MGIEasy

RNA Directional Library Prep Set User Manual

Cat. No.: 1000006385 (16 RXN) , 1000006386 (96 RXN)

Kit Version: V2.1

Manual Version: 5.0

Revision History

Manual Version	Kit Version	Date	Description
5.0	V2.1	Mar. 2022	◆ Update Manufacturer LOGO.
A3	V2.1	Jan. 2021	◆ Update contact information.
A2	V2.1	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 V2.1
A1	V2.0	Sep. 2019	◆ 1.3 Add DNBSEQ series sequencing platform ◆ 2.2 Add $OD_{260/230}$ requirement
A0	V2.0	Nov. 2018	◆ Initial release.

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

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Chapter 1 Product Description

1.1 Introduction

MGIEasy RNA Directional Library Prep Set is specifically designed for rapid library preparation for MGI high-throughput sequencing platforms. The library prep kit is optimized to convert 10 ng - 1 μg of eukaryotic total RNA into a single strand circularized DNA library for MGI sequencing platforms. Compared to the MGIEasy RNA Library Prep Kit, the library prepared using this set has accurate retention of transcript strand of origin information. This set can be used with rRNA Depletion kit to generate directional RNA library for non-coding RNA analysis. All reagents provided within this set have passed stringent quality control and functional verification procedures, ensuring performance stability and reproducibility.

1.2 Application

This library prep set is applicable to 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 can be expected.

1.3 Platform Compatibility

Constructed libraries are compatible with:

BGISEQ-500 (PE50/PE100/PE150)

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

1.4 Contents

MGIEasy RNA Directional 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 Directional Library Prep Set (16 RXN) (Cat. No.: 1000006385)

Modules and Cat. No.	Components	Color-Coded Screw Cap	Spec& Quantity
MGIEasy RNA Directional Library Prep Kit Cat. No.: 1000005270	Fragmentation Buffer	Green	93 μL/tube× 1 tube
	Directional RT Buffer 1	Green	88 μL/tube× 1 tube
	Directional RT Buffer 2	Brown	5 μL/tube× 1 tube
	RT Enzyme Mix	Green	24 μL/tube× 1 tube
	Directional Second Strand Buffer	Yellow	470 μL/tube× 1 tube
	Second Strand Enzyme Mix	Yellow	78 μL/tube× 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
	UDG	Blue	21 μL/tube× 1 tube
MGIEasy DNA Adapters-16 (Tube) Kit Cat. No.: 1000005284	DNA Adapters	White	10 μL/tube× 16 tubes
MGIEasy DNA Clean Beads Cat. No.: 1000005278	DNA Clean Beads	White	8 mL/tube× 1 tube
	TE Buffer	White	4 mL/tube× 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 Directional Library Prep Set (96 RXN) (Cat. No.: 1000006386)

Modules and Cat. No.	Components	Color-Coded Screw Cap	Spec& Quantity
MGIEasy RNA Directional Library Prep Kit Cat. No.: 1000005272	Fragmentation Buffer	Green	608 μL/tube× 1 tube
	Directional RT Buffer 1	Green	608 μL/tube× 1 tube
	Directional RT Buffer 2	Brown	20 μL/tube× 1 tube
	RT Enzyme Mix	Green	136 μL/tube× 1 tube
	Directional 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
	UDG	Blue	108 μL/tube× 1 tube
MGIEasy DNA Adapters-96 (Plate) Kit Cat. No.: 1000005282	DNA Adapters	White	10 μL/well× 96 wells
MGIEasy DNA Clean Beads Cat. No.: 1000005279	DNA Clean Beads	White	50 mL/tube× 1 tube
	TE Buffer	White	25 mL/tube× 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 Directional Library Prep Kit

- Storage Temperature: -25°C--15°C.
- Expiration Date: refer to the label.
- Transport Conditions: transported on dry ice.

MGIEasy DNA Adapters Kit

- Storage Temperature: -25°C--15°C.
- Expiration Date: refer to the label.
- Transport Conditions: transported on dry ice.

MGIEasy DNA Clean Beads

- Storage Temperature: 2°C-8°C.
- Expiration Date: refer to the label.
- Transport Conditions: transported with an ice pack.

