

ATOplex

RNA Universal Library Preparation General User Manual

Manual Version: A1

Revision History

Manual Version	Kit Version	Date	Description
A1	V1.0	Dec. 2020	<ul style="list-style-type: none"> • Add recommendations for extraction kits • Delete the description of quantitative RNA application and 960 BC.
A0	V1.0	May 2020	<ul style="list-style-type: none"> • 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

The user manual is designed for multiplex PCR-based library preparation of RNA on MGI ATOplex platforms. It is intended solely for the contractual use of its customer in connection with the use of the product(s) described herein. This user manual applies to the following products: ATOplex RNA Universal Library Preparation Module, Customized Primer Panel, ATOplex PCR Dual Barcode Primer Module (01-96), MGIEasy Dual Barcode Circularization kit and ATOplex Dual Barcoded Balance Library Reagent. The complete kit uses two-step multiplex PCR technology to amplify the RNA target region in one tube, prepares circularized single strand DNA (ssCirDNA) from RNA sample for subsequent sequencing on DNBSEQ™ sequencers, and obtains the target region sequences by high-throughput sequencing. Dual barcodes can reduce the ratio of barcode crosstalk and error rates. All reagents provided within this kit have passed stringent quality control and functional verification procedures, ensuring performance stability and reproducibility.

1.2 Application

This module can be used for the total RNA extracted from multiple sample types such as blood, tissue, swabs, etc.

1.3 Sequencing Platform Compatibility

Construct libraries with MGIEasy Dual Barcode Circularization kit (PN: 1000020570)

The constructed libraries must be sequenced with ATOplex Dual Barcoded Balance Library Reagent (PN: 1000022270)

Constructed libraries are compatible with PE100+10+10 sequencing on:

MGISEQ-200RS/ DNBSEQ-G50RS

MGISEQ-2000RS/ DNBSEQ-G400RS

DNBSEQ-T7RS

See Chapter 4 for the recommended sequencing kits.

1.4 Contents

The user manual applies to the following products, and further information of Cat. No., Components and Specifications are listed in table 1.

Table 1 Components and Specifications of related modules

Modules & Cat. No.	Components	Cap Color	Spec & Quantity
ATOplex RNA Universal Library Preparation Module (Box 1) Cat. No: 1000021624 Specification: 96 RXN	PCR Enzyme Mix	White	4,800 μ L/ bottle \times 1 bottle
	PCR Clean Enzyme	White	96 μ L/tube \times 1 tube
	PCR Additive	Yellow	96 μ L/tube \times 1 tube
	N6 Buffer	Green	384 μ L/tube \times 1 tube
	RT Buffer	Green	480 μ L/tube \times 1 tube
	RT Enzyme Mix	Green	96 μ L/tube \times 1 tube
ATOplex RNA Universal Library Preparation Module (Box 2) Cat. No: 1000021624 Specification: 96 RXN	DNA Clean Beads	White	3,360 μ L/tube \times 3 tubes
	Elution Buffer	White	4,800 μ L/tube \times 1 tube
Customized Primer Panel Specification: 96 RXN	PCR Primer Pool (customized)	Blue	384 μ L/tube \times 1 tube
	PCR Block (customized)	Blue	192 μ L/tube \times 1 tube
ATOplex PCR Dual Barcode Primer Module (01-96) Cat. No: 1000021626 Specification: 96 RXN	PCR Dual Barcode Primer Mix (01-96)	—	8 μ L/well \times 96 wells
MGEasy Dual Barcode Circularization kit (MGEasy Dual Barcode Circularization Module) Cat. No: 1000020570 Specification:16 RXN	Dual Barcode Split Buffer	Purple	225 μ L/tube \times 1 tube
	DNA Rapid Ligase	Purple	10 μ L/tube \times 1 tube
	Digestion Buffer	White	30 μ L/tube \times 1 tube
	Digestion Enzyme	White	50 μ L/tube \times 1 tube
	Digestion Stop Buffer	White	145 μ L/tube \times 1 tube
MGEasy Dual Barcode Circularization kit (MGEasy DNA Clean Beads) Cat. No: 1000020570 Specification:16 RXN	DNA Clean Beads	White	1600 μ L/tube \times 2 tubes
	TE buffer	White	1600 μ L/tube \times 1 tube
ATOplex Dual Barcoded Balance Library Reagent Cat. No: 1000022270	ATOplex Dual Barcoded Balance Library Reagent	Orange	26 ng /tube \times 1 tube

