VAHTSTM mRNA-seq V3 Library Prep Kit for Illumina®

Catalog # NR611

Version 5.1

Vazyme biotech co., ltd.

1 Introduction

VAHTSTM mRNA-seq V3 Library Prep Kit for Illumina® is specially designed for the libraries preparation of ready-to-use transcriptome for next generation sequencing (NGS) platforms of Illumina. The kit is suited for total RNA of animal, plant, fungal, and other eukaryotes with initial template input of 0.01 µg - 4 µg. The library is prepared through mRNA isolation (from total RNA), fragmentation, synthesis of cDNA, end-repair, adapter-ligation, size selection of library, and library amplification. Magnetic beads for sorting was used to get a specific-length library rapidly, meeting the personalized demands of different experiments. Three separate packages of NR1, NR21 and NR31, containing all enzymes and buffers required for library construction, have passed the quality control and function assay test , maximally promising a stable and repeatable output cDNA library.

As a novel version based on VAHTSTM mRNA-seq V2 Library Prep Kit for Illumina®, VAHTSTM mRNA-seq V3 Library Prep Kit for Illumina® simplifies operational processes, increases library transformation efficacy, and is compatible with lower starting inputs with uniform coverage for various starting amounts of RNA.

2 Contents of Kits

	Component	NR611-01 (24 rxn)	NR611-02 (96 rxn)
NR 1	mRNA Capture Beads	1.2 ml	4.8 ml
	Beads Binding Buffer	1.2 ml	4.8 ml
	Beads Wash Buffer	9.6 ml	38.4 ml
	Tris Buffer	1.2 ml	4.8 ml
NR 21	Frag/Prime Buffer	468 µl	2 ×936 µl
	1st Strand Buffer	144 µl	576 µl
	1st Strand Enzyme Mix 2	48 µl	192 µl
	2nd Strand Buffer	480 µl	2 ×960 µl
	2nd Strand Enzyme Mix 2	120 µl	480 µl
NR 31	End Prep Mix 3	360 µl	2 ×720 µl
	Rapid Ligation Buffer	600 µl	4 ×600 µl
	Rapid DNA Ligase	120 µl	480 µl
	PCR Primer Mix	120 µl	480 µl
	Amplification Mix 1	600 µl	4 ×600 µl

Notes: The color indicated in above table represents the cap color of each component, and the components (≥ 1.5 ml) are stored in the HDPE bottle.

3 Storage

NR 1: store at 2 - 8℃.
NR 21: store at -20℃.
NR 31: store at -20℃.

4 Applications

VAHTSTM mRNA-seq V3 Library Prep Kit for Illumina® is applicable for total RNA of animal, plant, fungal, and other eukaryotes with initial template input of 0.01 µg - 4 µg. As the content of mRNA in total RNA of different samples varies greatly, enough total RNA need to be inputted to make sure the sufficient mRNA for library construction. Degraded total RNA used for library construction will lead to 3' bias in RNA-seq. It is recommended to use an Agilent 2100 Bioanalyzer to analyze the integrity of total RNA, and the RIN value should be ≥ 7.0.

This kit is applicable to mRNA-related analysis, while for stranded transcriptome library construction, the VAHTS Stranded mRNA-seq Library Prep Kit for Illumina® (Vazyme, #NR602) is recommended; for non-coding RNA (i.e. Inc-RNA) of human, rat, mice or other animals, the VAHTS Total RNA-seq (H/M/R) Library Prep Kit for Illumina® (Vazyme, #NR603) is needed.

Main fields of mRNA-related analysis:
◇Gene expression analysis
◇Single nucleotide variation calling
◇Alternative splicing detection
◇Gene fusion detection
◇Target transcriptome analysis

5 Additional Materials Required

◇DNA Clean Beads: VAHTSTM DNA Clean Beads (Vazyme #N411) or
Agencourt® AMPure® XP reagent (Beckman #A63880, #A63881, #A63882).
◇RNA Analysis: Agilent RNA 6000 Pico Kit (Agilent #5067-1513).
◇Library Analysis: Agilent DNA 1000 Kit (Agilent #5067-1504).
◇Adaptors: VAHTSTM RNA Adapters set 1- set 2 for Illumina® (Vazyme #N803, Vazyme#N804)
or VAHTSTM RNA Adapters set 3 - set 6 for Illumina® (Vazyme #N809, Vazyme#N810, Vazyme #N811, Vazyme #N812).
◇Other Materials: Fresh 80% Ethanol, Nuclease-free H_2O, Nuclease-free PCR tubes, Low absorption EP tubes (Eppendorf #022431021), Agilent Technologies 2100 Bioanalyzer or other equal products, PCR instrument, Magnetic stand.

6 Tips

6.1 Store the Components at Indicated Condition

1. NR 1 contains mRNA Capture Beads, must be stored at 2 - 8℃, otherwise the capture efficacy of the beads will be influenced.
2. NR 21 and NR 31 contain different Enzyme Mix, must be stored at -15 ~ -20℃. Put the enzyme on ice when in use and store at indicated condition after use, otherwise the enzyme activity will be reduced.
3. Buffers in Beads Binding, NR 21 and NR 31 may form precipitate when stored at low temperature, which doesn't influence normal use if equilibrating the buffers to room temperature and mixing thoroughly by vortexing.
4. To avoid repeated freezing and thawing or enzyme activity decrease after long-time use, it is recommended to store the residual regents in small subpackages after first time use.

