Part No.:H-940-000890-00-01

User Manual

Version:1.0

MGIEasy Fast RNA Library Prep Set

Cat. No.: 940-000890-00 (16 RXN)
940-000889-00 (96 RXN)
940-000961-00 (192 RXN)

Set version: V1.0

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About the user manual

Manufacturer information

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Revision history

Manual version	Set version	Date	Description
1.0	1.0	Jun. 2023	Initial release

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

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1 Product overview

1.1 Introduction

The MGIEasy Fast RNA Library Prep Set is designed to prepare libraries for MGI high-throughput sequencing platforms.

The library prep set is optimized to convert 10 ng to 1 μg of total RNA into a DNA library for gene expression profiling, transcriptome analysis, or detection of pathogenic microorganisms in high-throughput RNA sequencing by using MGI devices. This kit is optimized with high-quality enzyme and buffer system to combine cDNA second strand synthesis and end repair steps, which greatly shortens the time of library construction. The second strand synthesis is equipped with two kinds of buffers that can be used to prepare a common or directional library according to your needs . All reagents provided in this set have passed strict quality control and functional verification procedures, ensuring stability and reproducibility.

Tips
- MGIEasy Fast RNA Library Prep Set is paired with MGIEasy UDB Primers Adapter Kit for library construction.
- The constructed dsDNA library can be combined with the MGIEasy Dual Barcode Circularization Kit (Cat. No.: 1000020570) for circularization and DNB preparation “Circularization and DNB preparation” on page 28.
- Or, it can be combined with DNBSEQ OneStep DNB Make Reagent Kit V2.0 Model: OS-DB (Cat. No.: 940-000036-00) for rapid DNB preparation “Onestep DNB preparation” on page 33.

1.2 Intended use

This library prep set is applicable to 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.

In addition, this library prep set is also used for whole blood, saliva, fresh tissue, and other type of human samples for the detection of pathogenic microorganisms.

1.3 Applicable sequencing platform

Select the appropriate DNB prep kit, sequencing platform and sequencing type based on application requirements.

Table 1 Sequencing platform and sequencing type recommendation

Reagent kit	Sequencing platform and type
MGIEasy Dual Barcode Circularization Kit	MGISEQ-200RS, DNBSEQ-G50RS (SE50/PE100/PE150) MGISEQ-2000RS, DNBSEQ-G400RS (SE50/PE100/PE150) DNBSEQ-G99RS (SE50/PE100/PE150) DNBSEQ-T7RS (SE50/PE100/PE150) DNBSEQ-T10x4RS (SE50/PE100/PE150)
DNBSEQ Onestep DNB Make Reagent Kit V2.0	MGISEQ-2000RS, DNBSEQ-G400RS (PE100/PE150) DNBSEQ-T7RS (PE100) DNBSEQ-G99RS (SE50/PE100/PE150) MGISEQ-200RS, DNBSEQ-G50RS (PE100)

Tips When the library insert is 200 bp, it is recommended that you use a sequencing read length of PE100 or SE50; when the library insert is 270 bp, it is recommended that you use a sequencing read length of PE150.

1.4 Components

This library prep set comes in three specifications: 16 RXN, 96 RXN, and 192 RXN. Three separate boxes are included for each specification. For component details, refer to the following table.

Each library prep set contains an information card. Relevant manuals and MSDS files can be downloaded from the MGI website provided on this card.

Table 2 MGIEasy Fast RNA Library Prep Set (16 RXN) (Cat. No.: 940-000890-00)

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy Fast RNA Library Prep Kit Cat. No.: 940-000887-00 Specification: 16 RXN	Fragmentation Buffer	Green	80 μL/tube × 1
	RT Buffer	brown	64 μL/tube × 1
	RT Enzyme Mix	Green	16 μL/tube × 1
	Second Strand Buffer (with dNTP)	Yellow	405 μL/tube × 1
	Directional Second Strand Buffer (with dUTP)	Orange	405 μL/tube × 1
	Second Strand Enzyme Mix	Yellow	76 μL/tube × 1

Item & Cat. No.	Component	Cap color	Spec & Quantity
	Ligation Buffer	Red	375 μL/tube × 1
	DNA Ligase	Red	26 μL/tube × 1
	PCR Enzyme Mix	Blue	400 μL/tube × 1
MGIEasy UDB Primers Adapter Kit Cat. No.: 1000022800 Specification: 16 RXN	UDB Adapter	White	80 μL/tube × 1
	UDB PCR Primer Mix-57- 64,89-96	Blue	12 μL/tube × 16
MGIEasy DNA Clean Beads Cat. No.: 940-001176-00 Specification: 3.2 mL	DNA Clean Beads	White	3.2 mL/tube × 1
	TE Buffer	White	3.2 mL/tube × 1

Table 3 MGIEasy Fast RNA Library Prep Set (96 RXN) (Cat. No.: 940-000889-00)

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy Fast RNA Library Prep Kit Cat. No.: 940-000888-00 Specification: 96 RXN	Fragmentation Buffer	Green	480 μL/tube × 1
	RT Buffer	brown	384 μL/tube × 1
	RT Enzyme Mix	Green	96 μL/tube × 1
	Second Strand Buffer (with dNTP)	Yellow	1215 μL/tube × 2
	Directional Second Strand Buffer (with dUTP)	Orange	1215 μL/tube × 2
	Second Strand Enzyme Mix	Yellow	452 μL/tube × 1
	Ligation Buffer	Red	1124 μL/tube × 2
	DNA Ligase	Red	154 μL/tube × 1
	PCR Enzyme Mix	Blue	1200 μL/tube × 2
MGIEasy UDB Primers Adapter Kit B Cat. No.: 1000022802 Specification: 96 RXN	UDB Adapter	White	480 μL/tube × 1
	UDB PCR Primer Mix-97- 192	Colorless	12 μL/well × 96
MGIEasy DNA Clean Beads Cat. No.: 940-001174-00 Specification: 15 mL	DNA Clean Beads	White	15 mL/tube × 1
	TE Buffer	White	17 mL/tube × 1

