ATOPlex

RNA Multiplex PCR-based Library Preparation Set V3.1 User Manual

Cat. No.: 940-000132-00（16RXN），940-000133-00（96RXN）

Kit Version: V3.1

Manual Version: 2.0

For Research Use Only.

Revision History

Manual Version	Kit Version	Date	Description
2.0	V3.1	Apr. 2022	◆ Upgraded Internal Control Primer Pool V3.1. ◆ Modified PCR reaction conditions. ◆ Deleted the flowchart for library preparation. ◆ Reduced the ligation product input for DNB preparation.
1.0	V3.0	Dec. 2021	◆ Initial release.

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

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

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

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Chapter 1 Product Description

1.1 Introduction

This manual is for the ATOPlex RNA multiplex PCR library Preparation Set V3.1 based on the MGI ATOPlex customized platform. The product uses multiplex RT-PCR technology to complete the RNA target region capture and amplification in two tubes. MGIEasy Fast PCR-FREE FS DNA Library Prep Set for specific library preparation and One Step DNB Make Reagent Kit for DNB making are then used before sequencing. The combination of RNA multiplex PCR-based amplification method, Fast PCR-FREE library preparation and one-step DNB preparation method simplify the library preparation process and shorten the operating time. All reagents provided within these kits have passed stringent quality control and functional verification procedures, ensuring performance stability and reproducibility.

1.2 Application

This set can be used for the total RNA extracted from multiple sample types such as blood, tissue, throat swab, nasal swab, etc.

The DNB is prepared by DNBSEQ One Step DNB Make Reagent Kit (OS-DB) (Cat. No: 1000026466) and it is compatible with SE100+10+10 sequencing on DNBSEQ-G50RS and DNBSEQ-G400RS. See Chapter 4 for the recommended sequencing kits.

1.3 Kit Contents

The user manual applies to the following products, further information on Cat. No.:, Components and Specifications are listed below.

Table 1-1 ATOPlex RNA Multiplex PCR-based Library Preparation Set V3.1 (16 RXN)

(Cat. No.: 940-000132-00)

Modules & Cat. No.	Modules & Cat. No.	Components	Cap Color	Spec & Quantity
ATOPlex RNA Multiplex PCR Amplification Set V3.1 Cat. No.: 940-000119-00	ATOPlex RNA Multiplex PCR Amplification Module Cat. No.: 940-000128-00	PCR Enzyme Mix	Blue	384 μL / tube × 1tube
		PCR Clean Enzyme	White	16 μL / tube × 1 tube
		PCR Additive	Yellow	16 μL / tube × 1 tube
		N6 Buffer	Green	64 μL / tube × 1 tube
		RT Buffer	Green	80 μL / tube × 1 tube
		RT Enzyme Mix	Green	16 μL / tube × 1 tube
	Internal Control Primer Pool V3.1 Cat. No.: 940-000333-00	PCR Primer Pool 1 V3.1	Blue	32 μL/tube × 1 tube
		PCR Primer Pool 2 V3.1	Blue	32 μL/tube × 1 tube
MGIEasy Fast PCR-FREE FS DNA Library Prep Set Cat. No: 940-000019-00	MGIEasy Fast PCR-FREE FS DNA Library Prep Kit Cat. No.: 940-000017-00	20x Elute Enhancer	Black	5 μL / tube x 1 tube
		Fast FS Buffer	Green	180 μL/ tube × 1 tube
		Fast FS Enzyme	Green	90 μL/ tube × 1 tube
		Fast Ligation Buffer	Red	391 μL/ tube × 1 tube
		Ad Ligase	Red	85 μL/ tube × 1 tube
		Ligation Enhancer	Brown	34 μL/ tube × 1 tube
	MGIEasy UDB PF Adapter Kit Cat. No.: 940-000018-00	UDB Adapters	Blue	5 μL/ tube × 16 tubes
	MGIEasy DNA Clean Beads Cat. No.: 1000005278	DNA Clean Beads	White	8 mL/ tube × 1 tube
		TE Buffer	White	4 mL/ tube × 1 tube

Table 1-2 ATOPlex RNA Multiplex PCR-based Library Preparation Set V3.1 (96 RXN)
(Cat. No.: 940-000133-00)

