not removed, will interfere with efficient dual bead-based size selection and gel-based size selection. Therefore, if size selection is performed after Adapter Ligation, it is important to perform at least one step of bead-based clean-up (Refer to **08/ Standard Protocol for Library Preparation, Step 02. Adapter Ligation, Step 6. Purify the Adapter Ligation products using VAHTS DNA Clean Beads**) prior to performing bead- or electrophoresis-based size selection. If size selection is performed after Library Amplification, the original clean-up step can be directly replaced by dual bead-based size selection or gel-based size selection.

06-6/ Library Amplification

◇The PCR Primer Mix 3 is suitable for the amplification of all Illumina® libraries flanked by the P5 and P7 flow cell sequences. User-supplied primer mixes may be used in combination with incomplete or custom adapters. Each primer should be used at a final concentration of 5 μM-20 μM.

◇In library amplification reactions, primers are typically depleted before dNTPs. When DNA synthesis can no longer take place due to substrate depletion, subsequent rounds of DNA denaturation and annealing result in the formation of improperly annealed, partially double-stranded, heteroduplex DNA. These species migrate slower and are observed as secondary, higher molecular weight peaks during the electrophoretic analysis of amplified libraries. However, they typically comprise library molecules of the desired length, which are individualized during denaturation prior to cluster amplification or probe hybridization. Since these heteroduplexes contain significant portions of single-stranded DNA, over-amplification leads to the under-quantification of library molecules with assays employing dsDNA-binding dyes (i.e. Euqalbit dsDNA HS Assay Kit, Vazyme, #EQ111). qPCR-based library quantification methods, such as the VAHTS Library Quantification Kit for Illumina® (Vazyme, #NQ101-NQ106), quantify DNA by denaturation and amplification, thereby providing an accurate measure of the amount of adapter-ligated molecules in a library - even if the library was over-amplified.

◇The number of amplification cycles for library amplification should be limited as much as possible. Insufficient library amplification leads to insufficient library output. Excessive library amplification can result in other unwanted artifacts such as amplification bias, PCR duplicates, chimeric library inserts and amplified mutations. **Table 3** provides recommended cycle numbers for libraries prepared from 100 pg-1 μg high-quality input DNA, to obtain approximately 100 ng or 1 μg of amplified library.

Table 3. Recommended cycle numbers for 100 pg–1 μg of input DNA

Input DNA	Number of cycles required to generate	
	100 ng	1 μg
100 pg	13 - 15	15 - 17
1 ng	9 - 11	12 - 14
10 ng	4 - 6	7 - 9
50 ng	2 - 3	4 - 6
100 ng	0 - 2	3- 4
250 ng	0	2 - 4
500 ng	0	2 - 3
1 μg	0	0 - 2

▲The above table is the recommended cycle numbers using a high-quality mouse gDNA fragmented at 37 °C for 15 min to construct a library. If the input DNA quality is low or the fragmentation time is different, please adjust cycle numbers appropriately to obtain sufficient library.

▲If size selection is proceeded after adapter ligation, please choose larger cycle numbers for Library Amplification. Otherwise, please choose the small numbers.

◇If the adapters are complete (e.g. Vazyme, #N801/N802 or #N331/N332/N333/N334) and a sufficient amount of library is available, it may be possible to skip library amplification to obtain PCR-Free libraries.

◇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/ Evaluation of Library Quality

Normally, a constructed library can be evaluated for quality by size distribution and concentration detection.

◇ Size Distribution

▲The size distribution of final libraries can be confirmed with an electrophoretic-based method. A LabChip ® GX, GXII or GX Touch (PerkinElmer), Bioanalyzer or Tapestation (Agilent Technologies), Fragment Analyzer™(Advanced Analytical) or similar instrument is recommended.

▲Please note that libraries prepared with “forked” adapters in PCR-free workflows will appear to have a longer than expected mode fragment length, and/or may display a broad or bimodal size distribution when analyzed electrophoretically. To accurately determine the size distribution of an unamplified library, an aliquot of the library may be subjected to a few cycles of amplification prior to electrophoretic analysis, to ensure that all adapter-ligated molecules are fully double-stranded. Alternatively, size information may be obtained by electrophoretic analysis of library quantification products generated with VAHTS Library Quantification Kit (Vazyme, #NQ101-NQ106).

