VAHTS Universal V8 RNA-seq Library Prep Kit for Illumina

NR605

Instruction for Use

Version 24.1

Vazyme

Contents

For Research Use Only. Not for use in diagnostic procedures.

01/Product Description

VAHTS Universal V8 RNA-seq Library Prep Kit for Illumina is specially designed for the preparation of transcriptome libraries for Illumina platform. The kit is universal and suitable for library construction of RNA that have been obtained by Poly(A)-based mRNA capture or rRNA depletion. The kit contains two types of 2nd strand cDNA synthesis buffer, which can be chosen for library construction for non-strand-specific or strand-specific transcriptome analysis.

This kit combines 2nd strand cDNA synthesis, end-repair and dA-tailing into one step with no purification, which greatly simplifies the process of library construction and shortens the operation time. The optimized reaction system improves the library construction efficiency, is compatible with lower input, and has uniform coverage for different amounts of input.

Libraries of specific sizes can be obtained after size selection with magnetic beads for meeting customized needs. All the components provided in the kit have undergone rigorous quality control and functional testing to ensure the optimal stability and repeatability.

02/Components

Components	NR605-01 (24 rxns)	NR605-02 (96 rxns)
2 × Frag/Prime Buffer	240 µl	960 µl
1st Strand Buffer 3	168 µl	672 µl
1st Strand Enzyme Mix 3	48 µl	192 µl
2nd Strand Buffer 2 (with dNTP)	600 µl	4 × 600 µl
2nd Strand Buffer 2 (with dUTP)	600 µl	4 × 600 µl
2nd Strand Enzyme Super Mix 2	360 µl	2 × 720 µl
Rapid Ligation Buffer 4	600 µl	4 × 600 µl
Rapid DNA Ligase 4	120 µl	480 µl
PCR Primer Mix 4	120 µl	480 µl
2 × HF Amplification Mix	600 µl	4 × 600 µl

03/Storage

Store at -30 ~ -15°C and ship at ≤0°C.

04/Applications

VAHTS Universal V8 RNA-seq Library Prep Kit for Illumina is suitable for RNA library preparation of total RNA with good integrity, RNA enriched by rRNA depletion or mRNA capture by Poly(A) method from eukaryotes such as animals, plants, fungi, etc. The input is related to the mRNA enrichment module:

VAHTS mRNA Capture Beads 2.0 (Vazyme #N403): 0.01 - 4 μg
Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N406): 0.01 - 1 μg
Ribo-off rRNA Depletion Kit V2 (Bacteria) (Vazyme #N417): 0.01 - 1 μg
Ribo-off Globin & rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N408): 0.01 - 1 μg
Ribo-off rRNA Depletion Kit (Plant) (Vazyme #N409): 1 - 5 μg
Ribo-MagOff rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N420): 0.01 - 1 μg
Purified mRNA or Ribosomal-depleted RNA: 0.5 - 100 ng.

It is recommended to use Agilent 2100 Bioanalyzer to analyze the integrity of total RNA. mRNA enriched with VAHTS mRNA Capture Beads 2.0 (Vazyme #N403) must be high quality RNA (RIN≥7). Degraded total RNA used for library construction will lead to bias of the 3' ends of the RNA molecules. For RNA samples with RIN<7, rRNA can be removed using the Ribo-off method (Vazyme #N406/417/408/409/420).

Main fields of RNA-related analysis:

- ◇ gene expression analysis
- ◇ single nucleotide variation calling
- ◇ alternative splicing detection
- ◇ gene fusion detection
- ◇ target transcriptome analysis

05/Self-prepared Materials

◇ **RNA Analysis:**

Equalbit RNA HS Assay Kit (Vazyme #EQ211)
Equalbit RNA BR Assay Kit (Vazyme #EQ212)
Agilent RNA 6000 Pico Kit (Agilent #5067-1513)

◇ **mRNA Enrichment & rRNA Depletion:**

VAHTS mRNA Capture Beads 2.0 (Vazyme #N403)
Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N406)
RRibo-off rRNA Depletion Kit V2 (Bacteria) (Vazyme #N417)
Ribo-off Globin & rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N408)
Ribo-off rRNA Depletion Kit (Plant) (Vazyme #N409)
Ribo-MagOff rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N420)

◇ **RNA and DNA Clean Beads:**

VAHTS DNA Clean Beads (Vazyme #N411)
VAHTS RNA Clean Beads (Vazyme #N412)

◇ **Adapters:**

VAHTS RNA Adapters Set 3 - Set 6 for Illumina (Vazyme #N809/N810/N811/N812) or VAHTS RNA Multiplex Oligos Set 1 - Set 2 for Illumina (Vazyme #N323/N324)

◇ **Library Quality Control:**

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

Agilent DNA 1000 Kit (Agilent #5067-1504) or Agilent High Sensitivity DNA Kit (Agilent #5067 - 4626)

◇ **Other Materials:**

Freshly prepared Ethanol (80%), Nuclease-free ddH_2O, Low-adsorption Nuclease-free PCR Tubes and Pipette Tips, PCR Instrument, Magnetic Rack, Qubit, Agilent 2100 Bioanalyzer.

06/Notes

06-1/Quality Control of RNA Samples

To ensure the library quality, quality control must be performed before the experiment. The total amount and purity of RNA samples must meet the following conditions.

1. The initial 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 2.1. If the ratio>2.1, the RNA samples may have been contaminated with genomic DNA. If the ratio<1.8, the RNA samples may have been contaminated with protein. The ratio of OD230/OD260 should be between 0.4 and 0.5. If the ratio>0.5, the RNA samples may have been contaminated with salt or small molecule. If the ratio<0.4, the RNA samples may have been contaminated with genomic DNA.

06-2/RNA Sample Preparation

1. Pay attention to mixing solution containing RNA by pipetting gently. **Do Not vortex**, avoid unexpected size of library caused by RNA breaking.
2. The mRNA enriched by Poly(A) method or the RNA with rRNA depletion should be performed to subsequent operations as soon as possible to avoid RNA degradation.
3. For the Input RNA with low concentration, it can be concentrated using lyophilization, ethanol-precipitation, column-based or beads-based purification (e.g. VAHTS RNA Clean Beads (Vazyme #N412)).

