Part No.:H-T-123

User Manual

Version:2.0

ATOPlex Metabarcoding Library Prep

Panel: 16S V3V4/18SV4/ITS1/COI/ Ac12S/MiFish
Kit Version: V1.0

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About the user manual

Manufacturer information

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Revision history

Manual version	Kit version	Date	Description
2.0	V1.0	Apr. 2024	• Updated manufacturer information • Updated user manual style • Optimized partial description
1.0	V1.0	Oct. 2023	First 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 ATOPlex Metabarcoding User Manual provides guidance for the preparation of multiple PCR amplification for sequencing on the customized product platform by MGI. Metabarcoding is commonly employed for species classification, abundance analysis, and comparative studies of various biological samples. The barcodes frequently used for biodiversity assessment include prokaryotic 16S ribosomal DNA (for bacteria and archaea), eukaryotic 18S ribosomal DNA (for diverse eukaryotes such as plants, protists, and fungi), eukaryotic ITS ribosomal DNA (for fungi), mitochondrial COI gene (for a wide range of eukaryotes including animals and protists), and mitochondrial 12S DNA (specifically for fish). This user manual is only applicable to the use of the library construction products described in this document: ATOPlex 16S V3V4 rDNA Primer Pool, ATOPlex 18SV4 rDNA Primer Pool, ATOPlex ITS1 rDNA Primer Pool, ATOPlex COI mtDNA Primer Pool, ATOPlex Ac12S mtDNA Primer Pool, ATOPlex MiFish Primer Pool, and ATOPlex DNA Dual Barcode Library Preparation Sequencing Kit.

The ATOPlex Library Prep Set uses a two-step PCR amplification method to capture, amplify DNA barcode regions and add sequencing adapters in a single tube and generate libraries compatible with MGI DNBSEQ sequencing platform. This library prep set also uses PCR contamination removal technology to reduce PCR aerosols contamination. All reagents provided within these kits have passed stringent quality control and functional verification procedures, ensuring performance stability and reproducibility.

1.2 Application

This kit is suitable for the extracted DNA from environmental samples such as soil, water, sediment, and air.

1.3 Applicable sequencing platforms

Constructed libraries are compatible with the following platforms for the sequencing and bioinformation analysis:

- DNBSEQ-G99ARS
- DNBSEQ-E25RS
- DNBSEQ-G400RS

1.4 Components

The kit includes ATOPlex Metabarcoding Library Prep Primer Pool and ATOPlex DNA Dual BC Library Prep Set in two different sizes: 96 RXN and 576 RXN. For component details, refer to the following table.

Table 1 ATOPlex Metabarcoding Library Prep Primer Pool (96 RXN)

Modules & Cat. No.	Components	Cap Color	Spec & Quantity
ATOPlex 16S V3V4 rDNA Panel Cat. No: 940-001260-00	16S rDNA PCR Primer Pool	Blue	192 µL/tube x 1
	16S rDNA PCR Block	Blue	96 µL/tube x 1
ATOPlex 18SV4 rDNA Panel Cat. No: 940-001533-00	18SV4 rDNA PCR Primer Pool	Blue	192 µL/tube x 1
	18SV4 rDNA PCR Block	Blue	96 µL/tube x 1
ATOPlex ITS1 rDNA Panel Cat. No: 940-001536-00	ITS1 rDNA PCR Primer Pool	Blue	192 µL/tube x 1
	ITS1 rDNA PCR Block	Blue	96 µL/tube x 1
ATOPlex COI mtDNA Panel Cat. No: 940-001534-00	COI mtDNA PCR Primer Pool	Blue	192 µL/tube x 1
	COI mtDNA PCR Block	Blue	96 µL/tube x 1
ATOPlex Ac12S mtDNA Panel Cat. No: 940-001535-00	Ac12S mtDNA PCR Primer Pool	Blue	192 µL/tube x 1
	Ac12S mtDNA PCR Block	Blue	96 µL/tube x 1
ATOPlex MiFish Panel Cat. No: 940-001539-00	MiFish PCR Primer Pool	Blue	192 µL/tube x 1
	MiFish PCR Block	Blue	96 µL/tube x 1

Table 2 ATOPlex DNA Dual BC Library Prep Set (96 RXN) (Cat No: 940-001191-00)

