Part No.:SOP-013-B02-105

User Manual

Version:6.0

MGIEasy RNA Directional Library Prep Set

Cat. No.: 1000006385 (16 RXN)
1000006386 (96 RXN)
Set Verion: V2.1

Leading Life Science Innovation

Research Use Only

About the user manual

©2024 All rights reserved by Shenzhen MGI Biological Electronic Technology Co., Ltd. (hereinafter referred to as "MGI").

This user manual and the information contained within are proprietary to MGI and are intended solely for the contractual use of its customers in connection with the use of the product described herein and for no other purpose. Any person or organization cannot entirely or partially reprint, copy, revise, distribute, or disclose to others the user manual without the prior written consent of MGI. Any unauthorized person should not use this user manual.

MGI does not make any promise of this user manual, including (but not limited to) any special commercial purpose and any reasonable implied guarantee. MGI has taken measures to guarantee the correctness of this user manual. However, MGI is not responsible for any missing parts in the manual and reserves the right to revise the manual and the reagent to improve the reliability, performance, or design.

All the pictures in this user manual are schematic diagrams and are for reference only. The content of the pictures may be slightly different from the actual product or the actual layout.

DNBSEQ™, MGISEQ™, Agilent®, Agilent Technologies®, ALPAQUA®, Ambion®, Axygen®, Advanced Analytical®, Eppendorf®, Invitrogen®, PerkinElmer®, Qubit®, Thermo Fisher®, Vazyme®, or any other company, product names, and trademarks are the property of their respective owners.

Manufacturer information

Company	Shenzhen MGI Biological Electronic Technology Co., Ltd.
Address	2/F, Building 11, Beishan Industrial Zone, No.146, Beishan Road, Yantian Street, Yantian District, Shenzhen, 518083, P.R. China
Service hotline	(+86) 4000-688-114
Email	MGI-service@mgi-tech.com
Website	http://en.mgi-tech.com

Revision history

Manual version	Set version	Date	Description
6.0	V2.1	Mar. 2024	• Update the manufacturer information • Update the manual style
5.0	V2.1	Mar. 2022	Update the manufacturer LOGO
A3	V2.1	Jan. 2021	Update the 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 4.1 • Set 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	Release the V2.0 set

Tips Please download the latest version of the manual and use it with the corresponding kit. Search for the manual by Cat. No. or product name from the following website:

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

Contents

1 Product overview

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 in this set have passed strict quality control and functional verification procedures, ensuring stability and reproducibility.

1.2 Intended use

This library prep set is applicable for the preparation of libraries for samples from common animals, plants, fungus, bacteria, including human, mouse, rice, *Arabidopsis*, yeast, *E. coli*, and so on. Stable performance across all such sample types is expected.

1.3 Applicable sequencing platforms

The prepared libraries are applicable to the following sequencing platforms.

- BGISEQ-500RS (PE50/PE100)
- MGISEQ-2000RS (PE100/PE150), DNBSEQ-G400RS (PE100/PE150)

Based on the intended sequencing kit read length, please choose the appropriate fragmented RNA insert size. Recommended insert size:

- 150 bp: PE50, PE100
- 250 bp: PE100, PE150

1.4 Components

This library preparation set comes in two specifications: 16 RXN and 96 RXN. Four separate boxes are included for each specification. For component details, refer to the following table. Each library preparation set contains an information card. Relevant manuals and SDS files can be downloaded from the MGI website provided on the information card.

Table 1 MGIEasy RNA Directional Library Prep Set (16 RXN) (Cat. No.: 1000006384)

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy RNA Directional Library Prep Kit Cat. No.: 1000005270	Fragmentation Buffer	Green	93 µL/tube × 1
	Directional RT Buffer 1	Green	88 µL/tube × 1
	Directional RT Buffer 2	Brown	5 µL/tube × 1
	RT Enzyme Mix	Green	24 µL/tube × 1
	Directional Second Strand Buffer	Yellow	470 µL/tube × 1
	Second Strand Enzyme Mix	Yellow	78 µL/tube × 1
	ERAT Buffer	Orange	132 µL/tube × 1
	ERAT Enzyme Mix	Orange	55 µL/tube × 1
	Ligation Buffer	Red	450 µL/tube × 1
	DNA Ligase	Red	34 µL/tube × 1
	PCR Enzyme Mix	Blue	470 µL/tube × 1
	PCR Primer Mix	Blue	90 µL/tube × 1
	UDG	Blue	21 µL/tube × 1
MGIEasy DNA Adapters-16 (Tube) Kit Cat. No.: 1000005284	DNA Adapters	White	10 µL/tube × 16
MGIEasy DNA Clean Beads Cat. No.: 1000005278	DNA Clean Beads	White	8 mL/tube × 1
	TE Buffer	White	4 mL/tube × 1

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy Circularization Module Cat. No.: 1000005260	Splint Buffer	Purple	186 μL/tube × 1
	DNA Rapid Ligase	Purple	8 μL/tube × 1
	Digestion Buffer	White	23 μL/tube × 1
	Digestion Enzyme	White	42 μL/tube × 1
	Digestion Stop Buffer	White	120 μL/tube × 1

Table 2 MGIEasy RNA Directional Library Prep Set (96 RXN) (Cat. No.: 1000006386)

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy RNA Directional Library Prep Kit Cat. No.: 1000005272	Fragmentation Buffer	Green	608 μL/tube × 1
	Directional RT Buffer 1	Green	760 μL/tube × 1
	Directional RT Buffer 2	Brown	20 μL/tube × 1
	RT Enzyme Mix	Green	136 μL/tube × 1
	Directional Second Strand Buffer	Yellow	1496 μL/tube × 2
	Second Strand Enzyme Mix	Yellow	448 μL/tube × 1
	ERAT Buffer	Orange	872 μL/tube × 1
	ERAT Enzyme Mix	Orange	325 μL/tube × 1
	Ligation Buffer	Red	1300 μL/tube × 2
	DNA Ligase	Red	173 μL/tube × 1
	PCR Enzyme Mix	Blue	1340 μL/tube × 1
	PCR Primer Mix	Blue	448 μL/tube × 1
	UDG	Blue	108 μL/tube × 1
MGIEasy DNA Adapters-96 (Plate) Kit Cat. No.: 1000005282	DNA Adapters	White	10 μL/well × 96

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy DNA Clean Beads Cat. No.: 1000005279	DNA Clean Beads	White	50 mL/tube × 1
	TE Buffer	White	25 mL/tube × 1
MGIEasy Circularization Module Cat. No.: 1000005260	Splint Buffer	Purple	186 µL/tube × 1
	DNA Rapid Ligase	Purple	8 µL/tube × 1
	Digestion Buffer	White	23 µL/tube × 1
	Digestion Enzyme	White	42 µL/tube × 1
	Digestion Stop Buffer	White	120 µL/tube × 1

1.5 Storage and transportation

Table 3 Kit storage and transportation temperatures

Item	Storage temperature	Transportation temperature
MGIEasy RNA Directional Library Prep Kit	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃
MGIEasy DNA Adapters-16 (Tube) Kit		
MGIEasy DNA Adapters-96 (Plate) Kit		
MGIEasy Circularization Module		
MGIEasy DNA Clean Beads	2 ℃ to 8 ℃	

Tips
- Production date and expiration date: refer to the label.
- For ice packs or dry ice shipments, ensure that there is enough ice or dry ice remaining after transportation.
- With proper transport, storage, and use, all components can maintain complete activity within their shelf life.

