MGI

MGIEasy

Small RNA Library Prep Kit User Manual

Cat. No.: 940-000196-00 (24 RXN)

Kit Version: V2.0

Manual Version: 1.0

Revision History

Manual Version	Kit Version	Date	Description
1.0	V2.0	Mar. 2022	• Update Cat. No.

Note: Please download the latest version of the manual and use it with the corresponding kit.

Search manual by Cat. No. or product name from website:

https://en.mgi-tech.com/download/files.html

Contents

Chapter 1 Product Description

1.1 Introduction

The MGIEasy Small RNA Library Prep Kit is specifically designed for rapid library preparation for MGI high-throughput sequencing platforms. This library prep kit is optimized to convert 10 ng – 1 μg eukaryotic total RNA into a single-stranded circularized DNA library for MGI sequencing platforms. This kit supports high-throughput small RNA sequencing for identifying known small RNA and discovering novel miRNA. All reagents provided within this kit have passed stringent quality control and functional verification procedures, ensuring performance, stability, and reproducibility.

1.2 Application

This library prep kit is applicable for samples from all common animals and plants, including human, mouse, rice, and Arabidopsis. Stable performance across all sample types is expected.

1.3 Platform Compatibility

Constructed libraries can be sequenced on BGISEQ-500, MGISEQ-2000, DNBSEQ-G400.

1.4 Contents

MGIEasy Small RNA Library Prep Kit has a single unit size, 24 RXN. Each kit consists of 2 boxes of reagents. Further information on Components and Specifications are listed below.

Table 1 MGIEasy Small RNA Library Prep Kit (24 RXN) (Cat. No. 940-000196-00)

Boxes	Components	Cap Color	Spec & Quantity
MGIEasy Small RNA Library Prep Kit (Box 1) Cat. No. 940-000179-00	3′ Adapter	Green	24 μL/tube × 1 tube
	RNAse Inhibitor	Green	108 μL/tube × 1 tube
	3′ Ligation Enzyme	Green	48 μL/tube × 1 tube
	3′ Ligation Buffer Mix	Green	240 μL/tube × 1 tube
	Adapter Depletion Enzyme I	Colorless	60 μL/tube × 1 tube
	Adapter Depletion Enzyme II	Colorless	41 μL/tube × 1 tube
	Adapter Depletion Buffer	Colorless	20 μL/tube × 1 tube
	5′ Adapter	Red	24 μL/tube × 1 tube
	5′ Ligation Enzyme	Red	48 μL/tube × 1 tube
	5′ Ligation Buffer Mix	Red	96 μL/tube × 1 tube
	RT Primer (1-4,13-16)	Colorless	3 μL/tube × 8 tubes

	RT Primer (25-32)	Colorless	3 μL/tube × 8 tubes
	RT Primer (97-104)	Colorless	3 μL/tube × 8 tubes
	FS Reaction Buffer Mix	Black	300 μL/tube × 1 tube
	RT Enzyme	Black	24 μL/tube × 1 tube
	PCR Enzyme Mix	Blue	1200 μL/tube × 1 tube
	Block oligo	Blue	24 μL/tube × 1 tube
	PCR Primer Mix(HC)	Blue	96 μL/tube × 1 tube
	Splint Buffer	Purple	93 μL/tube × 1 tube
	DNA Rapid Ligase	Purple	5 μL/tube × 1 tube
	Digestion Buffer	White	12 μL/tube × 1 tube
	Digestion Enzyme	White	21 μL/tube × 1 tube
MGIEasy Small RNA Library Prep Kit (Box 2) Cat. No. 940-000180-00	Stop Buffer(HC)	Blue	144 μL/tube × 1 tube
	Loading Buffer	Yellow	240 μL/tube × 1 tube
	10 bp DNA Ladder	Yellow	72 μL/tube × 1 tube
	10x TNE Buffer	Yellow	960 μL/tube × 1 tube
	Library Purification Beads	White	864 μL/tube × 1 tube
	Digestion Stop Buffer	White	60 μL/tube × 1 tube
	TE Buffer	White	600 μL/tube × 1 tube
	Nuclease-Free Water	Purple	1100 μL/tube × 1 tube

1.5 Storage Conditions and Shelf Life

MGIEasy Small RNA Library Prep Kit (Box 1)

- Storage temperature: -25°C ~ -15°C
- Expiration date: refer to the label
- Transport conditions: transported on dry ice

MGIEasy Small RNA Library Prep Kit (Box 2)

- Storage temperature: 2°C ~ 8°C
- Expiration date: refer to the label
- Transport conditions: transported with ice packs.

* Please ensure that an abundance of dry ice remains after transportation.

* Performance of products are guaranteed until the expiration date under appropriate transport, storage, and usage conditions.

