MGIEasy

RNA Library Prep Set User Manual

Cat. No.: 1000006383 (16RXN) , 1000006384 (96 RXN)

Kit Version: V3.1

Manual Version: A4

Revision History

Manual Version	Kit Version	Date	Description
A4	V3.1	Jan. 2021	◆ Update contact information.
A3	V3.1	Jul. 2020	◆ Kit specification is adapted to the requirements of MGISP-100 and MGISP-960 automated library construction ◆ Modified the description of sample multiplexing in 3.10 and 3.11 ◆ Kit version update to V3.1
A2	V3.0	Sep. 2019	◆ 1.3 Add DNBSEQ series sequencing platform ◆ 2.2 Add OD260/230 requirement
A1	V3.0	Mar. 2019	◆ 1.1 Introduction. "and detection of pathogenic microorganisms" ◆ 1.2 Application. "In addition, this library prep set is also applicable to samples from whole blood, intestinal and others type of human for the detection of pathogenic microorganisms." ◆ 1.3 Platform Compatibility "Sequencing platform." ◆ Chapter 3 Library Construction Protocol "For library construction of FFPE RNA sample, please refer to Appendix E. For library construction of Pathogenic microorganisms RNA sample, please refer to Appendix F." ◆ Add "Appendix F Library Construction from RNA pathogen sample"
A0	V3.0	Nov. 2018	◆ Release.V3.0 set

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 RNA Library Prep Set was designed to prepare libraries for MGI high-throughput sequencing platforms. The library prep set is optimized to convert 10 ng -1 μg of total RNA into a single stranded circularized DNA library for gene expression profiling, transcriptome analysis, or detection of pathogenic microorganisms by high-throughput RNA sequencing on MGI instruments. All reagents provided within this set have passed stringent quality control and functional verification procedures to ensure high performance, stability, and reproducibility.

1.2 Application

This library prep set can be used for samples from a variety of common animals, plants, fungus, bacteria. More specifically, the set can be used on human, mouse, rice, Arabidopsis, yeast and E. *coli* samples. Stable performance across all such sample types is expected. In addition, this library prep set can also be used for whole blood, intestinal, and others type of human samples for the detection of pathogenic microorganisms.

1.3 Platform Compatibility

Constructed libraries are compatible with:

BGISEQ-500 (SE50/PE50/PE100),

MGISEQ-2000 (PE100/PE150), DNBSEQ-G400 (PE100/PE150),

MGISEQ-200 (SE50/SE100), DNBSEQ-G50 (SE50/SE100).

1.4 Contents

The MGIEasy RNA Library Prep Set is available in two specifications, 16 RXN and 96 RXN, and consists of 4 modules. Further information on Cat. No., Components and Specifications are listed below:

Table 1 MGIEasy RNA Library Prep Set (16 RXN) (Cat. No.: 1000006383)

Modules and Cat. No.	Components	Color-Coded Screw Caps	Spec & Quantity
MGIEasy RNA Library Prep Kit Cat. No.: 1000005274	Fragmentation Buffer	Green	93 µL/tube x 1 tube
	RT Buffer	Green	88 µL/tube x 1 tube
	RT Enzyme Mix	Green	24 µL/tube x 1 tube
	Second Strand Buffer	Yellow	470 µL/tube x 1 tube
	Second Strand Enzyme Mix	Yellow	78 µL/tube x 1 tube
	ERAT Buffer	Orange	132 µL/tube x 1 tube
	ERAT Enzyme Mix	Orange	55 µL/tube x 1 tube
	Ligation Buffer	Red	450 µL/tube x 1 tube
	DNA Ligase	Red	34 µL/tube x 1 tube
	PCR Enzyme Mix	Blue	470 µL/tube x 1 tube
	PCR Primer Mix	Blue	90 µL/tube x 1 tube
MGIEasy DNA Adapters-16 (Tube) Kit Cat. No.: 1000005284	DNA Adapters	White	10 µL/tube x 16 tubes
MGIEasy DNA Clean Beads Cat. No.: 1000005278	DNA Clean Beads	White	8 mL/tube x 1 tube
	TE Buffer	White	4 mL/tube x 1 tube
MGIEasy Circularization Module Cat. No.: 1000005260	Splint Buffer	Purple	186 µL/tube x 1 tube
	DNA Rapid Ligase	Purple	8 µL/tube x 1 tube
	Digestion Buffer	White	23 µL/tube x 1 tube
	Digestion Enzyme	White	42 µL/tube x 1 tube
	Digestion Stop Buffer	White	120 µL/tube x 1 tube

Table 2 MGIEasy RNA Library Prep Set (96 RXN) (Cat. No.: 1000006384)

Modules and Cat. No.	Components	Color-Coded Screw Caps	Spec & Quantity
MGIEasy RNA Library Prep Kit Cat. No.: 1000005276	Fragmentation Buffer	Green	608 µL/tube x 1 tube
	RT Buffer	Green	760 µL/tube x 1 tube
	RT Enzyme Mix	Green	136 µL/tube x 1 tube
	Second Strand Buffer	Yellow	1496 µL/tube x 2 tubes
	Second Strand Enzyme Mix	Yellow	448 µL/tube x 1 tube
	ERAT Buffer	Orange	872 µL/tube x 1 tube
	ERAT Enzyme Mix	Orange	325 µL/tube x 1 tube
	Ligation Buffer	Red	1300 µL/tube x 2 tubes
	DNA Ligase	Red	173 µL/tube x 1 tube
	PCR Enzyme Mix	Blue	1340 µL/tube x 2 tubes
	PCR Primer Mix	Blue	448 µL/tube x 1 tube
MGIEasy DNA Adapters-96 (Plate) Kit Cat. No.: 1000005282	DNA Adapters	White	10 µL/ well x 96 wells
MGIEasy DNA Clean Beads Cat. No.: 1000005279	DNA Clean Beads	White	50 mL/tube x 1 tube
	TE Buffer	White	25 mL/tube x 1 tube
MGIEasy Circularization Module Cat. No.: 1000005260	Splint Buffer	Purple	186 µL/tube x 1 tube
	DNA Rapid Ligase	Purple	8 µL/tube x 1 tube
	Digestion Buffer	White	23 µL/tube x 1 tube
	Digestion Enzyme	White	42 µL/tube x 1 tube
	Digestion Stop Buffer	White	120 µL/tube x 1 tube

1.5 Storage Conditions and Shelf Life

MGIEasy RNA Library Prep Kit

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

MGIEasy DNA Adapters Kit

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

MGIEasy DNA Clean Beads

- Storage temperature: 2°C to 8°C
- Expiration date: refer to the label
- Transport Conditions: transported with an ice pack

MGIEasy Circularization Kit

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

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

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

1.6 Equipment and Materials Required but not Provided

Table 3 Equipment and Materials Required but not Provided

Equipment	ThermoMixer (Eppendorf) 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)
Reagents	RNase Zap Decontamination Solution (Ambion, Cat. No. AM9780) mRNA enrichment kit or rRNA depletion kit. Recommended: Dynabeads® mRNA Purification Kit （Invitrogen, Cat. No. 61006）or Library Preparation VAHTS mRNA Capture Beads （Vazyme, Cat. No. N401-01/02）for mRNA enrichment. MGIEasy rRNA depletion kit （MGI, Cat. No. 1000005953）or equivalent for rRNA depletion RNA Clean Beads (Agencourt RNAClean XP 40 mL Kit, Agencourt, Cat. No. A63987) (Material required for rRNA depletion method) Nuclease free water (NF water) (Ambion, Cat. No. AM9937) 100% Ethanol (Analytic 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) Agilent DNA 1000 Kit (Agilent, Cat. No. 5067-1504)
Consumables	Pipette tips and RNase-free tips 1.5 mL non-stick tube (Ambion, Cat. No. AM12450) 1.5 mL tube (Axygen, Cat. No. MCT-150-C) 0.2 mL PCR tube (Axygen, Cat. No. PCR-02-C) or 96-well plate (Axygen, Cat. No. PCR-96M2-HS-C) Qubit® Assay Tubes (Invitrogen， Cat. No. Q32856) or 0.5mLThin Wall PCR Tubes (Axygen, Cat. No. PCR-05-C)

