VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina

Catalog# NR604-01/02

Version 9.1

Vazyme biotech co., ltd.

01/ Introduction

VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina is specially designed for the preparation of transcriptome libraries for next generation sequencing (NGS) platforms of Illumina®. 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 transcriptome or stranded 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 selections with magnetic beads.

02/ Components

Components	NR604-01 (24 rxn)	NR604-02 (96 rxn)
Frag/Prime Buffer	468 μl	2 × 936 μl
Actinomycin D (5mg/ml)	24 μl	96 μl
1st Strand Buffer 2	144 μl	576 μl
1st Strand Enzyme Mix 2	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	360 μl	2 × 720 μl
Rapid Ligation Buffer 3	600 μl	4 × 600 μl
Rapid DNA Ligase 2	120 μl	480 μl
PCR Primer Mix 3	120 μl	480 μl
VAHTS HiFi Amplification Mix	600 μl	4 × 600 μl
Heat-labile UDG	24 μl	96 μl

03/ Storage

All Components should be stored at -30℃ ~ -15℃ , transported at -20℃ ~ 0℃.

04/ Applications

VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina is suitable for RNA library construction of 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): 0.05μg - 4 μg;

Ribo-off rRNA Depletion Kit (H/R/M) (Vazyme #N406): 0.05μg - 1 μg;

Ribo-off rRNA Depletion Kit (Bacteria) (Vazyme #N407): 1 μg - 5 μg;

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

It is recommended to use an Agilent 2100 Bioanalyzer to analyze the integrity of total RNA. mRNA enrichment using VAHTS® mRNA Capture Beads (Vazyme #N401) needs high quality RNA samples (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 #N406/407/409).

Main fields of RNA-related analysis:

◇gene expression analysis

◇single nucleotide variation calling

◇alternative splicing detection

◇gene fusion detection

◇target transcriptome analysis

05/ Additional Materials Required

◇**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:**
VAHTS mRNA Capture Beads (Vazyme # N401);
Ribo-off rRNA Depletion Kit (H/R/M) (Vazyme # N406);
Ribo-off rRNA Depletion Kit (Bacteria) (Vazyme # N407);
Ribo-off rRNA Depletion Kit (Plant) (Vazyme # N409).

◇**RNA and DNA 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®RNAClean® XP Beads (Beckman #A63987).

◇**Adapters:**
VAHTS RNA Adapters set 1/2 for Illumina (Vazyme # N803/N804), VAHTS RNA Adapters set 3 - set 6 for Illumina (Vazyme # N809/N810/N811/N812) or VAHTS RNA Multiplex Oligos set 1/2 for Illumina (Vazyme # N323/N324).

◇**Library Quality:**
Equalbit® dsDNA HS Assay Kit (Vazyme #EQ111);
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 quality of constructed library, RNA samples' property in total amount, purity and integrity should be controlled as follows, before starting experiment.

◇The initial template input of total RNA should ≥ 50 ng, otherwise, the mRNA may be insufficient for following library construction.

◇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 salt or small molecular impurity / genetic DNA contamination.

06-2/ RNA Samples Prepared

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 total RNA with low concentration, it can be concentrated using lyophilization, ethanol-precipitation, column-based or bead-based clean-ups (e.g. VAHTS RNA Clean Beads, Vazyme # N412).

06-3/ Tips for DNA Purification with Magnetic Beads

◇Equilibrate the beads to room temperature before use to assure capture efficacy.

◇Mix the beads thoroughly every time before pipetting.

◇The time required for complete capture of beads varies according to the reaction vessel and magnet used. 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 clarified, and leave 2 μl - 3 μl of supernatant behind 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.

◇Always use 80% ethanol freshly prepared. Keep tubes on magnet stand without disturbing the beads during elution.

◇Do not leave any 80% ethanol supernatant behind in the second washing step to reduce the residual impurities.

◇It is important to remove all the ethanol before proceeding with subsequent reactions. However, over-drying of beads may make them difficult to resuspend, resulting in a dramatic loss of DNA. Normally, drying of beads for 5 min–10 min at room temperature should be sufficient. **Do not heat and dry** (such as oven drying at 37°C).

06-4/ Operational Attentions

◇Thaw all components of this kit on ice. Mix thoroughly upside down several times, centrifuge briefly and place on ice for use.

◇It is recommended to use filter pipette tips; change tips when varying samples.

◇Please use RNase-free materials before second Strand cDNA synthesis, while use DNase-free materials after that.

◇Be sure to use fresh Nuclease-free H2O during the experiment; it is recommended to dispense it into small tubes for use and discard after use.

◇Be sure to wear gloves; change gloves after touching the equipment outside the RNase-free space or other working areas.

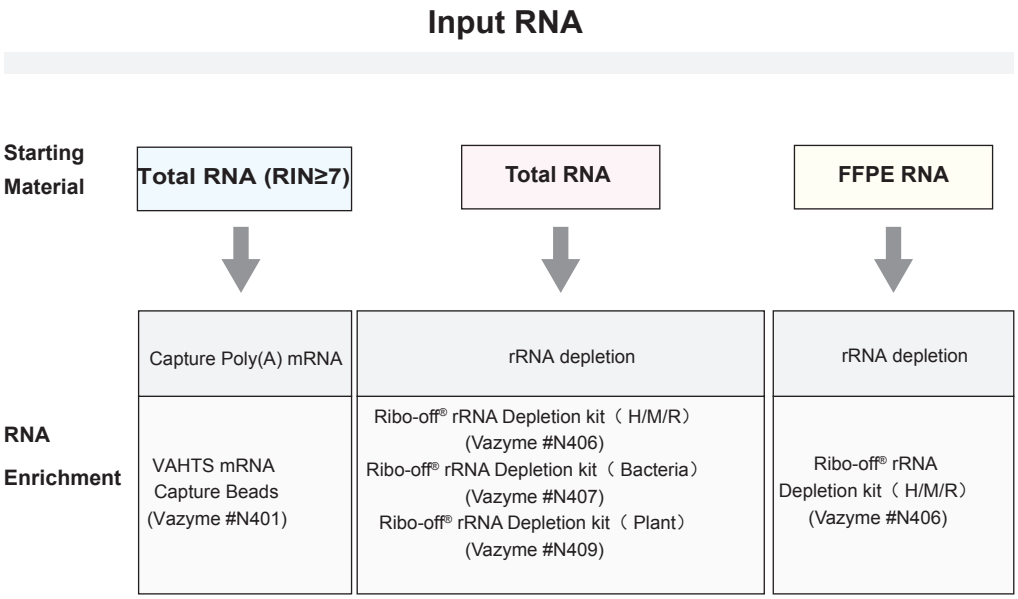
◇Please cover the reagent with lid to avoid contamination whenever finish use.