1.6 Equipment and Materials Required but not Provided

Table 2: Equipment and Materials Required but not Provided

Equipment	Common	Vortex mixer
		Desktop centrifuge
		Pipette
		Thermocycler
		Magnetic Separation Rack (ThermoFisher, Cat. No. 12321D)
		Qubit® 3.0 Fluorometer (ThermoFisher, Cat. No. Q33216)
		Agilent 2100 Bioanalyzer (Agilent Technologies, Cat. No. G2939AA)
	For Gel	Eppendorf® Refrigerated Microcentrifuge, Model 5417R (Eppendorf, Cat. No. 5417R)
		ThermoMixer(Eppendorf)
		PowerEase® 300W Power Supply (230 VAC) (Invitrogen, Cat. No. PS0301)
		Gel imaging system
		XCell *Sure Lock*® Mini-CE mark (Invitrogen, Cat. No. EI0001)
		Dark Reader transilluminator (Clare Chemical Research, Cat. No. D195M)
		SureCast™ Gel Handcast Bundle B - Hardware Only (Invitrogen, Cat. No. HC1000S)
Reagents	Common	RNase Zap Decontamination Solution (Ambion, Cat. No. AM9780)
		Nuclease free water (Ambion, Cat. No. AM9937)
		100% Ethanol (Analytical Grade)
		Qubit® ssDNA Assay Kit (Invitrogen, Cat. No. Q10212)
		Qubit® dsDNA HS Assay Kit (Invitrogen, Cat. No. Q32854)
		Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)
	For Beads	Agencourt® AMPure® XP Beads (Agencourt, Cat. No. A63881) or MGIEasy DNA Clean Beads (MGI, Cat. No. 1000005278 or 1000005279)
	For Gel	Glycogen (Ambion, Cat. No. AM9510)
		GelStar™ Nucleic Acid Gel Stain (Lonza, Cat. No. 50535)
Consumables	Common	Pipette tips and RNase-free tips
		1.5 mL microcentrifuge tube (Axygen, Cat. No. MCT-150-C)

	Qubit® Assay Tubes (Invitrogen, Cat. No. Q32856) or 0.5mLThin Wall PCR Tubes (Axygen, Cat. No. PCR-05-C)
	0.2 mL PCR tube (Axygen, Cat. No. PCR-02-C)
For Gel	Spin-X® Centrifuge Tube Filters (Corning, Cat. No. 8162)
	Novex® 6% TBE PAGE gel 1.0 mm (Invitrogen, Cat. No. EC6265BOX)
	2 mL microcentrifuge tube (Axygen, Cat. No. MCT-200-C)
	Plastic wrap
	Blade
	Lighter
	Alcohol burner
	Needle

1.7 Precautions and Warning

- Instructions provided in this manual are intended for general use only, and may require further adjustments to optimize performance. We recommend making adjustments depending on experimental design, sample characteristics, sequencing applications, and equipment for optimization.
- Remove the reagents from storage beforehand, and prepare them for use. For Enzymes, centrifuge briefly and place on ice for further use. For Buffer Mix reagents, thaw at room temperature and invert several times to mix properly, then centrifuge briefly and place on ice for use.
- When preparing mixtures and working solutions, we recommend pipetting at least 10 times to mix thoroughly. Note that vigorous shaking may cause a decrease in the yield of library preparation.
- To prevent cross-contamination, we recommend using filtered pipette tips. Use a new tip each time for pipetting different solutions.
- We recommend using the thermocyclers with heated lids for reactions. Preheat the thermocyclers to reaction temperature before use.
- Improper handling of samples and reagents may contribute to aerosol contamination of PCR products and may affect experimental accuracy. Therefore, we recommend physically separating two working areas in the laboratory for PCR reaction preparation and PCR product cleanup, respectively. Use designated equipment for each area and clean regularly to ensure a sterile working environment. (Use 0.5% Sodium Hypochlorite or 10% Bleach to clean working environments).
- If you have other questions, please contact MGI technical support MGI-service@mgi-tech.com

Chapter 2 Sample Preparation

2.1 Sample Compatibility and Requirement

This library prep kit is applicable for samples from all common animals and plants, including human, mouse, rice, and Arabidopsis. This protocol can be used with 10 ng to 1 μg of eukaryotic total RNA. When working with tissue samples, the recommended starting amount of total RNA is ≥ 100 ng.

2.2 Sample Quality Control

- RNA integrity: RNA integrity number (RIN) ≥ 7.5, 28 S/18 S ≥1.5, 5.8 S rRNA ≤50%.

Use Agilent 2100 Bioanalyzer to perform quality control of the total RNA samples.

- RNA purity: $OD_{260/280}$ = 1.8 – 2.0.

