Instruction Manual

VAHTS mRNA-seq v2 Library Prep Kit for Illumina®

Vazyme Cat #NR601

Vazyme Biotech Co., Ltd

Web: www.vazyme.com

Tel: 400-600-9335

Sales: Sales@vazyme.com

Support: Support@ vazyme.com

Address: Economic and Technological Development Zone, Red Maple Park Building C1-2, Kechuang Road, Nanjing, China

Contents

	Component	NR601-01 (24 rxn)	NR601-02 (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	1.872 ml
	1st Strand Buffer	144 µl	576 µl
	1st Strand Enzyme Mix	48 µl	192 µl
	2nd Strand Buffer	480 µl	1.92 ml
	2nd Strand Enzyme Mix	120 µl	480 µl
Box 3	End Prep Mix	960 µl	3.84 ml
	dA-Tailing Buffer Mix	240 µl	960 µl
	dA-Tailing Enzyme Mix	60 µl	240 µl
	Ligation Mix	60 µl	240 µl
	Stop Ligation Mix	120 µl	480 µl
	PCR Primer Mix	120 µl	480 µl
	Amplification Mix 1	600 µl	2.4 ml

Storage Conditions

Store Box 1 at 2-8℃ in the dark. Store Box 2 and Box 3 at -20℃ in the dark. Consider aliquoting the mix into smaller volumes to avoid repeatedly freeze-thaw cycles when used frequently.

Warranty

Box 1 warranty period is three years from purchase date, Box 2 and Box 3 warranty period is one and a half years from purchase date when stored under proper conditions.

Additional Required Reagents

100% ethanol.
Nuclease free water.
VAHTS DNA Clean Beads (Vazyme Cat#N411) or AMpure XP Beads (Beckman #A63881).
VAHTS RNA Adapters Set 1 for Illumina® (Adapter 1-12, Vazyme Cat#N803).
VAHTS RNA Adapters Set 2 for Illumina® (Adapter 13-27, Vazyme Cat#N804).

General Information

Introduction

VAHTS mRNA-seq v2 Library Prep Kit for Illumina® is designed for transcriptome library preparation ideal for high throughput sequencing. mRNA is purified from 0.1-1 µg total

RNA, fragmented, and reverse transcribed into double strand cDNA. VAHTS mRNA-seq v2 Library Prep Kit for Illumina® will also repair cDNA ends, add adenine tails, ligate adapters, and PCR during the cDNA library construction for Illumina® sequencing.

Quality Control

All the reagents provided in this kit undergo critical quality control and functional test, which guarantee the stability and repeatability of the constructed libraries.

Detailed Operating Instructions

Requirements for Initial Reagents

Initial template: 0.1-1 μg high quality total RNA. We recommend using Agilent 2100 Bioanalyzer to quantify the quality of extracted total RNA. RIN (RNA integrity number) value should be above 8. Using degraded total RNA will result in 3' preference of RNA-seq.

Transcripts: This kit is suitable for mRNA related analyses using high throughput sequencing including gene expression, single nucleotide variation, and alternative splicing/fusion gene and transcriptome analysis. If non-coding RNA is under consideration, please choose VAHTS Total RNA-seq (H/M/R) Library Prep Kit for Illumina® (Vazyme Cat#NR603).

Step 1: mRNA Purification and Fragmentation

1. Bring the mRNA Capture Beads to room temperature.
2. Prepare the RNA sample by dissolving 0.1-1 μg of total RNA in 50 μl nuclease free water in a nuclease free centrifuge tube, keep on ice.
3. Mix the mRNA Capture Beads by inverting or vortexing, add 50 μl of the beads to the total RNA tube, and mix by pipetting 6 times.
4. Incubate the sample in a thermostatic device (i.e. a PCR machine) at 65℃ for 5 minutes then hold at 4℃ to denature the RNA.
5. Allow the tube to rest at room temperature for 5 minutes, allowing the mRNA to bind to the Capture Beads.
6. Transfer the sample to a magnetic frame for 5 minutes then carefully remove the supernatant without disturbing the Capture Beads.
7. Remove the sample from the magnetic frame, add 200 μl of Beads Wash Buffer, and mix by pipetting 6 times. Return the sample to a magnetic frame, let rest for 5 minutes, and carefully remove the supernatant without disturbing the Capture Beads.
8. Remove the sample from the magnetic frame, add 50 μl of Tris Buffer, and mix by pipetting 6 times.

