VAHTS Universal DNA Library Prep Kit for Illumina® V3

ND607

Version 8.3

Vazyme biotech co., ltd.

Introduction

The Vazyme VAHTS Universal DNA Library Prep Kit for Illumina® V3 is specially designed for library preparation for next generation sequencing (NGS) on Illumina® platforms. This kit enables library preparation from 100 pg - 4 ug of input DNA. As a new updated version, the VAHTS Universal DNA Library Prep Kit for Illumina® V3 combines several handling steps, with a more streamlined workflow, higher library conversion rate, and improved compatibility. This kit can be used for DNA library preparation with or without PCR amplification of various kinds of input DNA, and it is also compatible with target capture systems. All kit components are subjected to stringent functional quality control, ensuring the consistency and reproducibility of library preparation.

Components

Comonent	ND607-01 (24 rxn)	ND607-02 (96 rxn)	ND607-03 (24 rxn)	ND607-04 (96 rxn)
End Prep Mix 4	360 µl	4 × 360 µl	360 µl	4 × 360 µl
Rapid Ligation Buffer 2	600 µl	4 × 600 µl	600 µl	4 × 600 µl
Rapid DNA Ligase	120 µl	480 µl	120 µl	480 µl
VAHTS HiFi Amplification Mix	600 µl	4 × 600 µl	------	------
PCR Primer Mix 3 for Illumina	120 µl	480 µl	------	------
Control DNA (264 bp, 50 ng/ul)	10 µl	10 µl	10 µl	10 µl

Storage

All components should be stored at -20℃.

Applications

Applicable to DNA library preparation for NGS on Illumina® platforms and compatible with various kinds of input samples, including Genomic DNA, cell-free DNA (cfDNA, ctDNA), formalin fixed paraffin-embedded DNA (FFPE DNA), Chromatin immuno-precipitation DNA (ChIP DNA), and Amplicons. For small genomes, cfDNA/ctDNA, ChIP DNA, and amplicons, the amount of input DNA can be as small as 100 pg. It is recommend to use this kit for:

- Whole genome sequencing
- Whole exome or targeted sequencing (using Roche® NimbleGen™ SeqCapTM EZ, Agilent® SureSelect, Illumina® TruSeq, or IDT xGen™ Lockdown™ Probes or other hybridization capture systems)
- Amplicon sequencing
- ChIP-seq
- Metagenome sequencing
- Methylation Sequencing (in combination with Phanta UC Super-Fidelity DNA Polymerase for Library Amplification,Vazyme, #P507)

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.

6-1. Input DNA & Fragmentation

◇ The recommended input DNA range is 100 pg - 4 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 - 4 μg
Targeted Sequencing	Complex gDNA	10 ng - 4 μg
Whole Genome/Targeted Sequencing	FFPE DNA	≥ 50 ng
Whole Genome/Targeted Sequencing	cfDNA/ctDNA	≥ 100 pg
Whole Genome Sequencing	Microbial genome	10 ng - 4 μg
Whole Genome Sequencing (PCR-Free)	Complex/Simple genome	≥ 50 ng (no size selection) ≥ 200 ng (with size selection)
Targeted Sequencing	Amplicons	≥ 100 pg
ChIP-seq	ChIP DNA	≥ 100 pg

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

◇ "Input DNA" typically refers to the input DNA added to the End Preparation, instead of the input amount for Fragmentation. If DNA was quantified before fragmentation, and fragmented DNA was subjected to cleanup or size selection prior to End Preparation, the actual input into library construction may need to be recalculated. Otherwise, low library amplification cycle number may lead to low library yield.

◇ DNA preparations containing high concentrations of EDTA, other chelating agents, or salts may affect the End Preparation reaction.

(1). Purify or size select fragmentation products and then dilute the purification products to 0.1× TE or ddH_2O (≤ 50 μl) before library construction.