Modules & Cat. No.	Components	Cap Color	Spec & Quantity
ATOPlex Multiplex PCR Spike-in Control Cat. No: 940-000950-00	Spike-in Control PCR Primer Pool	Bule	96 μL/tube x 1
	Spike-in Control PCR Block	Blue	96 μL/tube x 1
ATOPlex DNA Multiplex PCR Amplification Module Cat. No: 940-000124-00	PCR Enzyme Mix	Blue	1200 μL/tube x 2
	PCR Additive	Yellow	96 μL/tube x 1
	PCR Clean Enzyme	White	96 μL/tube x 1
MGIEasy DNA Clean Beads Cat. No: 1000005278	DNA Clean Beads	White	8 mL/tube x 1
	TE Buffer	White	4 mL/tube x 1
ATOPlex Dual Barcode Primer Module (01-96) V1.0 Cat. No: 1000021626	PCR Dual Barcode Primer Mix (01-96)	/	8 μL/well x 96

Table 3 ATOPlex Metabarcoding Library Prep Primer Pool (576 RXN)

Modules & Cat. No.	Components	Cap Color	Spec & Quantity
ATOPlex 16S V3V4 rDNA Panel Cat. No: 940-000724-00	16S rDNA PCR Primer Pool	Blue	576 μL/tube x 2
	16S rDNA PCR Block	Bule	576 μL/tube x 1
ATOPlex 18SV4 rDNA Panel Cat. No: 940-001541-00	18SV4 rDNA PCR Primer Pool	Bule	576 μL/tube x 2
	18SV4 rDNA PCR Block	Bule	576 μL/tube x 1
ATOPlex ITS1 rDNA Panel Cat. No: 940-001532-00	ITS1 rDNA PCR Primer Pool	Bule	576 μL/tube x 2
	ITS1 rDNA PCR Block	Bule	576 μL/tube x 1
ATOPlex COI mtDNA Panel Cat. No: 940-001537-00	COI mtDNA PCR Primer Pool	Bule	576 μL/tube x 2
	COI mtDNA PCR Block	Bule	576 μL/tube x 1
ATOPlex Ac12S mtDNA Panel Cat. No: 940-001540-00	Ac12S mtDNA PCR Primer Pool	Bule	576 μL/tube x 2
	Ac12S mtDNA PCR Block	Bule	576 μL/tube x 1
ATOPlex MiFish Panel Cat. No: 940-001538-00	MiFish PCR Primer Pool	Bule	576 μL/tube x 2
	MiFish PCR Block	Bule	576 μL/tube x 1

Table 4 ATOPlex DNA Dual BC Library Prep Set (576 RXN) (Cat No: 940-001190-00)

Modules & Cat. No.	Components	Cap Color	Spec & Quantity
ATOPlex Multiplex PCR Spike-in Control Cat. No: 940-000947-00	Spike-in Control PCR Primer Pool	Blue	576 μL/tube x 1
	Spike-in Control PCR Block	Blue	576 μL/tube x 1
ATOPlex DNA Multiplex PCR Amplification Module Cat. No: 940-000160-00	PCR Additive	Yellow	576 μL/tube x 1
	PCR Clean Enzyme	White	576 μL/tube x 1
	PCR Enzyme Mix	White	7.2 mL/tube x 2
MGIEasy DNA Clean Beads Cat. No: 1000005279	DNA Clean Beads	White	50 mL/tube x 1
	TE Buffer	White	25 mL/tube x 1
ATOPlex Dual Barcode Primer Module (48x96) Cat. No: 1000024935	Barcode 1(01-96)	/	12 μL/well x 96 wells
	Barcode 2(01-48)	/	24 μL/well x 48 wells

1.5 Storage and transportation

Table 5 Kit storage and transportation temperature

Item	Storage temperature	Transportation temperature
ATOPlex 16S V3V4 rDNA Panel	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃
ATOPlex 18SV4 rDNA Panel		
ATOPlex ITS1 rDNA Panel		
ATOPlex COI mtDNA Panel		
ATOPlex Ac12S mtDNA Panel		
ATOPlex MiFish Panel		
ATOPlex Multiplex PCR Spike-in Control		
ATOPlex DNA Multiplex PCR Amplification Module		
ATOPlex PCR Dual Barcode Primer Mix (01-96) V1.0		
ATOPlex Dual Barcode Primer Module (48x96)		
MGIEasy DNA Clean Beads	2 ℃ to 8 ℃	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.
- Under proper transport, storage, and use, all components will maintain its effectiveness within their shelf life.