Chapter 3 Library Construction Protocol

3.1 3′ Adapter Ligation

⚠ **Note 1: In the following procedures, do not vortex. Please use pipette to mix solution.**

⚠ **Note 2: If working with low RNA input samples, dilute the 3'Adapter using Nuclease-Free Water according to Table 3. Briefly centrifuge, pipette 10 times to mix, and centrifuge briefly again.**

Table 3 Dilution of 3′ Adapter

Input Amount (total RNA)	3′ Adapter Dilution
1000 ng	Use undiluted
500 ng	Use undiluted
100 ng	Dilute 1:1
10 ng	Dilute 1:5

3.1.1 Prepare the following components in a tube (see Table 4).

Table 4 The Denaturing Reaction Mixture

Components	Volume
Input RNA	x μL
3′ Adapter	1 μL
Nuclease-Free Water	(6-x) μL
Total	7 μL

3.1.2 Place the reaction tube into the thermocycler at 70°C for 2 min and immediately transfer the tube to ice for at least 1 min.

3.1.3 Remove 3′ Ligation Buffer Mix from -20°C and thaw at room temperature. Invert several times to mix, centrifuge briefly, and place on ice. Prepare the 3′ Adapter Ligation Reaction Mixture on ice (see Table 5).

Table 5 3′ Adapter Ligation Reaction Mixture

Components	Volume
3′ Ligation Buffer Mix	10 μL
RNAse Inhibitor	1 μL
3′ Ligation Enzyme	2 μL
Total	13 μL

3.1.4 Transfer 13 μL of 3′ Adapter Ligation Reaction Mixture to the tube in step 3.1.2 with a pipette. Pipette 10 times to mix. Centrifuge briefly to collect the solution to the bottom of the tube.

3.1.5 Place the reaction tube into the thermocycler at 25°C for 2 h, then hold at 4°C.

3.1.6 After the reaction is completed, centrifuge the tube 3 s and immediately proceed to 3′Adapter Digestion.

3.2 3′Adapter Digestion

⚠ **Note: In the following procedures, do not vortex. Please use pipette to mix solution.**

3.2.1 Remove Adapter Depletion Buffer from -20°C and thaw at room temperature. Invert several times to mix, centrifuge briefly, and place on ice. Prepare the 3′ Adapter Digestion Reaction Mixture on ice (see Table 6).

Table 6 3′ Adapter Digestion Reaction Mixture

Components	Volume
Adapter Depletion Enzyme Ⅰ	2.5 μL
Adapter Depletion Enzyme Ⅱ	1.7 μL
RNAse Inhibitor	1 μL
Adapter Depletion Buffer	0.8 μL
Total	6 μL

3.2.2 Transfer 6 μL of 3′ Adapter Digestion Reaction Mixture to the tube in step 3.1.6 with a pipette. Pipette 10 times to mix. Centrifuge briefly to collect the solution to the bottom of the tube.

3.2.3 Place the reaction tube into the thermocycler and run the following program in Table 7.

Table 7 The Reaction Conditions of 3′ Adapter Digestion

Temperature	Time
Heated lid	On
30°C	30 min
37°C	30 min
70°C	20 min
4°C	hold

3.2.4 After the reaction is completed, centrifuge the tube 3 s and immediately proceed to 5′Adapter Ligation.

3.3 5′Adapter Ligation

⚠ **Note: In the following procedures, do not vortex. Please use pipette to mix solution.**

3.3.1 Remove 5′ Adapter from -20°C and thaw on ice. Invert several times to mix, centrifuge briefly, and place on ice.

⚠ **Note: If working with low RNA input samples, dilute the 5′Adapter using Nuclease-Free Water according to Table 8. Pipette 10 times to mix, and centrifuge briefly again.**

Table 8 5′ Adapter Dilution

Input Amount (total RNA)	5′ Adapter Dilution
1000 ng	Use undiluted
500 ng	Use undiluted
100 ng	Dilute 1:1
10 ng	Dilute 1:5

3.3.2 Take (N+1) μL of 5′ Adapter, place the 5′ Adapter at 70°C for 2 min, and immediately transfer the tube to ice for 1 min (N is the number of reactions). Then add 1 μL of denatured 5′ Adapter to the reaction solution in step 3.2.4.

3.3.3 Remove 5′ Ligation Buffer Mix from -20°C and thaw at room temperature. Invert several times to mix, centrifuge briefly, and place on ice. Prepare the 5′ Adapter Ligation Reaction Mixture on ice (see Table 9).

Table 9 5′Adapter Ligation Reaction Mixture

Components	Volume
5′ Ligation Buffer Mix	4 μL
RNAse Inhibitor	1 μL
5′ Ligation Enzyme	2 μL
Total	7 μL

3.3.4 Transfer 7 μL of 5′ Adapter Ligation Reaction Mixture to the tube in step 3.3.2 with a pipette. Pipette 10 times to mix. Centrifuge briefly to collect the solution to the bottom of the tube.

