VAHTS Small RNA Library Prep Kit for Illumina V2

NB-54-0253-01

NB-54-0253-02

Contents

VAHTS Small RNA Library Prep Kit for Illumina V2

#Cat: NB-54-0253-01 Size: 24rxns
#Cat: NB-54-0253-02 Size: 96rxns

1/Product Description

VAHTS Small RNA Library Prep Kit for Illumina V2 is a small RNA-seq library preparation kit specifically designed for Illumina high-throughput sequencing platforms. This kit improves the stability and library yield at a lower-input amount through system optimisation. It is intended for animal and plant total RNA with a starting template amount of 10 ng - 1 μg, and ≥1 ng of RNA extracted and purified from serum, plasma, and exosomes. The universal adaptors are ligated to 3' and 5' ends of small RNAs, respectively. After steps such as reverse transcription, PCR amplification, and PAGE gel or magnetic bead purification, the sequencing library suitable for the Illumina platform can be obtained. This kit contains all the enzymes and buffers required for library preparation. All the reagents provided in the kit have undergone rigorous quality control and functional testing to ensure the optimal stability and repeatability of library preparation.

2/Components

	Components	NB-54-0253-01 (24rxns)	NB-54-0253-02 (96rxns)
■	RL3 Adaptor	24 μl	96 μl
■	RL3 Buffer V2	240μl	960μl
■	RL3 Enzyme mix V2	72 μl	288μl
■	RT Primer	24 μl	96 μl
■	RL5 Adaptor	24 μl	96 μl
■	RL5 Buffer	24 μl	96 μl
■	RL5 Enzyme mix	60 μl	240μl
■	RT Buffer	192 μl	768μl
■	RT Enzyme mix V2	48 μl	192μl
■	Amplification mix 3	1.2ml	4 × 1.2 ml
■	pBR322/MspI digest DNA Marker	60 μl	240μl
■	6 × Loading Buffer	500μl	2 × 1 ml
■	Co-precipitator	120μl	480μl
□	RNase-free dd_2OH	500μl	2 × 1 ml
	GE Buffer	6 ml	24 ml
	Filtration Column	24	2 × 48

▲Colored bullets represent the color of the cap of the tube containing the respective kit component.

3/Storage

RL5 Adaptor: Store at -85 ~ -65°C and ship on dry ice.

Filtration Column: Store at 15 ~ 25°C and transport at room temperature.

Other components: Store at -30 ~ -15°C and transport at ≤0°C.

4/Applications

1. 10 ng - 1 μg of total RNA from animal or plant samples with good integrity (RIN≥7). As the amount of small RNA in total RNA varies widely among different sample types, sufficient total RNA input is required to ensure successful library preparation. Libraries prepared from non-intact or degraded total RNA samples (RIN<7) may contain higher proportions of other RNA types, such as rRNA. Therefore, it is recommended touse Agilent RNA 6000 Pico Kit for quality control of total RNA sample to avoid using RNA samples with RIN<7.
2. ≥1 ng of RNA extracted and purified from serum, plasma, or exosomes.

5/Self-prepared Materials

Small RNA Index Primer: VAHTS Small RNA Index Primer Kit for Illumina (Neo Biotech # NB-54-0078/ NB-54-0079/ NB-54-0080).

RNA quality control: Agilent RNA 6000 Pico Kit

Library quality control: Agilent DNA 1000 Kit, High Sensitive Kit

Library purification: VAHTS DNA Clean Beads (Neo Biotech# NB-54-0060)

Size selection:

1. Size selection of PAGE gels:
   6% Novex TBE PAGE gel 1.0 mM 10-well, 5 × TBE, Ultra GelRed Nucleic Acid Stain (or SYBR Gold Nucleic Acid Gel Stain, 3 M sodium acetate (pH 5.2), ethanol absolute, freshly prepared 80% ethanol;

2. Size selection of beads: VAHTS DNA Clean Beads(Neo Biotech#NB-54-0060), freshly prepared 80% ethanol;

Other necessary materials:

Nuclease-free ddH_2O, RNase-free PCR tubes, low-binding EP tubes, Agilent 2100 Bioanalyzer or equivalent product, PCR instrument, magnetic rack, -80°C freezer, water bath, centrifuge.

6/Notes

For research use only. Not for use in diagnostic procedures.

