VAHTS ssDNA Library Prep Kit for Illumina

ND620

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Instruction for Use

Version 22.1

Vazyme

Contents

01/Product Description

VAHTS ssDNA Library Prep Kit for Illumina is a single-stranded DNA (ssDNA) library preparation kit that was specifically developed for the Illumina high-throughput sequencing platform. Based on high-efficiency single strand ligation technology, this kit can convert ssDNA into a sequencing library. At the same time, it is also suitable for double-stranded DNA (dsDNA) library preparation and mixed ssDNA and dsDNA library preparation. It is compatible with DNA samples as short as 40 bp and 10 pg - 250 ng of Input DNA. It is suitable for DNA samples with serious damage, denaturation or degradation. It is an ideal choice for library preparation for difficult and precious samples. The optimized library preparation process enables the library preparation to be completed within 2 h, and the manual operation duration is less than 30 min. It is suitable for automatic library preparation and compatible with the capture process. All the reagents in the kit have undergone rigorous quality control and function testing to ensure the optimal stability and repeatability of library preparation.

02/Componentss

	Components	ND620-01 (24 rxns)	ND620-02 (96 rxns)
BOX 1	Dilution Buffer	5 ml	20 ml
BOX 2	3' Ligation Buffer	240 µl	960 µl
	3' Ligation Enzyme Mix	120 µl	480 µl
	3' Adapter	120 µl	480 µl
	Extension Primer	120 µl	480 µl
	Extension Enzyme Mix	840 µl	4 × 840 µl
	5' Ligation Mix	240 µl	960 µl
	5' Adapter	240 µl	960 µl
	VAHTS HiFi Amplification Mix V1	600 µl	4 × 600 µl

03/Storage

BOX 1, Store at 2 ~ 8℃. Adjust the shipping method according to the destination.
BOX 2, Store at -30 ~ -15℃ and transport at ≤0℃.

04/Applications

This product is suitable for the preparation of Illumina high-throughput sequencing platform-specific libraries and is compatible with ssDNA, dsDNA and mixed ssDNA and dsDNA templates. It is compatible with a variety of sample types: genomic DNA, Formalin-Fixed and Paraffin-Embedded DNA (FFPE DNA), Chromatin immuno-precipitation DNA (ChIP DNA), cell-free DNA (cfDNA, ctDNA), microbial DNA, ancient DNA, etc. It can be compatible with DNA samples as short as 40 bp. It can be compatible with 10 pg - 250 ng of Input DNA.

05/Self-prepared Materials

◇ DNA quantification:

Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121);

Qubit ssDNA Assay Kit (Invitrogen #Q10212);

◇ Magnetic beads for purification:

VAHTS DNA Clean Beads (Vazyme #N411);

◇ PCR Index Primers:

VAHTS Dual UMI UDI Adapters Set 1 - Set 4 for Illumina (Vazyme #N351-N354);

#N351-N354 are 96 kinds of primer combinations with 8 bp Unique Indexed adapter at both ends of each primer, with 24 kinds for each catalog number.

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

#N321/N322 are combinations of primers with 8 bp of non-Unique Indexed adapters at both ends of each primer, which can be used to prepare libraries with up to 384 different index combinations.

◇ Library quality control:

Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121);

VAHTS Library Quantification Kit for Illumina (Vazyme #NQ101-NQ106);

Agilent Technologies 2100 Bioanalyzer or its equivalent.

◇ Other materials: 80% ethanol (freshly prepared), 0.1 × TE (pH 8.0), Nuclease-free ddH_2O, magnetic stand, PCR instrument, low binding Nuclease-free PCR tube, pipette tips and centrifuge tubes, etc.

06/Notes

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

Due to a number of factors such as the sample type, protocol, equipment and operations, process parameters for the library preparation may need to be adjusted according to the actual situation. In order to obtain a high-quality sequencing library, it is important to read the following notes carefully. In case of any questions during use, please contact Vazyme's technical support for help: info.biotech@vazyme.com.