06/ Notes

◇Quantification of Libraries

There are two methods of library quantification: one is based on dsDNA fluorescent dyes, i.e. 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:

▲When using full-length adapters, and once ligation has been completed, qPCR-based quantification kit can quantify libraries 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.

▲PCR-Free libraries contain some fragments with single-end adapters or without adapters. When using the method of double-stranded DNA dye, these fragments will be also measured. 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.

▲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.

06-8/ Other Notes

◇The size and distribution of the DNA fragmentation product is determined by a time-dependent enzymatic reaction, so the fragmentation reaction should be prepared on ice.

◇Thaw all the components at room temperature before use. Mix thoroughly by turning up and down multiple times after thawing. Centrifuge briefly and place on ice.

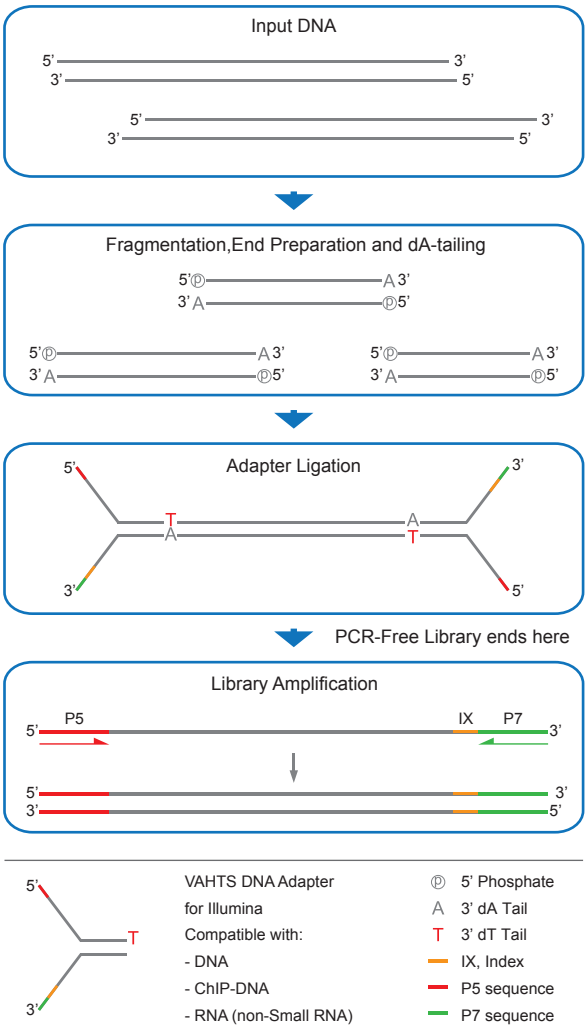
◇To avoid cross contamination, tips with filter are recommended. Change tips between samples.

◇It is recommended to use PCR instrument with heated lid. Preheat PCR instrument to reaction temperature in advance.

◇Aerosol contamination is easily to occur due to improper PCR operations, which affects experiment accuracy. Therefore, it is recommended to separate preparation area and clean-up area physically, use dedicated pipettor, and clean experimental region by 0.5% sodium hypochlorite or 10% decolorizer timely.