6-1/Storage and Usage

1. RL5 Adaptor is a 5' RNA adaptor and should be stored at -85 ~ -65°C.
2. All the enzymes included in the kit should be stored at -30 ~ -15°C. Mix well and centrifuge briefly before use to avoid loss of reagents from adhesion to the tube wall and cap. Place on ice during use and store promptly at -30 ~ -15°C after use to avoid decreasing the enzymatic activity.

6-2/Quality Control of RNA Samples

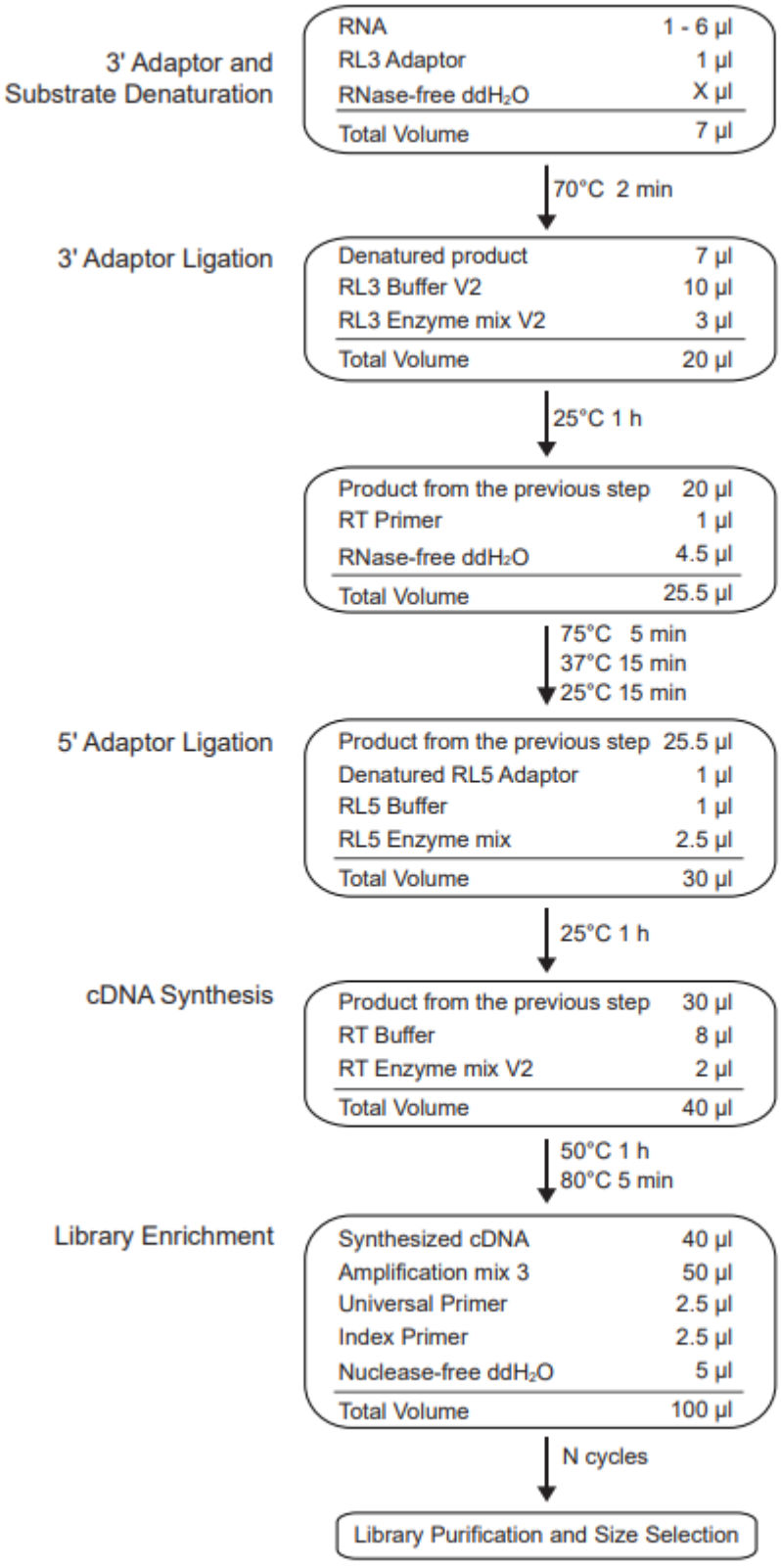
To ensure the quality of library preparation, please perform quality control on RNA samples before the experiment. The total amount, purity and integrity of RNA samples should meet the following requirements:

1. Using total RNA as the initial template, please use the precipitation method (e.g., the trizol method) or the small RNA extraction kit to extract RNA samples. Please ensure that the extraction method used does not lose small RNA.
2. The initial input amount of total RNA should be ≥10 ng. If the initial input amount is too low, it may lead to failure of library preparation.
3. The A260/A280 ratio should be within the range of 1.8 - 2.2. Use Bioanalyzer to detect RNA integrity, the RIN value should be ≥7; if the quality assessment is performed using agarose gel electrophoresis, 28S:18S should be ≥1.5 with no protein or genomic DNA contamination.
4. For 200 µl serum, plasma samples, use Qiagen extraction kit to extract RNA and 20 µl eluent for elution; for 1 ml plasma-extracted exosome samples, use Qiagen extraction kit to extract RNA and 14 µl eluent for elution; if the extracted product is lower than the detection limit of Qubit, it is recommended to use the maximum input volume of 6 µl.

6-3/Precautions for Operation

1. Use RNase-free pipette tips, EP tubes and PCR tubes during the experiment. Please change the pipette tip when pipetting different samples.
2. Please wear gloves and a mask for operation. After touching equipment outside the RNase-free space or other working areas, please change gloves.
3. Please cap the tubes of all reagents immediately after use to avoid contamination.
4. If you need to pause the experiment, please strictly follow the safe stopping points indicated in the manual and store the sample at a suitable temperature. Improper stopping may lower the success rate of library preparation.

7/Workflow



Library Preparation Workflow of VAHTS Small RNA Library Prep Kit for Illumina V2

8/Experiment Process

The amount of adaptors required varies with the initial input amount of RNA. Insufficient adaptors will result in poor yield, whereas excessive adaptors will lead to high levels of residual adaptor dimers. It is recommended to dilute the adaptors according to the table below. If the concentration of RNA-extracted products from serum, plasma or exosomes is lower than the detection limit of Qubit, use the maximum input volume of 6 µl. It is recommended to dilute the adaptors by the adaptor dilution factor of 10 ng of total RNA.

Table 1. Adaptor Dilution Factors for Different Initial Input Amounts of Total RNA

Input RNA	Adaptor:ddH_2O (Volume:Volume)
1 µg - 200 ng	NA
200 ng - 100 ng	1:1
10 ng	1:19 - 1:29

▲ The above table is the adaptor dilution factor tested using high-quality mouse liver RNA as a template,and it is for reference only. The proportion of small RNA in different samples varies greatly, and the actualadaptor dilution factor can be adjusted according to the amount of residual adaptor dimers in the experiment.

▲ Dilute the RL3 Adaptor, RL5 Adaptor, and RT Primer according to the table above.

8-1/3' Adaptor Ligation

Take out the RL3 Adaptor, RL3 Buffer V2 and RL3 Enzyme mix V2, then thaw and mix well. Briefly centrifuge to the bottom of the tube and place it on ice for later use. Perform all the following steps on ice.

1. Template and adaptor denaturation: Unwind the secondary structure that may exist between the RNA substrate itself and the RNA substrate.
2. Please refer to Table 1, dilute the RL3 Adaptor based on the initial input amount of template RNA. Prepare the reaction system in an RNase-free PCR tube according to the following table.

Components	Volume	
RNA	1 - 6 µl	
RL3 Adaptor*	1 µl	■
RNase-free ddH_2O	X µl	□
Total	7 µl	

* Please dilute the adaptor based on the input amount of initial templates. Excessive adaptors mayresult in increased amounts of adaptor dimers in the final library.

3. Incubate the PCR tube in a preheated PCR instrument at 70°C for 2 min, and then immediately place the tube on ice for 2 min.

4. Add the following components to the reaction tube from Step 3 in the order listed:

Components	Volume
Product from Step 2	7 µl
RL3 Buffer V2	10 µl ■
RL3 Enzyme mix V2	3 µl ■
Totale	20 µl

5. Mix well by pipetting up and down 10 - 15 times, then centrifuge briefly to the bottom of the tube. Place the reaction tube into the PCR instrument with a heated lid and run the following program:

Temperature	Time
25°C	1 h
4°C	hold

▲ The RL3 Buffer V2 and RL3 Enzyme mix V2 are both viscous. Pipette slowly to ensure that accuratevolumes are dispensed.

