VAHTS® Universal V8 RNA-seq Library Prep Kit for MGI

NRM605

Vazyme

Instruction for Use

Version 21.1

Contents

01/Product Description

VAHTS Universal V8 RNA-seq Library Prep Kit for MGIis specially designed for the preparation of transcriptome libraries for next generation sequencing (NGS) platforms of MGI®. The kit is universal and suitable for RNA library construction of RNA that have been obtained by Poly(A)-based mRNA enrichment or rRNA depletion. The kit contains two types of cDNA 2nd Strand synthesis buffer, which can be chosen for library construction for non-stranded or stranded RNA-Seq transcriptome analysis.

This kit combines 2nd Strand cDNA synthesis, end-repair and dA-Tailing into one step, with no need of 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 RNA, and has uniform coverage for different amounts of input-RNA. Libraries of specific sizes, which can be customized, can be obtained after size selection with magnetic beads. All the enzymes and buffer provided in the kit have undergone rigorous quality control and functional testing to ensure the optimal stability and repeatability.

02/Components

Component	NRM605-01 (24 rxns)	NRM605-02 (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 5 for MGI	120 μl	480 μl
2 × HF Amplification Mix	600 μl	4 × 600 μl

03/Storage

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

04/Applications

VAHTS Universal V8 RNA-seq Library Prep Kit for MGI is suitable for RNA library construction of total RNA that have been enriched by Poly(A) (for RNA with good integrity from eukaryotes such as animals, plants and fungi) or rRNA depletion. 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. The amount of input-RNA is related to the mRNA enrichment module:

VAHTS mRNA Capture Beads (Vazyme #N401): 0.01 - 4 μg;

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

Ribo-off rRNA Depletion Kit (Bacteria) (Vazyme #N407): 0.01 - 5 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;

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 (Vazyme #N401) must be high quality RNA (RIN ≥7). Degraded total RNA used for library construction will lead to 3' bias in RNA-seq. For RNA samples with RIN value <7, rRNA removal can be performed using the Ribo-off method (Vazyme #N406/407/408/409).

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

◇ RNA pretreatment：
VAHTS mRNA Capture Beads (Vazyme #N401)
Ribo-off rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N406)
Ribo-off rRNA Depletion Kit (Bacteria) (Vazyme #N407)
Ribo-off Globin & rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N408)
Ribo-off rRNA Depletion Kit (Plant) (Vazyme #N409)

◇ Clean Beads：
VAHTS DNA Clean Beads (Vazyme #N411)
or Agencourt AMPure XP Reagent (Beckman #A63880/A63881/A63882)
VAHTS RNA Clean Beads (Vazyme #N412)
or Agencourt RNA Clean XP Beads (Beckman #A63987)

◇ RNA QC:

Equalbit RNA HS Assay Kit (Vazyme #EQ211)

Equalbit RNA BR Assay Kit (Vazyme #EQ212)

Adapter:

VAHTS RNA Adapters Set 8 for MGI (Vazyme #NM208)

◇ Library QC：

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：

Fresh Ethanol (80%), Nuclease-free Water, Low-absorption Nuclease-free PCR tubes and Pipette tips, Low-absorption EP tubes, Thermocycler (PCR instrument), Magnetic Stand, 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 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/<0.4, the RNA samples may have been contaminated with salt or small molecular/genomic DNA.

06-2/RNA Sample Preparation

1. Give care 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 operation as soon as possible to avoid RNA degradation.
3. For the initial RNA with low concentration, it can be concentrated using lyophilization, ethanol-precipitation, column-based or beads-based clean-ups (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 efficacy.
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 stand for separation. It is important not to discard or transfer any beads with the removal or transfer of supernatant. 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 drawn out with the supernatant. The yield will decrease. The effect of size selection will be poor, and even the subsequent enzymatic reaction will be affected. In this case, the magnetic beads can be mixed and placed on magnetic stand again to separate the beads completely.
4. Always use 80% ethanol freshly prepared. Keep tubes on magnet stand without disturbing the beads during elution.
5. Do not leave any 80% ethanol supernatant behind in the second washing step to reduce the residual impurities.
6. It is important to remove all the ethanol before proceeding with subsequent reactions.