◇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.

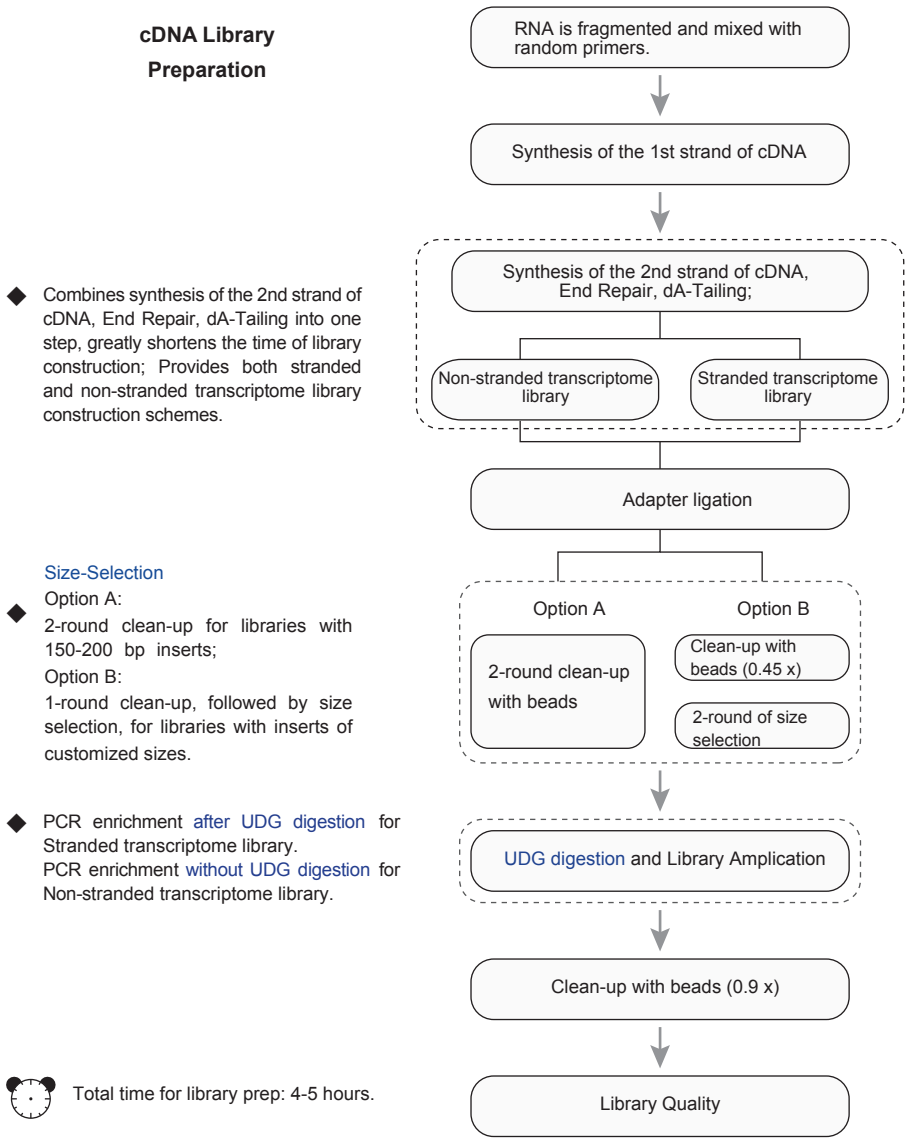
◇The PCR product will lead to aerosol contamination due to improper operation, affecting the accuracy of the experimental results. Therefore, it is recommended to isolate PCR reaction preparation area and PCR products detection area physically. Use a dedicated pipettor and other equipment, and regularly clean each experimental area to ensure the cleanliness of the experimental environment.

◇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/ Workflow



07/ Workflow



08/ Poly(A) enrichment protocol for Non-stranded mRNA Library

Taking VAHTS mRNA Capture Beads (Vazyme # N401) for mRNA enrichment as an example, this protocol is applicable for preparing non-stranded RNA libraries from 0.05 μg - 4 μg of total RNA of eukaryotes (e.g. animal, plant or fungal) with good integrity.

08-1/ mRNA Purification and Fragmentation

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 0.05 μg - 4 μg of total RNA in Nuclease-free H2O 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 binding of mRNA and magnetic beads:

Temperature	Time
65℃	5 min
25℃	5 min

08-1/ mRNA Purification and Fragmentation

5.Put the samples onto a magnetic stand. Wait until the solution clarifies (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. Put the samples back to the magnetic stand. Wait until the solution clarifies (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˚C	2 min
25˚C	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 clarifies (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 clarifies (about 5 min), then carefully discard the supernatant without disturbing the mRNA Capture Beads.

▲It is highly recommended to use a 10-µl pipettor to remove the residual supernatant in this step. The residual of Beads Wash Buffer will affect the fragmentation of mRNA.

13.Take the samples 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 samples in the PCR instrument and set programs according to fragment sizes:

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 stop or pause between steps from the fragmentation to the first 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°C ~ -15°C in advance and placed on ice for use.

14.Place the samples on the magnetic stand. Wait until the solution clarifies (about 5 min), and pipet 17 µl of supernatant into a new Nuclease-free PCR tube, then immediately proceed to synthesis of 1st Strand cDNA.

08-2/ Synthesis of Double Strand cDNA

1.The components for synthesis of double-stranded 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	17 µl
1st Strand Buffer 2	6 µl
1st Strand Enzyme Mix 2	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 2 and the 1st Strand Enzyme Mix 2 in a suitable size tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 x volumes of the actual volume to compensate for the loss.