(2). If fragmented DNA is not subjected to a bead-based cleanup or size selection prior to library construction:

For mechanical fragmentation, DNA should be fragmented in 0.1× TE. Fragmentation in water is not recommended.

For enzymatic fragmentation, make sure there is no EDTA or other chelating agents in the Stop Buffer.

6-2. Adapters

◇ For Illumina® platforms, please choose appropriate Indexed Adapters. Vazyme VAHTS Adapters are recommended to use with this kit:

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

This kit is also compatible with non-indexed, single-indexed, and dual-indexed adapters that are routinely used in Illumina® TruSeq®, Roche® NimbleGen™ SeqCap™ EZ, Agilent SureSelect, and other similar library construction and targeted capture workflows. Customized adapters that are of similar design and are compatible with "TA-ligation" of dsDNA may also be used.

◇ The quality and amount of Adapters directly affect the preparation efficiency and library quality. The recommended ratio of adapter : input is between 10 : 1 and 200 : 1. High Adapter input may lead to residual Adapter/Adapter Dimer. Low Adapter input may affect ligation efficiency and reduces 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 - 4 μg input DNA

Input DNA	Adapter : Input DNA Molar Ratio	Adapter concentration from other source	Vazyme Adapter Dilution Ratio
500 ng - 4 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	0.2 μM	1:30 - 1:100

▲ 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, two or three adapter concentrations should be evaluated: try the recommended adapter concentration (Table 2), as well as one or two 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-4 μg while default volume of adapter is 5 μl, please increase to 10 μl to enhance 5% - 15% library output.

6-3. Cleanup of Adapter Liagation 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 library amplification, the recommended elution volume is 22.5 μl.

◇ A second cleanup may be performed if post-ligation analysis reveals unacceptable levels of adapter and/or adapter-dimer carry-over after the first cleanup, using a 1× bead to DNA ratio. Make the volume of the purification products from the first round up to 50 μl with ddH_2O, 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 instruments. The adapter amount may also be decreased to eliminate the residual adapter and/or adapter-dimer.

6-4. Beads

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

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 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. 1.8× beads can purify DNA of 150 bp.

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

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

▲ 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. Capture times should be optimized accordingly.

▲ 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. With optimized aspiration of ethanol, drying of beads for 5 min–10 min at room temperature should be sufficient. Drying of beads at 37℃ is not recommended.

▲ DNA should be eluted from beads with elution buffer (10 mM Tris-HCl, pH 8.0–pH 8.5). Elution of DNA in PCR-grade water is not recommended, as DNA is unstable in unbuffered solutions. However, libraries for target capture must be eluted and stored in PCR-grade water to facilitate drying of DNA prior to probe hybridization.

▲ Purified DNA in elution buffer should be stable at 4℃ for 1 week, or at -20℃ for long-term storage. Avoid excessive freezing and thawing cycles.

6-5. 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 double-sided 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 Repair and dA-tailing of fragmented DNA;

▲ after the post-ligation cleanup;

▲ after library amplification.

The standard protocol of this manual (Refer to 8. 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. These losses can be 60% - 95%. 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. 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 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.

◇ Rapid Ligation Buffer 2 contains a high concentration of PEG 6000, which, if not removed, will interfere with efficient double-sided size selection and can affect the efficiency of other size selection techniques. Therefore, if size selection is performed after Adapter Ligation, it is important to perform at least one bead-based clean-up prior to performing bead- or electrophoresis-based size selection.

6-6. 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 ìM-20 ìM each.

◇ 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 extent of 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 nucleotide substitutions. 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 (Into End Preparation)	Number of cycles required to generate	
	100 ng	1 µg
100 pg	15 - 17	16 - 19
1 ng	9 - 11	12 - 15
5 ng	7 - 9	10 - 14
10 ng	6 - 8	8 - 12
50 ng	4 - 6	7 - 10
100 ng	2 - 4	5 - 8
250 ng	1 - 3	4 - 7
500 ng	0	2 - 5
1 µg	0	2 - 5

▲ The table above is the recommended cycle numbers for high-quality input DNA of 200 bp. If the input DNA quality is low or library length is long, please increase cycle numbers to obtain sufficient library.