1.6 User-supplied materials

Table 6 Order information for MGI products(Optional)

Cat. No.	Model	Name
1000020570	16 RXN	MGIEasy Dual Barcode Circularization Kit
1000026466	4 RXN	DNBSEQ OneStep DNB Make Reagent Kit
940-000637-00	40 ng/tube	ATOPlex E450 Dual Barcode Balanced Library Reagent
1000027585	1500 ng/tube	Standard Library Reagent (PCR Product) V4.0
940-000410-00	G99 SM FCL PE150	DNBSEQ-G99RS High-throughput Sequencing Set (G99 SM FCL PE150)
940-000415-00	G99 SM FCL PE300	DNBSEQ-G99RS High-throughput Sequencing Set (G99 SM FCL PE300)
940-000567-00	FCL PE150	DNBSEQ-E25RS High-throughput Sequencing Set (FCL PE150)

Table 7 User-supplied equipment list

Equipment	Recommended brand
Plate sealing machine	/
Vortex mixer	/
Desktop centrifuge	/
Pipette	/
Thermocycler	/
DynaMag™-96 Side Magnet	Thermo Fisher Scientific (Cat. No.: 12331D), or equivalent
Magnetic rack DynaMag -2	Thermo Fisher Scientific (Cat. No.: 12321D), or equivalent
Qubit 4 Fluorometer	Thermo Fisher Scientific (Cat. No.: Q33238), or equivalent
Microplate Reader	BMG Labtech, FLUOstar Omega, or equivalent
Agilent 2100 Bioanalyzer	Agilent Technologies (Cat. No.: G2939AA), or equivalent

Table 8 Recommended reagent/consumable list

Reagent/consumable	Recommended brand
100% Ethanol (Analytical Grade)	/
Sterilizing Pipette tips	/
Pipette tips with filter element	/
1.5 mL tube	/
Plastic sealing film	/
Nuclease-Free (NF) water	Ambion (Cat. No.: AM9937), or equivalent
1 x TE Buffer, pH 8.0	Ambion (Cat. No.: AM9858), or equivalent
Qubit® ssDNA Assay Kit	Invitrogen (Cat. No.: Q10212), or equivalent
Qubit® dsDNA HS Assay Kit	Invitrogen (Cat. No.: Q32854), or equivalent
Quant-iT PicoGreen dsDNA Assay Kit	Invitrogen (Cat. No.: P7589), or equivalent
Agilent DNA 1000 Kit	Agilent Technologies (Cat. No. 5067-1504), or equivalent
0.2 mL PCR tube or 96-well plate	Axygen (Cat. No.: PCR-02-C), or Axygen (Cat. No.: PCR-96M2-HS-C)
Qubit Assay Tubes or 0.5mL Thin Wall PCR Tubes	Invitrogen or Axygen, or equivalent
Aluminum sealing film	VITL (Cat. No.: V901002)
UV-Star 96-Well Microplates	GREINER BIO-ONE (Cat. No.: 655801)
High Sensitivity DNA Kits	Agilent Technologies(Cat. No.: 5067-4626) or equivalent Agilent DNA 1000 Kit (Cat. No. 5067-1504)

1.7 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 required.
- Instructions provided in this manual are intended for general use only and may require optimization for specific applications. We recommend adjusting it according to the experimental design, sample types, sequencing application, and alternatvie equipment.
- 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 in procedure reverse transcription, preparation PCR amplification mixture and transfer cDNA. Use a new tip each time for pipetting different solutions. If the reverse transcription, preparation PCR amplification mixture and transfer cDNA do not use filter tips, there may be a risk of contamination.
- We recommend using thermocyclers with heated lids for reactions. Preheat to reaction temperature before use.

  CAUTION 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 and the PCR reaction mix preparation in the Pre-PCR room, performing the PCR reaction, PCR product cleanup and PCR free library construction 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.)