Modules & Cat. No.	Modules & Cat. No.	Components	Cap Color	Spec & Quantity
ATOPlex RNA Multiplex PCR Amplification Set V3.1 Cat. No.: 940-000135-00	ATOPlex RNA Multiplex PCR Amplification Module Cat. No.: 940-000127-00	PCR Enzyme Mix	Blue	1,200 µL / tube × 2 tubes
		PCR Clean Enzyme	White	96 µL / tube × 1 tube
		PCR Additive	Yellow	96 µL / tube × 1 tube
		N6 Buffer	Green	384 µL / tube × 1 tube
		RT Buffer	Green	480 µL / tube × 1 tube
		RT Enzyme Mix	Green	96 µL / tube × 1 tube
	Internal Control Primer Pool V3.1 Cat. No.: 940-000334-00	PCR Primer Pool 1 V3.1	Blue	192 µL / tube × 1 tube
		PCR Primer Pool 2 V3.1	Blue	192 µL / tube × 1 tube
	MGIEasy DNA Clean Beads Cat. No.: 1000005278	DNA Clean Beads	White	8 mL/ tube × 1 tube
		TE Buffer	White	4 mL/ tube × 1 tube
MGIEasy Fast PCR-FREE FS DNA Library Prep Set Cat. No: 940-000021-00	MGIEasy Fast PCR-FREE FS DNA Library Prep Kit Cat. No.: 940-000020-00	20x Elute Enhancer	Black	15 µL / tube x 1 tube
		Fast FS Buffer	Green	1,360 µL/ tube × 1 tube
		Fast FS Enzyme	Green	600 µL/ tube × 1 tube
		Fast Ligation Buffer	Red	1,360 µL/ tube × 3 tubes
		Ad Ligase	Red	600 µL/ tube × 1 tube
		Ligation Enhance	Brown	320 µL/ tube × 1 tube
		TE Buffer	White	4 mL / tube x 2 tubes
	MGIEasy UDB PF Adapter Kit Cat. No.: 940-000023-00	UDB Adapters A	Clear	5 µL/ tube × 96 tubes
	MGIEasy DNA Clean Beads x 2 Cat. No.: 1000005278	DNA Clean Beads	White	8 mL/ tube × 1 tube
		TE Buffer	White	4 mL/ tube × 1 tube

1.4 Storage Conditions and Shelf Life

ATOPlex RNA Multiplex PCR Amplification Module

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

Internal Control Primer Pool V3.1

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

MGIEasy Fast PCR-FREE FS DNA Library Prep Kit

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

⚠ **Note: Keep 20x Elute Enhancer and Ligation Enhancer in dark under room temperature to avoid repeated freezing and thawing after using for the first time.**

MGIEasy UDB PF Adapter Kit

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

MGIEasy UDB PF Adapter Kit A

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

MGIEasy DNA Clean Beads

- Storage Temperature: 2 °C to 8 °C
- Production Date and Expiration Date: refer to the label
- Transport Conditions: transported with ice packs

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

⚠ **Note: Performance of products is guaranteed until the expiration date, and under appropriate**

transport, storage, and usage conditions.

1.5 Equipment and Materials Required but not Provided

Table 1-4 Equipment and Materials Required but not Provided

Equipment	Vortex Mixer Desktop Centrifuge Microplate Centrifuge Pipets Thermocycler Magnetic rack DynaMagTM-2 (Thermo Fisher Scientific，Cat. No.: 12321D) or equivalent Qubit® 3 Fluorometer (Thermo Fisher Scientific, Cat. No.: Q33216)
Reagents	Nuclease free water (NF water) (Ambion, Cat. No.: AM9937) 100% Ethanol (Analytical Grade) 1xTE Buffer, pH8.0 (Ambion, Cat. No.: AM9858) Qubit ssDNA Assay Kit (Invitrogen, Cat. No.: Q10212) Qubit dsDNA HS Assay Kit (Invitrogen, Cat. No.: Q32854)
Consumables	Pipette Tips 1.5 mL centrifuge tubes (Axygen, Cat. No.: MCT-150-C) 0.2 mL PCR tubes (Axygen, Cat. No.: PCR-02-C) or 96-well PCR plate (Axygen, Cat. No.: PCR-96M2-HS-C) Qubit Assay Tubes (Invitrogen, Cat. No.: Q32856) or 0.5 mL Thin Wall PCR Tubes (Axygen, Cat. No.: PCR-05-C)

1.6 Precautions and Warnings

- This product is for research use only. Please read this manual carefully before use.
- Before the experiment, please be familiar with the operation methods and precautions of various instruments to be used.
- Remove the reagents from storage beforehand and prepare them for use: Mix enzymes by inverting, then centrifuge briefly and place on ice for use. Thaw other reagents at room temperature and vortex several times to mix properly, then centrifuge briefly and place on ice until further use.
- To prevent cross contamination, we recommend using filter pipette tips for all steps except DNB preparation. Use a new tip each time for pipetting different solutions. There is a risk of contamination if filter tips are not used.