07/ Mechanism & Workflow

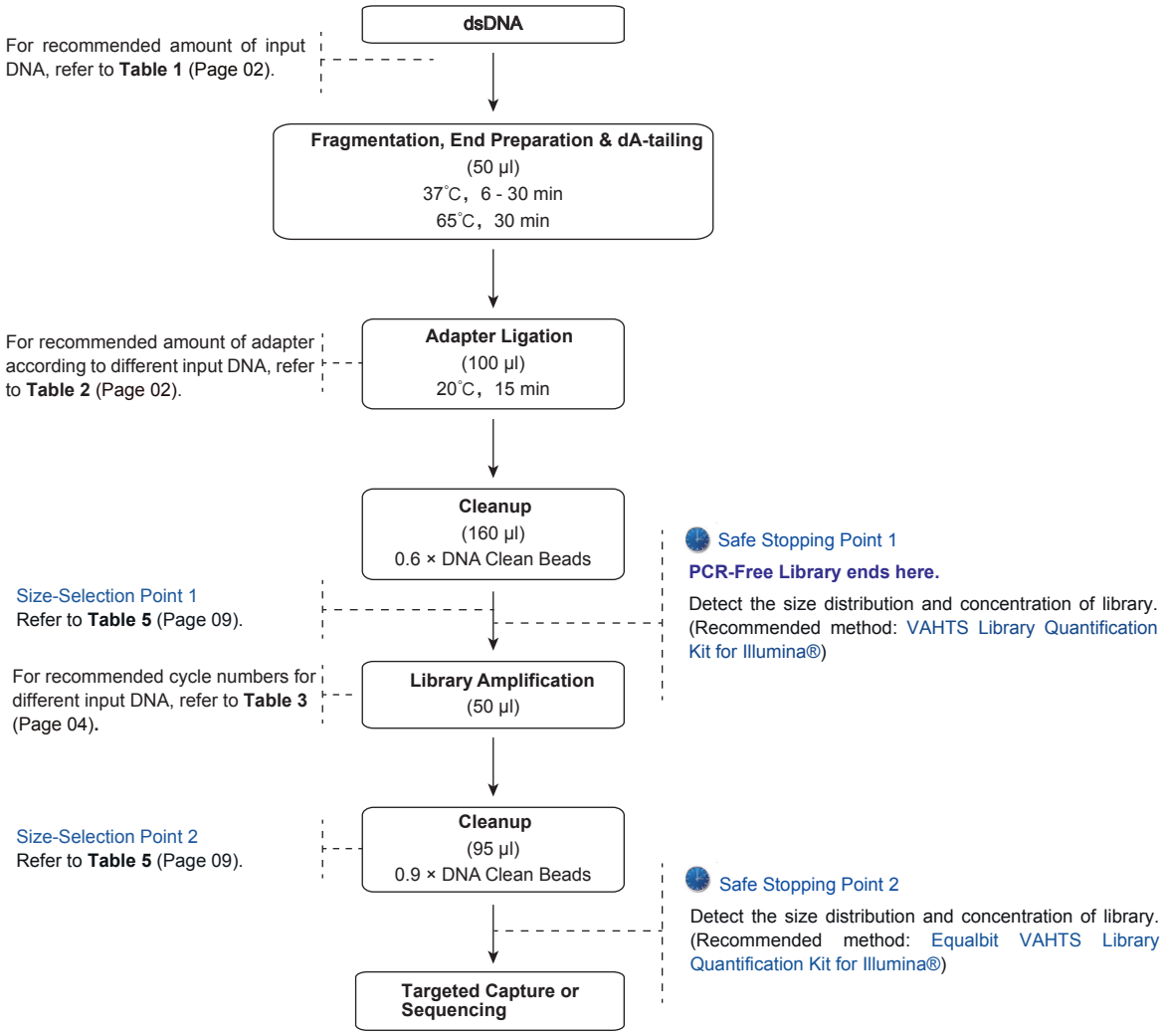
07-1/ Mechanism



Mechanism of VAHTS Universal Plus DNA Library Prep Kit for Illumina®

07/ Mechanism & Workflow

07-2/ Workflow



Workflow of VAHTS Universal Plus DNA Library Prep Kit for Illumina®

08/ Standard Protocol for Library Preparation

Step 01. Fragmentation, End Preparation & dA-tailing

This step is for Input-DNA Fragmentation, End Repair, 5' phosphorylated, and dA-tailing.

1.Before starting the experiment, please confirm which solvent the template DNA is dissolved in (sterilized-ultrapure water is recommended), whether the solvent contains EDTA. If it does not contain EDTA, proceed directly to **Step 3**; if it contains EDTA, the sample is pre-treated according to **Step 2**.

2.If solvent contains EDTA, the template DNA can be purified using 2.2 × magnetic beads and eluted by sterilized-ultrapure water. Alternatively, EDTA can be neutralized by adding corresponding volume of Neutralization Buffer according to the final concentration of EDTA in the fragmentation system.

▲Final concentration of EDTA in Fragmentation system = Concentration of EDTA in DNA solution × DNA usege volume / 50 μl; e.g., DNA is dissolved in TE buffer containing 1 mM EDTA, and 10 μl is used in one reaction. The final concentration of EDTA is 1 mM × 10 μl / 50 μl = 0.2 mM.

The reference table for the volume of Neutralization Buffer

Final concentration of EDTA in Fragmentation system	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.Take out the FEA Buffer and FEA Enzyme Mix, thaw and mix, briefly centrifuge to collect to the bottom of the tube, and place on ice until use. All the following steps are performed on ice.