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 second strand cDNA should be performed immediately after the synthesis of the first strand of cDNA.

08-2/ Synthesis of Double Strand cDNA

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)*	25 µl
2nd Strand Enzyme Super Mix	15 µl
Total	65 µl

▲Use 2nd Strand Buffer 2 (with dNTP) for Non-stranded mRNA Library. Do Not use 2nd Strand Buffer 2 (with dUTP)!

▲For multiple samples, it is recommended to prepare a mixture of the 2nd Strand Marking Buffer 2 and the 2nd Strand Enzyme Super Mix in a suitable size tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 x 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 **Step 08-3/** can be taken out from -30°C ~ -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 3	25 µl
Rapid DNA Ligase 2	5 µl
RNA Adapter*	x µl
Nuclease-free H2O	To 100 µl

▲The mixture of Rapid Ligation Buffer 3 and Rapid DNA ligase 2 can be stored at 2°C-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 3 and Rapid DNA Ligase 2.

▲If VAHTS RNA Multiplex Oligos Set 1- Set 2 for Illumina (Vazyme #N323/N324) is used, the RNA Adapter should be the RNA adapter-S for Illumina in the kit.

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

Initial input RNA	Volume of Adapter
1 - 4 µg	5 µl
100 - 999 ng	2 µl
50 - 99 ng	0.8 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°C - 8°C in advance and placed at room temperature.

The adapter ligation products can be temporarily stored at 2°C - 8°C for 1 hr.

08-4/ Purification of Adapter-Ligated DNA

This process is applicable for libraries with 150 bp-200 bp inserts (suitable for mRNA fragmented by incubation at 94˚C for 8 min)

▲For libraries with > 200 bp inserts, please refer to **Appendix 1** for size selection.

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 samples (adapter ligation products, 100 µl). Mix thoroughly by pipetting up and down for 10 times.

08-4/ Purification of Adapter-Ligated DNA

3. Incubate at room temperature for 10 min.

4. Place the samples on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on magnetic stand, and carefully discard the supernatant without disturbing the beads.

5. Keep the samples on the magnetic stand, add 200 µl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend 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 samples 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 remove the residual supernatant in this step.

▲Avoid over-drying of beads, resulting in a dramatic loss of DNA.

8. Take the samples out of the magnetic stand. Add 52.5 µl of Nuclease-free Water 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 50 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

9. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 50 µl (1×) of the suspended beads to the products above. Mix thoroughly by pipetting up and down for 10 times.

10. Incubate at room temperature for 10 min.

11. Place the samples on the magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand, and carefully discard the supernatant without disturbing the beads.

12. Keep the samples on the magnetic stand, and add 200 µl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend the beads!** Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

13. Repeat the **Step 12**.

14. Keep the sample 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 remove the residual supernatant in this step.

▲Avoid over-drying of beads, resulting in a dramatic loss of DNA.

15. Take the samples out of magnetic stand. Add 22.5 µl of nuclease-free water 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 clarifies (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 a trace amount of beads will affect the quality of the final library.

▲The reagents for **08-5/ (Step 1)** can be taken out from -30°C ~ -15°C in advance and placed on ice for use.

08-5/ Library Amplification

1.Prepare the PCR reaction system as follows:

Components	Volume
Purified Ligation Products	20 µl
PCR Primer Mix 3	5 µl
VAHTS HiFi 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 x volumes of the actual volume to compensate for the loss.

▲This reaction is applicable for the VAHTS RNA Adapters set 1- set 2 for Illumina® (Vazyme, #N803/N804) or VAHTS RNA Adapters set 3 - set 6 for Illumina® (Vazyme, #N809/N810/N811/N812).

▲If using VAHTS RNA Multiplex Oligos set1/2 for Illumina® (Vazyme, #N323 /N324), the i5 PCR Primer (RM5XX) and i7 PCR Primer (RM7XX) should be used in an amount of 2.5 µl, respectively.

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

08-5/ Library Amplification

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℃	30 sec	1
Denaturation	98℃	10 sec	
Annealing	60℃	30 sec	10 - 17
Extension	72℃	30 sec	
Complete Extension	72℃	5 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 10 - 17 cycles.

Initial input RNA	PCR cycles
2 - 4 μg	10 - 12
1 - 2 μg	12 - 13
100 - 999 ng	14 - 15
50 - 99 ng	16 - 17

4.Clean-up of the PCR product with VAHTS DNA Clean Beads.

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 samples (ligation products). Mix thoroughly by pipetting up and down for 10 times.

3/ Incubate at room temperature for 10 min.

4/ Place the samples on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep on magnetic stand, and carefully discard the supernatant without disturbing the beads.

5/ Keep the samples on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. DO NOT re-suspend 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 samples 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 remove the residual supernatant in this step.

▲Avoid over-drying the beads, which may lead to a dramatic loss of DNA.

8/ Take the samples out of the magnetic stand. Add 25 μl of nuclease-free water 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 clarifies (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.

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 128 bp indicates the contamination of adapter-dimers. To eliminate this contamination, dilute the library to 50 μl with Nuclease-free Water and repeat **08-5/Step4** for another clean-up.

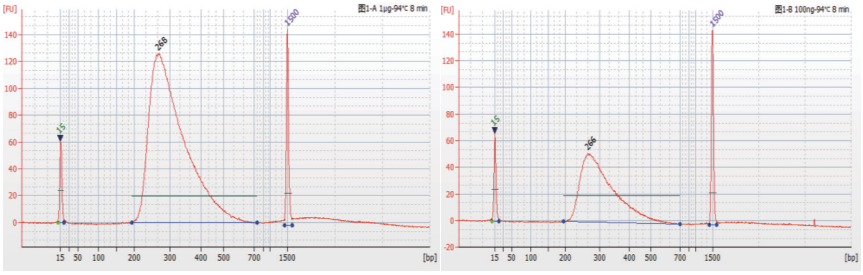


Fig. 1. Universal human reference RNA of 293T cells (1 μg - left, 100 ng - right) was fragmented at 94℃ for 8 min and purified in two rounds with VAHTS DNA Clean Beads (0.45 ×/1.0 ×), respectively.

