

Ne  *Biotech*

EpiArt DNA Methylation
Library Kit for Illumina
V3

NB-54-0307-01

NB-54-0307-02

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EpiArt DNA Methylation Library Kit for Illumina V3

#Cat: NB-54-0307-01 Size: 24rxns

#Cat: NB-54-0307-02 Size: 96rxns

1/Product Description

EpiArt DNA Methylation Library Kit for Illumina V3 for Illumina is a methyl-seq DNA 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 10 pg - 250 ng of converted DNA into a sequencing library. It is compatible with DNA samples as short as 40 bp. The optimized library preparation process enables the library preparation to be completed within 2h, 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.

2/ Components

Components		NB-54-0307-01 (24 rxns)	NB-54-0307-02 (96 rxns)
BOX 1	<input type="checkbox"/> Dilution Buffer	5 ml	20 ml
	<input type="checkbox"/> 3' Ligation Buffer	240 µl	960 µl
	<input type="checkbox"/> 3' Ligation Enzyme Mix	120 µl	480 µl
	<input type="checkbox"/> 3' Adapter	120 µl	480 µl
BOX 2	<input type="checkbox"/> Extension Primer	120 µl	480 µl
	<input type="checkbox"/> Extension Enzyme Mix	840 µl	4 × 840 µl
	<input type="checkbox"/> 5' Ligation Mix	360 µl	2 × 720 µl
	<input type="checkbox"/> 5' Adapter	120 µl	480 µl
	<input type="checkbox"/> VAHTS HiFi Amplification Mix V3	600 µl	4 × 600 µl

3/ Storage

BOX 1, Store at 2~8°C, Adjust the shipping method according to the destination.

BOX 2, Store at -30~-15°C and transport at ≤0°C.

4/Applications

This product is suitable for the preparation of Illumina high-throughput sequencing platform-specific libraries and is compatible with converted DNA 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, 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.

5/Self-prepared Materials

- DNA quantification:
Equalbit 1x dsDNA HS Assay Kit (Neo Biotech# NB-54-0027)
Qubit ssDNA Assay kit
- Magnetic beads for purification:
VAHTS DNA Clean Beads (Neo Biotech # NB-54-0060)
- PCR Index Primers:
VAHTS Dual UMI UDI Adapters Set 1 -Set 4 for Illumina (Neo Biotech # NB-54-0229 - NB-54-0232)
NB-54-0229 - NB-54-0232 are 96 kinds of primer combinations with 8bp 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 (Neo Biotech # NB-54-0047 / NB-54-0048);
NB-54-0047 / NB-54-0048 are combinations of primers with 8bp of non-Uniqu indexed adapter at both ends of each primer, with can be used to prepare libraries with up to 384 different index combinations.
- Library quality control:
Equalbit 1x dsDNA HS Assay Kit (Neo Biotech# NB-54-0027)
VAHTS Library Quantification Kit for Illumina (Neo Biotech# NB-54-0110- NB-54-0115)
Agilent Technologies 2100 Bioanalyzer or its equivalent.
- Other materials:80% ethanol (freshly prepared), 0.1x TE (ph 8.0), Nuclease-free ddH₂O, magnetic stand, PCR instrument, low binding Nuclease-free PCR tube, Pipette tips and centrifuge tubes, etc.

6/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 Neo Biotech's technical support for help: info@neo-biotech.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 1x dsDNA HS Assay Kit (Neo Biotech# NB-54-0027) is recommended for quantification of double-stranded DNA (dsDNA) and Qubit ssDNA Assay Kit for single-stranded DNA (ssDNA).
 - ▲ Input DNA amount refers to the amount of DNA input for the 3' Adapter Ligation step. If the DNA sample is pretreated, the amount of DNA before conversion cannot be directly used as the Input DNA amount, and the DNA concentration should be re-assayed. Otherwise, the library yield may be low due to insufficient number of 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. When sonication is used for fragmentation, please dilute the DNA in 0.1 x TE (ph 8.0) for fragmentation. Please do not dilute in sterile ddH₂O. For converted DNA, operators should ensure that no high-concentration metal ion chelating agent or other salts are introduced into the eluent, otherwise the efficiency of the 3' adapter ligation step may be affected. 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 xTE (pH8.0) before library preparation.