1.6 User-supplied materials

Table 4 Order information for MGI products

Catalog number	Model	Name
1000005953	32 RXN	MGIEasy rRNA Depletion Kit

Table 5 User-supplied equipment list

Equipment	Recommended brand
ThermoMixer	Eppendorf or equivalent
Vortex mixer	/
Desktop centrifuge	/
Pipettes	/
Thermocycler	/
Magnetic rack DynaMag -2 or equivalent	Thermo Fisher Scientific, Cat. No. 12321D
Qubit Fluorometer or equivalent	Thermo Fisher, Cat. No. Q33216
Agilent 2100 Bioanalyzer or equivalent	Agilent Technologies , Cat. No. G2939AA
LabChip GX, GXII, GX Touch or equivalent	PerkinElmer

Table 6 Recommended reagent/consumable list

Reagent/consumable	Recommended brand
Nuclease free (NF) water	Ambion, Cat. No. AM9937 or equivalent
TE Buffer, pH 8.0	Ambion, Cat. No. AM9858 or equivalent
100% Ethanol (Analytical Grade)	/
RNase Zap Decontamination Solution	Ambion, Cat. No. AM9780
rRNA depletion kit	/
AMPure XP beads, for rRNA Cleanup	Beckman Technologies or equivalent
Dynabeads mRNA Purification Kit, for mRNA enrichment	Invitrogen, Cat. No. 61006
Library Preparation VAHTS mRNA Capture Beads, for mRNA enrichment	Vazyme, Cat. No. N401-01/02
PicoGreen dsDNA Assay Kit	Invitrogen, Cat. No. P7589
Qubit ssDNA Assay Kit	Invitrogen, Cat. No. Q10212, or equivalent
Qubit dsDNA HS Assay Kit	Invitrogen, Cat. No. Q32854) or equivalent
Agilent High Sensitivity DNA Kit	Agilent, Cat. No. 5067-4626, or equivalent
Agilent DNA 1000 Kit	Agilent, Cat. No. 5067-1504, or equivalent
Pipette tips and RNase-free tips	/
1.5 mL tube	/
0.2 mL PCR tube or 96-well plate	/
Qubit Assay Tubes or 0.5mL Thin Wall PCR Tubes	Invitrogen or Axygen or equivalent

1.7 Precautions and warnings

- This product is for research use only, not for in vitro diagnosis. Please read this manual carefully before use.
- Familiarize yourself with the precautions and operation methods of various instruments before performing the experiment.
- This manual aims to provide a standard protocol. Changes can be made for different applications, but changes must be tested prior to starting the protocol.
- It is recommended that you use pipette tips with filters to prevent cross-contamination. Use a new tip each time for pipetting different solutions or samples.
- It is recommended that you use the thermocyclers with heated lids for reactions. Preheat the thermocyclers to reaction temperature before use. If the thermocycler does not allow for lid temperature adjustments, the preset lid temperature of 105 ℃ is sufficient.
- Aerosol contamination may cause inaccurate results. It is recommended that you prepare separate working areas in the laboratory for PCR reaction preparation, PCR reaction, and PCR product cleanup. Use designated equipment for each area and clean the area regularly to ensure a sterile working environment (use 0.5% Sodium Hypochlorite or 10% bleach to clean the working area).
- Avoid skin and eyes contact with samples and reagents. Do not eat or drink the samples and reagents. In case of contact with skin and eyes, rinse immediately with plenty of water and seek medical advice.
- Conform to the law and regulations when disposing of all samples and reagents.
- If you have questions, contact Technical Support: **MGI-service@mgi-tech.com**.

1.8 Workflow

Section	Workflow	Total time	Hands-on time
3.1	RNA enrichment	1 h - 2 h 20 min	20 - 50 min
3.2	RNA fragmentation	13 - 15 min	7 min
3.3	Reverse transcription and second strand synthesis ⏸	2 h 10 min	15 - 20 min
3.4	Cleanup of second strand synthesis product ⏸	30 - 40 min	20 - 30 min
3.5	End repair	50 min	15 min
3.6	Adapter ligation	40 min	10 min
3.7	Cleanup of adapter-ligated product ⏸	30 - 60 min	20 - 30 min
3.8	PCR	50 min	10 min
3.9	Cleanup of PCR product ⏸	30 - 40 min	20 - 30 min
3.10	QC of PCR product ⏸	15 - 60 min	10 - 20 min
4.1	Denaturation and single strand circularization	45 - 50 min	15 min
4.2	Digestion	35 - 40 min	10 min
4.3	Cleanup of digestion product ⏸	50 min	10 - 15 min
4.4	QC of digestion product ⏸	15 - 20 min	10 - 15 min

Tips
- Total time: The theoretical use time of 8 reactions. The time will be extended if the number of reactions increases.
- Hands-on time: The total required hands-on time in the process.
- ⏸ Stop point.

2 Sample preparation

2.1 Applicable sample 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 μg - 2.5 μg.

2.2 QC of sample

- 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 "Library preparation from low quality FFPE sample" on page 37.
- 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.

3 Library preparation protocol

This protocol is designed for a total RNA input of 200 ng with RIN ≥7.

If a different amount of RNA input is used, identify optimal adapter ligation and PCR reaction conditions by adjusting according to the recommended conditions in Section 3.8.

For library construction of FFPE RNA samples, please refer to "Library preparation from low quality FFPE sample" on page 37.

3.1 RNA enrichment

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

- "rRNA Depletion Kit" on page 9.
- "Dynabeads mRNA Purification Kit " on page 9.
- "Library Preparation VAHTS mRNA Capture Beads" on page 11.

3.1.1 rRNA Depletion Kit

Follow the *MGIEasy rRNA Depletion Kit User Manual* to perform rRNA depletion and proceed to "RNA fragmentation" on page 12.

3.1.2 Dynabeads mRNA Purification Kit

CAUTION

- Use non-stick tubes for the mRNA enrichment. Do not shake or vortex the sample in the following procedures. Mix the sample by gently pipetting.

- Do not touch or pipette the beads when adding reagents or transferring supernatant. If you accidentally pipette the beads, pipette all of the solution and beads back into the tube and restart the separation process.

3.1.2.1 Preparation

Table 7 Preparing the reagents

Reagent	Requirement
Beads	User-supplied. Take out 30 min in advance to equilibrate to room temperature (RT). Mix thoroughly by vortexing before each use.
Binding Buffer	User-supplied. Mix by vortexing, centrifuge briefly, and place at RT.
Washing Buffer	
10 mM Tris-HCl	
NF Water	

3.1.2.2 Resuspend the beads

According to the desired reaction number, resuspend the beads. The preparation volume of reagents listed blow is enough for 1 sample.

1. Mix the magnetic beads by vortexing for 1 min. Transfer 50 μL of beads to a new 1.5 mL non-stick tube. Place the tube on the magnetic rack for 2 min until the liquid is clear. Carefully remove and discard the supernatant.
2. Remove the non-stick tube from the magnetic rack and add 50 μL of Binding Buffer to the tube. Gently pipette at least 10 times until all beads are suspended. Place the tube on the magnetic rack for 2 min until the liquid is clear. Carefully remove and discard the supernatant.
3. Repeat step 2.
4. Add 25 μL of Binding Buffer to the tube and pipette 10 times to mix thoroughly.

3.1.2.3 mRNA purification

1. Preheat the Thermomixer to 65 ℃. 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 NF Water to make a total volume of 25 μL.
2. Place the sample tube(s) on the Thermomixer for denaturation at 65 ℃ for 5 min. Take out the tube(s) and immediately add 25 μL of resuspended beads to the sample. **Pipette** 10 times to mix it well.
3. Incubate at room temperature for 5 min. Set the Thermomixer to 80 ℃ .
4. Place the tube(s) on the magnetic rack for 2 min until the liquid is clear. Carefully remove and discard the supernatant.
5. Remove the non-stick tube(s) from the magnetic rack and add 50 μL of Washing Buffer to each sample. Gently **pipette** at least 10 times to mix it well. Place the tube(s) on the magnetic rack for 2 min. Carefully remove and discard the supernatant.

6. Repeat step 5.
7. Add 25 μL of 10 mM Tris-HCl to each sample tube and mix it well by gently **pipetting**. Incubate the tube(s) on the Thermomixer at 80 ℃ for 2 min to elute mRNA from magnetic beads.
8. Take out the tube and immediately add 25 μL of Binding Buffer to each sample. **Pipette** 10 times to mix it well and incubate at room temperature for 5 min. Place the tube(s) on the magnetic rack for 2 min. Carefully remove and discard the supernatant.
9. Repeat step 5 two more times.
10. Add 12 μL of 10 mM Tris-HCl to each sample tube and mix it well by gently **pipetting**. Incubate the tube(s) on the Thermomixer at 80 ℃ for 2 min.
11. Immediately place the tube(s) on the magnetic rack for 1 to 2 min until the liquid is clear. Carefully transfer 10 μL of supernatant to a new 0.2 mL PCR tube.

