VAHTS Stranded mRNA-seq Library Prep Kit for Illumina® V2

Catalog# NR612

Version 9.2

Vazyme biotech co., ltd.

01/ Introduction

VAHTS Stranded mRNA-seq Library Prep Kit for Illumina® V2 is specially designed for the preparation of stranded transcriptome libraries for next generation sequencing (NGS) platforms of Illumina®. This kit is suitable for the library preparation starting from 50 ng-4 μg of intact total RNA of eukaryotes (e.g. animal, plant, or fungal). Different from non-stranded transcriptome library prep kits, this kit enables the insertion of dUTP during the 2nd strand synthesis of cDNA. The double-strand cDNA is digested with uracil-DNA glycosylase (UDG) to remove the second strand containing dUTP before library enrichment. As a result, only information from the 1st strand cDNA is retained. In addition to standard transcriptome information, strand-specific (e.g. from sense or anti-sense DNA) information can also be obtained from NGS data analysis.

This kit is upgraded from VAHTS Stranded mRNA-seq Library Prep Kit for Illumina®. It significantly simplifies the operation process and improves library preparation efficiency. It is compatible with lower-input RNA. Libraries of specific sizes, which can be customized, can be obtained after size selections with magnetic beads.

02/ Components

	Components	NR612- 01 (24 rxn)	NR612- 02 (96 rxn)
Box 1	mRNA Capture Beads	1.2 ml	4.8 ml
	Beads Binding Buffer	1.2 ml	4.8 ml
	Beads Wash Buffer	9.6 ml	38.4 ml
	Tris Buffer	1.2 ml	4.8 ml
Box 2	Frag/Prime Buffer	468 μl	2 × 936 μl
	Actinomycin D (5 mg/ml)	24 μl	96 μl
	1st Strand Buffer 2	144 μl	576 μl
	1st Strand Enzyme Mix 2	48 μl	192 μl
	2nd Strand Marking Buffer 2	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

▲ Both BOX 1 and BOX 2 can be purchased separately. Their catalogue# are # N401 and # NR604, respectively.

03/ Storage

Box 1: contains mRNA Capture Beads and should be stored and transported at 2℃ - 8℃ . **DO NOT FREEZE!**
Box 2: should be stored at -30℃ ~ -15℃ , transported at -20℃ ~ 0℃ .

04/ Applications

◇ Requirements for Starting Materials: 50 ng-4 μg total RNA of eukaryotes (e.g. animal, plant, or fungal).

▲ The eukaryotic mRNA contains a ploy-(A) tail and can be isolated and purified by magnetic beads with poly-T. The mRNA content of total RNA varies in different samples. Low input of total RNA may fail to provide enough amount of mRNA for library preparation.

◇ This kit is suitable for RNA samples with good integrity (RIN value ≥ 7).

▲ Using degraded RNA for library preparation will lead to 3'-bias in RNA-seq by bead-based mRNA enrichment. For RNA samples with RIN values < 7, please use the probe-based approaches to remove rRNA (e.g. VAHTS Total RNA-seq (H/M/R) Library Prep Kit for Illumina®, Vazyme # NR603).

▲ For RNA-seq library prep of lnc-RNA and cirRNA of human, rat, mouse and other animals, please use VAHTS Total RNA-seq (H/M/R) Library Prep Kit for Illumina® (Vazyme # NR603).

◇ 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) or Agilent RNA 6000 Pico Kit (Agilent, #5067-1513).

◇ **DNA Clean Beads:**
VAHTS DNA Clean Beads (Vazyme, # N411) or Agencourt® AMPure® XP Beads (Beckman Coulter, #A63880, #A63881, #A63882).

