VAHTS Universal DNA Library Prep Kit for Illumina V4

ND610

Instruction for Use

Version 22.1

Vazyme

Contents

01/Product Description 02
02/Components 02
03/Storage 02
04/Applications 02
05/Self-prepared Materials 03
06/Notes 03
  06-1/Input DNA & Fragmentation 03
  06-2/Adapters 04
  06-3/Adapter Ligation Product Purification 05
  06-4/Magnetic Beads 05
  06-5/Size Selection 06
  06-6/Library Amplification 07
  06-7/Library Quality Control 08
  06-8/Other Notes 10
07/Workflow 11
08/Experiment Process 12
  08-1/End Preparation 12
  08-2/Adapter Ligation 12
  08-3/Library Amplification 14
  08-4/Library Quality Control 15
Appendix I: Two Rounds Beads Selection 15
Appendix II: cfDNA Library Preparation 19
Appendix III: FFPE DNA Library Preparation 20
Appendix IV: Target Capture Library Preparation 22

01/Product Description

VAHTS Universal DNA Library Prep Kit for Illumina V4 is a library preparation kit specifically optimized for the Illumina high-throughput sequencing platform. It can convert 100 pg - 1 μg Input DNA into a specialized Illumina library. As an upgraded version, the kit features an increased conversion rate of low-quality template library and a lower library duplication rate with optimized end-repair module, ligation module and library amplification module. The kit is widely applicable to PCR or PCR-free library construction from multiple sample types, and compatible with the targeted capture process. 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

	Component	ND610-01 (24 rxns)	ND610-02 (96 rxns)	ND610-03 (24 rxns)	ND610-04 (96 rxns)
■ (blue)	End Prep Buffer 2	168 μl	672 μl	168 μl	672 μl
■ (orange)	End Prep Enzyme 2	72 μl	288 μl	72 μl	288 μl
■ (red)	Rapid Ligation Buffer 5	720 μl	4 × 720 μl	720 μl	4 × 720 μl
□	Rapid DNA Ligase 2	240 μl	4 × 240 μl	240 μl	4 × 240 μl
■ (green)	VAHTS HiFi Amplification Mix 2	600 μl	4 × 600 μl	/	/
■ (purple)	PCR Primer Mix 3 for Illumina	120 μl	480 μl	/	/
■ (yellow)	Control DNA (264 bp, 50 ng/μl)	10 μl	10 μl	/	/

03/Storage

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

04/Applications

The kit is suitable for preparing a dedicated library for sequencing on the Illumina high-throughput sequencing platform. It is compatible with multiple sample types: Genomic DNA, cell-free DNA (cfDNA, ctDNA), Formalin-Fixed and Paraffin-Embedded DNA (FFPE DNA), Chromatin immuno-precipitation DNA (ChIP DNA) and Amplicons, etc. For samples with limited complexity such as Small Genome, cell-free DNA (cfDNA, ctDNA), ChIP DNA, and Amplicons, the amount of Input DNA can be as low as 100 pg.

It is recommend to use this kit for:

◇Whole-genome sequencing

◇Whole-exome sequencing or other targeted capture sequencing (compatible with Roche NimbleGen SeqCap EZ, Agilent SureSelect, Illumina TruSeq, IDT xGen Lockdown Probes, VAHTS Target Capture Core Exome Panel (Vazyme #NC001) or other hybrid capture systems)

◇Amplicons sequencing

◇ChIP sequencing

◇Metagenome sequencing

◇Methylation sequencing (in combination with Phanta UC Super-Fidelity DNA Polymerase for Library Amplification, Vazyme #P507; EpiArt DNA Methylation Bisulfite Kit, Vazyme #EM101)

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05/Self-prepared Materials

Magnetic Beads: VAHTS DNA Clean Beads (Vazyme #N411);
DNA Quality Control: Agilent Technologies 2100 Bioanalyzer or other equivalent instruments;
DNA Adapters:
VAHTS DNA Adapters Set 1 - Set 6 for Illumina (Vazyme #N801/N802/N805/N806/N807/N808);
VAHTS Multiplex Oligos Set 4/Set 5 for Illumina (Vazyme #N321/N322);
VAHTS Dual UMI UDI Adapters Set 1 - Set 4 for Illumina (Vazyme #N351/N352/N353/ N354);
Other materials:absolute ethanol, ddH$_2$O, 0.1 × TE, eluent buffer (10 mM Tris-HCl, pH 8.0 - pH 8.5), low-adsorption EP tube, PCR tube, magnetic stand, PCR instrument, etc.

06/Notes

For research use only. Not for use in diagnostic procedures.
Due to multiple factors such as sample, protocol, equipment and operation, it may be necessary to adjust the parameters of library preparation procedures 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:
info.biotech@vazyme.com

06-1/Input DNA & Fragmentation

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

Table 1. Recommended Input DNA amount for conventional applications

Application	Sample type	Recommended amount of Input DNA
Whole-genome sequencing	Complex gDNA	50 ng - 1 μg
Targeted capture sequencing	Complex gDNA	10 ng - 1 μg
Whole-genome/targeted capture sequencing	FFPE DNA	≥50 ng
Whole-genome/targeted capture sequencing	cfDNA/ctDNA	≥100 pg
Whole-genome sequencing	Microbial genome	1 ng - 1 μg
Whole-genome sequencing (PCR-free library)	Complex/Small Genome	≥50 ng (no size selection) ≥200 ng (with size selection)
ChIP sequencing	ChIP DNA	≥100 pg
Targeted sequencing	Amplicon	≥100 pg

▲The above table presents the recommended amount of Input DNA for high-quality DNA samples. The amount should be increased as appropriate if the quality of Input DNA is low.

◇ Input DNA refers specifically to DNA used in the End Preparation step. If the DNA sample has been purified or subjected to size selection following Fragmentation, its pre-fragmentation amount cannot be directly used as the amount of Input DNA, and its concentration should be redetermined. Otherwise, the library yield may be low due to insufficient amplification cycles.