⚠ **Note: Improper handling of samples and reagents may contribute to aerosol contamination of**

PCR products and may decrease the accuracy of results. Therefore, we recommend physically separating two working areas for the Pre-PCR room and the Post-PCR room, respectively. We recommend performing the reverse transcription, the PCR reaction mix preparation, PCR product cleanup mix preparation and PCR free library preparation mix preparation in the Pre-PCR room, performing the PCR reaction, PCR product cleanup and PCR free library preparation in the Post-PCR room. Library pooling and DNB preparation can be proceeded in the Post-PCR room. Use designated equipment for each area and perform cleaning regularly to ensure a sterile working environment. (Use 0.5% Sodium Hypochlorite or 10% Bleach to clean working environment.)

- We recommend using thermocyclers with heated lids for reactions. Preheat to reaction temperature before use.
- Avoid direct contact with skin and eyes. Do not swallow.
- All samples and wastes should be treated as potential contaminants in accordance with relevant regulations.
- If you have other questions, please contact MGI technical support MGI-service@mgi-tech.com

Chapter 2 Sample Preparation

2.1 Sample Requirements

2.1.1 Sample Type

This kit can be used for the total RNA extracted from multiple sample types, including blood, throat swab, nasal swab, etc.

2.1.2 RNA Requirement

It is recommended to use the following extraction kits for RNA extraction from sample:

QIAamp Viral RNA Mini Kit (QIAGEN, Cat. No.: 52904/ 52906)

MGIEasy Nucleic Acid Extraction Kit (MGI, Cat. No.: 1000023879 (VDR02P-96), 1000023935(VDR03P-32))

⚠ **Note: The RNA extracted by other non-recommended extraction kits has not been verified, and the success of library preparation or sequencing cannot be guaranteed. Please choose the above recommended extraction kits first.**

2.2 Sample Storage and Transport

Samples should be stored under freezing conditions: no more than 1 week at-20°C; no more than 6 months under -70°C. Isolated RNA samples should be stored under -70°C no more than 1 week.

Transport samples under freezing condition. Avoid the degradation of RNA samples due to repeated freezing and thawing during transport.

Chapter 3 Library Preparation Protocol

3.1 Reverse Transcription

⚠ **Note: Proceed preparation procedure under RNase-free environment in Pre-PCR area. Avoid vortex for RNA samples or Master Mixes with RNA, pipette mixing and flick mixing recommended.**

⚠ **Note: After reverse transcription, the cDNA should be divided into two equal parts, and PCR Primer Pool 1 and PCR Primer Pool 2 are used for PCR reaction respectively. After the PCR reaction is completed, they are combined into one tube for purification.**

3.1.1 Take out ATOPlex RNA Multiplex PCR Amplification Module for use. Take out N6 Buffer and RT Buffer from kit, mix it up and down after thawing. Prepare reverse transcription reaction mixture on ice (see Table 3-1) to a new 0.2 mL PCR tube.

Table 3-1 Reverse Transcription Reaction Mixture

Components	Volume
N6 Buffer	4 μL
RT Buffer	5 μL
RT Enzyme Mix	1 μL
Total	10 μL

3.1.2 Take out a new 0.2 mL PCR tube and mark sample ID. Transfer 10 μL of the reverse transcription reaction mixture to the PCR tube, and then transfer 10 μL RNA sample to the PCR tube. Mix it by pipetting up and down for 10 times and centrifuge briefly to collect the solution at the bottom of the tube.

3.1.3 Place the PCR tube from step 3.1.2 into the thermocycler and run the program in Table 3-2.

Table 3-2 Reverse Transcription Reaction Conditions

Temperature	Time
Heated lid	on
25°C	5 min
42°C	20 min
95°C	5 min
4°C	Hold

3.1.4 Put the tube on ice when the reaction is complete. Centrifuge briefly to collect the solution at the bottom of the tube.

3.2 PCR Amplification

3.2.1 For each sample, take two new PCR tubes and mark them as Sample ID-1, Sample ID-2, and use PCR Primer Pool 1 V3.1 or PCR Primer Pool 2 V3.1 for amplification, respectively.