3.1.3 Library Preparation VAHTS mRNA Capture Beads

⚠ CAUTION

- Use non-stick tubes for the mRNA enrichment. Do not shake or vortex the sample in the following procedures. Mix the sample by gently pipetting.
- Do not touch or pipette the beads when adding reagents or transferring supernatant. If you accidentally pipette the beads, pipette all of the solution and beads back into the tube and restart the separation process.

3.1.3.1 Preparation

Table 8 Preparing the reagents

Reagent	Requirement
mRNA Capture Beads	User-supplied. Take out 30 min in advance to equilibrate to RT. Mix thoroughly by vortexing before each use.
Beads Wash Buffer	User-supplied. Mix by vortexing, centrifuge briefly, and place at RT.
Tris Buffer	
Beads Binding Buffer	
NF Water	

3.1.3.2 mRNA capture

1. Preheat the Thermomixer to 65 ℃. 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 NF Water to make a total volume of 50 μL.
2. Mix the mRNA Capture Beads by vortexing for 1 min. Add 50 μL of mRNA Capture Beads to each sample tube and **pipette** 10 times to mix it well.
3. Place the tube(s) on the Thermomixer for denaturation at 65 ℃ for 5 min.

4. Take out the tube(s) and incubate at room temperature for 5 min. At the same time, set the Thermomixer to 80 ℃ .
5. Place the tube(s) on the magnetic rack for 5 min until the liquid is clear. Carefully remove and discard the supernatant.
6. Remove the non-stick tube(s) from the magnetic rack and add 200 μL of Beads Wash Buffer to each sample. Gently **pipette** at least 10 times to mix it well. Place the tube(s) on the magnetic rack for 5 min. Carefully remove and discard the supernatant.
7. Remove the tube(s) from the magnetic rack and add 50 μL of Tris Buffer to each sample. Gently pipette at least 10 times to mix it well. Incubate the tube(s) on the Thermomixer at 80 ℃ for 2 min.
8. Take out the tube(s) and immediately add 50 μL Beads Binding Buffer to the sample. **Pipette** 10 times to mix it well and incubate at room temperature for 5 min.
9. Place the tube(s) on the magnetic rack for 5 min until the liquid is clear. Carefully remove and discard the supernatant.
10. Repeat step 6.
11. Add 12 μL of Tris Buffer to each sample tube and mix it well by gently **pipetting**. Incubate the tube(s) on the Thermomixer at 80 ℃ for 2 min.
12. Immediately place the tube(s) on the magnetic rack for 5 min until the liquid is clear. Carefully transfer 10 μL of supernatant to a new 0.2 mL PCR tube.

3.2 RNA fragmentation

⚠ CAUTION
- Do not shake or vortex the sample in the following procedures. Mix the sample by gently pipetting.

3.2.1 Preparation

Mix the reagents before using and store the remaining reagents immediately after use.

Table 9 Preparing the reagent

Reagent	Requirement
Fragmentation Buffer	Thaw at RT, mix by vortexing, centrifuge briefly and place at RT.

3.2.2 RNA fragmentation

1. Add 4 μL of Fragmentation Buffer to each sample tube (from section 3.1). Gently **pipette** the solution at least 10 times, centrifuge briefly, and place on ice.
2. Place the PCR tube(s) into the thermocycler. Incubate according to the recommended fragmentation protocol for your target insert size (105 ℃ heated lid).

Table 10 Recommended Conditions for RNA Fragmentation (Volume: 14 μL)

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

3. When the program is completed, immediately place the PCR tube(s) on ice for 2 min. Centrifuge the tube(s) for 10 sec and immediately proceed to the reverse transcription step.

3.3 Reverse transcription and second strand synthesis

⚠ CAUTION Do not shake or vortex the sample in the following procedures. Mix the sample by gently pipetting.

3.3.1 Preparation

Mix the reagents before using and store the remaining reagents immediately after use.

Table 11 Preparing the reagents

Reagent	Requirement
Directional RT Buffer 2	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
NF Water	Place at RT.
Directional RT Buffer 1	Thaw at RT, invert the tube gently, centrifuge briefly, and place on ice.
RT Enzyme Mix	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.
Directional Second Strand Buffer	Take out during step 4. Thaw at RT, mix well, centrifuge briefly, and place on ice.
Second Strand Enzyme Mix	Take out during step 4. Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

3.3.2 Reverse transcription and second strand synthesis

1. (Freshly prepared) Dilute the Directional RT Buffer 2 with NF Water for 17 times on ice. Mix it well, centrifuge briefly, and place on ice.

Table 12 Dilution of Directional RT Buffer 2

Reagent	Volume per reaction
NF Water	16 μL
Directional RT Buffer 2	1 μL
Total	17 μL

Tips The diluted Directional RT Buffer 2 should be used immediately. Discard any unused diluent. Prepare fresh diluted Directional RT Buffer 2 for each library construction.

2. According to the desired reaction number, prepare the reverse transcription mixture in a 0.2 mL PCR tube on ice. Mix it well by vortexing 3 times (3 sec each). Centrifuge briefly and place on ice.

Table 13 Reverse transcription mixture

Reagent	Volume per reaction
Directional RT Buffer 1	4 μL
Diluted Directional RT Buffer 2	1 μL
RT Enzyme Mix	1 μL
Total	6 μL

3. Add 6 μL of reverse transcription mixture to each sample tube (from step 3 in section 3.2.2). Gently **pipette** at least 10 times to mix well. Centrifuge briefly and place on ice.
4. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 14 Reverse transcription reaction conditions (Volume: 20 μL)

Temperature	Time
75 ℃ Heated lid	On
25 ℃	10 min
42 ℃	30 min
70 ℃	15 min
4 ℃	Hold

5. When the program is completed, centrifuge the tube(s) for 10 s and place on ice.
6. According to the desired reaction number, prepare the second strand synthesis mixture in a 0.2 mL PCR tube on ice. Mix it well by vortexing 3 times (3 sec each). Centrifuge briefly and place on ice.

Table 15 Second strand synthesis mixture

Reagent	Volume per reaction
Directional Second Strand Buffer	26 μL
Second Strand Enzyme Mix	4 μL
Total	30 μL

7. Add 30 μL of second strand synthesis mixture to each sample tube(from step 3). Gently **pipette** 10 times to mix well. Centrifuge briefly and place on ice.
8. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 16 Second strand synthesis reaction conditions (Volume: 50 μL)

Temperature	Time
25 ℃ Heated lid	On
16 ℃	60 min
4 ℃	Hold

9. When the program is completed, centrifuge the tube(s) for 10 sec.

Stop point After reaction, the product(s) can be stored at -20 ℃ for no more than 16 h.

3.4 Cleanup of second strand synthesis product

Tips Do not touch or pipette the beads when adding reagents or transferring supernatant. If you accidentally pipette the beads, pipette all of the solution and beads back into the tube and restart the separation process.

3.4.1 Preparation

Table 17 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied. Freshly prepared.
TE Buffer	Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.4.2 Cleanup of second strand synthesis product

Tips Transfer all liquid to a new 1.5 mL centrifuge tube (one tube per reaction) in advance if using a 1.5 mL centrifuge tube and a corresponding magnetic rack for purification.

1. Mix the DNA Clean Beads thoroughly. Add 75 μL of DNA Clean Beads to each sample tube (from step 9 in 3.3.2), and gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing.
2. Incubate the sample(s) at room temperature for 5 min.
3. Centrifuge the sample tube(s) briefly, and place on the magnetic rack for 2 to 5 min until the liquid becomes clear. Carefully remove and discard the supernatant.

4. While keeping the centrifuge tube(s) on the magnetic rack, add 200 µL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
5. Repeat step 4. Try to remove all liquid from the tube. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.
6. Keep the centrifuge tube(s) on the magnetic rack. Open the tube cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the beads' surface. There should be no visible cracking on the surface of the beads.

   Tips Over-drying the beads will result in reduced yield.