1.5 Storage Conditions and Shelf Life

ATOPlex RNA Universal Library Preparation Module (Box 1)

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

ATOPlex RNA Universal Library Preparation Module (Box 2)

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

Customized Primer Panel

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

ATOPlex PCR Dual Barcode Primer Mix (01-96)

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

MGEasy Dual Barcode Circularization Module

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

MGEasy DNA Clean Beads

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

ATOPlex Dual Barcoded Balance Library Reagent

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

* Please ensure that an abundance of dry ice during transportation.

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

1.6 Equipments and Materials Required but not Provided

Table 2 Equipments and Materials Required but not Provided

Equipments	Vortex Mixer
	Desktop Centrifuge
	Pipets
	Thermocycler
	Magnetic rack for 96-well plate (ALPAQUA, Part#A00400)
	Magnetic rack DynaMag™-2 (Thermo Fisher Scientific™, Cat. No. 12321D)
	Qubit™ 3 Fluorometer (Thermo Fisher Scientific™, Cat. No. Q33216)
Reagents	Agilent 2100 Bioanalyzer (Agilent Technologies™, Cat. No. G2939AA) / LabChip® GX, GXII, GX Touch (PerkinElmer), or Fragment Analyzer™ (Advanced Analytical)
	Nuclease free water (NF water) (Ambion, Cat. No. AM9937)
	100% Ethanol (Analytical Grade)
	1x TE Buffer, pH 8.0 (Ambion, Cat. No. AM9858)
	Qubit® ssDNA Assay Kit (Invitrogen, Cat. No. Q10212)
	Qubit® dsDNA HS Assay Kit (Invitrogen, Cat. No. Q32854)/ Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Cat. No. P7589)
	Agilent DNA 1000 Kit (Agilent, Cat. No. 5067-1504)
Consumables	Pipette Tips (RNase free)
	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)

1.7 Precautions and Warnings

- This product is for research use only. Please read this manual carefully before use.
- Please familiarize yourself with the operation methods and precautions of the various instruments to be used before the experiment.
- The library preparation process can be adjusted and optimized based on experimental design, sample characteristics, sequencing applications, and available equipment.
- Remove the reagents from storage beforehand and prepare them for use: for enzymes, centrifuge briefly and place on ice until further use. For other reagents, first thaw at room temperature and invert several times to mix properly, then centrifuge briefly and place on ice until further use.
- To prevent cross-contamination, we recommend using filtered pipette tips. Use a new tip each time for pipetting different solutions.
- We recommend using thermocyclers with heated lids for reactions. Preheat to reaction temperature before use.



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 three working areas in the laboratory for the first area, the second area and the third area, respectively. We recommend performing the reverse transcription and the 1st PCR reaction mix preparation in the first area, performing the 1st PCR reaction, 1st PCR product cleanup and the 2nd PCR reaction mix preparation in the second area, performing the 2nd PCR reaction, and 2nd PCR product cleanup in the third area. Library pooling and ssDNA circularization can be completed in the third area. Use designated equipment for each area and perform regular cleaning regularly to ensure a sterile working environment. (Use 0.5% Sodium Hypochlorite or 10% Bleach to clean working environment)

- Avoid direct contact with skin and eyes. Do not swallow. If accidentally ingested, rinse immediately with plenty of water and seek medical attention.
- All samples and wastes should be disposed of 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 user manual can be used for the total RNA extracted from multiple sample types such as blood, tissue, swabs, etc.

