EpiArt® DNA Methylation Library Kit for Illumina V3

NE103

Vazyme

Instructions for Use

Version 22.1

Contents

01/Product Description

EpiArt DNA Methylation Library Kit for Illumina V3 is a dedicated methylation library preparation kit developed specifically for the Illumina high-throughput sequencing platform. Based on efficient single-strand ligation technology, this kit can convert 10 pg to 250 ng of methylated DNA into a dedicated library especially for the Illumina platform and is compatible with DNA samples as short as 40 bp. With the optimized library preparation process, library preparation can be completed within 2 hours and the manual preparation time is less than 30 minutes. This kit 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/Components

	Component	NE103-01 (24 rxns)	NE103-02 (96 rxns)
BOX1	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	360 µl	2 × 720 µl
	5' Adapter	120 µl	480 µl
	VAHTS HiFi Amplification Mix V3	600 µl	4 × 600 µl

03/Storage

Box 1, store at 2 to 8°C and adjust the transportation method according to destination;
Box 2, store at -30 to -15°C and transport at ≤ 0°C.

04/Applications

This product is suitable for preparing a dedicated library for Illumina high-throughput sequencing platform and is compatible with DNA templates after methylation. It is suitable for a variety of sample types: genomic DNA, FFPE DNA, ChIP DNA, cfDNA, ctDNA, microbial DNA etc. It is compatible with DNA samples as short as 40 bp, as well as input DNA of 10 pg–250 ng.

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 is a primer combination of 96 kinds of double-end 8 bp Unique Indexed adapter, and each item number contains 24 kinds.
VAHTS Multiplex Oligos Set 4 - Set 5 for Illumina (Vazyme #N321/N322);
#N321/N322 is a primer combination of double-end 8 bp non-Unique Indexed adapters, 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 equivalent;
◇ Other materials: 80% ethanol (freshly prepared), 0.1 × TE (pH 8.0), nuclease-free ddH_2O, magnetic rack, PCR thermal cycler, low adsorption nuclease-free PCR tubes, pipette tips and centrifuge tubes, etc.

06/Notes

Due to a number of factors such as the sample type, plan, equipment and operations, it may be necessary to adjust the process parameters for the library preparation 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 the process, please contact Vazyme technical support for help: support@vazyme.com

06-1/Input DNA and Fragmentation

1. The kit is compatible with input DNA amounts ranging from 10 pg to 250 ng. Accurate quantification of input DNA is very important for subsequent selection of library amplification cycle number. Qubit ssDNA assay kit (Invitrogen #q10212) is recommended for ssDNA quantification; and Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121) for dsDNA quantification.
   ▲ Input DNA amount refers to the amount of DNA input in the 3' adapter ligation step. If the DNA sample is pretreated, its concentration needs to be determined again. The amount of DNA before methylation cannot be directly taken as the input DNA amount. Otherwise, library yield may be low due to an insufficient number of library amplification cycles.
2. If the DNA sample is seriously fragmented, like ChIP DNA and cfDNA, there is no need for fragmentation.
3. If the DNA sample is genomic DNA with good integrity, fragmentation is recommended. Ultrasonication or enzyme digestion can be selected to fragment genomic DNA. When using ultrasonication for fragmentation, please dilute the DNA in 0.1 × TE (pH 8.0) for fragmentation, rather than in sterilized ddH_2O. For methylated DNA, ensure that no high-concentration metal ion chelating agent or other salts are introduced into the eluent. Otherwise, the efficiency of the 3' ligation step may be affected. If the above conditions cannot be met, it is acceptable to first dissolve the purified product in 0.1 ×TE (pH 8.0) (<22.5 μl) 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 from any other source are used, it may be required to make some changes to the purification conditions!
2. General notes for magnetic bead operations:
   a. The number of magnetic beads used is generally marked by multiplier "×," which is the volume factor of magnetic beads used relative to the original sample volume. For example, if the original volume of sample is 100 μl, 1 × beads used for purification is 1 × 100 μl = 100 μl. 0.6 ×/0.2 × size selection means the volume of magnetic beads used in the first round is 0.6 × 100 μl = 60 μl, and the volume of magnetic beads used 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 first be equilibrated to room temperature (by placing them at room temperature for 30 min). Otherwise, the yield will be decreased and the selection effect will be poor;
   d. All operations with magnetic beads should take place at room temperature. Do not freeze the magnetic beads to a temperature below 0°C;
   e. The beads should be fully vortexed or fully 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 removed. There should be 2–3 μl left after it has been removed. Accidentally removing the magnetic beads along with it can result in reduced yields or poor selection effect, and may even cause subsequent enzyme reactions. After removing 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 racks and other reasons, the default separation time may have to be prolonged sometimes in order to separate the magnetic beads thoroughly from the solution;
   g. The bead rinse should be performed using 80% ethanol that is freshly prepared and equilibrated to room temperature. The EP tube should always be placed in the magnetic stand during the rinse process and care should be taken to not disturb the magnetic beads. After the magnetic beads are washed, residual ethanol should be completely drawn 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. Excessive drying can cause the magnetic beads to crack, which reduces the purification yield rate. Usually, the magnetic beads can be thoroughly dried at room temperature for 3 to 5 min. Do not heat the magnetic beads to dry them, for example, drying at 37°C in an oven.