7. Remove the centrifuge tube(s) from the magnetic rack and add 42 µL of TE Buffer to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended.
8. Incubate the sample(s) at room temperature for 5 min.
9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid becomes clear. Carefully transfer 40 µL of supernatant to a new 0.2 mL PCR tube.

   Stop point After cleanup, the product(s) can be stored at -20 ℃.

3.5 End repair

3.5.1 Preparation

Mix the reagents before using and store the remaining reagents immediately after use.

Table 18 Preparing the reagents

Reagent	Requirement
ERAT Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
ERAT Enzyme Mix	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

3.5.2 End repair

1. According to the desired reaction number, prepare the end repair mixture in a 0.2 mL PCR tube on ice. Mix it well by vortexing, centrifuge briefly, and place on ice.

Table 19 End repair mixture

Reagent	Volume per reaction
ERAT Buffer	7.1 µL
ERAT Enzyme Mix	2.9 µL
Total	10 µL

2. Add 10 μL of end repair mixture to each sample tube (from step 9 in section 3.4.2) and vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.
3. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 20 End repair reaction conditions (Volume: 50 μL)

Temperature	Time
70 ℃ Heated lid	On
37 ℃	30 min
65 ℃	15 min
4 ℃	Hold

4. When the program is completed, centrifuge the PCR tube(s) briefly to collect the liquid to the bottom of the tube.

WARNING
- Do not stop at this step. Please proceed to next reaction.
- If the operation stops here, store the end repair product(s) at −20 ℃ overnight with a risk of 20% decrease in yield.

3.6 Adapter ligation

Tips The amount of Adapter used in Adapter Ligation depends on the amount of RNA input. Read “Using adapters” on page 31 carefully before operation.

Adapter quality as well as quantity directly affects the efficiency and quality of the library construction. Refer to the table below and the actual amount of total RNA to determine the corresponding times of adapter dilution. When the adapter needs to be diluted, use the TE Buffer in the kit .

Table 21 Recommended adapter input according to the amount of total RNA

RNA Sample (ng)	Adapter dilution ratio	MGI adapter input after dilution (μL)
201-5000	1 : 5	5
51-200	1 : 10	5
10-50	1 : 20	5

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

3.6.1 Preparation

Mix the reagents before using and store the remaining reagents immediately after use.

Table 22 Preparing the reagents

Reagent	Requirement
TE Buffer	User-supplied. Place at RT.

Reagent	Requirement
Adapters	Thaw on ice, mix by vortexing, centrifuge briefly, and place on ice.
Ligation Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
DNA Ligase	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

Tips
- Mix the adapter(s) well before use. Adapters should not be mixed directly with the adapter ligation mixture.
- The Ligation Buffer is highly viscous. Mix it by vortexing 6 times (3 sec each) and centrifuge briefly. When pipetting the Ligation Buffer, slowly aspirate to ensure that the volume is accurate.

3.6.2 Adapter ligation

1. Dilute the adapter(s) in a 1:10 dilution with TE Buffer, and mix well by vortexing 3 times (3 sec each). Centrifuge the diluted adapter(s) briefly and place on ice.

Table 23 Dilution of adapters

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

2. **Add 5 μL of diluted adapter(s) to the corresponding sample tube** (from step 4 in section 3.5.2). Vortex 3 times (3 sec each), centrifuge briefly, and place on ice.
3. According to the desired reaction number, prepare the adapter ligation mixture in a 0.2 mL PCR tube on ice. Mix it well by vortexing 6 times (3 sec each). Centrifuge briefly and place on ice.

Table 24 Adapter ligation mixture

Reagent	Volume per reaction
Ligation Buffer	23.4 μL
DNA Ligase	1.6 μL
Total	25 μL

4. Slowly pipette 25 μL of adapter ligation mixture to each sample tube and vortex 6 times (3 sec each). Centrifuge briefly to collect the liquid to the bottom of the tube and place on ice.

   Tips The adapter ligation mixture is highly viscous. Slowly aspirate to ensure the volume is accurate.

5. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 25 Adapter ligation reaction conditions (Volume: 80 μL)

Temperature	Time
30 ℃ Heated lid	On
23 ℃	30 min
4 ℃	Hold

6. When the program is completed, centrifuge the PCR tube(s) briefly and place on ice.
7. Add 20 μL of TE Buffer to make a total volume of 100 μL. Mix it well and centrifuge briefly.

Stop point Adapter-ligated DNA can be stored at -20 ℃ for no more than 16 hours.

3.7 Cleanup of adapter-ligated product

Tips

- For a 150 bp insert size, refer to section 3.7.2.
- For a 250 bp insert size, refer to section 3.7.3.
- Do not disturb or pipette the beads when adding reagents or transferring supernatant. If you accidentally disturb or pipette the beads, pipette the solution and beads back into the tube and restart the separation process.

3.7.1 Preparation

Table 26 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied. Freshly prepared.
TE Buffer	Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

3.7.2 Cleanup (150 bp insert size)

Tips Transfer all liquid to a new 1.5 mL centrifuge tube (one tube per reaction) in advance if using the 1.5 mL centrifuge tube and a corresponding magnetic rack for purification.

1. Mix the DNA Clean Beads thoroughly. Add 50 μL of DNA Clean Beads to each sample tube (from step 7 in section 3.6.2). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
2. Incubate the sample(s) at room temperature for 5 min.

3. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
4. While keeping the centrifuge tube(s) on the magnetic rack, add 200 µL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
5. Repeat step 4. Try to remove all liquid from the tube. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.
6. Keep the centrifuge tube(s) on the magnetic rack. Open the tube cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the beads' surface. There should be no visible cracking on the surface of the beads.

   Tips Over-drying the beads will result in reduced yield.

7. Remove the centrifuge tube(s) from the magnetic rack and add 22 µL of TE Buffer to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
8. Incubate the sample(s) at room temperature for 5 min.
9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 20 µL of supernatant to a new 0.2 mL PCR tube.

   Stop point After cleanup, the adapter-ligated product(s) can be stored at -20 °C.

3.7.3 Cleanup (250 bp insert size)

Tips Transfer all liquid to a new 1.5 mL centrifuge tube (one tube per reaction) in advance if using the 1.5 mL centrifuge tube and a corresponding magnetic rack for purification.

1. Perform step 1 to 6 above in step *3.7 operation (150 bp insert size)*.
2. Remove the centrifuge tube(s) from the magnetic rack, and add 52 µL of TE Buffer to elute the DNA. Gently pipette at least 10 times until all beads are suspended. Or, mix with a vortexer.
3. Incubate the sample(s) at room temperature for 5 min.

   Tips In the next step, keep the supernatant and discard the beads.

4. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 50 µL of supernatant to a new 1.5 mL centrifuge tube.
5. Add 32.5 µL of DNA Clean Beads to each sample tube (containing 50 µL supernatant). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
6. Incubate the sample(s) at room temperature for 5 min.

   Tips In the next step, keep the supernatant and discard the beads.

7. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 80 µL of supernatant to a new 1.5 mL centrifuge tube.

8. Add 10 μL DNA Clean Beads to each sample tube (containing 80 μL supernatant, from 3.7.3 step 7). Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
9. Incubate the sample(s) at room temperature for 5 min.
10. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
11. While keeping the centrifuge tube(s) on the magnetic rack, add 200 μL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
12. Repeat step 11. Try to remove all liquid from the tube. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. Remove all liquid by using a low volume pipette.
13. Keep the centrifuge tube(s) on the magnetic rack. Open the tube cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the beads' surface. There should be no visible cracking on the surface of the beads.

    Tips Over-drying the beads will result in reduced yield.

14. Remove the centrifuge tube(s) from the magnetic rack and add 22 μL of TE Buffer to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
15. Incubate the sample(s) at room temperature for 5 min.
16. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 20 μL of supernatant to a new 0.2 mL PCR tube.

    Stop point After cleanup, the adapter-ligated product(s) can be stored at -20 °C.

3.8 PCR

The number of PCR cycles should be strictly controlled.

- Insufficient cycles may lead to a reduced library yield.
- Excessive cycles may also lead to adverse effects such as over amplification, an increase in bias, PCR duplicates, chimeric sequences, and accumulated mutations.

