VAHTS Universal V10 RNA-seq Library Prep Kit (Plate)

NRB616

Instruction for Use

Version 25.1

Contents

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

01/Product Description

VAHTS Universal V10 RNA-seq Library Prep Kit (Plate) is a 96-well plate transcriptome library preparation kit, which is compatible with Illumina and MGI high-throughput sequencing platforms. The kit includes two types of second strand cDNA synthesis reagents, selectable according to the requirements for non-strand-specific transcriptome or strand-specific transcriptome library preparation.

The kit streamlines the process via pre-mixed reagents, and the optimized reaction system improves the library preparation efficiency, input compatibility and uniform coverage across different inputs. All the reagents provided in the kit have undergone rigorous quality control and function testing to ensure the optimal stability and repeatability of library preparation.

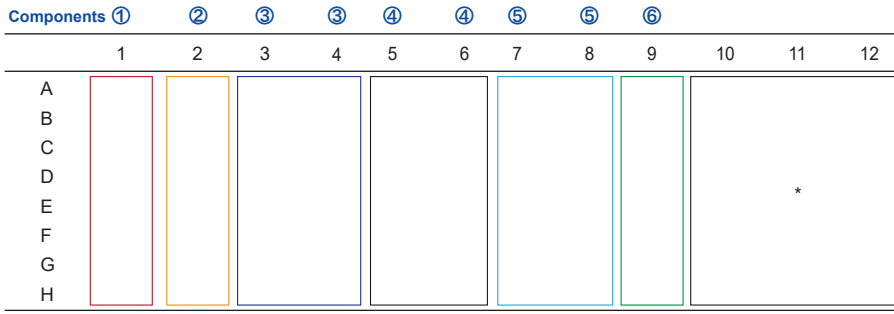
02/Components

Components	NRB616-01	NRB616-02
RNA Reagents (Prepackaged for NRB616)	48 rxns	96 rxns

Component details

	Components	NRB616-01 (48 rxns)	NRB616-02 (96 rxns)
①	Frag/Prime Buffer 2	164 µl each	285 µl each
②	1st Synthetic Master Mix 2	112 µl each	177 µl each
③	2nd Synthetic Master Mix 2 (with dNTP)	164 µl each	284 µl each
④	2nd Synthetic Master Mix 2 (with dUTP)	164 µl each	284 µl each
⑤	Ligase Master Mix 2	134 µl each	252 µl each
⑥	VAHTS HiFi Amplification Mix 3	180 µl each	360 µl each

Layout information of NRB616



* Plates 10 - 12 are vacant and can be used to prepare reaction mixes or reagents such as magnetic beads and adapters, depending on experimental requirements.

03/Storage

Store at -30 ~ -15°C and protect from light. Ship at ≤0°C.

04/Applications

VAHTS Universal V10 RNA-seq Library Prep Kit (Plate) is suitable for RNA library preparation for total RNA, ribosomal-depleted RNA or mRNA enriched by Poly(A) method from eukaryotes such as animals, plants, and fungi. The RNA amount varies significantly across different samples, and the initial total RNA inputs can be adjusted according to the sample type and the recommended input of the upstream mRNA enrichment module.

The detailed recommendations are as follows:
VAHTS mRNA Capture Beads (Ultrapure Plate) (Vazyme #RNB702): 0.01 - 4 μg
Ribo-clean rRNA Depletion Kit Mega (Prevalent Species) (Vazyme #RN416): 0.01 - 1 μg
Ribo-clean rRNA Depletion Kit Mega (Bacteria) (Vazyme #RN417): 0.01 - 1 μg
Ribo-clean Globin mRNA Depletion Kit Mega (Prevalent Species) (Vazyme #RN418): 0.01 - 1 μg
Ribo-clean rRNA Depletion Kit Mega (Model Species) (Vazyme #RN426): 0.01 - 1 μg
Ribo-clean rRNA Depletion Kit Mega (R/M) (Vazyme #RN411): 0.01 - 1 μg
Ribo-MagOff rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N420): 0.01 - 1 μg
Purified mRNA or Ribosomal-depleted RNA: 0.5 - 100 ng

It is recommended to use Agilent 2100 Bioanalyzer or other equivalent products to analyze RNA integrity. Please make sure that RNA samples are of high quality (RIN ≥7) when using VAHTS mRNA Capture Beads (Ultrapure Plate) (Vazyme #RNB702). The degraded sample is usually subjected to RNA fragmentation, causing a 3' bias during library preparation. For RNA samples with RIN <7, the Ribo-off method (Vazyme #RN416/RN417/RN418/RN426/RN411) is recommended.