06-1/About Input DNA and Fragmentation

1. The kit is compatible with 10 pg - 250 ng of Input DNA. Accurate quantification of Input DNA is very important for subsequent selection of library amplification cycle number. Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121) is recommended for quantification of dsDNA and Qubit ssDNA Assay Kit (Invitrogen #Q10212) for ssDNA.
   ▲ Input DNA amount refers to the amount of DNA input for the 3' Adapter Ligation step. If the DNA sample has undergone purification or size selection after fragmentation, its concentration should be measured again, and DNA amount before fragmentation cannot be directly used as Input DNA amount. Otherwise, library yield may be low due to an insufficient number of library amplification cycles.
2. If the DNA sample is severely fragmented DNA, such as ChIP DNA and cfDNA, there is no need for fragmentation.
3. If the DNA sample is genomic DNA with good integrity, the sample must be subjected to fragmentation. Sonication or enzyme digestion method can be selected to fragment genomic DNA. It is recommended that the fragment length of the product after fragmentation to be about 250 bp and the elution volume is 22.5 μl. Do not introduce high-concentration metal ion chelating agent or other salts during the preparation of Input DNA, otherwise the efficiency of the 3' Adapter Ligation step may be affected.
   ▲ When sonication is used for fragmentation and the product is directly used for library preparation without purification or size selection, please dilute the DNA in 0.1 × TE (pH 8.0) for fragmentation. Please do not dilute in sterile ddH_2O.
   ▲ When an enzyme digestion method is adopted for fragmentation and the resulting product is directly used for library preparation without purification or size selection, please make sure that the stop buffer does not contain excessive metal ion chelating agent. If the above conditions cannot be met, the fragmentation product can be purified or size selected and dissolved in less than 22.5 μl of 0.1 × TE (pH 8.0) before library preparation.

06-2/Use of Magnetic Beads

1. It is recommended to use VAHTS DNA Clean Beads (Vazyme #N411) for purification.
   ▲ If magnetic beads of any other source are used, some changes to the purification conditions may be required!
2. General notes for magnetic beads operations:
   a. The amount of magnetic beads used is generally marked by multiplier "×", which is the volume factor of magnetic beads used relative to the original volume of sample. For example, if the original volume of sample is 100 μl, for 1 × purification, the volume of magnetic beads used is 1 × 100 μl = 100 μl; if for 0.6 ×/0.2 × selection, the volume of magnetic beads used in the first round is 0.6 × 100 μl = 60 μl, and in the second round is 0.2 × 100 μl = 20 μl.
   b. The amount of magnetic beads used directly affects the lower limit of the DNA length that can be purified. The higher the multiplier, the smaller the lower limit of the DNA length that can be purified. Likewise, the lower the multiplier, the higher the lower limit. For instance, 1 × magnetic beads can only efficiently purify DNA longer than 250 bp, and will lead to the loss of a large amount of shorter DNA during purification; and when the volume factor is increased to 1.8 ×, 150 bp DNA can also be efficiently purified.
   c. Before use, magnetic beads should be equilibrated to room temperature (by incubation at room temperature for 30 min) first. Otherwise, yield will be decreased, and the selection effect will be poor.
   d. All magnetic beads operations should be performed at room temperature. Do not freeze the magnetic beads at a temperature below 0℃.
   e. The beads should be fully vortexed or mixed using a pipette.

f. When the sample is fully mixed with the beads and placed on the magnetic stand to separate, the solution must be completely clear before the supernatant is discarded. There should be 2 - 3 μl left after the supernatant has been removed. Accidental removal of the magnetic beads with the supernatant can result in reduced yield or poor selection effect, and may even influence subsequent enzyme reactions. After discarding the supernatant, the magnetic beads can then be mixed well again and placed back on the magnetic stand for further separation. Due to different attractive forces of magnetic stands and other reasons, the default separation duration may sometimes have to be prolonged to separate the magnetic beads thoroughly from the solution.

g. The beads washing should be performed using 80% ethanol that is freshly prepared and equilibrated to room temperature. The centrifuge tube should always be placed on the magnetic stand during the wash process, and care should be taken not to disturb the magnetic beads. After the magnetic beads are washed, residual ethanol should be completely removed as much as possible.

h. The beads should be allowed to dry at room temperature before elution. Insufficient drying can lead to residual absolute ethanol that will affect the subsequent reaction. Overdrying can lead to the cracking of magnetic beads, which reduces the purification yield. Usually, the magnetic beads can be thoroughly dried at room temperature for 3 - 5 min. Do not dry the magnetic beads by heating, for example, at 37℃ in an oven.

06-3/Size Selection

1. The size selection steps are not included in the standard experimental protocol. Sometimes it is necessary to choose between the library size distribution (with two rounds of size selection) and the library complexity (without two rounds of size selection).

2. Over-amplification typically results in the observation of higher molecular weight trailing band or tail peaks in the electrophoresis-based profiles of amplified libraries. The corresponding products are mostly non-complementary strand cross-annealing products (refer to 06-4/Library Amplification), which have no significant impact on sequencing. The recommended solution is to adjust the number of amplification cycles to avoid over-amplification. It is not recommended to remove trailing band or tail peaks by size selection.