3.3.5 Place the reaction tube into the thermocycler at 25°C for 1 h, then hold at 4°C.

3.3.6 After the reaction is completed, centrifuge the tube for 3 s and immediately proceed to Reverse Transcription.

3.4 Reverse Transcription

3.4.1 Remove RT Primer from -20°C and thaw at room temperature. Centrifuge briefly, and place on ice.

⚠ **Note 1: If working with low RNA input samples, dilute the RT Primer using Nuclease-Free Water according to Table 10. Pipette 10 times to mix, and centrifuge briefly again.**

⚠ **Note 2: RT Primers contain barcodes. Different barcodes are required to label samples for pooled sequencing. This kit provides 24 barcoded RT Primers. Recommended pooling strategies are listed in Appendix B.**

Table 10 RT Primer Dilution

Input Amount (total RNA)	RT Primer Dilution
1000 ng	Use undiluted
500 ng	Use undiluted
100 ng	Dilute 1:1
10 ng	Dilute 1:5

3.4.2 Add 1 μL of RT Primer to the reaction solution in step 3.3.6. Pipette 10 times to mix. Centrifuge briefly to collect the solution to the bottom of the tube.

3.4.3 Place the reaction tube into the thermocycler at 65°C for 3 min and immediately transfer the tube to ice for at least 1 min.

3.4.4 Remove FS Reaction Buffer Mix from -20°C and thaw at room temperature. Invert several times to mix, centrifuge briefly, and place on ice. Prepare the Reverse Transcription Reaction Mixture on ice (see Table11).

Table 11 Reverse Transcription Reaction Mixture

Components	Volume
FS Reaction Buffer Mix	12.5 μL
RNAse Inhibitor	1.5 μL
RT Enzyme	1 μL
Total	15 μL

3.4.5 Transfer 15 μL of Reverse Transcription Reaction Mixture to the tube in step 3.4.3 with a pipette. Pipette 10 times to mix. Centrifuge briefly to collect the solution to the bottom of the tube.

3.4.6 Place the reaction tube into the thermocycler and run the program in Table 12.

Table 12 The Reaction Conditions of Reverse Transcription

Temperature	Time
Heated lid	On
42°C	60 min
70°C	15 min
4°C	Hold

3.4.7 After the reaction is complete, centrifuge the tube for 3 s to collect the solution to the bottom of the tube.

3.5 PCR Amplification

⚠ **Note 1: PCR reaction specification has two options, 100 μL and 50 μL. The select of reaction specification depends on the subsequent size selection method.**

⚠ **Note 2: For human, mouse, and rat samples, we recommend size selection using beads, which corresponds to 100 μL PCR reaction volume. 6% TBE PAGE gel size selection is also applicable. For other samples, we recommend size selection using 6% TBE PAGE gel, which corresponds to 50 μL PCR reaction volume (see Table 13).**

Table 13 The Size Selection Method and PCR Reaction Volume

Size Selection Method	PCR Reaction Volume
Beads	100 μL
6% TBE PAGE Gel	50 μL

3.5.1 100 μL PCR Reaction Volume

a) Take 23 μL of reverse transcription product to do PCR amplification.

b) Remove PCR Primer Mix (HC), PCR Enzyme Mix and Block oligo from -20°C, and thaw at room temperature. Invert several times to mix, centrifuge briefly, and place on ice. Prepare the 100 μL PCR Amplification Mixture on ice (see Table 14).

Table 14 100 μL PCR Amplification Mixture

Components	Volume
cDNA	23 μL
PCR Primer Mix (HC)	4 μL
PCR Enzyme Mix	50 μL
Block oligo	1 μL
Nuclease-Free Water	22 μL
Total	100 μL

3.5.2 50 μL PCR Reaction Volume

a) Take 23 μL of reverse transcription product to do PCR amplification.

b) Remove PCR Primer Mix (HC) and PCR Enzyme Mix from -20°C, and thaw at room temperature. Invert several times to mix, centrifuge briefly, and place on ice. Prepare the 50 μL PCR Amplification Mixture on ice (see Table 15).

Table 15 50 μL PCR Amplification Mixture

Components	Volume
cDNA	23 μL
PCR Primer Mix(HC)	2 μL
PCR Enzyme Mix	25 μL
Total	50 μL

3.5.3 Vortex the 100 μL or 50 μL of PCR Amplification Mixture 3 times (3 s each), and centrifuge briefly to collect the solution to the bottom of the tube.

3.5.4 Place the reaction tube into the thermocycler and run the following program in Table 16.

Table 16 The Reaction Conditions of PCR Amplification

Temperature	Time	Cycles
Heated lid	on	
95°C	3 min	1 cycle
98°C	20 s	
56°C	15 s	N * cycles
72°C	15 s	
72°C	10 min	1 cycle
4°C	Hold	

* Please choose the different cycle number referring to the following table.

Table 17 Recommended PCR cycles for different input amount

Input Amount (total RNA)	Cycle Number
1000 ng	16 - 17
500 ng	17 - 19
100 ng	20 - 22
10 ng	22 - 25

Note 1: The number of cycles is based on the reference value obtained from test samples of human brain total RNA. The amount of small RNA varies depending on the sample species and cell type. Cycling parameters can be optimized based on the specific sample type.

Note 2: It is normal if there are some micro-precipitation after PCR amplification. This will not affect the subsequent experiments.

