VAHTS Small RNA Library Prep Kit for Illumina®

Catalog # NR801

Version 7.1

Vazyme Biotech Co., Ltd.

1. Introduction

VAHTS Small RNA Library Prep Kit for Illumina® is specially designed for the construction of small RNA library for next generation sequencing (NGS) on Illumina platforms. The input template for this kit can be 0.1 μg-1 μg of animal or plant total RNA or purified small RNA. Small RNA libraries applicable for Illumina NGS platforms are prepared by the ligation of universal adapter on the 3'-end and 5'-end of the small RNA, respectively, followed by reverse transcription, PCR amplification, and purification by PAGE gel or magnetic beads. This kit contains all the necessary enzymes and buffers for library preparation and purification. All kit components are subjected to stringent functional quality control, ensuring the consistency and reproducibility of library preparation.

2. Contents of Kits

Components	NR801–01 (24 rxn)	NR801–02 (96 rxn)
RL3 Adaptor	24 μl	96 μl
RL3 Buffer	240 μl	960 μl
RL3 Enzyme mix	72 μl	288 μl
RT Primer	24 μl	96 μl
RL5 Adaptor	24 μl	96 μl
RL5 Buffer	24 μl	96 μl
RL5 Enzyme mix	60 μl	240 μl
RT Buffer	192 μl	768 μl
RT Enzyme mix	48 μl	192 μl
Amplification mix 3	1.2 ml	4 × 1.2 ml
pBR322/MspI digest DNA Marker	120 μl	480 μl
6 × Loading Buffer	500 μl	2 × 1 ml
Co-precipitator	120 μl	480 μl
RNase-free H_2O	500 μl	2 × 1 ml
GE Buffer	6 ml	24 ml
Filtration Column	24	96

▲ The color of each component in the table is the same as the cap color of that in the kit.

3. Storage

RL5 Adaptor: store at -70°C;
Filtration Column: store at room temperature;
Other components: store at -20°C.

4. Applications

VAHTS Small RNA Library Prep Kit for Illumina® is applicable to input template with 0.1-1 μg of animal or plant total RNA with good integrity (RIN ≥ 7) or purified small RNA. The content of small RNA in total RNA of different samples varied greatly. If the input of total RNA is too low, it may lead to the unsuccessful library construction. The proportion of rRNA or other RNA in the library will increase if incomplete or degraded (RIN < 7) total RNA are used. It is recommended to use Agilent RNA 6000 Pico Kit to control the quality of the input total RNA and to avoid using RNA samples with RIN < 7.

5. Additional Materials Required

Small RNA Index Primer: VAHTS Small RNA Index Primer Kit for Illumina® (Vazyme, #N813-816);

RNA quality control: Agilent RNA 6000 Pico Kit (Agilent, #5067-1513);

Library quality control: Agilent DNA 1000 kit (Agilent, #5067-1504), Agilent High Sensitivity Kit (Agilent, # 5067-4626);

Library purification: VAHTS DNA Clean Beads (Vazyme, #N411);

Library selection:

1. Selection with PAGE Gel: 6% Novex® TBE PAGE gel 1.0 mM 10-well (Life Technologies, #EC6265BOX), 5× TBE, Ultra GelRed Nucleic Acid Stain (Vazyme, #GR501) or SYBR® Gold Nucleic Acid Gel Stain (Life Technologies, #S11494), sodium acetate (3 M, pH 5.2), absolute ethanol, 80% freshly prepared ethanol;

2. Selection with magnetic beads: VAHTS DNA Clean Beads (Vazyme, #N411), 80% freshly prepared ethanol;

Other materials: Nuclease-free H_2O, RNase-free PCR tubes, low adhesive EP tube (Eppendorf, #022431021);

Instruments: Agilent 2100 Bioanalyzer or other equivalent product, PCR instrument, magnetic stand, -80℃ refrigerator, water bath, and centrifuge.

6. Application Notes

6-1. Store all the components properly according to the indicated conditions

1. RL5 Adaptor is a RNA adapter and should be stored at -70°C.

2. All the enzymes in the kit should be stored at -20°C. Mix thoroughly and spin briefly to avoid the adhesion to the wall and cap of the tube, which may cause a loss of the reagent. Put the enzyme on ice and store it at indicated conditions after use to avoid possible reduce in enzyme activity.

3. RL3 Buffer is sticky, please spin it briefly to bring the sample to the bottom of the tube, avoid the adhesion to the wall and cap of the tube, which may cause a loss of the reagent.