06-3/Tips for DNA Purification with Magnetic Beads

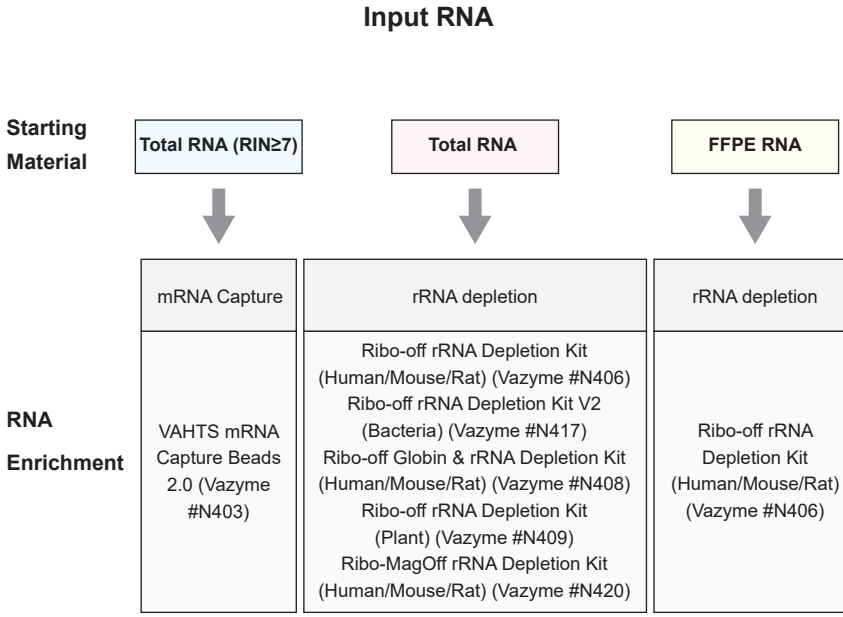
1. Equilibrate the beads to room temperature before use to assure capture efficiency.
2. Mix the beads thoroughly every time before pipetting.
3. The sample and the magnetic beads are thoroughly mixed and then placed on a magnetic rack for separation. Please transfer the supernatant after the solution is completely clear, and leave 2 - 3 μl of supernatant to avoid disturbing the beads. If the magnetic beads are accidentally pipetted with the supernatant, resulting in low yield, poor effect of size selection and even affecting the subsequent enzymatic reaction. In this case, the magnetic beads can be mixed and placed on magnetic rack again to separate the beads completely.
4. Always use 80% ethanol freshly prepared. Keep tubes on magnetic rack without disturbing the beads during elution.
5. Do not leave any 80% ethanol supernatant after the second washing step to reduce the residual impurities.
6. It is important to dry all the ethanol at room temperature before proceeding with subsequent reactions. Over-drying the beads may result in low yield. It is recommended to air-dry the beads at room temperature for 5 - 10 min. Do not heat to dry the beads (e.g. drying at 37°C in an oven).

06-4/Operational Attentions

1. Thaw all components of this kit on ice. Mix thoroughly upside down several times, centrifuge briefly and place on ice for use.
2. It is recommended to use filter pipette tips and change tips when pipetting different samples.
3. Please use RNase-free materials before 2nd strand cDNA synthesis, while use DNase-free materials after that.
4. Be sure to use fresh Nuclease-free ddH_2O during the experiment. It is recommended to dispense it into small tubes for use and discard after use.
5. Be sure to wear gloves and change gloves after touching the equipment outside the RNase-free space or other working areas.
6. Please cover the reagent with lid to avoid contamination whenever finish use.
7. It is recommended to use a PCR instrument with a heated lid when carrying out the reaction in each step. Choose the preheated lid option and set to the reaction temperature before use.

8. PCR products are highly susceptible to aerosol contamination caused by improper handling, which can affect the accuracy of the experiment results. Therefore, we recommend physically isolating the PCR reaction preparation area and the PCR product purification testing area, using equipment such as specialist pipettes, and periodically cleaning each laboratory area to ensure proper cleanliness of the laboratory environment.
9. If you need to pause during the experiment, please store the samples at the appropriate temperature according to the stop point indicated in protocol. Improper storage may reduce the success rate of the library construction.

07/Mechanism & Workflow



cDNA Library Preparation

- 2nd strand cDNA synthesis, End Repair and dA-Tailing are combined into one step, which greatly shortens the time of library construction. Both non-strand-specific and strand-specific transcriptome library construction schemes are provided.

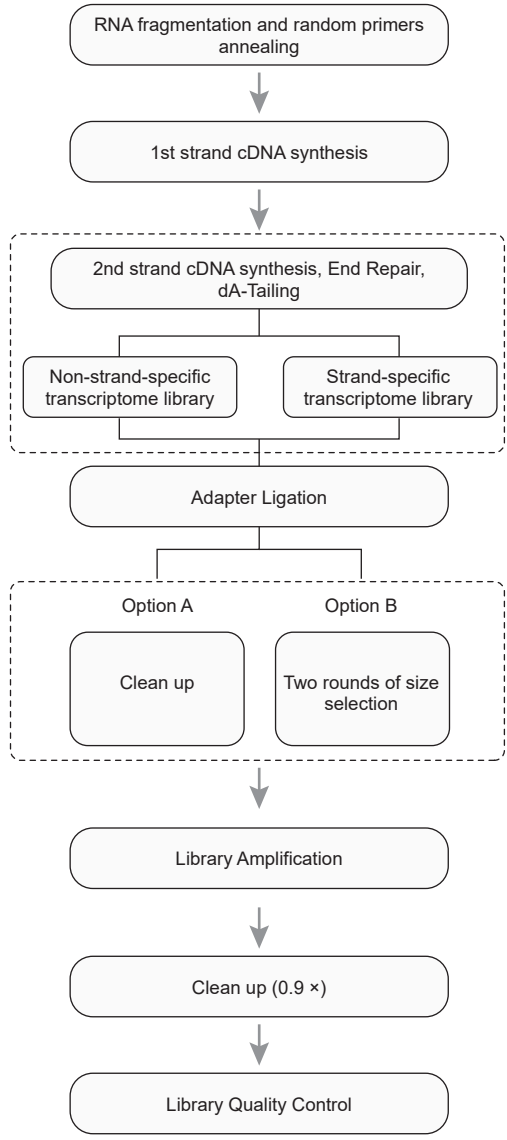
Size-Selection

Option A:

150 - 200 bp inserts can be obtained;

Option B:

Libraries with inserts of customized sizes can be obtained after two rounds of size selection.