06-4/Library Amplification

1. The 3' Adapter and 5' Adapter of this kit are incomplete adapters. The Index and P5/P7 sequences need to be introduced into the library molecules through primer amplification in the subsequent Library Amplification step.

2. Vazyme #N351-N354 or Vazyme #N321/N322 are recommended as the primer containing Index during library amplification. Selection is made according to different usage requirements and the number of pooling samples:

   VAHTS Dual UMI UDI Adapters Set 1 - Set 4 for Illumina (Vazyme #N351-N354).

   #N351-N354 are 96 kinds of primer combinations with 8 bp Unique Indexed adapter at both ends of each primer, with 24 kinds for each catalog number. The 96 indexes at i5 end and i7 end of this series are unique, which can effectively reduce the data pollution caused by Index hopping and facilitate the effective splitting of real data.

   VAHTS Multiplex Oligos Set 4 - Set 5 for Illumina (Vazyme #N321/N322).

   #N321/N322 are combinations of primers with 8 bp of non-Unique Indexed adapters at both ends of each primer, which can prepare libraries with up to 384 different Index combinations. It should be noted that the indexes of this series are combined. Actually, there are up to 16 indexes at the i5 end and up to 24 indexes at the i7 end. When used on NovaSeq and other sequencers that are prone to cause Index hopping, the impact caused by Index hopping cannot be accurately evaluated and the quality of offline data will be affected if only this series of indexes are used.

3. In the late stage of PCR reactions, primers are usually depleted before dNTP. At this point, too many cycles can cause non-specific annealing of the amplification products after denaturing, resulting in non-complementary strand cross-annealing products. These products migrate slower and show diffuse distribution in higher molecular weight bands in electrophoresis-based analysis. They are made up of single-stranded libraries with correct sizes, and can normally bind to Flow Cell and be sequenced after denaturation. Therefore, their presence or absence does not have a significant impact on sequencing. However, the existence of such products has a decisive impact on the choice of the library's quantification methods. As the products do not have a complete double-stranded structure, when library quantification is performed using fluorescent dye (Equalbit 1 × dsDNA HS Assay Kit, Vazyme #EQ121, etc) for dsDNA identification, quantification results will be lower than actual values. However, the qPCR-based library quantification system (such as VAHTS Library Quantification Kit for Illumina, Vazyme #NQ101-NQ106) involves a denaturation process in the process of quantification, and therefore can still accurately quantify such over-amplified libraries.

4. The Library Amplification step requires strict control of the number of amplification cycles. An insufficient number of cycles will lead to insufficient library yield. In contrast, an excessive number of cycles will lead to various adverse effects such as over-amplification, amplification bias, increased duplications, increased chimeric products, and increased nucleotide substitutions. Refer to 08-5/Library Amplification for the number of amplification cycles.

06-5/Library Quality Control

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

1. Library size distribution analysis:

   Library size distribution analysis can be performed by devices based on the principle of electrophoretic separation, e.g., LabChip GX, GXII, GX Touch (PerkinElmer); Bioanalyzer, Tapestation (Agilent Technologies); Fragment Analyzer (Advanced Analytical).

2. Library concentration analysis:

   Precise determination of library concentration is recommended for high-quality sequencing results, and absolute quantification using a qPCR-based method is recommended, such as VAHTS Library Quantification Kit for Illumina (Vazyme #NQ101-NQ106). In addition, library concentrations can be measured using a fluorescent dye method based on specific recognition of dsDNA, such as the Qubit method. It is recommended to use the Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121).

06-6/Other Notes

1. Thaw all the kit Componentss at room temperature before use. After thawing, mix well by inverting a few times, then centrifuge briefly and place on ice.
2. During the preparation of reaction solution in each step, it is recommended to use a pipette for full mixing, as violent shaking may decrease library yield.
3. To avoid cross-contamination of samples, it is recommended to use tips with a filter and to replace the tip between samples.
4. It is recommended to use a PCR instrument with a heated lid when carrying out the reaction in each step. Preheat the PCR instrument close to the reaction temperature before use.
5. Prevent contamination of PCR products:
   a. Physically isolate the PCR reaction solution preparation area from the PCR product purification area.
   b. Use special pipette and other devices.
   c. Clean the experimental areas regularly (wipe with RNase and nucleic acid remover, such as Vazyme #R504).