◇ If library size selection is performed subsequently, the recommended elution volume is 105 μl; otherwise, the elution volume is recommended to be 22.5 μl.

◇ If high-concentration metal ion chelators or other salts are introduced during Input DNA preparation, the efficiency of the End Preparation step may be affected. When Fragmentation is performed using mechanical methods, and library preparation is performed without product purification or size selection, DNA should be diluted in 0.1 × TE instead of ddH_2O for Fragmentation. When Fragmentation is performed using enzyme digestion, and library preparation is performed without product purification or size selection, ensure that no excess metal ion chelators are introduced in stop buffer. If the above conditions are not met, Fragmentation products should be purified or subjected to size selection, and then dissolved in 0.1 × TE or ddH_2O(≤50 μl) before library preparation.

06-2/Adapters

◇ Vazyme offers two sets of indexed Adapters for the Illumina sequencing platform, which can be selected according to the application scenario and the number of pooling samples. For the required amounts of different types of Adapters, refer to the respective Instructions for Use or, if it is not available, refer to **Table 2**.

◇ The quality and amount of Adapters used directly affect the preparation efficiency and library quality. The recommended molar ratio of Adapter: Input DNA ranges from 10:1 to 200:1. Too high Input of Adapters may lead to residual Adapters or Adapter dimer; insufficient Input may affect the ligation efficiency and reduce library yield. **Table 2** lists the recommended Adapter usage for different amount of Input DNA.

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

Input DNA	Adapter: Input DNA Molar ratio	Adapters from other Sources Working Concentration	Vazyme Adapter Pre-dilution Factor
1 μg	10:1	10 μM	Undiluted
100 ng	30:1	5 - 10 μM	1:2 or undiluted
10 ng	100:1	2 μM	1:5
1 ng	200:1	0.5 μM	1:30
100 pg	300:1	0.1 μM	1:200

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

▲It is recommended to pre-dilute the Adapter with 0.1 × TE according to the above table or using the dilution factor of the Vazyme Adapter. It ensures that the Adapters are used at a fixed volume (5 μl) during library preparation, thus avoiding pipetting error.

▲The Adapter quality directly affects the molar ratio of Adapter to Input DNA, which in turn affects the ligation efficiency and library yield. High-quality Adapters should be used; 0.1 × TE should be used to dilute and store the adapter solution. Repeated freeze-thaw cycles should be avoided.

▲ Increasing the amount of Adapters used can improve library yields to some extent, especially when the Input DNA is ≤25 ng. When you need to optimize the preparation efficiency, you may increase the Adapter Input (by 2 - 10 folds, preferably) under the recommended conditions in the table above. If the use amount is limited by the concentration of Adapters, volume could be increased. For example, with a default Adapter volume of 5 μl, when the Input DNA is 500 ng - 1 μg, the use amount of Vazyme Adapter can be increased to 10 μl to increase the library yield by 5% - 15%. However, it should be noted that increasing the Adapter concentration may increase the residual Adapters in the library, resulting in sequencing data wasting.

06-3/Adapter Ligation Product Purification

◇ After Adapter Ligation, the ramaining Adapters must be removed before Library Amplification (PCR library) or direct sequencing (PCR-free library). The default purification condition of 0.8 × (110 μl products and 88 μl magnetic beads) is applicable to most cases. To obtain libraries with larger insert size, you can reduce the amount of magnetic beads to reduce the content of small DNA fragments. However, this adjustment can only roughly change the position of the main peak of the library. If accurate control of the library size distribution is required, size selection can be performed after this purification process.

◇ If library size selection is being performed later, the recommended elution volume is 105 μl; otherwise, the elution volume is recommended to be 22.5 μl.

◇ If the data shows the purification products are heavily contaminated with unwanted Adapters or Adapter dimers, they can be re-purified using magnetic beads: Make up the volume of the initial purified product to 50 μl using ddH_2O, and add 50 μl magnetic beads (1 ×) for the second purification. This can significantly reduce the residual Adapters or Adapter dimers, especially for PCR-free library. Sometimes, the amount of Adapters used also needs to be reduced to completely eliminate the residual Adapters or Adapter dimers.

06-4/Magnetic Beads

◇ VAHTS DNA Clean Beads (Vazyme #N411) is recommended for purification.

▲Notes: Purification conditions may need to be changed if magnetic beads from other sources are used!

◇ General precautions for magnetic beads usage:

▲ The amount of magnetic beads used is indicated by the usual multiplier "×", expressed as a multiplier of the original sample volume. For example, if the original volume of the sample is 100 μl, the volume of magnetic beads used for 1 × purification is 1 × 100 μl=100 μl. For 0.6 ×/0.2 × size selection, the amount of magnetic beads used in the first round is 0.6 × 100 μl=60 μl, and 0.2 × 100 μl=20 μl in the second round.

▲ The amount of magnetic beads used directly affects the lower limit of the length of DNA that can be purified. The higher the multiplier, the shorter the purified DNA length is, and vice versa. For example, 1 × beads can only efficiently purify DNA longer than 250 bp, and shorter DNA will be lost in large quantities during purification; with the multiplier increased to 1.8 ×, DNA at 150 bp can also be efficiently purified.