Wokflow of VAHTS Universal V8 RNA-seq Library Prep Kit for Illumina

08/Experiment Process

08-1/mRNA Purification and Fragmentation

Protocol A: Poly(A)-based mRNA enrichment

Taking VAHTS mRNA Capture Beads 2.0 (Vazyme #N403) for mRNA enrichment as an example, this protocol is applicable for preparing RNA libraries from 0.01 - 4 μg of total RNA of eukaryotes (e.g. animal, plant or fungi) with good integrity.

1. Take out mRNA Capture Beads 2.0, Beads Wash Buffer 2.0, Tris Buffer 2.0 and Beads Binding Buffer 2.0 from 2 ~ 8°C, and equilibrate to room temperature.
2. Dissolve 0.01 - 4 μg of total RNA with Nuclease-free ddH_2O to a total volume of 50 μl in a Nuclease-free PCR tube. Keep the tube on ice and proceed to the next step as soon as possible.
3. Softly resuspend mRNA Capture Beads 2.0 thoroughly by inverting. Add 50 μl beads to prepared RNA sample and mix thoroughly by pipetting up and down 10 times.
   ▲mRNA Capture Beads 2.0, Beads Wash Buffer 2.0 and Beads Binding Buffer 2.0 contain detergent. DO NOT vortex violently when mixing. Avoid foaming when pipetting.
4. Run the following program in the PCR instrument to make the first time binding of mRNA andmagnetic beads:

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

5. Place the tube onto a magnetic rack until the supernatant is clear (about 5 min), carefully discard the supernatant without disturbing the beads.
6. Remove the tube from the magnetic rack. Add 200 μl of Beads Wash Buffer 2.0 and mix thoroughly by pipetting up and down 10 times. Place the tube back to the magnetic rack until the supernatant is clear (about 5 min), carefully discard the supernatant without disturbing the beads.
   ▲ Step 4 - 6 are the first round of mRNA isolation and purification, and Step 7 - 12 are the second round of mRNA isolation and purification to ensure the removal efficiency of rRNA.
   ▲ For some special samples, please repeat Step 6 and wash again to ensure the removal efficiency of rRNA.
7. Remove the tube from the magnetic rack. Add 50 μl of Tris Buffer 2.0 and resuspend the beads thoroughly by pipetting up and down 10 times.
8. Run the following program in the PCR instrument to release mRNA.

Temperature	Time
80°C	2 min
25°C	Hold

9. Add 50 µl of Beads Binding Buffer 2.0 and mix thoroughly by pipetting up and down 10 times.
10. Incubate at room temperature for 5 min to make the mRNA bind to the beads.
11. Keep the tube on the magnetic rack to isolate the mRNA from total RNA until the supernatant is clear (about 5 min), carefully discard the supernatant.
12. Remove the tube from the magnetic rack. Add 200 µl of Beads Wash Buffer 2.0 and mix thoroughly by pipetting up and down 10 times. Place the tube on the magnetic rack until the supernatant is clear (about 5 min), carefully discard the supernatant.
    ▲ It is highly recommended to completely remove the residual supernatant in this step. The residual of Beads Wash Buffer 2.0 will affect the fragmentation of mRNA.
13. Prepare the Frag/Prime Buffer (1 ×) in Nuclease-free tubes as follows:

Components	Volume
Nuclease-free ddH_2O	10 µl
2 × Frag/Prime Buffer	10 µl
Total	20 µl

14. Remove the tube from the magnetic rack. Add 18.5 µl of Frag/Prime Buffer (1 ×) and resuspend the beads thoroughly by pipetting up and down 10 times. Incubate in the PCR instrument and set programs according to insert size:

Insert size (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

    ▲ Do not pause between steps from the fragmentation to the 1st strand cDNA synthesis, as mRNA is easy to degrade under this system.
    ▲ The reagents for 08-2/Second Strand cDNA Synthesis/Step 1 can be taken out from -30 ~ -15°C in advance and place on ice for use.
15. Place the tube on the magnetic rack until the supernatant is clear (about 5 min). Transfer 16 µl of supernatant to a new Nuclease-free PCR tube, then immediately proceed to synthesis of 1st strand cDNA.

Protocol B: rRNA depletion method

Taking Ribo-off rRNA Depletion Kit (Human/Mouse/Rat)(Vazyme #N406) as an example, this protocol is applicable to the generation of total RNA transcriptome library of human, rat, mouse and other species with 0.01 - 1 µg of Input RNA.

1. Dissolve 0.01 - 1 µg of total RNA with Nuclease-free ddH_2O to a total volume of 11 µl in a Nuclease-free PCR tube, keep on ice for use.

2. rRNA and Probe hybridization:

A. rRNA and Probe hybridization

Components	Volume
rRNA Probe (H/M/R)	1 μl
Probe Buffer	3 μl
Total RNA	11 μl
Total	15 μl

Mix thoroughly by gently pipetting up and down 10 times.

▲For multiple samples, it is recommended to prepare a mixture of the rRNA Probe (H/M/R) and the Probe Buffer first, and then dispense into each PCR tube. It is recommended to prepare 1.1 × volumes of the actual volume to compensate for the loss.

B. Briefly centrifuge to collect the liquid at the bottom of the tube. Place the tube into a PCR instrument and run the following program:

Temperature	Time
Hot lid of 105°C	On
95°C	2 min
95 ~ 22°C	0.1°C/sec
22°C	5 min

▲This step takes about 15 - 20 min, which may vary with different types of PCR instruments.