06-3/Size Selection

1. The size selection steps are not included in the standard experiment protocol. If necessary, it can be selected 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 trailing band or tail peaks appeared at the high molecular weight position. The corresponding products are mostly non-complementary strand cross-annealing products (see 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 bank or tail peak 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 is 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 is a primer combination of 96 kinds of double-end 8 bp Unique Indexed adapter, and each item number contains 24 kinds 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 is a primer combination of double-end 8 bp non-Unique Indexed adapters, which can be used to prepare libraries with up to 384 different index combinations. It should be noted that this series of indexes are combined. In fact, there are at most 16 indexes at the i5 end and 24 indexes at the i7 end. When used on NovaSeq are prone to 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 later stage of PCR reaction, primers are usually depleted before dNTPs. At this point, too many cycles can cause non-specific annealing of the amplification product after denaturing, resulting in non-complementary strand cross-annealing products. These products migrate slower and show diffuse distribution in higher molecular weight bands in electrophoretic analysis. They are made up of single-stranded libraries with correct sizes, and can normally bind to flow cells and be sequenced after denaturation. Therefore, their presence or absence does not have a significant impact on sequencing. However, the existence of such a product has a decisive impact on the library's quantification methods. As the product does not have a complete double-stranded structure, when library quantification is performed using fluorescent dye (Equalbit 1 × dsDNA HS Assay Kit, Vazyme #EQ121) for double-stranded DNA identification, the results of quantification will be lower than the 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, but 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 an insufficient library yield, while an excessive number of cycles will lead to various adverse effects such as over-amplification, increased amplification bias, increased duplications, chimeric products, and increased amplification mutations. Refer to 08-5/Library Amplification for the number of amplification cycles.

06-5/Library Quality Control

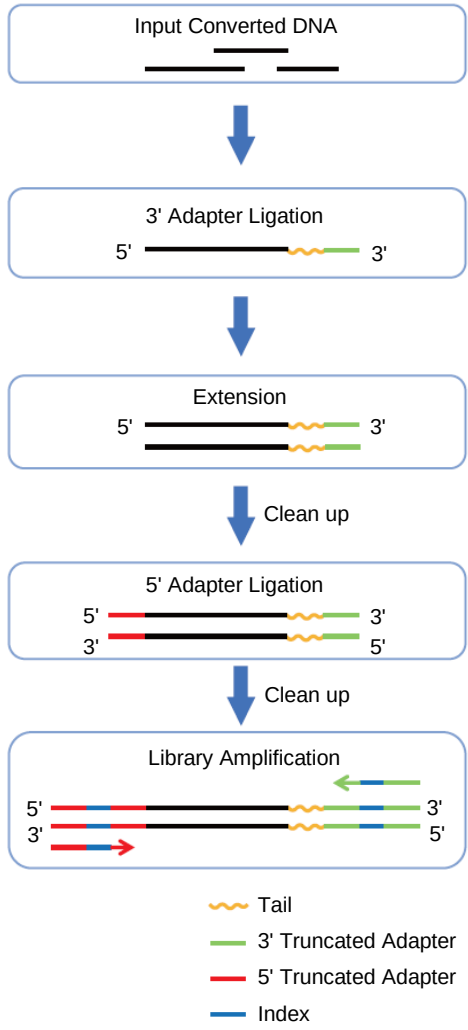
Typically, a well-constructed library can be evaluated through size distribution and concentration analysis.