6-2. Quality control of input RNA samples

To ensure high-quality library construction, quality control of input RNA samples is highly recommended. The quality of input RNA (i.e. total amount, purity, and integrity) should meet the following requirements:

1. Please use methods based on precipitation (i.e. Trizol) to get the total RNA, or use extraction kits specially designed for small RNA to get small RNA, in order to make sure that no loss of small RNA during RNA isolation.

2. The amount of input total RNA should be ≥ 100 ng. Less input of total RNA may lead to the unsuccessful library construction.

3. The ratio of OD260/OD280 should between 1.8 and 2.0. Please use a Bioanalyzer to analyze the integrity of total RNA. The RIN (RNA integrity number) value should be ≥ 7.0. Please use agarose gel electrophoresis to example 28S and 18S RNA. The ratio of 28S : 18S should be ≥ 1.5; and no protein or genome DNA contamination should be detected.

6-3. Attention

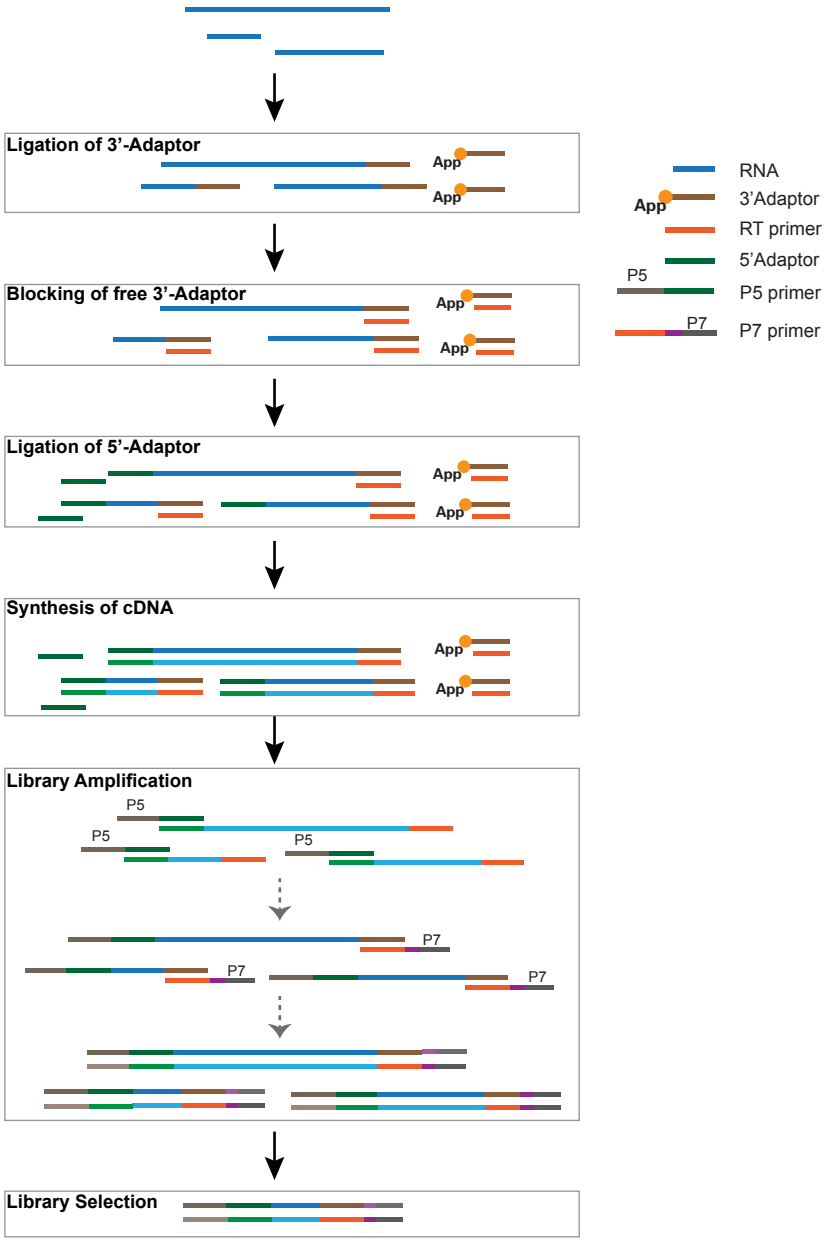
1. Please use RNase-free tips, EP tubes, and PCR tubes. Don't forget to change tips between samples.