08/ Standard Protocol for Library Preparation

4.Prepare the following solution in a sterile PCR tube:

Components	Volume	
Input DNA	x μl	
Neutralization Buffer	y μl	□
FEA Buffer	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 will lead to over-fragmentation.

▲For multiple samples contained EDTA, it is necessary to calculate the volumes of Neutralization Buffer added. Please refer to Appendix 3, Multi-sample Fragmentation Scheme.

5.Add 10 μl of FEA Enzyme Mix 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! ! !

▲The fragmentation reaction is a time-dependent enzymatic reaction, and the size of the fragmented products depend on the reaction time. Therefore, it is recommended to add FEA Enzyme Mix to the reaction system separately at last, and mix and perform the subsequent reaction immediately.

▲The fragmentation reaction system is sensitive to oxidation. Therefore, the lid of FEA Buffer and FEA Enzyme Mix tube should be tightened as soon as possible after the reaction system is prepared and stored at -20 °C.

6.Put the tube in a PCR instrument and run the following PCR program:

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

* Fragmentation time depends on the quality of Input-DNA and the expected-insert size:

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 above recommended time was validated using high-quality human placenta gDNA as a template. When using high-quality human placenta gDNA for library construction, within the recommended input range (100 pg - 1 μg), the different-input DNA has the same Fragmentation time, and the distribution range of fragmentation products is not much different (the distribution range is basically the same, but the main peak position may be slightly different).

If the input DNA is of poor quality or the fragmentation size is not in the expected range, it is recommended to adjust the fragmentation time up and down of 2 - 5 min. For FFPE DNA, the fragmentation time should be reduced accordingly, depending on its integrity. Two experimental examples are provided in **Appendix 2** (Page.10/11).

Step 02. Adapter Ligation

This step is to ligate the product of Fragmentation, End Preparation & dA-tailing from **Step 01** to the adapter.

1.Dilute the Adapter to the appropriate concentration according to the amount of Input DNA, please refer to **Table 2** (Page 02).

2.Take out Rapid Ligation Buffer 3 and Rapid DNA Ligase from -20°C, thaw and mix, briefly centrifuge to collect to the bottom of the tube, and place on ice for use.

3.Prepare the reaction system according to the following table:

Components	Volume	
Product from Step 01	50 μl	
Rapid Ligation Buffer 3	25 μl	■
Rapid DNA Ligase	5 μl	■
DNA Adapter X	5 μl	
ddH2O	15 μl	

▲If VAHTS Multiplex Oligos Set 4/5 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 μl.

4.Mix thoroughly by gently pipetting up and down. And collect the reaction solution to the bottom of the tube by briefly centrifuging.

5.Put the tube in a PCR instrument and run the following PCR program:

Temperature	Time
Hot lid of 105℃	On
20℃	15 min
4℃	Hold

▲For the low-amount of Input DNA, try to double the ligation time. However, extending the ligation time may result in increase of the Adapter Dimer. If necessary, the Adapter concentration should be adjusted at the same time.

08/ Standard Protocol for Library Preparation

6.Purify the Adapter Ligation products using VAHTS DNA Clean Beads:

1/ Equilibrate the VAHTS DNA Clean Beads to room temperature. Suspend the beads thoroughly by vortexing.

2/ Pipet 60 µl of beads into 100 µl of the Adapter Ligation products. Mix thoroughly by vortexing or pipetting up and down for 10 times.

3/ Incubate at room temperature for 5 min.

4/ Place the sample on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on magnetic stand and carefully discard the supernatant without disturbing the beads.

5/ Keeping the sample on magnetic stand, add 200 µl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend the beads!** Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

6/ Repeat the **Step 5/**.

7/ Keep the tube on the magnetic stand, open the tube, and air-dry the beads for 5 - 10 min (avoid over-drying) until there is no ethanol residue.

8/ Take the tube out of the magnetic stand for elution:

▲For products with no need for size-selection: Add 22.5 µl of elution buffer (10 mM Tris-HCl, pH 8.0 - pH 8.5) to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 20 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

▲For products with need for size-selection: Add 105 µl of elution buffer (10 mM Tris-HCl, pH 8.0 - pH 8.5) to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 100 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads. Proceed to size-selection according to **Table 5** (Page 09).