07/Mechanism & Workflow

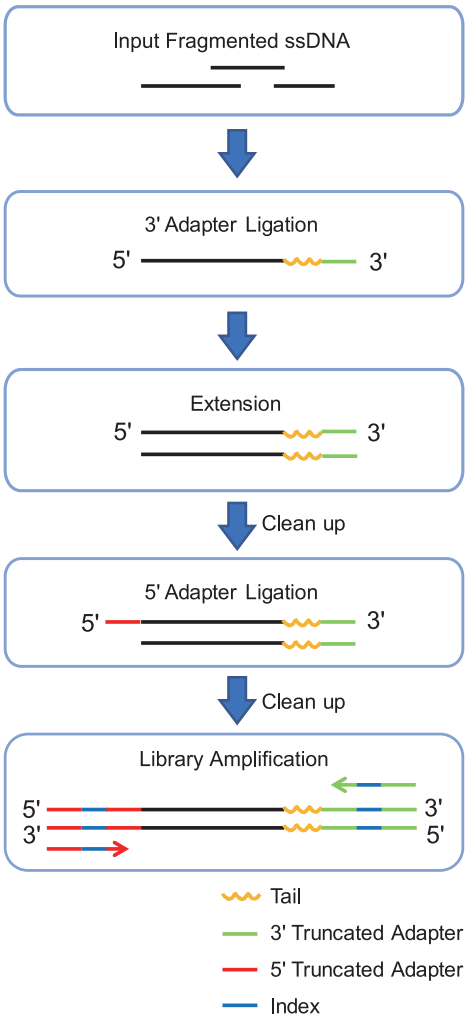

Fig 1. Library preparation principle with VAHTS ssDNA Library Prep Kit for Illumina

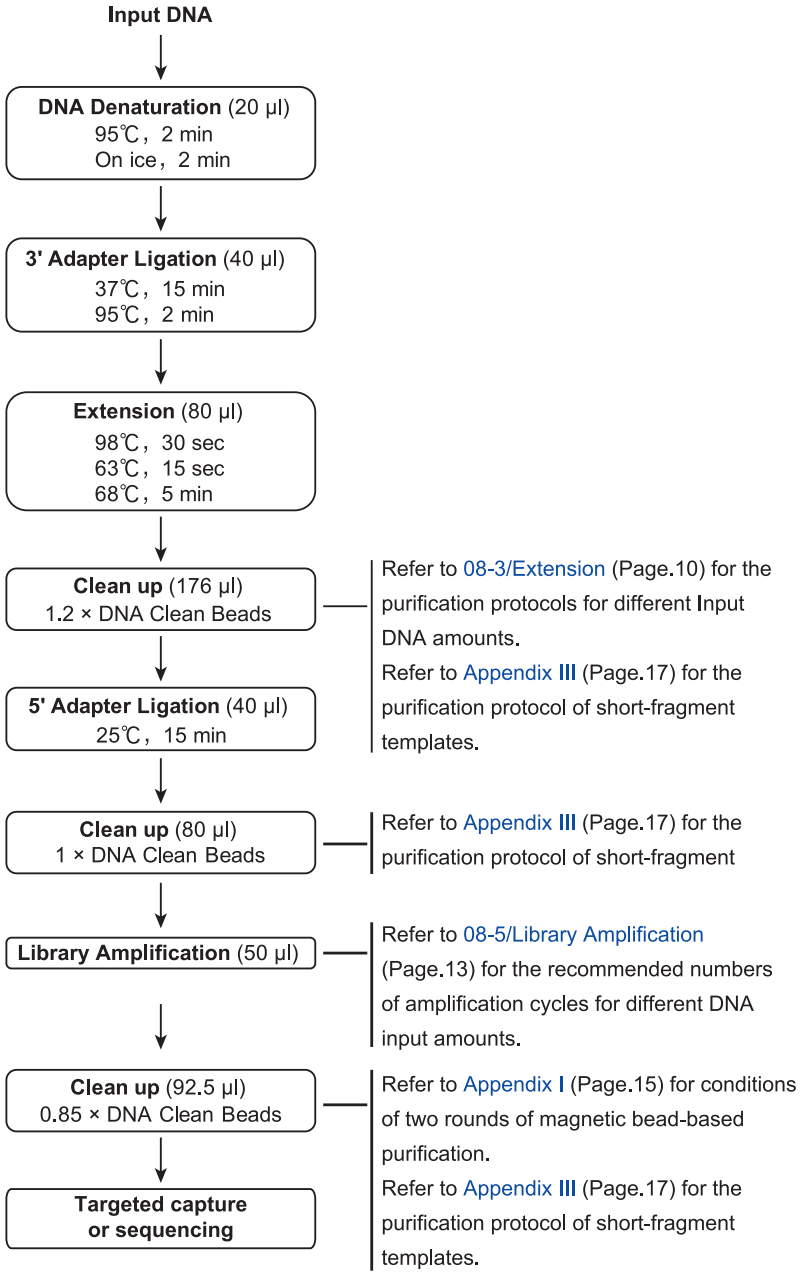

Fig 2. Workflow of VAHTS ssDNA Library Prep Kit for Illumina

08/Experiment Process

08-1/DNA Denaturation

In this step, dsDNA is denatured to become ssDNA.