6.2 Quality Control of RNA Samples

To ensure the quality of constructed library, RNA samples' property in total amount, purity and integrity should be controlled as follows, before starting experiment.
1. The initial template input of total RNA should ≥ 10 ng, otherwise, the mRNA may be insufficient for following library construction.
2. The ratio of OD260/OD280 should be between 1.8 and 2.0; if the radio >2.1 / <1.8, the RNA samples may have been contaminated with genomic DNA / protein. The ratio of OD230/OD260 should be between 0.4 and 0.5; if the radio >0.5 / <1.8, the RNA samples may have salt or small molecular weight impurity / genetic DNA contamination.
3. The RIN (RNA integrity number) should ≥ 7.0, analyzed by Bioanalyzer; the 28S:18S ratio of total RNA should ≥1.5, evaluated by agarose gel electrophoresis, which means no protein or genomic DNA pollution.

6.3 RNA Samples Prepared

1. RNA samples with high RIN and less impurities are highly recommended to assure the quality of library construction.
2. Give care to mixing solution containing RNA by pipetting gently. Do Not vortex, avoid unwanted size of library caused by RNA breaking.
3. Dilute RNA with Nuclease-free H_2O to 50 µl, put on ice and perform immediately to avoid RNA degradation.
4. If the initial volume of RNA > 50 µl, a RNA concentration by freeze-drying, ethanol precipitation, column recovery or magnetic beads purification (VAHTSTM RNA Clean Beads, Vazyme #N412) etc. is recommended.

6.4 Correctly Select Fragmentation Program

Fragment sizes of mRNA varies according to fragmentation conditions. Carefully select the appropriate break temperature and time according to the required size of insert library, otherwise the size and yield of library fragment will be affected.

6.5 Tips for DNA Purification with Magnetic Beads

1. Take magnetic beads out from 2 - 8℃, equilibrate to room temperature to assure capture efficacy.
2. Mix magnetic beads thoroughly by vortexing before pipetting.
3. Use fresh 80% ethanol to wash magnetic beads, the unfresh will lead to residual impurities.
4. Make sure the beads are fully dried before eluting (the surface color changed from bright brown to frosted brown), but avoid over dry (cracking), which will lead to DNA damage. Avoid ethanol residues that will affect subsequent experiments.

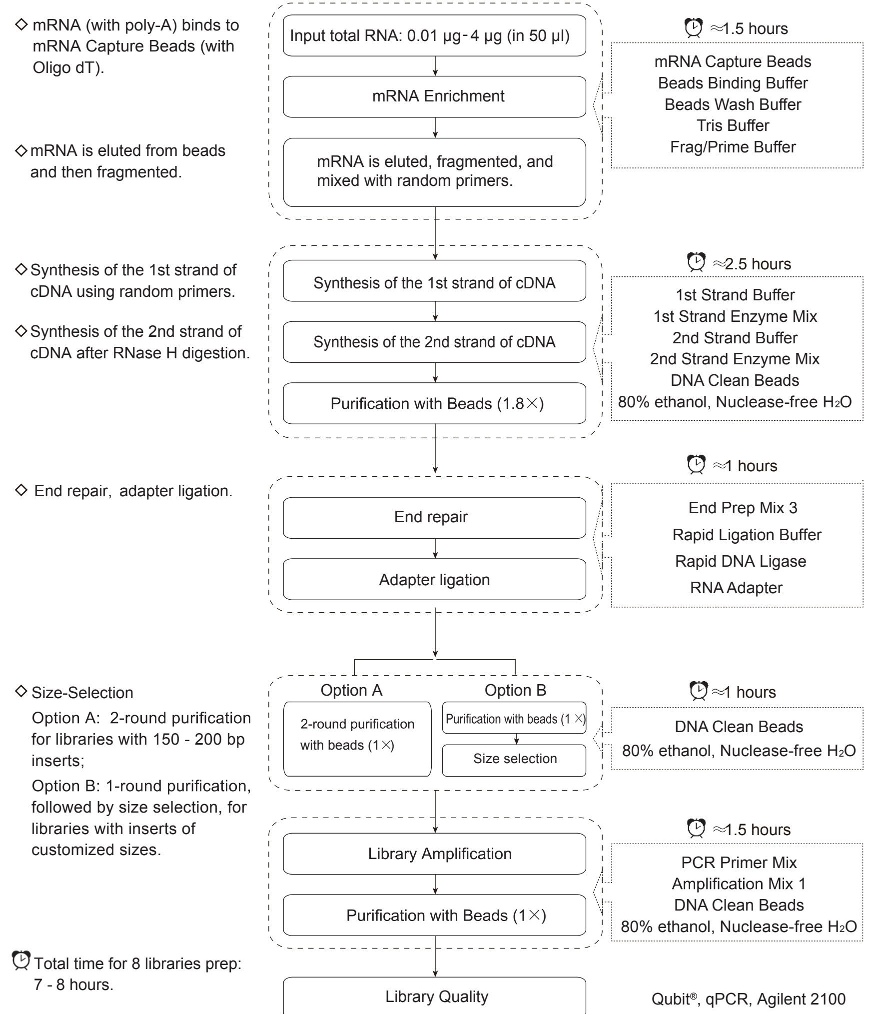
6.6 Operational Attentions

1. It is recommended to use filter pipette tips; change tips when varying samples.
2. Please use RNase-free materials before second Strand cDNA synthesis, while use DNase-free materials after that.
3. Be sure to use fresh Nuclease-free H_2O during the experiment; it is recommended to dispense it into small tubes for use and discard after use.
4. Be sure to wear gloves; change gloves after touching the equipment outside the RNase-free space or other working areas.
5. Please cover the reagent with lid to avoid contamination whenever finish use.
6. Reagents in this kit are sufficiently provided. However, for high sticky reagents with small amount used in every single reaction (such as 1st Strand Enzyme Mix 2), a briefly spin is needed to bring the sample to the bottom of the tube, avoiding the adhesion to the wall and cap of the tube, which may cause reagent loss.