09/ Poly(A) enrichment protocol for Stranded mRNA Library

Taking VAHTS mRNA Capture Beads (Vazyme # N401) for mRNA enrichment as an example, this protocol is applicable for preparing stranded RNA libraries from 0.05 μg - 4 μg of total RNA of eukaryotes (e.g. animal, plant or fungal) with good integrity.

09-1/ mRNA Enrichment and Fragmentation

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 0.05 μg - 4 μg of total RNA in Nuclease-free H2O 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 binding of mRNA and magnetic beads:

Temperature	Time
65℃	5 min
25℃	5 min

5.Put the samples onto a magnetic stand. Wait until the solution clarifies (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. Put the samples back to the magnetic stand. Wait until the solution clarifies (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 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.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, 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 clarifies (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 clarifies (about 5 min), then carefully discard the supernatant without disturbing the mRNA Capture Beads.

▲It is highly recommended to use a 10-μl pipettor to remove the residual supernatant in this step. The residual of Beads Wash Buffer will affect the fragmentation of mRNA.

13.Take the samples out of the magnetic stand, add 18.5 μl of Frag/Prime Buffer to re-suspend the beads thoroughly by pipetting up and down for 10 times. Incubate the samples in a PCR device 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 first strand cDNA synthesis, as mRNA is easy to degrade under this system.

▲The reagents for **09-2/ (Step 1)** can be taken out from -30°C ~ -15°C in advance and placed on ice for use.

14.Place the samples on the magnetic stand. Wait until the solution clarifies (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.

09-2/ Synthesis of Double Strand cDNA

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

1.Dilute Actinomycin D (5 mg / ml) to 0.12 mg / ml according to the following table:

Components	Volume
Nuclease-free H2O	48.8 µl
Actinomycin D (5 mg/ml)	1.2 µl
Total	50 µl

▲The Diluted Actinomycin D solution is highly sensitive to light and will attach to the surfaces of plastic and glass. Discard unused diluted solution.

2.Prepare the reaction solution to synthesize the 1st strand of cDNA as follows:

Components	Volume
Fragmented mRNA	16 µl
Actinomycin D (0.12 mg/ml)	1 µl
1st Strand Buffer 2	6 µl
1st Strand Enzyme Mix 2	2 µl
Total	25 µl

3.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 2 and the 1st Strand Enzyme Mix 2 in a suitable size tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 x volumes of the actual volume to compensate for the loss.

4.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 second strand cDNA should be performed immediately after the synthesis of the first strand of cDNA.

5.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)*	25 µl
2nd Strand Enzyme Super Mix	15 µl
Total	65 µl

▲Use 2nd Strand Buffer 2 (with dUTP) for Stranded mRNA Library. Do Not use 2nd Strand Buffer 2 (with dNTP)!

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

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

7.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 **Step 9-3/** can be taken out from -30°C ~ -15°C in advance and placed on ice for use.

09-3/ Adapter Ligation

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

Components	Volume
ds cDNA	65 µl
Rapid Ligation buffer 3	25 µl
Rapid DNA Ligase 2	5 µl
RNA Adapter*	x µl
Nuclease-free H2O	To 100 µl

▲The mixture of Rapid Ligation Buffer 3 and Rapid DNA ligase 2 can be stored at 2°C-8°C for no more than 24 h.

09-3/ Adapter Ligation

▲It is recommended to add the RNA adapter to the ds cDNA first and mix thoroughly, then add the mixture of Rapid Ligation Buffer 3 and Rapid DNA Ligase 2.

▲If VAHTS RNA Multiplex Oligos Set 1- Set 2 for Illumina (Vazyme #N323/N324) is used, the RNA Adapter should be the RNA adapter-S for Illumina in the kit.

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

Initial input RNA	Volume of Adapter
1 - 4 μg	5 μl
100 - 999 ng	2 μl
50 - 99 ng	0.8 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℃	On
20℃	15 min
4℃	Hold

▲VAHTS DNA Clean Beads for **Step 09-4** can be taken out from 2°C - 8°C in advance and placed at room temperature.

The adapter ligation products can be temporarily stored at 2°C - 8°C for 1 hr.

09-4/ Purification of Adapter-Ligated DNA

This process is applicable for libraries with 150 bp-200 bp inserts (suitable for mRNA fragmented by incubation at 94℃ for 8 min)

▲For libraries with > 200 bp inserts, please refer to **Appendix 1** for size selection.

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 samples (adapter ligation products, 100 μl). Mix thoroughly by pipetting up and down for 10 times.

3. Incubate at room temperature for 10 min.

4. Place the samples on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on magnetic stand, and carefully discard the supernatant without disturbing the beads.

5. Keep the sample on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend 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 samples 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 remove the residual supernatant in this step.

▲Avoid over-drying of beads, resulting in a dramatic loss of DNA.

8. Take the samples out of the magnetic stand. Add 52.5 μl of Nuclease-free Water 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 50 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

9. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 50 μl (1×) of the suspended beads to the products above. Mix thoroughly by pipetting up and down for 10 times.

10. Incubate at room temperature for 10 min.

11. Place the samples on the magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand, and carefully discard the supernatant without disturbing the beads.

12. Keep the samples on the magnetic stand, and add 200 μl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend the beads!** Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

13. Repeat the **Step 12**.

14. Keep the sample 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 remove the residual supernatant in this step.

▲Avoid over-drying of beads, resulting in a dramatic loss of DNA.

09-4/ Purification of Adapter-Ligated DNA

15. Take the samples out of magnetic stand. Add 21.5 μl of nuclease-free water 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 clarifies (about 5 min). Carefully transfer 19 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 a trace amount of beads will affect the quality of the final library.

▲The reagents for **09-5/ (Step 1)** can be taken out from -30°C ~ -15°C in advance and placed on ice for use.

09-5/ Library Amplification

1.Prepare the PCR reaction system as follows:

Components	Volume
Purified Ligation Products	19 μl
PCR Primer Mix 3	5 μl
VAHTS HiFi Amplification Mix	25 μl
Heat-labile UDG	1 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 x volumes of the actual volume to compensate for the loss.