MGIEasy Circularization Module

- Storage Temperature: -25°C--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 are 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 Warnings

- Instructions provided in this manual are intended for general use only, and may require further adjustments to optimize 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, let thaw at 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 Heated lid would be 105°C if there is no special illustration.
- 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 cleaning working 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 applicable to 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 total RNA input of 10 ng - 1 μg. For low-abundance mRNA species such as plants, it is recommended to use total RNA input of 1 - 2.5 μg.

2.2 Sample Quality Control

- Use Agilent 2100 Bioanalyzer to perform quality control of extracted total RNA sample. RIN value should be ≥7. If RIN< 7, use 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 sample, please refer to Appendix E.
- RNA integrity: $OD_{260/280}$=1.8~ 2.0, $OD_{260/230}$≥2.
- If DNA contamination is found in RNA sample, perform DNase I digestion to remove DNA before proceeding to the next procedures.

Chapter 3 Library Construction Protocol

This protocol is designed for 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 the recommended conditions in Appendix C.

3.1 RNA Enrichment

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

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 in the mRNA enrichment. Do not shake or vortex the sample in the following procedures. Please gently pipette to mix.**

3.1.2.1 Vortex magnetic beads provided in the mRNA Purification Kit for 1 minute to mix. Transfer 50 μL 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. Ensure that the solution and beads are fully dispensed from the pipette tip into the bottom of the tube. Pipette 10 times to mix thoroughly. Place the tube on the Magnetic Separation Rack for 2 minutes and remove the supernatant.

3.1.2.3 Repeat step3.1.2.2 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) total RNA sample to a 1.5 mL non-stick tube and add NF Water to 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 and remove 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 all beads and solution up and down 10 times to mix thoroughly. Place the tube on the Magnetic Separation Rack for 2 minutes and remove the supernatant.

3.1.2.10 Repeat step3.1.2.9 once.

3.1.2.11 Use 25 μL of 10 mM Tris-HCl 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 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 sample species) total RNA sample to a 1.5 mL non-stick tube and add NF Water to a total volume of 50 μL.

3.1.3.3 Vortex to mix mRNA Capture Beads thoroughly. Transfer 50 μL beads to 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 up 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 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 to resuspend 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 supernatant to a new PCR tube.

3.2 RNA Fragmentation

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

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

Table 4 The Recommended Conditions of RNA Fragmentation

Insert Size	Temperature of RNA Fragmentation	Time of RNA Fragmentation
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.

3.3 Reverse Transcription and Second Strand Synthesis

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

3.3.1 Remove Directional RT Buffer 2 from -20°C and thaw it at room temperature. Vortex to mix and centrifuge briefly to collect the solution to the bottom of the tube. Dilute Directional RT Buffer 2 for 17 times following Table 5.

Table 5 Dilution of Directional RT Buffer 2

Components	Volume
Directional RT Buffer 2	1 μL
NF water	16 μL
Total	17 μL

⚠ **Note: The diluted Directional RT Buffer 2 should be used immediately. Please discard any unused diluent. Prepare fresh diluted Directional RT Buffer 2 for each library construction.**

3.3.2 Remove Directional RT Buffer 1 from -20°C and thaw it at room temperature. Invert several times to mix. Prepare the Reverse Transcription mixture on ice (see Table 6):

Table 6 Reverse Transcription Mixture

Components	Volume
Directional RT Buffer 1	4 μL
Diluted Directional RT Buffer 2	1 μL
RT Enzyme Mix	1 μL
Total	6 μL

3.3.3 Transfer 6μL of Reverse Transcription Mixture to the RNA Fragmentation product in step 3.2.2 using a pipette. Pipette 10 times to mix. Centrifuge briefly to collect the solution at the bottom of the tube.

3.3.4 Place the PCR tube in step 3.3.3 into the thermocycler and run the following program in Table 7:

Table 7 The Reaction Conditions of Reverse Transcription

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

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

3.3.6 Remove Directional 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 8):

Table 8 Second Strand Synthesis Mixture

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

3.3.7 Transfer 30μL Second Strand Synthesis mixture to the Reverse Transcription product in step 3.3.5. Pipette 10 times to mix and centrifuge briefly to collect the solution at the bottom of the tube.

