VAHTS AmpSeq Library Prep Kit V3

NA210

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Instruction for Use

Version 22.2

Contents

01/Product Description

VAHTS AmpSeq Library Prep Kit V3 is based on ultra-multiplex PCR and several core technologies such as end primer digestion, used to construct amplicon libraries. The whole library construction workflow can be finished in one tube. The whole process takes less than 5 h, of which the manual operation time is less than 1.5 h. It is compatible with genomic DNA, FFPE DNA, cfDNA, etc, and amount of Input DNA is 1 - 100 ng. This kit is intended for both the Illumina and Ion Torrent sequencing platforms. And corresponding adapters (Vazyme #NA111/NA121) are also provided. It is compatible with panels including Ion AmpliSeq Series Panel and AmpliSeq for Illumina Series Panel, as well as the corresponding individually customized panels.

This kit is comprised of multiplex amplification module, digestion module and ligation module, which has great amplification performance for different input amounts and customized panels, great efficiency of library ligation, and high coverage and high uniformity of amplicon library. By using this kit, researchers and technical personnel could prepare high quality library quickly and easily, and get more stabel and reliable sequencing data. All the reagents provided in the kit have undergone rigorous quality control and functional testing, to ensure the optimal stability and repeatability of library construction.

02/Components

Components	NA210-01 (24 rxns)	NA210-02 (96 rxns)
4 × VAHTS Multi-PCR Mix	120 μl	480 μl
VAHTS Digest Mix 2	60 μl	240 μl
VAHTS Ligation Enhancer	144 μl	576 μl
VAHTS Ligation Enzyme Mix 2	24 μl	96 μl
VAHTS HiFi Amplification Mix	600 μl	4 × 600 μl
PCR Primer Mix for Ion Torrent	120 μl	480 μl
PCR Primer Mix for Illumina	120 μl	480 μl
TE	1 ml	4 × 1 ml

03/Storage

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

04/Applications

This product is suitable for amplicon library preparation with 1 - 100 ng Input DNA, and is compatible with DNA templates derived from different sources:

◇ Cells or tissues;

◇ FFPE samples;

◇ cfDNA, etc.

05/Self-prepared Materials

◇ **Library preparation reagents:**

Ion Ampliseq Series Panel, AmpliSeq for Illumina Series Panel, or other equivalent products;
VAHTS AmpSeq Adapters for Illumina/Ion Torrent (Vazyme #NA111/NA121);

◇ **Magnetic beads for purification:**

VAHTS DNA Clean Beads (Vazyme #N411);

◇ **Evaluation of libraries:**

Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121);
VAHTS Library Quantification Kit for Illumina (Vazyme #NQ101-106);
Agilent Technologies 2100 Bioanalyzer or other equivalent products;

◇ **Other materials:**

Freshly prepared 80% ethanol, Nuclease-free ddH_2O;
RNase-free PCR tubes, low-binding EP tubes;
PCR instrument, magnetic stand, etc.

06/Notes

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

1. This product has high detection sensitivity. Multiplex PCR and other procedures should be performed in a separate clean area to avoid contamination.
2. All components of this product should be stored in an environment free of nucleic acids and nuclease contamination.
3. If carrying out the protocol for the first time, it is recommended to set up positive and negative controls within the same experiment.
4. Thaw 4 × VAHTS Multi-PCR Mix, VAHTS Digest Mix 2, VAHTS Ligation Enzyme Mix 2, etc., on ice; with the exception of the VAHTS Ligation Enhancer, all components should be kept on ice throughout the experiment.
5. Do not keep adapters above room temperature, in order to prevent the double-stranded adapters from unwinding.
6. If there is visible precipitate in the VAHTS Ligation Enhancer after thawing, mix and re-suspend by repeatedly inverting at room temperature, and use until the precipitate has been completely dissolved. If precipitate is still present, it can be dissolved in a 55℃ water bath.
7. 4 × VAHTS Multi-PCR Mix and VAHTS Ligation Enhancer are viscous. Prior to use, please mix it by inversing, centrifuge briefly, and pipette slowly.