The indicated table shows the number of PCR cycles required to yield 200 ng of library from 10 ng to 1000 ng high quality RNA sample (150 bp). For lower quality or longer RNA fragments, PCR cycles should be increased appropriately to generate sufficient yield.

Table 27 PCR cycles required to yield 300 ng libraries

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

3.8.1 Preparation

Mix the reagents before using and store the remaining reagents immediately after use.

Table 28 Preparing the reagents

Reagent	Requirement
PCR Enzyme Mix	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
PCR Primer Mix	Thaw at RT, mix by vortexing, centrifuge briefly, and place at RT.
UDG	

3.8.2 PCR

1. According to the desired reaction number, prepare the PCR mixture in a 0.2 mL PCR tube on ice. Mix it well by vortexing, centrifuge briefly, and place on ice.

Table 29 PCR mixture

Reagent	Volume per reaction
PCR Enzyme Mix	25 μL
PCR Primer Mix	4 μL
UDG	1 μL
Total	30 μL

2. Add 30 μL of PCR mixture to each sample tube (from step 9 in 3.7.2 or step 16 in 3.7.3), and vortex 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly to collect the liquid to the bottom of the tube.
3. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 30 PCR reaction conditions (Volume: 50 μL)

Temperature	Time	Cycles
105 ℃ Heated lid	On	-
37 ℃	20 min	1
95 ℃	3 min	1
95 ℃	30 sec	14*
56 ℃	30 sec	
72 ℃	1 min	
72 ℃	5 min	1
4 ℃	Hold	-

Tips *: If a different amount of RNA input is used, refer to Table 27 to adjust the number of cycles.

4. When the program is completed, centrifuge the tube(s) briefly.

3.9 Cleanup of PCR product

Tips Do not disturb or pipette the beads when adding reagents or transferring supernatant. If you accidentally disturb or pipette the beads, pipette the solution and beads back into the tube and restart the separation process.

3.9.1 Preparation

Table 31 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied. Freshly prepared.
TE Buffer	Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before every use.

3.9.2 Cleanup of PCR product

Tips Transfer all liquid to a new 1.5 mL centrifuge tube (one tube per reaction) in advance if using the 1.5 mL centrifuge tube and a corresponding magnetic rack for purification.

1. Mix the DNA Clean Beads thoroughly. Add 60 µL of DNA Clean Beads to each sample tube (from step 4 in section 3.8.2). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
2. Incubate the sample(s) at room temperature for 5 min.
3. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
4. While keeping the centrifuge tube(s) on the magnetic rack, add 200 µL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
5. Repeat step 4. Try to remove all liquid from the tube. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.

6. Keep the centrifuge tube(s) on the magnetic rack. Open the tube cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the beads' surface. There should be no visible cracking on the surface of the beads.

   Tips Over-drying the beads will result in reduced yield.

7. Remove the tube(s) from the magnetic rack and add 32 μL of TE Buffer to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
8. Incubate the sample(s) at room temperature for 5 min.
9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 30 μL of supernatant to a new 0.2 mL PCR tube.

   Stop point After cleanup, PCR product can be stored at -20 ℃.

3.10 QC of PCR product

- **dsDNA fluorescence quantification method**: Quantify purified PCR products with dsDNA fluorescence assay kits and instructions.
- **Electrophoresis method**: Validate the size range of purified PCR products with electrophoresis based equipment and instructions.

Table 32 Different QC methods and standards for library

Method	Equipment/Reagent	Standard
dsDNA fluorescence quantification method	Qubit dsDNA HS Assay Kit, Quant-iT PicoGreen dsDNA Assay Kit	Yield for PCR products: ≥ 1 pmol
Electrophoresis method	Tapestation (Agilent Technologies), Bioanalyzer, LabChip GX, GXII, GX Touch (PerkinElmer), Fragment Analyzer (Advanced Analytical)	150 bp insert size: Main size: 230 bp
		250 bp insert size: Main size: 330 bp

Formula 1 Conversion between 1 pmol of dsDNA sample and mass in ng

Mass corresponding to 1 pmol PCR product (ng) = PCR product peak size (bp) × 0.66

Table 33 The corresponding yield in 1 pmol for PCR products with different fragment sizes

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

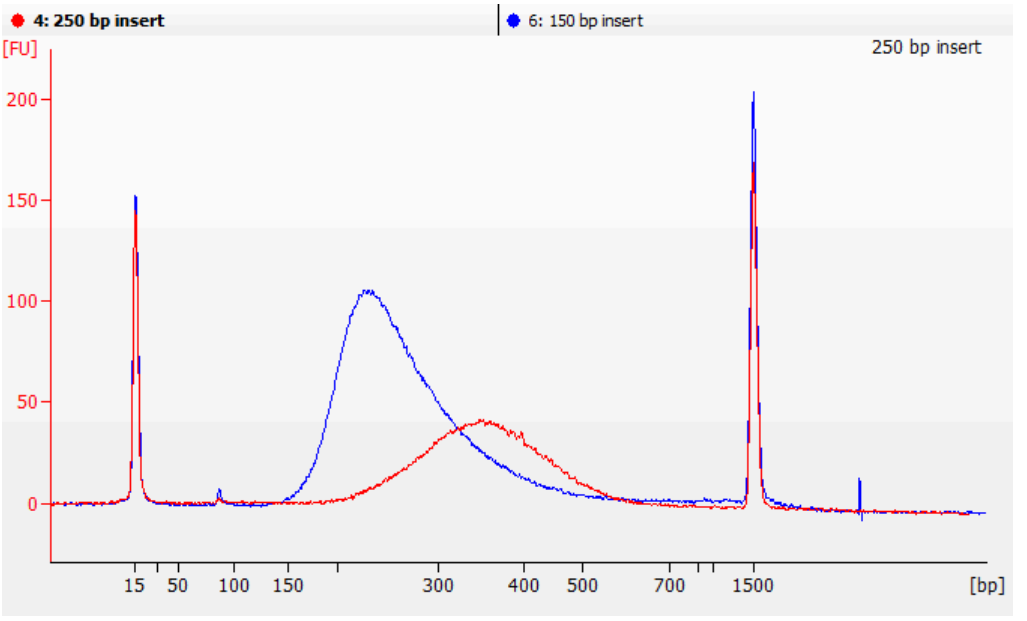

Figure 1 The Agilent 2100 bioanalyzer results of purified PCR product

Tips

- Stop here if the library will be delivered to a service lab for sequencing.
- Proceed to next reaction if the library will be sequenced in your lab.

4 Circularization and digestion

4.1 Denaturation and single strand circularization

Tips
- Calculate the required purified PCR product volume based on the main fragment size of the purified PCR product , concentration of the sample, and Formula 1.
- For multiple samples pooled sequencing, please follow the instructions provided by MGIEasy DNA Adapters Kitorreference “Using adapters” on page 31. Detailed information shows how to plan samples pooling. Quantify the purified PCR products before pooling. The total yield after pooling should be 1 pmol, with a total volume ≤ 48 μL.
  - For example. For 8 samples (belong to 150 bp insert size libraries) pooled sequencing, add 19 ng of the PCR product of each sample into a new 0.2 mL PCR tube. The PCR product should have a total mass of 152 ng and be equal to a total yield of 1 pmol. Then add TE Buffer to make a total volume of 48 μL.

4.1.1 Preparation

Mix the reagents before using and store the remaining reagents immediately after use.

Table 34 Preparing the reagents

Reagent	Requirement
TE Buffer	User-supplied. Place at RT
Splint Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
DNA Rapid Ligase	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

4.1.2 Denaturation

1. Add 1 pmol of PCR product into a new 0.2 mL PCR tube and add TE Buffer to make a total volume of 48 μL.

2. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 35 Denaturation reaction conditions (Volume: 48 μL)

Temperature	Time
105 ℃ Heated lid	On
95 ℃	3 min

3. When the program is completed, immediately place the PCR tube(s) on ice for 2 min. Centrifuge briefly and place on ice.

4.1.3 Single strand circularization

1. According to the desired reaction number, prepare the single strand circularization mixture in a 0.2 mL PCR tube on ice. Mix it well by vortexing 3 times (3 sec each). Centrifuge briefly and place on ice.