1. Preheat the PCR instrument: set the heated lid temperature to 105℃ and the reaction temperature to 95℃. Prepare the master mix of 08-2/3' Adapter Ligation in an Nuclease-free PCR tube, gently mix well with a pipette, and put it on ice for use.
2. Prepare the following mixture in a new Nuclease-free PCR tube:

Components	Volume
Input DNA(10 pg - 250 ng)	x μl
Dilution Buffer	To 20 μl ☐

3. Heat at 95℃ for 2 min, quickly place on ice and leave to rest for 2 min.

▲ After DNA denaturation, immediately place the sample on ice to avoid the slow renaturation of denatured ssDNA.

08-2/3' Adapter Ligation

In this step, a truncated adapter is ligated to the 3' end of ssDNA.

1. Thaw 3' Ligation Buffer, 3' Ligation Enzyme Mix and 3' Adapter at room temperature, invert them and mix well for use.
2. Prepare the master mix of 3' Adapter Ligation in an Nuclease-free PCR tube:

▲ This master mix needs to be prepared before DNA denaturation, so that the denatured DNA can be subjected to 3' Adapter Ligation immediately.

Components	Volume
3' Ligation Buffer	10 μl
3' Ligation Enzyme Mix	5 μl
3' Adapter	5 μl
Total	20 μl

3. Mix 20 μl of the master mix of 3' Adapter Ligation with the denatured DNA by gently pipetting up and down, and briefly centrifuge until the reaction solution has sunk to the bottom of the tube.
4. Place the PCR tube in the PCR instrument and perform the following reaction:

Temperature	Time
Heating lid at 105℃	On
37℃	15 min
95℃	2 min
4℃	Hold

08-3/Extension

In this step, the ssDNA with a truncated adapter at the 3' end is extended into a complete double strand by extension of primers.

1. Thaw Extension Primer and Extension Enzyme Mix at room temperature, invert them to mix well for use.

2. Prepare the following mixture in an Nuclease-free PCR tube:

Components	Volume	
Reaction solution from the previous step	40 μl	
Extension Primer	5 μl	■
Extension Enzyme Mix	35 μl	■
Total	80 μl	

3. Mix by gently pipetting up and down and briefly centrifuge until the reaction solution has sunk to the bottom of the tube.

4. Place the PCR tube in the PCR instrument and perform the following reaction:

Temperature	Time
Heating lid at 105℃	On
98℃	30 sec
63℃	15 sec
68℃	5 min
4℃	Hold

5. Purify the reaction product using 1.2 × VAHTS DNA Clean Beads after the reaction has been completed:

▲ If the template as short as 40 bp needs to be retained, refer to **Appendix III: Short Fragment Template Purification Protocol** for purification method.

a. After equilibrating magnetic beads to room temperature, fully mix VAHTS DNA Clean Beads by inversion or vortex.

b. Pippet 96 μl of VAHTS DNA Clean Beads to 80 μl of extension product. Mix well by vortexing or pipetting up and down 10 times.

c. Incubate for 5 min at room temperature.

d. Briefly centrifuge the PCR tube and put it on a magnetic stand. When the solution becomes clear (about 5 min), carefully remove the supernatant.

e. Keep the PCR tube always on the magnetic stand, and slowly add 200 μl of freshly prepared 80% ethanol (equilibrated to room temperature) to wash the magnetic beads. Incubate for 30 sec at room temperature, and carefully remove the supernatant.

f. Repeat step e to wash the magnetic beads twice in total.

g. Keep the PCR tube always on the magnetic stand, and open the lid to air-dry the magnetic beads for 3 - 5 min until there is no ethanol residue.

▲ Due to the difference in the degree of dryness and humidity in different regions, the drying time of magnetic beads varies. When the beads are just dried, the surface changes from shiny brown to frosted brown. Overdrying will lead to difficulty in elution, and incomplete drying will leave alcohol residue to affect subsequent experimental reactions.

h. Remove the PCR tube from the magnetic stand for the elution step:

▲ In case of Input DNA ≥1 ng, add 22.5 μl of the Dilution Buffer (equilibrated to room temperature) into the PCR tube, and mix well by vortexing or gently pipetting up and down. Incubate at room temperature for 5 min. Briefly centrifuge the PCR tube, and leave to stand on the magnetic stand. When the solution becomes clear (about 5 min), carefully transfer 20 μl of supernatant into a new Nuclease-free PCR tube without disturbing the magnetic beads.