▲ If size selection is proceeded during library construction, please choose larger cycle numbers for Library Amplification. Otherwise, please choose the small numbers.

▲ When using incomplete adapters (i.e. Vazyme, #N321/N322), a minimum number of amplification cycles (at least 2) is required to complete adapter sequences.

◇ If the adapters are complete (such as Vazyme, #N801/N802 or #N805/806/807/808) and a sufficient amount of library is available, it may be possible to skip library amplification to obtain PCR-Free libraries.

6-7. Evaluation of Library Quality

◇ Size Distribution

▲ The size distribution of final libraries can be confirmed with an electrophoretic method. A LabChip® GX, GXII or GX Touch (Perkin Elmer), Bioanalyzer or Tapestation (Agilent Technologies), Fragment Analyzer™(Advanced Analytical) or similar instrument is recommended. Typical electrophoretic profiles for libraries prepared with the VAHTS Universal DNA Library Prep Kit for Illumina® V3 are shown in Fig. 1.

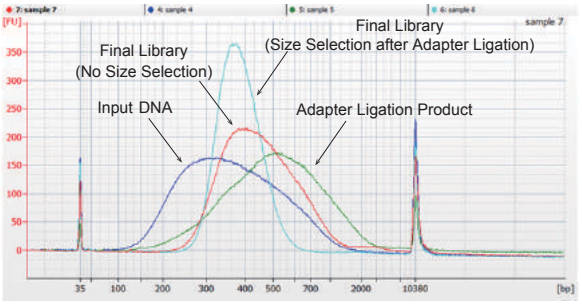


Fig. 1. Length Distribution of Products during Library Preparation Using VAHTS Universal DNA Library Prep Kit for Illumina® V3

▲ 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).

◇ 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 two 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.

6-8. Other Notes

◇ 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.

◇ Mixing with pipetting is recommended when preparing solutions. Shaking excessively may lead to reduced yield.

◇ 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.