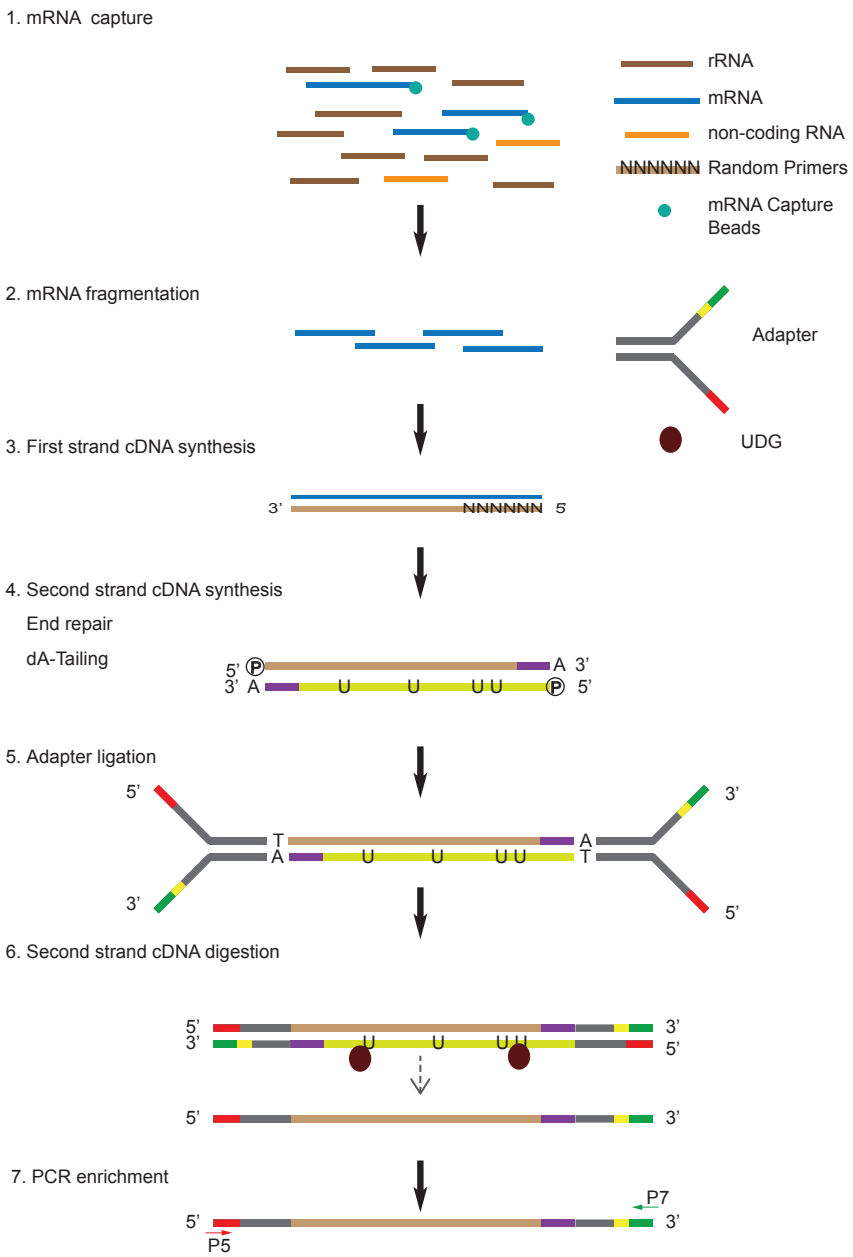
◇ **Adapters:**
VAHTS RNA Adapters set 1/2 for Illumina® (Vazyme #N803/N804), VAHTS RNA Adapters set 3-set 6 for Illumina® (Vazyme, #N809/N810/N811/N812), VAHTS RNA Multiplex Oligos set 1/2 for Illumina® (Vazyme #N323/N324).

◇ **Library Quality:**
Equalbit dsDNA HS Assay Kit (Vazyme, #EQ111) or Agilent DNA 1000 Kit (Agilent, #5067-1504).