▲ In case of Input DNA <1 ng, add 52.5 μl of the Dilution Buffer (equilibrated to room temperature) into the PCR tube, and mix well by vortexing or gently pipetting up and down. Incubate at room temperature for 5 min. Briefly centrifuge the PCR tube, and leave to stand on the magnetic stand. When the solution becomes clear (about 5 min), carefully transfer 50 μl of supernatant into a new Nuclease-free PCR tube without disturbing the magnetic beads. Purify the supernatant using 1.2 × VAHTS DNA Clean Beads (60 μl) (repeat steps b - g). Finally, add 22.5 μl of Dilution Buffer (equilibrated to room temperature) and carefully transfer 20 μl of supernatant into a new Nuclease-free PCR tube for subsequent steps.

▲ In this step, the sample can be temporarily stored at -20℃ for 24 h.

08-4/5' Adapter Ligation

In this step, a truncated adapter is ligated to the 5' end of the original template chain of the extended dsDNA.

1. Thaw 5' Ligation Mix and 5' Adapter at room temperature, invert them and mix well for use.

2. Prepare the following mixture in an Nuclease-free PCR tube:

Components	Volume	
The purification product from previous step	20 μl	
5' Ligation Mix	10 μl	■
5' Adapter	10 μl	■
Total	40 μl	

▲ Do not mix 5' Ligation Mix and 5' Adapter in advance to avoid self-connection of adapters.

3. Mix by gently pipetting up and down and briefly centrifuge until the reaction solution has sunk to the bottom of the tube.

4. Place the PCR tube in the PCR instrument and perform the following reaction:

Temperature	Time
Heating lid at 105℃	On
25℃	15 min
4℃	Hold

5. Purify the reaction product using 1 × VAHTS DNA Clean Beads after the reaction has been completed:

▲ If the template as short as 40 bp needs to be retained, refer to **Appendix III: Short Fragment Template Purification Protocol** for purification method.

a. After equilibrating magnetic beads to room temperature, fully mix VAHTS DNA Clean Beads by inversion or vortex.

b. Add 40 μl of VAHTS DNA Clean Beads to 40 μl of 5' Adapter Ligation product, and mix well by vortexing or gently pipetting up and down 10 times.

c. Incubate for 5 min at room temperature.

d. Briefly centrifuge the PCR tube and place it on a magnetic stand. When the solution becomes clear (about 5 min), carefully remove the supernatant.

e. Keep the PCR tube always on the magnetic stand, and slowly add 200 μl of freshly prepared 80% ethanol (equilibrated to room temperature) to wash the magnetic beads. Incubate for 30 sec at room temperature, and carefully remove the supernatant.

f. Repeat step e to wash the magnetic beads twice in total.

g. Keep the PCR tube always on the magnetic stand, and open the lid to air-dry the magnetic beads for 3 - 5 min until there is no ethanol residue.

▲ Due to the difference in the degree of dryness and humidity in different regions, the drying time of magnetic beads varies. When the beads are just dried, the surface changes from shiny brown to frosted brown. Overdrying will lead to difficulty in elution, and incomplete drying will leave alcohol residue to affect subsequent experimental reactions.

h. Remove the PCR tube from the magnetic stand for elution: add 22.5 μl of the Dilution Buffer (equilibrated to room temperature) into the PCR tube, and mix well by vortexing or gently pipetting up and down. Incubate at room temperature for 5 min. Briefly centrifuge the PCR tube, and leave to stand on the magnetic stand. When the solution becomes clear (about 5 min), carefully transfer 20 μl of supernatant into a new Nuclease-free PCR tube without disturbing the magnetic beads.

▲ In this step, the sample can be temporarily stored at -20℃ for 24 h.

08-5/Library Amplification

In this step, the complete library can be obtained by primer amplification.

1. Thaw VAHTS HiFi Amplification Mix V1 at room temperature and mix well by inverting.

2. Prepare the following mixture in an Nuclease-free PCR tube:

Components	Volume
The purification product from previous step	20 μl
Index Primers*	5 μl
VAHTS HiFi Amplification Mix V1	25 μl
Total	50 μl

*Index Primers are amplification primers containing Index. If VAHTS Dual UMI UDI Adapters Set 1 - Set 4 for Illumina (Vazyme #N351-N354) is used, the volume of UDIXX is 5 μl. 24 kinds of UDIXX are provided for each of catalog number from #N351-N354. If VAHTS Multiplex Oligos Set 4 - Set 5 for Illumina (Vazyme #N321/N322) is used, the volume of either DM5XX or DM7XX is 2.5 μl. #N321/#N322 provides 8 kinds of DM5XX and 12 kinds of DM7XX respectively, which can be selected according to the number of samples and Index matching strategy.