9. Incubate the sample in a thermostatic device (i.e. a PCR machine) at 80 ℃ for 2 minutes and then hold at 25 ℃.
10. Add 50 µl of the Beads Binding Buffer, mix by pipetting 6 times.
11. Incubate at room temperature for 5 minutes.
12. Return the sample to a magnetic frame, let rest for 5 minutes, and carefully remove the supernatant without disturbing the Capture Beads.
13. Remove the sample from the magnetic frame, add 200 µl of the Beads Wash Buffer, and mix by pipetting 6 times. Let rest for 5 minutes on a magnetic frame then carefully remove the supernatant without disturbing the Capture Beads. Remove as much liquid as possible using a 10 µl pipette.
14. Remove the sample from the magnetic frame, add 19.5 µl of Frag /Prime Buffer, mix by pipetting 6 times. Incubate the sample in a PCR device and set programs according to the fragment size required:
    - 150-200 bp insertion: 8 minutes at 94 ℃, 4℃ hold.
    - 200-300 bp insertion: 5 minutes at 94 ℃, 4℃ hold.
    - 250-450 bp insertion: 6 minutes at 85 ℃, 4℃ hold.
    - 450-550 bp insertion: 5 minutes at 85 ℃, 4℃ hold.
    - Please refer to Table 1 for more information on fragmentation.
15. Place the sample on a magnetic frame, transfer 17 µl of the supernatant to a new nuclease free tube and proceed to Step 2: Synthesis of Double Strand cDNA.

Step 2: Synthesis of Double Strand cDNA

1. Invert the thawed 1st Strand Buffer several times and combine the reaction below in a sterile PCR tube.

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

2. Mix the tube by gently pipetting (DO NOT VORTEX) followed by a briefly centrifugation so the reaction volume collects at the bottom of the PCR tube.
3. Place the reaction tube in the PCR instrument and operate under the following condition:
    i. 10 minutes at 25℃
    ii. 15 minutes at 42℃
    iii. 15 minutes at 70℃
    iv. Hold at 4℃
4. Immediately Perform 2nd Strand Synthesis steps below.
5. Invert the thawed 2st Strand Buffer several times and combine the reaction below in sterile PCR tube.

Component	Volume
1st Strand cDNA (from step 3 above)	25 µl
2nd Strand Buffer	20 µl
2nd Strand Enzyme Mix	5 µl
Total	50 µl

6. Mix the tube by gently pipetting (DO NOT VORTEX) followed by a briefly centrifugation so the reaction volume collects at the bottom of the PCR tube.
7. Place the reaction tube in a PCR instrument and operate under the following conditions:
    i. 60 minutes at 16℃
    ii. Hold at 4℃

8. Purify and sort the reaction products according to size using VAHTS DNA Clean Beads (Vazyme Cat#N411).

    1) Incubate the VAHTS DNA Clean Beads at room temperature for 30 minutes prior to purification.
    2) Vortex the VAHTS DNA Clean Beads.
    3) Add 90 µl of VAHTS DNA Clean Beads (1.8x) to the sample above. Mix thoroughly by pipetting 10 times.
    4) Incubate at room temperature for 10 minutes.
    5) Briefly centrifuge the reaction tube and place in the magnetic frame. Let the tube rest until the solution clarifies (about 5 minutes) and discard supernatant.
    6) Keep the EP tube in the magnetic frame and add 200 µl of freshly prepared 80% ethanol to wash the beads. Incubate at room temperature for 30 seconds and discard the supernatant.
    7) Repeat step 6.
    8) Keep the EP tube in the magnetic frame, open the EP tube lid and air-dry the beads for 5-10 minutes.
    9) Remove the EP tube from the magnetic frame and add 62.5 µl of nuclease-free water. Mix by vortex or pipetting. Briefly centrifuge the tube and return the reaction tube to the magnetic frame. Let the tube rest until the solution clarifies (about 5 minutes). Carefully remove 60 µl of the supernatant to a new PCR tube and carefully avoid touching the VAHTS DNA Clean Beads.
    NOTE: the dilution can be stored at -20℃.