▲The reagents for step 3 can be taken out from -30 ~ -15°C in advance and place on ice for use.

3. Digestion with RNase H:

A. Prepare the following reaction solution on ice:

Components	Volume
RNase H Buffer	4 μl
RNase H	1 μl
Products of previous step	15 μl
Total	20 μl

Mix thoroughly by gently pipetting up and down 10 times.

▲For multiple samples, it is recommended to prepare a mixture of the RNase H Buffer and the RNase H first, and then dispense into each PCR tube. It is recommended to prepare 1.1 × volumes of the actual volume to compensate for the loss.

B. Put the sample into a PCR instrument and run the following program:

Temperature	Time
37°C	30 min
4°C	Hold

▲The reagents for Step 4 can be taken out from -30 ~ -15°C in advance and place on ice for use.

4. Digestion with DNase I:

A. Prepare the following reaction solution on ice:

Components	Volume
DNase I Buffer	29 μl
DNase I	1 μl
RNase H Digested Products	20 μl
Total	50 μl

Mix thoroughly by gently pipetting up and down 10 times.

▲ For multiple samples, it is recommended to prepare a mixture of the DNase I Buffer and the DNase I in a centrifuge tube of appropriate size first, and then dispense into each PCR tube. It is recommended to prepare 1.1 × volumes of the actual volume to compensate for the loss.

B. Place the tube into a PCR instrument and run the following program:

Temperature	Time
37°C	30 min
4°C	Hold

Briefly centrifuge to collect the liquid at the bottom of the tube. Place the tube on ice and immediately proceed to the next program.

5. Purification of ribosomal-depleted RNA with VAHTS RNA Clean Beads.
   a. Resuspend the VAHTS RNA Clean Beads thoroughly by inverting or vortexing, piptte 110 μl (2.2 ×) of beads into the RNA sample of previous step. Mix thoroughly by pipetting up and down 10 times.
   b. Incubate the sample on ice for 15 min to bind RNA to the beads.
   c. Keep the tube on the magnetic rack until the supernatant is clear (about 5 min), carefully discard the supernatant without disturbing the beads.
   d. Keep the tube on the magnetic rack and add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate at room temperature for 30 sec and carefully discard the supernatant without disturbing the beads.
   e. Repeat Step d.
   f. Keep the tube on the magnetic rack and air-dry the beads for 5 - 10 min.
      ▲ Do not disturb the beads when adding 80% ethanol.
      ▲ It is highly recommended to use a 10 μl pipettor to remove the residual supernatant in this step.
      ▲ Avoid over-drying of beads, which resulting in the reduction of recovery efficiency.

6. Prepare the Frag/Prime Buffer (1 ×) in a Nuclease-free tube as follows:

Components	Volume
Nuclease-free ddH_2O	10 μl
2 × Frag/Prime Buffer	10 μl
Total	20 μl

7. Remove the tube from the magnetic rack. Add 18.5 μl of Frag/Primer Buffer (1 ×) and mix thoroughly by pipetting up and down 10 times. Incubate at room temperature for 2 min. Put the tube back on the magnetic rack until the supernatant is clear (about 5 min), carefully transfer 16 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

8. Incubate the tube in a PCR instrument and set programs according to the fragment size:

Insert size (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

▲ Do not perform fragmentation for samples with RIN<3.0.

▲ Do not pause between steps from the fragmentation to the 1st strand cDNA synthesis, as mRNA is easy to degrade under this system.

▲ The reagents for 08-2/Second Strand cDNA Synthesis/Step 1 can be taken out from -30 ~ -15°C in advance and place on ice for use.

Protocol C: Use purified mRNA or Ribosomal-depleted RNA as templates

This protocol is applicable for using 0.5 - 100 ng of purified mRNA or Ribosomal-depleted RNA as templates for library preparation.

1. Prepare the reaction system as follows:

Components	Volume
RNA	8 μl
2 × Frag/Prime Buffer	8 μl
Total	16 μl

2. Mix thoroughly by pipetting up and down 10 times. Incubate the tube in the PCR instrument and set programs according to insert size:

Insert size (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

▲ Do not perform fragmentation for fragmented or short RNA.

▲ Do not pause between steps from the fragmentation to the 1st strand cDNA synthesis, as mRNA is easy to degrade under this system.

▲ The reagents for 08-2/Second Strand cDNA Synthesis/Step 1 can be taken out from -30 ~ -15°C in advance and place on ice for use.

08-2/Second Strand cDNA Synthesis

1. The components for synthesis of double strand cDNA should be dissolved on ice and mixed by inverting. Briefly centrifuge to collect the solution at the bottom of the tube. Place on ice for use. Prepare the reaction solution to synthesize the 1st strand of cDNA as follows:

Components	Volume
Fragmented mRNA	16 μl
1st Strand Buffer 3	7 μl
1st Strand Enzyme Mix 3	2 μl
Total	25 μl

2. Adjust the pipettor to a 20 μl range and mix thoroughly by gently pipetting up and down 10 times.

▲ For multiple samples, it is recommended to prepare a mixture of the 1st Strand Buffer 3 and the 1st Strand Enzyme Mix 3 in a tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 × volumes of the actual volume to compensate for the loss. The mixture should be kept away from light.

▲ Before add the mixture to the reaction system, keep it away from light.

3. Run the following program in a PCR instrument for the synthesis of 1st strand cDNA:

Temperature	Time
Hot lid of 105°C	On
25°C	10 min
42°C	15 min
70°C	15 min
4°C	Hold

▲The synthesis of 2nd strand cDNA should be performed immediately after the synthesis of the 1st strand of cDNA.

▲The reagents for Step 4 can be taken out from -30 ~ -15°C in advance and place on ice for use.