07/Workflow

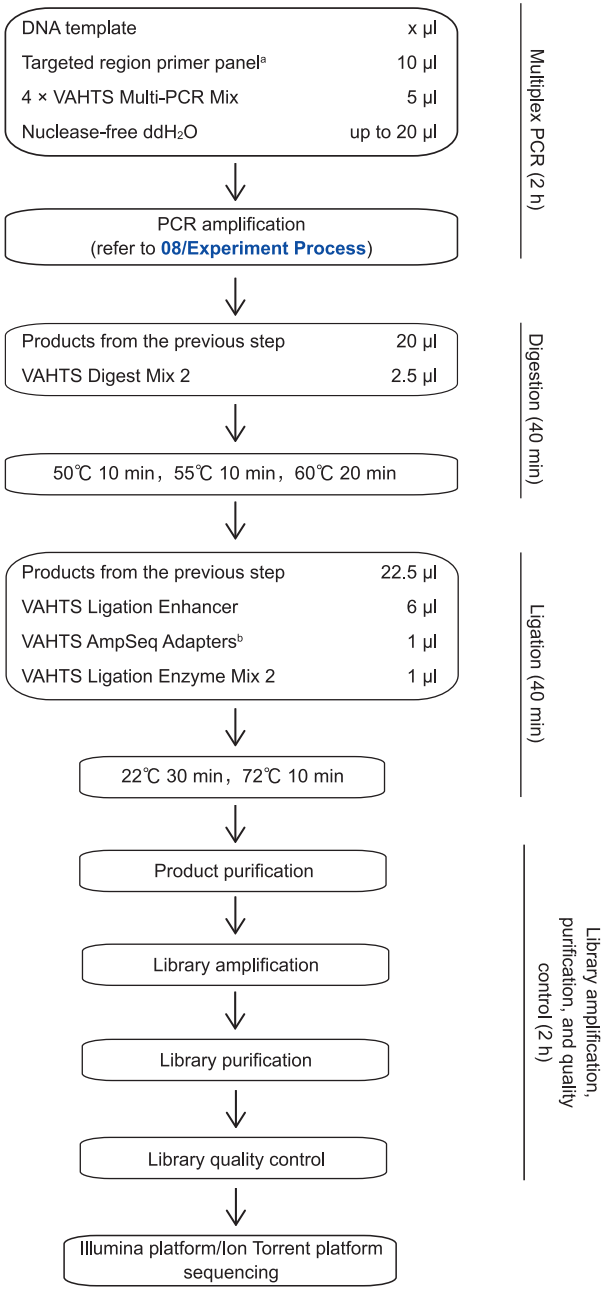

Fig 1. Workflow of VAHTS AmpSeq Library Prep Kit V3

a. For the panel, there is the option to use Ion AmpliSeq Panel, AmpliSeq for Illumina Series Panel, as well as a corresponding customized panel. This process takes 2 × Panel as an example.

b. For the Illumina platform, please use VAHTS AmpSeq Adapters for Illumina (Vazyme #NA111). For the Ion Torrent platform, please use VAHTS AmpSeq Adapters for Ion Torrent (Vazyme #NA121).

08/Experiment Process

Please read the instruction manual carefully before conducting the experiment.

This kit is suitable for the efficient construction of libraries for target regions using 1 - 100 ng DNA templates, together with primer panels such as Ion AmpliSeq Panel, AmpliSeq for Illumina Series Panel, as well as corresponding customized panels.

We recommend using the Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121) to quantify the template DNA concentration. The use of spectrophotometry is not recommended. In order to cover a larger area of the genome, the panel may contain several primer pools. Each primer pool requires at least 0.5 ng of DNA template. Panels corresponding to the general genome may not be suitable for amplifying FFPE DNA or cfDNA. High quality kits are recommended for the extraction of DNA.

08-1/Multiplex PCR

The purpose of this step is to obtain targeted amplicons from a DNA template using a targeted primer panel. 2 × Panel is used as an example in the following steps.

▲ To avoid contamination, operate in an ultra-clean workbench. This step should not be carried out in the same lab area as that used for the subsequent steps.

1. Thaw the required components on ice. Mix thoroughly and centrifuge briefly, then place on ice.

   ▲ The 4 × VAHTS Multi-PCR Mix is viscous and needs to be mixed thoroughly and briefly centrifuged. Pipette the solution slowly.