6.7 Other Tips

1. Mix the enzyme component thoroughly and spin briefly to avoid the adhesion to the wall and cap of the tube, which may cause a reagent loss.
2. If the experiment need to be paused, please put the samples at right temperature according to the storage indication. Incorrect putting may reduce the efficacy of library construction.

7 Mechanisms & Workflow



8 Protocol

8.1 mRNA Purification and Fragmentation

1. Take NR1 (mRNA Capture Beads, Beads Wash Buffer, Tris Buffer, and Beads Binding Buffer) from 2 - 8℃, set sit and equilibrate to room temperature.
2. Prepare the RNA sample carefully: dissolve 0.01 μg – 4 μg of total RNA in 50 μl Nuclease-free H_2O in a Nuclease-free PCR tube. Keep the tube on ice and proceed to the next step as soon as possible.
3. Softly suspend RNA Capture Beads thoroughly by inverting, aspirate 50 μl beads into prepared RNA sample, mix thoroughly by pipetting up and down for 10 times.

▲mRNA Capture Beads, Beads Wash Buffer, and Beads Binding Buffer contain detergent, so DO NOT vortexing or oscillating at high speed when mixing, and avoid foaming when pipetting.

4. Incubate the sample in PCR machine for first binding of mRNA:

Temperature	Time
65 °C	5 min
25 °C	5 min

5. Put the sample on a magnetic stand. Wait until the solution clarifies (about 5 min), then carefully discard the supernatant.
6. Take the sample out of the magnetic stand. Add 200 μl of Beads Wash Buffer to re-suspend beads, and mix thoroughly by pipetting up and down for 10 times. Put the sample back to the magnetic stand. Wait until the solution clarifies (about 5 min), carefully discard the supernatant.

▲Step 4 - 6 is the first-round mRNA Purification, step 7 - 12 is the second-round mRNA Purification to guarantee the removal efficacy of rRNA.

7. Take the sample out of the magnetic stand, add 50 μl of Tris Buffer to resuspend the beads thoroughly by pipetting up and down for 10 times.
8. Incubate the sample in a PCR machine to release mRNA:

Temperature	Time
80 °C	2 min
25 °C	hold

9. Add 50 μl of Beads Binding Buffer, mix thoroughly by pipetting up and down for 10 times.
10. Set sit at room temperature for 5 min to make mRNA bind to beads.
11. Place the sample on the magnetic stand to isolate the mRNA from total RNA. Wait until the solution clarifies (about 5 min), then carefully discard the supernatant.
12. Take the sample out of the magnetic stand, add 200 μl of Beads Wash Buffer to resuspend the beads, mix thoroughly by pipetting up and down for 10 times. Place the tube on the magnetic separation rack. Wait until the solution clarifies (about 5 min), then carefully discard the supernatant.

▲It is highly recommended to use a 10 μl pipette to remove the residual supernatant in this step, residual Beads Wash Buffer will influence mRNA fragmentation.

13. Take the sample out of the magnetic stand, add 19.5 μl of Frag/Prime Buffer to re-suspend the beads thoroughly by pipetting up and down for 10 times. Incubate the sample in a PCR device and set programs according to the fragment size required:

Fragment size required (bp)	Temperature	Time
150 - 200	94°C	8 min , 4°C hold
200 - 300	94°C	5 min , 4°C hold
250 - 450	85°C	6 min , 4°C hold
450 - 550	85°C	5 min , 4°C hold

▲Please do not stop from fragmentation to the synthesis of first strand cDNA, because mRNA is easy to degrade during these procedures.

▲Take reagents required for 8.2 (step1) from -20℃ in advance, keep on ice and proceed to the next step.

14. Place the sample on the magnetic stand. Wait until the solution clarifies (about 5 min), and carefully aspirate 17 μl of supernatant into a new Nuclease-free PCR tube, then immediately proceed to synthesis of first-strand cDNA.

8.2 Synthesis of Second Strand cDNA

1. Thaw the 1st Strand Buffer (from -20℃) and mix thoroughly by inverting the tube. Prepare the reaction solution to synthesize the first strand cDNA as follows:

Component	Volume
Fragmented mRNA	17 µl
1st Strand Buffer	6 µl
1st Strand Enzyme Mix 2	2 µl
Total	25 µl

2. Mix thoroughly by gently pipetting up and down for 10 times.
▲If multiple samples are to be processed at the same time, the mixture of 1st Strand Buffer and 1st Strand Enzyme Mix 2 can be prepared in a suitable size centrifuge tube and then dispensed into each PCR tube. It is recommended that the actual reaction volume to be prepared should be 1.1 times to the logical one for making up possible loss.
3. Put the sample in a PCR instrument and run the following program:(first strand cDNA synthesis)

Temperature	Time
Hot Lid Temperature: 105℃	On
25 ℃	10 min
42 ℃	15 min
70 ℃	15 min
4 ℃	Hold