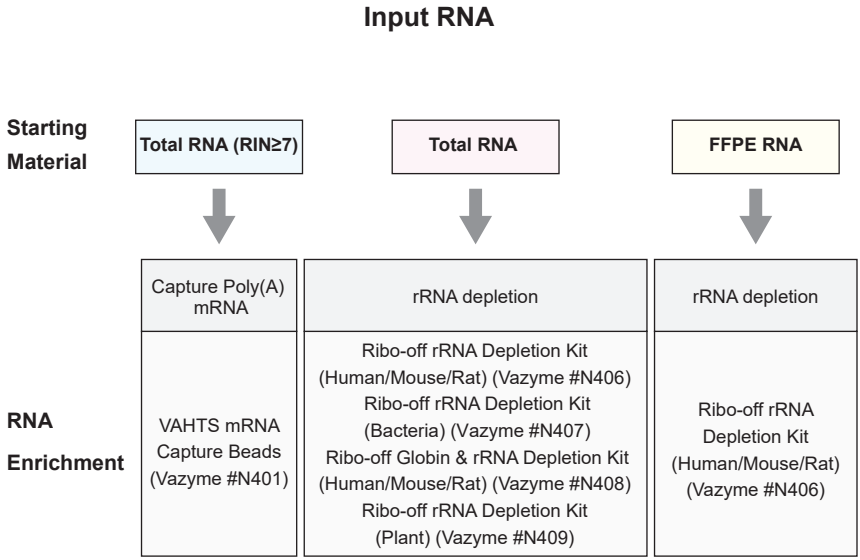
   Over-drying the beads may result in reduced yield. Normally, drying of beads for 5 - 10 min at room temperature should be sufficient. Drying of beads at 37℃ is not recommended.

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; change tips when varying 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; 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 perform the reaction in a PCR instrument with a hot lid, and preheat the PCR instrument to near the reaction temperature before use.

8. Aerosol contamination is easily to be caused by PCR products due to improper operation, affecting the accuracy of the experimental results. Therefore, it is recommended to isolate PCR reaction preparation area and PCR products detection area physically. Use a dedicated pipettor and other equipment, and regularly clean each experimental area to ensure the cleanliness of the experimental 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 stranded and non-stranded transcriptome library construction schemes are provided.

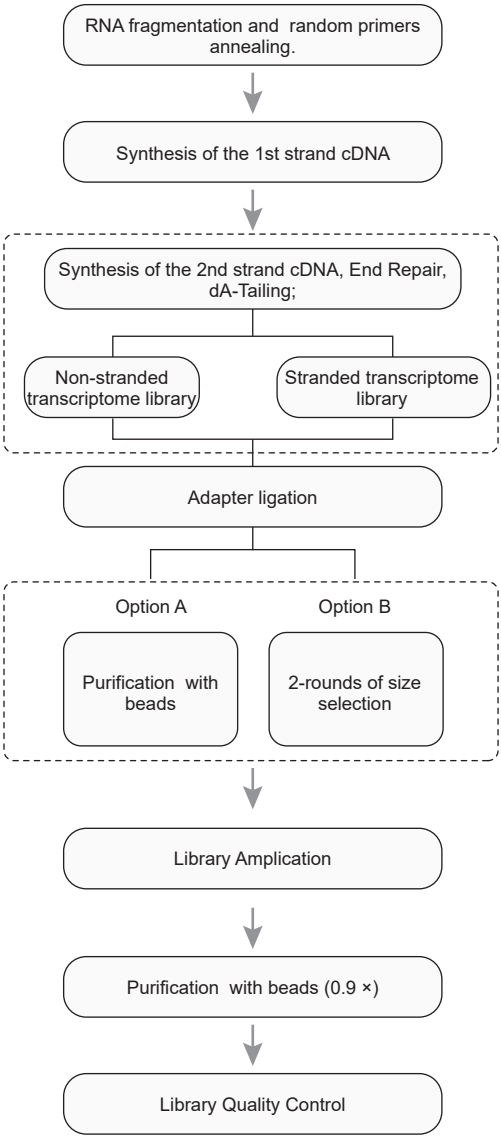
Size-Selection

Option A:

150 - 200 bp inserts can be obtained;

Option B:

Libraries with inserts of customized sizes can be obtained after 2-rounds size selection.



08/Experiment Process

08-1/mRNA Purification and Fragmentation

Protocol A: Poly(A)-based mRNA enrichment

Taking VAHTS mRNA Capture Beads (Vazyme #N401) for mRNA enrichment as an example, this protocol is applicable for preparing non-stranded RNA libraries from 0.01 - 4 μg of total RNA of eukaryotes (e.g. animal, plant or fungal) with good integrity.

1. Take out mRNA Capture Beads, Beads Wash Buffer, Tris Buffer, and Beads Binding Buffer from 2 ~ 8℃, and equilibrate to room temperature.
2. Prepare the RNA sample carefully: dissolve 0.01 - 4 μg of total RNA in Nuclease-free ddH_2O in a Nuclease-free PCR tube, to a total volume of 50 μl. 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, and mix thoroughly by pipetting up and down for 10 times.
   ▲mRNA Capture Beads, Beads Wash Buffer, and Beads Binding Buffer contain detergent. DO NOT vortex or oscillate violently when mixing. Avoid foaming when pipetting.
4. Run the following program in the PCR instrument to make the first time binding of mRNA and magnetic beads:

Temperature	Time
65℃	5 min
25℃	5 min

5. Place the tube onto a magnetic stand. Wait until the solution is clear (about 5 min), then carefully discard the supernatant without disturbing the beads.
6. Take the samples out of the magnetic stand. Add 200 μl of Beads Wash Buffer, and mix thoroughly by pipetting up and down for 10 times. Place the tube back to the magnetic stand. Wait until the solution is clear (about 5 min), then carefully discard the supernatant without disturbing the beads.
   ▲Steps 4 - 6 are the first round of mRNA isolation and purification, and Steps 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. Take the samples 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. Run the following procedure in the PCR instrument to release mRNA.

Temperature	Time
80℃	2 min
25℃	Hold

9. Add 50 μl of Beads Binding Buffer, and mix thoroughly by pipetting up and down for 10 times.

10. Incubate at room temperature for 5 min to make the mRNA bind to the beads.

11. Place the samples on the magnetic stand to isolate the mRNA from total RNA. Wait until the solution is clear (about 5 min), then carefully discard the supernatant without disturbing the mRNA Capture Beads.

12. Take the samples out of the magnetic stand, add 200 μl of Beads Wash Buffer, and mix thoroughly by pipetting up and down for 10 times. Place the tube on the magnetic stand. Wait until the solution is clear (about 5 min), then carefully discard the supernatant without disturbing the mRNA Capture Beads.

▲It is highly recommended to completely remove the residual supernatant in this step. The residual of Beads Wash Buffer will affect the fragmentation of mRNA.

13. Prepare the Frag/Prime Buffer (1 ×) as follows in Nuclease-free tubes:

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

14. Take the samples out of the magnetic stand, add 18.5 μl of Frag/Prime Buffer (1 ×) to resuspend the beads thoroughly by pipetting up and down for 10 times. Incubate the samples in the PCR instrument and set programs according to fragment sizes:

Insert size (bp)	Temperature	Time
150 - 200	94℃	8 min, 4℃ hold
200 - 300	94℃	5 min, 4℃ hold
250 - 450	85℃	6 min, 4℃ hold
450 - 550	85℃	5 min, 4℃ hold

▲Do not stop or 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/ (Step 1) can be taken out from -30 ~ -15℃ in advance and placed on ice for use.