Table 36 Single strand circularization mixture

Reagent	Volume per reaction
Splint Buffer	11.6 μL
DNA Rapid Ligase	0.5 μL
Total	12.1 μL

2. Add 12.1 μL of single strand circularization mixture to each sample tube (from step 3 in section 4.1.2). Vortex it 3 times (3 sec each) to mix well. Centrifuge the tube(s) briefly and place on ice.
3. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 37 Single strand circularization reaction conditions (Volume: 60.1 μL)

Temperature	Time
45 ℃ Heated lid	On
37 ℃	30 min
4 ℃	Hold

4. When the program is completed, place the PCR tube(s) on ice, centrifuge briefly, and immediately proceed to the next step.

4.2 Digestion

4.2.1 Preparation

Mix the reagents before using and store the remaining reagents immediately after use.

Table 38 Preparing the reagents

Reagent	Requirement
Digestion Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Digestion Enzyme	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.
Digestion Stop Buffer	Thaw at RT, mix by vortexing, centrifuge briefly, and place at RT.

4.2.2 Digestion

1. According to the desired reaction number, prepare the digestion mixture in a 0.2 mL PCR tube on ice. Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.

Table 39 Digestion mixture

Reagent	Volume per reaction
Digestion Buffer	1.4 μL
Digestion Enzyme	2.6 μL
Total	4.0 μL

2. Add 4 μL of digestion mixture to each sample tube (from step 4 in section 4.1.3). Vortex 3 times (3 sec each), centrifuge briefly, and place on ice.
3. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 40 Digestion reaction conditions (Volume: 64.1 μL)

Temperature	Time
45 ℃ Heated lid	On
37 ℃	30 min
4 ℃	Hold

4. When the program is completed, centrifuge the tube(s) briefly. Immediately add **7.5 μL of Digestion Stop Buffer** to each sample tube.
5. Vortex the tube(s) 3 times (3 sec each) and centrifuge briefly. Transfer all liquid to a new 1.5 mL centrifuge tube (one tube per reaction).

4.3 Cleanup of digestion product

Tips Do not disturb or pipette the beads when adding reagents or transferring supernatant. If you accidentally disturb or pipette the beads, pipette the solution and beads back into the tube and restart the separation process.

4.3.1 Preparation

Table 41 Preparing the reagents

Reagent	Requirement
80% ethanol	User-supplied. Freshly prepared.
TE Buffer	Place at RT.
DNA Clean Beads	Allow 30 min to equilibrate to RT before use. Mix thoroughly by vortexing before each use.

4.3.2 Cleanup of digestion product

1. Mix the DNA Clean Beads thoroughly. Add 170 µL of DNA Clean Beads to each sample tube (from step 5 in 4.2.2). Gently pipette at least 10 times until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing. Or, mix with a vortexer.
2. Incubate the sample(s) at room temperature for 10 min.
3. Centrifuge the sample tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
4. While keeping the centrifuge tube(s) on the magnetic rack, add 500 µL of 80% ethanol to each tube to wash the beads and tube wall. Wait for 30 sec. Carefully remove and discard the supernatant.
5. Repeat step 4. Try to remove all liquid from the tube. If some liquid remains on the tube wall, centrifuge the tube briefly and place it on the magnetic rack for separation. Remove all liquid by using a low-volume pipette.
6. Keep the centrifuge tube(s) on the magnetic rack. Open the tube cap and air-dry the beads at room temperature until no wetness or glossiness is visible on the beads' surface. There should be no visible cracking on the surface of the beads.

   Tips Over-drying the beads will result in reduced yield.

7. Remove the tube(s) from the magnetic rack and add 22 µL of TE Buffer to elute the DNA. Gently pipette the liquid at least 10 times until all beads are suspended. Or, mix with a vortexer.
8. Incubate the sample(s) at room temperature for 5 min.

9. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer 20 μL of supernatant to a new 1.5 mL centrifuge tube.

Stop point After cleanup, the digestion product(s) can be stored at -20 °C.

4.4 QC of digestion product

Quantify the purified digestion product by following the instructions of the Qubit ssDNA Assay Kit.

- The yield of digestion product should be not less than 80 fmol (enough for two sequencing runs).

Formula 2 Circular ssDNA fmol to ng conversion

Mass corresponding to 80 fmol circular ssDNA (ng) = 0.08 × PCR product peak size (bp) × 0.33

Table 42 The corresponding mass yield in 80 fmol for different PCR product size (ssDNA)

Input size (bp)	PCR product size (bp)	Corresponding mass yield in 80 fmol (ng)
150	230	6.07
250	330	8.71

5 Appendix

5.1 Using adapters

MGI currently offers the Adapter Reagent Kits with two specifications based on the number of reactions: the MGIEasy DNA Adapters-16 (Tube) Kit and 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 numbers of barcode adapter are not continuous. For optimal performance, read the instructions of Adapter Reagent Kits carefully before use.

- Adapters from the two kits contain overlapping barcodes and cannot be sequenced in the same lane.
- All adapters are double stranded. To prevent structural changes, such as denaturation, which might affect performance, do not place the adapters above 30℃.
- Before use, mix the adapter(s) well and centrifuge to collect the liquid at the bottom of tubes or plates.
- Change tips when pipetting different adapters to prevent cross-contamination.
- For MGIEasy DNA Adapters-16 (Tube) Kit, carefully open the tube cap to prevent spills or cross-contamination and close the cap immediately after use.
- For the MGIEasy DNA Adapters-96 (Plate) Kit, spray 75% alcohol and wipe the surface of the aluminum film of the plate with absorbent paper. The aluminum film is penetrable and do not touch the surface of the aluminum film with sharp objects. Pierce the aluminum film to pipette solutions for first-time use. After use, separately transfer the remaining reagents to 1.5 mL centrifuge tube(s) or 0.2 mL PCR tube(s), label the tubes clearly, and store them at -20 ℃.
- Adapters from other MGI Library Prep Kits are designed differently and cannot be mixed with the adapters described here. Otherwise, errors will occur during barcode demultiplexing procedures on DNBSEQ platforms.

5.1.1 Instructions for DNA Adapters-16 (Tube)

Based on the principles of balanced base composition, adapters must be used in specific groups. Please follow the instructions below to use the adapters in the proper combinations.

- 2 sets of 4 adapters: (01-04) and (13-16)
- 1 set of 8 adapters: (97-104)

If the sequencing data output requirement is the same for all samples in one lane, choose the barcode adapter combinations in the table below.

CAUTION The number of the adapter should not be repeated between samples in one lane.

Table 43 Instructions for DNA Adapters-16 (Tube)

Sample/lane	Instruction (Example)
1	Requires at least 1 set of adapters: • For a set of 4 adapters, add 4 adapters to each sample. For example: 01-04. Mix 4 adapters with equal volume and add the mixture to the sample. • Or, for a set of 8 adapters, add 8 adapters to each sample. For example: 97-104. Mix 8 adapters with equal volume and add the mixture to the sample.
2	Requires at least 1 set of adapters: • For a set of 4 adapters, add 2 adapters to each sample. For example: 01-04. Mix 01 and 02 with equal volume and add the mixture to sample 1; Mix 03 and 04 with equal volume and add the mixture to sample 2. • Or, for a set of 8 adapters, add 4 adapters to each sample. For example: 97-104. Mix 97-100 with equal volume and add the mixture to sample 1; Mix 101-104 with equal volume and add the mixture to sample 2.
3	Requires at least 2 sets of Adapters: 1. For samples 1 and 2, use the method for (2 samples/lane) above. 2. For sample 3, use the method for (1 sample/lane) above. Tips Use different adapter sets for samples 1, 2, and 3.
4	Requires at least 1 set of adapters: • For a set of 4 adapters, add 1 adapter to each sample. For example: 01-04. Add adapter 01, 02, 03, 04 to sample 1, 2, 3, 4, in that order. • Or, for a set of 8 adapters, add 2 adapters to each sample. For example: 97-104. Mix 97-98, 99-100, 101-102, and 103-104 with equal volume. Add the mixture to sample 1, 2, 3, 4, in that order.