2. Please wear gloves and masks during operation. When you contact equipment or working place which is not RNase-free, please change your gloves.

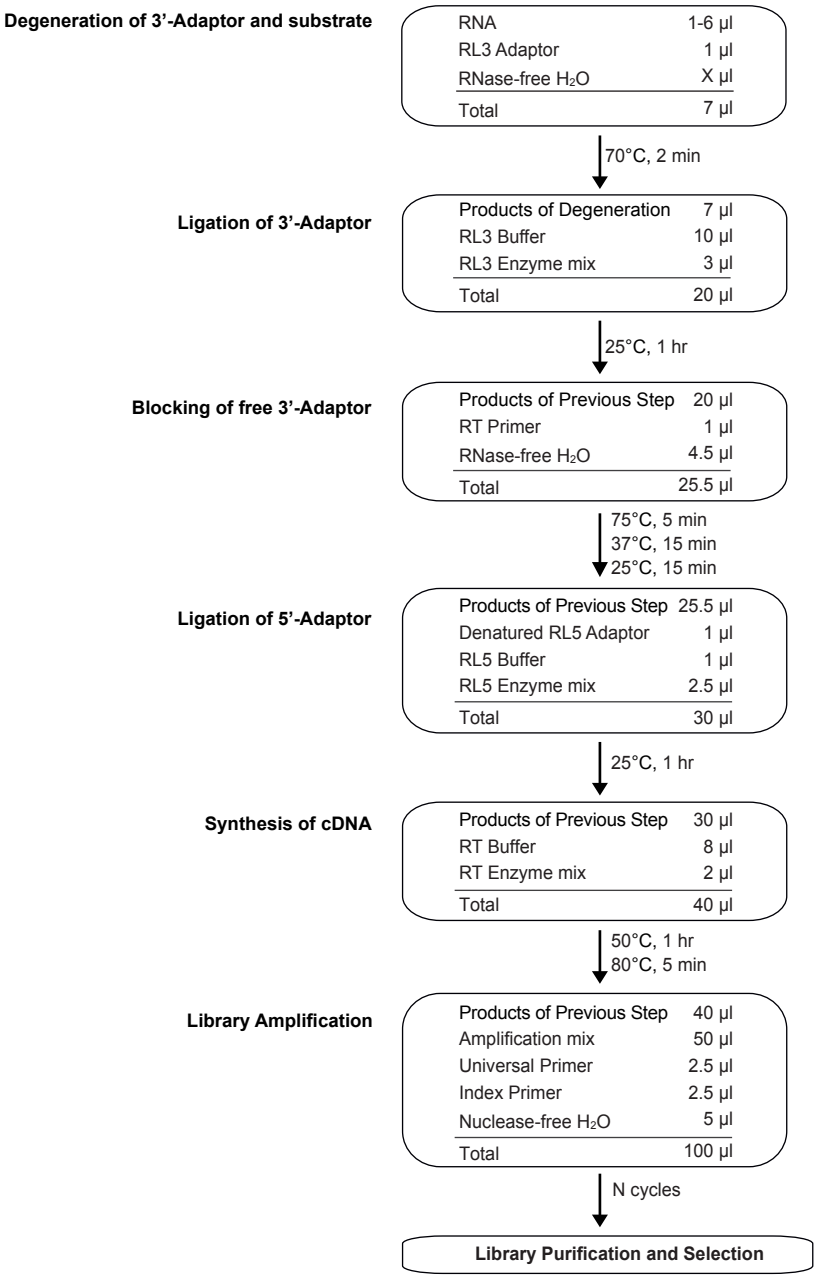
3. Close the cap and seal the tube once finishing using the reagent, in order to avoid possible contamination.

4. To pause during library preparation, please put the samples at proper temperature according to the stop point indicated in this manual. Inappropriate stop point may reduce the efficacy of library construction.

7. Mechanism and Workflow Overview



Workflow of library construction with VAHTS Small RNA Library Prep Kit for Illumina®.



Workflow overview of VAHTS Small RNA Library Prep Kit for Illumina®.

8. Protocol

8-1. Ligation of 3'-Adaptor

Take the RL3 Adaptor, RL3 Buffer, and RL3 Enzyme mix out of the kit, thaw and mix thoroughly, spin briefly to bring the sample to the bottom of the tube and put them on ice. All steps below should be on ice.