▲ The reaction mix in this step is viscous. Please mix well by pipetting up and down.

Take out the RT Primer, then thaw and mix well. Briefly centrifuge to the bottom of the tube and place it onice for later use. Perform all the following steps on ice

6. Please refer to Table 1, dilute the RT Primer based on the initial input amount of template RNA. Prepare the reaction system according to the following table.

Components	Volume
Product from Step 5 in 08-1	20 µl
RT Primer	1 µl ■
RNase-free ddH_2O	4.5 µl □
Totale	25.5 µl

7. Mix well by pipetting up and down 10 - 15 times, then centrifuge briefly to the bottom of the tube. Place the tube into the PCR instrument with a heated lid and run the following program:

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

8-2/5' Adaptor Ligation

Take out the RL5 Adaptor, RL5 Buffer and RL5 Enzyme mix, then thaw and mix well. Briefly centrifuge to thebottom of the tube and place it on ice for later use. Perform all the following steps on ice.

1. 5' adaptor denaturation: The RL5 Adaptor itself may form the secondary structure, which needs to be unwound by denaturation before use.
2. Please refer to Table 1, dilute the RL5 Adaptor based on the input amount of template RNA. Incubate the diluted RL5 Adaptor in the PCR instrument at 70°C for 2 min and then immediately place the tube on ice.
3. Prepare the reaction system of 5' adaptor ligation according to the following table:

Components	Volume
Product from Step 7 in 08-1	25.5 µl
Denatured RL5 Adaptor	1 µl
RL5 Buffer	1 µl
RL5 Enzyme mix	2.5 µl
Total	30 µl

4. Mix well by pipetting up and down 10 - 15 times, then centrifuge briefly to the bottom of the tube. Place the reaction tube into the PCR instrument with a heated lid and run the following program:

Temperature	Time
25°C	1h
4°C	hold

8-3/cDNA Synthesis

Take out the RT Buffer and RT Enzyme mix V2, then thaw and mix well. Briefly centrifuge to the bottom ofthe tube and place it on ice for later use. Perform all the following steps on ice.

1. Prepare the reaction system of reverse transcription according to the following table:

Components	Volume
Product from Step 4 in 08-2	30 µl
RT Buffer	8 µl
RT Enzyme mix V2	2 µl
Total	40 µl

2. Mix well by pipetting up and down 10 - 15 times, then centrifuge briefly to the bottom of the tube. Place the reaction tube into the PCR instrument with a heated lid and run the following program:

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

▲ The products of this step can be stored at -30 ~ -15°C for 24 h.

8-4/Library Enrichment

Take out the Universal Primer, Index Primer and Amplification mix 3, then thaw and mix well. Place the tube on ice for later use.

▲ VAHTS Small RNA Index Primer Kit for Illumina contains one unique UniversalPrimer and 48 different Index Primers.

1. Prepare the reaction system according to the following table.

Components	Volume
Product from Step 2 in 08-3	40µl
Amplification mix 3	50 µl
Universal Primer	2.5 µl
Index Primer	2.5 µl
Nuclease-free ddH_2O	5 µl
Total	100µl

2. Mix the mixture well, then centrifuge briefly to the bottom of the tube. Place the reaction tube into the PCR instrument with a heated lid and run the following program:

Temperature	Time	
94°C	3 min	
94°C	15 sec	N cycles
65°C	15 sec	
72°C	15 sec	
72°C	1 min	
4°C	hold	

The number of reaction cycles refers to the following table:

Template Input	Number of Cycles
1 µg	11 - 12
500 ng	12 - 13
200 ng	13 - 14
100 ng	14 - 15
10 ng	20 - 22

▲ The amount of small RNA varies widely among different sample types. The numbers of PCR cycles listed in the table above are derived from tests using total RNA from 293T cells and mouse liver tissues.If the concentration of RNA extracted from 200 µl of serum, plasma or exosomes or from 1 ml of plasmais below the lower limit of detection of RNA Qubit, the maximum input volume of 6 µl can be used with20- to 30-fold dilution of adaptors and 20 - 22 cycles.

▲ The products of this step can be stored at -30 ~ -15°C for 24 h.