Sample/lane	Instruction (Example)
5	Requires at least 2 sets of Adapters: • For samples 1-4, use the method for (4 samples/lane) above. • For sample 5, use the method for (1 sample/lane) above. Tips Use different adapter sets for samples 1-4 and for sample 5.
6	Requires at least 2 sets of adapters: 1. For samples 1-4, use the method for (4 samples/lane) above. 2. For samples 5-6, use the method for (2 sample/lane) above. Tips Use different adapter sets for samples 1-4 and for samples 5-6.
7	Requires all 3 Adapter sets and follow 3 steps: 1. For samples 1-4, use the method for (4 samples/lane) above (use the first adapter set). 2. For samples 5-6, use the method for (2 samples/lane) above (use the second adapter set). 3. For sample 7, use the method for (1 sample/lane) above (use the third adapter set). ▪ Add a single adapter within the adapter set. ▪ Or, add the adapter mix which is mixed from all adapters within the adapter set with an equal volume. Tips Use different adapter sets for samples 1-4, for samples 5-6 and for sample 7.
8	Requires at least 1 set of adapters: • For a set of 8 adapters, add 1 adapter to each sample. For example: 97-104. Add adapters 97-104 to samples 1-8, in that order. • Or, for 2 sets of 4 adapters, add 1 adapter to each sample. For example: 01-04 and 13-16. Add 1 adapter to each sample.
8+x (x=1-8, Total 9-16)	Perform the following 3 steps: 1. For samples 1 to 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 X, according to the value of X, use the methods above for 1-8 sample/lane accordingly. Tips Use different adapter sets for steps 1 and 2.

For situations in which sequencing data output requirements are different among samples, any sample with a data output of more than 20% for each lane should use a separate set of adapters.

For example, 9 samples are pooled into one lane, one sample of which requires 30% of the total data output.

1. 8 samples may use adapter set (97-104).
2. The final sample should use a full adapter set instead of using only a single adapter. For example: adapter set (01-04) or (13-16).

5.1.2 Instructions for DNA Adapters-96 (Plate)

Based on the principles of balanced base composition, adapters should be used in specific groups. Follow the instructions below to use the adapters in the proper combinations.

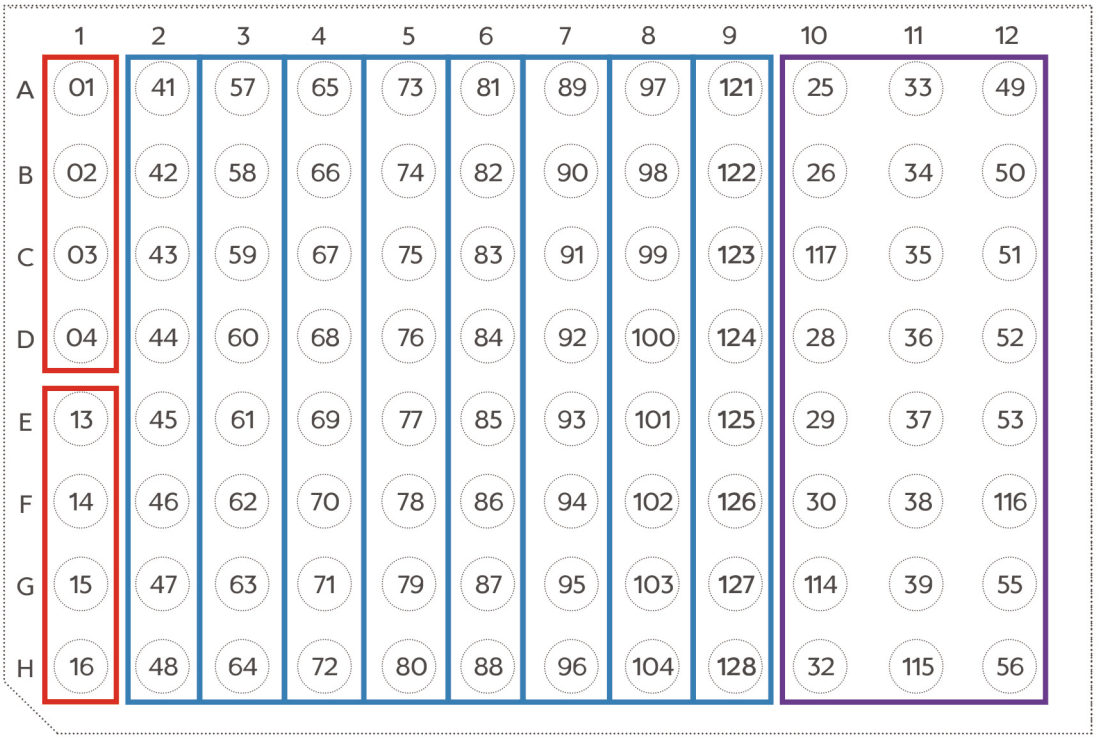

Figure 2 DNA adapters-96 (plate) adapters layout and combination instructions

- 2 sets of 4 adapters: Column 1 (01-04, 13-16) (see the red box in the figure above)
- 8 sets of 8 adapters: Columns 2-9 (41-48, 57-64, 65-72, 73-80, 81-88, 89-96, 97-104, and 121-128) (see the blue box in the figure above)
- 1 set of 24 adapters: Columns 10-12 (see the purple box in the figure above)

If the sequencing data output requirement is the same for all samples in a lane, please refer to the table below to organize your barcode adapter combinations.

CAUTION The number of the adapter should not be repeated between samples in one lane.

Table 44 Instructions for DNA Adapters-96 (Plate)

Sample/lane	Instruction (Example)
1	• For a set of 4 adapters, add 4 adapters to each sample. For example: 01-04. Mix 4 adapters with equal volume and add the mixture to the sample. • Or, for a set of 8 adapters, add 8 adapters to each sample. For example: 41-48. Mix 8 adapters with equal volume and add the mixture to the sample.

Sample/lane	Instruction (Example)
2	• For a set of 4 adapters, add 2 adapters to each sample. For example: 01-04. Mix 01 and 02 with equal volume and add the mixture to sample 1; Mix 03 and 04 with equal volume and add the mixture to sample 2. • Or, for a set of 8 adapters, add 4 adapters to each sample. For example: 41-48. Mix 41-44 with equal volume and add the mixture to sample 1; Mix 45-48 with equal volume and add the mixture to sample 2.
3	1. For samples 1 and 2, use the method for (2 samples/lane) above. 2. For sample 3, use the method for (1 sample/lane) above. Tips Use different adapter sets for samples 1, 2, and 3.
4	• For a set of 4 adapters, add 1 adapter to each sample. For example: 01-04. Add adapters 01, 02, 03, 04 to samples 1, 2, 3, 4, in that order. • Or, for a set of 8 adapters, add 2 adapters to each sample. For example: 41-48. Mix 41-42, 43-44, 45-46, and 47-48 with equal volume. Add the mixture to sample 1, 2, 3, 4, in that order.
5	1. For samples 1-4, use the method for (4 samples/lane) above. 2. For sample 5, use the method for (1 sample/lane) above. Tips Use different adapter sets for samples 1-4 and 5.
6	1. For samples 1-4, use the method for (4 samples/lane) above. 2. For samples 5-6, use the method for (2 sample/lane) above. Tips Use different adapter sets for samples 1-4 and 5-6.

Sample/lane	Instruction (Example)
7	1. For samples 1-4, use the method for (4 samples/lane) above (use the first adapter set). 2. For samples 5-6, use the method for (2 samples/lane) above (use the second adapter set). 3. For sample 7, use the method for (1 sample/lane) above (use the third adapter set). Tips Use different adapter sets for samples 1-4, samples 5-6, and sample 7.
8	• For a set of 8 adapters, add 1 adapter to each sample. For example: 41-48. Add adapters 41-48 to samples 1 - 8, in that order.
8n+x (n=1 or 2, x=1-8, total 9-24)	Perform the following 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. Tips Use different adapter sets for steps 1, 2, and 3.
8n+x (3≤n<11, x=1-8, total 25-96)	Perform the following 3 steps: 1. For samples 1-24, use a set of 24 adapters and add 1 adapter to each sample. 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. Tips Use different adapter sets for steps 1, 2, and 3.