Table 4 MGIEasy Fast RNA Library Prep Set (192 RXN) (Cat. No.: 940-000961-00)

Item & Cat. No.	Component	Cap color	Spec & Quantity
MGIEasy Fast RNA Library Prep Kit Cat. No.: 940-000888-00 Specification: 96 RXN x2	Fragmentation Buffer	Green	480 μL/tube × 1
	RT Buffer	brown	384 μL/tube × 1
	RT Enzyme Mix	Green	96 μL/tube × 1
	Second Strand Buffer (with dNTP)	Yellow	1215 μL/tube × 2
	Directional Second Strand Buffer (with dUTP)	Orange	1215 μL/tube × 2
	Second Strand Enzyme Mix	Yellow	452 μL/tube × 1
	Ligation Buffer	Red	1124 μL/tube × 2
	DNA Ligase	Red	154 μL/tube × 1
	PCR Enzyme Mix	Blue	1200 μL/tube × 2
MGIEasy UDB Primers Adapter Kit A Cat. No.: 1000022801 Specification: 96 RXN	UDB Adapter	White	480 μL/tube × 1
	UDB PCR Primer Mix-01- 96	Colorless	12 μL/well × 96
MGIEasy UDB Primers Adapter Kit B Cat. No.: 1000022802 Specification: 96 RXN	UDB Adapter	White	480 μL/tube × 1
	UDB PCR Primer Mix-97- 192	Colorless	12 μL/well × 96
MGIEasy DNA Clean Beads Cat. No.: 940-001174-00 Specification: 15 mL x 2	DNA Clean Beads	White	15 mL/tube × 1
	TE Buffer	White	17 mL/tube × 1

1.5 Storage and transportation

Table 5 Kit storage and transportation temperatures

Item	Storage temperature	Transportation temperature
MGIEasy Fast RNA Library Prep Kit	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃
MGIEasy UDB Primers Adapter Kit		
MGIEasy UDB Primers Adapter Kit A		

Item	Storage temperature	Transportation temperature
MGIEasy UDB Primers Adapter Kit B	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃
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 6 Order information for MGI products

Catalog number	Model	Name
1000005953	32 RXN	MGIEasy rRNA Depletion Kit
1000020570	16 RXN	MGIEasy Dual Barcode Circularization Kit
940-000036-00	OS-DB, 4 RXN	DNBSEQ Onestep DNB Make Reagent Kit V2.0

Table 7 User-supplied equipment list

Equipment	Recommended brand
Vortex mixer	/
Desktop centrifuge	/
Pipettes	/
Thermocycler	/
Magnetic rack DynaMag -2, or equivalent	Thermo Fisher Scientific, Cat. No. 12321D
Qubit Fluoromete, or equivalent	Thermo Fisher, Cat. No. Q33216
Agilent 2100 Bioanalyzer, or equivalent	Agilent Technologies , Cat. No. G2939AA

Table 8 Recommended reagent/consumable list

Reagent/consumable	Recommended brand
Dynabeads mRNA Purification Kit	Invitrogen, Cat. No. 61006
Library Preparation VAHTS mRNA Capture Beads	Vazyme, Cat. No. N403-02
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)	/

Reagent/consumable	Recommended brand
Qubit ssDNA Assay Kit	YEASEN, Cat. No.12645ES60/12645ES76 ,Invitrogen, Cat. No. Q10212, or equivalent
Qubit dsDNA HS Assay Kit	YEASEN, Cat. No.12640ES60/12640ES76, 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	/
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, please contact Technical Support: **MGI-service@mgi-tech.com.**

1.8 Workflow

Section	Workflow	Total time	Hands-on time
3.1	RNA enrichment	1 hr - 2 hr 20 min	20 - 50 min
3.2	RNA fragmentation	11 min	5 min
3.3	Reverse transcription	50 - 55 min	10 - 15 min
3.4	Second strand synthesis and End repair	55 - 60 min	10 - 15 min
3.5	Adapter ligation	25 min	10 min
3.6	Cleanup of adapter-ligated product ⏸	30 - 50 min	20 - 40 min
3.7	PCR ⏸	50 min	10 min
3.8	Cleanup of PCR product ⏸	30 - 40 min	20 - 30 min
3.9	QC of PCR product ⏸	30 - 40 min	10 - 20 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.
- ⏸ : The stop point.

2 Sample preparation

2.1 Applicable sample and requirement

- It is strongly recommended that you use a total RNA input of 10 ng to 1 μg.
- For low-abundance mRNA species such as plants, a total RNA input of 1 - 2.5 μg is recommended.
- For pathogenic microorganisms, human whole blood samples and intestinal samples need to be treated with *MGIEasy rRNA Depletion Kit*, and the input total RNA amount of the samples used is 200 ng.

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.
- RNA integrity: $OD_{260/280}$ = 1.8 - 2.0, $OD_{260/230}$ ≥ 2.
- For library construction of FFPE RNA samples, refer to “Library preparation from low quality FFPE sample” on page 35.
- For library construction of Pathogenic microorganism RNA samples, refer to “Library preparation for RNA pathogen sample” on page 38.
- If DNA contamination is visible in the RNA sample, perform a DNase I digestion to remove DNA before starting the procedure below.

3 Library preparation protocol

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.

3.1.2.1 Preparation

Table 9 Preparing the reagents

Reagent	Requirement
Beads	User-supplied. Take out 30 min in advance to equilibrate to room temperature (RT) and 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.
  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. 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 a 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 a 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(s) 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 a 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 a 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.

3.1.3.1 Preparation

Table 10 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 each 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.
- RNA fragmentation, Reverse transcription, Second strand synthesis and End repair are recommended to be performed without interruption.

3.2.1 Preparation

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

Table 11 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 **5 μL of Fragmentation Buffer** to each sample tube (from 3.1 RNA enrichment). Gently **pipette** the solution at least 5 times, centrifuge briefly, and place on ice.