Mechanism & Workflow

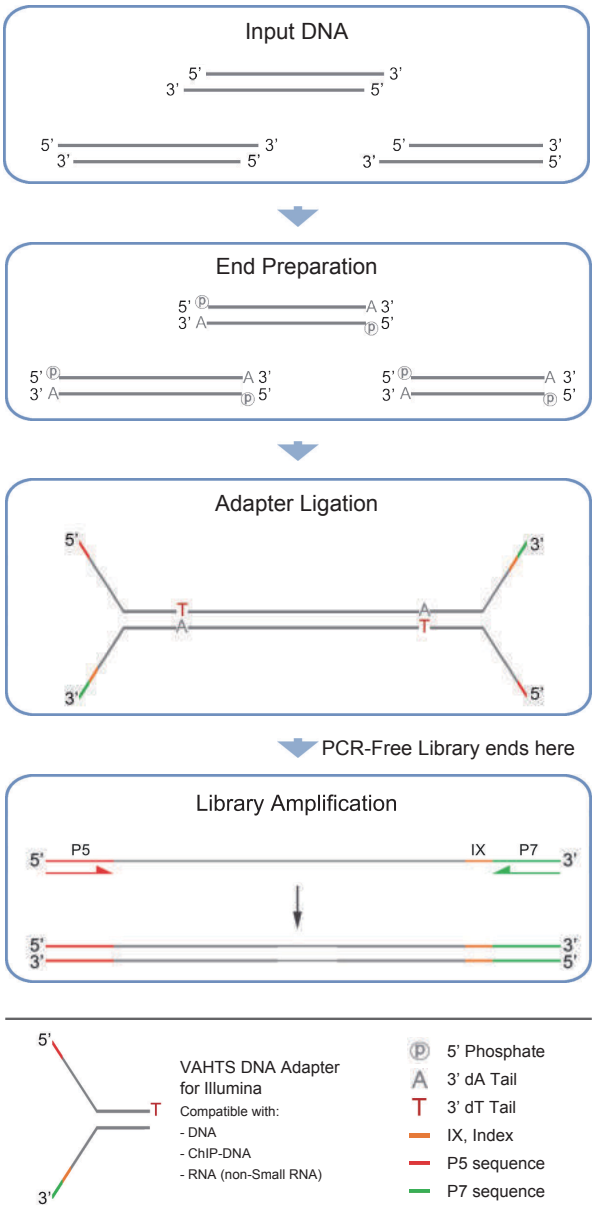


Fig. 2. Mechanism of VAHTS Universal DNA Library Prep Kit for Illumina® V3

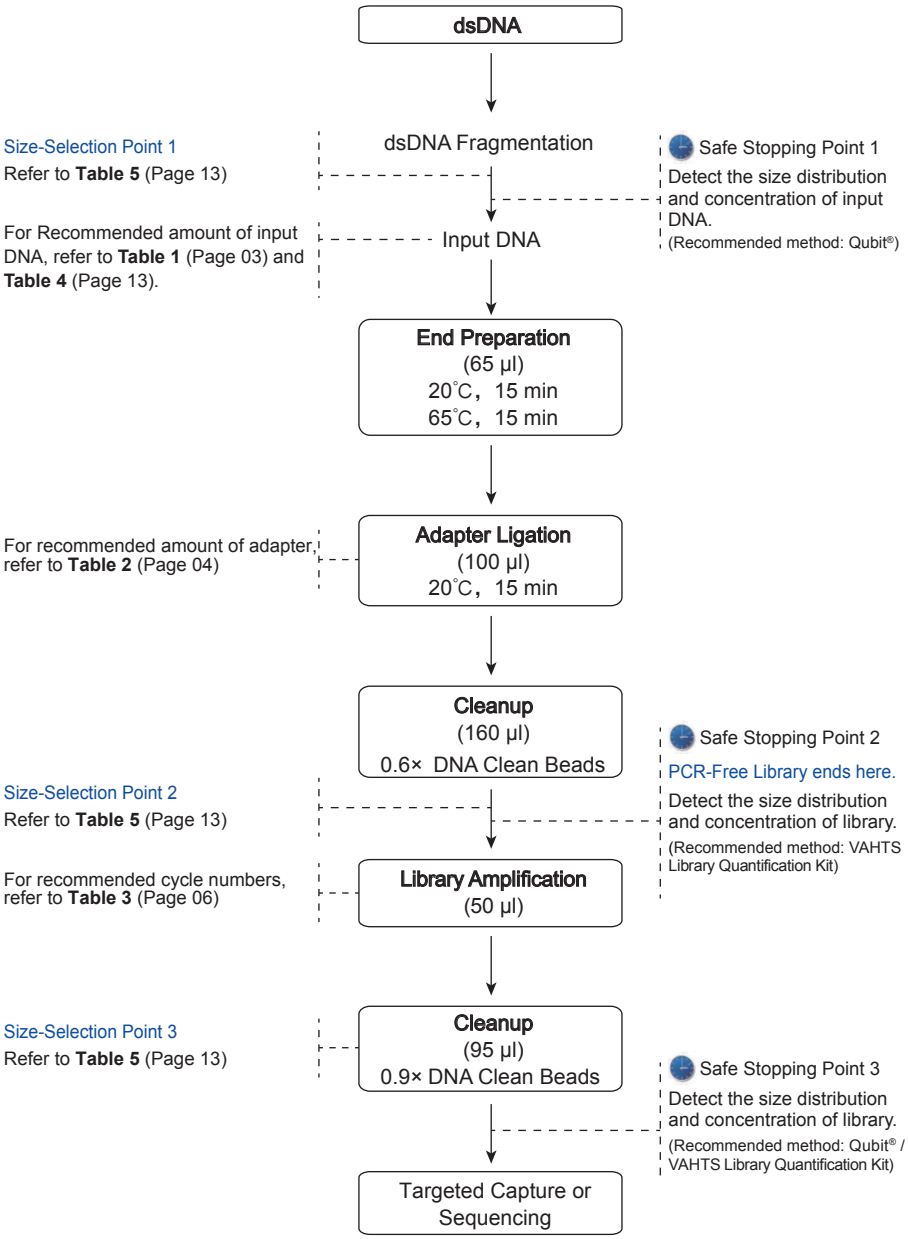


Fig. 3. Workflow of VAHTS Universal DNA Library Prep Kit for Illumina® V3

Standard Protocol for Library Preparation

Step 1. End Preparation

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

1.Thaw the End Prep Mix 4 and spin down briefly. Prepare the reaction solution in a PCR tube as follows:

Input DNA	x µl	
End Prep Mix 4	15 µl	■
ddH_2O	To 65 µl	

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: 105℃):

20℃	15 min
65℃	15 min
4℃	Hold

Step 2. Adapter Ligation

This process is to ligate End Preparation products to Adapters.

1.Dilute adapter stocks to the appropriate concentration, as outlined in Table 2 (Page 04).

2.Thaw the Rapid Ligation Buffer and mix thoroughly. Place on ice.

3.Prepare the reaction solution in a sterile PCR tube as follows:

End Preparation Products	65 µl	
Rapid Ligation buffer 2	25 µl	■
Rapid DNA ligase	5 µl	■
DNA Adapter X	5 µl	
Total Volume	100 µl	

▲ The premixes of Rapid Ligation Buffer 2 and Rapid DNA Ligase are stable for less than 24 hrs at 4℃.

▲ If VAHTS Multiplex Oligos Set 4/5 for Illumina (Vazyme, #N321/N322) is used, the DNA Adapter should be 5 µl of DNA adapter-S for Illumina.

4.Mix thoroughly by gently pipetting up and down. DO NOT Vortex! Spin down briefly.

5.Put the tube in a PCR instrument and run the following PCR program (Hot Lid Temperature: 105℃):

20℃	15 min
4℃	Hold

▲ For low-input samples, consider doubling the ligation time. However, longer ligation times may lead to increased levels of adapter-dimers. If necessary, the adapter concentrations may also need to be optimized.