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); and 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 double-stranded DNA, such as the Qubit method. It is recommended to use the Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121).

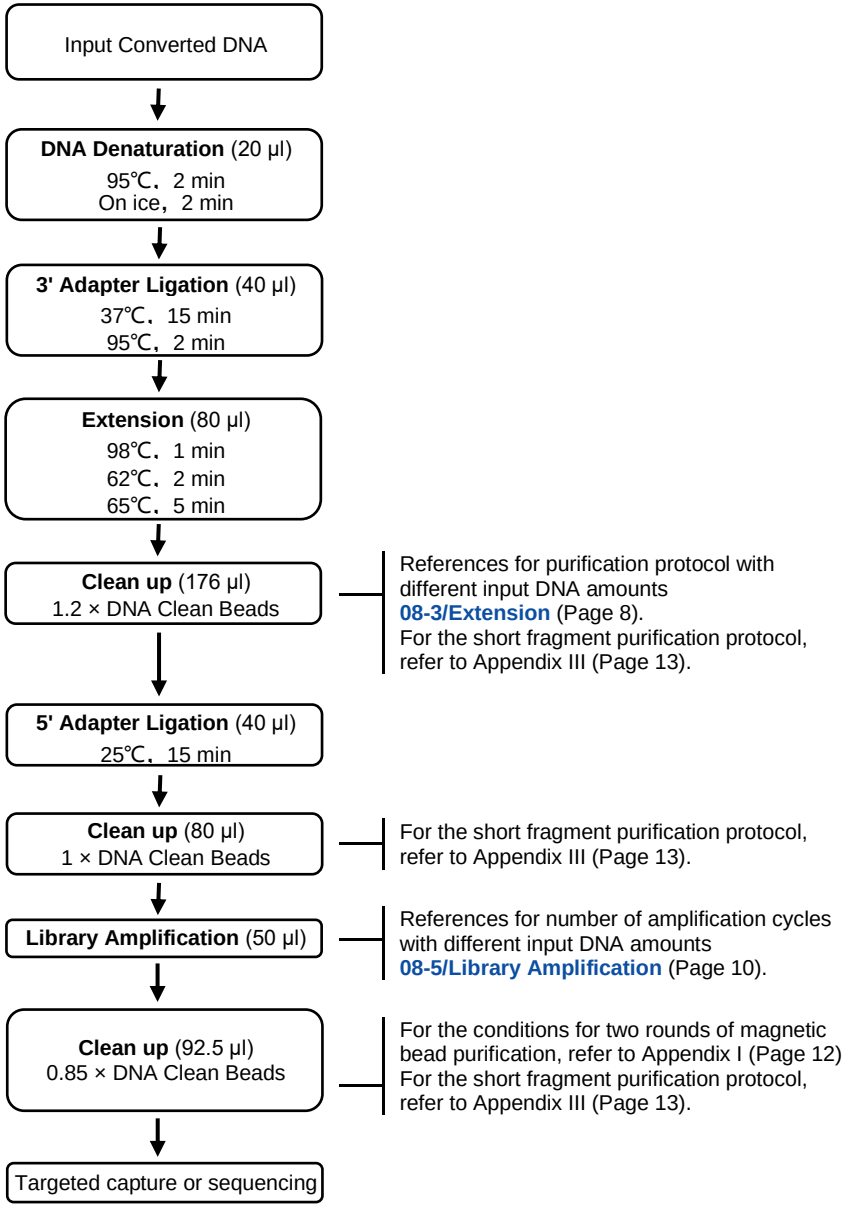
06-6/Further Notes

1. Thaw all the kit components at room temperature before use. After thawing, mix well by inverting a few times, then centrifuge briefly and place on ice.
2. During 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 machine with a heated lid when carrying out the reaction in each step. Preheat the PCR machine 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 (Vazyme #R504)).

07/Mechanism & Workflow



Library preparation principle with EpiArt DNA Methylation Library Kit for Illumina V3



Outline of library preparation process with EpiArt DNA Methylation Library Kit for Illumina V3

08/Experiment Process

08-1/DNA Denaturation

In this step, Input DNA is denatured to become single-stranded DNAs.