1.7 Precautions and Warning

- Instructions provided in this manual are intended for general use only, and may require adjustments for optimal performance. Adjust the protocol according to your experimental design, sample characteristics, sequencing application and equipment limitations as you deem necessary.
- Remove the reagents from storage beforehand and prepare them for use: For enzymes, centrifuge briefly and place on ice for further use. For other reagents, bring to room temperature and invert several times to mix thoroughly. Centrifuge briefly and place on ice for use.
- When preparing mixtures and working solutions, pipette at least 10 times to mix thoroughly. Vigorous shaking may cause a decrease in library yield.
- To prevent cross-contamination, we recommend using filtered pipette tips. Use a new tip each time for pipetting different solutions. Pipette carefully to avoid spillage.
- Use thermocyclers equipped with heated lids for reactions. Preheat the lid to reaction temperature before use. The temperature of the heated lid will be 105°C unless otherwise specified.
- Avoid contamination of PCR products due to aerosolization of samples and reagents by:
  - Preparing PCR reactions and cleaning up PCR products in separate working spaces.
  - Using only equipment designated for either process in their respective area.
  - Regularly clean the work environment with 0.5% Sodium Hypochlorite or 10% Bleach.
- 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 set is used for samples from a variety of common animals, plants, fungus, and bacteria. More specifically, the kit can be used on human, mouse, rice, Arabidopsis, and yeast samples. It is strongly recommended to use a total RNA input of 10 ng - 1 μg. For low-abundance mRNA species such as plants, a total RNA input of 1 - 2.5 μg is recommended.

2.2 Sample Quality Control

- Use an Agilent 2100 Bioanalyzer to perform quality control of extracted total RNA samples. RIN value should be ≥7. If RIN< 7, use an RNA input of no more than 2.5 μg and appropriately increase the number of PCR cycles in the library construction. For library construction of FFPE RNA samples, please refer to Appendix E. For library construction of Pathogenic microorganism RNA samples, please refer to Appendix F.
- RNA integrity: $OD_{260/280}$=1.8~ 2.0, $OD_{260/230}$≥2.
- If DNA contamination is found in the RNA sample, perform a DNase I digestion to remove DNA before starting the procedure below.

Chapter 3 Library Construction Protocol

This protocol is designed for a total RNA input of 200ng with RIN ≥7. If a different amount of RNA input is used, please identify optimal adapter ligation and PCR reaction conditions by adjusting according to the recommended conditions in Appendix B and Appendix C. For library construction of FFPE RNA samples, please refer to Appendix E. For library construction of Pathogenic microorganism RNA samples, please refer to Appendix F.

3.1 RNA Enrichment

Select one of the following three RNA Enrichment methods based on your needs:

3.1.1 rRNA Depletion Kit

Please follow the rRNA Depletion Kit User Manual to perform rRNA depletion and proceed to step 3.2 RNA Fragmentation.

3.1.2 Dynabeads® mRNA Purification Kit for mRNA enrichment

⚠ **Note: Use non-stick tubes for the mRNA enrichment. Do not shake or vortex the sample in the following procedures. Please pipette gently to mix.**

3.1.2.1 Vortex magnetic beads provided in the mRNA Purification Kit for 1 minute to mix. Transfer 50 μL of beads to a new 1.5 mL non-stick tube. Place on the Magnetic Separation Rack for 2 minutes and remove the supernatant.

3.1.2.2 Remove the tube from the rack and add 50 μL of Binding Buffer to the non-stick tube containing beads. Pipette 10 times to mix thoroughly. Ensure that the solution and beads are fully dispensed from the pipette tip into the bottom of the tube. Place the tube on the Magnetic Separation Rack for 2 minutes and remove the supernatant.

3.1.2.3 Repeat step 3.1.2.2 once.

3.1.2.4 Add 25 μL of Binding Buffer to the tube and pipette 10 times to mix thoroughly.

3.1.2.5 Preheat the ThermoMixer to 65°C.

3.1.2.6 Add 200 ng (determined by the species and concentration of sample) of total RNA sample to a 1.5 mL non-stick tube and add enough NF Water to reach a total volume of 25 μL.

3.1.2.7 Place the tube on the ThermoMixer for denaturation for 5 minutes. When the reaction completes, immediately add 25 μL of resuspended beads to the sample and pipette 10 times to mix. Place

the non-stick tube on the bench and incubate at room temperature for 5 minutes to allow the RNA to bind to the beads. During the incubation, set the ThermoMixer to 80°C.

3.1.2.8 Place the tube on the Magnetic Separation Rack for 2 minutes. Remove and discard the supernatant using a pipette.

3.1.2.9 Remove the tube from the Magnetic Separation Rack. Add 50 µL of Washing Buffer to the non-stick tube containing beads. Pipette the beads and solution up and down 10 times to mix thoroughly. Place the tube on the Magnetic Separation Rack for 2 minutes, then remove the supernatant.

3.1.2.10 Repeat step 3.1.2.9 once.

3.1.2.11 Use 25 µL of 10 mM Tris-HCl from mRNA Purification Kit to resuspend beads and mix thoroughly. Incubate the tube on the ThermoMixer at 80°C for 2 minutes to elute mRNA from magnetic beads.

3.1.2.12 Immediately add 25 µL of Binding Buffer and pipette 10 times to mix thoroughly. Incubate at room temperature for 5 minutes. Place the tube on the Magnetic Separation Rack for 2 minutes and remove the supernatant.

3.1.2.13 Repeat step 3.1.2.9 twice.

3.1.2.14 Use 12 µL of 10 mM Tris-HCl from mRNA Purification Kit to resuspend magnetic beads. Heat the tube on the ThermoMixer at 80°C for 2 minutes to elute mRNA from magnetic beads. Immediately place the tube on the Magnetic Separation Rack for 1-2 minutes. Transfer 10 µL of the supernatant to a new PCR tube.

3.1.3 Library Preparation VAHTS mRNA Capture Beads for mRNA enrichment

3.1.3.1 Remove mRNA Capture Beads from 4°C and bring to room temperature.

3.1.3.2 Add 200 ng (determined by the species and concentration of the sample) of total RNA sample to a 1.5 mL non-stick tube and add enough NF Water to reach a total volume of 50 µL.

3.1.3.3 Vortex to mix mRNA Capture Beads thoroughly. Transfer 50 µL of beads into the tube containing the total RNA sample and pipette 10 times to mix thoroughly.

3.1.3.4 Place the tube on the ThermoMixer for denaturation at 65°C for 5 minutes.

3.1.3.5 Incubate at room temperature for 5 minutes to facilitate the binding of mRNA to beads. Set the ThermoMixer to 80°C.

3.1.3.6 Place the tube on the Magnetic Separation Rack for 5 minutes to separate mRNA from total RNA. Carefully remove the supernatant.