6.Cleanup of Adapter Ligation products with 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 soultion 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 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 13).

Note: The products can be stable for one week at 4˚C. Keep at -20˚C for long-stem storage. Avoid uncessary freeze-and-thaw cycles.

Step 3. Library Amplification

This process 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 the need of application. If adapters are not in complete length (i.e. Vazyme, #N321/N322), this step is necessary. If adapters are in complete length (i.e. Vazyme, #N801/N802/N805-N805), for input DNA < 50 ng, amplification is recommended. Skip this step 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:

Purified or Size-Selected Adapter-Ligated Library	20 µl	
PCR Primer Mix 3 for Illumina	5 µl	■
VAHTS HiFi Amplification Mix	25 µl	■
Total Volume	50 µl	

▲ If VAHTS Multiplex Oligos Set 4/5 for Illumina (Vazyme, #N321/N322) is used, the primer mix should be 2.5 µl of i5 PCR Primer DM5XX and 2.5 µl of i7 PCR Primer DM7XX.

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	20 sec	According to Table 3 (Page 06)
60˚C	15 sec	
72˚C	30 sec	
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 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 soultion 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 ddH_2O 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.

Note: The products can be stable for one week at 4˚C. Keep at -20˚C for long-stem storage. Avoid uncessary freeze-and-thaw cycles.

Step 4. Quality Control of Library

Please refer to 6-7. Evaluation of Library Quality for details.