3.5.5 After the reaction is completed, centrifuge the tube for 3 s to collect the solution to the bottom of the tube.

3.6 Size Selection of PCR Product

Note: Different size selection methods fit different species. Please choose the following methods based on your samples.

3.6.1 Size Selection by using beads

Note 1: Please read Appendix A carefully before you begin.

Note 2: The following beads used are AMPure® XP beads or MGIEasy DNA Clean Beads. Other

beads are not compatible.

3.6.1.1 Remove beads from refrigerator and bring to room temperature for 30 min beforehand. Vortex and mix thoroughly before use.

3.6.1.2 Transfer 180 μL of beads and 50 μL of ethanol to 1.5 mL tube.

3.6.1.3 Add 6 μL of Stop Buffer (HC) to the PCR tube in step 3.5.5. Mix thoroughly and transfer 100 μL to the tube in step 3.6.1.2. Gently pipette at least 10 times to mix thoroughly. Ensure that all solution and beads are expelled from the tip into the tube.

⚠ **Note: Make sure the volume added to the 1.5 mL tube is 100 μL.**

3.6.1.4 Incubate at room temperature for 10 min, and gently pipette 5 times with a pipette every 5 min to prevent precipitation of the beads.

3.6.1.5 Centrifuge briefly and place the tube on the Magnetic Separation Rack for 2-5 min until liquid becomes clear. Transfer supernatant to a new 1.5 mL tube.

⚠ **Note: Do not discard the supernatant.**

3.6.1.6 Add 40 μL of beads to the supernatant. Gently pipette at least 10 times to mix thoroughly. Ensure that all solution and beads are expelled from the tip into the tube.

3.6.1.7 Incubate at room temperature for 10 min.

3.6.1.8 Centrifuge briefly and place the tube on the Magnetic Separation Rack for 2-5 min until liquid becomes clear. Carefully remove and discard the supernatant with a pipette.

3.6.1.9 With the tube on the Magnetic Separation Rack, add 500 μL of freshly prepared 80% ethanol to the tube without disturbing the beads. Incubate for 30 s. Carefully remove and discard the supernatant.

3.6.1.10 Repeat step 3.6.1.9 once. Remove all liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid to the bottom, separate beads magnetically, then remove remaining liquid using a small volume pipette.

3.6.1.11 Keep the tube on the Magnetic Separation Rack with the lid open, air-dry beads until no wetness (glossiness) is visible but before the pellet cracks.

3.6.1.12 Remove the tube from the Magnetic Separation Rack. Add 16 μL of TE Buffer to elute DNA. Gently pipette the entire volume up and down 10 times to mix thoroughly or until the beads are fully resuspended.

3.6.1.13 Incubate at room temperature for 10 min.

3.6.1.14 Centrifuge briefly, place the tube on the Magnetic Separation Rack for 2-5 min until liquid becomes clear. Transfer 14 µL supernatant to a new 1.5 mL tube.

✔ **Stop point: The purified product can be stored at -20°C.**

3.6.2 Separation of PCR product using 6% TBE PAGE gels

3.6.2.1 Material Preparation

a) Seal the tube cap of 2 mL tube with plastic wrap to prevent contamination.

b) Pierce the bottom of 0.5 mL tube 6-7 times with a heated needle and then place it into the 2 mL tube.

3.6.2.2 Protocols

a) Take 6% TBE PAGE gel, lock the gel wedge and place it in the electrophoresis tank, add 1× TBE buffer and pull out the comb.

b) Add 10 µL of Loading Buffer to each 50 µL of PCR product. Mix thoroughly, and load 3 wells with 20 µL each of mixed product. Add 3 µL of 10 bp DNA Ladder (see Appendix E) to a separate well.

c) Run the electrophoresis at 185 V. Terminate the run when the blue dye reaches the bottom of the gel. Stain the gel with GelStar™ Nucleic Acid Gel Stain for 10 min. Capture image with a gel imaging system (see Figure 1).

⚠ **Prepare GelStar™ Nucleic Acid Gel Stain:**

Add 5 µL of GelStar™ Nucleic Acid Gel Stain to 50 mL 1x TBE buffer and mix thoroughly. Avoid exposure of stain mixture to direct light. This mixture is for one gel only. It is recommended to stain each gel separately to avoid cross-contamination.

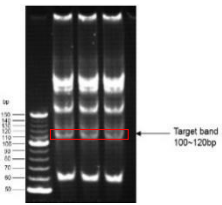

Figure 1 Gel electrophoresis of PCR products

d) Extract the sections with bands around 100 – 120 bp from the gel (from the upper end of the 100 bp marker to lower end of 120 bp marker). Place the gel into the 0.5 mL tube prepared in step 3.6.2.1. Centrifuge at 13600 rpm for 2 min to force the gel through the holes, which will shred it into smaller pieces.

e) Add 400 µL of 1× TNE Buffer to the shredded gel. Incubate at 37°C for at least 2 h on ThermoMixer at 650 rpm to extract DNA.