It is recommended to use the following extraction kits for sample RNA extraction under different scenarios:

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

The spin column extraction method is only suitable for manual operation.

- **Nucleic Acid Extraction Kit** (MGI, Cat No.: 1000023877 (VDR02P-96))

The magnetic bead extraction method is only suitable for automated operations, and 96 samples can be automatically extracted by MGISP-960 at a time.



Note: Other non-recommended extraction kits have not been verified. Therefore, we cannot guarantee the success of library construction or sequencing for RNA extracted by these kits. Please choose the above recommended extraction kits if possible.

2.1.2 Input

RNA input: 10 μ L; cDNA input: \leq 20.5 μ L without fragmentation.

2.2 Sample Storage and Transport

Samples should be stored no more than 1 week under -20°C , and no more than 6 months under -70°C . The extracted RNA samples should be stored below -70°C , or reverse transcribed to cDNA and stored in -20°C within 1 week. Avoid repeated freezing and thawing.

Chapter 3 Library Construction Protocol

RNA sample is reverse transcribed to cDNA first. Then DNA library is prepared by two-step PCR amplification, circularization and digestion.

3.1 Reverse Transcription



Note: The reverse transcription and the 1st PCR reaction mixture preparation should be in the first area. Avoid vortexing in these steps, please mix the sample by pipetting. If the sample is cDNA, please start the operation from step 3.2 and the cDNA input should be less than 20 μ L.

- 3.1.1 Take out the ATOplex RNA Universal Library Preparation Module for use. Transfer 10 μ L RNA sample to a new 0.2 mL PCR tube.
- 3.1.2 Take out N6 Buffer and RT Buffer from -20°C , mix it up and down after thawing. Prepare reverse transcription reaction mixture on ice (see Table 3).

Table 3 Reverse Transcription Reaction Mixture

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

- 3.1.3 Transfer 10 μ L of the reverse transcription reaction mixture to the PCR tube from step 3.1.1. Mix it by pipetting up and down for 10 times and centrifuge briefly to collect the solution at the bottom of the tube.
- 3.1.4 Place the PCR tube from step 3.1.3 into the thermocycler and run the program in Table 4.

Table 4 Reverse Transcription Reaction Conditions

Temperature	Time
Heated lid (105°C)	On
25°C	10 min
42°C	30 min
70°C	15 min
4°C	Hold

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

3.2 1st PCR Amplification

- 3.2.1 According to the number of reactions, prepare 1st PCR amplification mixture on ice (see Table 5).

Table 5 1st PCR Amplification Mixture

Components	Volume
PCR Enzyme Mix	25 μ L
PCR Clean Enzyme	0.5 μ L
PCR Primer Pool	4 μ L
Total	29.5 μ L



Note: PCR Primer Pool is the component of customized Primer Panel, please mix the PCR Primer Pool thoroughly before use. Vortex 5–6 times, 3–5 s each time.

- 3.2.2 Transfer 29.5 μ L of the 1st PCR amplification mixture to the PCR tube from step 3.1.5. Vortex three times (3s each) and centrifuge briefly to collect the solution at the bottom of the tube.
- 3.2.3 Place the PCR tube from step 3.2.2 into the thermocycler and run the program in Table 6.



Note: From the 1st PCR reaction to the 2nd PCR reaction mixture preparation, it should be completed in the second area.

Table 6 1st PCR Amplification Reaction Conditions

Temperature	Time	Cycles
Heated lid (105°C)	on	
37°C	5 min	1 cycle
95°C	10 min	
95°C	15 s	13 cycles
64°C	1 min	
60°C	1 min	
72°C	30 s	
4°C	Hold	

- 3.2.4 Centrifuge briefly to collect the solution at the bottom of the tube. Cleanup of 1st PCR Product.

3.3 Cleanup of 1st PCR Product



Note: Please read Appendix A carefully before you begin.