Standard Protocol for Library Preparation

◇ To meet the needs of different sequencing applications, 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
Prior to End Preparation	Sufficient input DNA,with broad size distribution or unexpected insert size; input DNA with low purity.	Selected length is concentrated; accurate amount of input DNA; increased DNA purity to increase library prep success rate;	Loss of DNA;size distribution is broad*	gDNA with insufficient or excess fragmentation
After Library Amplification	Sufficient input DNA with proper size distribution***	Decrease the loss of short input DNA; applicable to almost cases	Size distribution is broad*	Proper fragmentation of gDNA or FFPE DNA with broad size distribution
After Adapter Ligation (Recommended)	Low input DNA***	Decrease the loss of input DNA during workflow,increase library complesity	Size distribution is broad**	cfDNA
No Size-selection	Size distribution of input DNA is proper;low input DNA	Decrease the loss of input DNAduring workflow,increase library complexity	Can't control the insert size	Multiple PCR products; fragmented FFPE DNA

*DNA ends affect the result of size selection. Single end of input DNA and non-complementary arms of "Y" adapters ligated to DNA fragments may result in a broader size distribution.

**Compared with other selection points, the library distribution will be further centralized after the library Amplification of other location sorting products.

***If the amount of input DNA is ≥ 50 ng, size selection after Adapter Ligation is recommended. If the amount of input DNA is ＜50 ng, or the copies of samples is limited, size selection after Library Amplification is recommended.

◇ 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	550	700
Before End Preparation (Sample volume is added to 100 µl	1st-Round X(µl)	100	90	80	70	60	55	52	50	48	43
	2nd-Round Y(µl)	30	20	20	20	20	20	15	15	15	12
After Adapter Ligation (Sample volume is added to 100 µlĎ	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 added to 100 µlĎ	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 (i.e. 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 added to 100 µlĎ	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 can select DNA with insert size＞700 bp Please choose electrophoretic method instead.

▲ 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, the initial sample volume should be 100 ul. If the volume is 100 ul, please add ddH_2O to make up to 100 ul

▲ 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 ul directly without pretreatment

◇ Protocol for Size Selection [Refer to **Table 5** (Page 13) 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 ul of beads into 100 Ml 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 ul of beads into the supernatant of 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 ul 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 ul 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 ul of the supernatant to a new Nuclease-free PCR tube without disturbing the beads

▲ For products with need for Targeted Capture: Add 22.5 ul of ddH_2O 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 ul of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

Appendix 2. cfDNA Library Preparation

Cell-free DNA (cDNA) is a highly fragmented(~180 bp) DNA in blood with low content. cfDNA is of great importance in noninvasive prenatal testing(NIPT) and liquid biopsy. VAHTS Universal DNA Library Prep Kit for illumina provides an easy and optimized solution for the library preparation of cfDNA.

Notes

◇ “Input DNA" is the DNA added to End Preparation with volume ≤ 50 ul.

◇ cfDNA is highly fragmented. No fragmentation is needed for cfDNA.

◇ To ensure the quality of library, it is recommended to detect the size distribution (by an Agilent 2100 Bioanalyzer) and concentration (by Qubit) of input DNA.

Library Preparation Procedures

Step 1: End Preparation (Refer to **8. Standard Protocol of Library Preparation,** Step1)
Input DNA: 100 pg-100 ng.

Step 2: Adapter Ligation (Refer to **8. Standard Protocol of Library Preparation,** Step 2)

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

Cleanup: use 0. 6 x beads, elute DNA with 22.5 ul of elution buffer, transfer 20 ul supernatant for next step.

Step 3: Library Amplification (Refer to **8. Standard Protocol of Library Preparation,** Step 3)

Number of cycles: 12 - 17 is recommended. Adjust according to requirement of library yield.

Cleanup: whether to perform a size-selection depends on the sample situation and data analysis requirements.

