# Small RNA-seq Lib Prep Kit for Illumina V3

RK20312



Instruction Manual

Version: N18B19v1.1

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# 1. Overview

The kit is compatible with the Illumina sequencing platform.

The kit is applicable to animal, plant, and eukaryote (e.g., fungi) total RNA templates as well as the isolated and purified small RNA templates. The recommended total RNA input is 100 ng-1 μg.

Small RNAs to be analyzed usually include miRNAs, piRNAs, and tsRNAs. Different types of small RNAs may vary in length, so gel extraction and library purification vary depending on the target library.

Small RNA library preparation procedures include: ligation with the respective universal adapters at the 3' and 5' ends of the small RNA, reverse transcription, amplification by PCR, purification with magnetic beads, PAGE gel extraction, etc. The obtained target library is sequenced on the Illumina sequencing platform. For standard miRNA libraries, the fragment size is about 150 bp. The fragment at about 130 bp is the adapter dimer.

The adapters provided in the kit are truncated, so they need to be used with the Small RNA Adapter Module for Illumina (RK20358/RK20359/RK20360), and PCR is required to ensure an intact library structure and enrich the library.

All reagents (including all enzymes and buffers) provided in the kit have undergone rigorous quality control and have been verified through library preparation and sequencing, ensuring stability and repeatability.

## 2. Kit Compents

| Module Name | Tube Color & Name | 24 RXN | 96 RXN |
|---|---|---|---|
| Box-1 | 3′ Adapter II | 24 μL | 96 μL |
| | 3′ Adapter Ligation Buffer II | 192 μL | 768 μL |
| | 3′ Adapter Ligation Enzymes II | 48 μL | 192 μL |
| | Adapter Depletion Mix | 48 μL | 192 μL |
| | 5′ Adapter Ligation Buffer | 24 μL | 96 μL |
| | 5′ Adapter Ligation Enzymes | 36 μL | 144 μL |
| | 5X First Strand Synthesis Buffer | 192 μL | 768 μL |
| | First Strand Synthesis Enzyme Mix | 48 μL | 192 μL |
| | 2X LA PCR Mix | 1.2 mL | 4.8 mL |
| | Nuclease-free Water | 1 mL | 1 mL X 2 |
| | Quick Load pBR322-MspI Digest Marker | 240 μL | 960 μL |
| | 6X Loading Buffer | 120 μL | 480 μL |
| | Linear Acrylamide | 24 μL | 96 μL |
| Box-2 | 5′ Adapter | 24 μL | 96 μL |
| Box-3 | DNA PAGE Gel Elution Buffer | 6 mL | 24 mL |

**Note: denotes the color of the tube cap.**

During small RNA library preparation, the ABclonal Small RNA Lib Prep Kit for Illumina® can be used with one of the following kits for library enrichment and index labeling of each sample library.

| Kit Name | Cat. No. |
|---|---|
| Small RNA Adapter Module 96 Index for Illumina MidiSet (24 indices) | RK20358 |
| Small RNA Adapter Module 96Index for Illumina Set_A (48 indices) | RK20359 |
| Small RNA Adapter Module 96 Index for Illumina Set_B (48 indices) | RK20360 |

**Note: The Small RNA Index Primer contains a total of 96 index primers (primers 1-96). Each index primer is used at 2.5 μL/sample for library PCR. See the Appendix for specific index sequences.**

## 3. Storage

Small RNA-seq Lib Prep Kit for Illumina V3 includes three packages:

| Module Name | Storage |
|---|---|
| Box-1 | -20℃ |
| Box-2 | -80℃ |
| Box-3 | Room Temperature |

## 4. Additional Materials

### 4.1. Small RNA Adapter Module for Illumina

Small RNA Adapter Module 96 Index for Illumina MidiSet (24 indices) (ABclonal，Cat.NO. RK20358)

Small RNA Adapter Module 96Index for Illumina Set_A (48 indices) (ABclonal，Cat.NO. RK20359)

Small RNA Adapter Module 96 Index for Illumina Set_B (48 indices) (ABclonal，Cat.NO. RK20360)

### 4.2. RNA sample concentration and quality evaluation

Qubit fluorometers.

Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Cat. Q32855).

Nanodrop.

Agilent RNA 6000 Pico chip (Agilent #5067-1513).

### 4.3. Magnetic beads for purification:

AFTMag NGS DNA Clean Beads (ABclonal，Cat.NO. RK20257)，or other equivalent magnetic beads for nucleic acid purification.

### 4.4. Library quality control

Qubit fluorometers.

ABQubit dsDNA Quantitation Kit (ABclonal, Cat. RK30140).

Agilent high sensitivity DNA Chips (Agilent #5067-4626).

Agilent DNA 1000 chip (Agilent #5067-1504).

### 4.5. Size Selection Materials

6% or 10% TBE PAGE gel (self prepared) and its supporting electrophoresis device.

3M sodium acetate，pH5.5.