The products can be stable for one week at 4°C. Keep at -20°C for long-stem storage. Avoid unnecessary freeze-and-thaw cycles.

Step 03. Library Amplification

This step is to amplify the purified or size-selected adapter ligation products. 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 04**, if input DNA is ≥ 50 ng or there is no need for library amplification.

1.Thaw PCR Primer Mix 3 and VAHTS HiFi Amplification Mix, and mix thoroughly. Prepare the reaction solution in a sterile PCR tube as follows:

Components	Volume
Purified or size-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 VAHTS Multiplex Oligos set 4/5 for Illumina® (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 thoroughly by gently pipetting up and down. DO NOT Vortex! Spin down briefly.

3.Put the tube in a PCR instrument and run the following PCR program (Hot Lid Temperature: 105˚C):

Temperature	Time	Cycles
95˚C	3 min	1
98˚C 60˚C 72˚C	20 sec 15 sec 30 sec	According to **Table 3** (Page 04)
72˚C	5 min	1
4˚C	Hold	

4.For size selection, please refer to Appendix 1. If there is no need for size selection, please purify the products with VAHTS DNA Clean Beads as follows:

1/ Equilibrate the VAHTS DNA Clean Beads to room temperature. Suspend the beads thoroughly by vortexing.

2/ Pipet 45 µl (0.9x) of beads into 50 µl of the Library Amplification products. Mix thoroughly by vortexing or pipetting up and down for 10 times.

3/ Incubate at room temperature for 5 min.

4/ Place the tube on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand and carefully discard the supernatant without disturbing the beads.

5/ Keeping the sample on the magnetic stand, add 200 µl of freshly prepared 80% ethanol to rinse the beads. DO NOT re-suspend the beads! Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

6/ Repeat the **Step 5/**.

7/ Keep the tube on the magnetic stand, open the tube and air-dry the beads for 5 - 10 min.

8/ Take the Tube out of the magnetic stand for elution:

▲For products with no need for Targeted Capture: Add 22.5 µl of elution buffer (10 mM Tris-HCl, pH 8.0 - pH 8.5) to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 20 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

▲For products with need for Targeted Capture: Add 22.5 µl of ddH2O to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 20 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

Step 04. Quality Control of Library

Please refer to **06-7. Evaluation of Library Quality** for details.

Appendix 1. Dual bead-based size selection

◇ To meet the needs of different sequencing applications, dual bead-based size-selection is necessary to control the distribution of Insert Size. Generally, the size-selection is recommended to be carried out after the post-ligation cleanup. It also can be arranged prior to End Preparation or after library amplification. Ensure the point of size selection is unique, for two or more times of size selection will lead to dramatic decrease in library complexity and yields. Please refer to **Table 4** for the choice of size selection points and advantages/disadvantages of each point.

Table 4. The Choice of Size Selection Point

Size Selection Point	Applicable Conditions	Advantages	Disadvantages	Applicable Samples
After Adapter Ligation	Sufficient input DNA with proper size distribution**	Decrease the loss of short input-DNA	Cannot accurately estimate the size distribution*	Broad fragmentation of gDNA or FFPE DNA with broad size distribution
After Library Amplification	Low input DNA**	Decrease the loss of input DNA during workflow, increase library complexity	Size distribution is broad	
No Size-selection	Size distribution of input DNA is proper; low input DNA	Decrease the loss of input DNA during workflow, increase library complexity	Can't control the insert size	Proper fragmentation of gDNA

*DNA ends affect the result of size selection. Single-stranded portion at the end of the Input DNA and single-stranded non-complementary arms of “Y” adapters ligated to DNA fragments may result in a broader size distribution.

** If the amount of input DNA is ≥ 100 ng, size selection after Adapter Ligation is recommended. If the amount of input DNA is ＜100 ng, or the copies of samples is limited, size selection after Library Amplification is recommended.

◇ Dual bead-based size selection is used to select DNA fragments of expected length by controlling the amount of beads used. The mechanism of size-selection is: (1) during the first round, DNA with larger fragments bind to beads and are discarded with these beads (2) during the second round, DNA of expected length bind to bead while DNA with smaller fragments in the supernatant are discarded. Components in the initial DNA may affect the result of size selection. Therefore, according to the point of size selection, the amount of beads differs. Please refer to **Table 5** to choose the volume of beads according to expected insert size and selection points.