3.1.3.7 Remove the tube from the Magnetic Separation Rack. Add 200 μL of Beads Wash Buffer and pipette 10 times to mix thoroughly. Place the tube on the Magnetic Separation Rack for 5 minutes and carefully remove the supernatant.

3.1.3.8 Remove the sample from the Magnetic Separation Rack. Add 50 μL of Tris Buffer from mRNA enrichment kit to resuspend magnetic beads and pipette 10 times to mix thoroughly. Incubate the tube on the ThermoMixer at 80°C for 2 minutes to elute mRNA from beads.

3.1.3.9 Immediately add 50 μL of Beads Binding Buffer to the tube and pipette 10 times to mix thoroughly. Incubate at room temperature for 5 minutes to facilitate the binding of mRNA to beads.

3.1.3.10 Place the tube on the Magnetic Separation Rack for 5 minutes to separate mRNA from total RNA. Carefully remove the supernatant.

3.1.3.11 Remove the tube from the Magnetic Separation Rack. Add 200 μL of Beads Wash Buffer and pipette 10 times to mix thoroughly. Place the tube on the Magnetic Separation Rack for 5 minutes. Carefully remove the supernatant.

3.1.3.12 Add 12 μL of Tris Buffer from mRNA enrichment kit to resuspend the magnetic beads. Incubate the tube at 80°C for 2 minutes to elute mRNA from beads. Immediately place the tube on the Magnetic Separation Rack for 5 minutes. Transfer 10 μL of supernatant to a new PCR tube.

3.2 RNA Fragmentation

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

3.2.1 Add 4 μL of Fragmentation Buffer to 10 μL of RNA Elution product from step 3.1 and pipette 10 times to mix. Centrifuge briefly and place the tube into the thermocycler. Incubate according to the recommended fragmentation protocol for your target insert size. Use the table 4 as a guide.

Table 4 Recommended Conditions for RNA Fragmentation

Insert Size	RNA Fragmentation Temperature	RNA Fragmentation Time
150 bp	94°C	8min
250 bp	87°C	6min

3.2.2 When the reaction completes, immediately place the tube on ice for 2 minutes. Centrifuge for 10 seconds and immediately proceed to the Reverse Transcription step.

3.3 Reverse Transcription and Second Strand Synthesis

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

3.3.1 Remove the RT Buffer from -20°C and thaw it at room temperature. Invert several times to mix. Prepare the Reverse Transcription mixture on ice (see Table 5).

Table 5 Reverse Transcription Mixture

Components	Volume
RT Buffer	5 μL
RT Enzyme Mix	1 μL
Total	6 μL

3.3.2 Transfer 6μL of Reverse Transcription Mixture to the fragmentation product from step 3.2.2 using a pipette. Pipette 10 times to mix. Centrifuge briefly to collect the solution at the bottom of the tube.

3.3.3 Place the PCR tube from step 3.3.2 into the thermocycler and run the program in Table 6:

Table 6 Reverse Transcription Reaction Conditions

Temperature	Time
Heated lid	On
25°C	10 min
42°C	30 min
70°C	15 min
4°C	Hold

3.3.4 When the reaction completes, place the product on ice and centrifuge for 10 seconds.

3.3.5 Remove the Second Strand Buffer from -20°C and thaw at room temperature. Invert several times to mix. Prepare the Second Strand Synthesis mixture on ice (see Table 7).

Table 7 Second Strand Synthesis Mixture

Components	Volume
Second Strand Buffer	26 μL
Second Strand Enzyme Mix	4 μL
Total	30 μL

3.3.6 Transfer 30μL of the Second Strand Synthesis mixture to the Reverse Transcription product from step 3.3.4 using a pipette. Pipette 10 times to mix. Centrifuge briefly to collect the solution at the bottom of the tube.

3.3.7 Place the PCR tube from step 3.3.6 into the thermocycler and run the program in Table 8.

Table 8 Second Strand Synthesis Reaction Conditions

Temperature	Time
Heated lid	On
16°C	60 min
4°C	Hold

3.3.8 When the reaction completes, place the product on ice and centrifuge for 10 seconds. Transfer the Second Strand Synthesis product to a new 1.5 mL tube and place it on ice for the next reaction.

✔ **Stopping Point: Second strand synthesis product can be stored at -20°C for no more than 16 hours.**

3.4 Cleanup of Second Strand Synthesis Product

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

3.4.1 Remove DNA Clean Beads from the refrigerator and incubate at room temperature for 30 min before use. Vortex and mix thoroughly before use.

3.4.2 Use a pipette to transfer 75 μL of DNA Clean Beads to the Second Strand Synthesis product from step 3.3.8. Gently pipette at least 10 times to mix thoroughly. Ensure that all of the solution and beads are fully dispensed from the tip into the tube.

3.4.3 Incubate at room temperature for 5 minutes.

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

3.4.5 With the 1.5 mL tube on the Magnetic Separation Rack, add 200 μL of freshly prepared 80%

ethanol to the tube without disturbing the beads. Incubate 30 seconds. Carefully remove and discard the supernatant.

3.4.6 Repeat step 3.4.5 once. Remove all of the liquid from the tube without disrupting the beads. Centrifuge briefly to collect any remaining liquid at the bottom, separate beads magnetically and then remove any remaining liquid using a small volume pipette.

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

3.4.8 Remove the 1.5 mL tube from the Magnetic Separation Rack and add 42 µL of TE Buffer to elute the DNA. Gently pipette at least 10 times to mix thoroughly and centrifuge briefly.

3.4.9 Incubate at room temperature for 5 minutes.

3.4.10 Centrifuge briefly and place the 1.5 mL tube back onto the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Transfer 40 µL of supernatant to a new 0.2 mL PCR tube.

✔ **Stopping Point: Purified second strand synthesis product can be stored at -20°C overnight.**

3.5 End Repair and A-tailing

3.5.1 Prepare the End Repair and A-tailing mixture on ice (see Table 9).

Table 9 End Repair and A-tailing Mixture

Components	Volume
ERAT Buffer	7.1 µL
ERAT Enzyme Mix	2.9 µL
Total	10 µL

3.5.2 Use a pipette to transfer 10µL of End Repair and A-tailing mixture to the purified Second Strand Synthesis product from step 3.4.10. Gently pipette at least 10 times to mix thoroughly. Centrifuge briefly to collect the solution at the bottom of the tube.

3.5.3 Place the PCR tube from step 3.5.2 into the thermocycler and run the program in Table 10.

Table 10 End Repair and A-tailing Reaction Conditions

Temperature	Time
Heated lid	On
37°C	30 min
65°C	15 min
4°C	Hold

3.5.4 Centrifuge briefly to collect the solution at the bottom of the tube.

⚠ **Note: We do not recommend stopping at this point. Please continue to step 3.6. If the operation must be put on hold, the End Repair product can be stored at -20°C overnight with a risk of 20% decrease in yield.**

3.6 Adapter Ligation

⚠ **Note: The amount of Adapter used in Adapter Ligation depends on the amount of RNA input. Please read Appendix B carefully before you begin.**

3.6.1 Dilute the Adapter in a 1:10 dilution according to Table 11. Mix and centrifuge briefly for further use.

Table 11 Adapter Dilution

Components	Volume
Adapter	1 μL
TE Buffer	9 μL
Total	10 μL

3.6.2 Please refer to the instructions for MGIEasy DNA Adapters (see Appendix B). Add 5 μL of diluted DNA Adapters to the PCR tube from step 3.5.4. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube.