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

Component	Volume	
Input DNA (10 pg - 250 ng)	× 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 single-stranded DNA.

08-2/3' Adapter Ligation

In this step, a truncated adapter is ligated to the 3' end of single-stranded DNA.

1. Thaw 3' Ligation Buffer, 3' Ligation Enzyme Mix and 3' Adapter at room temperature, invert the tube and mix well for use.
2. Prepare the master mix of 3' Adapter Ligation in a 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.

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

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

Temperature	Time
Heated lid 105°C	On
37°C	15 min
95°C	2 min
4°C	Hold

08-3/Extension

In this step, the single-stranded DNA with a truncated adapter at the 3' end is extended into a complete double-stranded DNA by primer extension.

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

2. Prepare the following mixture in a nuclease-free PCR tube:

Component	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 machine and perform the below reaction:

Temperature	Time
Heated lid 105°C	On
98°C	1 min
62°C	2 min
65°C	5 min
4°C	Hold

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

▲ If a short-fragment template needs to be retained, refer to **Appendix III**: Short Fragment Template Purification Protocol for purification method.

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

b. Pipette 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 minutes at room temperature;

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

e. Keep the PCR tube always on the magnetic stand and slowly add 200 μL 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 in the magnetic stand and open the lid to air-dry the magnetic beads for 3 to 5 min until there is no ethanol residue;

▲ Due to the difference in the degree of dryness and humidity in different regions, the time for the magnetic beads to dry will vary. 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 residual alcohol that will affect subsequent experimental reactions.

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

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

1. Thaw 5' Ligation Mix and 5' Adapter at room temperature, invert them and mix well for later use.
2. Prepare the following mixture in a nuclease-free PCR tube:

Component	Volume
The purification product from previous step	20 μl
5' Ligation Mix	15 μl
5' Adapter	5 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 machine and perform the below reaction:

Temperature	Time
Heated lid 105°C	Off
25°C	15 min
4°C	Hold

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

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

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

b. Add 40 μl VAHTS DNA Clean Beads to 40 μl 5' adapter ligation product and mix well by vortexing or gently pipetting up and down 10 times;

c. Incubate for 5 minutes at room temperature;

d. Briefly centrifuge the PCR tube and place it in a magnetic rack. When the solution becomes clear (approximately 5 min), carefully remove the supernatant;

e. Keep the PCR tube always on the magnetic rack, and slowly add 200 μL freshly prepared 80% ethanol (conditioned 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 in the magnetic rack, and open the lid to air-dry the magnetic beads for 3 to 5 min until there is no ethanol residue;

▲ Due to the different dryness degree in different regions, the time for the magnetic beads to dry will vary. 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 residual alcohol that will affect subsequent experimental reactions.

h. Remove the PCR tube from the magnetic rack for elution: Add 22.5 μl of Dilution Buffer (equilibrated to room temperature) into the PCR tube and mix well by vortexing or gently pipetting. Incubate at room temperature for 5 minutes. Briefly centrifuge the PCR tube and leave it stand. When the solution becomes clear (about 5 min), carefully transfer 20 μl of supernatant into a new nuclease-free PCR tube without touching 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 V3 and mix well by inverting.
2. Prepare the following mixture in a nuclease-free PCR tube:

Component	Volume
The purification product from previous step	20 μl
Index Primers*	5 μl
VAHTS HiFi Amplification Mix V3	25 μl
Total	50 μl

*Index primers are amplification primers containing an index sequence. 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 UDIXX0 are provided for each catalog no. from N351 to 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 machine and perform the below reaction:

Temperature	Time	Number of cycles
Heated lid 105°C	On	-
95°C	3 min	1
98°C	20 sec	2–23
60°C	15 sec	
72°C	30 sec	
72°C	5 min	1
4°C	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	18 - 19	21 - 23
100 pg	13 - 15	18 - 20
1 ng	10 - 12	14 - 16
10 ng	7 - 9	10 - 12
50 ng	5 - 7	8 - 10
100 ng	3 - 5	7 - 8
250 ng	2 - 4	5 - 7

- ▲ The above table specifies the cycle numbers which is measured with high-quality input DNA. When DNA quality is poor or library size is large, the number of cycles should be appropriately increased in order to obtain a sufficient number of libraries.