3.2.2 According to the number of reactions, prepare two tubes of PCR amplification mixture on ice, respectively. (see Table 3-3).

Table 3-3 PCR Amplification Mixture

Components	Volume
PCR Enzyme Mix	12 μL
PCR Clean Enzyme	0.5 μL
PCR Additive	0.5 μL
PCR Primer Pool 1 V3.1 or PCR Primer Pool 2 V3.1	2 μL
Total	15 μL

⚠ **Note: Please mix the PCR Primer Pool 1 V3.1 and PCR Primer Pool 2 V3.1 thoroughly before use. Vortex 5-6 times, 3-5 s each time.**

3.2.3 Transfer 10 μL of the reverse transcribed product from step 3.1.4 to each PCR tube from step 3.2.1, and transfer 15 μL of PCR Amplification Mixture 1 and PCR Amplification Mixture 2 to the two PCR tubes of step 3.2.1 respectively. Vortex three times (3s each) and centrifuge briefly to collect the solution at the bottom of the tube.

3.2.4 Place the PCR tube from step 3.2.3 into the thermocycler and run the program in Table 3-4.

⚠ **Note: Proceed PCR reaction and following procedures in Post-PCR area.**

Table 3-4 PCR Amplification Reaction Conditions

Temperature	Time	Cycles
Heated lid (105°C)	on	
37°C	5 min	1 cycle
95°C	5 min	
95°C	10 s	
62°C	1 min	35 cycles
58°C	1 min	
72°C	20 s	
72°C	1 min	1 cycle
4°C	Hold	

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

⚠ **Note: DO NOT STOP AT THIS STEP. Please proceed to step 3.3 PCR Product Purification immediately.**

3.3 PCR Product Purification

⚠ **Note: Please read Appendix A carefully before procedure.**

3.3.1 Allow 30 minutes for DNA Clean Beads to warm to room temperature. Vortex and mix thoroughly before use.

3.3.2 Take out a new 0.2 mL PCR tube and mark sample ID. Transfer 75 μL DNA Clean Beads to the PCR tube.

3.3.3 Then transfer product 1 and product 2 of the same sample in step 3.2.5 to the tube (50 μL product in total). Turn the volume of the pipette to 100 μ L and pipette up and down at least 10 times to mix thoroughly. Ensure that all of the liquid and beads are fully dispensed from the pipette tips into the centrifuge tube before proceeding and no beads hanging on the tube wall.

3.3.4 Incubate the mixture for 5 minutes at room temperature.

3.3.5 Centrifuge briefly and place the tube onto the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully aspirate the supernatant out and discard.

3.3.6 Keep the tube on Magnetic Separation Rack. Add 160 μL freshly prepared 80% ethanol to wash the beads and tube wall. Allow to stand for 30 s, then aspirate the ethanol out and discard.

3.3.7 Repeat step 3.3.6. Pipette up as much ethanol as possible. Centrifuge briefly if there is some

ethanol hanging on tube wall, and then use small range pipette to pipette up all solution at the bottom of the tube after separation on rack.

3.3.8 Keep the 0.2 mL PCR tube on the Magnetic Separation Rack with the lid open. Air-dry beads at room temperature until no wetness (reflectiveness) is observed but before the pellet begins to crack.

3.3.9 Remove the tube from the rack. Add 32 µL TE buffer to elute DNA, then pipette up and down at least 10 times to mix thoroughly.

3.3.10 Incubate the solution for 5 minutes at room temperature.

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

3.3.12 Quantify the purified RT-PCR product with dsDNA Fluorescence Assay Kits such as Qubit® dsDNA HS Assay Kit or Quant-iT™ PicoGreen® dsDNA Assay Kit. The required concentration of RT-PCR products is ≥ 5 ng/µL.

⚠ **Note: PCR purification product can be stored at -20°C for one week.**

3.4 PCR-FREE Library Prep Reagent Preparation

3.4.1 Take out the MGIEasy Fast PCR-FREE FS DNA Library Prep Kit for use. Prepare 1x Elute Enhancer (see Table 3-5), which is available within 7 days at room temperature. 20x Elute Enhancer should be stored at room temperature after initial use.