⚠ CAUTION Do not vortex. When mixing with a pipette for the last time, gently lift the pipette tip to near the liquid surface and slowly inject all of the liquid to ensure that there is no liquid left in the pipette tip.

2. Place the PCR tube(s) into the thermocycler when the temperature reaches the reaction temperature. Incubate according to the recommended fragmentation protocol for your target insert size (105 ℃ heated lid).

CAUTION Preheat the thermocycler to the reaction temperature in advance.

Table 12 Recommended conditions for RNA fragmentation (Volume: 15 μL)

Insert size	RNA fragmentation temperature	RNA fragmentation time
200 bp	94 ℃	6 min
270 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

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 13 Preparing the reagents

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

3.3.2 Reverse transcription

1. 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 14 Reverse transcription mixture

Reagent	Volume per reaction
RT Buffer	4 μL
RT Enzyme Mix	1 μL
Total	5 μL

2. Add **5 μL of reverse transcription mixture** to each sample tube (from step 3 in section 3.2.2). Gently **pipette** at least 5 times to mix it well. Centrifuge briefly and place on ice.

CAUTION Do not vortex. When mixing with a pipette for the last time, gently lift the pipette tip to near the liquid surface and slowly inject all of the liquid to ensure that there is no liquid left in the pipette tip.

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

Table 15 Reverse transcription reaction conditions (Volume: 20 μL)

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

Tips Prepare the "Second strand synthesis and End repair" on page 14 in advance of this step.

4. When the program is completed, centrifuge the tube(s) for 5 sec and place on ice.

3.4 Second strand synthesis and End repair

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

3.4.1 Preparation

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

Table 16 Preparing the reagents

Reagent	Requirement
Second Strand Buffer (with dNTP) or Directional Second Strand Buffer (with dUTP)	Thaw at RT, mix well, centrifuge briefly and place on ice.
Second Strand Enzyme Mix	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

CAUTION The Second Strand Buffer can be selected according to actual needs: If constructing a common RNA library, use the Second Strand Buffer (withdNTP). If constructing a directional RNA library, use the Directional Second Strand Buffer (with dUTP).

3.4.2 Second strand synthesis and End repair

1. 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 17 Second strand synthesis and End repair mixture

Reagent	Volume per reaction
Second Strand Buffer (with dNTP) or Directional Second Strand Buffer (with dUTP)	25.3 μL
Second Strand Enzyme Mix	4.7 μL
Total	30 μL

2. Add **30 μL** of Second strand synthesis and End repair mixture to each sample tube (from step 4 in section 3.3.2). Gently **pipette** at least 10 times to mix well. Centrifuge briefly and place on ice.

CAUTION
- Do not vortex. Set the pipette to 40 μL for mixing. To prevent bubbles from forming, do not pour out all of the liquid each time.
- When mixing with a pipette for the last time, gently lift the pipette tip to near the liquid surface and slowly inject all of the liquid to ensure that there is no liquid left in the pipette tip.

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

Table 18 Second strand synthesis and End repair reaction conditions (Volume: 50 μL)

Temperature	Time
70 ℃ Heated lid	On
16 ℃	30 min
65 ℃	15 min
4 ℃	Hold

Tips Prepare the “Adapter ligation ” on page 16 in advance of this step.

4. When the program is completed, centrifuge the tube(s) for 10 sec and place on ice.

3.5 Adapter ligation

Tips
- The amount of adapter used in Adapter Ligation depends on the amount of RNA input. Read "UDB PCR Primer Mix pooling guide" on page 26carefully 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.

3.5.1 Preparation

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

Table 19 Preparing the reagents

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

CAUTION
- Mix the adapter well before use. Adapter should not be mixed directly with the adapter ligation mixture.
- The Ligation Buffer is highly viscous. Mix it well by vortexing 6 times (3 sec each) and centrifuge briefly for 10 sec. If white precipitation is visible inside the tube cap, cover the tube cap and turn it upside down and then shake and mix well to dissolve the white precipitate.

3.5.2 Adapter ligation

1. Dilute the adapter according the table below with **TE Buffer** and mix well by vortexing 3 times (3 s each). Centrifuge the diluted adapter(s) briefly and place on ice.

Table 20 Recommended adapter input according to the amount of total RNA

total RNA (ng)	UDB Adapter	
-	Adapter dilution ratio	Adapter input after dilution (μL)
201-2500	No dilution	5
51-200	5	5
10-50	10	5

Tips The table above lists the corresponding dilution factor of the UDB Adapter when high-quality total RNA samples of varying quality are input. For samples with different degrees of degradation, refer to "Recommended amount of FFPE sample input" on page 36

2. **Add 5 μL of diluted adapter to the corresponding sample tube** (from step 4 in section 3.4.2). 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 21 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. Be careful to aspirate and not to extend the tip too far below the liquid surface to prevent it from sticking to the tip wall; when adding liquid, add the liquid slowly, and there should be no liquid left in the tip.

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

Table 22 Adapter ligation reaction conditions (Volume: 80 μL)

Temperature	Time
30 ℃ Heated lid	On
23 ℃	15 min
4 ℃	Hold

6. When the program is completed, centrifuge the PCR tube(s) briefly and place on ice.

CAUTION
- Do not stop at this step. Proceed to next reaction.
- If you need to stop here, store the product(s) at -20 ℃ for no longer than 16 hr. There is a risk of reduced yield.

3.6 Cleanup of adapter-ligated product

Tips
- For a 200 bp insert size, refer to section 3.6.2. For a 270 bp insert size, refer to section 3.6.3.

- 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.

3.6.1 Preparation

Table 23 Preparing the reagents

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

3.6.2 Cleanup (200 bp insert size)

1. Add **40 µL of TE Buffer** into each sample tube (from step 6 in section 3.5.2) to 120 µL.

   CAUTION
   - 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.
   - When adding TE Buffer and DNA Clean Beads, the liquid should be added slowly with a single tip to ensure accurate pipetting. Otherwise, the final product yield and fragment size may be affected.