2. Prepare the reaction system on ice:

Components	Volume	
DNA template	x μl	
2 × Panel	10 μl	
4 × VAHTS Multi-PCR Mix	5 μl	■
Nuclease-free ddH_2O	To 20 μl	

3. Gently pipette up and down to mix (do not vortex), and briefly centrifuge.

4. Place the PCR tube into the PCR instrument, and perform the following procedure:

Temperature	Time	Number of cycles
99℃	2 min	
99℃	15 sec	x*
60℃	4 min	
72℃	10 min	
4℃	Hold	

*The number of amplification cycles “x” can be found in the following table:

Primer pairs per pool	General DNA	FFPE/cfDNA
10 - 50	22 - 24	25 - 27
50 - 200	20 - 22	23 - 25
200 - 1,000	17 - 20	20 - 23
≥1,000	15 - 17	18 - 20

Amount of Input DNA	Adjustment to cycle number
1 ng (300 Copies)	+3
10 ng (3,000 Copies)	0
100 ng (30,000 Copies)	-3

▲ When the numbers of primer pairs per pool are above 1,000 and 3,000, increase the annealing/ extension time to 8 min and 16 min, respectively.

▲ If the sample DNA quality is poor, increase the number of cycles appropriately.

▲ If multiple primer pools for a single panel fall into different pair categories, use the set up the program according to the most primer pair number.

▲ If using a panel with 2 primer pools, 10 μl of the reaction solution may be used for each tube of primers. Once the multiplex amplification reaction is complete, mix the two reaction solution together to proceed to the next digestion reaction.

Prepare the reaction system on ice as follows:

a. 10 × Panel:

Components	Volume	
DNA template	x μl	
10 × Panel	2 μl	
4 × VAHTS Multi-PCR Mix	5 μl	■
Nuclease-free ddH_2O	To 20 μl	

b. 5 × Panel:

Components	Volume	
DNA template	x μl	
5 × Panel	4 μl	
4 × VAHTS Multi-PCR Mix	5 μl	■
Nuclease-free ddH_2O	To 20 μl	

Refer to the protocol for 2 × Panel for operation process and amplification program.

08-2/Amplicons Digestion

1. Mix the VAHTS Digest Mix 2 by flicking, centrifuge briefly, place on ice, and prepare the following reaction solution on ice.

Components	Volume	
Reaction products from step 08-1	20 μl	
VAHTS Digest Mix 2	2.5 μl	■

▲ Adjust the pipette to approximately 70% of the total reaction solution volume (approximately 15 μl), and mix by gently pipetting up and down about five times. Avoid producing bubbles. Do not vortex or centrifuge. When the number of primer pairs is above 5,000, add 5 μl VAHTS Digest Mix 2.

2. Place the PCR tubes in the PCR instrument. Set the hot lid temperature at 105℃ and run the following program:

Temperature	Time	
	Panel<1,000	Panel≥1,000
50℃	10 min	20 min
55℃	10 min	20 min
60℃	20 min	20 min
4℃	Hold	Hold

08-3/Amplicon Adapter Ligation and Library Purification

1. Thaw the required components on ice. Mix thoroughly and centrifuge briefly, then place on ice.

▲ If there is visible precipitate in the VAHTS Ligation Enhancer after thawing, mix and re-suspend by repeatedly inverting. If precipitate is still present, it can be dissolved in a 55℃ water bath.

▲ When multiple samples are loaded at the same time, adapters with a barcode are required.

Add the following components successively to the reaction products from step 08-2 on ice:

Components	Volume	
Products from step 08-2	22.5 μl	
VAHTS Ligation Enhancer[a]	6 μl	■
VAHTS Ampseq Adapters[b]	1 μl	
VAHTS Ligation Enzyme Mix 2	1 μl	■

a. After adding Ligation Enhancer, adjust the pipette to approximately 70% of the total reaction solution volume (approximately 20 μl), and mix by gently pipetting up and down about five times.

b. Use the corresponding VAHTS AmpSeq Adapters for this step according to the requirements of the sequencing platform used. For the Illumina platform, please use VAHTS AmpSeq Adapters for Illumina (Vazyme #NA111). For the Ion Torrent platform, please use VAHTS AmpSeq Adapters for Ion Torrent (Vazyme #NA121).

▲ After adding all components in sequence, adjust the pipette to approximately 70% of the total reaction system volume (approximately 21 μl), and mix by gently pipetting up and down about five times. Do not centrifuge, and do not mix AmpSeq Adapters with Ligation Enzyme Mix 2 before adding.