- 3.3.1 Take out DNA Clean Beads from refrigerator and allow 30 minutes for the solution to come to room temperature. Vortex and mix thoroughly before use.
- 3.3.2 Transfer 60 μ L DNA Clean Beads to the centrifuge tube from step 3.2.4. 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 tube before proceeding.
- 3.3.3 Incubate at room temperature for 5 minutes.
- 3.3.4 Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant.
- 3.3.5 Keep the tube on the Magnetic Separation Rack and add 150 μ L freshly prepared 80% ethanol to wash the beads and the walls of the tube. Incubate for 30 seconds, then carefully remove and discard the supernatant.
- 3.3.6 Repeat step 3.3.5 once, remove all of the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom of the centrifuge tube, separate the beads magnetically and then remove any remaining liquid using a small volume pipette.
- 3.3.7 Keep the centrifuge tube on the Magnetic Separation Rack with the lid open, and air-dry beads at room temperature until no wetness (reflectiveness) is observed. Do not over-dry the beads (cracks can be observed on pellet).
- 3.3.8 Remove the centrifuge tube from the Magnetic Separation Rack and add 14 μ L of Elution Buffer to elute the DNA. Make sure that the Elution Buffer fully wets the magnetic beads to prevent the magnetic beads from drying out and causing loss of PCR products.
- 3.3.9 Incubate at room temperature for 5 minutes.



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.



Stop Point: After cleanup, purified 1st PCR Products can be stored at -20°C.

3.4 2nd PCR Amplification



Note: Please read Appendix B carefully before you begin.

- 3.4.1 Take out ATOplex PCR Dual Barcode Primer Module (01-96). Add 8 μL PCR Dual Barcode Primer Mix (01-96) to the corresponding position in the PCR tube from step 3.3.9.
- 3.4.2 Prepare 2nd PCR amplification mixture on ice (see Table 7).

Table 7 2nd PCR Amplification Mixture

Components	Volume
PCR Enzyme Mix	25 μL
PCR Clean Enzyme	0.5 μL
PCR Additive	1 μL
PCR Block	2 μL
Total	28.5 μL



Note: PCR Block is the component of customized Primer Panel, please mix the PCR Block thoroughly before use by vortex 5–6 times for 3–5 s each time.

- 3.4.3 Transfer 28.5 μL of the 2nd PCR amplification mixture to the PCR tube from step 3.3.9. Vortex three times (3s each) and centrifuge briefly to collect the solution at the bottom of the tube.
- 3.4.4 Place the PCR tube from step 3.4.3 into the thermocycler and run the program in Table 8.



Note: The 2nd PCR reaction and the subsequent step should be completed in the third area. Please implement Step 3.5 immediately to purify the PCR product. Do not keep the PCR product in the thermocycler overnight after the reaction is over.

Table 8 2nd PCR Amplification Reaction Conditions

Temperature	Time	Cycles
Heated lid (105°C)	on	
37°C	5 min	1 cycle
95°C	10 min	
95°C	15 s	27 cycles
64°C	1 min	
60°C	1 min	
72°C	30 s	
4°C	Hold	

- 3.4.5 Centrifuge briefly to collect the solution at the bottom of the tube.

3.5 Cleanup of 2nd PCR Product



Note: Please read Appendix A carefully before you begin.

- 3.5.1 Take out DNA Clean Beads from the refrigerator and allow 30 minutes for the solution to come to room temperature. Vortex and mix thoroughly before use.
- 3.5.2 Transfer 45 μ L of DNA Clean Beads to the centrifuge tube from step 3.4.5. 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 tube before proceeding.
- 3.5.3 Incubate at room temperature for 5 minutes.
- 3.5.4 Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant with a pipette.
- 3.5.5 Keep the tube on the Magnetic Separation Rack and add 150 μ L freshly prepared 80% ethanol to wash the beads and the walls of the tube. Incubate for 30 seconds, then carefully remove and discard the supernatant.
- 3.5.6 Repeat step 3.5.5 once, remove all of the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom of the centrifuge tube, separate the beads magnetically and then remove any remaining liquid using a small volume pipette.
- 3.5.7 Keep the centrifuge tube on the Magnetic Separation Rack with the lid open, and air-dry beads at room temperature until no wetness (reflectiveness) is observed. Do not over-dry the beads (cracks can be observed on pellet).
- 3.5.8 Remove the centrifuge tube from the Magnetic Separation Rack and add 25 μ L of Elution Buffer to elute the DNA. Pipette up and down at least 10 times to mix thoroughly.
- 3.5.9 Incubate at room temperature for 5 minutes.
- 3.5.10 Centrifuge briefly and place the centrifuge tube back onto the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Transfer 23 μ L of supernatant to a new 1.5 mL centrifuge tube.