6-2/ Use of magnetic beads

1. It is recommended to use VAHTS DNA Clean Beads (Neo Biotech # NB-54-0060) 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 "x" 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 100µl for 1x purification, the volume of magnetic beads used is 1x100µl=100µl if for 0.6x/0.2 selection, the volume of magnetic beads used in the first round is 0.6 x 100µl=60µl, and in the second round is 0.2x100µl=20µl.
 - 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 x 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 a, 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°C.
 - 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 3-5min. Do not dry the magnetic beads by heating, for example, at 37°C in an oven.

6-3/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 6-4/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.

6-4/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. Neo Biotech # NB-54-0229- NB-54-0232 or Neo Biotech # NB-54-0047 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 Illumine (Neo Biotech # NB-54-0229- NB-54-0232).
NB-54-0229- NB-54-0232 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 Illumine (Neo Biotech # NB-54-0047/ NB-54-0048).
NB-54-0047/ NB-54-0048 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 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 a dsDNA HS Assay Kit, Neo Biotech # NB-54-0027, 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 Illumine) 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 **8-5/Library Amplification** for the number of amplification cycles.

6-5/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 ; Bioanalyzer, TapeStation Fragment Analyzer

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 Illumine (Neo Biotech # NB-54-0428). 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 (Neo Biotech # NB-54-0027).

6-6/Other 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 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 Neo Biotech # **NB-54-0405**).