Immediately proceed to 2nd Strand cDNA Synthesis.
▲Take reagents required for step4 out from -20℃, keep the RNA solution on ice and proceed to the next step.
4. Thaw the 2nd Strand Buffer and mix thoroughly by inverting. Prepare the reaction solution to synthesize the 2nd strand cDNA as follows:

Component	Volume
1st Strand cDNA	25 µl
2nd Strand Buffer	20 µl
2nd Strand Enzyme Mix 2	5 µl
Total	50 µl

5. Mix thoroughly by gently pipetting up and down for 10 times.
▲If multiple samples are to be processed at the same time, the mixture of 2nd Strand Buffer and 2nd Strand Enzyme Mix 2 can be prepared in a suitable size centrifuge tube and then dispensed into each PCR tube. It is recommended that the actual reaction volume to be prepared should be 1.1 times to the logical one for making up possible loss.
6. Put the sample in a PCR instrument and run the following program:(2nd strand cDNA synthesize)

Temperature	Time
Hot Lid Temperature: 30℃	On
16 ℃	60 min
4 ℃	Hold

▲Hot lid temperature in this reaction is 30℃, but if the PCR instrument cannot be set so, just cover the lid with a clean tissue.
▲Take VAHTS DNA Clean Beads required for step7 out from 2 - 8℃ in advance, keep on ice and proceed to the next step.
The double Strand cDNA can be stored at 4℃ for 1 hour.

7. Purification of the double strand cDNA
a. Equilibrate the VAHTS DNA Clean Beads from 2 - 8℃ to room temperature 30 min early.
b. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 90 µl (1.8x) of beads into the above sample. Mix thoroughly by pipetting up and down for 10 times.
c. Incubate at room temperature for 10 min for DNA binding.
d. Place the sample on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on magnetic stand, carefully discard the supernatant.
e. Keep the sample on the magnetic stand, add 200 µl of freshly prepared 80% ethanol to rinse the beads. Incubate at room temperature for 30sec and carefully discard the supernatant without disturbing the beads.
f. Repeat the Step e.
g. Keep the sample on the magnetic stand, open the tube and air-dry the beads for 5 - 10 min.
▲Add freshly prepared 80% ethanol to rinse the beads but DO NOT resuspend the beads!
▲It is highly recommended to use a 10 µl pipette to remove the residual supernatant in this step.
▲Avoid over drying the magnetic beads (cracks), which can decrease recovery efficiency.

h. Take the sample off the magnetic stand. Add 52.5 μl of Nuclease-free H_2O to elute the cDNA. Mix thoroughly by vortexing or pipetting, incubate at room temperature for 2 min. Put the tube on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 50 μl supernatant to a new Nuclease-free PCR tube,

▲Carefully transfer supernatant without disturbing the beads. Even trace amount of beads will affect the quality of the final library.

▲Take reagents required for 8.3 (step1) out from -20℃, keep on ice and proceed to the next step.

The dilution can be stored at -20℃ for 24 hours.

8.3 End Repair

1. Thaw the End Prep Mix 3 and mix thoroughly by inverting the tube. Prepare the reaction solution as follows:

Component	Volume
ds cDNA	50 μl
End Prep Mix 3	15 μl
Total	65 μl

2. Mix thoroughly by gently pipetting up and down for 10 times.
3. Put the sample in a PCR instrument and run the following program for end-repair:

Temperature	Time
Hot Lid Temperature: 105℃	On
20 ˚C	15 min
65 ˚C	15 min
4 ˚C	Hold

The end-repair product can be stored at 4°C for 60 min.

8.4 Adapter Ligation

1. Thaw the RNA Adapter and mix thoroughly by inverting the tube.
2. Thaw the Rapid Ligation Buffer and mix thoroughly by inverting the tube. Keep on ice and proceed to the next step.
3. Prepare the reaction solution as follows:

Component	Volume
End Preparation Products	65 μl
Rapid Ligation Buffer	25 μl
Rapid DNA Ligase	5 μl
RNA Adapter*	2.5 μl
Nuclease-free H_2O	2.5 μl
Total	100 μl

*RNA Adapter must be prepared separately. See Troubleshooting 4 (Page 11).

4. Mix thoroughly by gently pipetting up and down for 10 times.
5. Put the sample in a PCR instrument and run the following program for adapter ligation:

Temperature	Time
Hot Lid Temperature: 105℃	On
20 ˚C	15 min
4 ˚C	Hold

▲Take VAHTS DNA Clean Beads required for 8.5 out from -20℃ in advance, keep on ice and proceed to the next step.

The adapter-ligation products can be stored at 4℃ for 60 min.

8.5 Purification and Size Selection of Adapter-ligated DNA

This step provides two options. Please select carefully according to your needs:

Option A: Two-round purification were performed without sorting operation. This option can effectively obtain inserts of 150 bp - 200 bp at 94°C for 8 min, and remove the residual adapters.

Option B: One round of purification and then followed by two-round sorting; 200 bp of inserts can be obtained according to the different sorting conditions in Table 2 without residual adapters.