▲This reaction is applicable for the VAHTS RNA Adapters set 1- set 2 for Illumina® (Vazyme, #N803/N804) or VAHTS RNA Adapters set 3 - set 6 for Illumina® (Vazyme, #N809/N810/N811/N812).

▲If using VAHTS RNA Multiplex Oligos set1/2 for Illumina® (Vazyme, #N323 /N324), the i5 PCR Primer (RM5XX) and i7 PCR Primer (RM7XX) should be used in an amount of 2.5 μl, respectively.

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	
UDG Digestion	37℃	10 min	1
Pre-denaturation	98℃	30 sec	1
Denaturation	98℃	10 sec	10 - 17
Annealing	60℃	30 sec	
Extension	72℃	30 sec	
Complete Extension	72℃	5 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 10 - 17 cycles.

Initial input RNA	PCR cycles
2 - 4 μg	10 - 12
1 - 2 μg	12 - 13
100 - 999 ng	14 - 15
50 - 99 ng	16 - 17

4.Clean-up of the PCR product with VAHTS DNA Clean Beads.

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 samples (ligation products). Mix thoroughly by pipetting up and down for 10 times.

3/ Incubate at room temperature for 10 min.

4/ Place the samples on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep on magnetic stand, and carefully discard the supernatant without disturbing the beads.

5/ Keep the samples on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend 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 samples 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 remove the residual supernatant in this step.

▲Avoid over-drying the beads, which may lead to a dramatic loss of DNA.

09-5/ Library Amplification

8/ Take the samples out of the magnetic stand. Add 25 µl of nuclease-free water 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 clarifies (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.

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.2.**, a library with high quality should exhibit a narrow peak at the expected size. A narrow peak at 128 bp indicates the contamination of adapter-dimers. To eliminate this contamination, dilute the library to 50 µl with Nuclease-free Water and repeat **09-5/Step4** for another clean-up.

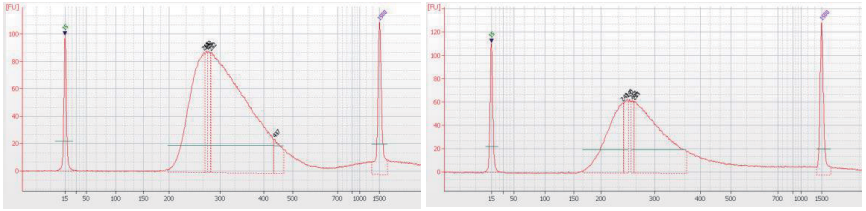


Fig. 2. Universal human reference RNA of 293T cells (1 µg - left, 100 ng - right) was fragmented at 94˚C for 8 min and purified in two rounds with VAHTS DNA Clean Beads (0.45 ×/1.0 ×), respectively.

10/ rRNA depletion protocol for Stranded Transcriptome Library

Taking Ribo-off rRNA Depletion Kit (H/R/M) as an example, this protocol is applicable to the generation of Total RNA strand-specific full transcriptome library of human, Rat, Mouse and other species with a starting template amount of 0.05 - 1 µg.

10-1/ rRNA depletion and Fragmentation

1.Dilute 0.05 µg - 1 µg of total RNA with 11 µl of Nuclease-free Water in a Nuclease-free PCR tube and keep on ice for use.

The reagents for **Step 2** can be taken out from -30°C ~ -15°C in advance and placed on ice for use.

2.rRNA/Probe hybridization

1/ rRNA/Probe hybridization

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

2/ Adjust the pipettor to a 10 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 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 x volumes of the actual volume to compensate for the loss.

3/ Collect the liquid to the bottom of the tube by a brief centrifugation. Put the sample 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 minutes, and different types of PCR instruments may be biased.

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

3.Digestion with RNase H

1/ Prepare the following reaction solution on ice:

Components	Volume
RNase H Buffer	4 µl
RNase H	1 µl
Products of last step (**Step2 3/**)	15 µl
Total	20 µl

10-1/ rRNA depletion and Fragmentation

Adjust the pipettor to a 15 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 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 x volumes of the actual volume to compensate for the loss.

2/ 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°C ~ -15°C in advance and placed on ice for use.

4.Digestion with DNase I

1/ 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

Adjust the pipettor to a 40 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 DNase I Buffer and the DNase I in a suitable size tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 x volumes of the actual volume to compensate for the loss.

2/ Put the sample into a PCR instrument and run the following program:

Temperature	Time
37℃	30 min
4℃	Hold

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

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

1/ Suspend the VAHTS RNA Clean Beads thoroughly by inverting or vortexing, pipet 110 μl (2.2×) of beads into the RNA sample of **Step 4 2/**. Mix thoroughly by pipetting for 10 times.

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

3/ Put the sample onto a magnetic stand. Wait until the solution clarifies (about 5 min). Then carefully discard the supernatant without disturbing the beads.

4/ Keep the sample on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. DO NOT re-suspend the beads! Incubate at room temperature for 30 sec and carefully discard the supernatant without disturbing the beads.

5/ Repeat **Step 4/**.

6/ Keep the sample 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 remove the residual supernatant in this step.

▲Avoid over-drying of beads, resulting in a dramatic loss of DNA.

6.Take the sample out of magnetic stand. Add 18.5 μl of Frag/Primer Buffer and mix thoroughly by pipetting for 6 times. Incubate at room temperature for 2 min. Put the tube back on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 16 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads, then **immediately proceed to synthesis of 1st Strand cDNA**.

Incubate the sample in a PCR device and set programs according to the fragment size required:

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 first strand cDNA synthesis, as mRNA is easy to degrade under this system.

▲ The reagents for **10-2/ (Step 1)** can be taken out from -30°C ~ -15°C in advance and placed on ice for use.

10-2/ Synthesis of Double Strand cDNA

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

1.Dilute Actinomycin D (5 mg / ml) to 0.12 mg / ml according to the following table:

Components	Volume
Nuclease-free H_2O	48.8 µl
Actinomycin D (5 mg/ml)	1.2 µl
Total	50 µl

▲The Diluted Actinomycin D solution is highly sensitive to light and will attach to the surfaces of plastic and glass. Discard unused diluted solution.