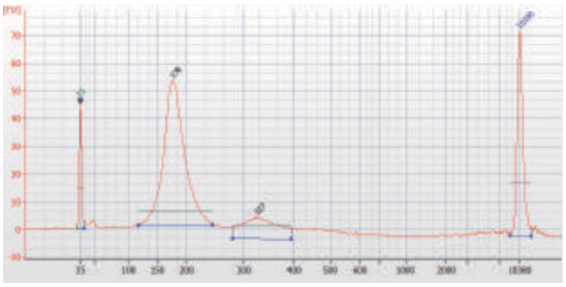
▲ No size-selection: purify with 0.9x beads, elute DNA with 22.5 ul of elution buffer. Transfer 20 ul of supernatant to a new Nuclease-free PCR tube and store at -20℃.

▲ Size-selection: size-select with 0.73x / 0.25x beads, elute DNA with 22.5 ul of elution buffer. Transfer 20 ul of supernatant to a Nuclease-free PCR tube and store at -20℃.

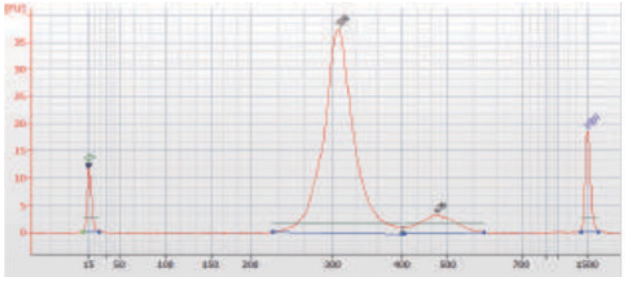
Step 4: Library Quality Control

Library concentration determination: it is recommended to use fluorescent dye (Qubit or Picogreen) or QPCR for library quantification.

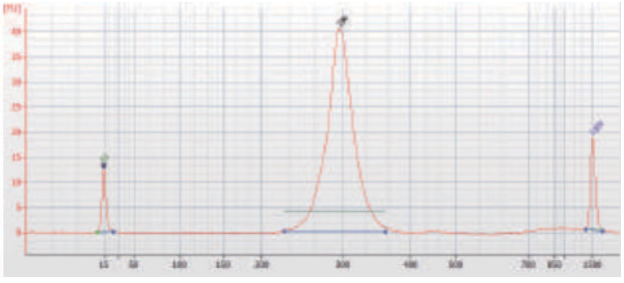
Library size distribution detection: detect by an Agilent 2100 Bioanalyzer.



cfDNA extracted with beads



cfDNA Library (without size-selection)



cfDNA Library (after size-selection)

Appendix 3. FFPE DNA Library Preparation

FFPE DNA is DNA from formalin - fixed and paraffin-embedded (FFPE) tissues. FFPE DNA is difficult to isolate (cross inked tightly with histone) and with low quality (degrade severely) . FFPE samples are of great applicable value in medical area because they are easy to store and with extensive sources. VAHTS Universal DNA Library Prep Kit for Illumina V3 provides an easy and optimized solution for the library preparation of FFPE DNA.

Notes

◇ input DNA is the DNA added to end preparation with volume < 50 ul.

◇ The quality of extracted FFPE DNA varies due to the differences in tissues types , embedding quality, and storage time. For FFPE DNA with low quality, please increase the amount of input DNA or increase amplification cycles accordingly.

◇ To ensure the quality of library, it is recommended to detect the size distribution by an agilent 2100 bioanalyzer) and concentration (by Qubit) of input DNA. Qpcr-based methods are also optional.

◇ For FFPE DNA with high molecular weight and not fragmented enough , fragmentation is necessary before library preparation.

Library Preparation Procedures

Step 1: End Preparation (Refer to **8. Standard Protocol of Library Preparation,** Step 1)

Input DNA: ≥ 50ng

Step 2: Adapter Ligation (Refer to **8. Standard Protocol of Library Preparation,** Step 2)

Adapter: dilute according to Table 2 (Page 04)

Cleanup: with 0.6x beads

▲ No size-selection: elute DNA with 22.5 ul of elution buffer. Transfer 20 ul of supernatant to a new Nuclease-free PCR tube.