15. Place the tube on the magnetic stand. Wait until the solution is clear (about 5 min), and pipet 16 μl of supernatant into 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 a starting template amount of 0.01 - 1 μg.

1. Dilute 0.01 - 1 μg of total RNA with 11 μl of Nuclease-free ddH_2O in a Nuclease-free PCR tube and keep on ice for use.

2. rRNA/Probe hybridization.

A. rRNA/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 for 10 times.

▲For multiple samples, it is recommended to prepare a mixture of the rRNA Probe (H/M/R) and the Probe Buffer in a suitable size 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.

B. Collect the liquid to the bottom of the tube by a brief centrifugation. Place the tube into a PCR instrument and run the following program:

Temperature	Time
Hot lid of 105℃	On
95℃	2 min
95 ~ 22℃	0.1℃/sec
22℃	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℃ in advance and placed 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 last step	15 μl
Total	20 μl

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

▲For multiple samples, it is recommended to prepare a mixture of the RNase H Buffer and the RNase H in a suitable size 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.

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

Temperature	Time
37℃	30 min
4℃	Hold

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

4. Digestion with DNase I

A. Prepare the following reaction solution on ice:

Component	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 for 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

Collect the liquid to the bottom of the tube by a brief centrifugation. Place the tube on ice and immediately proceed to the next procedure.

5. Purification of ribosomal-depleted RNA with VAHTS RNA Clean Beads.

a. Suspend the VAHTS RNA Clean Beads thoroughly by inverting or vortexing, pipet 110 μl (2.2 ×) of beads into the RNA sample of last Step. Mix thoroughly by pipetting up and down for 10 times.

b. Incubate the sample on ice for 15 min to make the RNA bind to the beads.

c. Place the tube onto a magnetic stand. Wait until the solution is clear (about 5 min). Then carefully discard the supernatant without disturbing the beads.

d. Keep the tube on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. DO NOT resuspend 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 stand, open the lid 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 reduce of recovery efficiency.

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

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

7. Take the sample out of magnetic stand. Add 18.5 μl of Frag/Primer Buffer (1 ×) and mix thoroughly by pipetting up and down for 10 times. Incubate at room temperature for 2 min. Put the tube back on the magnetic stand and wait until the solution 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℃	8 min，4℃ hold
200 - 300	94℃	5 min，4℃ hold
250 - 450	85℃	6 min，4℃ hold
450 - 550	85℃	5 min，4℃ hold

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

▲Do not stop or 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/ (Step 1) can be taken out from -30 ~ -15℃ in advance and placed 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 for 10 times. Incubate the tube in the PCR instrument and set programs according to fragment sizes:

Insert size (bp)	Temperature	Time
150 - 200	94℃	8 min，4℃ hold
200 - 300	94℃	5 min，4℃ hold
250 - 450	85℃	6 min，4℃ hold
450 - 550	85℃	5 min，4℃ hold

▲Do not perform fragmentation for fragmented or short RNA.

▲Do not stop or 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/(Step 1) can be taken out from -30 ~ -15℃ in advance and placed on ice for use.

08-2/Synthesis of Double Strand cDNA

1. The components for synthesis of double strand cDNA should be dissolved on ice, mixed upside down, briefly centrifuged to the bottom of the tube, and placed 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 for 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.

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

Temperature	Time
Hot lid of 105℃	On
25℃	10 min
42℃	15 min
70℃	15 min
4℃	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℃ in advance and placed 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-stranded mRNA Library. Use 2nd Strand Buffer 2 (with dUTP) for stranded 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 for 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/(Step 1) can be taken out from -30 ~ -15˚C in advance and placed on ice for use.