8-5/PCR Product Purification

1. Take out the VAHTS DNA Clean Beads (stored at 2 ~ 8°C) 30 min in advance and equilibrate to room temperature.
2. Resuspend the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Add 180 µl (1.8 ×) of the beads to PCR products, and then mix well by gently pipetting up and down 10 times.
3. Incubate the mixture at room temperature for 10 min to allow the DNA to bind to the beads.
4. Place the sample on a magnetic rack until the solution is clear (about 10 min). Then, carefully remove the supernatant.
5. Keep the sample on the magnetic rack. Add 200 µl of freshly prepared 80% ethanol to rinse the beads. Incubate at room temperature for 30 sec, and then carefully discard the supernatant.
6. Repeat Step 5 once.
7. Keep the sample on the magnetic rack. Uncap the tube and air-dry the beads at room temperature for 5 min.

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

▲ Use a 10 µl pipette to remove all the residual liquid. Avoid over drying the beads (appear cracked), which may result in low recovery.

8. Take out the sample from the magnetic rack and add 30 µl of Nuclease-free ddH_2O. Mix well by gently pipetting up and down, and then stand at room temperature for 2 min. Place the sample on a magnetic rack until the solution is clear (about 10 min). Carefully transfer 28 µl of the supernatant to a new Nuclease-free PCR tube.

▲ The elution product of this step can be stored at -30 ~ -15°C for one week.

9. Library QC (Agilent 2100 Bioanalyzer): Analyze 1 µl of the purified PCR product with the Agilent DNA 1000 chip. Affected by the migration rate of the Agilent 2100 Bioanalyzer, the main peak of the final library may have a bias of about 6 - 8 bp. As shown in Fig 1: the peak of the miRNA library is at 143 - 147 bp, and the peak of the piRNA library is at 153 - 156 bp.

▲ The amount and type of small RNAs vary among different sample types, as well as varying proportions ofmiRNA, piRNA, and other small RNAs of different sizes, leading to a bias of several bases for the main peakof the final library.

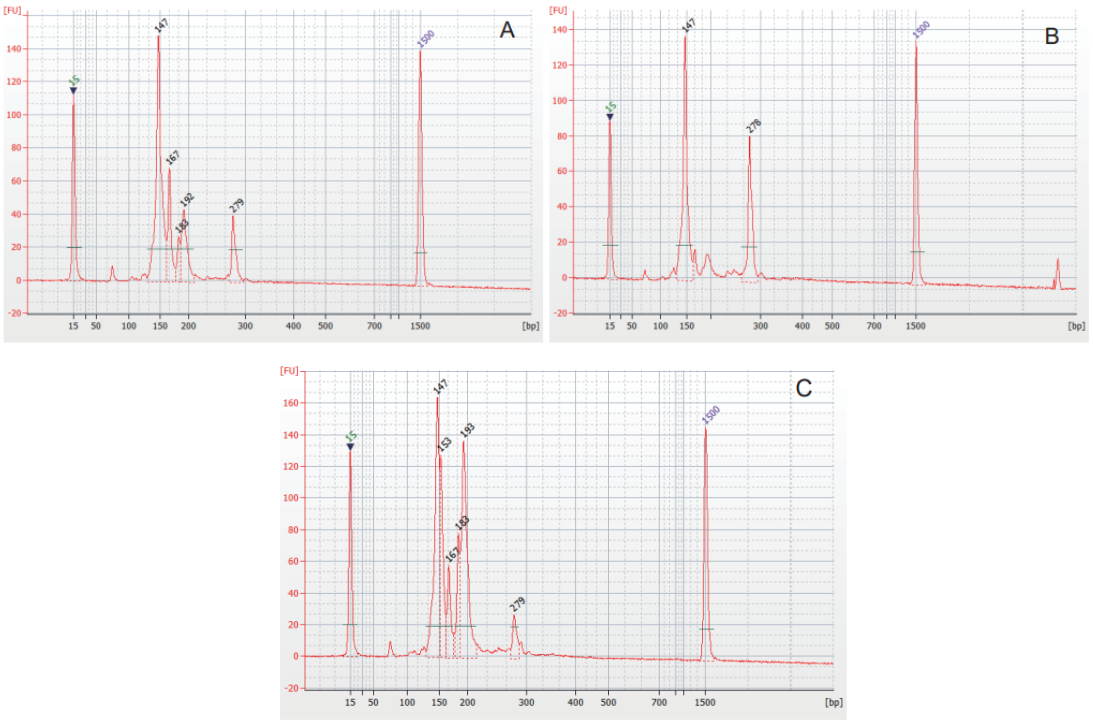


Fig 1. Panels A and B show the small RNA libraries prepared from 1 µg and 100 n g of mouse liver total RNA, respectively; the main peak at 147 bp corresponding to miRNA. Panel C shows the small RNA libraryprepared from 1 µg of mouse kidney total RNA; the main peaks at 147 bp and 153 bp corresponding tomiRNA and piRNA, respectively.