### 4.6. Other reagents and consumables

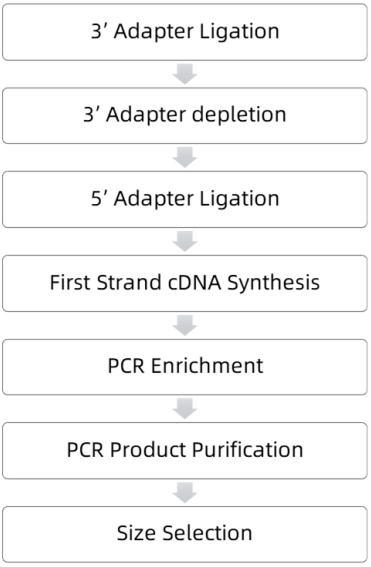
Freshly prepared 80% ethanol.

Magnetic rack.

PCR system.

Thermostatic metal bath or thermostatic water bath

## 5. Workflow




## 6. Precautions

### 6.1. Quality Control of RNA Samples

6.1.1. This kit is suitable for quantifying Total RNA with a starting amount of 100-1000 ng. It is recommended to use the Qubit RNA HS Assay Kit (Thermo Fisher SCIENTIFIC, Cat. Q32855) kit for quantifying Total RNA. A lower input amount can also be used to construct the library normally, but the gel cutting purification step may be necessary.

6.1.2. It is recommended to use Total RNA samples with high integrity for library construction (RIN value>7). Small RNA libraries are only used for analysis of small RNA fragments. If total RNA degradation is severe, this kit cannot guarantee the analysis of the type of Small RNA.

6.1.3. If it is a low-quality RNA sample from sources such as plasma, serum, or exosomes, it is recommended to increase the 3 'adapter connection time, or even stay overnight at 16 ℃, to enhance connection efficiency and avoid failure in library construction due to the failure to capture small RNA.

### 6.2. Use of Magnetic Beads

6.2.1. The magnetic beads should be taken out half an hour in advance and brought to room temperature.

6.2.2. During purification with AFTMag NGS DNA Clean Beads, the Low EDTA TE must be added for elution after ethanol has fully evaporated, namely when the bead color changes from bright brown to frosted brown. Residual ethanol or over-drying (leading to cracks) of beads may reduce the library yield.

### 6.3. Library Quality Control

6.3.1. The normal miRNA library size is about 150bp, and a fragment of approximately 130bp in size is a adapter dimer. The starting amount of Total RNA is between 100 ng-1 μg , and the library has almost no adapter dimers.

6.3.2. Try to increase the RNA input in Total RNA library construction, or enrich Small RNA before building the library. Additionally, it is also possible to increase the ligation time of the 3 'adapter, or even incubate overnight at 16℃.

### 6.4. Adapters for Library Preparation

6.4.1. The adapter quality and concentration directly affect the ligation efficiency and library yield. Excessive adapters may produce many adapter dimers, while insufficient adapters may compromise the ligation efficiency and library yield. Table 1 lists the recommended dilution ratios of 3 'and 5' adapters using Nuclease free Water at different total RNA inputs. If working with low RNA inputs or serum, plasma and exosomes sample, the RNA inputs volume can be added at a maximum volume of 9 μL.

Table 1. Recommended dilution ratios used for adapter

| Input RNA | Adapter Dilution |
|---|---|
| 1μg | undiluted |
| 100 ng | Dilute 1:2 |
| 10 ng | Dilute 1:5/1:10 |
| Serum/plasma / exosomes | Dilute 1:5/1:10 |

### 6.5. Operating Procedures

6.5.1. During the experiment, please use RNase free pipette tips, EP tubes, PCR tubes, and replace the pipette tips when taking different samples.

6.5.2. Please wear gloves and masks when operating. After coming into contact with equipment outside the RNase-free space or other work areas, please replace gloves.

6.5.3. The kit components are sensitive to temperature and must be stored under the aforesaid storage conditions. The reagents stored at -20℃ or -80℃ should be placed on ice after being taken out of the refrigerator.

6.5.4. Before pipetting the reagent, the reagent should be centrifuged instantaneously to concentrate the solution at the bottom of the reagent tube; Please immediately cover all reagents with caps after use to avoid contamination.

6.5.5. If there is a need to pause during the experiment, please strictly follow the safe stopping points specified in the usage method to store the samples at appropriate temperatures. Incorrect pause may reduce the success rate of database construction.

6.5.6. When using PCR Index, please pay attention to avoid cross contamination between reagents and samples.

6.5.7. The reagent for each step can be pre-mixed in advance, and its volume should be 1.1 times the sample volume to make up for the natural loss.

# 7. Protocols

## 7.1. 3′ Adapter Ligation

7.1.1. Thaw the 3 'Adapter II, 3' Adapter Ligation Buffer II, 3 'Adapter Ligation Enzymes II, and Nuclease-free Water on ice and mix well. Centrifuge them briefly to collect residual liquid from the tube wall, and keep on ice. All the following operations are performed on ice.