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 no more 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:

Initial input RNA		Volume of Adapter
Total RNA	Purified mRNA	
1 - 4 µg	100 ng	5 µl
100 - 999 ng	10 - 99 ng	3 µl
10 - 99 ng	0.5 - 9.9 ng	1 µl

2. Adjust the pipettor to an 80 µl range and mix thoroughly by gently pipetting up and down for 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 placed 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.

Protocol 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 incubation at 94℃ for 8 min); It is recommended to adopt this protocol when input RNA <100 ng.

Protocol B is two-rounds of size selection. Different size of inserts (>200 bp) can be obtained according to table1/2 when input RNA ≥100 ng.

Protocol A: For libraries with 150 - 200 bp inserts (suitable for mRNA fragmented by incubation at 94℃ for 8 min or input RNA <100 ng)

1. Equilibrate the VAHTS DNA Clean Beads to room temperature.
2. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 45 μl (0.45 ×) of beads into the above adapter ligation products. Mix thoroughly by pipetting up and down for 10 times.
3. Incubate at room temperature for 10 min.
4. Place the tube on a magnetic stand. Wait until the solution is clear (about 5 min). Keep it on magnetic stand, and carefully discard the supernatant without disturbing the beads.
5. Keep the tube on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. **DO NOT resuspend the beads!** Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.
6. Repeat the **Step 5**.
7. Keep the tube on the magnetic stand, open the lid 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 reduce of recovery efficiency. Please avoid over-drying.
8. Take the tube out of the magnetic stand. 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 stand and wait until the solution is clear (about 5 min). Carefully transfer 20 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

Protocol B: For libraries with >200 bp inserts (Suitable for mRNA fragmented by incubation at 94℃ for 5 min, 85℃ for 6 min, or 85℃ for 5 min)

When initial total input RNA <100 ng, after mRNA capture or rRNA depletion, it is recommended to do protocol A instead of protocol B. If want to acquire more concentrate library size distribution, please refer to the appendix.

When VAHTS RNA Adapters Set 8 for MGI(Vazyme #NM208) are used, please refer to Table 1 for size selection.

Table 1. Recommended conditions for beads-based size selection (applicable for complete adapters)

Insert Size (bp)	200 - 300	250 - 350	350 - 450	450 - 550
Library Size (bp)	280 - 380	330 - 430	430 - 530	530 - 630
Fragmentation Condition	94℃ 5 min	85℃ 6 min	85℃ 6 min	85℃ 5 min
Volume of beads for 1st round (μl)	14	11	8	6
Volume of beads for 2nd round (μl)	10	10	10	10

▲The size of the library here is the size of insert + the size of adapter. The volume of magnetic beads will affect the final library size.

Using 85℃ 6 min fragmentation, 350 - 450 bp insert as an example, for libraries with different insert size please refer to the above tables.

1. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Transfer 8 μl of beads into the samples above. Mix thoroughly by pipetting up and down for 10 times.
2. Incubate at room temperature for 10 min.
3. Place the tube on a magnetic stand. Wait until the solution is clear (about 5 min). Keep it on magnetic stand and carefully transfer 90 μl of the supernatant **(DO NOT discard)** into 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 10 μl of VAHTS DNA Clean Beads, mix thoroughly by pipetting up and down for 10 times.
5. Incubate at room temperature for 10 min.
6. Place the tube on the magnetic stand. Wait until the solution is clear (about 5 min). Keep the tube on magnetic stand, and carefully discard the supernatant without disturbing the beads.
7. Keep the tube on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. DO NOT resuspend the beads! Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.
8. Repeat the **Step 7**.

9. Keep the tube on the magnetic stand, open the tube 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 reduce of recovery efficiency. Please avoid over-drying.

10. Take the tube out of magnetic stand. Add 22.5 µl of Nuclease-free ddH_2O to elute the DNA. Mix thoroughly by vortexing or pipetting and place for 2 min at room temperature. Place the tube on the magnetic stand and wait until the solution is clear (about 5 min). Carefully transfer 20 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.