Step 3: End Repair

1. Invert the thawed End Prep Mix several times and combines the reaction below in a sterile PCR tube.

Component	Volume

Double stranded cDNA (from above)	60 µl
End Prep Mix	40 µl
Total	100 µl

2. Mix the reaction by gently pipetting (DO NOT VORTEX) followed by a briefly centrifugation so the reaction volume collects at the bottom of the PCR tube.
3. Place the reaction tube in a PCR instrument and operate under the following condition:
    i. 30 minutes at 30℃
    ii. Hold at 4℃
4. Purify and sort reaction products according to size using VAHTS DNA Clean Beads (Vazyme Cat#N411).

    1) Incubate the VAHTS DNA Clean Beads at room temperature for 30 minutes prior to purification.
    2) Vortex the VAHTS DNA Clean Beads.
    3) Add 90 µl of VAHTS DNA Clean Beads (1.8x) to the sample above. Mix thoroughly by pipetting 10 times.
    4) Incubate at room temperature for 10 minutes.
    5) Briefly centrifuge the reaction tube and place in the magnetic frame. Let the tube rest until the solution clarifies (about 5 minutes) and discard supernatant.
    6) Keep the EP tube in the magnetic frame and add 200 µl of freshly prepared 80% ethanol to wash the beads. Incubate at room temperature for 30 seconds and discard the supernatant.
    7) Repeat step 6.
    8) Keep the EP tube in the magnetic frame, open the EP tube lid and air-dry the beads for 5-10 minutes.
    9) Remove the EP tube from the magnetic frame and add 62.5 µl of nuclease-free water. Mix by vortex or pipetting. Briefly centrifuge the tube and return the reaction tube to the magnetic frame. Let the tube rest until the solution clarifies (about 5 minutes). Carefully remove 60 µl of the supernatant to a new PCR tube and avoid touching the VAHTS DNA Clean Beads.
    NOTE: the dilution can be stored at -20℃.

Step 4: Adding dA-Tails

1. Mix the thawed dA-Tailing Buffer by inverting and combine the reaction below in a sterile PCR tube.

Component	Volume
Purified end-repaired products	17.5 µl
dA-Tailing Buffer Mix	10 µl

dA-Tailing Enzyme Mix	2.5 µl
Final Volume	30 µl

2. Mix the tube by gently pipetting (DO NOT VORTEX) followed by a briefly centrifugation so the reaction volume collects at the bottom of the PCR tube.
3. Place the reaction tube in a PCR instrument and operate under the following condition:
    i. 30 minutes at 37℃
    ii. 5 minutes at 70℃
    iii. Hold at 4℃
4. Immediately Perform **Step 5: Adapter Ligation** below.

Step 5: Adapter Ligation

1. Mix the thawed Stop Ligation Mix by inverting and combine the reaction below in a sterile PCR tube.

Component	Volume
Purified dA-Tailing Products	30 µl
Ligation Mix	2.5 µl
RNA Adapter	2.5 µl
Total	35 µl

2. Mix the reaction by gently pipetting (DO NOT VORTEX) followed by a briefly centrifugation so the reaction volume collects at the bottom of the PCR tube.
3. Place the reaction tube in the PCR instrument and operate under the following condition:
    i. 10 minutes at 30℃
    ii. Hold at 4℃
4. Add 5 µl of Stop Ligation Mix to the reaction tube and mix by gently pipetting.

Step 6: Purification of Ligation Product and Size Fractioning

Solution A: 150 – 200bp Library Construction

Requires mRNA incubation at 98℃ for 8 minutes for fragmentation.