Option A for libraries with inserts of 150 bp - 200 bp (suitable for mRNA fragmented by incubation at 94℃ for 8 min)

A1. Take the VAHTS DNA Clean Beads out from 2 - 8℃ 30min early and equilibrate to room temperature.
A2. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing, and pipet 100 μl (1 x) into the above ligation products. Mix thoroughly by pipetting up and down for 10 times.
A3. Incubate at room temperature for 10 min for DNA binding.
A4. Place the sample on a magnetic stand. Wait until the solution clarifies (about 5 min). Carefully discard the supernatant.
A5. Keep the sample on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30sec at room temperature and carefully discard the supernatant.
A6. Repeat the Step A5.
A7. Keep the sample on the magnetic stand, open the tube and air-dry the beads for 5 - 10 min.
▲Add freshly prepared 80% ethanol to rinse the beads, but DO NOT resuspend the beads!
▲It is highly recommended to use a 10 μl pipette to remove the residual supernatant in this step.
▲Avoid excessive dry of magnetic beads (cracks) for that will lead low recovery efficiency.
A8. Take the sample out of the magnetic stand. Add 52.5 μl of Nuclease-free H_2O to elute DNA. Mix thoroughly by vortexing or pipetting and set sit for 2 min at room temperature. Place the tube back on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 50 μl of the supernatant to a new Nuclease-free PCR tube.
A9. Suspend the VAHTDNA Clean Beads thoroughly by inverting or vortexing. Pipet 50 μl (1 ×) of the suspended beads to the product above. Mix thoroughly by pipetting up and down for 10 times.
A10. Incubate at room temperature for 10 min for DNA binding.
A11. Place the sample on the magnetic stand. Wait until the solution clarifies (about 5 min) and carefully discard the supernatant without disturbing the beads .
A12. Keep the sample on the magnetic stand, and add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30sec at room temperature and carefully discard the supernatant.
A13. Repeat the Step A12.
A14. Keep the sample on the magnetic stand, open the tube and air-dry the beads for 5 - 10 min.
▲Add freshly prepared 80% ethanol to rinse the beads, but DO NOT resuspend the beads!
▲It is highly recommended to use a 10 μl pipette to remove the residual supernatant in this step.
▲Avoid excessive dry of magnetic beads (cracks) for that will lead low recovery efficiency.
A15. Take the sample off the magnetic stand. Add 22.5 μl of nuclease-free H_2O to elute the DNA. Mix thoroughly by vortexing or pipetting and set sit for 2 min at room temperature. Place the tube on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 20 μl of supernatant to a new Nuclease-free PCR tube for PCR reaction.
▲DO NOT disturb the beads while aspirating the supernatant. Even trace amount of beads will affect the quality of the final library.
▲Take reagents required for 8.6 (step1) out from -20℃ in advance, keep it on ice and proceed to the next step.

The elution can be stored at -20℃ for 24 hours.

Option B for libraries with inserts > 200 bp (for mRNA fragmented by incubation at 94℃ for 5min, 85℃ for 6 min, or 85℃ for 5 min).

B-1. Purification of ligation products by 1× VAHTS DNA Clean Beads
B1. Take the VAHTS DNA Clean Beads out from 2 - 8℃ 30 min early. Equilibrate to room temperature.
B2. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 100 μl (1 ×) of beads into the sample above. Mix thoroughly by pipetting up and down for 10 times.
B3. Incubate at room temperature for 10 min for DNA binding.
B4. Place the sample on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand and carefully discard the supernatant.
B5. Keep the sample on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30sec at room temperature and carefully discard the supernatant.
B6. Repeat the Step B5.
B7. Keep the sample on the magnetic stand, open the tube and air-dry the beads for 5 - 10 min.
▲Add freshly prepared 80% ethanol to rinse the beads but DO NOT resuspend the beads!
▲It is highly recommended to use a 10 μl pipette to remove the residual supernatant in this step.
▲Avoid excessive dry of magnetic beads (cracks) for that will lead low recovery efficiency.
B8. Take the sample out of the magnetic stand. Add 102.5 μl of Nuclease-free H_2O to elute DNA. Mix thoroughly by vortexing or pipetting and incubate at room temperature for 2 min. Place the tube back on the magnetic stand. Wait until the solution clarifies (about 5 min). Carefully transfer 100 μl of the supernatant to a new Nuclease-free PCR tube.

B-2. Size selection with two-round VAHTS DNA Clean Beads.

The following protocol is an example for a library with inserts of 350 bp - 450 bp obtained at 85℃ for 6min. Please refer to Table 1 for the appropriate volume of beads for libraries with inserts of other sizes.

Table 1 Recommended conditions for bead-based size selection

Insertion Length (bp)	200 - 300	250 - 350	350 - 450	450 - 550
Library Length (bp)*	320 - 420	370 - 470	470 - 570	570 - 670
Fragmentation Condition	94℃, 5 min	85℃, 6 min	85℃, 6 min	85℃, 5 min
Volume of beads for 1st round (μl)	70 (0.7×)	65 (0.65×)	60 (0.6×)	55 (0.55×)
Volume of beads for 2nd round (μl)	10 (0.1×)	10 (0.1×)	10 (0.1×)	10 (0.1×)

▲The full library length, equaling to insertion length plus adapter length (120 bp), is the range of peak size determined by Agilent 2100 Bioanalyzer. Please refer to 8.6 (step 4) for further information.

▲Any deviation of the loading volume in each step will affect the size of the final library insert.

▲The volume of magnetic bead used for the sorting is relative to the initial DNA volume, for example: for initial DNA solution of 100 μl, the volume of first round magnetic bead is 60 μl, the 60% of 100 μl (0.6×), and the volume of second round magnetic bead is 10 μl, the 10% of 100 μl (0.1×).