2. Add **30 µL of DNA Clean Beads** to each sample tube. Mix with a vortexer until all beads are suspended. Centrifuge lightly, and pay attention to maintain uniform dispersion of magnetic beads.
3. Incubate the sample(s) at room temperature for 5 min.
4. 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.

   CAUTION If the liquid is difficult to clarify, gently rotate the sample tube on a magnetic rack to make the magnetic beads adsorb more concentratedly.

5. 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.
6. Repeat step 5. 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.

7. 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.

8. Remove the centrifuge tube(s) from the magnetic rack and add **23 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.
9. Incubate the sample(s) at room temperature for 5 min.
10. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer **21 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.

3.6.3 Cleanup (270 bp insert size)

1. Add **60 μL of TE Buffer** into each sample tube (from step 6 in 3.5.2) to 140 μL.

   CAUTION
   - 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.
   - When adding TE Buffer and DNA Clean Beads, the liquid should be added slowly with single tip to ensure accurate pipetting, otherwise the final product yield and fragment size may be affected.

2. Add **23 μL of DNA Clean Beads** to each sample tube. Mix with a vortexer until all beads are suspended. Ensure that all of the solution and beads in the tip are transferred into the tube after mixing.
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 **153 μL** of supernatant to a new PCR or centrifuge tube.
5. Add **15 μL of DNA Clean Beads** to each sample tube (containing 153 μL supernatant). Mix with a vortexer until all beads are suspended. Centrifuge lightly, pay attention to maintain uniform dispersion of magnetic beads.

   CAUTION When adding DNA Clean Beads, ensure that you are pipetting accurately. Otherwise, the final product yield and fragment size may be affected.

6. Incubate the sample(s) at room temperature for 5 min.
7. 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.
8. 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.

9. Repeat step 8. 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.
10. 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.

11. Remove the centrifuge tube(s) from the magnetic rack and add **23 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.
12. Incubate the sample(s) at room temperature for 5 min.
13. Centrifuge the tube(s) briefly and place on the magnetic rack for 2 to 5 min until the liquid is clear. Carefully transfer **21 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 ℃.

3.7 PCR

3.7.1 Preparation

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

Table 24 Preparing the reagents

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

3.7.2 PCR

1. Add **25 µL PCR Enzyme Mix** to each sample tube (from step 10 in section 3.6.2 or step 13 in section 3.6.3).
2. Add **4 µL** of the corresponding **UDB PCR Primer Mix** according to "Notes for UDB PCR Primer Mix" on page 25 . Vortex 3 times (3 sec each) and centrifuge briefly to collect the solution at the bottom of the tube.

Table 25 PCR mixture

Reagent	Volume per reaction
Adapter-ligated product (from step 10 in section 3.6.2 or step 13 in section 3.6.3)	21 μL
PCR Enzyme Mix	25 μL
Corresponding UDB PCR Primer Mix	4 μL
Total	50 μL

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

Table 26 PCR reaction conditions (Volume: 50 μL)

Temperature	Time	Cycles
105 ℃ Heated lid	On	-
95 ℃	3 min	1
95 ℃	15 s	X (see table 27)
60 ℃	30 s	
72 ℃	30 s	
72 ℃	5 min	1
4 ℃	Hold	-

Table 27 The recommended PCR cycles for 420 ng PCR yield

Total RNA (ng)	Directional PCR cycles	Common PCR cycles
10	17-19	16-18
50	16-17	15-16
200	14-15	13-14
1000	12-13	11-12

Tips The table above lists the number of PCR cycles required when inputting 10-1000 ng of high-quality sample total RNA(the requirements of high-quality total RNA refer to "QC of sample" on page 8). For samples of different species and different degrees of degradation, the number of PCR cycles should be adjusted according to the actual situation.

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

Stop point PCR product(s) can be stored at -20 ℃.

3.8 Cleanup of PCR 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.

3.8.1 Preparation

Table 28 Preparing the reagents

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

3.8.2 Cleanup of PCR product

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

1. Mix the DNA Clean Beads thoroughly. Add **40 μL of DNA Clean Beads** to each sample tube (from step 4 in 3.7.2). 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 ℃.

3.9 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 29 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: ≥ 1.5 pmol
Electrophoresis method	Tapestation (Agilent Technologies), Bioanalyzer, LabChip GX, GXII, GX Touch (PerkinElmer), Fragment Analyzer (Advanced Analytical)	200 bp insert size: Main size: 280 bp - 370 bp
		270 bp insert size: Main size: 380 bp - 470 bp

Formula 1 Conversion between N pmol and mass in ng of PCR product

$$\text{N pmol PCR product (ng)} = N\times \frac{\text{PCR product peak size (bp)}}{1000\ \text{bp}} \times 660\ \text{ng}$$

Tips Set N = 1.5 in Formula 1 when calculating the mass of 1.5 pmol PCR product.