1. Degeneration of 3'-Adaptor and template:

Prepare the reaction solution in a RNase-free PCR tube. If the amount of input RNA is > 100 ng, prepare the reaction solution as follows, while if 100 ng of input RNA is used, please dilute the RL3 Adaptor with RNase-free H_2O at a ratio of 1 : 1 first, and then prepare the reaction solution as follows.

RNA	1-6 μl	
*RL3 Adaptor	1 μl	■
RNase-free H_2O	X μl	□
Total	7 μl	

▲*Please dilute adaptor refer to the amount of input RNA. Excess adaptors may lead to the increase of adaptor dimers in the library.

2. Incubate in a PCR instrument (pre-heated) at 70°C for 2 min, then immediately chill on ice for 2 min.

3. Add the following reagents to the product of previous step:

Products of 8-1 Step 2	7 µl
RL3 Buffer	10 µl
RL3 Enzyme mix	3 µl
Total	20 µl

4. Mix thoroughly by pipetting up and down for 10-15 times, spin briefly to bring the sample to the bottom of the tube. Put the sample in a PCR instrument and run the following program:

Temperature	Time
25°C	1 hr
4°C	hold

▲ RL3 buffer and RL3 Enzyme mix are sticky, please pipet as slowly as possible to ensure the accuracy of pipetting.

▲ The reaction solution is sticky, please use a pipette to mix thoroughly.

8-2. Blocking of free 3'-Adaptor

Take the RT Primer out of the kit, thaw and mix thoroughly, spin briefly to bring the sample to the bottom of the tube and put them on ice. All steps below should be on ice.

1. If the amount of input RNA is > 100 ng, prepare the reaction solution as follows, while if 100 ng of input RNA is used, please dilute the RT Primer with RNase-free H_2O at a ratio of 1 : 1 first, and then prepare the reaction solution as follows.

Products of 8-1 Step 4	20 µl
RT Primer	1 µl
RNase-free H_2O	4.5 µl
Total	25.5 µl

2. Mix thoroughly by pipetting up and down for 10-15 times, spin briefly to bring the sample to the bottom of the tube. Put the sample in a PCR instrument and run the following program:

Temperature	Time
75°C	5 min
37°C	15 min
25°C	15 min
4°C	hold

8-3. Ligation of 5'-Adaptor

Take the RL5 Adaptor, RL5 Buffer, and RL5 Enzyme mix out of the kit, thaw and mix thoroughly, spin briefly to bring the sample to the bottom of the tube and put them on ice. All steps below should be on ice.

1. Degeneration of 5'-Adaptor:

The RL5 Adaptor should be denatured before use to avoid possible secondary structure formed by itself. To compensate possible loss during pipetting, if the sample number is N, add N × 1.1 µl of RL5 Adaptor into a RNase-free PCR tube.

If 100 ng of input RNA is used, please dilute the RL5 Adaptor with RNase-free H_2O at a ratio of 1:1.

Incubate in a PCR instrument (pre-heated) at 70°C for 2 min, then immediately chill on ice for 2 min.

2. Prepare the following reaction solution for the ligation of 5'-Adaptor:

Products of 8-2 Step 2	25.5 µl
Denatured RL5 Adaptor	1 µl
RL5 Buffer	1 µl
RL5 Enzyme mix	2.5 µl
Total	30 µl

3. Mix thoroughly by pipetting up and down for 10-15 times, spin briefly to bring the sample to the bottom of the tube. Put the sample in a PCR instrument and run the following program:

Temperature	Time
25°C	1 hr
4°C	hold

8-4. Synthesis of cDNA

Take the RT Buffer and RT Enzyme mix out of the kit, thaw and mix thoroughly, spin briefly to bring the sample to the bottom of the tube and put them on ice.

1. Prepare the following reaction solution for reverse transcription:

Products of 8-3 Step 3	30 µl
RT Buffer	8 µl
RT Enzyme mix	2 µl
Total	40 µl

Mix thoroughly by pipetting up and down for 10-15 times, spin briefly to bring the sample to the bottom of the tube. Put the sample in a PCR instrument and run the following program:

Temperature	Time
50°C	1 hr
80°C	5 min
4°C	hold

Stop point: the cDNA can be stored at-20°C for 24 hours.

8-5. Library Amplification

Take the Universal Primer, Index Primer, and Amplification mix out of the kit, thaw and mix thoroughly and put them on ice.

▲ VAHTS Small RNA Index Primer Kit for Illumina® (Vazyme, #N813-816) includes one kind of Universal Primer and 48 different kinds of Index Primer.