2. Place the PCR tubes in the PCR instrument. Set the hot lid temperature at 105℃ and run the following program.

Temperature	Time
22℃	30 min
72℃	10 min
4℃	Hold

3. Library purification

a. Before use, mix the VAHTS DNA Clean Beads by vortex, and equilibrate to room temperature. Prepare a sufficient quantity of fresh 80% ethanol, approximately 400 μl is required for each sample.

b. Prior to carrying out this purification step, make the sample volume up to 60 μl using sterile Nuclease-free ddH_2O. Vortex the VAHTS DNA Clean Beads to fully mix. Add 60 μl (1 ×) VAHTS DNA Clean Beads to the PCR solution mentioned above. Mix the reaction thoroughly by gently pipetting up and down about 10 times. Incubate at room temperature for 8 min enabling the DNA fragments to bind to the magnetic beads.

▲ The beads are viscous. Pipette the beads slowly and ensure accurate usage.

c. Briefly centrifuge the reaction tubes, and place them on a magnetic stand to separate the magnetic beads from supernatant.

d. Keep the PCR tubes on the magnetic stand, and after the solution is clear (approximately 5 min), carefully remove and discard the supernatant without disturbing the magnetic beads.

e. Keep the PCR tubes on the magnetic stand, and add 200 μl of freshly prepared 80% ethanol. Take care not to disturb the magnetic beads when adding the ethanol. Incubate for 30 sec, then carefully remove the supernatant.

f. Repeat step e, rinsing twice in total.

g. Centrifuge briefly, and place them on the magnetic stand for 30 sec. Remove all residual ethanol using a pipette.

h. Open the lids to air dry for 3 - 5 min.

▲ Ensure the beads are dried properly. They should not appear glossy. If the beads are not dried completely, the residual ethanol in the sample will decrease the DNA elution rate, and may interfere with downstream reactions. If the beads are over-dried, they will crack. It is recommended that the incubation time in step i should be extended to allow the beads to sufficiently rehydrate, otherwise this will reduce the DNA elution effect, and ultimately decrease yields.

i. After the beads are dried, remove the PCR tubes from the magnetic stand and add 22 μl Elution Buffer (or Nuclease-free ddH_2O). Mix the beads by pipetting up and down. Incubate at room temperature for 2 min. If the beads are over-dried, extend the incubation time appropriately.

j. Briefly centrifuge the PCR tubes, and place them in a magnetic stand to separate the magnetic beads from supernatant until the solution becomes clear (approximately 5 min).

▲ If a small number of beads are no longer attached to the magnetic stand, mix the unattached beads in the supernatant by pipetting to re-suspend them. Continue to incubate until no beads are remained in the supernatant.

k. Carefully transfer 20 μl of the supernatant into a new EP tube, and store at -20℃.

08-4/Library Amplification and Purification

Thaw the required components on ice. Mix thoroughly and centrifuge briefly, then place on ice.

1. Prepare the reaction solution on ice as follows:

Components	Volume	
Purified library	20 μl	
VAHTS HiFi Amplification Mix	25 μl	■
PCR Primer Mix	5 μl	■■

▲ Please select the appropriate primers according to library type. For the Illumina platform, use PCR Primer Mix for Illumina. For the Ion Torrent platform, use PCR Primer Mix for Ion Torrent.

2. Place the PCR tubes in the PCR instrument. Set the hot lid temperature at 105℃ and run the following program:

Temperature	Time	Number of cycles
95℃	3 min	
98℃	20 sec	5
60℃	15 sec	
72℃	30 sec	
72℃	10 min	
4℃	Hold	

3. Library purification

a. Before use, mix the VAHTS DNA Clean Beads by vortex, and equilibrate to room temperature. Prepare a sufficient quantity of fresh 80% ethanol, approximately 400 μl is required for each sample.

b. Vortex the VAHTS DNA Clean Beads to fully mix. Add 45 μl (0.9 ×) VAHTS DNA Clean Beads to the PCR system mentioned above. Mix the reaction thoroughly by gently pipetting up and down about 10 times. Incubate at room temperature for 8 min enabling the DNA fragments to bind to the magnetic beads.