Table 3-5 1X Elute Enhancer Preparation

Components	Volume
20x Elute Enhancer	2 µL
Nuclease-Free Water	38 µL
Total	40 µL

3.4.2 Prepare En-TE (see Table 3-6), available within 7 days at 4°C.

Table 3-6 En-TE Preparation

Components	Volume
1x Elute Enhancer	4.8 µL
TE Buffer	2,395.2 µL
Total	2,400 µL

3.4.3 Prepare En-Beads (see Table 3-7), which is available within 7 days at 4°C.

Table 3-7 En-Beads Preparation

Components	Volume
1x Elute Enhancer	30 µL
DNA Clean Beads	2,970 µL
Total	3,000 µL

Note: The reagents in Table 3-5 to Table 3-7 are sufficient for about 20 samples. If there are more samples, the reagents can be enlarged in proportion.

3.5 Fragmentation

3.5.1 Mix Fast FS Enzyme by inverting 10 times and flicking the bottom gently, make sure no residual reagent was left at the bottom, then centrifuge briefly and place it on ice for use. DO NOT vortex Fast FS Enzyme. Mix Fast FS Buffer by vortexing 3 times (3 s each), then centrifuge briefly and place it on ice for use.

Note: Please strictly follow the instructions on the manual or insufficient mixing would affect the fragmentation process.

3.5.2 Set and run the following program on the thermocycler in advance (See Table 3-8). **First step: 4℃, Hold**, reaction volume: 60 µL.

Table 3-8 Fragmentation Reaction Conditions

Temperature	Time
Heated lid (70°C)	on
4°C	Hold
4°C	1 min
30°C	12 min
65°C	20 min
4°C	Hold

3.5.3 Prepare the fragmentation mixture on ice (see Table 3-9). Vortex the tube 3 times (3 s each). Centrifuge the tube briefly and place it on ice.

Table 3-9 Fragmentation Mixture

Components	Volume
Fast FS Buffer	10 μL
Fast FS Enzyme	5 μL
Total	15 μL

3.5.4 Take a new 0.2 mL PCR tube and transfer 15 μL fragmentation mixture to the tube. According to the concentration determined by Qubit® dsDNA HS Assay Kit (See step 3.3.11), calculate the volume needed for 200 ng of PCR purification product. Transfer 200 ng PCR purification product to the tube. Use all of the product if the total yield is 100-200 ng. Add TE buffer to bring a total volume of 60 μL. Vortex the tube 3 times (3 s each), then centrifuge the tube briefly and place it on ice.

3.5.5 Make sure the thermocycler from step 3.5.2 has cooled to 4°C. Place the 0.2 mL PCR tube into the thermocycler. **Skip the first step of program (4°C Hold)** to start the reaction.

3.5.6 After reaction, centrifuge the tube briefly and put it on ice for later use.

⚠ **Note: DO NOT STOP AT THIS STEP. Please proceed to step 3.6 Adapter-Ligated DNA Cleanup.**

3.6 Cleanup of Fragmentation Product

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

3.6.1 Allow 30 minutes for En-Beads to come to room temperature. Vortex and mix thoroughly before use.

3.6.2 Transfer 60 μL En-Beads to the 0.2 mL PCR tube from step 3.5.6. Turn the volume of the pipette to 100 μL and pipette up and down at least 10 times to mix thoroughly. Ensure that all of the liquid and beads are fully dispensed from the pipette tips into the centrifuge tube.

3.6.3 Incubate the mixture for 5 minutes at room temperature.

3.6.4 Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully aspirate the supernatant out and discard.

3.6.5 Keep the centrifuge tube on the Magnetic Separation Rack and add 160 μL of freshly prepared 80% ethanol to wash the beads and the walls of the tube. Incubate for 30 seconds at room temperature, and then aspirate out the ethanol and discard.

3.6.6 Repeat step 3.6.5. Pipette up as much solution as possible. Centrifuge briefly if there is some ethanol hanging on tube wall. Use small range pipette to pipette up all solution at the bottom

of the 0.2 mL PCR tube after separation on rack

3.6.7 Keep the 0.2 mL PCR tube on the Magnetic Separation Rack with the lid open. Air-dry beads at room temperature until no wetness (reflectiveness) is observed but before the pellet begins to crack.

3.6.8 Remove the tube from the rack. Add 45 µL of En-TE buffer and pipette up and down at least 10 times to mix thoroughly.