8-6/Size Selection

Choose the appropriate size selection and purification method based on the library QC results in Step 9 of 08-5/PCR Product Purification. If the Agilent 2100 Bioanalyzer trace identifies abundant adaptor dimers at ~ 120 bp, residual primers at ~ 70 - 80 bp, or rRNA at ~ 160 bp and
190 bp, it is recommended to use PAGE gel for size selection. If there are few adaptor dimers,
residual primers or rRNA, size selection can be performed to use magnetic beads. It is recommendedto use PAGE gel for size selection to ensure a sufficient proportion of valid final libraries.

Protocol A: Size Selection (6% Non-denaturing PAGE Gel):

1. Place a 6% 10-well non-denaturing PAGE gel in an electrophoresis tank, and then add an appropriate amount of TBE (1 ×) electrophoresis buffer.
2. Add 5 µl of Loading Buffer (6 ×) to the purified PCR product. Mix well, and then centrifuge to the bottom of the tube.
3. Gently transfer 5 µl of pBR322/MspI Digest DNA Marker to one sample well of the PAGE gel.
4. Gently transfer each PCR product (mixed with Loading Buffer) to two sample wells of the PAGE gel at 15 µl per well.
5. Run the gel at 120 - 150 V for 1 h. The run time may vary depending on the migration rate of the electrophoresis apparatus. Stop electrophoresis 3 - 5 min after the blue indicator of the Loading Buffer runs off the gel.

6. Take out the plate from the electrophoresis apparatus, and then remove the PAGE gel from the plate. Stain the gel with GelRed nucleic acid dye for 10 min and view the stained gel with a UV transilluminator. As shown in Fig 2, the bands at 140 bp and 150 bp correspond to miRNA and piRNA, respectively.

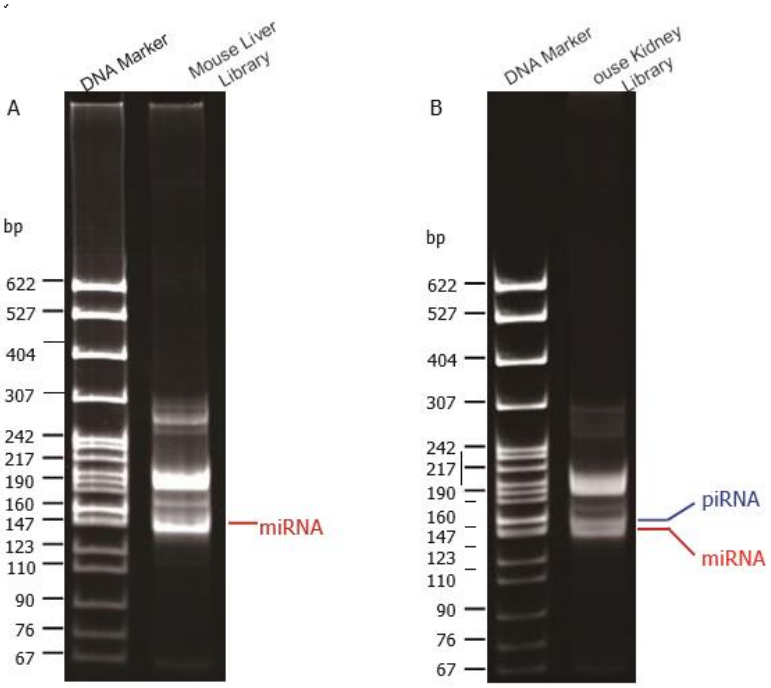


Fig 2. Panel A shows the small RNA library prepared from 1 µg of mouse liver total RNA; the band indicated by the red line is the miRNA library. Panel B shows the small RNA library prepared from 1 µg ofmouse kidney total RNA; the bands indicated by the red and blue lines are the miRNA and piRNA libraries, respectively.

7. Excise the corresponding bands with a razor and place the gel slices into a pre-treated 500 µl low-binding EP tube (the bottom of the EP tube has been poked into several small holes with a 1 ml sterile syringe needle). Place the 500 µl EP tube into a 1.5 ml low-binding EP tube, and then centrifuge at 12,000 rpm (13,800 × g) for 2 min to crush the gel slices.