2.Prepare the reaction solution to synthesize the 1st strand of cDNA as follows:

Components	Volume
Fragmented mRNA	16 µl
Actinomycin D (0.12 mg/ml)	1 µl
1st Strand Buffer 2	6 µl
1st Strand Enzyme Mix 2	2 µl
Total	25 µl

3.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 2 and the 1st Strand Enzyme Mix 2 in a suitable size tube first, and then dispense into each PCR tube. It is recommended to prepare 1.1 x volumes of the actual volume to compensate for the loss.

4.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 second strand cDNA should be performed immediately after the synthesis of the first strand of cDNA.

5.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 dUTP)*	25 µl
2nd Strand Enzyme Super Mix	15 µl
Total	65 µl

▲Use 2nd Strand Buffer 2 (with dUTP) for Stranded mRNA Library. Do Not use 2nd Strand Buffer 2 (with dNTP)!

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

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

7.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 **Step 10-3/** can be taken out from -30°C ~ -15°C in advance and placed on ice for use.

10-3/ Adapter Ligation

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

Components	Volume
ds cDNA	65 µl
Rapid Ligation Buffer 3	25 µl
Rapid DNA Ligase 2	5 µl
RNA Adapter*	x µl
Nuclease-free H_2O	To 100 µl

▲The mixture of Rapid Ligation Buffer 3 and Rapid DNA ligase 2 can be stored at 2°C-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 3 and Rapid DNA Ligase 2.

10-3/ Adapter Ligation

▲If VAHTS RNA Multiplex Oligos Set 1- Set 2 for Illumina (Vazyme #N323/N324) is used, the RNA Adapter should be the RNA adapter-S for Illumina in the kit.

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

Initial input RNA	Volume of Adapter
1 μg	5 μl
100 - 999 ng	2 μl
50 - 99 ng	0.8 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℃	On
20℃	15 min
4℃	Hold

▲VAHTS DNA Clean Beads for **Step 10-4** can be taken out from 2°C - 8°C in advance and placed at room temperature.

The adapter ligation products can be temporarily stored at 2°C - 8°C for 1 hr.

10-4/ Purification of Adapter-Ligated DNA

This process is applicable for libraries with 150 bp-200 bp inserts (suitable for mRNA fragmented by incubation at 94℃ for 8 min)

▲For libraries with > 200 bp inserts, please refer to **Appendix 1** for size selection.

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 samples (adapter ligation products, 100 μl). Mix thoroughly by pipetting up and down for 10 times.

3. Incubate at room temperature for 10 min.

4. Place the samples on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on magnetic stand, and carefully discard the supernatant without disturbing the beads.

5. Keep the sample on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend 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 samples 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 remove the residual supernatant in this step.

▲Avoid over-drying of beads, resulting in a dramatic loss of DNA.

8. Take the samples out of the magnetic stand. Add 52.5 μl of Nuclease-free Water 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 50 μl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

9. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 50 μl (1×) of the suspended beads to the products above. Mix thoroughly by pipetting up and down for 10 times.

10. Incubate at room temperature for 10 min.

11. Place the samples on the magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand, and carefully discard the supernatant without disturbing the beads.

12. Keep the samples on the magnetic stand, and add 200 μl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend the beads!** Incubate for 30 sec at room temperature and carefully discard the supernatant without disturbing the beads.

13. Repeat the **Step 12**.

14. Keep the sample 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 remove the residual supernatant in this step.

▲Avoid over-drying of beads, resulting in a dramatic loss of DNA.

10-4/ Purification of Adapter-Ligated DNA

15. Take the samples out of magnetic stand. Add 21.5 μl of nuclease-free water 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 clarifies (about 5 min). Carefully transfer 19 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 a trace amount of beads will affect the quality of the final library.

▲The reagents for **10-5/ (Step 1)** can be taken out from -30°C ~ -15°C in advance and placed on ice for use.

10-5/ Library Amplification

1.Prepare the PCR reaction system as follows:

Components	Volume
Purified Ligation Products	19 μl
PCR Primer Mix 3	5 μl
VAHTS HiFi Amplification Mix	25 μl
Heat-labile UDG	1 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 x volumes of the actual volume to compensate for the loss.

▲This reaction is applicable for the VAHTS RNA Adapters set 1- set 2 for Illumina® (Vazyme, #N803/N804) or VAHTS RNA Adapters set 3 - set 6 for Illumina® (Vazyme, #N809/N810/N811/N812).

▲If using VAHTS RNA Multiplex Oligos set1/2 for Illumina® (Vazyme, #N323 /N324), the i5 PCR Primer (RM5XX) and i7 PCR Primer (RM7XX) should be used in an amount of 2.5 μl, respectively.

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	
UDG Digestion	37℃	10 min	1
Pre-denaturation	98℃	30 sec	1
Denaturation	98℃	10 sec	} 10 - 17
Annealing	60℃	30 sec	
Extension	72℃	30 sec	
Complete Extension	72℃	5 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 12 - 17 cycles.

Initial input RNA	PCR cycles
1 μg	12 - 13
100 - 999 ng	14 - 15
50 - 99 ng	16 - 17

4.Clean-up of the PCR product with VAHTS DNA Clean Beads.

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 (0.9 ×) of beads into the above samples (ligation products). Mix thoroughly by pipetting up and down for 10 times.

3/ Incubate at room temperature for 10 min.

4/ Place the samples on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep on magnetic stand, and carefully discard the supernatant without disturbing the beads.

5/ Keep the samples on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend 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 samples 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 remove the residual supernatant in this step.

▲Avoid over-drying the beads, which may lead to a dramatic loss of DNA.

10-5/ Library Amplification

8/ Take the samples out of the magnetic stand. Add 25 µl of nuclease-free water 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 clarifies (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.

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.3.**, a library with high quality should exhibit a narrow peak at the expected size. A narrow peak at 128 bp indicates the contamination of adapter-dimers. To eliminate this contamination, dilute the library to 50 µl with Nuclease-free Water and repeat **10-5/Step4** for another clean-up.