3.6.3 Prepare the Adapter Ligation reaction mixture on ice (see Table 12).

Table 12 Adapter Ligation Reaction Mixture

Components	Volume
Ligation Buffer	23.4 μL
DNA Ligase	1.6 μL
Total	25 μL

3.6.4 Pipette slowly to transfer 25 μL of Adapter Ligation reaction mixture to the PCR tube from step 3.6.2. Vortex 6 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube.

3.6.5 Place the PCR tube from step 3.6.4 into the thermocycler and run the following program in Table 13.

Table 13 Adapter Ligation Reaction Conditions

Temperature	Time
Heated lid	On
23°C	30 min
4°C	Hold

3.6.6 Centrifuge briefly to collect the solution at the bottom of the tube.

3.6.7 Add 20 µL of TE Buffer for a total volume of 100 µL and transfer the entire volume to a new 1.5 mL tube.

⚠ **Stopping Point: Adapter Ligation product can be stored at -20°C for a maximum of 16 hours.**

3.7 Cleanup of Adapter Ligation Product

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

If the insert size is 150bp (The conditions of RNA Fragmentation are 94°C 8min), please cleanup the Adapter Ligation product following the instructions below:

3.7.1 Remove DNA Clean Beads from the refrigerator and bring to room temperature for 30 min beforehand. Vortex and mix thoroughly before use.

3.7.2 Use pipette to transfer 50 µL of DNA Clean Beads to the Adapter Ligation product from step 3.6.7. Gently pipette at least 10 times to mix thoroughly. Ensure that all of the solution and beads are fully dispensed from the tip into the tube.

3.7.3 Incubate at room temperature for 5 minutes.

3.7.4 Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant using a pipette.

3.7.5 With the 1.5 mL tube on the Magnetic Separation Rack, add 200 µL of freshly prepared 80% ethanol to the tube without disturbing the beads. Incubate for 30 seconds. Carefully remove and discard the supernatant.

3.7.6 Repeat step 3.7.5 once. Remove all of the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom of the tube. Separate beads magnetically by replacing the tube on the Magnetic Separation Rack and then remove any remaining liquid using a small volume pipette.

3.7.7 Keep the 1.5 mL tube on the Magnetic Separation Rack with the lid open and allow the beads

to air-dry until no wetness (glossiness) is visible but before the pellet begins to crack.

3.7.8 Remove the 1.5 mL tube from the Magnetic Separation Rack. Add 23 µL of TE Buffer to elute the DNA. Gently pipette at least 10 times to mix thoroughly.

3.7.9 Incubate at room temperature for 5 minutes.

3.7.10 Centrifuge briefly and place the 1.5 mL tube on the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Transfer 21 µL of supernatant to a new 0.2 mL PCR tube.

✔ **Stopping Point: Purified adapter ligation product can be stored at -20°C.**

If the insert size is 250bp (The conditions of RNA Fragmentation are 87°C 6min), please follow the instructions below:

3.7.11 Perform step 3.7.1 to 3.7.7 above. In step 3.7.8, add 52 µL of TE Buffer to elute DNA. In step 3.7.10, transfer 50 µL of supernatant to a new 1.5 mL tube.

3.7.12 Transfer 32.5 µL of DNA Clean Beads into the tube containing 50 µL of supernatant from step 3.7.11. Gently pipette at least 10 times to mix thoroughly. Ensure that all of the solution and beads are fully dispensed from the tip into the tube.

3.7.13 Incubate at room temperature for 5 minutes.

⚠ **Note: In the next step, keep the supernatant and discard the beads.**

3.7.14 Centrifuge briefly and place the 1.5 mL tube onto a Magnetic Separation Rack for 2-5 min until the liquid becomes clear. Carefully transfer the supernatant to a new 1.5 mL tube.

3.7.15 Transfer 10 µL DNA Clean Beads to the supernatant from step 3.7.14. Gently pipette at least 10 times to mix thoroughly. Ensure that all of the solution and beads are fully dispensed from the tip into the tube.

3.7.16 Incubate at room temperature for 5 minutes.

3.7.17 Centrifuge briefly and place the 1.5 mL tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant using a pipette.

3.7.18 With the 1.5 mL tube on the Magnetic Separation Rack, add 200 µL of freshly prepared 80% ethanol to the tube without disturbing the beads. Incubate 30 seconds. Carefully remove and discard the supernatant.

3.7.19 Repeat step 3.7.18 once. Remove all of the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom, separate beads

magnetically and then remove the remaining liquid using a small volume pipette.

3.7.20 Keep the 1.5 mL tube on the Magnetic Separation Rack with the lid open and allow the beads to air-dry until no wetness (glossiness) is visible but before the pellet begins to crack.

3.7.21 Remove the 1.5 mL tube from the Magnetic Separation Rack. Add 23 µL of TE Buffer to elute DNA. Gently pipette at least 10 times to mix thoroughly.

3.7.22 Incubate at room temperature for 5 minutes.

3.7.23 Centrifuge briefly and place the 1.5 mL tube on the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Transfer 21 µL of supernatant to a new 0.2 mL PCR tube.

✔ **Stopping Point: Purified Adapter Ligation product can be stored at -20°C.**

3.8 PCR Amplification

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

3.8.1 Prepare the PCR Amplification mixture on ice (see Table 14)

Table 14 PCR Amplification Mixture

Components	Volume
PCR Enzyme Mix	25 µL
PCR Primer Mix	4 µL
Total	29 µL

3.8.2 Transfer 29 µL of PCR Amplification mixture to the PCR tube from step 3.7.23. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube.

3.8.3 Place the PCR tube from step 3.8.3 into the thermocycler and run the program in Table 15:

Table 15 PCR Amplification Reaction Conditions

Temperature	Time	Cycles
Heated lid	on	
95°C	3 min	1 cycle
95°C	30 seconds	
56°C	30 seconds	14 cycles
72°C	1 min	
72°C	5 min	1 cycle
4°C	Hold	

3.8.4 Centrifuge briefly to collect the solution at the bottom of the tube.

3.8.5 Transfer all the solution to a new 1.5 mL tube.

3.9 Cleanup of PCR Product

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

3.9.1 Remove DNA Clean Beads from the refrigerator and incubate at room temperature for 30 min. Vortex and mix thoroughly before use.

3.9.2 Transfer 60 µL of DNA Clean Beads into the tube containing 50 µL of PCR product from step 3.8.6. Gently pipette at least 10 times to mix thoroughly. Ensure that all of the solution and beads are fully dispensed from the tip into the tube.

3.9.3 Incubate at room temperature for 5 minutes.

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

3.9.5 With the 1.5 mL tube on the Magnetic Separation Rack, add 200 µL of freshly prepared 80% ethanol to the tube without disturbing the beads. Incubate for 30 seconds. Carefully remove and discard the supernatant.

3.9.6 Repeat step 3.9.5 once. Remove all of the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom of the tube, separate the beads magnetically and then remove the remaining liquid using a small volume pipette.

3.9.7 Keep the 1.5 mL tube on the Magnetic Separation Rack with the lid open and allow beads to air-dry until no wetness (glossiness) is visible but before the pellet begins to crack.

3.9.8 Remove the 1.5 mL tube from the Magnetic Separation Rack. Add 32 µL of TE Buffer to elute the DNA. Gently pipette at least 10 times to mix thoroughly.