7.1.2. Thaw the RNA sample on ice, add the following components in a sterile nuclease-free PCR tube.

| | Reagent | Volume |
|---|---|---|
| | RNA sample | 1-9 μL |
| | Nuclease-free Water | To 9 μL |

7.1.3. Gently mix, briefly centrifuge to collect residual liquid from the tube wall, and place on ice.

7.1.4. Place in a thermocycler，and run the following program（ Lid preheated to ≥75℃）:

| Temperature | Time |
|---|---|
| 70℃ | 2 min |
| Transfer the tube to ice | 2 min |

7.1.5. Prepare the 3 'Adapter ligation reaction according to the table below.

| | Reagent | Volume |
|---|---|---|
| | Denaturation RNA sample (from 7.1.4) | 9 μL |
| | 3′ Adapter II* | 1 μL |
| | 3′ Adapter Ligation Buffer II** | 8 μL |
| | 3′ Adapter Ligation Enzymes II | 2 μL |
| | Total volume | 20 μL |

**Note 1: Please dilute 3 'Adapter II according to the amount of RNA input, refer to Table 1 Recommended dilution ratios used for adapter.**

**Note 2: The 3 'Adapter Ligation Buffer II is very viscous, please pipet it slowly.**

7.1.6. Gently mix by pipetting up and down 15-20 times, and centrifuge briefly. Place the tube in a thermocycler，and run the following program (with the heating lid disabled).

| Temperature | Time |
|---|---|
| 30℃ | 1 hr |
| 4℃ | Hold |

**Note: For low RNA inputs or serum, plasma and exosomes sample, it is recommended to incubate at 30 ℃ overnight to enhance ligation efficiency.**

## 7.2. 3′ Adapter depletion

7.2.1. Thaw the Adapter Depletion Mix on ice and mix well. Centrifuge them briefly to collect residual liquid from the tube wall, and keep on ice. All the following operations are performed on ice

7.2.2. Prepare the 3 ' Adapter depletion reaction according to the table below.

| | Reagent | Volume |
|---|---|---|
| | 3 ' Adapter ligation reaction (from 7.1.6) | 20 μL |
| ● | Adapter Depletion Mix | 2 μL |
| ○ | Nuclease-free Water | 4.5 μL |
| | Total volume | 26.5 μL |

7.2.1. Gently mix by pipetting up and down 15-20 times, and centrifuge briefly. Place the tube in a thermocycler，and run the following program (Lid preheated to ≥75℃).

| Temperature | Time |
|---|---|
| 30℃ | 15 min |
| 37℃ | 15 min |
| 75℃ | 5 min |
| 4℃ | Hold |

### 7.3. 5′ Adapter Ligation

7.3.1. Thaw the 5′ Adapter, 5′ Adapter Ligation Buffer on ice and mix well. Centrifuge them briefly to collect residual liquid from the tube wall, and keep on ice. All the following operations are performed on ice.

7.3.2. According to the number of samples (N), transfer (N + 1) μL of 5' Adapter into a sterile nuclease-free PCR tube, and run the following program for denaturation (Lid preheated to ≥75℃).

| Temperature | Time |
|---|---|
| 70℃ | 2 min |
| Transfer the tube to ice | 2 min |

7.3.3. Prepare the 5 'Adapter ligation reaction according to the table below.

| | Reagent | Volume |
|---|---|---|
| | 3' Adapter depletion reaction (from 7.2.1) | 26.5 μL |
| ● | 5′ Adapter（denaturation） | 1 μL |
| ● | 5′ Adapter Ligation Buffer | 1 μL |
| ● | 5′ Adapter Ligation Enzymes | 1.5 μL |
| | Total volume | 30 μL |

**Note: Please dilute 5 'Adapter according to the amount of RNA Input, refer to Table 1 Recommended dilution ratios used for adapter.**

7.3.4. Gently mix by pipetting up and down 15-20 times, and centrifuge briefly. Place the tube in a thermocycler，and run the following program（with the heating lid disabled）.

| Temperature | Time |
|---|---|
| 25℃ | 1 hr |
| 4℃ | Hold |

### 7.4. First Strand cDNA Synthesis

7.4.1. Thaw the 5X First Strand Synthesis Buffer, First Strand Synthesis Enzyme Mix on ice and mix well. Centrifuge them briefly to collect residual liquid from the tube wall, and keep on ice. All the following operations are performed on ice.