1. Prepare the reaction solution as follows:

Products of 8-4 Step 2	40 μl	
Amplification mix 3	50 μl	
Universal Primer	2.5 μl	
Index Primer	2.5 μl	
Nuclease-free H_2O	5 μl	
Total	100 μl	

2. Mix thoroughly and spin briefly to bring the sample to the bottom of the tube. Put the sample in a PCR instrument and run the following program:

Temperature	Time	Cycles
94°C	3 min	
94°C 65°C 72°C	15 sec 15 sec 15 sec	N (Refer to the following table)
72°C	1 min	
4°C	Hold	

Cycle numbers for different starting templates for reference:

Input Template	Cycles
1 μg	12-13
500 ng	13-14
200 ng	14-15
100 ng	15-16

▲ The content of small RNA in total RNA of different samples varied greatly. The cycles numbers in this table is according to data obtained with total RNA of 293T cells and mouse liver tissues.

Stop point: the library can be stored at -20°C for 24 hr.

8-6. Purification of PCR Products

1. Take the VAHTS DNA Clean Beads out from 2-8°C 30 min before use, and equilibrate it to room temperature.

2. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Add 180 μl (1.8 ×) of beads into the above sample. Mix thoroughly by pipetting up and down for 10 times.

3. Incubate at room temperature for 10 min.

4. Place the sample on a magnetic stand. Wait until the solution clarifies (about 10 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. Incubate at room temperature for 30 sec and carefully discard the supernatant without disturbing the beads.

6. Repeat the previous step (8-6 Step 5).

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

▲ DO NOT re-suspend the beads when adding 80% ethanol.

▲ To completely discard the supernatant, it is recommended to use a 10 μl pipette to pipet residual liquid. Avoid excessive drying of magnetic beads (cracks) which may lead to low recovery efficiency.

8. Take the sample out of the magnetic stand. Add 30 μl of Nuclease-free H_2O to elute the cDNA. Mix thoroughly by vortexing or pipetting, and incubate for 2 min at room temperature. Put the tube on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 27.5 μl of supernatant to a new Nuclease-free PCR tube without disturbing the beads.

Stop Point: the dilution can be stored at -20°C for one week. Except for purification with magnetic beads, PCR products can also be purified using a PCR Purification Kit with high recovery efficacy of DNA fragments less than 200 bp.

9. Library Quality Determination Using an Agilent Technologies 2100 Bioanalyzer: Analyze 1 μl of purified PCR product using an Agilent DNA 1000 chip. Due to the influence of 2100 mobility, the peak of the library may have a deviation with 6 - 8 bp. As shown in **Fig. 1**, the peak of miRNA library is at 143-147 bp and the peak of piRNA library is at 153 - 156 bp.

▲ The content of small RNA in total RNA of different samples varied greatly, therefore, the proportion of miRNA, piRNA, and other small RNA with different length is different and the peak of their library may have a deviation with several bps.