4. Prepare the reaction solution to synthesize the 2nd strand of cDNA as follows:

Components	Volume
1st Strand cDNA	25 μl
2nd Strand Buffer 2 (with dNTP or dUTP)*	25 μl
2nd Strand Enzyme Super Mix 2	15 μl
Total	65 μl

* **Use 2nd Strand Buffer 2 (with dNTP) for non-strand-specific mRNA Library. Use 2nd Strand Buffer 2 (with dUTP) for strand-specific mRNA library.**

▲ For multiple samples, it is recommended to prepare a mixture of the 2nd Strand Buffer 2 (with dNTP or dUTP) and the 2nd Strand Enzyme Super Mix 2 in PCR tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 × volumes of the actual volume to compensate for the loss.

5. Adjust the pipettor to a 50 μl range and mix thoroughly by gently pipetting up and down 10 times.

6. Run the following program in a PCR instrument for the synthesis of 2nd strand cDNA:

Temperature	Time
Hot lid of 105°C	On
16°C	30 min
65°C	15 min
4°C	Hold

▲The reagents for 08-3/Adapter Ligation/Step 1 can be taken out from -30 ~ -15°C in advance and place on ice for use.

The ds cDNA synthesis products can be stored at -30 ~ -15°C for 24 h.

08-3/Adapter Ligation

1. Prepare the reaction solution of Adapter Ligation as follows:

Components	Volume
ds cDNA	65 µl
Rapid Ligation Buffer 4	25 µl
Rapid DNA Ligase 4	5 µl
RNA Adapter*	x µl
Nuclease-free ddH_2O	To 100 µl

▲ The mixture of Rapid Ligation Buffer 4 and Rapid DNA Ligase 4 can be stored at 2 ~ 8°C for less than 24 h.

▲ It is recommended to add the RNA adapter to the ds cDNA first and mix thoroughly, then add the mixture of Rapid Ligation Buffer 4 and Rapid DNA Ligase 4.

* Please refer to the following table for the amount of Adapter:

Input RNA		Volume of Adapter
Protocol A/B	Protocol C	
1 - 4 µg	100 ng	3.5 µl
100 - 999 ng	10 - 99 ng	1 µl
10 - 99 ng	0.5 - 9.9 ng	0.5 µl

2. Adjust the pipettor to an 80 µl range and mix thoroughly by gently pipetting up and down 10 times.
3. Run the program of ligation reaction in the PCR instrument:

Temperature	Time
Hot lid of 105°C	On
20°C	15 min
4°C	Hold

▲ VAHTS DNA Clean Beads for Step 08-4 can be taken out from 2 ~ 8°C in advance and place at room temperature.

The adapter ligation products can be temporarily stored at 2 ~ 8°C for 1 h or -85 ~ -65°C for 12 h.

08-4/Purification/Size-selection Protocol

Two options are provided in this step, please select a suitable solution according to the actual situation.

Option A is one round of purification and no size selection. It is applicable for libraries with 150 - 200 bp inserts (suitable for mRNA fragmented by 94°C for 8 min). When Input RNA is <100 ng, this option is suggested.

Option B is one round of purification and two-rounds of size selection. This protocol is suitable for library preparation after mRNA capture or rRNA removal when Input RNA is ≥100 ng. Different sizes of inserts (>200 bp) can be obtained according to Table 1/2 when Input RNA is ≥100 ng. The library of the desired size can also be directly obtained in the Appendix.

Option A: For libraries with 150 - 200 bp inserts (suitable for mRNA fragmented by incubation at 94°C for 8 min or Input RNA <100 ng)

1. Equilibrate the VAHTS DNA Clean Beads to room temperature.
2. Resuspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Add 45 μl (0.45 ×) of beads into the above adapter ligation products. Mix thoroughly by pipetting up and down 10 times.
3. Incubate at room temperature for 10 min.
4. Place the tube on a magnetic rack until the supernatant is clear (about 5 min), carefully discard the supernatant without disturbing the beads.
5. Keep the tube on the magnetic rack and add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.
6. Repeat Step 5.
7. Keep the tube on the magnetic rack and air-dry the beads for 5 - 10 min.
   ▲ Do not disturb the beads when adding 80% ethanol.
   ▲ It is highly recommended to use a 10 μl pipettor to completely remove the residual supernatant in this step.
   ▲ Over-drying of beads will result in the reduction of recovery efficiency. Please avoid over-drying.
8. Remove the tube from the magnetic rack. Add 22.5 μl of Nuclease-free ddH_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 rack until the supernatant is clear (about 5 min), and carefully transfer 20 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

Option B: For libraries with >200 bp inserts (suitable for mRNA fragmented by incubation at 94°C for 5 min, 85°C for 6 min, or 85°C for 5 min)

Purify the ligation products with 0.45 × VAHTS DNA Clean Beads.

1. Equilibrate the VAHTS DNA Clean Beads to room temperature.
2. Resuspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Add 45 μl (0.45 ×) of beads into the above adapter ligation products. Mix thoroughly by pipetting up and down 10 times.
3. Incubate at room temperature for 10 min.
4. Place the tube on a magnetic rack until the supernatant is clear (about 5 min), carefully discard the supernatant without disturbing the beads.
5. Keep the tube on the magnetic rack add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.
6. Repeat Step 5.
7. Keep the tube on the magnetic rack and air-dry the beads for 5 - 10 min.
   ▲ Do not disturb the beads when adding 80% ethanol.
   ▲ It is highly recommended to use a 10 μl pipettor to completely remove the residual supernatant in this step.
   ▲ Over-drying of beads will result in the reduction of recovery efficiency. Please avoid over-drying.
8. Remove the tube from the magnetic rack. Add 102.5 μl of Nuclease-free ddH_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 rack until the supernatant is clear (about 5 min), and carefully transfer 100 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

Conduct two rounds of size selection with VAHTS DNA Clean Beads.

When VAHTS RNA Adapters Set 3 - Set 6 for Illumina (Vazyme #N809/N810/N811/N812) are used, please refer to Table 1 for size selection.