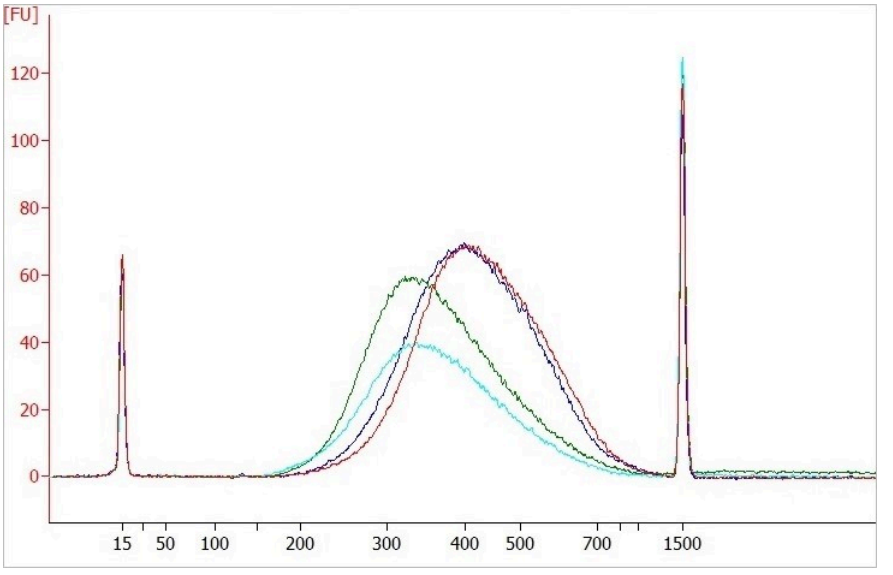

Figure 1 The Agilent 2100 bioanalyzer results of purified PCR product

Tips
- Stop here if the library will be delivered to a service lab for sequencing.
- Proceed to "Circularization and DNB preparation" on page 28 or "Onestep DNB preparation" on page 33 if the library will be sequenced in your lab.

4 Appendix

4.1 MGIEasy UDB Primers Adapter Kit Instructions

MGIEasy UDB Universal Library Prep Sets (16 RXN) provides primers in tubes, 96 RXN provides primers in plates, and 192 RXN provides two plates of primers. This kit was developed to meet requirements for batch processing of library construction and multiplex sequencing. The maximum 192-well of UDB PCR Primer Mix and one tube of UDB Adapter Mix are provided, which provides 192 samples of multiplex sequencing.

4.1.1 Notes for UDB Adapter

- UDB Adapter is double stranded. To prevent structural changes, such as denaturation, which might affect performance, do not place the adapters in an area that exceeds 30 ℃.
- Before use, centrifuge UDB Adapter to collect liquid to the bottom of the tubes. Mix UDB Adapter thoroughly before use. Close the cap immediately after use.
- The length of UDB Adapter is 132 bp.

4.1.2 Notes for UDB PCR Primer Mix

- The MGI dual-barcode library can be used for both single-barcode sequencing and dual-barcode sequencing. Please refer to the corresponding single-barcode/dual-barcode sequencing user manual.
- Before the use, centrifuge to collect liquid at the bottom of tubes or plates.
- Change tips when pipetting different solutions to prevent cross-contamination.
- For tubes, gently remove the cap to prevent liquid from spilling and cross-contamination. Cover the tube immediately after use.
- For 96-well plate, spray 75% alcohol and wipe the surface of the aluminum film of the plate with absorbent paper. The aluminum film is penetrable. 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 ℃.

- Based on the principles of balanced base composition, UDB PCR Primer Mix must be used in specific groups. Follow the instructions below to use UDB PCR Primer Mix in proper combination:

1. For Tube: 2 sets of 8 UDB PCR Primers, which are UDB PCR Primer Mix-57 _ UDB PCR Primer Mix-64 and UDB PCR Primer Mix-89 _ UDB PCR Primer Mix-96 separately.
2. For Plate: 96 UDB PCR Primer Mix per plate, 8 wells of each column are preset as a balanced dual barcode combination. The detailed layout is as follows.

Table 30 Set A barcode layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDB1	UDB9	UDB17	UDB25	UDB33	UDB41	UDB49	**UDB57**	UDB65	UDB73	UDB81	**UDB89**
B	UDB2	UDB10	UDB18	UDB26	UDB34	UDB42	UDB50	**UDB58**	UDB66	UDB74	UDB82	**UDB90**
C	UDB3	UDB11	UDB19	UDB27	UDB35	UDB43	UDB51	**UDB59**	UDB67	UDB75	UDB83	**UDB91**
D	UDB4	UDB12	UDB20	UDB28	UDB36	UDB44	UDB52	**UDB60**	UDB68	UDB76	UDB84	**UDB92**
E	UDB5	UDB13	UDB21	UDB29	UDB37	UDB45	UDB53	**UDB61**	UDB69	UDB77	UDB85	**UDB93**
F	UDB6	UDB14	UDB22	UDB30	UDB38	UDB46	UDB54	**UDB62**	UDB70	UDB78	UDB86	**UDB94**
G	UDB7	UDB15	UDB23	UDB31	UDB39	UDB47	UDB55	**UDB63**	UDB71	UDB79	UDB87	**UDB95**
H	UDB8	UDB16	UDB24	UDB32	UDB40	UDB48	UDB56	**UDB64**	UDB72	UDB80	UDB88	**UDB96**

Table 31 Set B barcode layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDB97	UDB105	UDB113	UDB121	UDB129	UDB137	UDB145	UDB153	UDB161	UDB169	UDB177	UDB185
B	UDB98	UDB106	UDB114	UDB122	UDB130	UDB138	UDB146	UDB154	UDB162	UDB170	UDB178	UDB186
C	UDB99	UDB107	UDB115	UDB123	UDB131	UDB139	UDB147	UDB155	UDB163	UDB171	UDB179	UDB187
D	UDB100	UDB108	UDB116	UDB124	UDB132	UDB140	UDB148	UDB156	UDB164	UDB172	UDB180	UDB188
E	UDB101	UDB109	UDB117	UDB125	UDB133	UDB141	UDB149	UDB157	UDB165	UDB173	UDB181	UDB189
F	UDB102	UDB110	UDB118	UDB126	UDB134	UDB142	UDB150	UDB158	UDB166	UDB174	UDB182	UDB190
G	UDB103	UDB111	UDB119	UDB127	UDB135	UDB143	UDB151	UDB159	UDB167	UDB175	UDB183	UDB191
H	UDB104	UDB112	UDB120	UDB128	UDB136	UDB144	UDB152	UDB160	UDB168	UDB176	UDB184	UDB192

4.1.3 UDB PCR Primer Mix pooling guide

It is recommended that you optimize base balance by planning dual barcode with diverse sequences when pooling libraries across DNBSEQ systems. Pooling combines at least eight libraries to sequence in one lane. Eight wells of each column are preset as a balanced dual barcode combination. Use this guide as a reference to plan X-plex pooling (X≥8) strategies showed in the following table.