3.3.8 Place the PCR tube in step 3.3.7 into the thermocycler and run the following program in Table 9:

Table 9 The Reaction Conditions of Second Strand Synthesis

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

3.3.9 When the reaction completes, place the product on ice and centrifuge for 10 seconds. Transfer all 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 no more than 16 h.**

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 bring to room temperature for 30 min beforehand. Vortex and mix thoroughly before use.

3.4.2 Use pipette to transfer 75 µL of DNA Clean Beads to the Second Strand Synthesis product in step 3.3.8. Gently pipette at least 10 times to mix thoroughly. Ensure that all solution and beads are expelled 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 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 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 liquid from the tube without disrupting the beads. Centrifuge briefly to collect any remaining liquid at the bottom, separate beads magnetically and then remove 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 liquid becomes clear. Transfer 40 µL 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& A-tailing

3.5.1 Prepare the End Repair& A-tailing mixture on ice (see Table 10):

Table 10 End-Repair& 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 End Repair& A-tailing mixture to purified Second Strand Synthesis product in 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 in step 3.5.2 into the thermocycler and run the following program in Table 11:

Table 11 The Reaction Conditions of End Repair& A-tailing

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 to 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 Adapter 10 times following Table 11. Mix and centrifuge briefly for further use.

Table 12 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 in step 3.5.4. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution to the bottom of the tube.

3.6.3 Prepare the Adapter Ligation mixture on ice (see Table 13):

Table 13 Adapter Ligation 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 mixture to the PCR tube in step 3.6.2. Vortex 6 times (3 s each) and centrifuge briefly to collect the solution to the bottom of the tube.

3.6.5 Place the PCR tube in step 3.6.4 into the thermocycler and run the following program in Table 14:

Table 14 The Reaction Conditions of Adapter Ligation

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

3.6.6 Centrifuge briefly to collect the solution to 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 overnight for a maximum of 16 hours.**

3.7 Cleanup of Adapter Ligation

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

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

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

3.7.2 Use pipette to transfer 50 µL DNA Clean Beads to the Adapter Ligation product in step 3.6.7. Gently pipette at least 10 times to mix thoroughly. Ensure that all solution and beads are expelled 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 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 freshly prepared 80% Ethanol to the tube without disturbing the beads. Incubate 30 seconds. Carefully remove and discard the supernatant.

3.7.6 Repeat step 3.7.5 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 and then remove 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 beads to air dry until no wetness (glossiness) is visible but before the pellet shows cracks.

3.7.8 Remove the 1.5 mL tube from the Magnetic Separation Rack. Add 23 µL TE Buffer to elute 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 liquid becomes clear. Transfer 21 µL 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 is 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 DNA Clean Beads to the tube in step 3.7.11. Gently pipette at least 10 times to mix thoroughly. Ensure that all solution and beads are expelled 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 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 in step 3.7.14. Gently pipette at least 10 times to mix thoroughly. Ensure that all solution and beads are expelled 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 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 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 liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid to the bottom, separate beads magnetically and then remove 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 beads to air dry until no wetness (glossiness) is visible but before the pellet shows cracks.

3.7.21 Remove the 1.5 mL tube from the Magnetic Separation Rack. Add 23 µL 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 liquid becomes clear. Transfer 21 µL 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 20 µL of purified Adapter Ligation product.

3.8.2 Prepare the PCR Amplification mixture on ice (see Table 15):

Table 15 PCR Amplification Mixture

Components	Volume
PCR Enzyme Mix	25 µL
PCR Primer Mix	4 µL
UDG	1 µL
Total	30 µL

3.8.3 Transfer 30 µL of PCR Amplification mixture to the PCR tube in step 3.8.1. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution to the bottom of the tube.

3.8.4 Place the PCR tube in step 3.8.3 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	
37°C	20 min	1 cycle
95°C	3 min	1 cycle
95°C	30 s	
56°C	30 s	14 cycles
72°C	1 min	
72°C	5 min	1 cycle
4°C	Hold	

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

3.8.6 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 refrigerator and bring to room temperature for 30 min beforehand. Vortex and mix thoroughly before use.