Main aspects of RNA-related analysis:

◇ Gene expression analysis

◇ Single nucleotide variation calling

◇ Alternative splicing detection

◇ Gene fusion detection

◇ Target transcriptome analysis

05/Self-prepared Materials

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

◇ mRNA Enrichment Module:
VAHTS 2 × Frag/Prime Buffer V2 (Vazyme #N405)
VAHTS mRNA Capture Beads (Ultrapure Plate) (Vazyme #RNB702)
Ribo-clean rRNA Depletion Kit Mega (Prevalent Species) (Vazyme #RN416)
Ribo-clean rRNA Depletion Kit Mega (Bacteria) (Vazyme #RN417)
Ribo-clean Globin mRNA Depletion Kit Mega (Prevalent Species) (Vazyme #RN418)
Ribo-clean rRNA Depletion Kit Mega (Model Species) (Vazyme #RN426)
Ribo-clean rRNA Depletion Kit Mega (R/M) (Vazyme #RN411)
Ribo-MagOff rRNA Depletion Kit (Human/Mouse/Rat) (Vazyme #N420)

◇ DNA and RNA Purification:
VAHTS DNA Clean Beads (Vazyme #N411)
VAHTS RNA Clean Beads (Vazyme #N412)
DF Flowsizer Buffer (Vazyme #N001)

◇ Adapter:
VAHTS Maxi Unique Dual Index Primers Set 1 - Set 4 for Illumina (Plate) (Vazyme #NB34401 - NB34404)
VAHTS Maxi Unique Dual Barcode Primers Set 1 - Set 4 for MGI (Plate) (Vazyme #NMB34401 - NMB34404)

◇ Library Quality Control:
Equalbit 1 × dsDNA HS Assay Kit (Vazyme #EQ121)

◇ Other Materials:
80% ethanol (freshly prepared with Nuclease-free ddH_2O), Nuclease-free ddH_2O; low adsorption Nuclease-free PCR tubes and tips; PCR instrument, magnetic rack, qubit, and Agilent 2100 Bioanalyzer or other equivalent products.

06/Notes

06-1/About RNA Sample Quality Control

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

1. The initial total RNA inputs should be ≥10 ng; otherwise, the mRNA may be insufficient for library preparation.
2. The ratio of OD260/OD280 should be between 1.8 and 2.1. If the ratio is >2.1, the RNA samples may have been contaminated with genomic DNA. If the ratio is <1.8, the RNA samples may have been contaminated with protein. The ratio of OD230/OD260 should be between 0.4 and 0.5. If the ratio is >0.5, the RNA samples may have been contaminated with salt or small molecule. If the ratio is <0.4, the RNA samples may have been contaminated with genomic DNA.

06-2/About RNA Sample Preparation

1. Mix the reaction mix containing RNA by pipetting instead of vortexing to avoid unexpected library size caused by RNA fragmentation.
2. Proceed to library preparation as soon as possible using mRNA or ribosomal-depleted RNA to avoid RNA degradation.
3. If the initial concentration of RNA is low, the RNA can be concentrated by lyophilization, ethanol precipitation, column purification or magnetic bead purification (VAHTS RNA Clean Beads, Vazyme #N412).