7.4.2. Prepare the First Strand cDNA Synthesis reaction according to the table below.

| | Reagent | Volume |
|---|---|---|
| | 5 'Adapter ligation reaction (from 7.3.4) | 30 μL |
| ● | 5X First Strand Synthesis Buffer | 8 μL |
| ● | First Strand Synthesis Enzyme Mix | 2 μL |
| | Total volume | 40 μL |

7.4.3. Gently mix by pipetting up and down 15-20 times, and centrifuge briefly. Place the tube in a thermocycler, and run the following program (Lid preheated to ≥75℃).

| Temperature | Time |
|---|---|
| 50℃ | 1 hr |
| 4℃ | Hold |

**Safe Stopping Point: If you do not plan to proceed immediately to PCR amplification, then heat inactivate the RT reaction at 70℃ for 15 minutes. Samples can be safely stored at -20℃ for 24 hr.**

### 7.5. PCR Enrichment

7.5.1. Thaw the sRNA Universal Primer*、PCR Index XX* and 2X LA PCR Mix on ice and mix well. Centrifuge them briefly to collect residual liquid from the tube wall, and keep on ice. All the following operations are performed on ice.

**Note: sRNA Universal Prime and PCR Index XX are included in the Small RNA Adapter Module 96 Index for Illumina Kit.**

7.5.2. Prepare the PCR Enrichment reaction according to the table below.

| | Reagent | Volume |
|---|---|---|
| | First Strand cDNA Synthesis reaction (from 7.4.3) | 40 μL |
| ● | 2X LA PCR Mix | 50 μL |
| | sRNA Universal Primer | 2.5 μL |
| | PCR Index XX | 2.5 μL |
| ○ | Nuclease-free Water | 5 μL |
| | Total volume | 100 μL |

7.5.3. Gently mix by pipetting up and down 15-20 times, and centrifuge briefly. Place the tube in a thermocycler, and run the following program ( Lid preheated to 105℃).

| Temperature | Time | Cycles |
|---|---|---|
| 94℃ | 2 min | 1 |
| 94℃ | 15 s | 12-24* (Refer to Table 2) |
| 62℃ | 30 s | |
| 70℃ | 15 s | |
| 70℃ | 5 min | 1 |
| 4℃ | Hold | |

Table 2. Recommended Cycles of PCR

| Input RNA | Cycles |
|---|---|
| 1μg | 12 |
| 100 ng | 16 |
| 10ng | 20 |
| 1ng | 24 |

**Note 1: Amplification conditions may vary based on RNA input amount, tissue, and species. This conditions was optimized using total RNA from 293T cells/mouse liver tissue.**

**Note 2: For low RNA inputs or serum, plasma and exosomes sample, it is recommended to run 24 cycles of PCR.**

**Safe Stopping Point: It is safe to store the library at -20℃ for 24 hr after PCR. Avoid leaving the sample at 4℃ overnight if possible.**

## 7.6. PCR Product Purification

7.6.1. Take AFTMag NGS DNA Clean Beads out of the 2-8℃ environment 30 minutes in advance, let it stand until it reaches room temperature, and vortex-mix or shake it well prior to use.

7.6.2. Transfer PCR Enrichment reaction (from 7.5.3) into a 1.5 mL sterile nuclease-free PCR tube, add 180 μL of magnetic beads (1.8X) into each sample, mix them by pipetting, let the mixture stand at room temperature for 5 minutes.

7.6.3. Place the tube on the magnetic rack for another 5 minutes until the solution becomes clear, and cautiously remove and discard the supernatant.

7.6.4. Hold the tube on the magnetic rack, add 500 μL of 80% ethanol, let the tube stand for 30s, and cautiously remove and discard the supernatant.

7.6.5. Repeat Step 7.6.4 once.

7.6.6. Hold the tube on the magnetic rack, remove the residual ethanol with a 10 μL pipette, and open the tube cap for drying at room temperature for about 5 minutes (*bead color changes from bright brown to frosted brown*).

**Note: Residual ethanol or over-drying (leading to cracks) of beads may reduce the library yield.**

7.6.7. Take down the tube from the magnetic rack and add 26 μL of nuclease-free water, mix the solution thoroughly by pipetting, let the mixture stand on the magnetic rack at room temperature for 2 minutes until the solution becomes clear, and pipette 25 μL of the supernatant into a new sterile nuclease-free PCR tube.

7.6.8. Load 1 μl of the PCR product purification reaction (from 7.6.7) into the Agilent 2100 Bioanalyzer and analyze it with the Agilent D1000 DNA Chip. The peak size about 150 bp represents an enriched miRNA library or piRNA library, and the typical library product detection results are shown in Figure 1.