3.9.2 Transfer 60 μL (1.2x) DNA Clean Beads to 50 μL PCR product in step 3.8.6. Gently pipette at least 10 times to mix thoroughly. Ensure that all solution and beads are expelled 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 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 freshly prepared 80% Ethanol to the tube without disturbing the beads. Incubate 30 seconds. Carefully remove and discard the supernatant.

3.9.6 Repeat step 3.9.5 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 and then remove 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 shows cracks.

3.9.8 Remove the 1.5 mL tube from the Magnetic Separation Rack. Add 32 μL TE Buffer to elute 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 liquid becomes clear. Transfer 30 μL 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 Qubit® dsDNA HS Assay Kit or Quant-iT™ PicoGreen® dsDNA Assay Kit. Please follow the assay kit instructions to quantify the purified PCR products. The required yield for PCR products is ≥ 1 pmol. See Table 17 for the corresponding yield for different insert sizes.

Table 17 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)
150	230	152
250	330	218

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

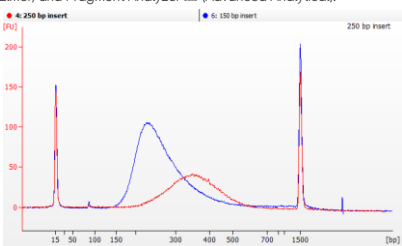

Figure 1 The Agilent 2100 Bioanalyzer Results of Purified PCR Product

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 17 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 in step 3.11.1 into the thermocycler and run the following program in Table 18:

Table 18 The Reaction Conditions of Denaturation

Temperature	Time
Heated lid	On
95°C	3 min

3.11.3 After the reaction is completed, 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 19):

Table 19 Single Strand Circularization 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 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 to the bottom of the tube.

3.12.3 Place the PCR tube into the thermocycler and run the following program in Table 18:

Table 20 The Reaction Conditions of Single Strand Circularization

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

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

3.13 Enzymatic Digestion

3.13.1 Prepare the Enzymatic Digestion mixture (see Table 21) on ice during the reaction in step 3.12.3

Table 21 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 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 to the bottom of the tube.

3.13.3 Place the PCR tube in step 3.13.2 into the thermocycler and run the following program in Table 22:

Table 22 The Reaction Conditions of Enzymatic Digestion

Temperature	Time
Heated lid	On
37°C	30 min

3.13.4 Centrifuge briefly to collect the solution to 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 to 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 refrigerator and bring to room temperature for 30 minutes beforehand. Vortex and mix thoroughly before use.

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

3.14.3 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 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 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.

3.14.7 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 or until the beads are fully resuspended.

3.14.9 Incubate at room temperature for 5 minutes.

3.14.10 Centrifuge briefly. Place the 1.5 mL tube on the Magnetic Separation Rack for 2-5 minutes until liquid becomes clear. Transfer 20 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.**

3.15 Quality Control of Enzymatic Digestion Product

Quantify the purified Enzymatic Digestion product with Qubit® ssDNA Assay Kit. The final yield should be ≥ 80 fmol (enough for two sequencing runs). Please refer to Table 23 or formula 2 in Appendix D for your calculations.

Table 23 The Corresponding Yield in 80 fmol for Different PCR Product Size (Circularized ssDNA)

Insert Size (bp)	PCR Product Size (bp)	Corresponding 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. No. 1000005278 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 let it stand at room temperature for 30 minutes 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

- If the sample volume decreases due to evaporation during incubation, add TE buffer to designated volume before using beads to purify. It 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 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. 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. Observe closely until the pellet appears sufficiently dry with a matt appearance and continue to the elution process with TE Buffer provided with this kit.

- 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 1.5 mL tubes on the Separation Rack. Strong vibrations may cause sample loss through spilled liquid or bead. 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 combination based on the principle of balanced base composition. However, the Barcode Adapters are not continuous. 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 place 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 liquid at the bottom of the tube. For MGIEasy DNA Adapters-16 (Tube) Kit, gently remove the cap to prevent spills and cross-contamination. After use, please close the cap immediately. For 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 construction efficiency.

Table 24 Recommended Adapter Input according to the Amount of total RNA

RNA Sample (ng)	MGI Adapter Dilution Ratio	MGI Adapter 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 25 to organize your Barcode Adapter combinations:

Table 25 MGIEasy DNA Adapters-16 (Tube) Kit Instruction

Samples /lane	Instructions (Example)
1	Requires at least 1 set of Adapter: 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 Adapter: 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 sample 1, 2 and 3.
4	Requires at least 1 set of Adapter: 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 sample 1-4 and 5.