7/ Mechanism and workflow

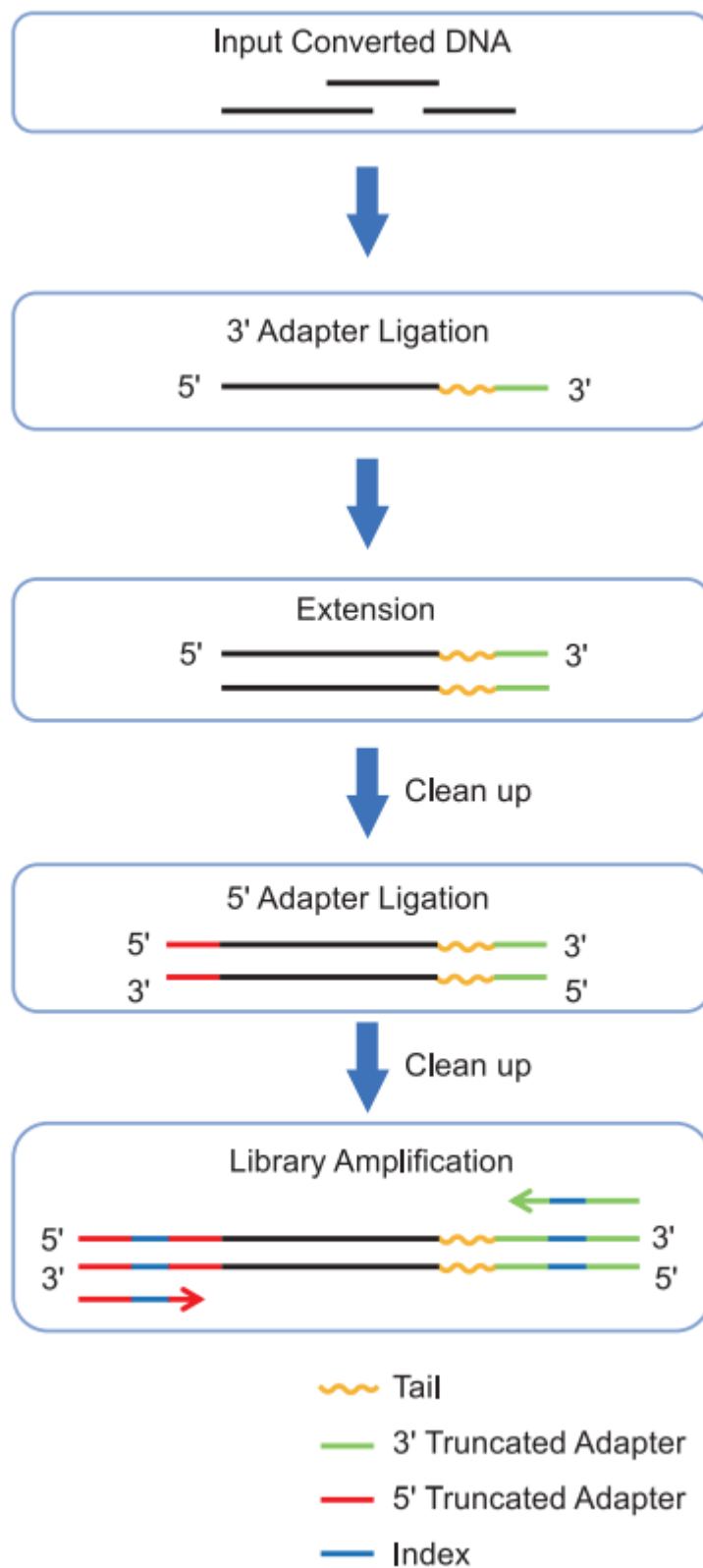


Fig 1. Library preparation principle with EpiArt DNA Methylation Library Kit for Illumina V3