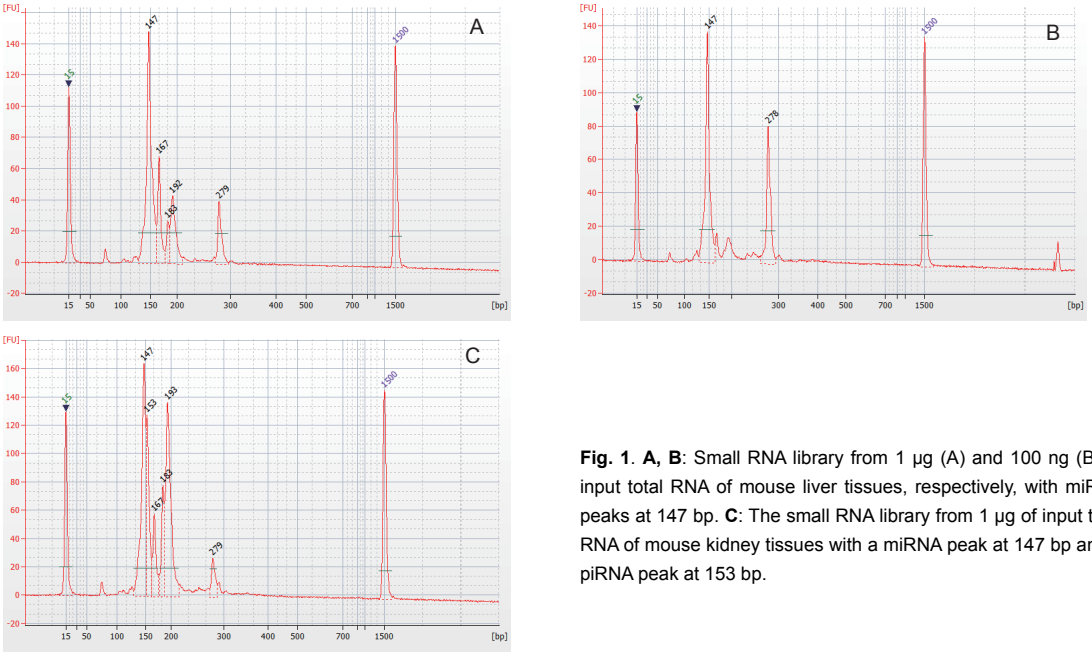


Fig. 1. **A, B**: Small RNA library from 1 μg (A) and 100 ng (B) of input total RNA of mouse liver tissues, respectively, with miRNA peaks at 147 bp. **C**: The small RNA library from 1 μg of input total RNA of mouse kidney tissues with a miRNA peak at 147 bp and a piRNA peak at 153 bp.

8-7. Library Selection

Select appropriate methods for library selection and purification according to the result of 08-6 Step 9 (Library Quality Control). As shown in **Fig. 2**, it is recommended to use PAGE Gel for library selection under the following circumstances: too much adaptor dimers at 120 bp, residual primers at 70 - 80 bp, or obvious rRNA at 160 bp and 190 bp. If there is less adaptor primer, primer and rRNA, library selection with magnetic bead is recommended. However, to ensure the ratio of the effective library, library selection with PAGE Gel is also recommended.

Option A: Library selection with a PAGE Gel (6%, non-denatured)

1. Put a PAGE gel (6%, non-denatured) with 10 sampling holes in an electrophoretic tank, and add 1 × TBE electrophoretic buffer.

2. Add 5 μl of 6 × Loading buffer into purified PCR products, mix them thoroughly and spin briefly to bring the sample to the bottom of the tube.

3. Gently load 5 μl of pBR322/MspI digest DNA Marker into sampling holes, then load 10 μl of 1 × Loading buffer for equilibration.

4. For each sample, gently load 15 μl of PCR products mixed with Loading Buffer into two separated sampling holes, respectively. If you need to select multiple libraries in one PAGE gel, a blank sampling hole (with 15 μl of 1 × Loading buffer for equilibration) should be set between different libraries.

5. After loading, electrophoresis at 120 - 150 V for 1 hr. Different electrophoresis apparatus may have different migration rate and electrophoresis time. You can stop electrophoresis until the blue indicators in the sample hole run out of the plastic board after 3 - 5 min.

6. Take the PAGE gel out and stain with Gel-Red for 10 min, observe it with a transilluminator. As shown in **Fig. 2**, the miRNA library and piRNA library is at 140 bp and 150 bp site, respectively.

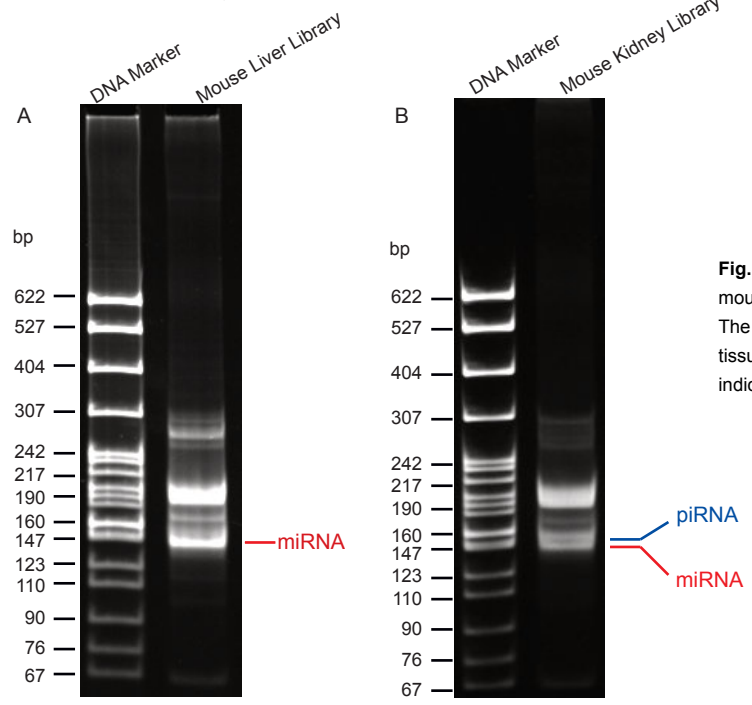


Fig. 2: (A) The small RNA library of 1 μg of total RNA of mouse liver tissues, the red line indicates miRNA library; (B) The small RNA library of 1 μg of total RNA of mouse kidney tissues, the red line indicates miRNA library and the blue line indicates piRNA library.