For situations in which sequencing data output requirements are different among samples, any sample with a data output of more than 20% for each lane should use a separate set of adapters.

For example, 9 samples are pooled into one lane, one sample of which requires 30% of the total data output.

1. 8 samples may use adapters (41-48).
2. The final sample should use a full adapter set instead of using only a single adapter. For example: adapter set (01-04) or (13-16).

5.2 Library preparation from low quality FFPE sample

This procedure is used for low-quality total RNA samples such as FFPE. However, as the result of 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.

5.2.1 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, 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 nucleotides in the sample. For severely degraded FFPE samples, the DV_{200} value is a reliable indicator of the sample quality.

5.2.1.1 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 below.

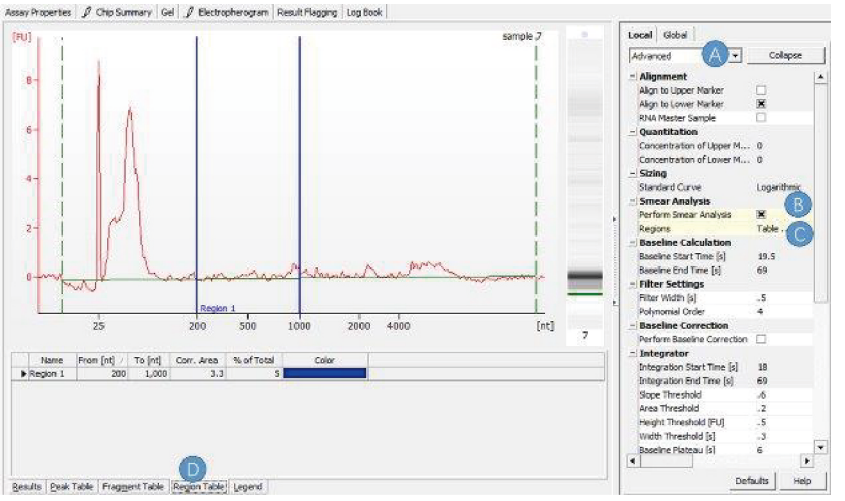

Figure 3 The calculation of DV_{200}

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 ***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, refer to *DV_{200} determination for FFPE RNA samples*. (https://www.agilent.com/en/promotions/dv200-determination)

5.2.2 Recommended amount of FFPE sample input

Use rRNA depleted RNA for NGS library construction.

- In "RNA Fragmentation" section, use different conditions of RNA fragmentation for different samples.
- In "Cleanup of Second Strand Synthesis Product" section, use 100 μL of Beads for cleanup.
- In "Adapter Ligation" section, pay attention to the amount of adapter.
- In "PCR" section, note the corresponding different number of PCR cycles for different DNA sample inputs. See indicated table for detailed conditions.

Table 45 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 ℃, 8 min	100 μL Beads	14
50-70%	200~400 ng	94 ℃, 8 min	100 μL Beads	16
30-50%	500 ng	94 ℃, 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 46 Recommended adapter of library construction from FFPE sample

FFPE DV_{200}	Recommended amount of total RNA input	MGI adapter dilution ratio	MGI adapter 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

5.2.3 Library preparation protocol from FFPE sample

5.2.3.1 RNA enrichment

Use the MGI rRNA Depletion Kit. Follow the instructions provided in the rRNA Depletion Kit User Manual to enrich RNA.

5.2.3.2 RNA fragmentation

Refer to "Table 45 Recommended conditions of library construction from FFPE sample" on page 38 to set up different conditions for RNA Fragmentation for samples with different levels of degradation.

If fragmentation is not required for the RNA Enrichment product, perform the following steps.

1. According to the desired reaction number, add 4 * (n+1) μL of Fragmentation Buffer to a new 0.2 mL PCR tube.
2. Incubate the RNA enrichment product(s) and the PCR tube (contains Fragmentation Buffer) at 65 ℃ for 5 min. Immediately place the sample and PCR tube on ice for 2 min and centrifuge for 10 sec for further use.
3. Add 4 μL of Fragmentation Buffer to each sample and immediately proceed to the next reaction.

5.2.3.3 Reverse transcription and second strand synthesis

Same as section 3.3.

5.2.3.4 Cleanup of second strand synthesis product

Refer to section 3.4.

Refer to "Table 45 Recommended conditions of library construction from FFPE sample" on page 38. Use 100 μL of beads to cleanup. Add 42 μL of TE Buffer to elute DNA. Transfer 40 μL of supernatant to a new 0.2 mL PCR tube.

5.2.3.5 End repair

Same as section 3.5.

5.2.3.6 Adapter ligation

Refer to section 3.6.

Refer to "Table 46 Recommended adapter of library construction from FFPE sample" on page 38. Use a different amount of adapter for different FFPE samples.

5.2.3.7 Cleanup of adapter-ligated product

Same as section 3.7.1 (150 bp insert size).

5.2.3.8 PCR

Refer to section 3.8.

Refer to "Table 45 Recommended conditions of library construction from FFPE sample" on page 38. Use different numbers of PCR cycles for different FFPE samples.

5.2.3.9 Cleanup of PCR product to QC of digestion product

Same as section 3.9 to 4.4.

5.3 Library preparation from RNA pathogen sample

5.3.1 Applicable types of RNA pathogen samples

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

5.3.2 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.

5.3.3 Library preparation from RNA pathogen samples

5.3.3.1 RNA enrichment

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

5.3.3.2 RNA fragmentation

Refer to section 3.2.

The RNA sample is incubated at 94 ℃ for 8 min according to the conditions for 150 bp.

5.3.3.3 Reverse transcription to Cleanup of adapter-ligated product

Same as section 3.3 to 3.6.
Same as section 3.7.1 (150 bp insert size).

5.3.3.4 PCR

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

5.3.3.5 Cleanup of PCR product

Same as section 3.9.

5.3.3.6 QC of PCR product

The standards of library quality control are shown in table below. Libraries that do not meet quality control requirements have a risk of failing in the sequencing run.

Table 47 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	Size range: 210-250 bp Average: 150-500 bp
Adapter residue	Agilent 2100 chip inspection	Visual observation, No obvious peak around 80 bp

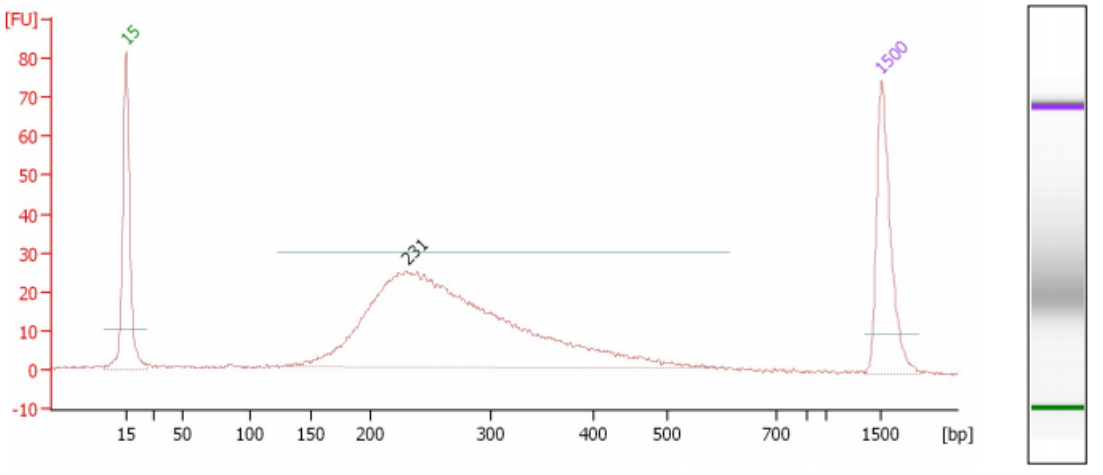

Figure 4 The agilent 2100 bioanalyzer results of purified PCR product

5.3.3.7 Denaturation to QC of digestion product

Same as section 4.1 to 4.4.