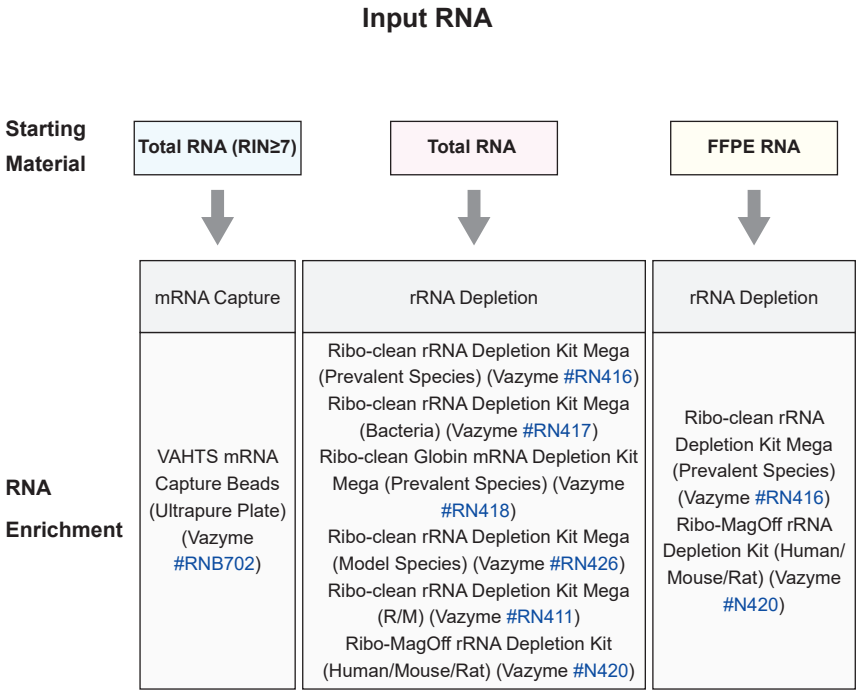
06-3/About DNA Magnetic Beads

1. Equilibrate magnetic beads to room temperature and mix well before use, or the recovery efficiency may be affected.
2. Transfer the supernatant when the solution becomes clear and do not disturb the beads! If the magnetic beads are accidentally aspirated, please replace the tube on the magnetic rack for separation.
3. Use 80% ethanol (freshly prepared with Nuclease-free ddH_2O) to wash the magnetic beads; otherwise, it may cause RNA loss and library preparation failure. Please discard as much of the supernatant as possible to minimize impurity residues after second wash.
4. Before elution, ensure that the magnetic beads are sufficiently dry (with the surface changing from shiny brown to matte brown) to prevent ethanol residue from affecting downstream experiments. Over-drying the beads may reduce recovery efficiency.

06-4/About Operation

1. Thaw all components on ice and mix well by inversion. Centrifuge briefly and place on ice for later use.
2. It is recommended to use pipette tips with filters and change the tips for different samples.
3. Consumables used should be Nuclease-free before second strand synthesis and DNase-free after second strand synthesis.
4. Be sure to use fresh Nuclease-free ddH_2O. It is recommended to aliquot the solution to separate tubes and discard each tube after a single use.
5. Be sure to wear gloves during operation, and change gloves after touching equipment or other work areas outside the RNase-free zone.
6. All reagents must be capped immediately after use to avoid contamination.
7. It is recommended to physically isolate the PCR reaction preparation area from the PCR product purification area to avoid aerosol contamination.

07/Mechanism & Workflow



cDNA Library Preparation

- 2nd Strand cDNA Synthesis, End Repair, and dA-Tailing are combined into one step, streamlining the library preparation.

- Purification and Size Selection
  Purification: insert DNA >200 bp
  Size Selection: insert DNA with customized size

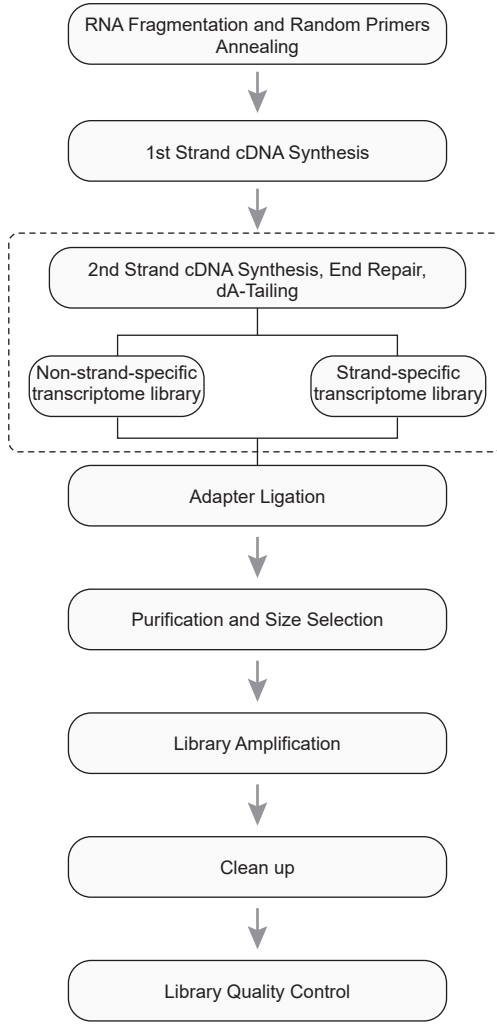


Fig 1. Workflow of VAHTS Universal V10 RNA-seq Library Prep Kit (Plate)

08/Experiment Process

08-1/mRNA Enrichment and Fragmentation

Option A: Poly(A)-based mRNA Capture

Take VAHTS mRNA Capture Beads (Ultrapure Plate) (Vazyme #RNB702) for mRNA capture as an example. It is applicable to RNA library preparation with 10 ng - 4 μg intact total RNA from eukaryotes (e.g., animal, plant, or fungi).