⚠ **Note: Next step is carried out with magnetic beads. Do not place the centrifuge tube back onto the magnetic rack or transfer the supernatant to a new tube.**

3.7 Adapter Ligation

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

3.7.1 Take out UDB Adapters and Fast Ligation Buffer in advance to dissolve and mix by vortexing. Mix Ad Ligase by inverting 10 times, then centrifuge briefly and place them on ice for use.

3.7.2 According to UDB Adapters protocol (See Appendix B), add 5 µL of UDB Adapter to each PCR tube from step 3.6.8.

3.7.3 Prepare the Adapter ligation mixture on ice (See Table 3-10). Vortex 6 times (3 s each) and centrifuge briefly.

Table 3-10 Adapter Ligation mixture

Components	Volume
Fast Ligation Buffer	23 µL
Ad Ligase	5 µL
Ligation Enhancer	2 µL
Total	30 µL

3.7.4 Pipette slowly and transfer 30 µL of Adapter ligation mixture to the 0.2 mL PCR tube from step 3.7.2. Vortex 6 times (3 s each) and centrifuge briefly.

⚠ **Note: Adapter Ligation Mixture must be prepared in advance, The reaction mixture is sticky, please mix thoroughly.**

3.7.5 Place the PCR tube from step 3.7.4 into the thermocycler and run the program in Table 3-11. Total volume: 80µL.

Table 3-11 Adapter Ligation Reaction Conditions

Temperature	Time
Heated lid (30°C)	on
25°C	10 min
4°C	Hold

3.7.6 After reaction, centrifuge the tube briefly.

⚠ **Note: DO NOT STOP AT THIS STEP. Please proceed to step 3.8 Adapter-Ligated DNA Cleanup.**

3.8 Adapter-Ligated DNA Cleanup

3.8.1 According to Appendix A, take out En-Beads and allow 30 minutes for the solution to warm to room temperature. Vortex and mix thoroughly before use.

3.8.2 Transfer 20 μL of En-TE and 20 μL of En-Beads to the 0.2 mL PCR tube from step 3.7.6. Turn the volume of the pipette to 100 μL and pipette up and down at least 10 times to mix thoroughly. Ensure that all of the liquid and beads are fully dispensed from the pipette tip into the centrifuge tube before proceeding.

3.8.3 Incubate the mixture for 5 minutes at room temperature.

3.8.4 Centrifuge briefly and place the PCR tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully aspirate the supernatant out and discard.

3.8.5 Keep the centrifuge tube on the Magnetic Separation Rack and add 160 μL freshly prepared 80% ethanol to wash the beads and the walls of the tube. Incubate for 30 seconds at room temperature, then aspirate out the ethanol and discard.

3.8.6 Repeat step 3.8.5. Centrifuge briefly if there is some ethanol hanging on tube wall. Use small range pipette to pipette up all solution at the bottom of the 0.2 mL PCR tube after separation on rack.

3.8.7 Keep the tube on the Magnetic Separation Rack with the lid open. Air-dry beads at room temperature until no wetness (reflectiveness) is observed but before the pellet begins to crack.

3.8.8 Remove the tube from the Magnetic Separation Rack and add 20 μL of En-TE Buffer to elute the DNA. Pipette up and down at least 10 times to suspend all the beads.

3.8.9 Add another 20 μL En-Beads to the tube from step 3.8.8, pipette up and down at least 10 times to mix thoroughly. Ensure that all of the liquid and beads are fully dispensed from the pipette tips into the centrifuge tube.

3.8.10 Incubate the mixture for 5 minutes at room temperature.

3.8.11 Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully aspirate the supernatant out and discard.

3.8.12 Keep the centrifuge tube on the Magnetic Separation Rack and add 160 µL of freshly prepared 80% ethanol to wash the beads and the walls of the tube. Incubate for 30 seconds at room temperature, then aspirate out the ethanol and discard.

3.8.13 Repeat step 3.8.12. Centrifuge briefly if there is some hanging on tube wall. Use small range pipette to pipette up all solution at the bottom of the tube.

3.8.14 Keep the tube on the Magnetic Separation Rack with the lid open. Air-dry beads at room temperature until no wetness (reflectiveness) is observed but before the pellet begins to crack.

3.8.15 Remove the tube from the Magnetic Separation Rack and add 27 µL of En-TE Buffer to elute the DNA. Pipette up and down at least 10 times to suspend all the beads.

3.8.16 Incubate the solution for 5 minutes at room temperature.

3.8.17 Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully transfer 25 µL supernatant into a new 1.5 mL tube.