⚠ **Note: 10× TNE Buffer can be diluted with Nuclease-Free Water to final 1× TNE Buffer.**

f) Transfer the shredded gel and buffer into Spin-X® Centrifuge Tube Filters and centrifuge at 13600 rpm for 3 min.

g) Add 3 µL of glycogen and 1200 µL of pre-chilled 100% ethanol to the tube in step 3.6.2.2.6. After mixing, place at −80°C for at least 30 min.

h) Remove the product from −80°C and centrifuge at 13600 rpm for 30 min at 4°C (pre-chill the centrifuge to 4°C in advance). Carefully remove the supernatant without disturbing the white pellet.

i) Add 700 µL of pre-chilled 80% ethanol. Centrifuge at 13600 rpm for 3 min at 4°C. Carefully remove the supernatant without disturbing the white pellet.

j) Centrifuge at high speed for 10 s to spin down the remaining ethanol. Carefully remove the supernatant and air-dry the pellet at room temperature.

k) Resuspend the pellet with 14 µL of TE buffer.

✔ **Stop point: The purified product can be stored at -20°C.**

3.7 Quality Control of PCR Product

3.7.1 Quantitate the purified PCR products with Qubit® dsDNA HS Assay Kit. Follow the assay kit instructions to quantitate the purified PCR products. The required yield for purified PCR products is ≥ 10 ng.

3.7.2 Determine the amounts of samples for the next reaction according to the quantitation results. Based on the sequencing data requirement of each sample, mix samples with the appropriate amount. The total DNA input amount of pooling samples should be 1 pmol, with a total volume ≤ 48 μL. See Table 18 and the formula 1 of Appendix D for the corresponding yield for different insert sizes.

Table 18 The Corresponding Yield in 1 pmol for PCR Products with Different Insert Sizes

Insert Size (bp)	PCR product size (bp)	Corresponding Yield in 1 pmol (ng)
22	105	70
30	113	75
50	133	88

3.7.3 Assess the fragment size distribution of purified PCR products with Agilent 2100 Bioanalyzer. The 105 and 116 bp bands correspond to miRNAs and piRNAs, respectively. The bands on the Bioanalyzer electropherograms can shift from sample to sample due to an incorrect identification of the marker by the bioanalyzer software. miRNA peak should be 103-109 bp.

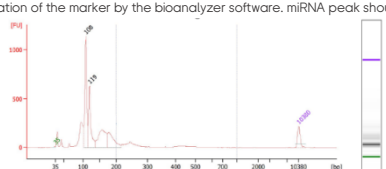

Figure 2 Agilent 2100 Bioanalyzer of bead size selected purified product from human brain total RNA.

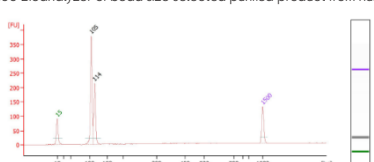

Figure 3 Agilent 2100 Bioanalyzer of gel size selected purified product from human brain total RNA.

3.8 Denaturation

⚠ **Note: Please read Appendix D carefully before you begin.**

3.8.1 According to the PCR product size and the formula 1 in Appendix D, transfer 1 pmol of PCR product to a new 0.2 mL PCR tube. Add TE Buffer to a total volume of 48 μL.

3.8.2 Place the PCR tube in step 3.8.1 into the thermocycler and run the following program in Table 19.

Table 19 The Reaction Conditions of Denaturation

Temperature	Time
Heated lid	On
95°C	3 min

3.8.3 After the reaction is completed, immediately place the tube on ice for 2 min and centrifuge briefly.

3.9 Single Strand Circularization

3.9.1 Prepare the Single Strand Circularization Reaction Mixture in a 0.2 mL PCR tube on ice (see Table 20).

Table 20 Single Strand Circularization Reaction Mixture

Components	Volume
Splint Buffer	11.6 μL
DNA Rapid Ligase	0.5 μL
Total	12.1 μL

3.9.2 Transfer 12.1 μL of Single Strand Circularization Reaction Mixture to the PCR tube in step 3.8.3. Vortex 3 times (3s each) and centrifuge briefly to collect the solution to the bottom of the tube.

3.9.3 Place the PCR tube into the thermocycler and run the program in Table 21.

Table 21 The Reaction Conditions of Single Strand Circularization

Temperature	Time
Heated lid	On
37°C	30 min
4°C	Hold

3.9.4 After the reaction is completed, immediately place the tube on ice for the next reaction.

3.10 Enzymatic Digestion

3.10.1 Prepare the Enzymatic Digestion Mixture (see Table 22) on ice during the reaction in step 3.9.3.

Table 22 Enzymatic Digestion Mixture

Components	Volume
Digestion Buffer	1.4 μL
Digestion Enzyme	2.6 μL
Total	4 μL

3.10.2 Transfer 4 μL of Enzymatic Digestion Mixture to the PCR tube in step 3.9.4. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution to the bottom of the tube.