1. Equilibrate the kit to room temperature.
2. Prepare RNA samples: Dilute 10 ng - 4 μg total RNA in Nuclease-free ddH_2O to a final volume of 50 μl, and place it on ice for later use.
3. Mix the Ultrapure mRNA Capture Beads well by inversion, add 50 μl of Ultrapure mRNA Capture Beads to the prepared RNA sample and mix well by pipetting.
4. Perform the following program in the PCR instrument, enabling mRNA to bind to magnetic beads:

Temperature	Time
65°C	5 min
25°C	5 min
4°C	Hold

5. Place the tube on the magnetic rack, and discard the supernatant when the solution is clear (about 5 min).
6. Remove the tube from the magnetic rack, add 150 - 200 μl of Ultrapure Beads Wash Buffer to resuspend the magnetic beads, and mix well by pipetting. Place the tube on the magnetic rack, and discard the supernatant when the solution is clear (about 5 min).
   ▲ Determine the final volume of Ultrapure Beads Wash Buffer used in the script based on the actual conditions of the automated workstation.
7. Remove the tube from the magnetic rack, add 52 μl of Ultrapure Tris Buffer and mix well by pipetting.
8. Perform the following program in the PCR instrument to elute mRNA:

Temperature	Time
80°C	2 min
25°C	Hold

9. Place the tube on the magnetic rack, then transfer 50 μl of supernatant to a new Nuclease-free tube when the solution is clear (about 5 min).
10. Add 50 μl of Ultrapure mRNA Capture Beads to the supernatant and mix well by pipetting.
11. Perform the following program in the PCR instrument, enabling mRNA to bind to magnetic beads:

Temperature	Time
65°C	5 min
25°C	5 min
4°C	Hold

12. Place the tube on the magnetic rack, and discard the supernatant when the solution is clear (about 5 min).

13. Remove the tube from the magnetic rack, add 150 - 200 µl of Ultrapure Beads Wash Buffer to resuspend the magnetic beads, and mix well by pipetting. Place the tube on the magnetic rack, and discard the supernatant when the solution is clear (about 5 min).

    ▲ Determine the final volume of Ultrapure Beads Wash Buffer used in the script based on the actual conditions of the automated workstation.

14. Remove the tube from the magnetic rack, add 18 µl of Frag/Prime Buffer 2 from VAHTS Universal V10 RNA-seq Library Prep Kit (Plate) (Vazyme #NRB616) to resuspend the magnetic beads, and mix well by pipetting. Place the tube in the PCR instrument, and perform the following program for fragmentation:

Temperature	Time
85°C	6 min
4°C	Hold

    ▲ Please refer to Appendix I/Table 1 for other fragmentation conditions.

    ▲ Do not pause during the process from fragmentation to first strand cDNA synthesis, as mRNA is prone to degradation under these conditions.

15. Place the tube on the magnetic rack, carefully transfer 16 µl of supernatant into a new Nuclease-free PCR tube when the solution is clear (about 5 min), and immediately proceed to the first strand cDNA synthesis reaction.

Option B: rRNA Depletion

Take Ribo-clean rRNA Depletion Kit Mega (Prevalent Species) (Vazyme #RN416) for rRNA depletion as an example. It is applicable to RNA library preparation with 10 ng - 1 µg total RNA from human, mouse, and rat. For other species, please refer to the corresponding rRNA depletion kit instruction.

1. Prepare RNA samples: Dilute 10 ng - 1 µg total RNA in Nuclease-free ddH_2O to a total volume of 9 µl, and place on ice for later use.

    ▲ Take out the required components from -30 ~ -15°C in advance for next step and place on ice for later use.