1. Incubate VAHTS DNA Clean Beads at room temperature for 30 minutes prior to purification.
2. Vortex the VAHTS DNA Clean Beads.
3. Add 40 µl of VAHTS DNA Clean Beads (1x) to the ligation product above. Mix thoroughly by pipetting 10 times.
4. Incubate at room temperature for 10 minutes.

5. Briefly centrifuge the reaction tube and place in the magnetic frame. Let the tube rest until the solution clarifies (about 5 minutes) and discard supernatant.
6. Keep the EP tube in the magnetic frame and add 200 µl of freshly prepared 80% ethanol to wash the beads. Incubate at room temperature for 30 seconds and discard the supernatant.
7. Repeat step 6.
8. Keep the EP tube in the magnetic frame, open the EP tube lid and air-dry the beads for 5-10 minutes.
9. Remove the EP tube from the magnetic frame and add 52.5 µl of nuclease-free water. Mix by vortex or pipetting. Briefly centrifuge the tube and return the reaction tube to the magnetic frame. Let the tube rest until the solution clarifies (about 5 minutes). Carefully remove 50 µl of the supernatant to a new PCR tube and avoid touching the VAHTS DNA Clean Beads.
10. Vortex the VAHTS DNA Clean Beads.
11. Add 50 µl of VAHTS DNA Clean Beads (1x) to the sample. Mix thoroughly by pipetting 10 times.
12. Incubate at room temperature for 10 minutes.
13. Briefly centrifuge the reaction tube and place in the magnetic frame. Let the tube rest until the solution clarifies (about 5 minutes) and discard supernatant.
14. Keep the EP tube in the magnetic frame and add 200 µl of freshly prepared 80% ethanol to wash the beads. Incubate at room temperature for 30 seconds and discard the supernatant.
15. Repeat step 6.
16. Keeping the EP tube in the magnetic frame, open the EP tube lid and air-dry the beads for 5-10 minutes.
17. Remove the EP tube from the magnetic frame and add 22.5 µl of nuclease-free water. Mix by vortex or pipetting. Briefly centrifuge the tube and return the reaction tube to the magnetic frame. Let the tube rest until the solution clarifies (about 5 minutes). Carefully remove 20 µl of the supernatant to a new PCR tube and carefully avoid touching the VAHTS DNA Clean Beads.
    NOTE: Immediate Perform **Step 7: Library Amplification**.
    NOTE: Do NOT disturb the beads while drawing samples from the supernatant; any trace amount of beads will affect the library quality.

Solution B: >200 bp Library Construction

Requires mRNA incubation at (SEE TABLE 1) for fragmentation.

1. Incubate the VAHTS DNA Clean Beads at room temperature for 30 minutes prior to purification.
2. Vortex the VAHTS DNA Clean Beads.
3. Add 40 µl of VAHTS DNA Clean Beads (1x) to the ligation product above. Mix thoroughly by pipetting 10 times.
4. Incubate at room temperature for 10 minutes.

5. Briefly centrifuge the reaction tube and place in the magnetic frame. Let the tube rest until the solution clarifies (about 5 minutes) and discard supernatant.
6. Keep the EP tube in the magnetic frame and add 200 µl of freshly prepared 80% ethanol to wash the beads. Incubate at room temperature for 30 seconds and discard the supernatant.
7. Repeat step 6.
8. Keep the EP tube in the magnetic frame, open the EP tube lid and air-dry the beads for 5-10 minutes.
9. Remove the EP tube from the magnetic frame and add 102.5 µl of nuclease-free water. Mix by vortex or pipetting. Briefly centrifuge the tube and return the reaction tube to the magnetic frame. Let the tube rest until the solution clarifies (about 5 minutes). Carefully remove 100 µl of the supernatant to a new PCR tube and avoid touching the VAHTS DNA Clean Beads.