7. Cut the target band with a knife and add it to a 500 µl EP tube with low adhesion. The bottom of the EP tube has been pricked several holes with a 1 ml syringe needle that has been burned with an alcohol lamp). Put the 500 µl EP tube into a 1.5 ml EP tube with low adhesion, centrifuge at 12000 rpm for 2 min to crush the gel.

▲ The crushed extent of the gel depends on the diameter of poles, so the syringe needle can be used to prick the bottom of EP tube.

8. Discard the 500 µl EP tube after centrifugation, add 250 µl of GE Buffer into the 1.5 ml EP tube with crushed gel and put it into water bath at 50°C for 1-2 hr.

9. Centrifuge the above tube at 12000 rpm for 2 min to bring the evaporative liquid to the bottom of the tube.

10. Transfer the above supernatant to Filtration Column and centrifuge at 12000 rpm for 2 min. Discard the column and transfer the liquid to a novel 1.5 ml EP tube with low adhesion.

11. Add 5 µl of Co-precipitator, 30 µl of 3 M Sodium Acetate (pH 5.2) and 1 ml of ethanol into the above supernatant, mix thoroughly by vortexing and precipitate it at -80°C for 1 hr.

12. Take the precipitates out from -80°C, centrifuge at 4°C, 12000 rpm for 30 min.

13. Carefully discard the supernatant, DO NOT pipette white precipitate. Add 1 ml of freshly prepared 80% ethanol, centrifuge at 4°C, 12000 rpm for 10 min.

14. Carefully discard the supernatant, DO NOT pipette white precipitate. Spin briefly to bring the sample to the bottom of the tube, carefully discard the supernatant and open the tube and air-dry for 10 min.

15. Add 15 µl of Nuclease-free H_2O to elute the precipitate until the residual ethanol evaporate completely.

16. Library Quality Determination Using an Agilent Technologies 2100 Bioanalyzer: Analyze 1 µl of purified PCR product using an Agilent DNA 1000 chip or Agilent High Sensitivity DNA Chip. As shown in **Fig. 3**, the peak of miRNA library is at 143 - 147 bp.

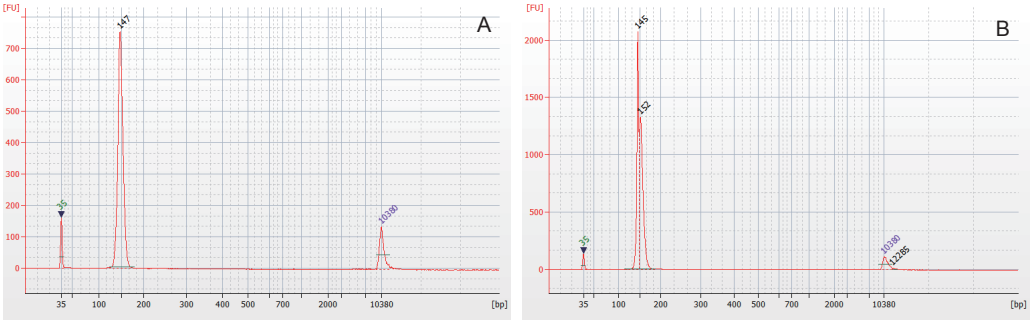


Fig. 3. (A) The small RNA library of 1 µg of total RNA of mouse liver tissues purified by PAGE Gel; (B) The small RNA library of 1 µg of total RNA of mouse kidney tissues purified by PAGE Gel.

Option B: Library selection with a PAGE Gel (6%, non-denatured)

1. Take VAHTS DNA Clean Beads out from 2 - 8°C 30 min before using and equilibrate it to room temperature.

2. Suspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Pipet 32.5 µl (1.3 ×) of beads into the above sample. Mix thoroughly by pipetting up and down for 10 times.

3. Incubate at room temperature for 10 min.

4. Place the sample 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. Add 22.5 µl (0.9 ×) of beads into the above sample, mix thoroughly by pipetting up and down for 10 times.