Table 5.Size Selection of Library

Point of size selection	Amount of Beads Added	Expected Insert Size (bp)							
		150	200	250	300	350	400	450	500
After Adapter Ligation (Sample volume is 100 µl)	1st-Round X(µl)	78	68	65	59	56	53	51	50
	2nd-Round Y(µl)	20	20	15	15	12	12	10	10
After Library Amplification (Sample volume is adjusted to 100 µl)	1st-Round X(µl)	78	70	63	55	50	46	45	44
	2nd-Round Y(µl)	20	20	20	20	20	20	20	15

◇ 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.

Point of size selection	Amount of Beads Added	Expected Insert Size(bp)							
		150	200	250	300	350	400	450	500
After Adapter Ligation (Sample volume is 100 µl)	1st-Round X(µl)	100	90	75	65	60	55	53	50
	2nd-Round Y(µl)	20	20	20	20	20	20	20	18

▲In bead-based size selection, the larger Insert Size, the broader size distribution. However, beads cannot select DNA with insert size＞700 bp , please choose electrophoretic method instead.

Appendix 1. Dual bead-based size selection

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

◇ Pretreatment of Samples (Important!)

▲For size-selection after purified Adapter Ligation or after Library Amplification, the initial sample volume should be 100 ul. If the volume is less than 100 µl, please adjust to 100 µl using ddH2O.

▲If no such pretreatment is preformed, please adjust the beads volume according to the actual sample volume. However, small volume of samples will lead to increasing inaccuracy in pipetting, resulting in inaccuracy in size selection. Therefore, it is NOT recommended to size-select samples of < 50 µl directly without pretreatment.

◇ Protocol for Size Selection [Refer to **Table 5** (Page 09) for the value of X and Y]

1. Equilibrate the VAHTS DNA Clean Beads to room temperature. Suspend the beads thoroughly by vortexing.
2. Add X µl of beads into 100µl of the DNA products. Mix thoroughly by vortexing or pipetting up and down for 10 times.
3. Incubate at room temperature for 5 min.
4. Place the sample on the magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand and carefully transfer the supernatant to a new Nuclease-free PCR tube without disturbing the beads.
5. Add Y µl of beads into the supernatant from Step 4. Mix thoroughly by vortexing or pipetting up and down for 10 times.
6. Incubate at room temperature for 5 min.
7. Place the sample on the magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic separation rack and carefully discard the supernatant without disturbing the bead.
8. Keep the tube on the magnetic stand, add 200 µl of freshly prepared 80% ethanol to the beads. **DO NOT re-suspend the beads!** Incubate at room temperature for 30 sec and carefully discard the supernatant without disturbing the beads.
9. Repeat the **Step 8**.
10. Keep the tube on the magnetic stand, open the tube and air-dry the beads for 5 - 10 min.
11. Take the Tube out of the magnetic stand for elution.

▲For products with no need for Targeted Capture: Add 22.5 µl of elution buffer (10 mM Tris-HCl, pH 8.0-pH 8.5) to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 20 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads

▲For products with need for Targeted Capture: Add 22.5 µl of ddH2O to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 20 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

Appendix 2. Experimental example

1.FFPE DNA Library Preparation.

FFPE DNA samples of different quality levels (as shown in Figure A) were as an initial template for library construction using this kit, according to the corresponding reaction conditions in the following table. The peaks of final library were shown in **Figure B**, respectively.

Sample	Type	Quality level	Input DNA	Fragmentation time	Cycles
Sample 1	Sample 1	A	100 ng	15 min	3
Sample 2	Sample 2	B	100 ng	10 min	6
Sample 3	Sample 3	C1	100 ng	8 min	6
Sample 4	Sample 4	C2	100 ng	8 min	6
Sample 5	Sample 5	D	100 ng	6 min	7

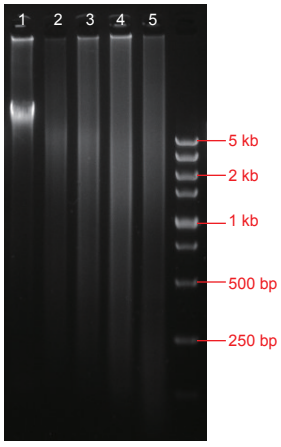


Figure A. Electrophoresis analysis of FFPE samples.