3. Mix by gently pipetting up and down and briefly centrifuge until the reaction solution has sunk to the bottom of the tube.

4. Place the PCR tube in the PCR instrument and perform the following reaction:

Temperature	Time	Cycles
Heating lid at 105℃	On	-
98℃	45 sec	1
98℃	15 sec	2 - 21
60℃	30 sec	
72℃	30 sec	
72℃	1 min	1
4℃	Hold	-

For number of amplification cycles, refer to the table below. The table below specifies the recommended number of amplification cycles to obtain a 100 ng or 1 μg of library when using 10 pg - 250 ng of high-quality Input DNA.

Input DNA (Into 3' Adapter Ligation)	Numbers of cycles required to generate 100 ng	1 μg
10 pg	16 - 18	19 - 21
100 pg	13 - 15	16 - 18
1 ng	10 - 12	13 - 15
10 ng	7 - 9	10 - 12
50 ng	5 - 7	8 - 10
100 ng	3 - 5	6 - 8
250 ng	2 - 4	5 - 7

▲ The above table specifies the cycle number parameter, which is measured when about 250 bp high-quality Input DNA is used. When DNA quality is poor or library size is large, the number of cycles should be appropriately increased in order to obtain sufficient amount of libraries.

5. Purify the reaction product using 0.85 × VAHTS DNA Clean Beads after the reaction has been completed:

▲ For a sequencer based on Patterned Flow Cell, it is recommended to carry out two rounds of magnetic bead purification (refer to **Appendix I: Two Rounds of Magnetic Bead Purification**) to remove residual primers and reduce Index hopping.

▲ If the template as short as 40 bp needs to be retained, refer to **Appendix III: Short Fragment Template Purification Protocol** for purification method.

a. After equilibrating magnetic beads to room temperature, fully mix VAHTS DNA Clean Beads by inversion or vortex.

b. Add 42.5 μl of VAHTS DNA Clean Beads to 50 μl of Library Amplification solution. Mix well by vortexing or pipetting up and down 10 times.

c. Incubate for 5 min at room temperature.

d. Briefly centrifuge the PCR tube and place it on a magnetic stand. When the solution becomes clear (about 5 min), carefully remove the supernatant.

e. Keep the PCR tube always on the magnetic stand, and slowly add 200 μl of freshly prepared 80% ethanol (equilibrated to room temperature) to wash the magnetic beads. Incubate for 30 sec at room temperature, and carefully remove the supernatant.

f. Repeat step e to wash the magnetic beads twice in total.

g. Keep the PCR tube always on the magnetic stand, and open the lid to air-dry the magnetic beads for 3 - 5 min until there is no ethanol residue.

▲ Due to the difference in the degree of dryness and humidity in different regions, the drying time of magnetic beads varies. When the beads are just dried, the surface changes from shiny brown to frosted brown. Overdrying will lead to difficulty in elution, and incomplete drying will leave alcohol residue to affect subsequent experimental reactions.

h. Remove the PCR tube from the magnetic stand for elution: add 22.5 μl of the Dilution Buffer (equilibrated to room temperature) into the PCR tube, and mix well by vortexing or gently pipetting up and down. Incubate at room temperature for 5 min. Briefly centrifuge the PCR tube, and leave to stand on the magnetic stand. When the solution becomes clear (about 5 min), carefully transfer 20 μl of supernatant into a new Nuclease-free PCR tube without disturbing the magnetic beads.

▲ In this step, the sample can be temporarily stored at -20℃ and long-term storage must be at -80℃ .Avoid unnecessary repeated freezing and thawing.

08-6/Library Quality Control

Refer to 06-5/Library Quality Control.

Appendix I: Two Rounds of Magnetic Bead Purification

1. For a sequencer based on Patterned Flow Cell, it is recommended to carry out two rounds of magnetic bead purification to remove primer residue and reduce the impact from Index hopping on correct data splitting.