- ▲ The magnetic beads should be brought to room temperature (30 min at room temperature) before use; otherwise, decreased yield and poor size selection effect may occur.
- ▲ The magnetic beads should be mixed well by shaking thoroughly or pipetting up and down before each use.
- ▲ After the sample and magnetic beads are thoroughly mixed, place the mixture on the magnetic stand for separation. When the solution becomes completely clear, pipette the supernatant, 2 - 3 μl supernatant should be left. Avoid disturbing the magnetic beads during pipetting; otherwise, 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 the magnetic stand again for separation. Given the varying performance of the magnetic stand, a longer separation time may be needed to separate the magnetic beads from the liquid completely.
- ▲ Magnetic bead should be rinsed using 80% ethanol that is freshly prepared and brought to room temperature. During rinsing, always keep the EP tube on the magnetic stand without disturbing the magnetic beads.
- ▲ Dry the magnetic beads at room temperature before elution. Insufficient drying may lead to absolute ethanol residue which will affect subsequent reactions. Excessive drying may cause cracking on the surface of beads and thereby reduce the purification yield. To get higher purification yield, incubation time may be doubled as appropriate. In general, the magnetic beads can be fully dried through air-drying at room temperature for 5 - 10 min. Do not dry by heating (e.g., drying in a 37℃ oven).
- ▲ It is generally recommended to use an elution buffer (10 mM Tris-HCl, pH 8.0 - pH 8.5) for product elution, which is more conducive to the stable preservation of the product. However, if targeted capture of the library is required later, product elution should be performed with ddH_2O to facilitate drying and concentration of the library before capture and to prevent any impact on subsequent capture reactions.
- ▲ The eluent can be stable at 4℃ for one week. Store at -20℃ for long-term preservation. Repeated freeze-thaw cycles should be avoided.

06-5/Size Selection

- ◇ If the Input DNA distribution range is wide, size selection is usually required during library preparation to control the final library size distribution. Two Rounds Beads Selection using magnetic beads are recommended. Size selection can also be performed by gel-based size selection technique.
- ◇ Size selection can be carried out before End Preparation, after Adapter Ligation, or after Library Amplification. No size selection step is included in the experiment procedure. If such step is required, refer to Appendix I: Two Rounds Beads Selection.

◇ The amount of DNA loss involved in size selection is about 60% - 95%. Sometimes it is necessary to choose between the library size distribution (with size selection) and the library complexity (no size selection), especially when Input DNA is limited. Two or more size selections can lead to a significant decrease in library complexity and yield.

◇ Over-amplification typically results in trailing band or tail peak appeared at the high molecular weight position. The corresponding products are mostly non-complementary strand cross-annealing products (refer to 06-6/Library Amplification). The recommended solution is to adjust the number of amplification cycles to avoid over amplification. It is not recommended to remove trailing or tailing peaks by size selection.

◇ The high-concentration PEG contained in Rapid Ligation Buffer 5 has a significant impact on the Two Rounds Beads Selection and gel extraction. Therefore, if size selection is performed after Adapter Ligation, the ligation product purification steps (refer to 08-2/Adapter Ligation/6. Purify the reaction products using VAHTS DNA Clean Beads) cannot be omitted, and the purified product should be eluted into a suitable volume of elution buffer, followed by Two Rounds Beads Selection or gel-based size selection technique. If size selection must be performed after Adapter Ligation, the size selection conditions need to be adjusted separately. If size selection is performed before End Preparation or after Library Amplification, the initial purification step can be directly replaced by Two Rounds Beads Selection or gel-based size selection technique.

06-6/Library Amplification

◇ PCR Primer Mix 3 is suitable for amplifying libraries containing complete Adapters for the Illumina high-throughput sequencing platform. Amplification primers need to be replaced for incomplete-length Adapters or libraries on other platforms, and the recommended final amplification concentration for each primer is 5 - 20 μM.

◇ During the late stage of PCR, primers are usually depleted before dNTP. In this case, excessive cycles can cause non-specific annealing of the amplified product after unwinding, resulting in non-complementary strand cross-annealing products. These products migrate slowly in electrophoresis-based assays, and diffuse in the high molecular weight region. These products are prepared from single-stranded libraries with the correct length, and can bind to the Flow Cell normally and be sequenced after denaturation. Therefore, their presence or absence has no significant impact on sequencing. However, the presence of such products has a decisive impact on how libraries are quantified. As the product is not a complete double-stranded structure, when a fluorochrome-based assay for double-stranded DNA (dsDNA) quantitation (e.g., Equalbit 1 × dsDNA HS Assay Kit, Vazyme #EQ121) is used for library quantification, the results will be lower than the actual values. However, qPCR-based library quantification systems (e.g., VAHTS Library Quantification Kit for Illumina, Vazyme #NQ101 - NQ106)

include a denaturation process, so that such over-amplified libraries can still be accurately quantified.

◇ The Library Amplification step requires strict control of the number of amplification cycles. Insufficient number of cycles will lead to insufficient library yield; excessive number of cycles will lead to over amplification, increased amplification bias, increased duplicates, increased chimeric products, cumulative amplification mutation, and other adverse consequences. **Table 3** lists the recommended number of amplification cycles to obtain approximately 1 μg of amplified library when using 100 pg - 1 μg of high-quality Input DNA.

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

Input DNA (Into End Preparation)	Number of cycles required to generate (1 μg)
1 μg	3 - 5
100 ng	6 - 8
10 ng	10 - 13
1 ng	13 - 15
100 pg	17 - 19

▲The above table shows the cycle number measured when using approximately 200 bp of high-quality Input DNA. When the DNA quality is poor and the library fragment length is long, it is necessary to increase the number of cycles as appropriate to obtain a sufficient library.

▲If size selection has been performed during library preparation, Library Amplification should be performed with the higher number of cycles; otherwise, the lower number of cycles is feasible.

◇ If complete Adapters (e.g., Vazyme #N801/N802 or N805/N806/N807/N808) are used during Adapter Ligation, and the library yield meets the application requirement, the Library Amplification step may be exempted to obtain PCR-free libraries.

◇ When incomplete Adapters (e.g., Vazyme #N321/N322 or N351/N352/N353/N354) are used during Adapter Ligation, a minimum number of amplification cycles (at least 2) is required to complete Adapter sequences.

06-7/Library Quality Control

Generally, the quality of the prepared libraries is evaluated through size distribution and concentration analysis.

◇ Library size distribution analysis:

▲ The size distribution of libraries can be determined by devices based on the electrophoretic separation principle such as LabChip GX, GXII, GX Touch (PerkinElmer); Bioanalyzer, Tapestation (Agilent Technologies); Fragment Analyzer (Advanced Analytical). The size distribution of the products in each step of library preparation using VAHTS Universal DNA Library Prep Kit for Illumina V4 is determined as shown in the figure below.