3.9 Quantification and Pooling

3.9.1 Quantify the purified ligation products with dsDNA Fluorescence Assay Kits such as Qubit® dsDNA HS Assay Kit or Quant-iT™ PicoGreen® dsDNA Assay Kit. The required concentration of the products is ≥ 0.8 ng/µL.

3.9.2 According to the barcode pooling guide in Appendix B3, the library is mixed in proportion to the required sequencing data, requiring a total mixing mass of > 15 ng to meet at least one-time DNB preparation need.

⚠ **For examples, there are N libraries that need to be mixed, and each library needs the same amount of sequencing data, then all libraries are mixed with the same mass, the pooling mass of a library (ng) = 15 ng/N, the pooling volume of a library (µL) = the pooling mass of a library (ng) / the concentration of a library (ng/µL).**

✔ **Stop Point: After cleanup, Adapter-ligated DNA can be stored at -20°C for one month.**

Chapter 4 Sequencing

4.1 DNB Preparation

By using the DNBSEQ One Step DNB Make Reagent Kit (OS-DB) (Cat. No.: 1000026466), the Adapter-ligated product is prepared into DNB, and 15 ng of purified ligation product is needed for DNB making each time.

Please follow the protocol described in "DNBSEQ DNB Make Reagent Kit User Manual" for DNB making.

4.2 Sequencing

DNB is compatible with SE100+10+10 sequencing on DNBSEQ-G50RS or DNBSEQ-G400RS for RNA genome sequencing.

⚠ **Note: Dual Barcode SE sequencing need to use SE sequencing kits with CPAS Barcode Primer 4 Reagent Kit (PN: 1000014048) (for SE sequencing).**

Please follow the protocol described in 'MGISEQ/DNBSEQ High-throughput Sequencing Set Instruction Manual" for sequencing.

⚠ **Note: When the library prepared by this kit is sequenced, it needs to be split with the barcode list file *UDB_PF_Adapter_A (385-480)* for this kit. Please check the software version of the sequencer and import the corresponding barcode list before sequencing.**

Appendix

Appendix A Magnetic Beads and Cleanup Procedures

For bead-based purification, we recommend using DNA Clean Beads included in the MGIEasy DNA Clean Beads (MGI, Cat. No.: 1000005278 or 1000005279). If you choose Magnetic Beads from other sources, please optimize the cleanup conditions before getting started.

Before You Use

- To ensure capture efficiency of the Magnetic Beads, remove beads from 4°C refrigerator storage and equilibrate to room temperature for 30 minutes before use. Vortex and mix thoroughly before use.
- Vortex or pipette up and down to ensure that the beads are thoroughly mixed before each use.
- The volume of the beads determines the lower limit of fragment size that can be purified.

Operation Notes

- If the sample volume decreases due to evaporation during incubation, add additional En-TE to reach the designated volume before using the beads to purify. This ensures that the correct ratio for the beads is used.
- In the magnetic separation step, please allow the solution to become completely clear before removing the supernatant. This process takes approximately 2-3 minutes. Consider the different magnetic strength of your specific Separation Plate or Rack and allow enough time for the solution to turn completely clear.
- Avoid touching the beads with pipette tips when pipetting. 2-3 μL of fluids can be left in the tube to avoid contact. In case of contact between the beads and the pipette tip, aspirate all of the solution and beads back into the tube and restart the separation process.
- Use freshly prepared 80% ethanol (at room temperature) to wash the beads twice. Keep the centrifuge tube on the Magnetic Separation Rack when washing. Do not shake or disturb the beads in any way.
- After the 2nd wash of beads with ethanol, try to remove all of the liquid in the tube. You may centrifuge briefly to collect any remaining liquid at the bottom, separate beads magnetically, and then remove the remaining liquid using a small volume pipette.
- After washing twice with ethanol, air-dry the beads at room temperature. Drying takes approximately 2-5 minutes, depending on your specific lab environment. Watch closely until the pellet appears sufficiently dry with a matte appearance, then continue to the elution step with EN-TE Buffer.

- During the elution step, do not touch the beads with the pipette tips when removing the supernatant. Contamination of DNA by the beads may affect subsequent purification. Therefore, the total volume of EN-TE buffer and the beads should be greater than the volume of the supernatant by 2 μL.
- Pay attention when opening/closing the lids of centrifuge tubes on the Separation Rack. Strong vibrations may cause sample loss by spilling liquid or beads from the tubes. Secure the tubes before opening the lids.