The purified products can be stored at -30 ~ -15℃ for 24 h.

08-5/Library Amplification

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

Components	Volume
Purified Ligation Products	20 µl
PCR Primer Mix 5 for MGI	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) in a suitable size 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.

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

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

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

▲The mRNA proportion varies between species and individuals. Please refer to the following Table for recommended PCR cycles, which should be generally set as 9 - 19 cycles.

Initial input RNA		Cycles
Total RNA	Purified mRNA	
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 samples (ligation products). Mix thoroughly by pipetting up and down for 10 times.

c. Incubate at room temperature for 10 min.

d. Place the tube on a magnetic stand. Wait until the solution is clear (about 5 min). keep it on magnetic stand, and carefully discard the supernatant without disturbing the beads.

e. Keep the tube on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. DO NOT resuspend the beads! Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

f. Repeat the **Step e**.

g. Keep the tube on the magnetic stand, open the lid 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 reduce of recovery efficiency. Please avoid over-drying.

h. Take the tube out of the magnetic stand. Add 25 μl of Nuclease-free ddH_2O to elute the DNA. Mix thoroughly by vortexing or pipetting and place for 2 min at room temperature. Place the tube on the magnetic stand and wait until the solution 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 Using an Agilent Technologies 2100 Bioanalyzer.

Analyze 1 μl of purified PCR products using an Agilent DNA 1000 chip (Agilent #5067-1504). For example, as shown in **Fig. 1**. A library with high quality should exhibit a narrow peak at the 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/Step4** for another purification.

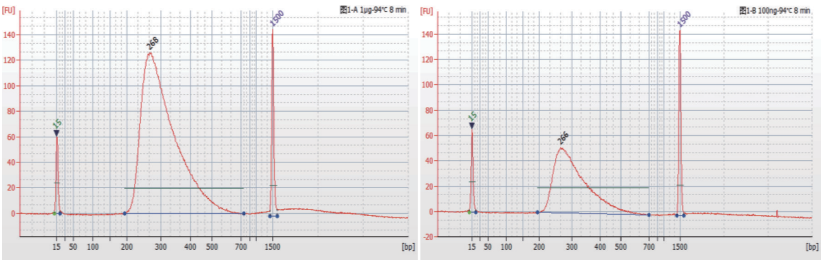
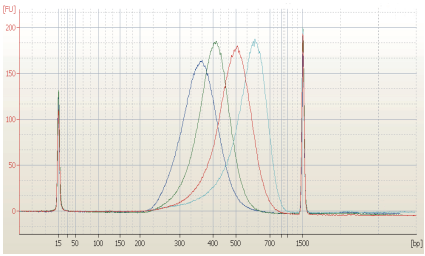


Fig. 1 1 μg/100 ng RNA of 293T cells was fragmented at 94˚C for 8 min and purified with 0.9 × VAHTS DNA Clean Beads, respectively.



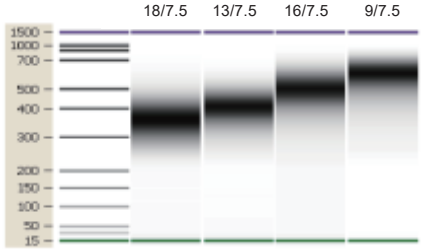


Fig. 2 200 ng RNA of 293T cells was fragmented under different conditions, and size selection was performed with VAHTS DNA Clean Beads according to Table 1, respectively.

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 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.
08-1/ Protocol A 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℃ 2 min, you can use magnetic stand. After adsorption, discard the supernatant and add Tris Buffer.
08-1/ Protocol A 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.
08-1/ Protocol A 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.
08-1/ Protocol A Step 14	Frag/Prime Buffer(1 ×) was added to break mRNA	After discarding Wash Buffer, 2 × 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.
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℃, 6 min as 94℃, 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.	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 libraries 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 Initial amount: Increase the initial amount.

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 and the homogeneity is also well.