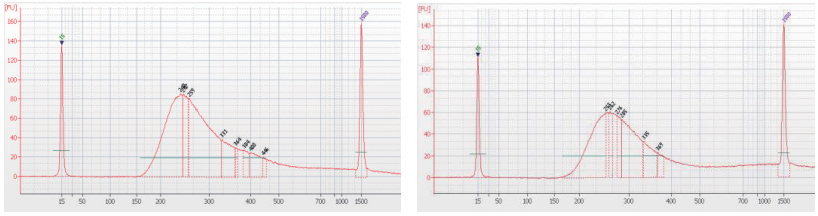


Fig. 3. RNA of 293T cells (1 µg - left, 100 ng - right) was fragmented at 94˚C for 8 min and purified in two rounds with VAHTS DNA Clean Beads (0.45 ×/1.0 ×), respectively.

Appendix 1. Size Selection

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)

▲Clean-up of adapter ligation products using 0.45 × VAHTS DNA Clean Beads.

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 samples (adapter ligation products, 100 µl). Mix thoroughly by pipetting up and down for 10 times.

3. Incubate at room temperature for 10 min.

4. Place the samples on a magnetic stand. Wait until the solution clarifies (about 5 min). Keep it on the magnetic stand, and carefully discard the supernatant without disturbing the beads.

5. Keep the samples on the magnetic stand, add 200 µl of freshly prepared 80% ethanol to rinse the beads. **DO NOT re-suspend 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 samples 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 remove the residual supernatant in this step.

▲Avoid over-drying of beads, resulting in a dramatic loss of DNA.

8. Take the sample out of the magnetic stand. Add 102.5 µl of Nuclease-free Water 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 100 µl of the supernatant to a new Nuclease-free PCR tube without disturbing the beads.

▲ For a library with 350 bp - 450 bp inserts (as an example). Please refer to **Table 1** or **Table 2** for the appropriate volume of beads for libraries with inserts of other sizes.

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

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˚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 ×)

▲The conditions for size selection in **Table 1** is applicable for VAHTS RNA Adapters set 1/2 for Illumina® (Adapters 1-27, Vazyme, #N803/N804) or VAHTS RNA Adapters set 3 - set 6 for Illumina® (Adapters 1-96, Vazyme, #N809/N810/N811/N812).

▲When using a non-complete adapter, such as VAHTS RNA Multiplex Oligos set1/2 for Illumina® (Vazyme, #N323/N324), please refer to Table 2 for size selection.

Appendix 1. Size Selection

Table 2. Recommended conditions for bead-based size selection (applicable for non-complete adapters)

Insertion Length (bp)	200 - 300	250 - 350	350 - 450	450 - 550
Library Length (bp)*	260 - 360	310 - 410	410 - 510	510 - 610
Fragmentation Condition	94℃ 5 min	85℃ 6 min	85℃ 6 min	85℃ 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 size of the library here is the size of insert + the size of adapter.

▲The volume ratio of the beads is based on the initial DNA volume. For example, if initial DNA volume is 100 μl, 1× beads means the volume of beads is 1 × 100 μl = 100 μl; 0.6× / 0.1× size selection means the first round of bead volume is 0.6 × 100 μl = 60 μl and the second round is 0.1 × 100 μl = 10 μl.

9. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Transfer 60 μl (0.6 ×) 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 samples 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 **(DO NOT discard)** into a new Nuclease-free PCR tube.

▲155 μl of the supernatant is taken from a total volume of 160 μl without disturbing the beads.

▲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.

▲For other size-selections (e.g. the total volume is not 160 μl), the volume of the transferred supernatant should be 5 μl less than the total volume.

12. Add 10 μl (0.1 ×) 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 samples on the magnetic stand. Wait until the solution clarifies (about 5 min). Keep them on magnetic stand, and carefully discard the supernatant without disturbing the beads.

15. Keep the samples on the magnetic stand, add 200 μl of freshly prepared 80% ethanol to rinse the beads. DO NOT re-suspend 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 samples 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 remove the residual supernatant in this step.

▲Avoid over-drying of beads, resulting in a dramatic loss of DNA.

18. Take the samples out of magnetic stand. Add 21.5 μl of nuclease-free water 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 clarifies (about 5 min). Carefully transfer 19 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.

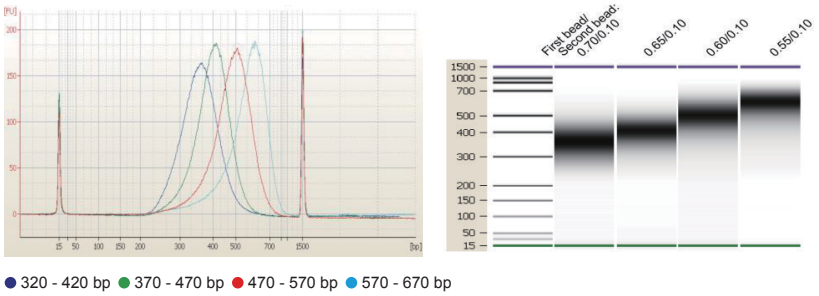


Fig. 4. Universal human reference RNA of 293T cells (200 ng) was fragmented under different conditions, and purified once with VAHTS DNA Clean Beads (0.45×), followed by size selection steps according to **Table 1**, respectively.

Appendix 2. FFPE sample or other degraded sample treatment instructions

1.The mRNA in this type of sample has different degrees of degradation, and the integrity is poor. It is recommended to increase the initial template input (>1 μg) to ensure a qualified sequencing library. If the amount of total RNA is insufficient, such as the template input is < 100 ng, it is recommended to use the 65 °C 5 min fragmentation condition and do not perform size selection, otherwise the library yield will be very low.

2.For this type of sample, the mapping rate may decrease as the degree of RNA degradation increases, which is unavoidable.

3.Solutions and case analysis for different RIN value of RNA samples are as follows:

Sample with RIN value > 3.0:

◇For 1 μg of template input, size selection or non-size selection are choosable.

◇If template input is < 100 ng, Do Not perform size selection, otherwise the library yield will be very low. It is recommended to use the condition of 65 °C 5 min fragmentation to ensure the library yield.