   ▲ The degree to which the gel slices are crushed depends on the size of the holes at the bottom of the 500 µl EP tube. Thus, the tube should be poked with a syringe needle.

8. Discard the 500 µl EP tube after centrifugation. Add 250 µl of GE Buffer to the 1.5 ml EP tube containing the crushed PAGE gel. Incubate in a 50°C water bath for 1 - 2 h.
9. Centrifuge the EP tube from Step 8 at 12,000 rpm (13,800 × g) for 2 min to collect the liquid on the tube wall to the bottom.
10. Transfer the supernatant from Step 9 to a Filtration Column. Centrifuge at 12,000 rpm (13,800 × g) for 2 min and collect the eluate. Discard the column and transfer the eluate to a new 1.5 ml low-binding EP tube.

11. Add 5 µl of Co-precipitator, 30 µl of 3 M sodium acetate (pH 5.2) and 1 ml of ethanol absolute to the supernatant from Step 10. Mix well by vortexing and precipitate at -80°C for 1 h.
12. Take out the -80°C pellet and centrifuge in a refrigerated centrifuge pre-cooled to 4°C at 12,000 rpm (13,800 × g) for 30 min.
13. Discard the supernatant, taking care not to pipette the white pellet. Add 1 ml of freshly prepared 80% ethanol to the EP tube. Centrifuge in a refrigerated centrifuge pre-cooled to 4°C at 12,000 rpm (13,800 × g) for 10 min.
14. Discard the supernatant, taking care not to pipette the white pellet. Briefly centrifuge to collect the residual liquid on the tube wall to the bottom. Carefully discard the residual liquid and air-dry the pellet at room temperature for 10 min.
15. After the residual ethanol in the product from Step 14 has completely evaporated, resuspend the pellet in 15 µl of Nuclease-free ddH_2O.
16. Library QC (Agilent 2100 Bioanalyzer): Analyze 1 µl of the size-selected purified PCR product with the Agilent high sensitive chip. The traces of good libraries should be as shown in Fig 3, and the corresponding miRNA library band is at 147 - 149 bp.

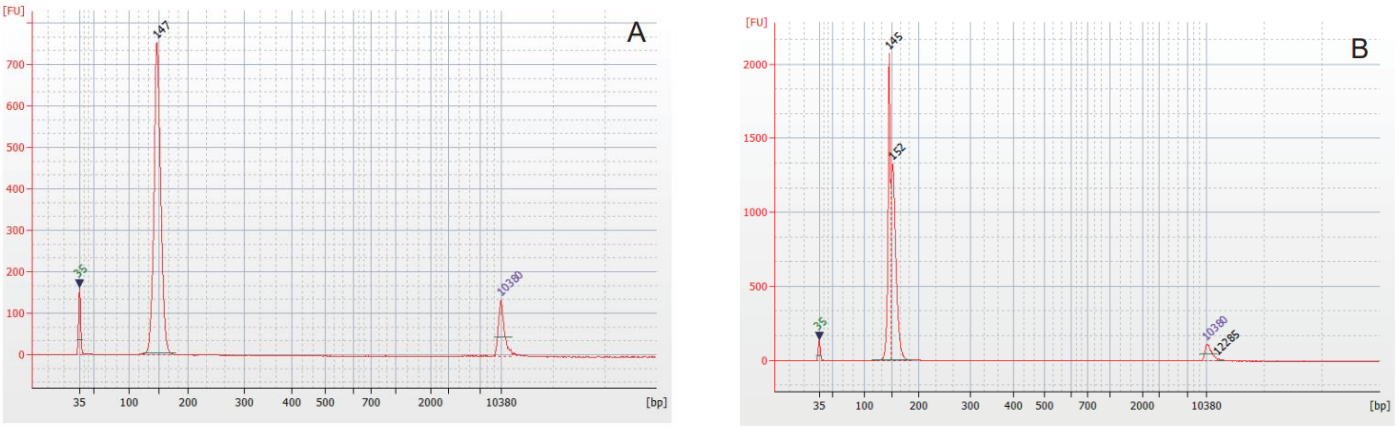


Fig 3. Panels A and B show the Agilent 2100 traces of the PAGE gel-purified small RNA libraries preparedfrom 1 µg of mouse liver total RNA and 1 µg of mouse kidney total RNA, respectively