3.10.3 Place the PCR tube in step 3.10.2 into the thermocycler and run the following program in Table 23.

Table 23 The Reaction Conditions of Enzymatic Digestion

Temperature	Time
Heated lid	On
37°C	30 min

3.10.4 Centrifuge briefly to collect the solution to the bottom of the tube.

3.10.5 Add 7.5 μL of Digestion Stop Buffer to the PCR tube. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution to the bottom of the tube. Transfer all of the solution into a new 1.5 mL tube.

3.11 Cleanup of Enzymatic Digestion Product

3.11.1 Remove Library Purification Beads from refrigerator and bring to room temperature for 30 min beforehand. Vortex and mix thoroughly before use.

Note: Library Purification Beads are included in the kit.

3.11.2 Transfer 108 μL of Library Purification Beads to Enzymatic Digestion product in step 3.10.5. Gently pipette at least 10 times to mix thoroughly. Ensure that all solution and beads are expelled from the tip into the tube.

3.11.3 Incubate at room temperature for 10 min.

3.11.4 Centrifuge briefly and place the 1.5 mL tube on the Magnetic Separation Rack for 2-5 min until liquid becomes clear. Carefully remove and discard the supernatant with a pipette.

3.11.5 With the 1.5 mL tube on the Magnetic Separation Rack, add 500 µL of freshly prepared 80% ethanol to the tube without disturbing the beads. Incubate for 30 s. Carefully remove and discard the supernatant.

3.11.6 Repeat step 3.11.5 once. Remove all liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid to the bottom, separate beads magnetically, then remove remaining liquid using a small volume pipette.

3.11.7 Keep the 1.5 mL tube on the Magnetic Separation Rack with the lid open, and air-dry beads until no wetness (glossiness) is visible but before the pellet cracks.

3.11.8 Remove the 1.5 mL tube from the Magnetic Separation Rack. Add 16 µL of TE Buffer to elute DNA. Gently pipette the entire volume up and down 10 times to mix thoroughly or until the beads are fully resuspended.

3.11.9 Incubate at room temperature for 10 min.

3.11.10 Centrifuge briefly. Place the 1.5 mL tube on the Magnetic Separation Rack for 2-5 min until liquid becomes clear. Transfer 14 µL supernatant to a new 1.5 mL tube. Take care not to disturb the beads.

✔ **Stopping Point: Purified Enzymatic Digestion products can be stored at -20°C for one month.**

3.12 Quality Control of Enzymatic Digestion Product

Quantitate the purified Enzymatic Digestion product with Qubit® ssDNA Assay Kit. The final yield should be ≥ 120 fmol (enough for two sequencing runs). Please refer to Table 24 or formula 2 in Appendix D for your calculations.

Table 24 The Corresponding Yield in 120 fmol for Different PCR Product Size (Circularized ssDNA)

Insert Size (bp)	PCR Product Size (bp)	Corresponding Yield in 120 fmol (ng)
22	105	4.16
30	113	4.47
50	133	5.27

Chapter 4 Sequencing

Small RNA library should be sequenced using a specific sequencing set on a particular sequencer. Please select the corresponding sequencing set following instructions.

For BGISEQ-500:

Sequencing set for small RNA library: BGISEQ-500RS High-throughput Sequencing Set (SE50) (Small RNA) for SE50+10 sequencing. Please refer to BGISEQ-500RS High-throughput Sequencing Set (SE50) (Small RNA) User Manual for sequencing instructions.

For MGISEQ-2000\DNBSEQ-G400:

Sequencing set for small RNA library: MGISEQ-2000RS High-throughput Sequencing Set (SE50) (Small RNA) or DNBSEQ-G400RS High-throughput Sequencing Set (Small RNA FCL SE50) for SE50+10 sequencing. Please refer to MGISEQ-2000RS High-throughput Sequencing Set User Manual or DNBSEQ-G400RS High-throughput Sequencing Set User Manual for sequencing instructions.

Appendix

Appendix A Magnetic Beads

For magnetic beads-based size selection after PCR amplification, we recommend using AMPure® XP (Agencourt, Cat. No. A63881) or DNA Clean Beads included in the MGIEasy DNA Clean Beads Kit (MGI, Cat. No1000005278 or 1000005279). For magnetic beads from other sources, size selection condition is not compatible.

Before You Use:

- Remove beads from 4°C storage and let it stand at room temperature for 30 min beforehand. Vortex and mix thoroughly before use.
- Vortex or pipette up and down to ensure that the beads are thoroughly mixed every time before use.