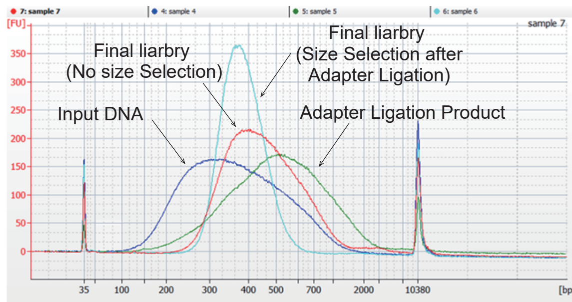

Fig 1. Size distribution of the products in each step of library preparation using VAHTS Universal DNA Library Prep Kit for Illumina V4

◇ Library concentration analysis:

There are two common methods for determining library concentration: one uses fluorescent dsDNA-binding dyes, such as Qubit, Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121) and PicoGreen, etc. The other is based on qPCR-based absolute quantification, such as VAHTS Library Quantification Kit for Illumina (Vazyme #NQ101-NQ106). Although the former is simple and easy to operate, the qPCR-based absolute quantification method is recommended for the following reasons:

▲ When complete Adapters are used and Adapter Ligation is completed, the qPCR-based absolute quantification method can quantify library concentration of products at any step. Thus this method enables monitoring the efficiency of Adapter Ligation, magnetic bead-based purification/size selection, and Library Amplification, to provide useful data for system optimization and analyzing the causes of abnormalities in library preparation.

▲ Since PCR-free libraries are not subject to Library Amplification, the prepared library contains a certain proportion of products with single-end Adapters or without Adapters. For methods using fluorescent dsDNA-binding dyes for concentration determination, such as Qubit and PicoGreen, these products cannot be effectively distinguished. However, qPCR absolute quantification is based on the principle of PCR amplification, and only quantifies libraries with an adapter at both ends in the sample (i.e., sequenceable libraries), which can rule out the interference of unsequenceable libraries with single-end Adapters or without Adapters. Thus, the concentration of PCR-free libraries can only be determined through the qPCR-based absolute quantification method.

▲ Methods using fluorescent dsDNA-binding dyes, such as Qubit and PicoGreen, are not applicable to overamplified libraries since they contain a large number of incomplete double-stranded structures. However, over-amplification does not impact qPCR-based absolute quantification (refer to 06-6/Library Amplification).

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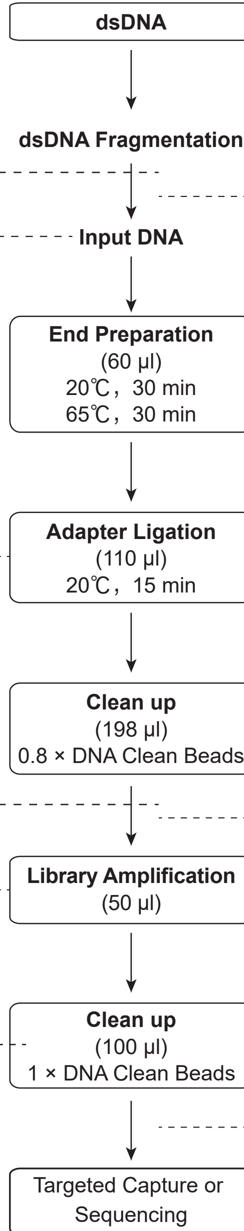
06-8/Other Notes

- ◇ Thaw all components at room temperature before use. After thawing, fully mix the components by turning upside down several times, centrifuge briefly, and place them on the ice for later use.
- ◇ It is recommended to thoroughly mix the reaction solution by pipetting up and down at each step (vigorous upside-down shaking may decrease library yields).
- ◇ To avoid cross-contamination of samples, it is recommended to use filter tips and change the pipette tip after each sample.
- ◇ It is recommended to use a PCR instrument with a heating lid. Pre-heat the PCR instrument to a temperature close to the reaction temperature before use.
- ◇ Improper operations make PCR products prone to occur aerosol contamination and thereby affect the accuracy of experiment results. Thus, it is recommended to physically separate the PCR reaction system preparation area from the PCR product purification and experiment area, use dedicated equipment (e.g., pipettes), and regularly clean each experiment area (wipe with 0.5% sodium hypochlorite or 10% bleach) to ensure clean experiment environments.

07/Workflow



Size selection time point 1
Refer to **Table 5** (Page 17) for size selection conditions

The recommended amounts of Input DNA for common applications are provided in **Table 1** (Page 03).
The time point for the size selection step in the overall workflow should be determined based on the application scenario and the DNA Input amount. Refer to **Table 4** (Page 16).

The recommended Adapter concentration for different DNA Input amounts is presented in **Table 2** (Page 04).

Size selection time point 2
Refer to **Table 5** (Page 17) for size selection conditions

The recommended number of amplification cycles for different DNA Input amounts is presented in **Table 3** (Page 08).

Size selection time point 3
Refer to **Table 5** (Page 17) for size selection conditions.

Safe Stopping Point 1
Input DNA size distribution and concentration determination may be performed.
(Recommended concentration determination method: Qubit)

Safe Stopping Point 2
PCR-free library preparation ends here
Library size distribution and effective concentration determination may be performed.
(Recommended concentration determination method: VAHTS Library Quantification Kit for Illumina)

Safe Stopping Point 3
Library size distribution and effective concentration determination may be performed.
(Recommended concentration determination method: Qubit/VAHTS Library Quantification Kit for Illumina)

Fig 2. Workflow of VAHTS Universal DNA Library Prep Kit for Illumina V4

08/Experiment Process

08-1/End Preparation

This step is for repairing the Input DNA end, and adding dA tail at 3' end.