B9. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Transfer 60 μl (0.6 ×) of beads into the purified ligation products above. Mix thoroughly by pipetting up and down for 10 times.

B10. Incubate at room temperature for 10 min for DNA binding.

B11. Place the sample on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on Magnetic stand and carefully transfer 155 μl of the supernatant (to be saved instead of discarded) into a new Nuclease-free PCR tube.

▲Take 155 μl of the supernatant from a total volume of 160 μl so as to avoid aspirating the beads bound with the large DNA fragments to the next step, which will result in wrong size fragments (large fragments) to be brought to final library. The total volume in other sorting conditions is not 160 μl, the amount of supernatant to be aspirated should be 5 μl less than the total one.

B12. Add 10 μl (0.1 ×) of VAHTS DNA Clean Beads, mix thoroughly by pipetting up and down for 10 times.

B13. Incubate at room temperature for 10 min for DNA binding.

B14. Place the sample on the magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand and carefully discard the supernatant.

B15. Keep the sample on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30sec at room temperature and carefully discard the supernatant.

B16. Repeat the Step B15.

B17. Keep the sample on the magnetic stand, open the tube and air-dry the beads for 10 min.

▲Add freshly prepared 80% ethanol to rinse the beads but DO NOT resuspend the beads!

▲It is highly recommended to use a 10 μl pipette to remove the residual supernatant in this step.

▲Avoid excessive dry of magnetic beads (cracks) for that will lead low recovery efficiency.

B18.Take the sample off the magnetic stand. Add 22.5 μl of nuclease-free H_2O to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 20 μl of the supernatant to a new Nuclease-free PCR tube.

▲DO NOT disturb the beads while aspirating samples from supernatant. Even trace amount of beads will affect the quality of final library.

▲Take reagents required for 8.6 (step1) from -20℃ in advance, keep the reagent on ice and proceed to the next step.

The elution can be stored at -20℃ for 24 hours.

8.6 Library Amplification

1. Thaw the PCR Primer Mix and Amplification Mix 1 thoroughly by inverting the tube. Prepare the reaction solution as follows:

Component	Volume
Purified Ligation Product	20 μl
PCR Primer Mix	5 μl
Amplification Mix 1	25 μl
Total	50 μl

Mix thoroughly by gently pipetting up and down for 10 times.

▲If multiple samples are to be processed at the same time, the mixture of 2nd Strand Buffer and 2nd Strand Enzyme Mix 2 can be prepared in a suitable size centrifuge tube and then dispensed into each PCR tube. It is recommended that the actual reaction volume to be prepared should be 1.1 times to the logical one for making up possible loss.

2. Put the sample in a PCR instrument and run the following PCR program:

Procedure	Temperature	Time	Cycle Numbers
Hot lid	105℃		
Pre-denaturation	98℃	30 sec	1
Denaturation	98℃	10 sec	12 - 17
Annealing	60℃	30 sec	
Extension	72℃	30 sec	
Complete Extension	72℃	5 min	1
Hold	4℃		

▲The mRNA content in a certain amount of total RNA varies in different species. According to the certain species, the number of PCR cycles can be appropriately adjusted, basically between 12 to17.

The initial amount of RNA	Cycle Numbers
1000 ng	12 - 13
100 ng	14 - 15
10 ng	16 - 17

3. Purification of the PCR product with VAHTS DNA Clean Beads.
a. Take the VAHTS DNA Clean Beads out from 2 - 8℃ 30 min early. Equilibrate to room temperature.
b. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Transfer 50 μl (1 ×) of the beads into the PCR product. Mix thoroughly by pipetting up and down for 10 times.
c. Incubate at room temperature for 10 min for DNA binding.
d. Place the sample on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand and carefully discard the supernatant.
e. Keep the sample on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature and carefully discard the supernatant.
f. Repeat the Step e.
g. Keep the sample on the magnetic stand, open the tube and air-dry the beads for 5 - 10 min.
▲Add freshly prepared 80% ethanol to rinse the beads DO NOT resuspend the beads!
▲It is highly recommended to use a 10 μl pipette to remove the residual supernatant in this step.
▲Avoid excessive dry of magnetic beads (cracks) for that will lead low recovery efficiency.
h. Take the sample out of the magnetic stand. Add 25 μl of Nuclease-free H_2O to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube back on the magnetic stand and wait until the solution clarifies (about 5min). Carefully transfer 22.5 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.
▲DO NOT disturb the beads while drawing samples from supernatant. Even trace amount of beads will affect the quality of final library.

The elution can be stored at -20℃ for 24 hours.

4. Library Quality Determination Analyzed by an Agilent Technologies 2100 Bioanalyzer.
Analyze 1 μl of purified PCR product by an Agilent DNA 1000 kit (Agilent, Cat.No. 5067-1504). As shown in Fig. 1, a library with high quality should show a narrow peak at expected site. while a narrow peak at around 128 bp suggests the contamination of adapter-dimer. To eliminate this contamination, dilute the library to 50 μl with Nuclease-free H_2O and repeat Step 8.6 (Step3) for further purification.