3.9.9 Incubate at room temperature for 5 minutes.

3.9.10 Centrifuge briefly and place the 1.5 mL tube on the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Transfer 30 µL of supernatant to a new 1.5 mL tube.

✔ **Stopping Point: Purified PCR Products can be stored at -20°C.**

3.10 Quality Control of PCR Product

3.10.1 Quantify the purified PCR products with dsDNA Fluorescence Assay Kits such as the Qubit® dsDNA HS Assay Kit or the Quant-iT™ PicoGreen® dsDNA Assay Kit. Please follow the assay kit instructions to quantitate the purified PCR products. The required yield for PCR products is ≥ 1 pmol. See Table 16 for the corresponding yield (in ng) for different insert sizes.

Table 16 The Corresponding Mass Yield in 1 pmol for PCR Products with Different Insert Sizes

Insert Size (bp)	PCR product size (bp)	Corresponding Mass Yield in 1 pmol (ng)
150	230	152
250	330	218

3.10.2 Assess the fragment size distribution of purified PCR products using a capillary electrophoresis instrument such as a Bioanalyzer, Tapestation (Agilent Technologies), LabChip® GX, GXII, GX Touch (PerkinElmer) or Fragment Analyzer™ (Advanced Analytical).

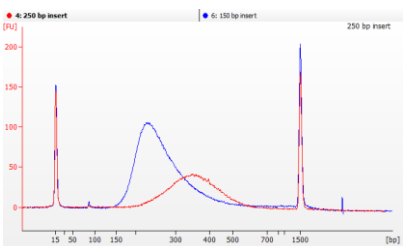

Figure 1 The Agilent 2100 Bioanalyzer Results of Purified PCR Product

⚠ **Note: If the library will be delivered to a service lab for sequencing, please stop here.**

⚠ **Note: If the library will be sequenced in your lab, please go to step 3.11.**

3.11 Denaturation

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

3.11.1 According to the PCR product size and table 16 or Formula 1 in Appendix D, 6-16 samples will

be multiplexed for circularization (Determine the number of pooling samples according to actual needs), please follow the instructions provided for the MGIEasy DNA Adapters Kit. See Appendix B for detailed information about how to plan your sample pooling. Quantify your purified PCR products before multiplexing. The total yield after multiplexing should be 1 pmol and add enough TE Buffer to reach a total volume of 48 μL. For example, if 150bp insert size libraries were prepared and 8 samples were multiplexed for equal data amount, 19 ng of the PCR product of each sample were multiplexed equally with a total of 152 ng into a new 0.2 mL PCR tube. Add enough TE Buffer to reach a total volume of 48 μL.

3.11.2 Place the PCR tube from step 3.11.1 into the thermocycler and run the program in Table 17.

Table 17 Denaturation Reaction Conditions

Temperature	Time
Heated lid	On
95°C	3 min

3.11.3 After the reaction is complete, immediately place the tube on ice for 2 minutes and centrifuge briefly.

3.12 Single Strand Circularization

3.12.1 Prepare the Single Strand Circularization reaction mixture on ice (see Table 18).

Table 18 Single Strand Circularization Reaction Mixture

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

3.12.2 Transfer 12.1 μL of Single Strand Circularization mixture to the PCR tube from step 3.11.3. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube.

3.12.3 Place the PCR tube into the thermocycler and run the program in Table 19.

Table 19 Single Strand Circularization Reaction Conditions

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

3.12.4 After the reaction is complete, immediately place the tube on ice for the next reaction.

3.13 Enzymatic Digestion

3.13.1 Prepare the Enzymatic Digestion mixture (see Table 20) on ice during the reaction in step 3.12.3.

Table 20 Enzymatic Digestion Mixture

Components	Volume
Digestion Buffer	1.4 µL
Digestion Enzyme	2.6 µL
Total	4.0 µL

3.13.2 Transfer 4 µL of Enzymatic Digestion mixture into the PCR tube from step 3.12.4. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube.

3.13.3 Place the PCR tube from step 3.13.2 into the thermocycler and run the program in Table 21.

Table 21 Enzymatic Digestion Reaction Conditions

Temperature	Time
Heated lid	On
37°C	30 min

3.13.4 Centrifuge briefly to collect the solution at the bottom of the tube.

3.13.5 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 at the bottom of the tube. Transfer all of the solution into a new 1.5 mL tube.

3.14 Cleanup of Enzymatic Digestion Product

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

3.14.1 Remove DNA Clean Beads from the refrigerator and allow 30 minutes for the solution to come to room temperature. Vortex and mix thoroughly before use.

3.14.2 Transfer 170 µL of DNA Clean Beads to the Enzymatic Digestion product from step 3.13.5. Gently pipette at least 10 times to mix thoroughly. Ensure that all of the solution and beads are fully dispensed from the tip into the tube.

3.14.3 Incubate at room temperature for 10 minutes.

3.14.4 Centrifuge briefly and place the 1.5 mL tube on the Magnetic Separation Rack for 2-5 minutes

until liquid becomes clear. Carefully remove and discard the supernatant using a pipette.

3.14.5 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 seconds. Carefully remove and discard the supernatant.

3.14.6 Repeat step 3.14.5 once. Remove all of the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom of the tube, separate beads magnetically, then remove the remaining liquid using a small volume pipette.

3.14.7 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.

3.14.8 Remove the 1.5 mL tube from the Magnetic Separation Rack. Add 22 µL of TE Buffer to elute DNA. Gently pipette the entire volume up and down 10 times to mix thoroughly.

3.14.9 Incubate at room temperature for 10 minutes.

3.14.10 Centrifuge briefly. Place the 1.5 mL tube on the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Transfer 20 µL of supernatant to a new 1.5 mL tube. Take care to not disturb the beads.

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

3.15 Quality Control of Enzymatic Digestion Product

Quantify the purified Enzymatic Digestion product with a Qubit® ssDNA Assay Kit. The final yield should be ≥ 80 fmol (enough for two sequencing runs). Please refer to Table 22 or Formula 2 in Appendix D for your calculations.

Table 22 The Corresponding Mass Yield in 80 fmol for Different PCR Product Size (Circularized ssDNA)

Insert Size (bp)	PCR Product Size (bp)	Corresponding Mass Yield in 80 fmol (ng)
150	230	6.07
250	330	8.71

Appendix

Appendix A Magnetic Beads and Cleanup Procedures

For magnetic bead-based purification, we recommend using the DNA Clean Beads included in the MGIEasy DNA Clean Beads Kit (MGI, Cat. No1000005278 or 1000005279) or AMPure® XP (Agencourt, Cat. No. A63882) (not provided). Magnetic beads from other sources may yield unexpected results.

Before You use:

- To ensure capture efficiency of the Magnetic Beads, remove beads from 4°C storage and allow 30 minutes for the solution to come to room temperature. 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:

- If the sample volume decreases due to evaporation during incubation, add TE buffer to reach the designated volume before using beads to purify. This ensures that the correct multiplier for the beads is applied.
- During the magnetic separation step, please allow the solution to become completely clear before removing the supernatant. This process takes approximately 2-3 minutes. 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 fluid can be left in the tube to avoid contact. In the event of contact between the beads and the pipette tip, expel all of the solution and beads back into the tube and restart the separation process.
- Use freshly prepared 80% ethanol (at room temperature) to wash the beads. The 1.5 mL tube should remain on the Magnetic Separation Rack while washing with ethanol. Do not shake or disturb the beads in any way.
- After the 2nd washing of beads with ethanol, try to remove all of the liquid within the tube. You may centrifuge briefly to collect any remaining liquid at the bottom of the tube. Separate beads magnetically and remove the remaining liquid by using a small volume pipette.
- After washing twice with ethanol, air-dry the beads at room temperature. Insufficient drying (visible by a reflective surface) will cause Anhydrous Ethanol to deposit, affecting subsequent reactions. Over-drying (pellet cracks) may cause a reduction in yield. Drying takes approximately 5-10 minutes

depending on your specific lab environment. Watch closely until the pellet appears sufficiently dry with a matte appearance and continue to the elution process with TE Buffer.