Table 32 UDB PCR Primer Mix Pooling Guide

Plexity	Combinations
8X	X entire column
8X+1	X entire column + 1 random well
8X+2	X entire column + 2 random well
8X+3	X entire colu mn + 3 random well
8X+4	X entire column + 4 random well
8X+5	X entire column + 5 random well
8X+6	X entire column + 6 random well
8X+7	X entire column + 7 random well

Under exceptional circumstances (for example, one well of barcode missed), when it cannot meet the requirement of at least one balanced barcode combination for standard pooling or the required data amount of each library pooled is not equal, make sure to determine the pooling strategy by calculating the content of each base in each sequencing cycle. It is necessary to ensure that each base content is not less than 12.5% and is not greater than 62.5% in single sequencing position in the same lane.

Table 33 perfect balanced 8 barcode Pooling strategy (8 barcode from one entire column)

Sample 1	A	G	G	A	C	G	T	A	G	A
Sample 2	C	T	G	A	A	C	C	G	A	A
Sample 3	G	A	A	C	G	T	G	T	C	G
Sample 4	T	C	C	G	T	G	A	C	T	C
Sample 5	A	A	T	T	C	A	C	T	G	T
Sample 6	C	C	T	G	A	A	G	G	A	T
Sample 7	T	T	C	C	T	T	A	C	T	G
Sample 8	G	G	A	T	G	C	T	A	C	C
Signal%	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0

Table 34 unacceptable 9 barcode Pooling strategy (barcodes from different column)

Sample 1	A	G	G	A	C	G	T	A	G	T
Sample 2	A	C	G	A	A	G	G	T	C	C
Sample 3	G	A	A	C	G	T	G	T	C	G
Sample 4	T	C	C	G	T	G	A	C	T	C
Sample 5	A	A	T	T	C	A	C	T	G	T
Sample 6	G	C	T	G	A	A	G	G	A	T
Sample 7	T	G	C	C	T	T	A	C	T	G
Sample 8	G	G	A	T	G	A	T	A	C	C

Sample 9	G	A	C	G	G	T	C	G	A	G
A signal%	33.3	33.3	22.2	22.2	22.2	33.3	22.2	22.2	22.2	0
T signal%	22.2	0	22.2	22.2	22.2	33.3	22.2	33.3	22.2	33.3
C signal%	0	33.3	33.3	22.2	22.2	0	22.2	22.2	33.3	33.3
G signal%	44.4	33.3	22.2	33.3	33.3	33.3	33.3	22.2	22.2	33.3

4.2 Circularization and DNB preparation

4.2.1 Denaturation and single strand circularization

Tips

- According to the distribution of the main band and concentration of the purified PCR product, refer to Formula 1 in section 3.9 (set N = 1.5) to calculate the required volume of the purified PCR product.
- For multiple samples pooled sequencing, refer to "UDB PCR Primer Mix pooling guide" on page 26. Quantify the purified PCR products before pooling. The total yield after pooling should be 1.5 pmol, with a total volume ≤ 48 μL.

  For example: For 8 libraries with main insert fragment of 200 bp (UDB Adapter is 132 bp, PCR product fragment is 332 bp), add 41.1 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 328.7 ng and be equal to a total yield of 1.5 pmol. Add TE Buffer to make a total volume of 48 μL.

4.2.1.1 Preparation

Reagent: For use with MGIEasy Dual Barcode Circularization Kit. User-supplied.

Table 35 Preparing the reagents

Reagent	Requirement
TE Buffer, pH 8.0	User-supplied; place at RT.
Dual Barcode Splint Buffer	Thaw at RT, mix well, centrifuge briefly, and place on ice.
DNA Rapid Ligase	Flick and/or invert the tube gently, centrifuge briefly, and place on ice.

4.2.1.2 Denaturation

1. Pipette 1.5 pmol of PCR purified product or mixed purified products into a new 0.2 mL PCR tube. Add **TE Buffer** to make a total volume of 48 μL.
2. Place the PCR tube into the thermocycler. Run the program with the following conditions.

Table 36 Denaturation reaction conditions (Volume: 48 μL)

Temperature	Time
100 ℃ Heated lid	On
95 ℃	3 min

3. After the reaction, immediately place the PCR tube(s) on ice for 2 min. Centrifuge briefly and place on ice.

4.2.1.3 Single strand circularization

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

Table 37 Circularization reaction mixture

Reagent	Volume per reaction
Dual Barcode Splint Buffer	11.5 μL
DNA Rapid Ligase	0.5 μL
Total	12.0 μL

2. Add **12.0 μL of circularization reaction mixture** to each sample tube (from step 2 in section 4.2.1.2). Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.
3. Place the PCR tube into the thermocycler. Run the program with the following conditions.

Table 38 Single strand DNA circularization reaction conditions (Volume: 60 μL)

Temperature	Time
42 ℃ Heated lid	On
37 ℃	10 min
4 ℃	Hold

Tips Prepare the "Table 40 Digestion mixture" on page 30 in advance of this step.

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

4.2.2 Digestion

4.2.2.1 Preparation

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

Table 39 Preparing the reagents

Reagent	Requirement
Digestion Buffer	Thaw at RT, mix well, 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 well, centrifuge briefly, and place at RT.

4.2.2.2 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 40 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 4.2.1.3). Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.
3. Place the PCR tube into the thermocycler. Run the program with the following conditions.

Table 41 Digestion reaction conditions (Volume: 64 μL)

Temperature	Time
42 ℃ Heated lid	On
37 ℃	10 min
4 ℃	Hold

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

CAUTION It is not recommended to stop here, please continue to complete the cleanup of digestion product. If it must be stopped, please place it in a -20 ℃ refrigerator for no more than 16 hr, but there is a risk of reduced yield or reduced performance.