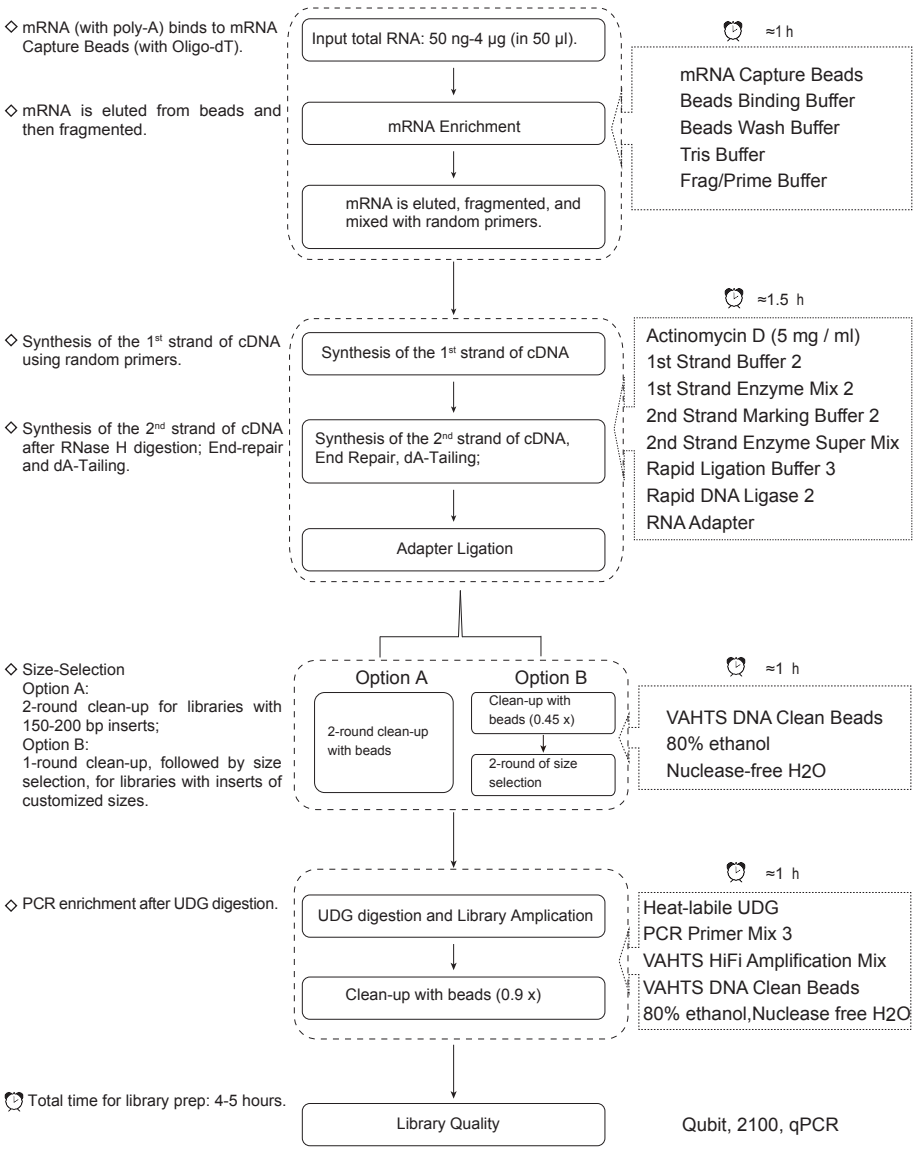
◇ **Other Materials:**
resh Ethanol (80%), Nuclease-free Water, Nuclease-free PCR tubes, Low-absorption EP tubes, Qubit, Agilent 2100 Bioanalyzer, Thermocycler (PCR instrument), Magnetic Stand.

06/ Mechanism & Workflow

06-1/ Mechanism



06-2/ Workflow



07/ Notes

◇ The Beads Binding Buffer and the Buffer in Box 2 may precipitate under low temperature conditions. It that happens, it is recommended to vortex after being equilibrated to room temperature to dissolve the precipitation completely.

◇ Before library prep, the RNA sample quality (e.g. the total amount, purity, and integrity) should be evaluated.

▲The initial input of the total RNA should be ≥ 50 ng. It cannot obtain enough mRNA for subsequent library preparation from low-input total RNA.

▲The OD260/OD280 ratio of total RNA should be between 1.8 and 2.1, and the OD230/OD260 ratio of total RNA should be between 0.4 and 0.5.

▲If using Bioanalyzer for RNA integrity analysis, the RIN value should be ≥ 7.0; if using electrophoresis analysis, the bands of 28 s and 18 s should not be significantly degraded, without protein and genomic contamination.

▲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).