2. See the following table for the volume of the two rounds of magnetic beads used in the two rounds of magnetic bead purification for library amplification product:

Number of purification rounds	Sample volume	Magnetic bead volume
1st round of purification	50 μl	42.5 μl (0.85 ×)
2nd round of purification	50 μl	42.5 μl (0.85 ×)

3. Purification protocol with two rounds of magnetic bead purification:

   a. After equilibrating magnetic beads to room temperature, fully mix VAHTS DNA Clean Beads by inversion or vortex.

   b. Add 42.5 μl (0.85 ×) of VAHTS DNA Clean Beads to 50 μl of Library Amplification solution. Mix well by vortexing or pipetting up and down 10 times.

   c. Incubate for 5 min at room temperature.

   d. Briefly centrifuge the PCR tube and place it on a magnetic stand. When the solution becomes clear (about 5 min), carefully remove the supernatant.

   e. Keep the PCR tube always on the magnetic stand, and slowly add 200 μl of freshly prepared 80% ethanol (equilibrated to room temperature) to wash the magnetic beads. Incubate for 30 sec at room temperature, and carefully remove the supernatant.

   f. Repeat step e to wash the magnetic beads twice in total.

   g. Keep the PCR tube always on the magnetic stand, and open the lid to air-dry the magnetic beads for 3 - 5 min until there is no ethanol residue.

   ▲ Due to the difference in the degree of dryness and humidity in different regions, the drying time of magnetic beads varies. When the beads are just dried, the surface changes from shiny brown to frosted brown. Overdrying will lead to difficulty in elution, and incomplete drying will leave alcohol residue to affect subsequent experimental reactions.

   h. Remove the PCR tube from the magnetic stand for elution: add 52.5 μl of the Dilution Buffer (equilibrated to room temperature) into the PCR tube, and mix well by vortexing or gently pipetting up and down. Incubate at room temperature for 5 min. Briefly centrifuge the PCR tube, and leave to stand on the magnetic stand. When the solution becomes clear (about 5 min), carefully transfer 50 μl of supernatant into a new Nuclease-free PCR tube without disturbing the magnetic beads.

   i. Add 42.5 μl (0.85 ×) of VAHTS DNA Clean Beads to the purification product in the previous step. Mix well by vortexing or pipetting up and down 10 times.

   j. Incubate for 5 min at room temperature.

   k. Briefly centrifuge the PCR tube and place it on a magnetic stand. When the solution becomes clear (about 5 min), carefully remove the supernatant.

   l. Keep the PCR tube always on the magnetic stand, and slowly add 200 μl of freshly prepared 80% ethanol (equilibrated to room temperature) to wash the magnetic beads. Incubate for 30 sec at room temperature, and carefully remove the supernatant.

   m. Repeat step l to wash the magnetic beads twice in total.

   n. Keep the PCR tube always on the magnetic stand, and open the lid to air-dry the magnetic beads for 3 - 5 min until there is no ethanol residue.

   ▲ Due to the difference in the degree of dryness and humidity in different regions, the drying time of magnetic beads varies. When the beads are just dried, the surface changes from shiny brown to frosted brown. Overdrying will lead to difficulty in elution, and incomplete drying will leave alcohol residue to affect subsequent experimental reactions.

   o. Remove the PCR tube from the magnetic stand for elution: add 22.5 μl of the Dilution Buffer (equilibrated to room temperature) into the PCR tube, and mix well by vortexing or gently pipetting up and down. Incubate at room temperature for 5 min. Briefly centrifuge the PCR tube, and leave to stand on the magnetic stand. When the solution becomes clear (about 5 min), carefully transfer 20 μl of supernatant into a new Nuclease-free PCR tube without disturbing the magnetic beads.

Appendix Ⅱ: Sequencing Notes and Data Output Preprocessing

1. As an additional Tail structure is added in the 3' Adapter Ligation step, the Tail structure is of low complexity and may lead to poor data quality.It is important to assess the need of no-less than 25% of PhiX or other balanced, high-complexity library spike-ins.
2. For the additional Tail structure added in the 3' Adapter Ligation step, it is recommended to perform reads trim before sequence alignment: when the inserted fragment is greater than the read size, it is only necessary to trim the first 10 bases at the beginning (5' end) of Read 2; when the inserted fragment is less than the read size, trim the first 10 bases at the beginning (5' end) of Read 2 and the end (3' end) of Read 1.

Appendix Ⅲ: Short Fragment Template Purification Protocol

1. When using a short fragment template to prepare a library, it is necessary to modify the standard VAHTS ssDNA Library Prep Kit for Illumina step and use different amounts of magnetic beads to ensure that the retained DNA fragments are as short as 40 bp.
2. When performing different purification steps, refer to the following table for the amount of magnetic beads used:

Steps	Sample volume	Magnetic bead volume	Elution volume
Purification after Extension	80 μl	144 μl (1.8 ×)	22.5 μl
Purification after 5' Adapter Ligation	40 μl	64 μl (1.6 ×)	22.5 μl
Purification after Library Amplification	50 μl	80 μl (1.6 ×)	22.5 μl