4.2.3 Cleanup of digestion 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.

4.2.3.1 Preparation

Table 42 Preparing the reagents

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

4.2.3.2 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 section 4.2.2.2). 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 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 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 10 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 ℃ for one month.

4.2.4 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 120 fmol (enough for two sequencing runs). Refer to Formula 2 for calculations.

Formula 2 Conversion between M pmol and mass in ng of ssDNA

$$\text{M pmol ssDNA (ng)} = \text{M}\times \frac{\text{PCR product peak size (bp)}}{\text{1000 bp}} \times 330 \text{ ng}$$

Tips Set M = 0.12 in Formula 2 to calculate the mass of 120 fmol ssDNA product.

4.2.5 DNB preparation

Refer to *MGISEQ-200RS High-throughput (Rapid)Sequencing Set User Manual*, *DNBSEQ-G400RS High-throughput (Rapid) Sequencing Set User Manual*, or *DNBSEQ-T7RS High-throughput Sequencing Set User Manual* to prepare DNB, 60 fmol (according to formula 2, set N=0.06) ssDNA product was prepared into a DNB library suitable for an MGI sequencer.

If multiple ssDNA libraries need to be pooled, it is recommended that you mix them based on their molar ratio.The molar ratio of pooled ssDNA depends on the expected data volume ratio of the different samples by the customers. However, the UDB corresponding to the pooled sample must comply with "UDB PCR Primer Mix pooling guide" on page 26.

4.3 Onestep DNB preparation

If the onestep DNB preparation method is choosen, DNBSEQ Onestep DNB Make Reagent Kit V2.0 (OS-DB, Cat. No.: 940-000036-00) is required for rapid DNB preparation.

Tips Library Pooling Considerations.

- When pooling library from same type of sample and same input quantities, libraries can be pooled by certain mass ratio based on the desired ratio of sequencing data output.
- Pooling library from different types of sample or different input quantities is not recommended as it could potentially result in non-uniformed data split across different barcode.

4.3.1 Preparation

- Samples: Place the product from step 9 in section 3.8.2 on ice.
- Mix the reagents before using and store the remaining reagents immediately after use.

Table 43 Preparing the reagents

Reagent	Requirement
Make DNB Buffer (OS-V2.0-DB)	Thaw at RT, mix well, centrifuge briefly, and place on ice.
Molecular Grade Water	
Make DNB Enzyme Mix I (OS-V2.0)	Centrifuge briefly and place on ice.
Make DNB Enzyme Mix II (OS)	
Stop DNB Reaction Buffer	Thaw at RT, mix well, centrifuge briefly, and place on ice.
Qubit ssDNA Assay Kit	User-supplied.

4.3.2 Operation

1. Based on the dsDNA library concentration, add **100 fmol dsDNA library** (calculate the corresponding mass according to Formula 1, set N = 0.1) to a new 0.2 ml 8-strip or PCR tube. Add the following reagents into the tube on ice. Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.

Table 44 DNB Making System 1

Reagent	Volume per reaction
dsDNA library	V μL
Molecular Grade Water	20-V μL
Make DNB Buffer (OS-V2.0-DB)	20 μL
Total	40 μL

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

Table 45 DNB Reaction Condition 1 (Volume: 40 μL)

Temperature	Time
105 ℃ Heated lid	On
95 ℃	3 min
57 ℃	3 min
4 ℃	Hold

3. According to the desired reaction number, prepare the DNB making system 2 on ice. Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.

Table 46 DNB Making System 2

Reagent	Volume per reaction
Make DNB Enzyme Mix I (OS-V2.0)	40 μL
Make DNB Enzyme Mix II (OS)	4 μL
Total	44 μL

4. Add **44 μL of DNB making system 2** to each sample tube (from step 2). Vortex it 3 times (3 sec each), centrifuge briefly, and place on ice.
5. Place the PCR tube into the thermocycler. Run the program with the following conditions.

Table 47 DNB Reaction Condition 2 (Volume: 84 μL)

Temperature	Time
35 ℃ Heated lid	On
30 ℃	25 min
4 ℃	Hold

Tips
- For certain thermocyclers from some manufacturers, it takes an extended time to achieve the desired temperature of the heated lids. For this type of thermocycler, preheat the lid in advance to ensure that the lid remains at working temperature during the reaction..

- The temperature of heated lids is suggested to be 35 ℃, or as close as possible to the lowest temperature of 35 ℃.

6. After the reaction, immediately add **20 μL of Stop DNB Reaction Buffer** to each sample tube. Mix gently by pipetting 5 to 8 times with a wide-bore tip. Do not vortex, shake the tube or pipette vigorously. After mixing, store samples at 4 ℃ for later use (use within 48 hours).

   CAUTION DNB must be pipetted gently with a wide-bore tip. Do not centrifuge, vortex, shake, or pipette vigorously.

7. Quantify the DNB with ssDNA Fluorescence Assay Kits, such as Qubit ssDNA Assay Kit. Sequencing requires a minimum DNB concentration of 8 ng/μL . If the concentration is lower than 8 ng/μL, the unqualified DNB should be prepared again.

   CAUTION A volume of 2 μL DNB is suggested to be measured. If the number of samples is large, it is recommended that they be quantified in batches. This helps avoid inaccurate measurement of DNB concentration.

4.4 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 Fast RNA Library Prep Set as an example and list the problems that you need to address in the library construction to account for different quality FFPE samples.

4.4.1 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.

4.4.1.1 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.