Table 1: Fractioning Conditions for different size insertions

Insertion Length (bp)	**200-300**	**250-350**	**350-450**	**450-550**
Library Length (bp)*	320-420	370-470	470-570	570-670
Fragmentation Condition	5 minutes at 94℃	6 minutes at 85℃	6 minutes at 85℃	5 minutes at 85℃
1st Round bead volume (µl)	70 (0.7x)	65 (0.65x)	60 (0.6x)	55 (0.55x)
2nd Round bead volume (µl)	10 (0.1x)	10 (0.1x)	10 (0.1x)	10 (0.1x)

*Library length here means the peak size range determined by Agilent 2100 Bioanalyzer. Library length is equal to insertion length plus adapter length (120 bp). Please see Step 7: Library Amplification for more information.

10. Vortex the VAHTS DNA Clean Beads.
11. Add 60 µl of VAHTS DNA Clean Beads (0.6x) to the purified sample. Mix thoroughly by pipetting 10 times.
12. Incubate at room temperature for 10 minutes.
13. Place the reaction tube in the magnetic stand. Let the tube rest until the solution clarifies (about 5 minutes) and carefully remove all but 155 µl of the supernatant to a new nuclease free tube.
14. Add 10 µl VAHTS DNA Clean Beads to this new tube and mix thoroughly by pipetting 10 times.
15. Incubate at room temperature for 10 minutes.
16. Place the reaction tube in the magnetic stand. Let the tube rest until the solution clarifies (about 5 minutes) and discard supernatant.
17. Keep the EP tube in the magnetic stand and add 200 µl of freshly prepared 80% ethanol to wash the beads. Incubate at room temperature for 30 seconds and discard the supernatant.
18. Repeat Step 17.

19. Keeping the EP tube in the magnetic stand, open the EP tube lid and air-dry the beads for 5-10 minutes.
20. Remove the EP tube from the magnetic stand and add 22.5 µl of nuclease-free water. Mix by vortexing or pipetting. Let rest at room temperature for 2 minutes and return the reaction tube to the magnetic stand. Let the tube rest until the solution clarifies (about 5 minutes). Remove 20 µl of the supernatant to a new PCR tube, careful to avoid touching the VAHTS DNA Clean Beads.
Note: Do NOT disturb the beads while drawing samples from the supernatant; any trace amount of beads will affect the library quality.

Step 7: Library Amplification

This step will amplify and enrich the dA-Tailing.

1. Invert the thawed PCR Primer Mix and combine the reaction below in sterile PCR tube.

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

2. Mix the reaction by gently pipetting (DO NOT VORTEX) followed by a briefly centrifugation so the reaction volume collects at the bottom of the PCR tube.

3. Place the reaction tube in the PCR instrument and operate under the following condition:
   i. 3 seconds at 98℃
   ii. 10 seconds at 98℃
   iii. 30 seconds at 60℃ } 15 Cycles
   iv. 30 seconds at 72℃
   v. 5 minutes at 72℃
   vi. Hold at 4℃

4. Purify and sort the reaction products according to size using VAHTS DNA Clean Beads (Vazyme Cat#N411).

   1) Incubate the VAHTS DNA Clean Beads at room temperature for 30 minutes prior to purification.
   2) Vortex the VAHTS DNA Clean Beads.
   3) Add 50 µl of VAHTS DNA Clean Beads to 50 µl of the PCR products above. Mix thoroughly by pipetting 10 times.

4) Incubate at room temperature for 5 minutes.
5) Briefly centrifuge the reaction tube and place in the magnetic frame. Let the tube rest until the solution clarifies (about 5 minutes) and remove the supernatant.
6) Keep the EP tube in the magnetic frame and add 200 µl of freshly prepared 80% ethanol to wash the beads. Incubate at room temperature for 30 seconds and discard the supernatant.
7) Repeat step 5 and completely rinse two additional times.
8) Keep the EP tube in the magnetic frame, open the EP tube lid and air-dry the beads for 10 minutes.
9) Remove the EP tube from the magnetic frame and add 25 µl of ultrapure sterile water. Mix by vortex or gently pipetting. Briefly centrifuge the tube and return the reaction tube to the magnetic frame. Let the tube rest until the solution clarifies (about 5 minutes). Carefully remove 22.5 µl of the supernatant to a new PCR tube and avoid touching the VAHTS DNA Clean Beads.

   Note: Sample can be stored at -20℃.