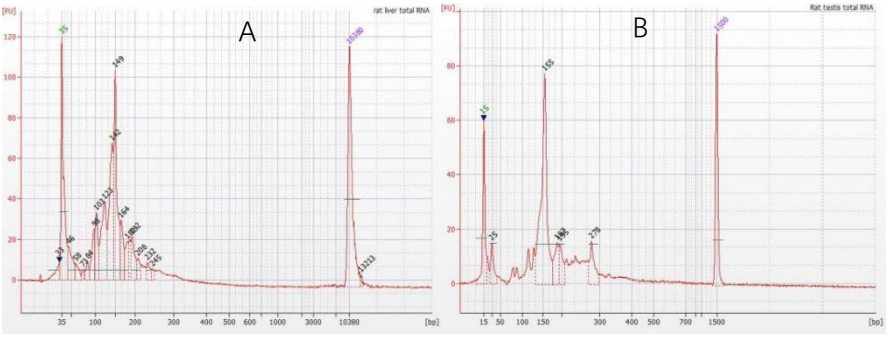



**Figure1. Library Fragment Size Distribution of Small RNA。** Use 1 μg total RNA from mouse liver tissue (A) and rat testicular tissue (B) to obtain Small RNA libraries. Dilute the libraries to 2 ng/μL and analyze their peak patterns using the Agilent 2100 Bioanalyzer.

### 7.7. Size Selection

**There are two methods for performing size selection. If you perform the QC check shows that the sample contains a significant amount of dimers (~130 bp) or residues of amplification primers (70-80 bp), it is recommended to use PolyAcrylamide Gel for size selection.**

**Protocol 1: Size selection using PolyAcrylamide Gel**

7.7.1. Place the 10% nature PAGE gel into the electrophoresis tank, and add an appropriate amount of 0.5X TBE electrophoresis buffer into the tank.

7.7.2. Add 5 μL of 6X Loading Buffer into 25 μL of the purified PCR product, mix them well by pipetting, and centrifuge the mixture instantaneously.

7.7.3. Add 10 μL of Quick Load pBR322-MspI Digest DNA Marker into a well of the PAGE gel, and slowly load the samples into the wells.

7.7.4. Run the gel at 100 V (10 V/1 cm) for about 85 minutes until the Marker is about 1 cm from the bottom of the gel plate.

7.7.5. Remove the PAGE gel from the electrophoresis system, stain it with Safe nucleic acid dye (Safe Red) (RM19009) for 10 minutes, and load it into a gel imager for observation.

7.7.6. Use a blade or a gel cutter to excise the band corresponding to the target library, place it into a new 1.5 mL sterile nuclease-free PCR tube, crush the gel band with a pipette tip, add 200 μL of DNA PAGE Gel Elution Buffer, and incubate the solution at 37°C for 3-4 hours.

Note: The selection of the target library can refer to 8. Appendix

7.7.7. Centrifuge the solution instantaneously, transfer the supernatant into a new 1.5 mL sterile nuclease-free PCR tube. Wash the gel band with 50 μL of DNA PAGE Gel Elution Buffer again, and pipette all the supernatant into the same PCR tube.

7.7.8. Add ethanol absolute (3X supernatant volume), 3 M sodium acetate (pH 5.5, 1/10 of the supernatant volume), and 1 μL of Linear Acrylamide to the above supernatant solution. Vortex-mix them well, and centrifuge briefly.

7.7.9. Vortex-mix them well, then precipitation at -80°C for at least 30 minutes.

7.7.10.Take out the precipitate product at -80 ℃ and place it in a pre-cooled refrigerated centrifuge at 4 ℃. Centrifuge at 12000 rpm for 10 minutes, and cautiously remove and discard the supernatant, be careful not to absorb white precipitate.

7.7.11.Add 500 μL of 80% ethanol, and centrifuge it at 4°C and 12,000 rpm for 5 minutes, and cautiously remove and discard the supernatant, be careful not to absorb white precipitate.

7.7.12.Repeat Step 7.7.11 once.

7.7.13.Open the tube cap for drying at room temperature for about 10 minutes.

7.7.14.Add 20 μL of nuclease-free water to dissolve the precipitate after alcohol volatilization.

7.7.15.Load 1 μL of size selected products into the Agilent 2100 Bioanalyzer and analyze peak patterns with the Agilent D1000 DNA Chip. The following figure shows a good library.

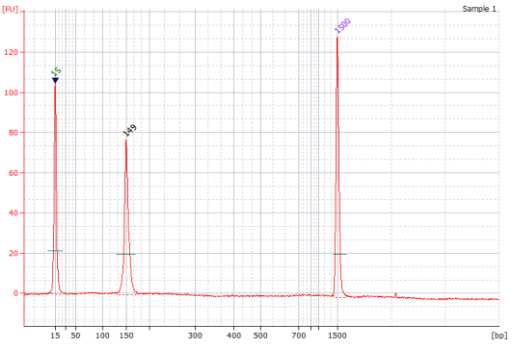



Figure 2. Analysis of Rat Liver Small RNA Library Obtained by Gel Purification (Agilent 2100 Bioanalyzer). 1

μg total RNA, 15 PCR cycles, clean-up with 1.8X beads, gel selection with 10% nature PAGE gel; analyze peak patterns of gel extraction product (1 μL) using the Agilent 2100 DNA 1000 Chip.

### Protocol 2: Size selection using Magnetic Beads

7.7.1. Take AFTMag NGS DNA Clean Beads out of the 2-8℃ environment 30 minutes in advance, let it stand until it reaches room temperature, and vortex-mix or shake it well prior to use.