08/ Protocol

08-1/ mRNA Enrichment and Fragmentation

1. Equilibrate the BOX 1 to room temperature, which contains mRNA Capture Beads, Beads Wash Buffer, Tris Buffer, and Beads Binding Buffer.

2. Prepare the reaction solution as follows:

Components	Volume
Diluted RNA	50 μl
mRNA Capture Beads	50 μl

▲ Mix thoroughly by pipetting up and down for 10 times. The mRNA Capture Beads, Beads Wash Buffer, and Beads Binding Buffer contains detergents. Don't vortex or shake violently when mixing up. Avoid air bubbles when pipetting.

3. Run the following program in a PCR instrument for the binding of mRNA to mRNA Capture Beads:

Temperature	Time
65˚C	5 min
25˚C	5 min

4. Put the samples onto a magnetic stand. Wait until the solution clarifies (about 5 min), then carefully discard the supernatant without disturbing the beads.

5. 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 3-5** are the first round of mRNA isolation and purification, and **Steps 6-11** are the second round of mRNA isolation and purification to ensure the removal efficiency of rRNA.

6. Take the samples out of the magnetic stand, and add 50 µl of Tris Buffer to re-suspend the beads thoroughly by pipetting up and down for 10 times.

7. Run the following procedure in the PCR instrument to release mRNA.

Temperature	Time
80˚C	2 min
25˚C	Hold

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

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

10. 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.

11. 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.

12. 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˚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.

13. 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.

08-2/ 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 120 ng / µl 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 (120 ng/µl)	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 Marking Buffer 2	25 µl
2nd Strand Enzyme Super Mix	15 µl
Total	65 µl

6. Adjust the pipettor to a 50 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 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.

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 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 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.

▲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
200 - 999 ng	2 µl
50 - 199 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/ Clean-up and Size Selection of Adapter-Ligated DNA

There are two options in this step, please choose carefully to follow as needed:

◇ **Option A** provides a 2-round clean-up without size-selection, and will generate fragments with 150 bp - 200 bp inserts (without residual adapters).

◇ **Option B** provides a 1-round clean-up and then a 2-round size-selection. According to different size-selection conditions in **Table 2 (on page 7)**, this option will generate size-selected fragments with > 200 bp interts (without residual adapters).

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

A1. Equilibrate the VAHTS DNA Clean Beads to room temperature.

A2. 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.

A3. Incubate at room temperature for 10 min.

A4. 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.

A5. Keeping 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.

A6. Repeat the **Step A5**.

A7. 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.

A8. 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.

A9. 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.

A10. Incubate at room temperature for 10 min.

A11. 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.

A12. 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.

A13. Repeat the Step **A12**.

A14. Keep the sample on the magnetic stand, open the tube and air-dry the beads for 5-10 min.

▲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.

A15. 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 08-5/ (Step 1) can be taken out from -30°C ~ -15°C in advance and placed on ice for use.

Option 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)

B-1. Clean-up of adapter ligation products using 0.45 × VAHTS DNA Clean Beads

B1. Equilibrate the VAHTS DNA Clean Beads to room temperature.

B2. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 45 μl (0.45 ×) of beads into the above sample (adapter ligation products, 100 μl). Mix thoroughly by pipetting up and down for 10 times.

B3. Incubate at room temperature for 10 min.

B4. 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.

B5. Keeping 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.

B6. Repeat the **Step B5**.

B7. 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.

B8. 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.

B-2. Size selection with VAHTS DNA Clean Beads

The following protocol is for a library with 350 bp - 450 bp inserts (as an example). Please refer to **Table 1** for the appropriate volume of beads for libraries with inserts of other sizes.

Table 1. Recommended conditions for bead-based size selection (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℃ 5 min	85℃ 6 min	85℃ 6 min	85℃ 5 min
Volume of beads for 1st round (µl)	70 (0.7 ×)	65 (0.65 ×)	60 (0.6 ×)	55 (0.55 ×)
Volume of beads for 2nd round (µl)	10 (0.1 ×)	10 (0.1 ×)	10 (0.1 ×)	10 (0.1 ×)

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

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 (30 bp).

▲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.

B9. 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.

B10. Incubate at room temperature for 10 min.

B11. 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.