6. Incubate at room temperature for 10 min.

7. Place the sample 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.

8. Keep the sample on a magnetic stand, add 200 µl of freshly prepared 80% ethanol to rinse the beads. Incubate at room temperature for 30 sec and carefully discard the supernatant without disturbing the beads.

9. Repeat the Step 8.

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

▲ DO NOT re-suspend the beads when add 200 µl of freshly prepared 80% ethanol.

▲ When discard the supernatant in last step, use 10 µl pipette to pipet residual liquid.

▲ Avoid excessive drying of magnetic beads (cracks), which may lead to low recycle efficiency.

11. Take the sample out of the magnetic stand. Add 17.5 µl of Nuclease-free H_2O to elute the cDNA. Mix thoroughly by vortexing or pipetting, and incubate for 2 min at room temperature. Put the tube on the magnetic stand and wait until the solution clarifies (about 5 min). Carefully transfer 15 µl of supernatant to a new Nuclease-free PCR tube without disturbing the beads.

12. Library Quality Determination Using an Agilent Technologies 2100 Bioanalyzer: Analyze 1 µl of purified PCR product using an Agilent DNA 1000 chip or Agilent High Sensitivity DNA Chip. As shown in **Fig. 4**, the peak of miRNA library is at 143 bp - 147 bp.

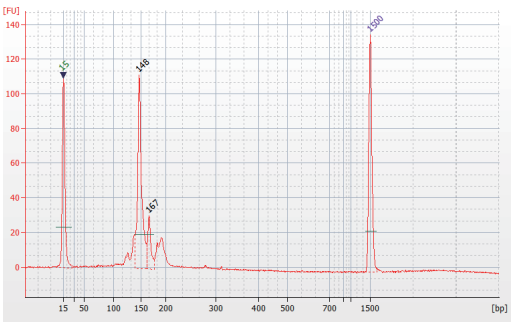


Fig. 4. The small RNA library of 1 µg of total RNA of mouse liver tissues by magnetic beads.

9. FAQs

1. The structure of small RNA library constructed with VAHTS Small RNA Library Prep Kit for Illumina®?

AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGACGATC——
Insert——AGATCGGAAGAGCACACGTCTGAACTCCAGTCACIIIIIIATCTCGTATGCCGTC
TTCTGCTTG Index

2. Can centrifugal column be used to extract RNA except TRIZOL?

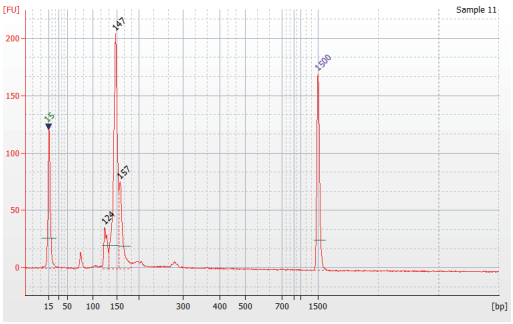
This Kit has no special requirements for RNA extraction. If use centrifugal column to extract RNA, please ensure that the kit used will not cause the loss of small RNA.

3. Can RT primer be added at the cDNA synthesis Step?

No. RT primers added after ligation of 3'-Adaptor can be complementary reversely with extra 3'-Adaptor to form a double chain structure, which effectively prevents the ligation between 5'-Adaptor and 3'-Adaptor, and reduces the adaptor dimers; Besides, RT primer complements reversely with RNA substrate with 3'-Adaptor can be the primer of reverse transcription, so RT primer must be added after 3'-Adaptor ligation instead of cDNA synthesis Step.

4. Can other total RNA be as initial template for library construction except total RNA of plants and animals?

Except total RNA of plants and animals and purified small RNA, the Kit is suitable for other total RNA, such as total RNA of exosomes. The following figure shows the miRNA library constructed by total RNA of exosomes in the supernatant of Hela cell (The initial template is 80 ng).



5. Formulas of additional Materials Required?

5 × TBE electrophoresis buffer (1L)

Components	Mass / Volume
Tris	54 g
Boric acid	27.5 g
EDTA-2Na	4.65 g
ddH2O	Up to 1 L

The buffer is 5 × TBE after completely dissolved, while 1 × TBE can be acquired by 5 times dilution.

3 M Sodium Acetate (pH 5.2, 10 ml)

Components	Mass / Volume
CH3COONa	2.46 g
ddH2O	8 ml

After completely dissolved, use Acetic acid to adjust pH to 5.2 and add ddH_2O to make 10 ml.