◇ High rRNA residue

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

① Poly(A) enrichment, VAHTS mRNA Capture Beads (Vazyme #N401): 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 (Bacteria) (Vazyme #N407): 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;

◇ 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 reflects 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.

◇ 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 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.

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

The number of cycles can be adjusted according to the initial amount; 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?

① 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℃ for 15 min for degradation test. If RNA is unqualified, please re-extract the RNA.

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

③ High-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.

Appendix：Purification and size selection plan

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

▲Use 0.45 × VAHTS DNA Clean Beads to purify the ligation product.

1. Equilibrate the VAHTS DNA Clean Beads to room temperature.
2. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 45 μl (0.45 ×) of beads into the above adapter ligation products. Mix thoroughly by pipetting up and down for 10 times.
3. Incubate at room temperature for 10 min.
4. Place the tube on a magnetic stand. Wait until the solution is clear (about 5 min). Keep it on magnetic stand, and carefully discard the supernatant without disturbing the beads.
5. Keep the tube on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. **DO NOT resuspend the beads!** Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.
6. Repeat the **Step 5**.
7. Keep the tube on the magnetic stand, open the lid 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 reduce of recovery efficiency. Please avoid over-drying.

8. Take the tube out of magnetic stand. Add 102.5 μl of Nuclease-free ddH2O to elute the DNA. Mix thoroughly by vortexing or pipetting and place for 2 min at room temperature. Place the tube on the magnetic stand and wait until the solution is clear (about 5 min). Carefully transfer 100 μl of supernatant to a new Nuclease-free PCR tube without disturbing the beads.

▲Use two round of VAHTS DNA Clean Beads to do the size selection (mRNA fragmented by incubation at 85℃ for 6 min, 350bp - 450 bp as example)

When VAHTS RNA Adapters Set 8 for MGI(Vazyme #NM208) are used, please refer to Table 1 for size selection.

Table 1. Recommended conditions for beads-based size selection (applicable for complete adapters)

Insert Size (bp)	200 - 300	250 - 350	350 - 450	450 - 550
Library Size (bp)	280 - 380	330 - 430	430 - 530	530 - 630
Fragmentation Condition	94℃ 5 min	85℃ 6 min	85℃ 6 min	85℃ 5 min
Volume of beads for 1st round (μl)	60 (0.6 ×)	55 (0.55 ×)	50 (0.5 ×)	45 (0.45 ×)
Volume of beads for 2nd round (μl)	20 (0.2 ×)	20 (0.2 ×)	15 (0.15 ×)	15 (0.15 ×)

▲The size of the library here is the size of insert + the size of adapter. The volume of magnetic beads will affect the final library size.

9. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Transfer 50 μl of beads into the samples above. Mix thoroughly by pipetting up and down for 10 times.

10. Incubate at room temperature for 10 min.

11. Place the tube on a magnetic stand. Wait until the solution is clear (about 5 min). Keep it on magnetic stand and carefully transfer 145 μl of the supernatant **(DO NOT discard)** into a new Nuclease-free PCR tube.

12. Add 15 μl (0.15 ×) of VAHTS DNA Clean Beads, mix thoroughly by pipetting up and down for 10 times.

13. Incubate at room temperature for 10 min.

14. Place the tube on the magnetic stand. Wait until the solution is clear (about 5 min). Keep the tube on magnetic stand, and carefully discard the supernatant without disturbing the beads.

15. Keep the tube on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. DO NOT resuspend the beads! Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

16. Repeat the **Step 15**.

17. Keep the tube on the magnetic stand, open the tube 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 reduce of recovery efficiency. Please avoid over-drying.

18. Take the tube out of magnetic stand. Add 22.5 µl of Nuclease-free ddH_2O to elute the DNA. Mix thoroughly by vortexing or pipetting and place for 2 min at room temperature. Place the tube on the magnetic stand and wait until the solution is clear (about 5 min). Carefully transfer 20 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.

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