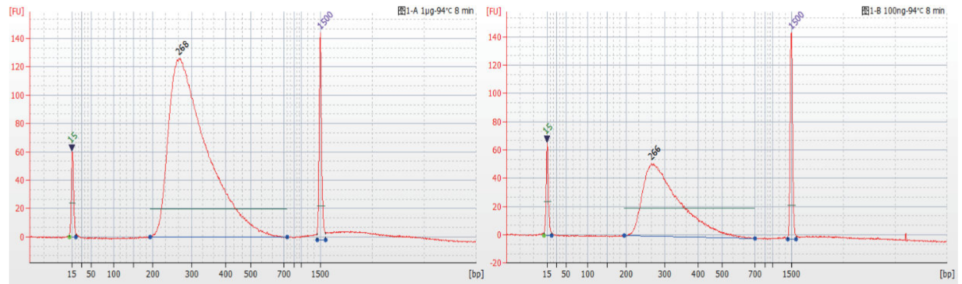


Fig 1. A/B 1 μg/100 ng universal human reference RNA, fragmented at 94℃ for 8 min and purified twice with VAHTS DNA Clean Beads (1×).

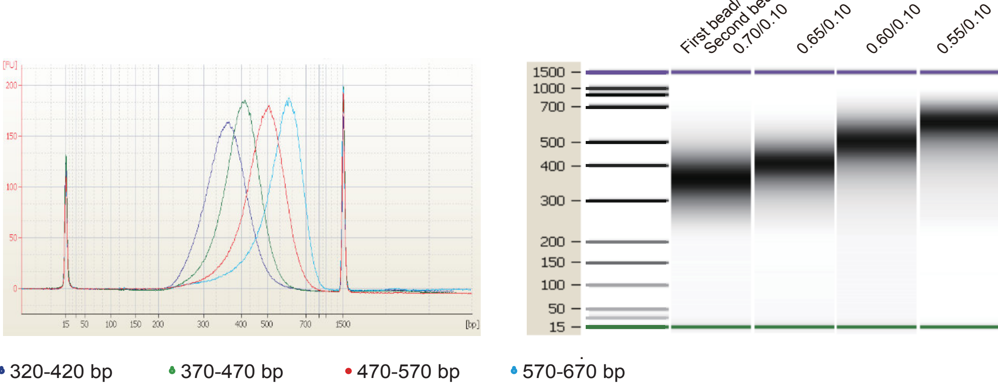


Fig 2. 200 ng universal human reference RNA, fragmented under different conditions, and purified once with VAHTS DNA Clean Beads (1×), followed by size selection steps according to different radios of VAHTS DNA Clean Beads.

9 Troubleshooting

1. Incorrect operation and remedy

Step	Correct Operation	Incorrect Operation	Remedy
8.1 Step 6	Add 200 μl of Beads Wash Buffer to rinse and wash the mRNA Capture beads.	Add 200 μl of 80% ethanol by mistake.	Discard the ethanol, dry it; resuspend the beads with 200 μl Beads Wash Buffer and continue the next step.
8.1 Step 7	Add 50 μl of Tris Buffer to resuspend mRNA Capture Beads.	Add Beads Binding Buffer by mistake.	If you did not treat it at 80 °C 2 min, you can use magnetic stand. After adsorption, discard the supernatant and add Tris Buffer.
8.1 Step 9	Add 50 μl of Beads Binding Buffer to make mRNA bind to mRNA Capture Beads.	Add Tris Buffer by mistake.	Amplify the reaction system and add Beads Binding Buffer, adding volume is equal to that of Tris Buffer.
8.1 Step 12	Add 200 μl of Beads Wash Buffer to rinse and wash the mRNA Capture beads.	Directly add Frag / Prime Buffer without adding Wash Buffer.	If it is not interrupted by heat, you can put it on the magnetic stand back , drop the Frag/Prime Buffer and add Wash Buffer.
8.1 Step 13	Add Frag/Prime Buffer to break mRNA.	Add Nuclease-free H_2O by mistake.	If condition permitted, add the same volume of 2× Frag/Prime Buffer to enlarge the reaction system until the purification step.
		After discarding Wash Buffer, the Frag/Prime Buffer is still in frozen.	Re-add Beads Wash Buffer to soak mRNA Capture Beads until the Frag/ prime buffer thaws, discard the Wash Buffer and continue the next step.
	Frag/Prime Buffer added sample was fragmented by high temperature.	Fragmentation conditions are not consistent with the initial settings, for example, set 85 °C, 6 min as 94 °C, 8 min.	Subsequent sorting steps must be selected corresponding to this breaking condition, otherwise the library will fail to build and the ultimate size of the inserted library fragment will change too.
8.1 Step 15	After breaking mRNA, transfer the supernatant to a new Nuclease-free PCR tube.	The volume of supernatant is less than 17 μl.	Add Nuclease-free H_2O to make up for 17 μl.
8.3/4/5	End repair and adapter ligation.	Add wrong reagent.	Add 1.8 DNA clean Beads for DNA purification, then continue the experiment.

8.2 Step 5-b	Use 1.8 x magnetic beads to purify Double Strand products.	Add wrong volume of DNA Clean Beads.	(1) Less: Make up for the volume (2) More: Add Nuclease-free H_2O to make up for the volume according to the radio of 1.8 x.
8.2 Step 5-h	Add 52.5 μl of Nuclease-free H_2O to elute purified fragments.	Add wrong volume of H_2O.	(1) For sufficient templates, discard extra volume of liquid (2) For insufficient templates, reuse 1.8 x magnetic beads for DNA purification, then continue the experiment.
8.5	Purification and Size Selection of adapter-ligated DNA .	Without purification, direct sorting.	The actual fragment will be slightly smaller, but the yield will not be affected. Continue the experiment according to the results of 2100 determining the library size.
	Without sorting conditions.	In first-round purification, add wrong volume of H_2O.	The amount of magnetic beads to be used in the subsequent step can be adjusted according to the radio.
8.6 Option A	Pipet 100 μl of purified products for sorting.	The supernatant is less than 100 μl.	Add Nuclease-free H_2O to make up for 100 μl.