- Avoid disturbing the beads when removing the supernatant. Contamination from the beads may affect subsequent reactions. Therefore, the total volume of TE Buffer and beads should be 2 μL more than the volume of the supernatant.
- Be attentive when opening / closing the lids of the 1.5 mL tubes on the Separation Rack. Strong vibrations may cause sample loss by spilling liquid or beads from the tubes. Secure the tubes well before opening with the lids.

Appendix B Using Barcode Adapters

- Two specifications of Adapter Reagent Kit are offered based on the number of reactions, the MGIEasy DNA Adapters-16 (Tube) Kit and the MGIEasy DNA Adapters-96 (Plate) Kit. Both kits were developed to meet requirements for batch processing of library construction and multiplex sequencing. We selected the best adapter combinations based on the principle of balanced base composition. However, not all barcode adapter combinations are compatible. For optimal performance, please refer to instructions in Appendix B-1 and B-2. Please note that Adapters from the two Kits above contain overlapping Barcodes and cannot be sequenced in the same lane.
- Please do not incubate the Adapters above room temperature to avoid structural changes such as denaturation which might affect performance.
- Before use, please mix the adapters by vortexing and centrifuge to collect the liquid at the bottom of the tube. For the MGIEasy DNA Adapters-16 (Tube) Kit, gently remove the cap to prevent spills and cross-contamination. After use, please close the cap immediately. For the MGIEasy DNA Adapters-96 (Plate) Kit, pierce the film to pipette solutions for first use. After use, please transfer the remaining reagents to individual 1.5 mL tubes or 0.2 mL PCR tubes, label and store at -20°C.
- Adapters from other MGI library kits (number 501-596) are designed differently and are incompatible for mixed use. Doing so will cause errors in barcode demultiplexing during data extraction.
- Adapter quality as well as quantity directly affect the efficiency and quality of the library construction. An excessive input of adapters may cause Adapter Dimers. Insufficient input may lower library yield and lower construction efficiency.

Table 23 Recommended Adapter Input according to the Amount of total RNA

RNA Sample (ng)	MGI Adapter	MGI Adapter
	Dilution Ratio	Input after Dilution (μL)
201-2500	5	5
51-200	10	5
10-50	20	5

- For other amounts of RNA sample input, please adjust the Adapter input appropriately.

B-1 MGIEasy DNA Adapters-16 (Tube) Kit Instruction

- Based on the principles of balanced base composition, Adapters must be used in specific combinations. Please follow the instructions bellow to use Adapters in proper combination:

  2 sets of 4 Adapters: (01-04) and (13-16)

  1 set of 8 Adapters: (97-104)

- Assuming data output requirement is the same for all samples in a lane, please refer to the Table 24 to organize your Barcode Adapter combinations:

Table 24 MGIEasy DNA Adapters-16 (Tube) Kit Instruction

Sample(s)/lane	Instructions (Example)
1	Requires at least 1 set of Adapters: 1. Take a set of 4 Adapters (01-04), mix all 4 Adapters in equal volume and add the mixture to the sample. Or 2. Take a set of 8 Adapters (97-104), mix all 8 Adapters in equal volume and add the mixture to the sample.
2	Requires at least 1 set of Adapters: 1. Take a set of 4 Adapters (01-04), mix equal volumes in pairs to obtain 2 mixtures of equal volume. Add 1 mixture to each sample. (e.g. Mix 01 & 02, then add to sample 1; Mix 03 & 04, then add to sample 2) Or 2. Take a set of 8 Adapters (97-104), mix equal volumes in groups of 4 to obtain 2 mixtures of equal volume. Add 1 mixture to each sample. (e.g. Mix 97-100, then add to sample 1; Mix 101-104, then add to sample 2)
3	Requires at least 2 sets of Adapters: For sample 1&2, use the method for (2 samples/lane) above. For sample 3, use the method for (1 sample/lane) above. Note that you should use different Adapter sets for samples 1- 2 and for sample 3.
4	Requires at least 1 set of Adapters: 1. Take a set of 4 Adapters (01-04), add each Adapter for each sample in equal volumes. (e.g. Add Adapters 01, 02, 03, 04 to samples 1, 2, 3, 4, respectively.) Or 2. Take a set of 8 Adapters (97-104), mix equal volumes in pairs to obtain 4 mixtures of equal volume. Add one mixture to each sample. (e.g. Mix 97-98, 99-100, 101-102, 103-104, then add respectively to samples 1, 2, 3, 4.)

5	Requires at least 2 Adapter sets: For samples 1-4, use the method for (4 samples/lane) above. For sample 5, use the method for (1 sample/lane) above. Note that you should use different Adapter sets for samples 1-4 and for sample 5.
6	Requires at least 2 Adapter sets: For samples 1-4, use the method for (4 samples/lane) above. For samples 5-6, use the method for (2 samples/lane) above. Note that you should use different Adapter sets for sample 1-4 and 5-6.
7	Requires all 3 Adapter sets, follow these 3 steps: 1) For samples 1-4, use the method for (4 samples/lane) above. (Use 1st Adapter set) 2) For samples 5-6, use the method for (2 samples/lane) above. (Use 2nd Adapter set) 3) For sample 7, use the method for (1 sample/lane) above. (Use 3rd Adapter set) Or add a single Adapter of the set to sample 7. Note that you should use different Adapter sets for samples 1-4, for sample 5-6 and for sample 7.
8	Requires at least 1 set of Adapter: 1. Take a set of 8 Adapters (97-104), add each Adapter for each sample in equal volume. Or 2. Take 2 sets of 4 Adapters (01-04 and 13-16), add one Adapter to one sample in equal volume.

- For situations in which the data output requirements are different between samples, any sample with a data output of more than 20% for each lane must use a separate set of Adapters. (e.g. 9 samples are pooled into 1 lane, one of which requires 30% of the total data output. In this case, the other 8 samples may use Adapters (97-104), whereas the final sample must use a full Adapter set of 01-04 or 13-16 instead of using only a single Adapter.)