Operation Notes:

- During the magnetic separation step, please allow the solution to become completely clear before removing the supernatant. This process takes approximately 2-3 min. Consider the varying magnetic strength of your specific Separation Plate / Rack, and allow enough time for the solution to turn completely clear.
- Avoid disturbing the beads while pipetting. 2-3 μL of fluids can be left in the tube to avoid contact. In case of contact between the beads and pipette tip, expel all solution and beads to the tube and restart the separation process.
- Use freshly prepared 80% ethanol (at room temperature) to wash the beads. The tube should remain on the Magnetic Separation Rack while washing. Do not shake or disturb the beads in any way.
- After the 2nd washing of beads with ethanol, try to remove all liquid within the tube. You may centrifuge briefly to collect any remaining liquid to the bottom, separate beads magnetically, and remove remaining liquid by using a small volume pipette.
- After washing twice with ethanol, air-dry the beads at room temperature. Drying takes approximately 2-5 min depending on your specific lab environment. Observe closely until the pellet appears sufficiently dry with a matte appearance, then continue to the elution step with TE Buffer.
- Avoid disturbing the beads when removing the supernatant. Contamination from the beads may affect subsequent reactions.
- Pay attention when opening / closing the lids of tubes on the Separation Rack. Strong vibrations may

cause sample loss through spilled liquid or beads. Secure the tubes well before opening the lids.

Appendix B The Combination Barcode Strategies of RT Primers.

- This kit provides 24 RT Primers with different barcodes (see Figure 4). The RT Primers provided were developed to meet requirements for batch processing of library construction and multiplex sequencing. We select the best barcode combination based on the principle of balanced base composition. We provide 4 different combination strategies to pool 8, 12, 16 and 24 samples together, respectively, to do circularization (See Table 25). However, the barcodes are not continuous.
- Before use, please centrifuge to collect liquid to the bottom of the tube. Gently remove the cap to prevent spills and cross-contamination. Mix the RT Primer by pipetting up and down before use. Remember to reseal the RT Primer immediately after use.
- Barcodes from MGIEasy Small RNA Library Prep Kits (No. 501-516) are designed differently and incompatible for mixed use. Mixed using will cause errors in barcode demultiplexing in data extraction procedures.

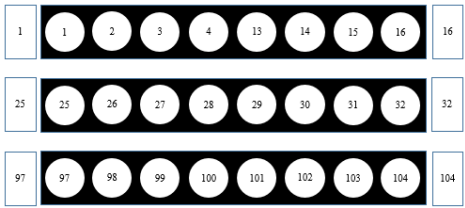

Figure 4 RT Primer (8-tube strip)

Table 25 Barcode pooling strategies

No.	The Number of Samples	RT Primer
1	8	1 – 4，13 – 16 or 25 – 32 or 97 – 104
2	12	1 – 4，25 – 32 or 1 – 4，97 – 104
3	16	1 – 4，13 – 16，25 – 32
4	24	1 – 4，13 – 16，25 – 32，97 – 104

Appendix C 6% TBE PAGE Gel Preparation

- 【Materials required】

Table 26 Materials required for PAGE gel

Equipment	Laminar flow hood
Consumables	Kimwipes Delicate Task Wiper (KIMBERLY-CLARK, Cat. No. 34120)
Reagents	Acryl/Bis 40% Solution (19:1) (Sangon Biotech, Cat. No. SD6012)
	10×TBE (Ambion, Cat. No. AM9863)
	Ammonium persulfate
	TEMED (Sangon Biotech, Cat. No. T0761)
	Nuclease Free Water (Ambion, Cat. No. AM9937)

- 【6% TBE PAGE gel recipe】

  Prepare 6% TBE PAGE gel in the laminar flow hood.

Table 27 6% TBE PAGE gel recipe

Nuclease Free Water	27.85 mL
10×TBE	3.75 mL
Acryl/Bis 40% Solution (19:1)	5.62 mL
10% Ammonium persulfate	270 μL
TEMED	24 μL
Total volume	37.5 mL
Number of gel	5.7 pcs

Appendix D Conversion between DNA Molecular Mass and Number of Moles

Formula 1 shows the calculation of the Mass in (ng) that corresponds to 1 pmol of dsDNA sample with varying fragment sizes. Please refer to formula 1 to calculate the amount of DNA needed.

Formula 1 dsDNA sample pmol and ng Conversion

$$\text{The Mass(ng) Corresponding to 1 pmol PCR Products (ng)} = \frac{\text{DNA Fragment Size(bp)}}{1000\ \text{bp}} \times 660\ \text{ng}$$

The yield for circularized ssDNA after cleanup must be at least 120 fmol or above for two sequencing runs. Please refer to Formula 2 below to calculate the number of mols needed.

Formula 2 Circular ssDNA fmol and ng Conversion.

$$\text{The Mass (ng) Corresponding to 120 fmol circular ssDNA} = 0.12 \times \frac{\text{DNA Fragment Size(bp)}}{1000\ \text{bp}} \times 330\ \text{ng}$$

Appendix E 10 bp DNA Ladder

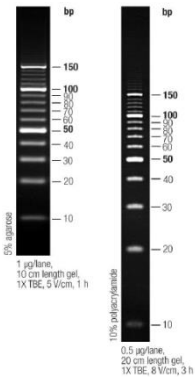

Figure 5 10 bp DNA Ladder

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