6	Requires at least 2 Adapter sets: For samples 1-4, use the method for (4 samples/lane) above. For sample 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 sample 1-4, sample 5-6 and sample 7.
8	Requires at least 1 set of Adapter: 1. Take a set of 8 Adapters (97-104), add each Adapter to every 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:

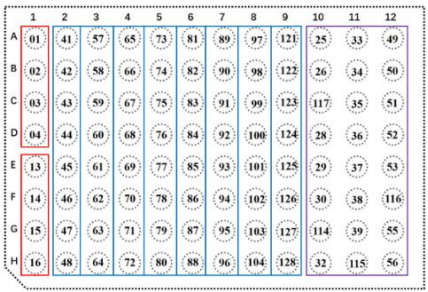

Figure 2 MGIEasy DNA Adapters-96 (Plate) Adapters

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 26 to organize your Barcode Adapter combinations:

Table 26 MGIEasy DNA Adapters-96 (Plate) Kit Instruction

Samples/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 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 mutation. 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). When RNA sample is of lower quality and consists of a longer fragment, more PCR cycles should be added to generate a sufficient yield.

Table 27 PCR Cycles required to yield 200 ng of PCR product

total RNA (ng)	PCR Cycles required for corresponding yield
	≥200 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 dsDNA sample with varying fragment sizes. Please refer to the Formula 1 to calculate the amount of DNA needed.

Formula 1 dsDNA sample pmol and ng Conversion

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

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

Formula 2 Circular ssDNA fmol and ng Conversion:

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

Appendix E Library Construction from Low Quality FFPE Sample

This procedure is applicable to low-quality total RNA samples such as FFPE. However, due to the large difference 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 using the "MGIEasy RNA Directional Library Prep Kit" as an example and list the problems that you need to pay attention to in the library construction from 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 RIN value of the FFPE samples is not always proportional to the overall success rate of library construction. Therefore, DV_{200} is also used for assessing the success rate of library construction from FFPE samples. The DV_{200} indicates the proportion of RNA fragments larger than 200 nt in the sample. For severely degraded FFPE samples, the DV_{200} value is a reliable indicator for the sample quality.

The calculation of DV_{200}

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

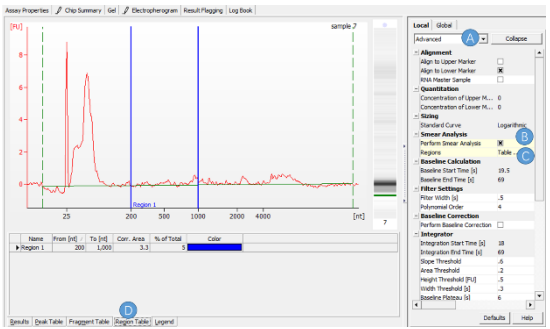

Figure 3 The Calculation of DV_{200}

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 bp to 1000 nt.

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

If you need to determine the DV_{200} of 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", please use 100 μL Beads for cleanup. In the "Adapter Ligation", pay attention to the amount of Adapter. In the "PCR Amplification", note the different corresponding PCR cycles for different DNA sample inputs. See Table 28 and 29 for detailed conditions.

Table 28. 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 min	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 29. 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. Please follow the instructions provided in the rRNA Depletion Kit User Manual to enrich RNA.

E-3.2 RNA Fragmentation

Refer to Table 28 to set up different conditions of 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 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 28. Use 100 μL beads to cleanup. Add 42 μL of TE Buffer to elute DNA and finally transfer 40 μL supernatant to a new 0.2 mL PCR tube.

E-3.5 End-Repair& A-tailing

Same as step 3.5.

E-3.6 Adapter Ligation

Refer to step 3.6.

Refer to Table 29. Use different amount of Adapter for different FFPE samples.

E-3.7 Cleanup of Adapter Ligation

Same as step 3.7.1 to 3.7.10.

E-3.8 PCR Amplification

Refer to step 3.8.

Refer to Table 28. 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.

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