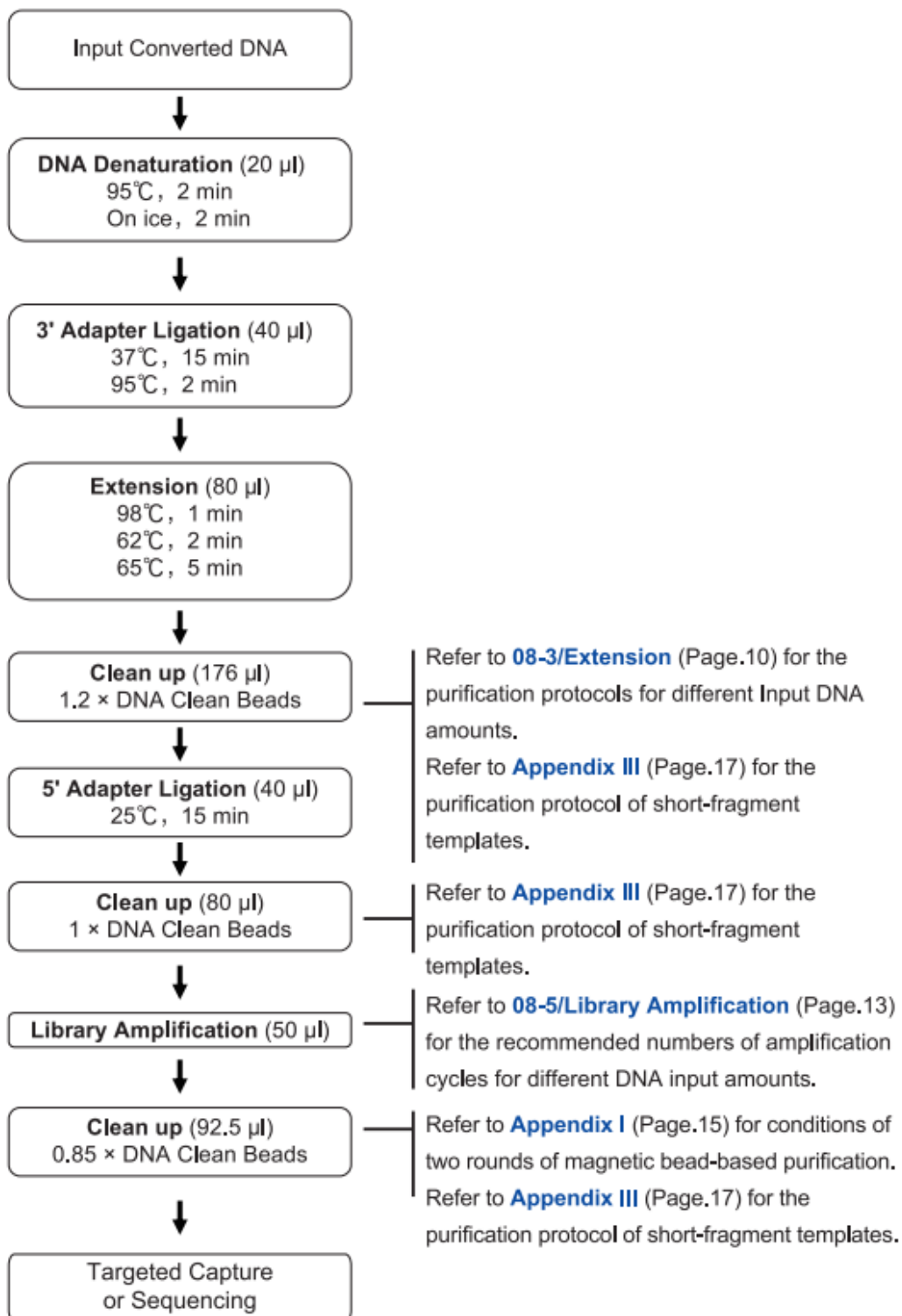


Fig 2. Workflow of EpiArt DNA methylation Library Kit for Illumina V3

8/Experiment Process

8-1/ DNA Denaturation

In this step, dsDNA, is denatured to become ssDNA.

1. Preheat the PCR instrument set the heated lid temperature to 105°C and the reaction temperature to 95°C. 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 <input type="checkbox"/>

3. Heat at 95°C 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.

8-2/3' Adapter Ligation

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

1. Thaw 3' Ligation Buffer, 3' a 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°C	On
37°C	15 min
95°C	2 min
4°C	Hold

8-3/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 Me 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°C	On
98°C	1 min
62°C	2 min
65°C	5 min
4°C	Hold

5. Purify the reaction product using 1.2x VAHTS DNA Clean Beads after the reaction has been completed:
 - ▲ If the template as short as 40bp 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 MARTS DNA Clean Beads, 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 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 Me 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 (5 min), carefully transfer 50 µl of supernatant into a new Nuclease-free PCR tube without disturbing the magnetic beads. Purify the supernatant using 1.2x VAHTS DNA Clean Beads (60 µl) (repeat steps b-g) Finally, add 22.5 µl of supernatant into a new Nuclease-free PCR tube for subsequent steps.
 - ▲ In this step, the sample can be temporarily stored at -20°C for 24h.

8-4/5' 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 μ l
5' Ligation Mix	15 μ l ■
5' Adapter	5 μ l ■
Total	40 μ l

▲ 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°C	off
25°C	15 min
4°C	Hold

5. Purify the reaction product using 1x VAHTS DNA Clean Beads after the reaction has been completed:
 - ▲ If the template as short as 40bp 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 μ l of VAHTS DNA Clean Beads to 40 μ l 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 μ l 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°C for 24 h.

8-5/ Library amplification

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

1. Thaw VAHTS HiFi Amplification Mix V3 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 V3	25 µl ■
Total	50 µl

*Index primers are amplification primers containing index. If VAHTS Dual UMI UDI adapters set 1-set 4 for Illumina (Neo Biotech# NB-54-0229-NB-54-0232) is used, the Volume of UDIXX is 5µl. 24 kinds of UDIXX are provided for each of catalog number from # NB-54-0229-NB-54-0232. If VAHTS multiplex oligos set 4-set 5 for Illumina (Neo biotech# NB-54-0047/NB-54-0048) is used, the volume of either DM5XX or DM7XX is 2.5µl. # NB-54-0047/# NB-54-0048 provides 8 kinds of DM5XX and 12kinds 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°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 1µg of library when using 10pg-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 number parameter, which is measured when about 250bp 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 x 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 by 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 20µl 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°C and long-term storage must be at -80°C. Avoid unnecessary repeated freezing and thawing.

8-6/ Library Quality control

Refer to 6-5 / 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 products:

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 μ l (0.85 x) of VAHTS DNA Clean Beads to 50 μ l 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 μ l 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 μ L 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 μ l of supernatant into a new Nuclease-free PCR tube without disturbing the magnetic beads.
 - i. Add 42.5 μ l (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 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 μ l 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 μ l 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 μ l 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 low-complexity Tail structure is added in the 3' Adapter Ligation step and converted DNA libraries are relatively low in base composition complexity due to C depletion, 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 Read1

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 EpiArt DNA Methylation Library Kit for Illumina V3 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