   Note: DO NOT disturb the VAHTS DNA Clean Beads when transferring supernatants because trace residues will affect subsequent steps of library construction.

5. Determine the library quality using an Agilent Technologies 2100 Bioanalyzer. Analyze 1 µl of purified PCR product using a DNA 100 chip. A good qualify library should exbit a nerrow peak at the expected size. A narrow peak shown at 128 bp suggests the contamination of adapter-dimer. Dilute the library with nuclease free water to 50 µl, repeat step 6-3 for furture purification.

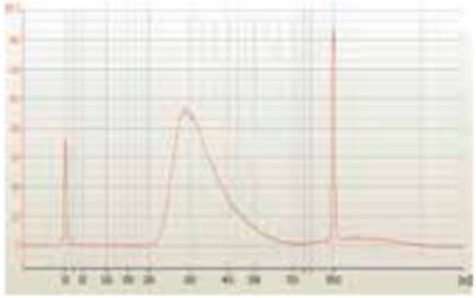

Figure 1. 100 ng of universal human reference RNA, fragmented at 94℃ for 8 minutes, and purified twice with 1x VAHTS DNA Clean Beads.

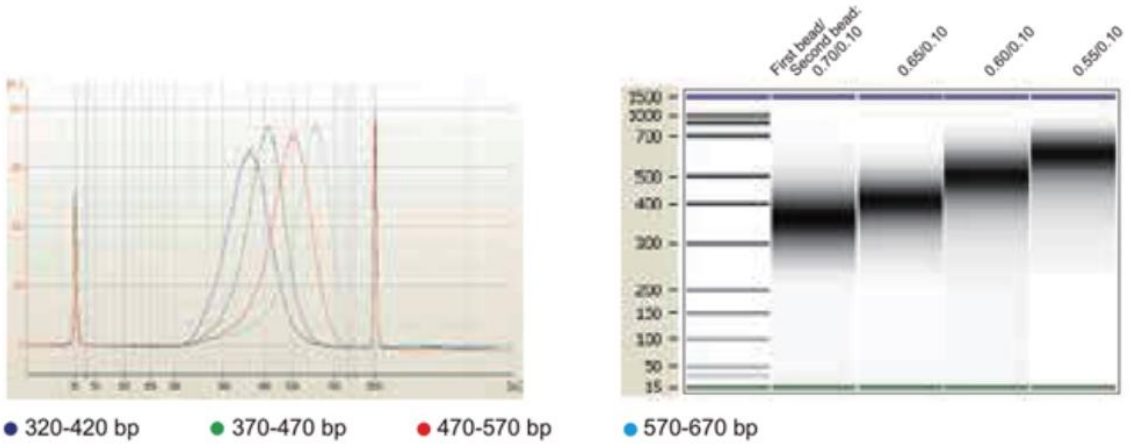

Figure 2. 200 ng of universal human reference RNA, fragmented at desired fragmentation condition, and purified once with 1x VAHTS DNA Clean Beads, followed by sorting steps according to TABLE 1.

FAQs and Troubleshooting

1. Aliquot Reagents in order to reduce frequent freeze thaw cycles.
2. VAHTS DNA Clean Beads (Vazyme Cat#N411) Tips.
    - Bring beads to room temperature before use.
    - Mix the beads thoroughly every time before removing beads.
    - Thoroughly mix beads with DNA samples.
    - Beads perform optimally at room temperature.
    - DO NOT disturb the VAHTS DNA Clean Beads when transferring supernatants.
    - Prepare fresh 80% ethanol and discard after use.
    - Try to sip up the beads after washed by 80% of the ethanol.
    - Thoroughly dry the beads before the wash step to avoiding residual ethanol effects on subsequent steps.
3. Avoid cross contamination of samples.
    - Change pipette tips between samples.
    - Use filtered pipette tips.
4. Prevent contamination of PCR products.
    - Physically isolate the experimental area and carefully clean all equipment and instruments (e.g., clean with 0.5% sodium hypochlorite or 10% bleach) in order to avoid contamination of the PCR reaction system.