1. Thaw End Prep Buffer 2, fully mix it with End Prep Enzyme 2 by turning upside down, and prepare the following reaction in a sterile PCR tube (**on ice**):

Component	Volume	
Input DNA	x μl	
End Prep Buffer 2	7 μl	■
End Prep Enzyme 2	3 μl	■
ddH_2O	To 60 μl	

2. Mix well by gentle pipetting up and down (**do not vortex**) and centrifuge briefly to collect the reaction solution to the bottom of the tube.

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

Temperature	Time
Heating lid at 105 ℃	On
20℃	30 min
65℃	30 min
4℃	Hold

08-2/Adapter Ligation

This step is for ligating Adapters to the ends of the End Preparation products.

1. Dilute the Adapter to the appropriate concentration based on the amount of Input DNA as specified in **Table 2** (Page 04).

2. Thaw the Rapid Ligation Buffer 5 and mix thoroughly by turning upside down several times before placing it on ice.

3. Prepare the following reaction solution in a PCR tube at the End Preparation step (**on ice**):

Component	Volume	
End Preparation Product	60 μl	
Rapid Ligation Buffer 5	30 μl	■
Rapid DNA Ligase 2	10 μl	□
DNA Adapter X	5 μl	
ddH_2O	5 μl	
Total	110 μl	

▲The pre-mixture of Rapid Ligation Buffer 5 and Rapid DNA Ligase 2 can be stored at 4℃ for up to 24 h.

▲If Vazyme #N321/N322 is used, the provided DNA Adapter-S for Illumina should be used, and the amount used remains 5 μl.

4. Mix well by gently pipetting up and down (**do not vortex**) and centrifuge briefly to collect the reaction solution to the bottom of the tube.

5. Place the PCR tube into the PCR instrument and perform the below reaction:

Temperature	Time
Heating lid at 105℃	On
20℃	15 min
4℃	Hold

▲ When the amount of Input DNA is low, the ligation time may be doubled as appropriate. However, longer reaction time may increase Adapter dimers. The concentration of Adapter needs to be adjusted if necessary.

6. Purify the reaction products using VAHTS DNA Clean Beads:

a/ Mix VAHTS DNA Clean Beads well by vortexing after equilibrating the magnetic beads to room temperature.

b/ Pipette 88 μl of VAHTS DNA Clean Beads into 110 μl of the Adapter Ligation products, and mix well by gently pipetting up and down 10 times.

c/ Incubate at room temperature for 5 min.

d/ Centrifuge the PCR tube briefly and place it on a magnetic stand to separate the magnetic beads from the solution. Wait until the solution becomes clear (about 5 min), and carefully **remove the supernatant**.

e/ Keep the PCR tube on the magnetic stand. Add 200 μl of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully **remove the supernatant**.

f/ Repeat step e/, rinse twice in total.

g/ Keep the PCR tube on the magnetic stand. Uncap the tube and air-dry the magnetic beads for 5 - 10 min until there is no residual ethanol.

h/ Remove the PCR tube from magnetic stand for elution:

▲ If the purified products are not subject to Two Rounds Beads Selection: Elute with 22.5 μl elution buffer (10 mM Tris-HCl, pH 8.0 - pH 8.5), mix well by gently pipetting up and down, and place the mixture at room temperature for 5 min; centrifuge the PCR tube briefly and place it on a magnetic stand without shaking; wait until the solution is clear (about 5 min), and carefully pipette 20 μl of the supernatant into a new EP tube without disturbing the magnetic beads.

▲ If the purified products are subject to Two Rounds Beads Selection: Elute with 105 μl elution buffer (10 mM Tris-HCl, pH 8.0 - 8.5), mix well by gently pipetting up and down, and place the mixture at room temperature for 5 min; centrifuge the PCR tube briefly and place it on a magnetic stand without shaking; wait until the solution is clear (about 5 min), and carefully pipette 100 μl of the supernatant into a new EP tube without disturbing the magnetic beads. Perform size selection according to **Table 5** (Page 17).

The samples can be stored for one week at 4℃, and for a longer time at -20℃. Repeated freeze-thaw cycles should be avoided.

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08-3/Library Amplification

This step is to amplify the purified or size-selected Adapter Ligation products. Whether this step is required depends on factors such as the amount of Input DNA, incomplete-length Adapter or not, and the application scenarios. This step is required if an incomplete-length Adapter (e.g., Vazyme #N321/N322) is used. If a complete Adapter is used, Library Amplification is recommended when Input DNA is less than 50 ng; this step may be skipped (directly proceed to 08-4/Library Quality Control instead) when Input DNA is more than 50 ng or there is no need for Library Amplification.

1. Thaw PCR Primer Mix 3 for Illumina and VAHTS HiFi Amplification Mix 2, mix thoroughly by turning upside down, and prepare the following reaction in a sterile PCR tube (**on ice**):

Component	Volume
Purified or Size-selected Adapter Ligation Product	20 μl
PCR Primer Mix 3 for Illumina	5 μl
VAHTS HiFi Amplification Mix 2	25 μl
Total	50 μl

▲ If the Vazyme #N321/N322 adapter-primer combination is used, the primers should be the provided i5 PCR Primer DM5XX and i7 PCR Primer DM7XX, with an amount of 2.5 μl for each primer.

2. Mix well by gentle pipetting up and down (**do not vortex**) and centrifuge briefly to collect the reaction solution to the bottom of the tube.