B-2 MGIEasy DNA Adapters-96 (Plate) Kit Instruction

- Based on the principles of balanced base composition, Adapters must be used in specific groups. Please follow the instructions bellow to use Adapters in proper combination:

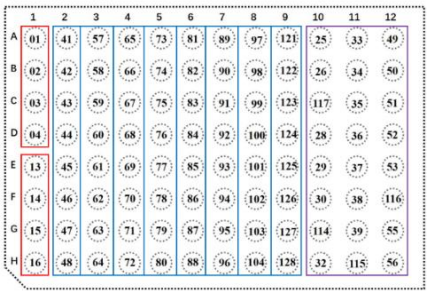

Figure 2 MGIEasy DNA Adapters-96 (Plate) Adapters

*2 sets of 4 Adapters: Column 1 (01-04, 13-16) (see the red box in Figure 2)

*8 sets of 8 Adapters: Column 2-9 (41-48, 57-64, 65-72, 73-80, 81-88, 89-96, 97-104, 121-128) (see the blue box in Figure 2)

*1 set of 24 Adapters: Column 10-12 (see the purple box in Figure 2)

- Assuming data output requirement is the same for all samples in a lane, please refer to the Table 25 to organize your Barcode Adapter combinations:

Table 25 MGIEasy DNA Adapters-96 (Plate) Kit Instruction

Sample(s)/lane	Instructions (Example)
1	1. Take a set of 4 Adapters (01-04), mix equal volumes, then add to the sample. Or 2. Take a set of 8 Adapters (41-48), mix equal volumes, then add to the sample.
2	1. Take a set of 4 Adapters (01-04), mix equal volumes in pairs to obtain 2 mixtures of equal volume. Add 1 mixture to each sample. (e.g. Mix 01 & 02, then add to sample 1; Mix 03 & 04, then add to sample 2) Or 2. Take a set of 8 Adapters (41-48), mix equal volumes in groups of 4 to obtain 2

	mixtures of equal volume. Add 1 mixture to each sample. (e.g. Mix 41-44, then add to sample 1; Mix 45-48, then add to sample 2)
3	For sample 1&2, use the method for (2 samples/lane) above. For sample 3, use the method for (1 sample/lane) above. Note that you should use different Adapter sets for sample 1,2 and 3.
4	1. Take a set of 4 Adapters (01-04), add 1 Adapter for each sample in equal volumes. (e.g. Respectively add Adapters 01, 02, 03, 04 to samples 1, 2, 3, 4.) Or 2. Take a set of 8 Adapters (41-48), mix equal volumes in pairs to obtain 4 mixtures of equal volume. Add 1 mixture to each sample. (e.g. Mix 41-42, 43-44, 45-46, 47-48, then add respectively to samples 1, 2, 3, 4.)
5	For samples 1-4, use the method for (4 samples/lane) above. For sample 5, use the method for (1 sample/lane) above. Note that you should use different Adapter sets for sample 1-4 and 5.
6	For samples 1-4, use the method for (4 samples/lane) above. For sample 5-6, use the method for (2 sample/lane) above. Note that you should use different Adapter sets for sample 1-4 and 5-6.
7	1) For samples 1-4, use the method for (4 samples/lane) above. (Use 1st Adapter set) 2) For samples 5-6, use the method for (2 samples/lane) above. (Use 2nd Adapter set) 3) For sample 7, use the method for (1 sample/lane) above. (Use 3rd Adapter set) Note that you should use different Adapter sets for sample 1-4, sample 5-6 and sample 7.
8	Take a set of 8 Adapters (41-48), add 1 Adapter for each sample in equal volumes.
8n+x (n=1,2, x=1-8, Total 9-24)	Follow these 3 steps: 1) For samples 1-8, use the method for (8 samples/lane) above. Or separate into 2 groups of 4 and use the method for (4 samples/lane) above for each group. 2) For samples 9-8n, separate samples into groups of 8, and use the method for (8 samples/lane) above. 3) For samples 8n+1 - 8n+X, according to the value of X, use the methods above for 1-8 sample/lane accordingly. Remember to use separate Adapter sets. Note that you should use different Adapter sets for steps 1), 2) and 3).

8n+x (3≤n<11, x=1-8, Total 25-96)	Follow these 3 steps: 1) For samples 1-24, take a set of 24 Adapters and add 1 Adapter for each sample in equal volumes. 2) For samples 25-8n, separate the samples into groups of 8, and use the method for (8 samples/lane) above. 3) For samples 8n+1 - 8n+X, according to the value of X, use the methods above for 1-8 sample/lane accordingly. Remember to use separate Adapter sets. Note that you should use different Adapter sets for steps 1), 2) and 3).

- For situations in which the data output requirements are different between samples, any sample with a data output of more than 20% for each lane must use a separate set of Adapters. (E.g. 9 samples are pooled into 1 lane, one of which requires 30% of the total data output. In this case, the other 8 samples may use Adapters (97-104), whereas the final sample must use a full Adapter set instead of using only a single Adapter. (e.g. Adapter set (01-04) or (13-16) or any other sets, with the exception of (97-104).)

Appendix C Adapter Ligation and PCR

- The Adapter Reaction mixture contains a high concentration of PEG which increases the viscosity of the mixture. Please pipette slowly and ensure that the correct amount has been used.
- The number of PCR cycles must be strictly controlled. Insufficient cycles may lead to a reduced library yield. Excessive cycles may also lead to adverse effects, such as over amplification and increases in bias, PCR duplicates, chimeric sequences, and accumulated mutations. Table 26 describes the number of PCR cycles required to yield 300 ng of library from 10-1000 ng high quality total RNA sample (150 bp). For lower quality, longer DNA fragments, more PCR cycles should be added to generate a sufficient yield.

Table 26 PCR Cycles required to yield 300 ng of PCR product

total RNA (ng)	PCR Cycles required for corresponding yield
	≥300 ng
10	17-18
50	15-16
200	13-14
1000	11-12

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 a dsDNA sample with varying fragment sizes. Please refer to Formula 1 to calculate the amount of DNA needed.

Formula 1 dsDNA sample pmol to ng Conversion

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

The yield for circularized ssDNA after cleanup must be at least 80 fmol or higher for two sequencing runs. Please refer to Formula 2 below to calculate the number of mols needed:

Formula 2 Circular ssDNA fmol to ng Conversion:

$$\text{Mass (ng) corresponding to 80 fmol circular ssDNA} = 0.08 \times \frac{\text{DNA Fragment Size (bp)}}{\text{1000 bp}} \times 330 \text{ ng}$$

Appendix E Library Construction from Low Quality FFPE Sample

This procedure is used for low-quality total RNA samples such as FFPE. However, due to the large differences between the quality of different FFPE samples, it is not guaranteed that libraries can be successfully prepared from all FFPE samples. The following instructions take the library construction from the "MGIEasy RNA Library Prep Kit" as an example and list the problems that you need to address in the library construction to account for different quality FFPE samples.

E-1. Quality Evaluation of FFPE Sample

The RIN value is the most common parameter for the evaluation of RNA quality. However, the RIN value cannot accurately assess the quality of the degraded FFPE samples. In particular, in the NGS library construction, the FFPE samples' RIN value is not always proportional to the overall success rate of library construction. Therefore, DV200 is also used for assessing the success rate of library construction from FFPE samples. The DV200 indicates the proportion of RNA fragments larger than 200 nucleotides in the sample. For severely degraded FFPE samples, the DV200 value is a reliable indicator of the sample quality.

The calculation of DV_{200}

Here is an Agilent 2100 Bioanalyzer result as an example for the DV_{200} calculation. The detailed calculation is shown in Figure 3.

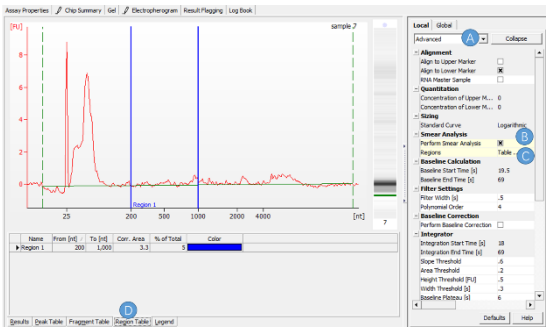

Figure 3 The Calculation of DV_{200}

A: In the Agilent 2100 Bioanalyzer result interface, choose *Advanced* under the *Local* tab

B: Check the Perform Smear Analysis option under *Smear Analysis*

C: Double-click on *Table* to enter the range of fragments to be calculated. The figure shows a range from 200 nt to 1000 nt.