2. RNA hybridization with probe:

    a. Prepare the following reaction mix in a Nuclease-free PCR tube and mix well by pipetting:

Components	Volume
rRNA Probe Mega (Prevalent Species)	3 µl
Probe Buffer	3 µl
Total RNA	9 µl
Total	15 µl

    ▲ When multiple samples are processed simultaneously, the rRNA Probe Mega and Probe Buffer can be pre-mixed, and then aliquoted into each PCR tube. It is recommended to prepare 1.1 times the actual number of reactions to compensate for losses.

b. Briefly centrifuge to collect the reaction mix at the tube bottom. Place the tube in the PCR instrument, and perform the following program for probe hybridization:

Temperature	Time
95°C	2 min
95 ~ 37°C	0.1°C/sec
37°C	5 min
4°C	Hold

3. RNase H digestion:

a. Take out the tube immediately after the reaction, and briefly centrifuge to collect the reaction mix at the tube bottom. Prepare the following reaction mix on ice and mix well by pipetting:

Components	Volume
RNase H Buffer 2	3 μl
RNase H Mix	2 μl
Previous step product	15 μl
Total	20 μl

b. Place the tube in the PCR instrument, and perform the following program for RNase H digestion:

Temperature	Time
50°C	15 min
4°C	Hold

4. DNase I digestion:

a. Briefly centrifuge to collect the solution at the tube bottom. Prepare the following reaction mix on ice and mix well by pipetting:

Components	Volume
DNase I Buffer	29 μl
DNase I	1 μl
RNase H digestion product	20 μl
Total	50 μl

b. Place the tube in the PCR instrument, and perform the following program for DNase I digestion:

Temperature	Time
37°C	10 min
4°C	Hold

c. Briefly centrifuge to collect the reaction mix at the tube bottom. Place it on ice and proceed to the next step immediately.

5. Ribosomal-depleted RNA purification:

a. Mix VAHTS RNA Clean Beads (Vazyme #N412) well by vortexing. Add 110 μl (2.2 ×) to the RNA sample from the previous step, and mix well by pipetting.

b. Incubate on ice for 15 min, enabling RNA to bind to magnetic beads.

c. Place the tube on the magnetic rack, and discard the supernatant when the solution is clear (about 5 min).

d. Keep the tube on the magnetic rack, and add 200 µl of 80% ethanol (freshly prepared with Nuclease-free ddH_2O) to wash magnetic beads. Incubate at room temperature for 30 sec, and discard the supernatant.

e. Repeat step d once.

f. Keep the tube on the magnetic rack, and air-dry the beads for 5 - 10 min.

▲ Do not disturb the magnetic beads when adding 80% ethanol.

▲ Discard the supernatant as much as possible with a 10 µl pipette.

▲ Avoid beads over-drying as this may reduce recovery efficiency.

6. Remove the tube from the magnetic rack, add 18 µl of Frag/Prime Buffer 2 from VAHTS Universal V10 RNA-seq Library Prep Kit (Plate) (Vazyme #NRB616), and mix well by pipetting. Incubate at room temperature for 2 min. Place the tube on the magnetic rack, then transfer 16 µl of supernatant to a new Nuclease-free tube when the solution is clear (about 5 min).

7. Place the tube in the PCR instrument, and perform the following program for fragmentation:

Temperature	Time
85°C	6 min
4°C	Hold

▲ The fragmentation conditions in the table above are for reference only. Please choose the fragmentation conditions according to Appendix I/Table 1.

▲ mRNA is prone to degradation. Please proceed to first strand cDNA synthesis as soon as possible.

▲ Take out 1st Synthetic Master Mix 2 from -30 ~ -15°C in advance and place it on ice for later use.

Option C: Purified mRNA or Total RNA

VAHTS 2 × Frag/Prime Buffer V2 (Vazyme #N405) is required for Option C. It is applicable to library preparation with 0.5 - 100 ng purified mRNA, ribosomal-depleted RNA or total RNA.

1. Prepare the reaction mix and mix well by pipetting:

Components	Volume
2 × Frag/Prime Buffer V2	8 µl
RNA	8 µl
Total	16 µl

2. Place the tube in the PCR instrument, and perform the following program for fragmentation:

Temperature	Time
85°C	6 min
4°C	Hold

▲ The fragmentation conditions in the table above are for reference only. Please choose the fragmentation conditions according to Appendix I/Table 1.

▲ mRNA is prone to degradation. Please proceed to first strand cDNA synthesis as soon as possible.

▲ Take out 1st Synthetic Master Mix 2 from -30 ~ -15°C in advance and place it on ice for later use.