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

Temperature	Time	Number of Cycles
98℃	45 sec	1
98℃	15 sec	Please refer to **Table 3** (Page 08) for the number of cycles.
60℃	30 sec	
72℃	30 sec	
72℃	1 min	1
4℃	Hold	

4. In case of size selection, refer to Appendix I: Two Rounds Beads Selection. If no size selection is required, purify the reaction products using VAHTS DNA Clean Beads:

a/ Mix VAHTS DNA Clean Beads well by vortexing after equilibrating the magnetic beads to room temperature.

b/ Pipette 50 μl of VAHTS DNA Clean Beads into 50 μl of the Library Amplification products, and mix well by gently pipetting up and down 10 times.

c/ Incubate at room temperature for 5 min.

d/ Centrifuge the PCR tube briefly and place it on a magnetic stand to separate the magnetic beads from the solution. Wait until the solution becomes clear (about 5 min), and carefully **remove the supernatant**.

e/ Keep the PCR tube on the magnetic stand. Add 200 μl of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully **remove the supernatant**.

f/ Repeat step e/, rinse twice in total.

g/ Keep the PCR tube on the magnetic stand. Uncap the tube and air-dry the magnetic beads for 5 - 10 min until there is no residual ethanol.

h/ Remove the PCR tube from magnetic stand for elution:

▲If no subsequent targeted capture is performed: Elute with 22.5 μl elution buffer (10 mM Tris-HCl, pH 8.0 - 8.5), mix well by gently pipetting up and down, and place the mixture at room temperature for 5 min; centrifuge the PCR tube briefly and place it on a magnetic stand without shaking; wait until the solution is clear about 5 min), and carefully pipette 20 μl of the supernatant into a new EP tube without disturbing the magnetic beads.

▲If subsequent targeted capture is performed: Elute with 22.5 μl ddH_2O. Mix well by gently pipetting up and down, and place the mixture at room temperature for 5 min; centrifuge the PCR tube briefly and place it on a magnetic stand without shaking; wait until the solution is clear (about 5 min), and carefully pipette 20 μl of the supernatant into a new EP tube without disturbing the magnetic beads.

The samples can be stored for one week at 4°C, and for a longer time at -20°C. Repeated freeze-thaw cycles should be avoided.

08-4/Library Quality Control

Please refer to 06-7/Library Quality Control.

Appendix I: Two Rounds Beads Selection

◇ In order to meet the needs of different application scenarios, Two rounds Beads Selection are usually required during library preparation to control the library insert size distribution. **Table 4** lists the time points for size selection and the advantages and disadvantages of each time point.

Table 4. Choosing the stage of the size selection

Time Point for Size Selection	Application Scenarios	Advantage	Disadvantage	Examples of Applicable Samples
Before End Preparation	Sufficient Input DNA, with broad distribution or inconsistency between the main peak size and the expected library insert size; low Input DNA purity	Centralized size distribution of size selection products; accurate amount of Input DNA; increased Input DNA purity to improve the success rate of library preparation	High DNA loss; slightly broad library distribution	Under- or over-fragmented genomic DNA
After Adapter Ligation **(Recommended)**	Sufficient Input DNA with appropriate distribution[a]	Reduced loss of short DNA fragments; applicable to most cases	Slightly broad library distribution	Moderately fragmented genomic DNA or FFPE DNA with broad distribution
After Library Amplification	Small amount of Input DNA[a]	Reduced loss of Input DNA during library preparation and increased library complexity	Broad library distribution[b]	cfDNA
No Size Selection	Small amount of Input DNA with appropriate distribution for library preparation	Reduced loss of Input DNA during library preparation and increased library complexity	Unable to control the library insert size	Multiple PCR products, highly fragmented FFPE DNA

a. Size selection after Adapter Ligation is recommended when the amount of Input DNA is ≥50 ng; size selection after Library Amplification is recommended when the amount of Input DNA is <50 ng or the number of copies of samples is limited.

b. For other size selection time points, the library distribution will be further centralized after the library amplification.

◇ The Two Rounds Beads Selection select DNA fragments of expected length by controlling the amount of magnetic beads used. The basic principle is, in the first round, DNA with larger molecular weight binds to magnetic beads and is discarded with these beads; in the second round, magnetic beads bind to DNA with larger molecular weight among the remaining products, and DNA with smaller molecular weight is discarded with the supernatant. Many components in the initial sample would interfere with the result of the Two Rounds Beads Selection Therefore, the amount of magnetic beads used in the Two Rounds Beads Selection varies across the time points for size selection. Please refer to **Table 5** to choose the optimal size selection parameters based on the expected library insert size and the time point for size selection.

Table 5. Library Size Selection

Time Point and Condition for Size Selection	Amount of Magnetic Beads Per Round	Expected Library Insert Size (bp)								
		200	250	300	350	400	450	500	550	700
Before End Preparation (Sample volume is made up to 110 μl)	1st Round X (μl)	98	88	77	66	60	57	55	53	47
	2nd Round Y (μl)	22	22	22	22	22	17	17	17	13
After Adapter Ligation (Sample volume is made up to 110 μl)	1st Round X (μl)	75	72	65	62	58	56	55	/	/
	2nd Round Y (μl)	22	17	17	13	13	11	11	/	/
After Library Amplification (Sample volume is made up to 110 μl)	1st Round X (μl)	77	69	61	55	51	50	48	/	/
	2nd Round Y (μl)	22	22	22	22	22	22	17	/	/

◇ When incomplete length Adapters (e.g., Vazyme #N321/N322) are used, the size selection after ligation is performed under different conditions compared with complete Adapters. Please refer to the following table to choose the optimal size selection parameters.

Time Point and Condition for Size Selection	Amount of Magnetic Beads Per Round	Expected Library Insert Size (bp)						
		200	250	300	350	400	450	500
After Adapter Ligation (Sample volume is made up to 110 μl)	1st Round X (μl)	99	83	72	66	60	58	55
	2nd Round Y (μl)	22	22	22	22	22	22	20

▲ In case of size selection using magnetic beads, the larger the Insert Size, the broader the final product distribution. When the Insert Size is >700 bp, Two Rounds Beads Selection make little difference. In this case, gel extraction is recommended for size selection.

▲ The volume ratio of the sample to the magnetic beads is critical for size selection. Please ensure the accuracy of the initial volume and pipetting volume of the sample.

4. Sample Pretreatment (Important!)

▲ If size selection is performed before End Preparation, the sample volume should be 110 μl, and if it is not, ddH_2O can be used to fill the gap.