Stop Point: After cleanup, purified 2nd PCR Products can be stored at -20°C.

3.6 Quality Control of 2nd PCR Product

- 3.6.1 Quantify the purified 2nd 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 2nd PCR products is ≥ 4 ng/ μ L.
- 3.6.2 Determine the fragment size distribution of purified 2nd PCR product with electrophoresis-based equipment such as Bioanalyzer, Tapestation (Agilent Technologies), LabChip[®] GX, GXII, GX Touch (PerkinElmer), or Fragment Analyzer[™] (Advanced Analytical). The final size distribution of purified 2nd PCR products should correspond with the size of the customized panel.
- 3.6.3 After the library passes the quality control metrics, pool the library according to the actual conditions. The total amount of 2nd PCR products after pooling is 400 ng and the total volume is ≤ 48 μ L.



For example: There are N libraries that need to be mixed, and each sample library needs the same amount of sequencing data, then all libraries are mixed with the same mass, the pooling mass of a library (ng) = 400 ng/N, the pooling volume of a library (μ L) = the pooling mass of a library (ng)/the concentration of a library (ng/ μ L).

3.7 Denaturation

By using MGIEasy Dual Barcode Circularization kit (Cat. No.: 1000020570), the pooled library is circularized and digested forming circularized single strand DNA (ssCirDNA) for subsequent sequencing on MGISEQ and DNBSEQ sequencers.

- 3.7.1 Transfer 400 ng of 2nd PCR products to a new 0.2 mL PCR tube. Add TE Buffer for a total volume of 48 μ L.
- 3.7.2 Place the 0.2 mL PCR tube from step 3.7.1 into the thermocycler and run the program in Table 9.

Table 9 The Reaction Conditions of Denaturation

Temperature	Time
Heated lid (105°C)	On
95°C	3 min
95°C	Hold

- 3.7.3 When the reaction is complete, immediately place the 0.2 mL PCR tube on ice for 2 minutes, then centrifuge briefly.

3.8 Single Strand DNA Circularization

- 3.8.1 Prepare the single strand DNA circularization mixture in a new 0.2 mL PCR tube on ice (see Table 10).

Table 10 Single Strand DNA Circularization Mixture

Components	Volume
Dual Barcode Splint Buffer	11.5 μ L
DNA Rapid Ligase	0.5 μ L
Total	12 μ L

- 3.8.2 Transfer 12 μ L single strand DNA circularization mixture to the 0.2 mL PCR tube from step 3.7.3 on ice. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube.
- 3.8.3 Place the PCR tube into the thermocycler and run the program in Table 11.

Table 11 Single Strand DNA Circularization Reaction Conditions

Temperature	Time
Heated lid (105°C)	On
37°C	30 min
4°C	Hold

- 3.8.4 After the reaction is complete, immediately place the tube on ice for the next step.

3.9 Enzymatic Digestion

- 3.9.1 Prepare the following enzymatic digestion mixture (see Table 12) in a new 0.2 mL PCR tube on ice during the reaction in step 3.8.3.

Table 12 Enzymatic Digestion Mixture

Components	Volume
Digestion Buffer	1.4 μ L
Digestion Enzyme	2.6 μ L
Total	4 μ L

- 3.9.2 Transfer 4 μ L of enzymatic digestion mixture into the PCR tube from step 3.8.4. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube.
- 3.9.3 Place the PCR tube from step 3.9.2 into the thermocycler and run the program in Table 13.