Table 1. Recommended conditions for size selection

Insert Size (bp)	200 - 300	250 - 350	350 - 450	450 - 550
Library Size (bp)	320 - 420	370 - 470	470 - 570	570 - 670
Fragmentation Condition	94°C 5 min	85°C 6 min	85°C 6 min	85°C 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 ×)

When VAHTS RNA Multiplex Oligos Set 1 - Set 2 for Illumina (Vazyme #N323/N324) are used, please refer to Table 2 for size selection.

Table 2. Recommended conditions for size selection

Insert size (bp)	200 - 300	250 - 350	350 - 450	450 - 550
Library size (bp)	320 - 420	370 - 470	470 - 570	570 - 670
Fragmentation Condition	94°C 5 min	85°C 6 min	85°C 6 min	85°C 5 min
Volume of beads for 1st round (μl)	80 (0.8 ×)	65 (0.65 ×)	60 (0.6 ×)	55 (0.55 ×)
Volume of beads for 2nd round (μl)	20 (0.2 ×)	20 (0.2 ×)	10 (0.1 ×)	10 (0.1 ×)

▲ The library size here is the final length and the volume of beads will affect the final size. The bead volume ratio used in size selection is relative to the initial DNA volume. Take the 350 - 450 bp insert size and the 100 μl of initial DNA as an example, the volume of beads for 1st round is 60 μl, that is the result of 0.6 × 100. And the volume of beads for 2nd round is 10 μl, that is the result of 0.1 × 100, rather than 10% of the absorbed 155 μl supernatant.

9. Resuspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Transfer 60 μl (0.6 ×) of the beads into the samples above. Mix thoroughly by pipetting up and down 10 times.
10. Incubate at room temperature for 10 min.
11. Place the tube on a magnetic rack until supernatant is clear (about 5 min), carefully transfer 155 μl of the supernatant (**DO NOT discard**) to a new Nuclease-free PCR tube.
12. Add 10 μl (0.1 ×) of VAHTS DNA Clean Beads and mix thoroughly by pipetting up and down 10 times.
13. Incubate at room temperature for 10 min.
14. Place the tube on a magnetic rack until supernatant is clear (about 5 min), carefully discard the supernatant without disturbing the beads.
15. Keep the tube on the magnetic rack and add 200 μl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.
16. Repeat Step 15.
17. Keep the tube on the magnetic rack and air-dry the beads for 5 - 10 min
    ▲ Do not disturb the beads when adding 80% ethanol.
    ▲ It is highly recommended to use a 10 μl pipettor to completely remove the residual supernatant in this step.
    ▲ Over-drying of beads will result in the reduction of recovery efficiency. Please avoid over-drying.

18. Remove the tube from the magnetic rack. Add 22.5 μl of Nuclease-free ddH_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 rack until supernatant is clear (about 5 min), carefully transfer 20 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

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

08-5/Library Amplification

1. Prepare the PCR reaction system according to the selected adapters as follows:

When VAHTS RNA Adapters Set 3 - Set 6 for Illumina (Vazyme #N809/N810/N811/N812) are used, please refer to Table 3.

Table 3. PCR reaction system for universal primers

Components	Volume	
Purified Ligation Products	20 μl	
PCR Primer Mix 4	5 μl	■
2 × HF Amplification Mix	25 μl	■
Total	50 μl	

When VAHTS RNA Multiplex Oligos Set 1 - Set 2 for Illumina (Vazyme #N323/N324) are used, please refer to Table 4.

Table 4. PCR reaction system for Index primers

Components	Volume	
Purified Ligation Products	20 μl	
VAHTS i5 PCR Primer*	2.5 μl	
VAHTS i7 PCR Primer*	2.5 μl	
2 × HF Amplification Mix	25 μl	■
Total	50 μl	

▲ For multiple samples, it is recommended to prepare a mixture of above components (except for Purified Ligation Product) first, and then dispense into each PCR tube. It is recommended to prepare 1.1 × volumes of the actual volume to compensate for the loss.

* VAHTS i5 PCR Primer and VAHTS i7 PCR Primer are provided in Set 1 - Set 2 for Illumina (Vazyme #N323/N324).

2. Adjust the pipettor to a 30 μl range and mix thoroughly by gently pipetting up and down 10 times.

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

Program	Temperature	Time	Cycles
Hot Lid	105°C	On	
Pre-denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	9 - 19
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	1 min	1
Hold	4°C	Hold	

The mRNA ratio in the equal amount of total RNA extracted from different species and individuals is not necessarily the same. According to the actual situation of species, the number of PCR cycles should be adjusted appropriately, generally 9 - 19 cycles. Refer to the following table for the number of cycles for each amount of Input RNA.

Input RNA		Cycles
Protocol A/B	Protocol C	
2 - 4 μg		9 - 10
1 - 2 μg	100 ng	10 - 12
100 - 999 ng	10 - 99 ng	13 - 15
10 - 99 ng	0.5 - 9.9 ng	16 - 19

4. Purification of the PCR products with VAHTS DNA Clean Beads.

   a. Equilibrate the VAHTS DNA Clean Beads to room temperature.

   b. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 45 μl (0.9 ×) of beads into the above amplification products. Mix thoroughly by pipetting up and down 10 times.

   c. Incubate at room temperature for 10 min.

   d. Place the tube on a magnetic rack until supernatant is clear (about 5 min), carefully discard the supernatant without disturbing the beads.

   e. Keep the tube on the magnetic rack and add 200 μl of freshly prepared 80% ethanol to rinse the beads Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

   f. Repeat Step e.

   g. Keep the tube on the magnetic rack and air-dry the beads for 5 - 10 min.
      ▲Do not disturb the beads when adding 80% ethanol.
      ▲It is highly recommended to use a 10 μl pipettor to completely remove the residual supernatant in this step.
      ▲Over-drying of beads will result in the reduction of recovery efficiency. Please avoid over-drying.

   h. Remove the tube from the magnetic rack. Add 25 μl of Nuclease-free ddH_2O to elute the DNA. Mix thoroughly by vortexing or pipetting and incubate 2 min at room temperature. Place the tube on the magnetic rack until supernatant is clear (about 5 min), carefully transfer 22.5 μl of supernatant to a new Nuclease-free PCR tube without disturbing the beads.
      ▲DO NOT disturb the beads while drawing samples from the supernatant. Even trace amount of beads will affect the quality of the final library.
      ▲Primer dimer is easily formed when 10 ng Input RNA is used for library preparation. 0.85 × beads purification can be additionally performed to remove the primer dimer.