▲ If size selection is performed after purification of the Adapter Ligation products, the sample volume should be 110 μl, and if it is not, ddH_2O can be used to fill the gap.

▲ If size selection is performed after Library Amplification, the sample volume should be 110 μl, and if it is not, ddH_2O can be used to fill the gap.

▲ If the sample is not pretreated by volume, please adjust the amount of magnetic beads in equal proportion to the actual sample volume. However, a too small sample volume may lead to less accurate pipetting and then less accurate size selection. Therefore, it is not recommended to size-select samples of <50 μl directly without pretreatment.

◇ Size Selection Protocol [Refer to **Table 5** (Page 17) for the values of X and Y]

1/ Mix VAHTS DNA Clean Beads thoroughly by vortexing after equilibrating the magnetic beads to room temperature.

2/ Pipette X μl of VAHTS DNA Clean Beads into 110 μl of the above products, and mix well by gently pipetting up and down 10 times.

3/ Incubate at room temperature for 5 min.

4/ Centrifuge the PCR tube briefly and place it on a magnetic stand to separate the magnetic beads from the solution. Wait until the solution becomes clear (about 5 min), and carefully transfer the supernatant to a new PCR tube, and **discard the magnetic beads**.

5/ Pipette Y μl of VAHTS DNA Clean Beads into the supernatant, and mix well by gently pipetting up and down 10 times.

6/ Incubate at room temperature for 5 min.

7/ Centrifuge the PCR tube briefly and place it on a magnetic stand to separate the magnetic beads from the solution. Wait until the solution becomes clear (about 5 min), and carefully **remove the supernatant**.

8/ Keep the PCR tube on the magnetic stand. Add 200 μl of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully **remove the supernatant**.

9/ Repeat step 8/, rinse twice in total.

10/ Keep the PCR tube on the magnetic stand. Uncap the tube and air-dry the magnetic beads for 5 - 10 min until there is no residual ethanol.

11/ Remove the PCR tube from magnetic stand for elution:

▲ If no subsequent targeted capture is performed: Elute with 22.5 μl elution buffer (10 mM Tris-HCl, pH 8.0 - 8.5). Mix well by gently pipetting up and down, and place the mixture at room temperature for 5 min. Centrifuge the PCR tube briefly and place it on a magnetic stand without shaking. Wait until the solution becomes clear (about 5 min), and carefully pipette 20 μl of the supernatant into a new EP tube without disturbing the magnetic beads.

▲ If subsequent targeted capture is performed: Elute with 22.5 μl ddH_2O. Mix well by gently pipetting up and down, and place the mixture at room temperature for 5 min. Centrifuge the PCR tube briefly and place it on a magnetic stand without shaking. Wait until the solution becomes clear (about 5 min), and carefully pipette 20 μl of the supernatant into a new EP tube without disturbing the magnetic beads.

Appendix II: cfDNA Library Preparation

Cell-free DNA (cfDNA) is highly fragmented DNA (about 180 bp) in blood with low content. cfDNA is of great value in non-invasive prenatal testing (NIPT) and liquid biopsy (ctDNA detection). VAHTS Universal DNA Library Prep Kit for Illumina V4 provides a highly simplified and optimized library preparation solution for cfDNA.

Notes

◇Input DNA refers specifically to the DNA added to End Preparation with a volume of ≤50 μl.

◇cfDNA is highly fragmented and does not require Fragmentation.

◇To ensure the quality of libraries, it is recommended to determine the size distribution (2100 Bioanalyzer) and concentration (Qubit) of cfDNA templates.

Library Preparation Procedure

Step 1: End Preparation (refer to 08-1/End Preparation)

Amount of Input DNA: 100 pg - 100 ng.

Step 2: Adapter Ligation (refer to 08-2/Adapter Ligation)

Adapter: Pre-dilute according to **Table 2** (Page 04).

Clean up: Purify with 0.8 × magnetic beads, elute DNA with 22.5 μl elution buffer, and pipette 20 μl of supernatant for the next step.

Step 3: Library Amplification (refer to 08-3/Library Amplification)

Number of cycles: Refer to **Table 3** (Page. 08) (adjustable based on library yield requirements).

Clean up: Whether to do size-selection of cfDNA library depends on the sample condition and data analysis requirements.

▲ If the amplified products are not subject to Two Rounds Beads Selection: Purify with 1 × magnetic beads, elute DNA with 22.5 μl elution buffer, and pipette 20 μl of supernatant into a new EP tube and store at -20°C.

▲ If the amplified products are subject to Two Rounds Beads Selection: Size-select with 0.73 ×/0.25 × magnetic beads, elute DNA with 22.5 μl elution buffer, and pipette 20 μl of supernatant into a new EP tube and store at -20°C.

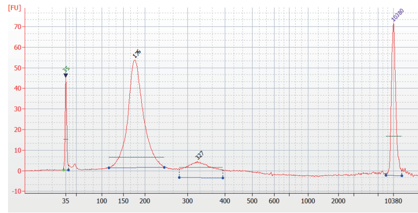
Step 4: Library Quality Control

Library concentration determination:

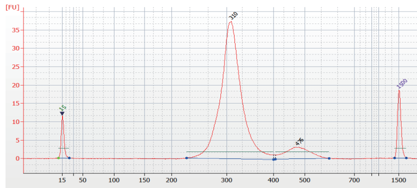
Fluorescent dye-based (Qubit or PicoGreen) or qPCR-based absolute quantification methods are recommended for library concentration determination.

Library size distribution determination:

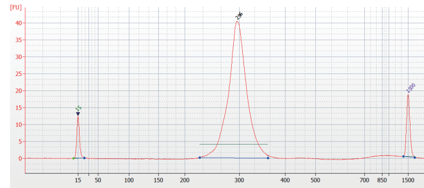
Library size distribution is determined using Agilent 2100 Bioanalyzer.



cfDNA extracted with magnetic beads



cfDNA library (without size selection)



cfDNA library (without size selection)

Appendix III: FFPE DNA Library Preparation

FFPE DNA is obtained from Formalin-Fixed and Parrffin-Embedded (FFPE) sections, which is characterized by difficult extraction (close cross-linking with histones) and low quality (serious degradation). FFPE samples are easy to store with a wide range of origins, and are of great applicable value in the medical field. VAHTS Universal DNA Library Prep Kit for Illumina V4 provides a highly simplified and optimized library preparation solution for FFPE DNA.