08-2/Double-Stranded cDNA Synthesis

1. Thaw the components for double-stranded cDNA synthesis on ice, mix by inversion and briefly centrifuge to collect the solution to the tube bottom. Prepare the following reaction mix for first strand cDNA synthesis:

Components	Volume
Fragmented mRNA	16 µl
1st Synthetic Master Mix 2	9 µl
Total	25 µl

2. Mix well by pipetting.

▲ Please protect 1st Synthetic Master Mix 2 from light before adding to the reaction mix.

3. Perform the following program in the PCR instrument for the first strand cDNA synthesis:

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

▲ Please proceed to the second strand cDNA synthesis immediately.

▲ Take out 2nd Synthetic Master Mix 2 from -30 ~ -15°C in advance, and place it on ice for later use.

4. Prepare the following reaction mix for second strand cDNA synthesis:

Components	Volume
1st Strand cDNA	25 µl
2nd Synthetic Master Mix 2 (with dNTP or dUTP) *	40 µl
Total	65 µl

* 2nd Synthetic Master Mix 2 (with dNTP) is for non-strand-specific transcriptome library, and 2nd Synthetic Master Mix 2 (with dUTP) is for strand-specific transcriptome library. Please choose the suitable component as needed.

5. Mix well by pipetting.

6. Perform the following program in the PCR instrument for second strand cDNA synthesis:

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

▲ Take out the components for 08-3/Adapter Ligation from -30 ~ -15°C in advance and place them on ice for later use.

▲ The double-stranded synthesis products can be stored at -30 ~ -15°C for 24 h.

08-3/Adapter Ligation

1. Prepare the following reaction mix, and mix well by pipetting:

Components	Volume
ds cDNA	65 μl
Adapter	5 μl*
Ligase Master Mix 2	30 μl
Total	To 100 μl

▲ It is recommended to add the Adapter to the ds cDNA and mix well. Then add Ligase Master Mix 2 to the mixture.

* The final volume of the adapter is 5 μl. Please dilute the adapter to the corresponding concentration based on the initial RNA input amount.

Total RNA Input		Adapter Concentration
Option A/B	Option C	
≥1 μg	100 ng	5 μM
500 ng	10 ng	3 μM
100 ng	1 ng	2 μM
10 ng	0.5 ng	1.5 μM

2. Perform the following program in the PCR instrument:

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

▲ Equilibrate VAHTS DNA Clean Beads to room temperature before use.

▲ Adapter Ligation products can be stored at 2 ~ 8°C for 1 h.

08-4/Purification and Size Selection

Two options are provided. Please choose the suitable one as needed.

Purification: A broad-range library size (>200 bp);

Size Selection: Both purification and size selection are needed. Library size is in a specific range. Please refer to Appendix I/Table 1 for the size selection conditions of different adapters.

Purification: A broad-range library size (>200 bp)

1. Equilibrate VAHTS DNA Clean Beads (Vazyme #N411) to room temperature.
2. Mix VAHTS DNA Clean Beads well by inversion or vortexing. Add 60 μl (0.6 ×) of beads to the ligation product and mix well by pipetting.
3. Incubate at room temperature for 10 min, enabling DNA to bind to magnetic beads.
4. Place the tube on the magnetic rack, and discard the supernatant when the solution is clear (about 5 min).
5. Keep the tube on the magnetic rack, and add 200 μl of 80% ethanol (freshly prepared with Nuclease-free ddH_2O) to wash magnetic beads. Incubate at room temperature for 30 sec, and discard the supernatant.

6. Repeat step 5 once.
7. Keep the tube on the magnetic rack, and air-dry the beads for 5 - 10 min.
   ▲ Do not disturb the beads when adding 80% ethanol.
   ▲ Avoid beads over-drying as this may reduce recovery efficiency.
8. Remove the tube from the magnetic rack, add 22 μl of Nuclease-free ddH_2O and mix well by pipetting. Incubate at room temperature for 2 min. Place the tube on the magnetic rack, and transfer 20 μl of the supernatant to a new Nuclease-free PCR tube when the solution is clear (about 5 min).

Size Selection: Both purification and size selection are needed. Please choose the suitable selection conditions for a specific library size.