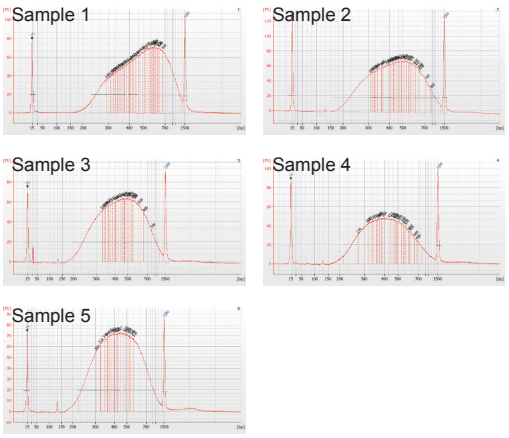
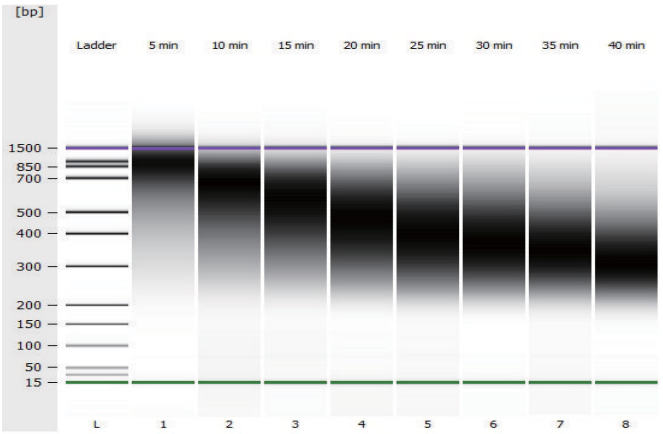


Figure B. The peaks distribution of final library on 2100 Bioanalyzer.

Appendix 2. Experimental example

2.Different fragmentation time for Library Preparation.

500 ng of human placental genome gDNA was as an initial template for library construction using this kit. The fragmentation conditions were at 37°C for 5/10/15/20/25/30/35/40 min, PCR amplification for 2 cycles, and the distribution of final libraries was shown as below.



Appendix 3. Protocol for Multi-sample fragmentation

For multiple samples contained EDTA, it is complicated but necessary to calculate the volume of the Neutralization Buffer. At this point, dilute the sample into the same concentration with the same solvent, to ensure that the volume of Neutralization Buffer is the same between samples, as well as the volume of samples. Taking the following table as an example, it is recommended to prepare a mixture of reaction reagents in a suitable size tube first, and then dispense into each PCR tube. Use a multi-channel pipettor or automated workstation to load DNA samples in a short time as possible, avoiding different fragment size between samples due to excessive loading time. Then immediately put the final mixture in a PCR instrument to run the reaction program.

1.E.g., after calculation and dilution, it is required to add 10 µl of DNA sample and 2.5 µl of Neutralization Buffer to a single reaction. The DNA samples are diluted according to the calculated results and arranged in 8-tube or 96-well plates.

2.Take out FEA Buffer, FEA Enzyme Mix, Neutralization buffer from -30°C ~ -15°C, thaw and mix, briefly centrifuge to collect to the bottom of the tube, and place on ice for use. **Note:** all of the following steps are performed on ice.

Components	Single reaction volume	96 reactions volume	
Neutralization Buffer	2.5 µl	250 µl	☐
FEA Buffer	5 µl	500 µl	■
FEA Enzyme Mix	10 µl	1000 µl	■
ddH_2O	22.5 µl	2250 µl	

▲For multiple samples, it is recommended to prepare a few more reactions than the actual number to compensate for the loss.

▲The fragmentation reaction mixture should be freshly prepared and should not be stored for a long time.

3.Mix thoroughly by gently pipetting up and down. And collect the reaction solution to the bottom of the tube by briefly centrifuging.

4.The reaction mixture is dispensed into a reaction tube or a 96-well plate at 40 µl per well.

5.Use a multi-channel pipettor or automated workstation to load DNA samples in a short time as possible, mix thoroughly by pipetting up and down for several times. **Then immediately put the final mixture in a PCR instrument to run the reaction program.**

▲The fragmentation reaction is a time-dependent enzymatic reaction, and the size of the fragmentation product depends on the reaction time. Therefore, load the multiple samples as soon as possible, then immediately mix thoroughly and run the subsequent reaction program.