Notes

◇ Input DNA refers specifically to the DNA added to End Preparation with a volume of ≤50 μl.

◇ The quality of extracted FFPE DNA is different due to tissue difference, embedding quality, storage time and other factors. When low-quality FFPE DNA is used for library preparation, the amount of Input DNA or the number of amplification cycles should be increased as appropriate.

◇ To ensure the quality of libraries, it is recommended to determine the size distribution (Agilent 2100 Bioanalyzer) and concentration (Qubit) of FFPE DNA templates. Templates may also be pre-tested using the qPCR-based FFPE DNA quality evaluation system.

◇ If the FFPE DNA degradation degree is insufficient and the average molecular weight is large, fragmentation should be conducted before library construction.

Library Preparation Procedure

Step 1: End Preparation (refer to 08-1/End Preparation)

Amount of Input DNA: ≥50 ng.

Step 2: Adapter Ligation (refer to 08-2/Adapter Ligation)

Adapter: Pre-dilute according to **Table 2** (Page 04).

Clean up: Purify with 0.8 × magnetic beads.

▲ If the purified products are not subject to Two Rounds Beads Selection: Elute DNA with 22.5 μl elution buffer, and pipette 20 μl of supernatant.

▲ If the purified products are subject to Two Rounds Beads Selection: Elute DNA with 105 μl elution buffer, pipette 100 μl of supernatant, and size-select the libraries based on the conditions for Two Rounds Beads Selection listed in **Table 5** (Page 17).

Step 3: Library Amplification (refer to 08-3/Library Amplification)

Number of cycles: Refer to **Table 3** (Page 08) (adjustable according to the sample quality).

Clean up:

▲ If the amplified products are not subject to Two Rounds Beads Selection: Purify with 1 × magnetic beads, elute DNA with 22.5 μl elution buffer, and pipette 20 μl of supernatant into a new EP tube and store at -20°C.

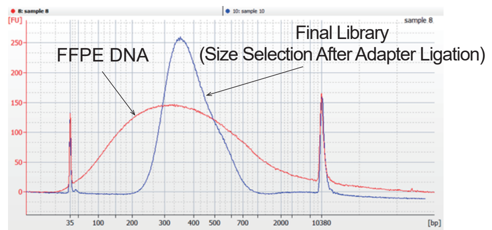
▲ If the amplified products are subject to Two Rounds Beads Selection: Add ddH_2O to make up the volume to 110 μl, and size-select the libraries based on the conditions for Two Rounds Beads Selection listed in **Table 5** (Page 17).

Step 4: Library Quality Control

Library concentration determination:

Fluorescent dye-based (Qubit or PicoGreen) or qPCR-based absolute quantification methods are recommended for library concentration determination.

Library size distribution determination: The library size distribution is determined using Agilent 2100 Bioanalyzer.



FFPE DNA Library (With Size Selection)

Appendix IV: Targeted Capture Library Preparation

In the capture process of NimbleGen SeqCap EZ, VAHTS Universal DNA Library Prep Kit for Illumina V4 can be used to prepare pre-capture libraries.

Notes

◇ Input DNA refers specifically to the DNA added to End Preparation with a volume of ≤50 μl.

◇ Input DNA should range from 180 - 220 bp in length. Refer to the Instructions for Use of Covaris or other DNA fragmentation equipment for fragmentation parameters.

◇ To ensure the quality of libraries, it is recommended to determine the size distribution (Agilent 2100 Bioanalyzer) and concentration (Qubit) of Input DNA.

Library Preparation Procedure

Step 1: End Preparation (refer to 08-1/End Preparation)

Amount of Input DNA: Refer to **Table 1** (Page 03) and based on the sample type.

Step 2: Adapter Ligation (refer to 08-2/Adapter Ligation)

Adapter: Pre-dilute according to **Table 2** (Page 04). VAHTS DNA Adapters for Illumina (Vazyme #N801/N802) are perfectly compatible with NimbleGen SeqCap EZ. When using Adapters or capturing reagents from other sources, choose Adapters based on the blocking reagents.

Clean up: Purify with 0.8 × magnetic beads, elute DNA with 105 μl elution buffer, and pipette 100 μl of supernatant for double rounds of size selection using magnetic beads (0.68 ×/0.2 ×), elute DNA with 22.5 μl elution buffer, and pipette 20 μl of supernatant for the next step.

Step 3: Library Amplification (refer to 08-3/Library Amplification)

Number of cycles: Refer to **Table 3** (Page 08). It is recommended to use the upper limit of the number of cycles, which is enough for a library yield of ≥1μg. If sample pooling is performed prior to capture, please ensure that the library yield is ≥1 μg/n (n=number of samples) for each sample. In this case, amplification cycles per sample can be reduced to increase the library complexity and decrease duplication rates.

Clean up: Purify with 1 × magnetic beads, elute DNA with 22.5 μl ddH_2O, and pipette 20 μl of supernatant into a new EP tube.

Step 4: Library Quality Control

Refer to SeqCap EZ Library SR User's Guide v5.1 (Step 5 in Chapter 4) (Roche document number 06588786001, 09/15) to perform library quality control.

Step 5: Targeted Enrichment

Refer to Chapters 5 - 8 of SeqCap EZ Library SR User's Guide v5.1 to complete the targeted capture process.

Vazyme

Nanjing Vazyme Biotech Co.,Ltd.

Tel: +86 25-83772625

Email: info.biotech@vazyme.com

Web: www.vazyme.com

Loc: Red Maple Hi-tech Industry Park, Nanjing, PRC

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www.vazyme.com