- 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

1.8 Workflow

Section	Workflow	Total time	Hands-on time
3.1	1st PCR reaction	1 hr 30 min	10 min
3.2	Cleanup of 1st PCR product ⏸	30 to 40 min	20 to 30 min
3.3	2nd PCR reaction	1 hr	20 min

Section	Workflow	Total time	Hands-on time
3.4	Cleanup of 2nd PCR product ⏸	30 to 40 min	20 to 30 min
3.5	QC of PCR product ⏸	15 to 60 min	10 to 20 min
3.6	Library pooling ⏸	40 min-1 hr	40 min-1 hr

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 Sample requirements

2.1.1 Sample type

This kit is suitable for the extracted DNA from environmental samples such as soil, water, sediment, and air.

2.1.2 DNA sample minimum requirement

The suitable amount of DNA input depends on the abundance of the target taxonomic group in the environmental sample. As shown in the table 9, as a general guideline, it is recommended to input 1-50 ng for 16S V3V4, 18SV4, ITS1, and COI primer library prep, and 50-100 ng for Ac12S and MiFish primer library prep, per reaction mix.

Table 9 Recommended starting amount of samples

Primer Pool	Input Amount Range	Recommended Input Amount
16S V3V4, 18SV4, ITS1, COI	1-50 ng	1-50 ng
Ac12S, MiFish	10-100 ng	50-100 ng

2.2 Sample storage and transportation

Store DNA samples at -20 ℃ for up to one year. If sample transportation is required, transport samples under freezing condition. Avoid frequent freezing and thawing of DNA to minimize DNA degradation.

3 Library preparation protocol

3.1 1st PCR reaction

3.1.1 Preparation

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

Table 10 Preparing the reagents

Reagent	Requirement
PCR Enzyme Mix	Thaw on ice, mix by vortexing, centrifuge briefly, and place on ice.
PCR Clean Enzyme	Flick the bottom to mix well, centrifuge briefly, and place on ice.
16S V3V4/18SV4/ITS1/COI/Ac12S/MiFish PCR Primer Pool	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Spike-in Control PCR Primer Pool (optional)	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.

Tips Please mix the PCR Primer Pool thoroughly before use. Vortex 5 to 6 times, 3 to 5 sec each time.

3.1.2 1st PCR reaction

1. According to the number of reactions, take out 0.2 mL tubes, prepare the 1st PCR mixture on ice. Mix it well by vortexing, centrifuge briefly, and place on ice.

Table 11 1st PCR mixture

Reagent	Volume per reaction
DNA	V
PCR Enzyme Mix	12.5 μL
PCR Clean Enzyme	0.5 μL
16S V3V4/18SV4/ITS1/COI/ Ac12S/MiFish PCR Primer Pool	2 μL
TE Buffer	10 μL-V
Total	25 μL

CAUTION The suitable amount of DNA input depends on the abundance of the target taxonomic group in the environmental sample. Please refer to the "Sample requirements" on page 10 for DNA sample requirements and recommended input amounts "V".

CAUTION Spike-in control PCR Primer Pool contains certain copies of spike-in DNA, it can be used in the following conditions:

① Do not use.

② Use Spike-in control as positive control: input 10 μL Spike-in control PCR Primer Pool instead of DNA sample to process the first PCR amplification (Table 11, V=10 μL).

2. After preparation, vortex 3 times (3 seconds each) and centrifuge briefly to collect the solution at the bottom of the tube.

CAUTION Make sure there are no air bubbles in the PCR tubes, as they can affect the efficiency of the PCR reaction.

3. Place the tube(s) into the thermocycler and run the program with the following conditions.

Table 12 1st PCR reaction conditions (Volume: 25 μL)

Temperature	Time	Cycles
105 ℃ Heated lid	On	/
37 ℃	5 min	1
95 ℃	10 min	1
95 ℃	20 sec	25-30 cycle
55 ℃ (16S V3V4/18SV4/ ITS1/COI) or 65 ℃ (Ac12S/MiFish)	30 sec	
72 ℃	30 sec	
12 ℃	Hold	/

CAUTION Depending on the sample, optimizations for the number of PCR cycles may be required to achieve the desired PCR product concentration. As a general guideline, it is recommended to use 25 cycles for 16S V3V4, 18SV4, ITS1, and COI amplicon sequencing, and 30 cycles for Ac12S and MiFish sequencing.