7.7.2. Add 32.5 μL of magnetic beads (1.3X) into 25 μL of the purified PCR products, mix them well by pipetting, let the mixture stand at room temperature for 5 minutes.

7.7.3. Take on the magnetic rack for another 5 minutes until the solution becomes clear, and pipette 57.5 μL of the supernatant into a new sterile nuclease-free PCR tube.

7.7.4. Add 22.5 μL of magnetic beads (0.9X) into the supernatant, mix them well by pipetting, let the mixture stand at room temperature for 5 minutes.

7.7.5. Place the tube on the magnetic rack for another 5 minutes until the solution becomes clear, and cautiously remove and discard the supernatant.

7.7.6. Hold the tube on the magnetic rack, add 200 μL of 80% ethanol, let the tube stand for 30s, and cautiously remove and discard the supernatant.

7.7.7. Repeat Step 7.7.6 once.

7.7.8. Hold the tube on the magnetic rack, remove the residual ethanol with a 10 μL pipette, and open the tube cap for drying at room temperature for about 5 minutes (*bead color changes from bright brown to frosted brown*).

7.7.9. Take down the tube from the magnetic rack and add 17 μL of nuclease-free water, mix the solution thoroughly by pipetting, let the mixture stand on the magnetic rack at room temperature for 5 minutes until the solution becomes clear, and pipette 15 μL of the supernatant into a new sterile nuclease-free PCR tube.

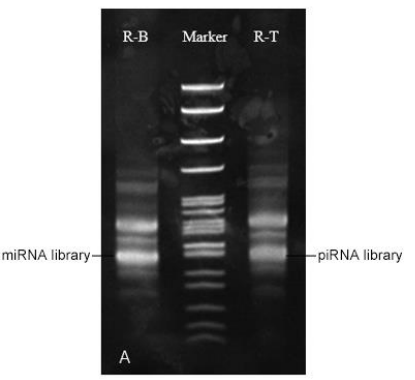
## 8. Appendix

### 8.1. Are there any other total RNA extraction methods in addition to Trizol lysis?

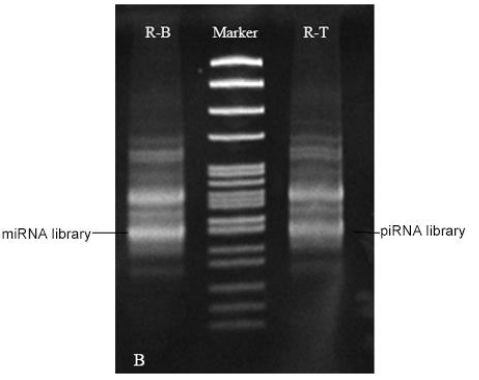
The total RNA needs to be extracted with commercial reagent kits that can extract small RNAs. During extraction, the RNA precipitation time needs to be prolonged to enrich more small RNAs.

### 8.2. Which gel band needs to be excised during size selection with the PAGE gel?

For PAGE gel extraction, the gel band at about 147 bp corresponds to the miRNA target library, and the band at about 153 bp corresponds to the piRNA target library. Alternatively, the target gel band can be excised after determining the positions of the target band and PAGE gel through extraction product analysis with the Agilent 2100 DNA 1000 Chip. Do not excise the gel band at about 130 bp (adapter dimers). For low-concentration libraries, 10% PAGE gel extraction can enhance the library resolution, avoiding excising the adapter dimer band.




6% PAGE Gel




10% PAGE Gel

**Figure 3. Gel Extraction of Small RNA Libraries.** Figure A shows libraries analyzed with the 6% PAGE gel. R-B: rat brain small RNA library; R-T: rat testis small RNA library. Figure B shows libraries analyzed with the 10% PAGE gel. R-B: rat brain small RNA library; R-T: rat testis small RNA library.

**Note: Compared with the 6% PAGE gel, the 10% PAGE gel delivers higher resolution, a narrower band distribution range, and easier gel band excision.**

### 8.3. How to improve the success rate of library construction for serum/plasma and exosome samples?

It is recommended to use commercial reagent kits for RNA extraction of corresponding samples. For example, for 200ul serum/plasma samples, QIAGEN's miRNeasy Serum/Plasma Advanced Kit can be used, and for 1ml serum/plasma extracted exosomal samples, QIAGEN's exoRNeasy Kit can be used. If the extracted product is below the lower limit of sensitivity detectable by Qubit, it is recommended to input the sample RNA volume according to a maximum of 9 uL.

### 8.4. Self-prepared Reagents

8.4.1. 5X TBE Buffer（1 L）

| Reagent | Amount |
|---|---|
| Tris-base | 54 g |
| Boric acid | 27.5 g |
| EDTA-2Na | 3.72 g |

Add the above reagents into a 1 L beaker, add 800 mL of deionized water, stir the solution thoroughly, adjust pH to 8.3 with 50% HCl, continue to add deionized water until the final volume reaches 1 L, and filter the solution with the 0.22 μm filter paper.