5. Purify the reaction product using 0.85 × VAHTS DNA Clean Beads after the reaction has 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 of short fragments needs to be retained, refer to **Appendix III**: Short Fragment Template Purification Protocol for purification method.

   a. After equilibrating the magnetic beads to room temperature, fully mix VAHTS DNA Clean Beads by inverting or vortexing;

   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 minutes at room temperature;

   d. Briefly centrifuge the PCR tube and place it in a magnetic rack. When the solution becomes clear (approximately 5 min), carefully remove the supernatant;

   e. Keep the PCR tube always on the magnetic rack, 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 in the magnetic rack, and open the lid to air-dry the magnetic beads for 3 to 5 min until there is no ethanol residue;
      - ▲ Due to the difference in the degree of dryness and humidity in different regions, the time for the magnetic beads to dry will vary. 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 residual alcohol that will affect subsequent experimental reactions.

   h. Remove the PCR tube from the magnetic rack 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 minutes. Briefly centrifuge the PCR tube, and leave to stand on the magnetic rack. When the solution becomes clear (about 5 min), carefully transfer 20 μl of supernatant into a new nuclease-free PCR tube without touching the magnetic beads;
      - ▲ In this step, the sample can be temporarily stored at -20℃. Long-term storage must be at -80°C to avoid unnecessary repeated freezing and thawing.

08-6/Library Quality Control

See 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 slightly or pipetting up and down 10 times.
   c. Incubate for 5 minutes at room temperature;
   d. Briefly centrifuge the PCR tube and place it in a magnetic rack. When the solution becomes clear (approximately 5 min), carefully remove the supernatant;
   e. Keep the PCR tube always on the magnetic rack 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 in the magnetic rack, and open the lid to air-dry the magnetic beads for 3 to 5 min until there is no ethanol residue;
      ▲ Due to the difference in the degree of dryness and humidity in different regions, the time for the magnetic beads to dry will vary. 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 residual alcohol that will affect subsequent experimental reactions.
   h. Remove the PCR tube from the magnetic rack 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 minutes. Briefly centrifuge the PCR tube, and leave to stand on the magnetic rack. When the solution becomes clear (about 5 min), carefully transfer 50 μl of supernatant into a new nuclease-free PCR tube without touching the magnetic beads;
   i. Add 42.5 μl (0.85 x) 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 minutes at room temperature;
   k. Briefly centrifuge the PCR tube and place it in a magnetic rack. When the solution becomes clear (approximately 5 min), carefully remove the supernatant;
   l. Keep the PCR tube always on the magnetic rack 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 I to wash the magnetic beads twice in total;
   n. Keep the PCR tube always in the magnetic rack, and open the lid to air-dry the magnetic beads for 3 to 5 min until there is no ethanol residue;
      ▲ Due to the difference in the degree of dryness and humidity in different regions, the time for the magnetic beads to dry will vary. 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 residual alcohol that will affect subsequent experimental reactions.

o. Remove the PCR tube from the magnetic rack 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 minutes. Briefly centrifuge the PCR tube and leave to stand on the magnetic rack. When the solution becomes clear (about 5 min), carefully transfer 20 μl of supernatant into a new nuclease-free PCR tube without touching the magnetic beads;

Appendix II: Sequencing Notes and Data Output Preprocessing

1. In the 3' adapter ligation step, an additional tail structure with low complexity is introduced and the bases are imbalanced in methylation library. Therefore, it is recommended to add at least 25% of Phix library or other libraries with high complexity for pooling and loading when sequencing.
2. For the additional tail structure added in the 3' adapter ligation step, it is recommended to trim reads before sequence alignment: When the inserted fragment is larger 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 smaller than the read size, it is necessary to trim the first 10 bases at the beginning (5' end) of Read 2 and the 10 bases at the end (3' end) of Read 1.

Appendix III:Short Fragment Template Purification Protocol

If a short-fragment template needs to be retained, the amount of magnetic beads used in the standard steps of EpiArt DNA Methylation Library Kit for Illumina V3 needs to be modified. Refer to the following table for the amount of beads to be used for purifying in various steps:

Step	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

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