2. Methods to solve the problem of low concentration of library

It is recommended to use high-quality RNA samples as templates for library construction to make library concentration meet the requirements of the machine sequencing. If you cannot provide qualified RNA samples, try to use the following methods to make up:

◇Initial amount: Increase the initial amount to 10 μg.

◇Do several duplicate samples, merge them at the two-strand synthesis purification step, or before PCR step.

◇Take option without sorting: Though RNA fragmented at 94°C for 8 min is short, its distribution is concentrated and the homogeneity is also well. However, some individualized samples have non-uniform fragments and this situation will be amplified by PCR, but this situation rarely occurs in the option of without sorting.

3. Questions for library quantification

There are two methods for library quantification: Qubit and qPCR, used for determining library mass concentration and library molarity, respectively. qPCR can truly reflects the number of DNA fragments used for machine sequencing owing to the theory of clustered primers performing amplification quantification. Therefore, the library quantitative results measured by qPCR are more reliable. These two methods can be used at the same time to quantify libraries and correct each other.

4. Instructions for adapter selection

At present, the adapters applicable to this kit is divided into two sets:

Set 1: VAHTSTM RNA Adapters set 1 - set 2 for Illumina® (Adapter 1-27, Vazyme #N803, Vazyme #N804) totally contains 24 different adapters, divided into two separate packages, each containing 12 different adapters according to the serial number.

Set 2: VAHTSTM RNA Adapters set 3 - set 6 for Illumina® (Adapter 1-96, Vazyme #N809, Vazyme #N810, Vazyme #N811, Vazyme #N812) totally contains 96 different adapters, divided into four separate packages, each containing 24 different adapters according to the serial number.

The above two sets of adapters cannot be mixed in the same batch of sequencing samples, refer to the following suggestions:

◇When the number of samples in the same batch of sequencing is <24, It is recommended to select set 1; When the number of samples is <12, the individual package of the set can be selected.

◇When the number of samples in the same batch of sequencing is between 24 and 96, it is recommended to select set 2, but the individual package of this set can also be selected according to the number of specific samples.

5. Is this kit suitable for small RNA library construction?

Not applicable. Considering the length of small RNA is only about 22nt and our magnetic beads capture RNA at least 100 bp long, this kit cannot efficiently enrich small RNA fragments.

6. Is the kit suitable for non-eukaryotic mRNA library construction?

Because the kit isolate mRNA via magnetic beads with Oligo dT directly capturing mRNA, it is not suitable for non-eukaryotes without poly-A.

7. Is FFPE samples suitable for library construction by this kit?

As the mRNA in FFPE sample typically have been degraded and with poor integrity, it is recommended to use VAHTSTM Total RNA-seq (H/M/R) Library Prep Kit for Illumina® (Vazyme #NR603) to construct library.

8. Can it still be used if NR1 is storied in -20 °C by mistake after receiving.

No. Low temperature will destroy the magnetic beads. You can purchase corresponding module VAHTSTM mRNA Capture Beads (Vazyme #N401) instead.

9. Why the sorting insertions is larger than the actual insert when operating as Instruction Manual?

There are various reasons that cause the amount of magnetic beads added less than the specified value, resulting in the larger sorting insertions: the magnetic beads are not equilibrated to room temperature or not mixed thoroughly; the pipette is inaccurate, and the tip of the pipette is severely attached.

10. How many cycle numbers at most can be used for library amplification?

The number of cycles can be adjusted according to the initial amount of input DNA; it is recommended to take 1 ul of input DNA for Qubit test and then make additional 1 - 2 cycles, but the maximum cycle numbers should no more than 17.

11. Why there are double peaks in the graph when the library was tested on the Agilent 2100 Bioanalyzer ?

◇There are residual impurities and degrades of RNA during library construction; the amount of effective template is low when PCR, causing non-specific amplification. It is recommended to heat RNA sample at 65°C for 15 min for degradation test. If RNA is unqualified, the RNA re-extraction must be taken.

◇The species itself is special. The RNA fragments are not continuous and uniform after fragmentation, and two ranges of fragments might be selected.

◇The mRNA abundance of specific species and the effective amount of template for PCR are insufficient. It is recommended to increase the amount of input or to mix repeated samples at the purification step after two-strand synthesis.

12. The explanations for over-amplified high-yield libraries after being tested on the Agilent 2100 Bioanalyzer, Qubit and qPCR.

High-yield libraries are often over-amplified in different degrees. Because at the later period of library amplification, primers are usually exhausted. Therefore, a large number of library fragments cannot be combined with primers, and the fragments are incorrectly annealed through incomplete matching. Thus, a hybrid strand mixed with partial double strand and partial single strand is formed in larger size. According to the corresponding principles of different detection methods, excessive amplified products show slight tailing after the upper marker in the analysis graph of Agilent 2100 Bioanalyzer; the single-stranded portion can not be detected by the Qubit, but can be effectively measured by qPCR, thus the concentration measured by Qubit is lower than that measured by qPCR at about 10% - 50%. The above phenomenon is normal and would not affect the library sequencing and data analysis.