Appendix B MGIEasy UDB Primers Adapter Kit Instructions

- Depending on the specification of the MGIEasy Fast PCR-FREE FS DNA Library Prep Set, UDB adapters are provided in different formats: in tubes for 16 RXN set and in one plate for 96 RXN set. This kit is developed to meet requirements for batch processing of library preparation and multiplex sequencing. For optimum performance, please refer to instructions in Appendix B1 to B3. Please note that among the adapters from the three sets, adapters with same ID number share same sequence, and thus cannot be sequenced in the same lane.
- UDB Adapter are double stranded. Please do not incubate above room temperature to avoid structural changes such as denaturation, which might affect performance.
- Before use, please centrifuge UDB Adapter to collect liquid at the bottom of tubes. Mix Adapters with a pipette before you use. Remember to close the cap immediately after use.

B-1 Note for MGIEasy UDB PF Adapter Kit (16 RXN)

- This kit contains 16 Adapters grouped into 2 sets: UDB Adapter-393 ~ UDB Adapter-400, UDB Adapter-401 ~ UDB Adapter-408. Each set of 8 adapters are balanced in base distribution.

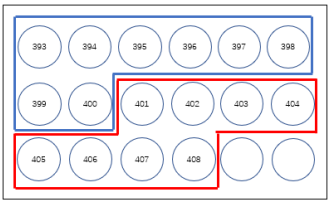

Figure B-1 MGIEasy UDB PF Adapter Kit (16 RXN) Adapter Layout and Combination Instructions

- Before use, please centrifuge UDB Adapter to collect liquid at the bottom of tubes. Mix Adapters

with a pipette before you use. Remember to close the cap immediately after use.

B-2 Note for MGIEasy UDB PF Adapter Kit A (96 RXN)

- One Adapter Plate A that contains 96 UDB adapters is included in each 96 RXN kit
- UDB Adapters A: 8 adapters within same row on the plate are grouped into same set and are balanced in base distribution. There are 12 rows on each plate and thus there are 12 sets of adapters on each plate.

Table B-1 MGIEasy UDB PF Adapter Kit A (96 RXN) Adapter Layout

UDB Adapters A	1	2	3	4	5	6	7	8	9	10	11	12
A	UDB-385	UDB-393	UDB-401	UDB-409	UDB-417	UDB-425	UDB-433	UDB-441	UDB-449	UDB-457	UDB-465	UDB-473
B	UDB-386	UDB-394	UDB-402	UDB-410	UDB-418	UDB-426	UDB-434	UDB-442	UDB-450	UDB-458	UDB-466	UDB-474
C	UDB-387	UDB-395	UDB-403	UDB-411	UDB-419	UDB-427	UDB-435	UDB-443	UDB-451	UDB-459	UDB-467	UDB-475
D	UDB-388	UDB-396	UDB-404	UDB-412	UDB-420	UDB-428	UDB-436	UDB-444	UDB-452	UDB-460	UDB-468	UDB-476
E	UDB-389	UDB-397	UDB-405	UDB-413	UDB-421	UDB-429	UDB-437	UDB-445	UDB-453	UDB-461	UDB-469	UDB-477
F	UDB-390	UDB-398	UDB-406	UDB-414	UDB-422	UDB-430	UDB-438	UDB-446	UDB-454	UDB-462	UDB-470	UDB-478
G	UDB-391	UDB-399	UDB-407	UDB-415	UDB-423	UDB-431	UDB-439	UDB-447	UDB-455	UDB-463	UDB-471	UDB-479
H	UDB-392	UDB-400	UDB-408	UDB-416	UDB-424	UDB-432	UDB-440	UDB-448	UDB-456	UDB-464	UDB-472	UDB-480

B-3 Barcode Pooling Guide

- It is recommended to 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 Table 2.

Table B-2 Dual Barcode 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 column + 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 not more than 62.5% in single sequencing position in the same lane. (See table 3 and table 4)

Table B-3 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 B-4 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

Appendix C Adapter Ligation

- The Adapter Reaction mixture contains a high concentration of PEG which increases the viscosity of the mixture. Please pipette slowly and ensure that the correct amount has been used.
- Due to the presence of PEG, the volume of beads required for the cleanup of Adapter-ligated DNA can be reduced. There is a risk of capturing Adapter dimers with a higher multiplier of beads. Therefore, we recommend using 20 μL Beads for the cleanup.

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