Case 1: FFPE sample of Mouse liver (RIN value = 5.2)

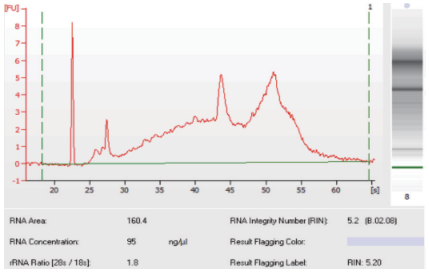


Table 3. The comparison results of constructed libraries from FFPE samples of mouse liver under different conditions.

Initial input RNA	Fragmentation condition	Size selection condition	Peak	Range of peak (bp)	Library concentration (ng/μl)
1 μg	65℃ 5 min	0.65 ×/0.1 ×	389	389	8.33
1 μg	65℃ 5 min	Two rounds of purification	267	267	51.28
1 μg	85℃ 5 min	0.65 ×/0.1 ×	395	395	8.57
1 μg	85℃ 5 min	Two rounds of purification	266	200 - 650	44.03
1 μg	94℃ 2 min	0.7 ×/0.1 ×	370	210 - 530	7.93
1 μg	94℃ 2 min	Two rounds of purification	266	150 - 700	36.46
100 ng	65℃ 5 min	Two rounds of purification	268	200 - 700	15.26

Sample with RIN value < 3.0:

◇Do not perform size selection, it is recommended to use the fragmentation condition of 65 °C 5 min to ensure the library yield.

Case 2: FFPE sample of Human prostate cancer tissue (PCa) (RIN value = 2.3)

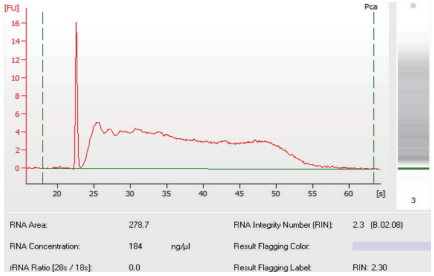


Table 4. The comparison results of constructed libraries from FFPE samples of Human prostate cancer tissue (PCa) under different conditions.

Initial input RNA	Fragmentation condition	Size selection condition	Peak	Range of peak (bp)	Library concentration (ng/μl)
100 ng	65℃ 5 min	Two rounds of purification	461	300 - 700	8.57
100 ng	65℃ 5 min	0.65 ×/0.1 ×	482	320 - 700	6.60

Appendix 2. FFPE sample or other degraded sample treatment instructions

Case 3: FFPE sample of Human lung cancer tissue (LC) (RIN value = 1.0)

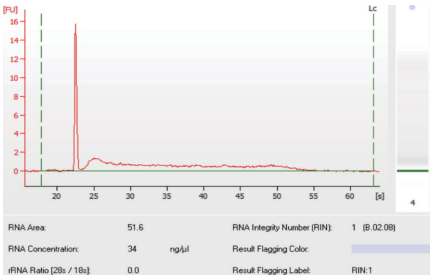


Table 5. The comparison results of constructed libraries from FFPE samples of Human lung cancer tissue (LC) under different conditions.

Initial input RNA	Fragmentation condition	Size selection condition	Peak	Range of peak (bp)	Library concentration (ng/µl)
100 ng	65℃ 5 min	Two rounds of purification	470	300 - 700	5.62
100 ng	65℃ 5 min	0.65 ×/0.1 ×	472	350 - 700	2.22

The non-size-selection protocol and milder fragmentation conditions are all conducive to obtaining higher library yields. In the above cases above, for samples with RIN > 3.0 (relatively less degraded), the size-selection will cause a large loss, and the concentration of the library obtained by the non-size-selection scheme is about 5 times higher than that of the size-selection scheme. So if the amount of the input template is insufficient, it is recommended to perform the non-size-selection scheme. For samples with RIN < 3.0 (seriously degraded), the library yield is very low, so a non-size-selection scheme and the mildest fragmentation conditions (65 ° C - 5 min) are recommended.

Troubleshooting

◇ Incorrect operation and remedy.

Step	Correct Operation	Incorrect Operation	Remedy
08-1/ 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.
08-1/ 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.
08-1/ 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.
08-1/ 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.
08-1/ Step 13	Add Frag/Prime Buffer to break mRNA.	Add Nuclease-free H2O 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 fragmentation condition, otherwise the library will fail to build and the ultimate size of the inserted library fragment will change too.
08-1/ 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 Nuclease-free H2O to make up for 16 µl.
08-2/ Step 2	Synthesis of the 1st strand of cDNA.	Actinomycin D is not diluted, or add wrong reagents.	Actinomycin D is not diluted, or add wrong reagents.
08-4/	Purification and Size Selection of adapter-ligated DNA	Without purification, direct size-selection.	Determine whether the library size meets the requirements based on the 2100 results.
08-4/	Without size selection conditions.	In first-round purification, add wrong volume of H2O.	The amount of magnetic beads to be used in the subsequent step can be adjusted according to the radio.

Troubleshooting

◇ 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.

Do Prepare several duplicate samples, merge them after the Fragmentation step, or before PCR step.

Take option without size selection: 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 size selection.

◇ 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): 0.05μg - 4 μg;

2.Ribo-off rRNA Depletion Kit (H/R/M) (Vazyme #N406): 0.05μg - 1 μg;

3.Ribo-off rRNA Depletion Kit (Bacteria) (Vazyme #N407): 1 μg - 5 μg;

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

◇ 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. 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 three 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 can 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.

Set 3: VAHTS RNA Multiplex Oligos set 1/2 for Illumina (Vazyme #N323, #N324) contains VAHTS RNA Adapter-S for Illumina, 8 kinds of VAHTS i5 PCR Primers and 12 kinds of VAHTS i7PCR Primers in each kit, allowing the construction of up to 384 different dual-Index libraries by adapter combination.

◇ 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.

◇ 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.

Troubleshooting

◇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.

◇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°C for 15 min for degradation test. If RNA is unqualified, the RNA re-extraction must be taken.

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

3.High-sensitivity chips were are used for high library concentrations. 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 HS kit.

◇ 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 above phenomenon is normal and would not affect the library sequencing and data analysis.