5. Library Quality Analysis based on Agilent Technologies 2100 Bioanalyzer.
   Analyze 1 μl of library using an Agilent DNA 1000 chip (Agilent #5067-1504). For example, as shown in **Fig 1 and Fig 2**. A library with high quality should exhibit a narrow peak as expected size. A narrow peak at 120 bp indicates the contamination of adapter dimers. To eliminate this contamination, dilute the library to 50 μl with Nuclease-free ddH_2O and repeat **08-5/Step 4** for one more purification.

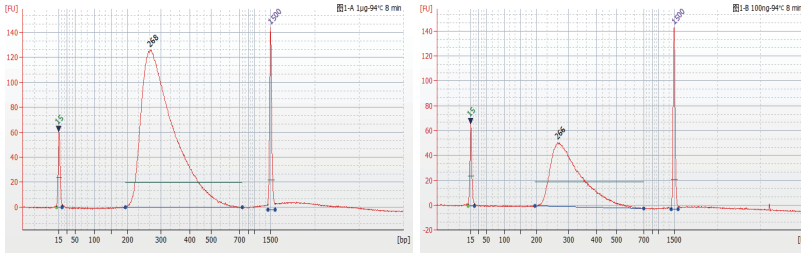


Fig 1-A/B. 1 μg/100 ng RNA of 293T cells were fragmented at 94°C for 8 min and purified with 0.9 × VAHTS DNA Clean Beads, respectively.

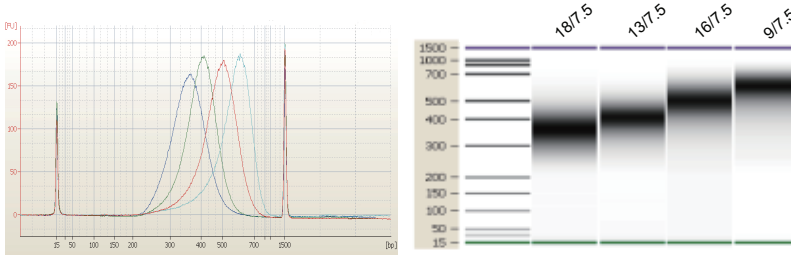


Fig 2. 200 ng RNA of 293T cells was fragmented under different conditions, and size selection was performed with VAHTS DNA Clean Beads according to Table 1, respectively.

09/FAQ & Troubleshooting

◇ Incorrect operation and remedy.

Step	Correct Operation	Incorrect Operation	Remedy
08-1/ Protocol A Step 6	Add 200 μl of Beads Wash Buffer 2.0, resuspend and wash the mRNA Capture Beads 2.0	Add 200 μl of 80% ethanol by mistake	Discard the ethanol, dry it; resuspend the beads with 200 μl Beads Wash Buffer 2.0 and continue the next step
08-1/ Protocol A Step 7	Add 50 μl of Tris Buffer 2.0, resuspend mRNA Capture Beads 2.0	Add Beads Binding Buffer 2.0 by mistake	If you did not treat it at 80°C 2 min, you can use magnetic rack. After adsorption, discard the supernatant and add Tris Buffer 2.0
08-1/ Protocol A Step 9	Add 50 μl of Beads Binding Buffer 2.0 to make mRNA bind to mRNA Capture Beads 2.0	Add Tris Buffer 2.0 by mistake	Amplify the reaction system and add Beads Binding Buffer 2.0, adding volume is equal to that of Tris Buffer 2.0
08-1/ Protocol A Step 12	Add 200 μl of Beads Wash Buffer 2.0, resuspend and wash the mRNA Capture Beads 2.0	Directly add Frag/Prime Buffer without adding Beads Wash Buffer 2.0	If it is not interrupted by heat, you can put it on the magnetic rack back, drop the Frag/Prime Buffer and add Beads Wash Buffer 2.0
08-1/ Protocol A Step 14	Frag/Prime Buffer (1 ×) was added to break mRNA	After discarding Beads Wash Buffer 2.0, 2 × Frag/Prime Buffer is still in frozen	Re-add Beads Wash Buffer 2.0 to soak mRNA Capture Beads 2.0 until the Frag/Prime buffer thaws, discard the Beads Wash Buffer 2.0 and continue the next step
Protocol B Step 8 Protocol C Step 2	Frag/Prime Buffer (1 ×) 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 fragmentation condition, otherwise the library will fail to construct and the ultimate size of the inserted library fragment will change too
08-1/ Protocol A Step 14	After fragmenting mRNA, transfer the supernatant to a new Nuclease-free PCR tube	The volume of supernatant is less than 16 μl	Add Frag/Prime Buffer(1 ×) to make up for 16 μl
08-1/ Protocol B Step 8	Fragmentation	Fragmentation was not performed, and 1st Synthesis Mix was added	Add 2.2 × VAHTS RNA Clean Beads for another round of purification
08-4/ Step 8	No size selection	In 1st round of purification, wrong volume of water was added	Amplification system can be adjusted or perform another round of purification

◇ 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 for sequencing. If you cannot provide qualified RNA samples, try to use the following methods to make up:

① Amount of Input RNA: Increase the amount of Input RNA.

② Prepare several duplicate samples, merge them after the fragmentation step or before PCR step.

③ Construct libraries without size selection: Though RNA fragmented at 94°C for 8 min is short, its distribution is concentrated.

◇ High rRNA residue.

Note: The amount of Input RNA is different according to the difference of mRNA enrichment methods. Please select the Input RNA within the specification range.