Purification with 0.6 × VAHTS DNA Clean Beads

1. Equilibrate VAHTS DNA Clean Beads (Vazyme #N411) to room temperature.
2. Mix VAHTS DNA Clean Beads well by inversion or vortexing. Add 60 μl (0.6 ×) of beads to the ligation product and mix well by pipetting.
3. Incubate at room temperature for 10 min, enabling DNA to bind to magnetic beads.
4. Place the tube on the magnetic rack, and discard the supernatant when the solution is clear (about 5 min).
5. Keep the tube on the magnetic rack, and add 200 μl of 80% ethanol (freshly prepared with Nuclease-free ddH_2O) to wash magnetic beads. Incubate at room temperature for 30 sec, and discard the supernatant.
6. Repeat step 5 once.
7. Keep the tube on the magnetic rack, and air-dry the beads for 5 - 10 min.
   ▲ Do not disturb the beads when adding 80% ethanol.
   ▲ Avoid beads over-drying as this may reduce recovery efficiency.
8. Remove the tube from the magnetic rack, add 102 μl of Nuclease-free ddH_2O and mix well by pipetting. Incubate at room temperature for 2 min. Place the tube on the magnetic rack, and transfer 100 μl of the supernatant to a new Nuclease-free PCR tube when the solution is clear (about 5 min).

Size selection with two rounds of VAHTS DNA Clean Beads

(In the following procedure, the fragmentation is performed at 85°C for 6 min, with an Illumina library insert size of 280 - 380 bp. These conditions are for reference only. Please refer to Appendix I/Table 1 for other size selection conditions.)

9. Mix VAHTS DNA Clean Beads well by inversion or vortexing. Add 60 μl (0.6 ×) of beads to the purified products and mix well by pipetting.
10. Incubate at room temperature for 10 min, enabling DNA to bind to magnetic beads.

11. Place the tube on the magnetic rack, and transfer 150 µl of supernatant to a new Nuclease-free PCR tube when the solution is clear (about 5 min).
    ▲ Do not disturb the beads when transferring the supernatant! Bead residues may cause large fragments in the final library.
12. Add 10 µl (0.1 ×) of VAHTS DNA Clean Beads, and mix well by pipetting.
13. Incubate at room temperature for 10 min, enabling DNA to bind to magnetic beads.
14. Place the tube on the magnetic rack, and discard the supernatant when the solution is clear (about 5 min).
15. Keep the tube on the magnetic rack, and add 200 µl of 80% ethanol (freshly prepared with Nuclease-free ddH_2O) to wash magnetic beads. Incubate at room temperature for 30 sec, and discard the supernatant.
16. Repeat step 15 once.
17. Keep the tube on the magnetic rack, and air-dry the beads for 5 - 10 min.
    ▲ Do not disturb the beads when adding 80% ethanol.
    ▲ Avoid beads over-drying as this may reduce recovery efficiency.
18. Remove the tube from the magnetic rack, add 22 µl of Nuclease-free ddH_2O and mix well by pipetting. Incubate at room temperature for 2 min. Place the tube on the magnetic rack, and transfer 20 µl of the supernatant to a new Nuclease-free PCR tube when the solution is clear (about 5 min).
    ▲ Do not disturb the beads when transferring the supernatant! Bead residues may cause reduced library yield.
    ▲ Purified products can be stored at -30 ~ -15°C for 24 h.

08-5/Library Amplification

1. Prepare the PCR reaction mix for corresponding adapter:

Components	Volume
Purified Ligation Products	20 µl
PCR Primer Mix 6 for Illumina/MGI*	5 µl
VAHTS HiFi Amplification Mix 3	25 µl
Total	50 µl

* Please use the PCR primer provided in the adapter kit.

2. Mix well by pipetting.
3. Place the tube in the PCR instrument, and perform the program for library amplification:

Program	Temperature	Time	Cycles
Heated Lid	105°C	On	
Pre-denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	9 - 18
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	1 min	1
	4°C	Hold	

The mRNA amount varies significantly across different samples. Please adjust the number of PCR cycles accordingly. Refer to the table below for recommended PCR cycle numbers.

Input RNA		Cycles	
Option A/B	Option C	Non-strand-specific Transcriptome	Strand-specific Transcriptome
>1 μg		9	9
1 μg	100 ng	10	11
500 ng	10 ng	12	13
100 ng	1 ng	14	15
10 ng	0.1 ng	17	18

4. PCR product purification:

a. Equilibrate VAHTS DNA Clean Beads to room temperature.

b. Mix VAHTS DNA Clean Beads well by inversion or vortexing. Add 45 μl (0.9 ×) of beads to the PCR product and mix well by pipetting.

c. Incubate at room temperature for 10 min, enabling DNA to bind to magnetic beads.

d. Place the tube on the magnetic rack, and discard the supernatant when the solution is clear (about 5 min).