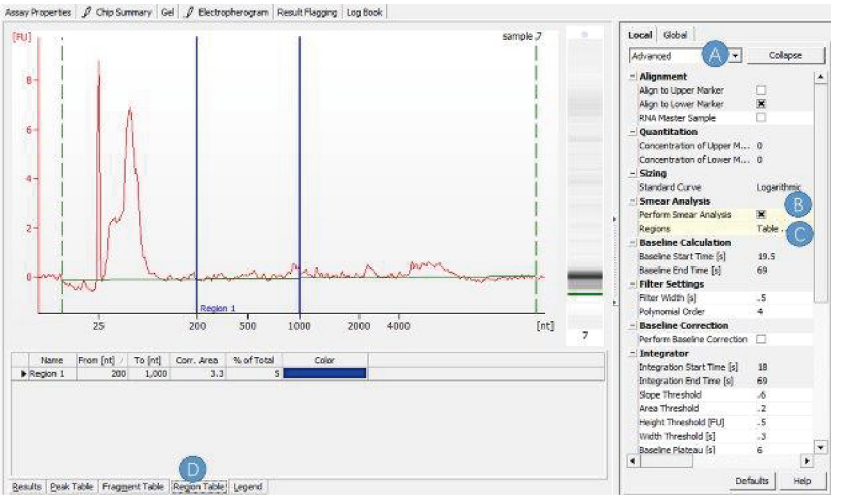

Figure 2 The calculation of DV_{200}

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).

4.4.2 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 "Adapter Ligation" section, pay attention to the amount of UDB Adapter.
- In "PCR" section, note the corresponding different number of PCR cycles for different DNA sample inputs. See the indicated table for detailed conditions.

Table 48 Recommended conditions of library construction from FFPE sample

FFPE DV_{200}	Recommended amount of total RNA input	RNA fragmentation	PCR cycles
>70%	200 ng	94 °C, 5 min	15
50-70%	200~400 ng	94 °C, 4 min	16
30-50%	500 ng	No fragmentation	16
<30%	0.5-1 μg (with a risk of failure of library construction)	No fragmentation	16

Table 49 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	10	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

4.4.3 Library preparation protocol for FFPE sample

4.4.3.1 RNA enrichment

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

4.4.3.2 RNA fragmentation

Refer to "Recommended amount of FFPE sample input" on page 36 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 5 * (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°C for 5 minutes. Immediately place the sample and PCR tube on ice for 2 minutes and centrifuge for 10 s for further use.
3. Add 5 μL Fragmentation Buffer to each sample and immediately proceed to the next reaction.

4.4.3.3 Reverse transcription

Same as section 3.3.

4.4.3.4 Second strand synthesis product and End repair

Refer to section 3.4.

4.4.3.5 Adapter ligation

Refer to section 3.5. Refer to "Recommended amount of FFPE sample input" on page 36. Use a different amount of adapter for different FFPE samples.

4.4.3.6 Cleanup of adapter-ligated product

Same as section 3.6 (200 bp insert size).

4.4.3.7 PCR

Refer to section 3.7. Refer to "Recommended amount of FFPE sample input" on page 36. Use different numbers of PCR cycles for different FFPE samples.

4.4.3.8 Cleanup to QC of PCR product

Same as section 3.8 to 3.9.

4.5 Library preparation for RNA pathogen sample

4.5.1 Applicable types of RNA pathogen samples

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

4.5.2 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.

4.5.3 Library preparation from RNA pathogen samples

4.5.3.1 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.

4.5.3.2 RNA fragmentation

Refer to section 3.2.
The RNA sample is incubated at 94 ℃ for 6 min according to the conditions for 200 bp.

4.5.3.3 Reverse transcription to Cleanup of adapter-ligated product

Same as section 3.3 to 3.6.
Same as section 3.6.2 (200 bp insert size).

4.5.3.4 PCR

Refer to section 3.7.
For rRNA depleted samples, do 15 cycles of PCR to amplify the sample.

4.5.3.5 Cleanup of PCR product

Same as section 3.8.

4.5.3.6 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 50 Standards of library quality control

QC	MGIEasy RNA Library Prep Set	Standards of library QC
PCR product yield	Quantify using Qubit dsDNA HS	≥ 420 ng
PCR product size	Agilent 2100 chip inspection	Size range: 280-370 bp, average: 200-700 bp
Adapter residue	Agilent 2100 chip inspection	Visual observation, No obvious peak around 130 bp

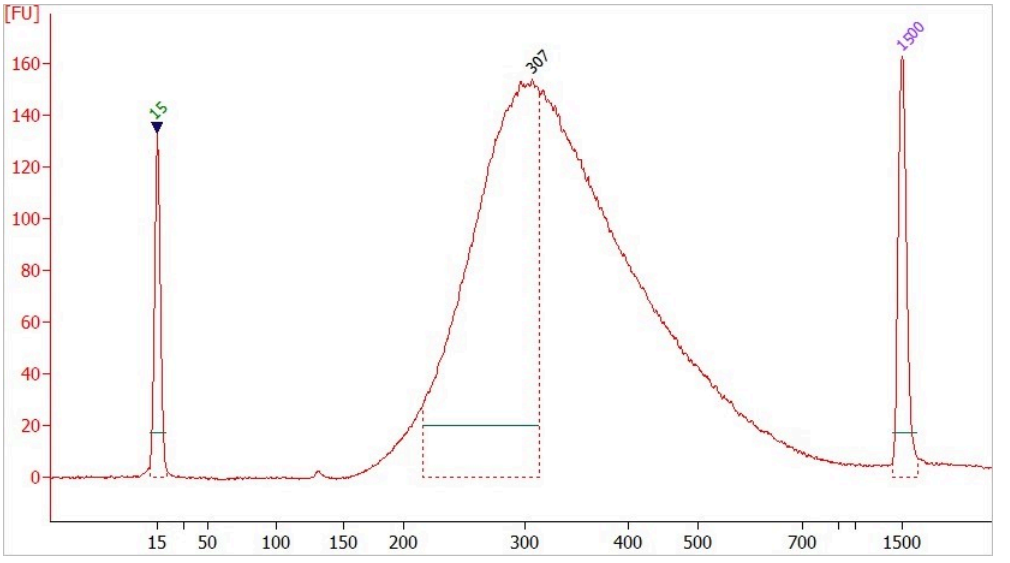

Figure 3 The agilent 2100 bioanalyzer results of purified PCR product