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

B13. Incubate at room temperature for 10 min.

B14. 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.

B15. Keeping 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.

B16. Repeat the **Step B15**.

B17. 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.

B18. 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 t**he beads while drawing samples from the supernatant. Even 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.

The eluates can be temporarily stored at -30°C ~ -15°C for 24 hr.

08-5/ 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	95℃	3 min	1
Denaturation	98℃	20 sec	} 10 - 17
Annealing	60℃	15 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
200 - 999 ng	14 - 15
50 - 199 ng	16 - 17

4. Clean-up of the PCR product 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 (0.45 ×) 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 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.

e. Keeping 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.

f. Repeat the **Step e**.

g. 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.

h. 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.

▲The eluted products can be stored at -30°C ~ -15°C.

The eluates can be stored at -30°C ~ -15°C.

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.

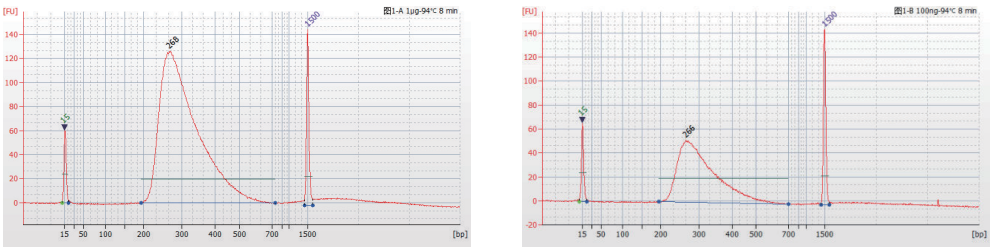


Fig. 1. Universal human reference RNA of 293T cells (1 μg - left, 100 ng - righ) was fragmented at 94℃ for 8 min and purified twice with VAHTS DNA Clean Beads (0.9×), respectively.

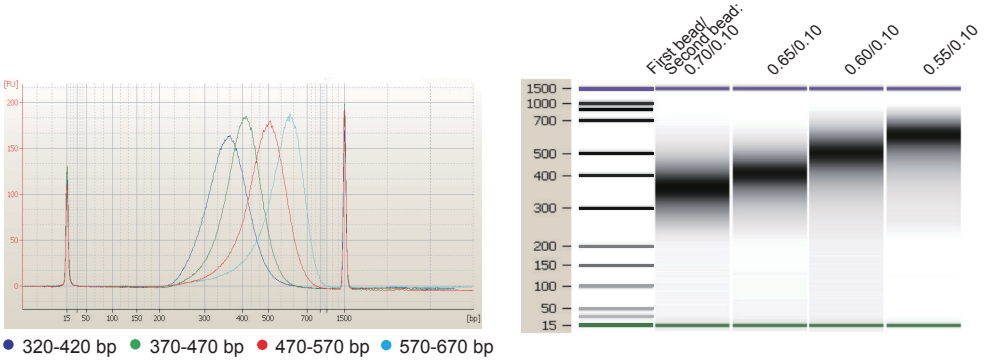


Fig. 2. Universal human reference RNA of 293T cells (200 ng) was fragmented under different conditions, and purified once with VAHTS DNA Clean Beads (0.45×), followed by size selection steps according to **Table 1**, respectivley.

Tips

1. VAHTS DNA Clean Beads Tips

Equilibrate the beads to room temperature before use.

Mix the beads thoroughly every time before pipetting.

Thoroughly mix the beads with DNA samples.

All the DNA size selection and procedures using beads should be performed at room temperature.

Do not pipet any **VAHTS DNA** Clean Beads when transferring the supernatant.

Prepare fresh 80% ethanol and discard after use.

Try to remove all the 80% ethanol after washing.

Thoroughly air-dry the beads before DNA elution.

2. Avoid cross contamination between samples.

Change tips between samples.

Use filtered pipette tips.

3. Aliquot reagents after the first use to avoid repeated freeze-thaw cycles.

4. Prevent contamination of PCR products.

Isolate the experimental area and carefully clean all equipments and instruments (e.g. clean with 0.5% sodium hypochlorite or 10% bleach) to avoid possible contamination in PCR system.

ISO 9001: 2015