① Poly(A) enrichment, VAHTS mRNA Capture Beads 2.0 (Vazyme #N403): 0.01 - 4 μg;

② Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N406): 0.01 - 1 μg;

③ Ribo-off rRNA Depletion Kit V2 (Bacteria) (Vazyme #N417): 0.01 - 5 μg;

④ Ribo-off Globin & rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N408) : 0.01 - 1 μg;

⑤ Ribo-off rRNA Depletion Kit (Plant) (Vazyme #N409): 1 - 5 μg;

⑥ Ribo-MagOff rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N420): 0.01 - 1 μg.

◇ Questions for library quantification.

There are two methods for library quantification: Qubit and qPCR are used for determining library concentration and library molarity, respectively. qPCR can truly detect the number of DNA fragments used for sequencing owing to the theory of clustered primers performing amplification quantification. Therefore, the library quantitative results measured by qPCR are more reliable. The single-stranded portion cannot 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%. These two methods can be used at the same time to quantify libraries and correct each other.

◇ Instructions for adapter selection.

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

Type 1: VAHTS RNA Adapters Set 3 - Set 6 for Illumina (Vazyme #N809/N810/N811/N812) totally contains 96 different adapters. The adapters are divided into four separate packages, each containing 24 different adapters according to the serial number.

Type 2: VAHTS RNA Multiplex Oligos Set 1 - Set 2 for Illumina (Vazyme #N323/N324) contain VAHTS RNA Adapter-S for Illumina, 8 kinds of VAHTS i5 PCR Primers and 12 kinds of VAHTS i7 PCR Primers in each kit, allowing the construction of up to 384 different dual-Index libraries by combination.

◇ Is this kit suitable for small RNA library construction?

Not applicable. Considering the length of small RNA is only about 22 nt and the captured RNA size by beads is at least 100 bp. This kit cannot efficiently enrich small RNA fragments.

◇ 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 Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N406) to construct library.

◇ Why the insert size is larger than the expected insert when operating size selection as 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.

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

The number of cycles can be adjusted according to the amount of Input RNA. It is recommended to take 1 μl for Qubit test and then make additional 1 - 2 cycles, but the maximum cycle numbers should be no more than 19.

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

①There 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, please re-extract the RNA.

②The species are special. The RNA fragments are not continuous and uniform after fragmentation, and two ranges of fragments might be obtained.

③High-sensitivity chips are used for high concentration detection. It is recommended to use the Agilent DNA 1000 kit for detection or to dilute the library to the appropriate concentration and test with the Agilent DNA High Sensitivity kit.

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

High-yield libraries usually exhibit varying degrees of over-amplification. Because at the later period of library amplification, primers are usually exhausted. Therefore, a large number of library fragments can't 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 above phenomenon is normal and would not affect the library sequencing and data analysis.

10/Appendix

For libraries with >200 bp inserts (suitable for mRNA fragmented by incubation at 94°C for 5 min, 85°C for 6 min, or 85°C for 5 min)

For library preparation with <100 ng Input RNA (mRNA capture or rRNA depletion), it is best to conduct only one round of 0.45 × purification and no size selection.

When VAHTS RNA Adapters Set 3 - Set 6 for Illumina (Vazyme #N809/N810/N811/N812) are used, please refer to Table 5 for size selection.

Table 5. Recommended conditions for size selection

Insert Size (bp)	200 - 300	250 - 350	350 - 450	450 - 550
Library Size (bp)	320 - 420	370 - 470	470 - 570	570 - 670
Fragmentation Condition	94°C 5 min	85°C 6 min	85°C 6 min	85°C 5 min
Volume of beads for 1st round (μl)	18	16	13	9
Volume of beads for 2nd round (μl)	7.5	7.5	7.5	7.5

When VAHTS RNA Multiplex Oligos Set 1 - Set 2 for Illumina (Vazyme #N323/N324) are used, please refer to Table 6 for size selection.

Table 6. Recommended size selection conditions for different insert sizes

Insert size (bp)	200 - 300	250 - 350	350 - 450	450 - 550
Library size (bp)	320 - 420	370 - 470	470 - 570	570 - 670
Fragmentation condition	94°C 5 min	85°C 6 min	85°C 6 min	85°C 5 min
Volume of beads for 1st round (μl)	25	19	13	9
Volume of beads for 2nd round (μl)	7.5	7.5	7.5	7.5

▲The size of the library here is the final size. The volume of added magnetic beads will affect the final library size.

Using 350 - 450 bp insert as an example, for libraries with different insert sizes please refer to the above tables.

1. Resuspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Transfer 13 μl of the beads into the samples above. Mix thoroughly by pipetting up and down 10 times.
2. Incubate at room temperature for 10 min.
3. Place the tube on a magnetic rack until the supernatant is clear (about 5 min), carefully transfer 100 μl of the supernatant (**DO NOT discard**) to a new Nuclease-free PCR tube.
   ▲ If the beads are drawn out with the supernatant, the large DNA fragment residuals on the beads will result in unexpected large fragments in the final library.
4. Add 7.5 μl of VAHTS DNA Clean Beads, mix thoroughly by pipetting up and down 10 times.
5. Incubate at room temperature for 10 min.
6. Place the tube on a magnetic rack until the supernatant is clear (about 5 min), carefully discard the supernatant without disturbing the beads.

7. Keep the tube on the magnetic rack and add 200 µl of freshly prepared 80% ethanol to rinse the beads. Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.
8. Repeat Step 7.
9. Keep the tube on the magnetic rack and air-dry the beads for 5 - 10 min.
   ▲ Do not disturb the beads when adding 80% ethanol.
   ▲ It is highly recommended to use a 10 µl pipettor to completely remove the residual supernatant in this step.
   ▲ Over-drying of beads will result in the reduction of recovery efficiency. Please avoid over-drying.
10. Remove the tube from the magnetic rack. Add 22.5 µl of Nuclease-free ddH_2O to elute DNA. Mix thoroughly by vortexing or pipetting and incubate for 2 min at room temperature. Place the tube on the magnetic rack until the supernatant is clear (about 5 min), carefully transfer 20 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.
    ▲ DO NOT disturb the beads while drawing samples from the supernatant. Even trace amout of beads will affect the quality of the final library.

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