8.4.2. 10% PAGE Gel（40 mL）

| Reagent | Amount |
|---|---|
| N,N′-Methylenebisacrylamide (30%) | 13.3 mL |
| 5X TBE | 8 mL |
| $ddH_2O$ | 18.4 mL |
| Ammonium persulfate (AP, 10%) | 400 μL |
| Tetramethylethylenediamine (TEMED) | 20 μL |

Successively add 10% AP and TEMED at the end of preparation, immediately mix the solution well, and quickly pour the mixture into the gel plate, place a comb in the gel, and wait for 30 minutes for PAGE gel formation.

8.4.3. 6% PAGE Gel（40 mL）

| Reagent | Amount |
|---|---|
| N,N′-Methylenebisacrylamide（30%） | 8 mL |
| 5X TBE | 8 mL |
| $ddH_2O$ | 24 mL |
| Ammonium persulfate (AP, 10%) | 400 μL |
| Tetramethylethylenediamine (TEMED) | 20 μL |

Successively add 10% AP and TEMED at the end of preparation, immediately mix the solution well, and quickly pour the mixture into the gel plate, place a comb in the gel, and wait for 30 minutes for PAGE gel formation.

8.4.4. 3 M Sodium Acetate (pH 5.5, 100 mL)

| Reagent | Amount |
|---|---|
| Anhydrous sodium acetate | 24.6 g |
| $ddH_2O$ | 80 mL |

After complete dissolution, adjust pH to 5.5 with glacial acetic acid.

8.4.5. Some Self-purchased Reagents

| Reagent | Brand | Cat. No. |
|---|---|---|
| Tris-base | Vetec | V900483 |
| Boric acid | Sinopharm | 10004818 |
| EDTA-2Na | Sinopharm | 10009717 |
| N,N′-Methylenebisacrylamide (30%) | Sangon Biotech | B546017-0500 |
| Ammonium persulfate (AP, 10%) | Sinopharm | 10002618 |
| Tetramethylethylenediamine (TEMED) | Beijing Dingguo Changsheng Biotechnolog Co., Ltd. | DH338-1 |
| Anhydrous sodium acetate | Sangon Biotech | A500827-0500 |

# 9. Appendix Table

9.1.1. Sequence Information of Adapter and PCR Index Primer

3′ Adapter:

5′ -rAppAGATCGGAAGAGCACACGTCT-3′

5′ Adapter:

5′ -rGrUrUrCrArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrC-3′

sRNA Universal Primer:

5′ -AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA-3′

PCR Index XX:

5′-Spc/C*A*A*GCAGAAGACGGCATACGAGATXXXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGA*T*C*T-3′

Note: XXXXXXXX is the base sequence of an index.

9.1.2. Index table 2. Small RNA Adapter Module 96 Index for Illumina® MidiSet (24 indices) (RK20358)

| sRNA Index Primer Name | Index | sRNA Index Primer Name | Index |
|---|---|---|---|
| PCR Index 1 | TTACCGAC | PCR Index 13 | TCGTCTGA |
| PCR Index 2 | AGTGACCT | PCR Index 14 | AGCCTATC |
| PCR Index 3 | TCGGATTC | PCR Index 15 | CTGTACCA |
| PCR Index 4 | CAAGGTAC | PCR Index 16 | AGACCTTG |
| PCR Index 5 | TCCTCATG | PCR Index 17 | AGGATAGC |
| PCR Index 6 | GTCAGTCA | PCR Index 18 | CCTTCCAT |
| PCR Index 7 | CGAATACG | PCR Index 19 | GTCCTTGA |
| PCR Index 8 | TCTAGGAG | PCR Index 20 | TGCGTAAC |
| PCR Index 9 | CGCAACTA | PCR Index 21 | CACAGACT |
| PCR Index 10 | CGTATCTC | PCR Index 22 | TTACGTGC |
| PCR Index 11 | GTACACCT | PCR Index 23 | CCAAGGTT |
| PCR Index 12 | CGGCATTA | PCR Index 24 | CACGCAAT |