Protocol B: Two Rounds of Size Selection Using Magnetic Beads

1. Take out the VAHTS DNA Clean Beads (stored at 2 ~ 8°C) 30 min in advance and equilibrate to room temperature.
2. Mix the VAHTS DNA Clean Beads thoroughly by inverting or vortexing. Transfer 25 µl of the purified PCR product to a 200 µl PCR tube and add 32.5 µl (1.3 ×) of the beads. Mix well by gently pipetting up and down 10 times.
3. Incubate the mixture at room temperature for 5 min to allow the DNA to bind to the beads.
4. Place the sample on a magnetic rack until the solution is clear (about 5 min). Carefully transfer the supernatant to a new Nuclease-free PCR tube.
5. Add 22.5 µl (0.9 ×) of the beads to the product from Step 4. Mix well by gently pipetting up and down 10 times.
6. Incubate the mixture at room temperature for 5 min to allow the DNA to bind to the beads.
7. Place the sample on a magnetic rack until the solution is clear (about 5 min). Then, carefully remove the supernatant.
8. Keep the sample on the magnetic rack. Add 200 µl of freshly prepared 80% ethanol to rinse the beads. Incubate at room temperature for 30 sec, and then carefully discard the supernatant.
9. Repeat Step 8 once.
10. Keep the sample on the magnetic rack. Uncap the tube and air-dry the beads at room temperature for 5 min.

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

    ▲Use a 10 µl pipette to remove all the residual liquid.

    ▲Avoid over drying the beads (appear cracked), which may result in low recovery.

11. Remove the sample from the magnetic rack and add 17.5 µl of Nuclease-free ddH_2O. Mix well by gently pipetting up and down 10 times, and then incubate at room temperature for 2 min. Place the tube on a magnetic rack until the solution is clear (about 5 min). Carefully transfer 15 µl of the supernatant to a new Nuclease-free PCR tube.

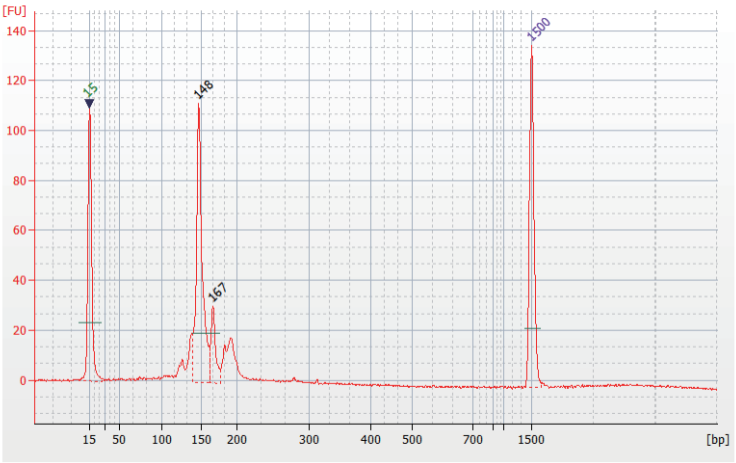


Fig 4. Agilent 2100 trace of the bead size-selected small RNA library prepared from 1 µg of mouse liver totalRNA.

9/FAQ & Troubleshooting

1. The structure of the small RNA library prepared using VAHTS Small RNA Library Prep Kit for Illumina V2:

AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGACGATC——Insert ——AGATCGGAAGAGCACACGTCTGAACTCCAGTCACIIIIIIATCTCGTATGCCGTCTTCTGCTTG

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2. Can I use total RNA extracted with the spin column method rather than the TRIzol method as the initial template?

   There are no special requirements regarding the extraction method of total RNA. If you extract totalRNA using a spin column extraction kit, please make sure that the kit used will not lead to loss of small RNA.

Appendix

TBE (5 ×) Electrophoresis Buffer (1 L)

Components	Mass/Volume
Tris	54 g
Boric acid	27.5 g
EDTA-2Na	4.65 g
ddH_2O	To 1 L

Dissolve completely to make TBE (5 ×) and dilute 5-fold to make TBE(1 ×).

3 M Sodium Acetate (pH 5.2; total 10 ml)

Component	Mass/Volume
Sodium acetate	2.46 g
ddH_2O	8 ml

Dissolve the sodium acetate, adjust the pH to 5.2 with glacial acetic acid, and dilute to 10 ml with ddH_2O