Table 13 Enzymatic Digestion Reaction Conditions

Temperature	Time
Heated lid (105°C)	On
37°C	30 min
4°C	Hold

- 3.9.4 Centrifuge briefly to collect the solution at the bottom of the tube.
- 3.9.5 Add 7.5 μ L Digestion Stop Buffer to the 0.2 mL PCR tube. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube. Transfer all of the solution into a new 1.5 mL centrifuge tube.

3.10 Enzymatic Digestion Product Cleanup



Note: Please read Appendix A carefully before you begin.

- 3.10.1 Take out DNA Clean Beads from the refrigerator and allow 30 minutes for the solution to come to room temperature. Vortex and mix thoroughly before use.
- 3.10.2 Transfer 170 μ L of DNA Clean Beads to the Enzymatic Digestion product from step 3.9.5. Gently pipette at least 10 times to mix thoroughly. Ensure that all of the solution and beads are fully dispensed from the tip into the tube.
- 3.10.3 Incubate at room temperature for 10 minutes.
- 3.10.4 Centrifuge briefly and place the 1.5 mL tube on the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant using a pipette.
- 3.10.5 With the 1.5 mL tube on the Magnetic Separation Rack, add 200 μ L of freshly prepared 80% ethanol to the tube without disturbing the beads. Incubate for 30 seconds. Carefully remove and discard the supernatant.
- 3.10.6 Repeat step 3.10.5 once. Remove all of the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom, separate the beads magnetically, then remove any remaining liquid using a small volume pipette.
- 3.10.7 Keep the 1.5 mL centrifuge tube on the Magnetic Separation Rack with the lid open, and air-dry beads at room temperature until no wetness (reflectiveness) is observed. Do not over-dry the beads (cracks can be observed on pellet).
- 3.10.8 Remove the 1.5 mL centrifuge tube from the Magnetic Separation Rack and add 22 μ L of Elution Buffer to elute the DNA. Gently pipette up and down at least 10 times to mix thoroughly or until

the beads are fully resuspended.

3.10.9 Incubate at room temperature for 10 minutes.

3.10.10 Centrifuge briefly and place the centrifuge tube back onto the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Transfer 20 μ L of supernatant to a new 1.5 mL centrifuge tube.



Stop Point: Purified Enzymatic Digestion products can be stored at -20°C .

3.11 Quality Control of Enzymatic Digestion Product

Quantify the purified Enzymatic Digestion product with Qubit[®] ssDNA Assay Kit. The final yield of the Enzymatic Digestion products should be ≥ 10 ng. If the final yield is more than 7 ng but less than 10 ng, it is recommended to make DNB, but data quality may be reduced.

Chapter 4 Sequencing

4.1 Make DNB

The constructed library need be sequenced with ATOplex Dual Barcoded Balance Library Reagent. Both the sample library and the balance library are performed "Make DNB" operation by the reagents in the sequencing kit:

- 4.1.1 Make DNBs of sample library: Transfer 10 ng digestion products from step 3.10 for "Make DNB" operation. If the final yield of ssDNA is more than 7 ng but less than 10 ng, input all digestion products for "Make DNB" operation. Add TE Buffer for a final volume of 20 μ L.
- 4.1.2 Make DNBs of ATOplex Dual Barcoded Balance Library: According to the concentration of Dual Barcoded Balance Library, transfer 6 ng library for "Make DNB" operation. Add TE Buffer for a final volume of 20 μ L.
- 4.1.3 Mix the DNBs of sample from step 4.1.1 and the DNB of balance library from step 4.1.2 with 3:1 in mass for subsequent sequencing. The mass of sample DNB (ng): the mass of standard library DNB (ng) = 3 : 1.

4.2 Sequencing

Constructed libraries match with PE100+10+10 sequencing on MGISEQ-200RS/ DNBSEQ-G50RS, MGISEQ-2000RS/ DNBSEQ-G400RS, DNBSEQ-T7RS for RNA full length genome sequencing.