▲The beads are viscous. Pipette the beads slowly and ensure accurate usage.

c. Briefly centrifuge the reaction tubes, and place them on a magnetic stand to separate the magnetic beads from supernatant. Keep the EP tubes on the magnetic stand, and after the solution is clear (approximately 5 min), carefully remove the supernatant without disturbing the magnetic beads.

d. Keep the EP tubes on the magnetic stand, and add 200 μl of freshly prepared 80% ethanol. Take care not to disturb the magnetic beads when adding the ethanol. Incubate for 30 sec, then carefully remove the supernatant.

e. Repeat step d, rinsing twice in total.

f. Centrifuge briefly, and place them on the magnetic stand for 30 sec. Remove all residual ethanol using a pipette.

g. Open the lids to air dry for 3 - 5 min.

▲ Ensure the beads are dried properly. They should not appear glossy. If the beads are not dried completely, the residual ethanol in the sample will decrease the DNA elution rate, and may interfere with downstream reactions. If the beads are over-dried, they will crack. It is recommended that the incubation time in step h should be extended to allow the beads to sufficiently rehydrate, otherwise this will reduce the DNA elution efficiency, and ultimately decrease yields.

h. After the beads are dried, remove the EP tubes from the magnetic stand and add 22 μl Elution Buffer (or Nuclease-free ddH_2O). Mix the beads by pipetting up and down. Incubate at room temperature for 2 min. If the beads are over-dried, extend the incubation time appropriately.

i. Briefly centrifuge the EP tubes, and place them in a magnetic stand to separate the magnetic beads from supernatant until the solution becomes clear (approximately 5 min).

▲ If a small number of beads are no longer attached to the magnetic stand, mix the unattached beads in the supernatant by pipetting to re-suspend them. Continue to incubate until no beads are remained in the supernatant.

j. Carefully transfer 20 μl of the supernatant into a new EP tube, and store at -20℃.

08-5/Library Quality Control

Library concentration determination:

It is recommended to use KAPA Library Quantification Kit Ion Torrent Platforms (KAPA #KK4827) or VAHTS Library Quantification Kit for Illumina (Vazyme #NQ101-106) to determine the library concentration. If Qubit Fluorometer is used to determine the concentration, the results only represent the DNA concentration in the purified products but not the valid library concentration.

Library size distribution detection:

It is recommended to use Agilent Technologies 2100 Bioanalyzer to detect the library size distribution. The library distribution range should be consistent with the panel design sequence length range.

09/FAQ & Troubleshooting

◇ Low library yield

① Requantify the Input DNA, and confirm whether the Input DNA is accurate. If the amount of Input DNA is below the lower limit (1 ng), please increase it.

② If the template is of poor quality, increase the number of cycles appropriately according to the instructions or use a high-quality template instead.

③ Please ensure that all steps and programs conform to the instructions, and pay attention to the operation details of each step.

④ The beads drying procedure during library purification should not be too long, to avoid over-drying. Over-drying the beads may lead to a reduced yield.

⑤ When carrying out library amplification, make sure that there are no magnetic beads remaining, otherwise the amplification reaction may be inhibited.

◇ High library yield

① Check whether the amount of Input DNA is more than 100 ng. If so, reduce the amount of Input DNA.

② The number of multiplex amplification cycles can be appropriately reduced according to the instructions.

◇ **Low library uniformity**

① Short amplicons are under-represented: increase the amount of magnetic beads (from 1.2 × to 1.5 ×).

② Long amplicons are under-represented: it may be caused by degraded sample DNA or inefficient PCR. Use a high-quality template or extend the annealing/extension time during the mutiplex PCR process.

③ AT-rich amplicons are under-represented: increasing the annealing time, or lower the annealing temperature from 60℃ to 58℃.

④ GC-rich amplicons are under-represented: adding 5% - 10% DMSO in PCR reaction solution.

◇ **High adapter dimers**

① Reduce the amount of magnetic beads for purification appropriately.

② Decrease the concentration of adapters appropriately.

◇ **Aerosol contamination**

PCR products are highly susceptible to aerosol contamination, which can lead to inaccurate and unreliable experimental results. Therefore, we recommend that you physically isolate the PCR preparation area and PCR products purification area, using equipment such as dedicated pipettes, and periodically clean each laboratory area (wipe down with 0.5% sodium hypochlorite or 10% bleach), to ensure the reliability of the experimental results.

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