9.1.3. Index table 3. Small RNA Adapter Module 96 Index for Illumina® Set_A (48 indices) (RK20359)

| sRNA Index Primer Name | Index | sRNA Index Primer Name | Index |
|---|---|---|---|
| PCR Index 1 | TTACCGAC | PCR Index 25 | TTCCAGGT |
| PCR Index 2 | AGTGACCT | PCR Index 26 | TCATCTCC |
| PCR Index 3 | TCGGATTC | PCR Index 27 | GAGAGTAC |
| PCR Index 4 | CAAGGTAC | PCR Index 28 | GTCGTTAC |
| PCR Index 5 | TCCTCATG | PCR Index 29 | GGAGGAAT |
| PCR Index 6 | GTCAGTCA | PCR Index 30 | AGGAACAC |
| PCR Index 7 | CGAATACG | PCR Index 31 | CAGTGCTT |
| PCR Index 8 | TCTAGGAG | PCR Index 32 | CTTGCTAG |
| PCR Index 9 | CGCAACTA | PCR Index 33 | TGGAAGCA |
| PCR Index 10 | CGTATCTC | PCR Index 34 | AGCTAAGC |
| PCR Index 11 | GTACACCT | PCR Index 35 | GAACGGTT |
| PCR Index 12 | CGGCATTA | PCR Index 36 | GGAATGTC |
| PCR Index 13 | TCGTCTGA | PCR Index 37 | TACGGTCT |
| PCR Index 14 | AGCCTATC | PCR Index 38 | CCAGTATC |
| PCR Index 15 | CTGTACCA | PCR Index 39 | TCTACGCA |
| PCR Index 16 | AGACCTTG | PCR Index 40 | GTAACCGA |
| PCR Index 17 | AGGATAGC | PCR Index 41 | GACGTCAT |
| PCR Index 18 | CCTTCCAT | PCR Index 42 | CTTACAGC |
| PCR Index 19 | GTCCTTGA | PCR Index 43 | TCCATTGC |
| PCR Index 20 | TGCGTAAC | PCR Index 44 | AGCGAGAT |
| PCR Index 21 | CACAGACT | PCR Index 45 | CAATAGCC |
| PCR Index 22 | TTACGTGC | PCR Index 46 | AAGACACC |
| PCR Index 23 | CCAAGGTT | PCR Index 47 | CCAGTTGA |
| PCR Index 24 | CACGCAAT | PCR Index 48 | TGGTGAAG |

9.1.4. Index table 4. Small RNA Adapter Module 96 Index for Illumina® Set_B (48 indices) (RK20360)

| sRNA Index Primer Name | Index | sRNA Index Primer Name | Index |
|---|---|---|---|
| PCR Index 49 | AAGACCGT | PCR Index 73 | TAGGAGCT |
| PCR Index 50 | TTGCGAGA | PCR Index 74 | CGAATTGC |
| PCR Index 51 | GCAATTCC | PCR Index 75 | GTCCTAAG |
| PCR Index 52 | GAATCCGT | PCR Index 76 | CTTAGGAC |
| PCR Index 53 | CCGCTTAA | PCR Index 77 | TCCACGTT |
| PCR Index 54 | TACCTGCA | PCR Index 78 | CAACACAG |
| PCR Index 55 | GTCGATTG | PCR Index 79 | GCCTTAAC |
| PCR Index 56 | TATGGCAC | PCR Index 80 | GTAAGGTG |
| PCR Index 57 | CTCGAACA | PCR Index 81 | AGCTACCA |
| PCR Index 58 | CAACTCCA | PCR Index 82 | CTTCACTG |
| PCR Index 59 | GTCATCGT | PCR Index 83 | GGTTGAAC |
| PCR Index 60 | GGACATCA | PCR Index 84 | GATAGCCA |
| PCR Index 61 | CAGGTTCA | PCR Index 85 | TACTCCAG |
| PCR Index 62 | GAACGAAG | PCR Index 86 | GGAAGAGA |
| PCR Index 63 | CTCAGAAG | PCR Index 87 | GCGTTAGA |
| PCR Index 64 | CATGAGCA | PCR Index 88 | ATCTGACC |
| PCR Index 65 | GACGAACT | PCR Index 89 | AACCAGAG |
| PCR Index 66 | AGACGCTA | PCR Index 90 | GTACCACA |
| PCR Index 67 | ATAACGCC | PCR Index 91 | GGTATAGG |
| PCR Index 68 | GAATCACC | PCR Index 92 | CGAGAGAA |
| PCR Index 69 | GGCAAGTT | PCR Index 93 | CAGCATAC |
| PCR Index 70 | GATCTTGC | PCR Index 94 | CTCGACTT |
| PCR Index 71 | CAATGCGA | PCR Index 95 | CTTCGGTT |
| PCR Index 72 | GGTGTACA | PCR Index 96 | CCACAACA |

9.1.5. Quick Load pBR322-MspI Digest DNA Marker Fragment Size(bp)

| Fragment No. | Fragment Size (bp) |
|---|---|
| 1 | 622 |
| 2 | 527 |
| 3 | 404 |
| 4 | 307 |
| 5 | 242 |
| 6 | 238 |
| 7 | 217 |
| 8 | 201 |
| 9 | 190 |
| 10 | 180 |
| 11,12 | 160 |
| 13,14 | 147 |
| 15 | 123 |
| 16 | 110 |
| 17 | 90 |
| 18 | 76 |
| 19 | 67 |
| 20,21 | 34 |
| 22,23 | 26 |
| 24 | 15 |
| 25,26 | 9 |

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