D: Obtain the proportion of selected fragments shown as *% of Total* in the *Region Table*

If you need to determine the DV_{200} of a FFPE sample, perform the Agilent 2100 Bioanalyzer analysis (using the RNA analysis chip) on the FFPE sample, and calculate DV_{200} according to the method above. For detailed information please see *DV200 determination for FFPE RNA samples.* (https://www.agilent.com/en/promotions/dv200-determination)

E-2. Recommended Amount of FFPE Sample Input

Please use rRNA depleted RNA for NGS library construction. In the "RNA Fragmentation", we recommend using different conditions of RNA Fragmentation for different samples. In the "Cleanup of Second Strand Synthesis Product" section, please use 100 μL of Beads for cleanup. In the "Adapter Ligation" section, pay attention to the amount of Adapter. In the "PCR Amplification", note the corresponding different number of PCR cycles for different DNA sample inputs. See Table 27 and 28 for detailed conditions.

Table 27. Recommended Conditions of Library Construction from FFPE Sample

FFPE DV_{200}	Recommended amount of total RNA input	RNA Fragmentation	Beads for second strand synthesis cleanup	PCR cycles
> 70%	200 ng	94°C, 8 min	100 μL Beads	14
50-70%	200-400 ng	94°C, 8 mins	100 μL Beads	16
30-50%	500 ng	94°C, 6 min	100 μL Beads	16
< 30%	0.5-1 μg (with a risk of failure of library construction)	No fragmentation	100 μL Beads	16

Table 28. Recommended Adapter of Library Construction from FFPE Sample

FFPE DV_{200}	Recommended amount of total RNA input	MGI Adapter	MGI Adapter
		Dilution Ratio	Input after Dilution (μL)
> 70%	200 ng	5	5
50-70%	200-400 ng	10	5
30-50%	500 ng	20	5
< 30%	0.5-1 μg (with a risk of failure of library construction)	50	5

E-3. The Workflow of Library Construction form FFPE Sample

E-3.1 RNA Enrichment

Use the rRNA Depletion Kit. Please follow the instructions provided in the rRNA Depletion Kit User Manual to enrich RNA.

E-3.2 RNA Fragmentation

Refer to Table 27 to set up different conditions for RNA Fragmentation for samples with different levels of degradation. If fragmentation is not required for RNA Enrichment product, please perform the following steps. Add 4 μL of Fragmentation Buffer to a new 0.2 mL PCR tube. Incubate the RNA Enrichment product and the PCR tube at 65°C for 5 minutes. Immediately place the sample and PCR tube on ice for 2 minutes and centrifuge for 10 seconds for further use. Transfer 4 μL of Fragmentation Buffer to the sample and immediately proceed to the next step, Reverse Transcription.

E-3.3 Reverse Transcription and Second Strand Synthesis

Same as step 3.3.

E-3.4 Cleanup of Second Strand Synthesis Product

Refer to step 3.4.

Refer to Table 27. Use 100 μL of beads to cleanup. Add 42 μL of TE Buffer to elute DNA and finally transfer 40 μL of supernatant to a new 0.2 mL PCR tube.

E-3.5 End Repair and A-tailing

Same as step 3.5.

E-3.6 Adapter Ligation

Refer to step 3.6.

Refer to Table 28. Use a different amount of Adapter for different FFPE samples.

E-3.7 Cleanup of Adapter Ligation Product

Same as step 3.7.1 to 3.7.10.

E-3.8 PCR Amplification

Refer to step 3.8.

Refer to Table 27. Use different numbers of PCR cycles for different FFPE samples.

E-3.9 Cleanup of PCR Product to Quality Control of Enzymatic Digestion Product

Same as step 3.9-3.15.

Appendix F Library Construction from RNA pathogen sample

F-1. Applicable types of RNA pathogen samples

The kit is suitable for the detection of RNA pathogenic microorganisms from human whole blood and intestinal samples.

F-2. Recommended Amount of RNA pathogen Sample Input

The recommended amount of RNA pathogen sample input is 200 ng. The rRNA of human whole blood or intestinal samples need to be removed with MGIEasy rRNA Depletion kit V1.1.

F-3. The Workflow of Library Construction from RNA pathogen samples

F-3.1 RNA Enrichment

Use the rRNA Depletion Kit to remove the rRNA of human whole blood or intestinal samples. Please follow the instructions provided by the rRNA Depletion Kit User Manual to enrich RNA.

F-3.2 RNA Fragmentation

The RNA sample is incubated at 94 ° C for 8 min according to the conditions for 150 bp in Table 4.

F-3.3 Reverse Transcription and Second Strand Synthesis

Same as step 3.3.

F-3.4 Cleanup of Second Strand Synthesis Product

Same as step 3.4.

F-3.5 End Repair and A-tailing

Same as step 3.5.

F-3.6 Adapter Ligation

Same as step 3.6.

F-3.7 Cleanup of Adapter Ligation Product

Same as step 3.7.1 to 3.7.10.

F-3.8 PCR Amplification

Refer to step 3.8. For rRNA depleted samples, do 15 cycles of PCR to amplify the sample.

F-3.9 Cleanup of PCR Product

Same as step 3.9.

F-3.10 Quality Control of PCR Product

Quantify the purified PCR products with dsDNA Fluorescence Assay Kits such as Qubit® dsDNA HS Assay Kit or Quant-iT™ PicoGreen® dsDNA Assay Kit. Please follow the assay kit instructions to quantitate the purified PCR products. The required yield for PCR products is ≥ 1 pmol. Table 16 shows the corresponding yield for different insert sizes. For multiplex sequencing, please follow the instructions provided for the MGIEasy DNA Adapters Kit. See Appendix B for detailed information about how to plan your sample pooling. Quantify your purified PCR products before multiplexing. The total yield after multiplexing should be 1 pmol, with a total volume ≤ 48 μL.

Assess the fragment size distribution of the purified PCR products by using a capillary electrophoresis instrument such as the Bioanalyzer, Tapestation (Agilent Technologies), LabChip® GX, GXII, GX Touch (PerkinElmer) or Fragment Analyzer™ (Advanced Analytical).

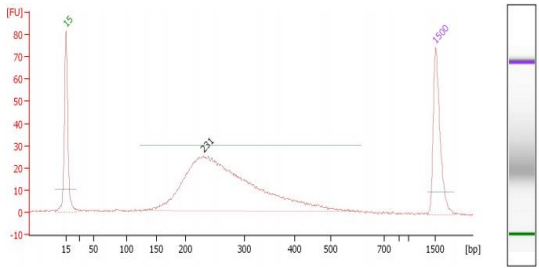

Figure 4 The Agilent 2100 Bioanalyzer Results of Purified PCR Product

The standards of library quality control are shown in Table 29. For the library not met the quality control requirements has a risk failing in the sequencing run.

Table 29 Standards of library quality control

QC	MGIEasy RNA Library Prep Set	Standards of library QC
PCR product yield	Quantify using Qubit dsDNA HS	≥ 200 ng
PCR product size	Agilent 2100 chip inspection	Main peak ： 210-250 bp，average:150-500bp
Adaptor residue	Agilent 2100 chip inspection	Visual observation，No obvious peak around 80bp

F-3.11 Denaturation to Quality Control of Enzymatic Digestion Product

Same as step 3.11-3.15.

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