The sequencing kits include:

CPAS Barcode Primer 3 Reagent Kit (PN:1000020834) (for PE sequencing)

DNBSEQ-G400RS High-throughput Rapid Sequencing Set (FCS PE100) or

DNBSEQ-G400RS High-throughput Sequencing Set (FCL PE100);

DNBSEQ-G50RS High-throughput Sequencing Set (FCL PE100) or

DNBSEQ-G50RS High-throughput Rapid Sequencing Set (FCS PE100);

DNBSEQ-T7RS High-throughput Sequencing Set (FCL PE100)



Note: CPAS Barcode Primer 3 Reagent Kit (PN:1000020834) (for PE sequencing)

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

Appendix

Appendix A Magnetic Beads and Cleanup Procedures

For bead-based purification, we recommend using DNA Clean Beads included in the kit. 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 TE buffer to reach the designated volume before using the beads to purify. It 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 / Rack, and allow enough time for the solution to turn completely clear.
- Avoid touching the beads with pipette tips when pipetting. 2-3 μ L of fluid can be left in the tube to avoid contact. In case of contact between the beads and the pipette tip, expel all of the solution and beads back into the tube and restart the separation process.
- Use freshly prepared 80% ethanol (at room temperature) to wash the beads. 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 within the tube. You may centrifuge briefly to collect any remaining liquid at the bottom, separate beads magnetically, and remove the remaining liquid by using a small volume pipette.
- After washing twice with ethanol, air-dry the beads at room temperature. 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 TE Buffer.

- During the elution step, do not touch the beads with the pipette tips when removing the supernatant. Contamination in DNA by the beads may affect subsequent purification. Therefore, the total volume of TE buffer and the beads should be 2 μL more than the volume of the supernatant.
- 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 The ATOplex PCR Dual Barcode Primer Module (01-96) Strategies

- The library should be constructed with dual barcode. It provides a 96-well plate of PCR Dual Barcode Primer Mix. Dual barcodes can reduce the ratio of barcode crosstalk and error rate. We selected the best barcodes combination based on the principle of balancing base composition. For optimum performance, please refer to instructions in Appendix B.
- Please do not incubate above room temperature to avoid structural changes such as degradation, which might affect performance.
- Before use, please centrifuge to collect liquid to the bottom. Gently remove the adhesive film to prevent spillage and cross contamination. Remember to reseal the PCR Dual Barcode Primer Mix immediately after use. If the adhesive film is contaminated, discard and use a new PCR sealing film to reseal the 96-well plate.

PCR Dual Barcode Primer usage rules

The layout of PCR Dual Barcode Primer Mix (01-96) is showed in Figure 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	01	09	17	25	33	41	49	57	65	73	81	89
B	02	10	18	26	34	42	50	58	66	74	82	90
C	03	11	19	27	35	43	51	59	67	75	83	91
D	04	12	20	28	36	44	52	60	68	76	84	92
E	05	13	21	29	37	45	53	61	69	77	85	93
F	06	14	22	30	38	46	54	62	70	78	86	94
G	07	15	23	31	39	47	55	63	71	79	87	95
H	08	16	24	32	40	48	56	64	72	80	88	96

Figure 1 PCR Dual Barcode Primer Mix (01-96) Layout

Based on the principles of balanced base composition, PCR Dual Barcode Primer Mix (01-96) must be used in specific groups. Please follow the instructions bellow to use in proper combination:

- 4 sets of 4 PCR Dual Barcode Primer Mix: 01-04, 05-08, 09-12 and 13-16.
- 10 sets of 8 PCR Dual Barcode Primer Mix: 17-24, 25-32, 33-40 and 41-48, 49-56, 57-64, 65-72, 73-80, 81-88, 89-96.

It is recommended that at least 4 samples be sequenced and ensure that the PCR Dual Barcode Primer Mix is used in above combination.

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