▲ DF Flowsizer Buffer (Vazyme #N001) is recommended for the removal of small fragments. The process is as follows:

(1) After finishing step d, remove the tube from the magnetic rack, add 50 μl of DF Flowsizer Buffer (Vazyme #N001), and mix well by pipetting. Incubate at room temperature for 5 min.

(2) Place the tube on the magnetic rack, and discard the supernatant when the solution is clear (about 5 min). Then proceed to step e.

e. Keep the tube on the magnetic rack, and add 200 μl of 80% ethanol (freshly prepared with Nuclease-free ddH_2O) to wash magnetic beads. Incubate at room temperature for 30 sec, and discard the supernatant.

f. Repeat step e once.

g. Keep the tube on the magnetic rack, and air-dry the beads for 5 - 10 min.

▲ Do not disturb the beads when adding 80% ethanol.

▲ Avoid beads over-drying as this may reduce recovery efficiency.

h. Remove the tube from the magnetic rack, add 25 μl of Nuclease-free ddH_2O and mix well by pipetting. Incubate at room temperature for 2 min. Place the tube on the magnetic rack, and transfer 23 μl of the supernatant to a new Nuclease-free PCR tube when the solution is clear (about 5 min).

▲ Do not disturb the beads when transferring the supernatant! Bead residues may affect sequencing results.

08-6/Library Quality Control

Use the Agilent DNA 1000 kit (Agilent, #5067 - 1504) to analyze the library size distribution. If a peak appears at 90 - 140 bp, it indicates adapter-dimer contamination in the library. Please dilute the library to 50 μl with Nuclease-free ddH_2O, and repeat step 4 of **08-5/Library Amplification** to re-purify the library.

09/FAQ & Troubleshooting

◇ If the library concentration is too low, how to improve it?

Library concentration is related to total RNA quality including the integrity and input amounts. If it is not possible to extract qualified RNA samples, the following compensatory methods may be taken:

① Increase total RNA inputs: up to 1 μg.

② Prepare several duplicate samples and merge them after purification.

◇ Is Vazyme #NRB616 suitable for Small RNA library preparation?

No. Small RNAs are about 22 nt and are too small to be captured by the beads used with Vazyme #NRB616.

◇ Is Vazyme #NRB616 compatible with FFPE samples?

Yes. However, mRNA in FFPE samples may be degraded; therefore, the Ribo-off rRNA depletion method is recommended for the mRNA enrichment module.

◇ How to choose PCR cycles at different RNA inputs?

Please refer to the recommendation shown in **08-5/library Amplification**. If uncertain, it is recommended to take 1 μl of amplification products (before purification) for Qubit detection, and then perform additional amplification cycles according to the actual yield.

◇ If there is a double peak in the library pattern, what could be the possible reasons?

① RNA degradation causes non-specific amplification.

② RNA fragments from special species may be not continuously and uniformly distributed after RNA fragmentation, resulting in two peaks in library size distribution.

③ Library is assessed using a high-sensitivity chip. It is recommended to use Agilent DNA 1000 Kit, or dilute the library to an appropriate concentration and use Agilent DNA HS Kit.

Appendix I: Size Selection Conditions for Full Length Adapter or Stubby Adapter on Illumina & MGI Platforms

Table 1. Size Selection Conditions for Ligation Products

Platform		Illumina			MGI	
Library Size (bp)		300 - 400	400 - 500	500 - 600	300 - 400	400 - 500
Fragmentation Condition		94°C 5 min	85°C 6 min	85°C 6 min	94°C 5 min	85°C 6 min
Insert Size (bp)		180 - 280	280 - 380	380 - 480	160 - 260	260 - 360
Stubby Adapter	1st Round Beads	0.65	0.6	0.5	0.5	0.5
	2nd Round Beads	0.1	0.1	0.1	0.2	0.15

▲ The final library length depends on the volume of magnetic beads added during size selection. The bead-to-DNA volume ratio used in the two rounds of size selection is relative to the initial DNA volume (100 μl).

▲ Stubby adapter: VAHTS Maxi Unique Dual Index Primers Set 1 - Set 4 for Illumina (Plate) (Vazyme #NB34401 - NB34404), VAHTS Maxi Unique Dual Barcode Primers Set 1 - Set 4 for MGI (Plate) (Vazyme #NMB34401 - NMB34404).

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