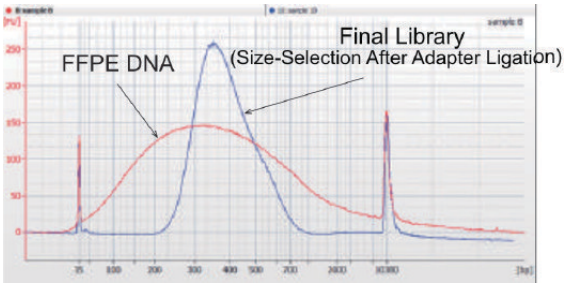
▲ Size-selection: elute DNA with 105 ul of elution buffer. Transfer 100 ul of supernatant to a new Nuclease-free EP tube.

Step 3: Library Amplification (Refer to **8 Standard Protocol of Library Preparation,** Step 3)
Number of cycles: according to Table 3 (Page 06)
Cleanup:

▲ No size-selection: purify by 0.9x beads, elute DNA with 22.5 ul of elution buffer. Transfer 20 ul of supernatant to a new Nuclease-free EP tube and store at -20℃.

▲ Size-selection: add ddH_2O to make the sample volume up to 100 ul, and size-select according to Table 5 (Page 13).

Step 4: Library Quality Control
Library concentration determination: it is recommended to use fluorescent dye (Qubit or Picogreen) or qPCR for library quantification.
Library size distribution detection: detect ban Agilent 2100 Bioanalyzer.



FFPE DNA Library (After Size Selection)

Appendix 4. Target Capture Library Preparation

Take NimbleGen SeqCap EZ capture library workflow as an example VAHTS Universal DNA Libraryep Kit for Illumina are suitable for the preparation of target capture library.

Notes

◇ Input DNA is the DNA added to End Preparation with volume ≤ 50 ul.

◇ The length of input DNA should be between 180 bp - 220 bp. Refer to the instruction of Covaris other fragmentation instruments for fragmentation parameter.

◇ To ensure the quality of library, it is recommended to detect the size distribution(by an Aqilent 2100 Bioanalyzer) and concentration (by Qubit)of input DNA.

Library Preparation Procedures

Step 1: End Preparation (Refer to **8. Standard Protocol of Library Preparation,** Step 1)
Input DNA: Refer to Table 1(Page 03)

Step 2 Adapter Ligation (Refer to **8 Standard Protocol of Library Preparation,** Step 2)
Adapter: dilute according to Table 2 (Page 04). VAHTS DNA Adapters for Illumina (Vazyme, # N801/N802) are perfectly compatible with Nimblegen Segcap EZ. When using capture reagents adapters from other sources, choose adapters according to the Blocking reagents
Cleanup: by 0.6x beads, elute DNA with 105 ul of elution buffer, transfer 100 ul of supernatant for size-selection (with 0.68/0.20 beads), then elute DNA with 22.5 ul of elution buffer and transfer 20 ul of supernatant for next step.

Step 3: Library Amplification (Refer to **8. Standard Protocol of Library Preparation,** Step 3)
Number of cycles: according to Table 3 (Page 06). It is recommended to use the upper limit of the cycle numbers, which is enough for a library yield ≥ 1ug. With sample pooling before target capture, pleasure ensure that all library yields are ≥ 1 ug/N (N is the sample number). Under this circumstance, reduce amplification cycles to increase the library complexity and decrease duplication rates.
Cleanup: purify by 0.9 beads, elute DNA with 22.5 Ul of ddH_2O (instead of Dilution Buffer)and transfer 20 ul of supernatant to a new Nuclease-free EP tube.

Step 4: Library Quality Control
Refer to Nimblegen Seqcap EZ Library SR Users Guide V5.1 (Chapter 4, Step 5)(Roche document number 06588786001, 09/15)

Step 5: Target Enrichment
Refer to Nimblegen Seqcap EZ Library SR Users' Guide V5.1(Chapter 5-8).

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