4. After the reaction, centrifuge briefly to collect the solution at the bottom of the tube for first purification.

3.2 Cleanup of 1st PCR product

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

3.2.1 Preparation

Table 13 Preparing the reagents

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

3.2.2 Cleanup of 1st PCR product

CAUTION If 1.5 mL centrifuge tube and adaptive magnetic rack are used for purification, transfer all the reaction liquid of each sample to a new 1.5 mL centrifuge tube first.

1. Mix the DNA Clean Beads thoroughly. Add 25 µL of DNA Clean Beads to each sample tube (from step 4 in section 3.1.2). Pipette at least 10 times to mix thoroughly. Ensure that all of the solution and beads in the pipette are transferred into the tube after mixing.
2. Incubate at room temperature for 5 min.
3. Centrifuge the tube briefly and place on a magnetic rack for 2 to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
4. While keeping the tube or 96-well plate on the magnetic rack, add 160 µL of 80% ethanol to wash the beads and tube wall. After slowly pipetting 3 to 5 times, wait for 30 sec. Carefully remove and discard the supernatant.
5. Repeat step 4. Remove all the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom of the tube, separate the beads magnetically and then remove any remaining liquid using a low-volume pipette.
6. Keep the tube or 96-well plate on the magnetic rack. Uncap the tube 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 or 96-well plate from the magnetic rack and add 6.5 µL of TE Buffer to elute the DNA. Pipette at least 10 times until all beads are suspended.
8. Incubate at room temperature for 5 min.
9. Centrifuge the tube or 96-well plate briefly. Proceed to the 2nd PCR reaction with beads.

   Tips The 2nd PCR reaction is carried out with beads, do not place the tube back onto the magnetic rack or transfer the supernatant to a new tube.

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

3.3 2nd PCR reaction

CAUTION There are two sets of barcodes for Dual BC Module. Select different strategy for sequencing and ensure that each library has at least one set of barcode.

- For 96 RXN, read "Usage rules of PCR Dual Barcode Primer Mix (01-96)" on page 24 for details before use.

- For 576 RXN, read "Usage rules of PCR Dual Barcode Primer (48X96)" on page 26 for details before use.

3.3.1 Preparation

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

Table 14 Preparing the 576 RXN reagents

Reagent	Requirement
PCR Enzyme Mix	Thaw on ice, mix by vortexing, centrifuge briefly, and place on ice.
PCR Clean Enzyme	Flick the bottom to mix well, centrifuge briefly, and place on ice.
PCR Additive	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
16S V3V4/18SV4/ITS1/COI/ Ac12S/MiFish PCR Block	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Spike-in Control PCR Block (optional)	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Barcode 1	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Barcode 2	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.

Table 15 Preparing the 96 RXN reagents

Reagent	Requirement
PCR Enzyme Mix	Thaw on ice, mix by vortexing, centrifuge briefly, and place on ice.
PCR Clean Enzyme	Flick the bottom to mix well, centrifuge briefly, and place on ice.
PCR Additive	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
16S V3V4/18SV4/ITS1/COI/ Ac12S/MiFish PCR Block	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
Spike-in Control PCR Block (optional)	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.
PCR Dual Barcode Primer Mix (01-96) V1.0	Thaw at RT, mix by vortexing, centrifuge briefly, and place on ice.

3.3.2 2nd PCR reaction

1. **For 96 RXN**, referring to section 5.1.1, pipette 4 μL of PCR Dual Barcode Primer Mix (01-96) to the corresponding sample tube (from step 9 in section 3.2.2). **For 576 RXN**, referring to section 5.1.2, pipette 2 μL of PCR Dual Barcode Primer F and 2 μL of PCR Dual Barcode Primer R respectively and add them to the corresponding sample tube (from step 9 in section 3.2.2).

2. According to the number of reactions, prepare the 2nd PCR mixture on ice. Mix it well by vortexing, centrifuge briefly, and place on ice.

Table 16 2nd PCR mixture

Reagent	Volume per reaction
PCR Enzyme Mix	12.5 μL
PCR Clean Enzyme	0.5 μL
PCR Additive	0.5 μL
16S V3V4/18SV4/ITS1/COI/ Ac12S/MiFish PCR Block or Spike-in control PCR Block	1 μL
Total	14.5 μL

⚠ CAUTION Spike-in control PCR Block can be used when choose to use Spike-in control PCR Primer Pool in 1st PCR. It also can be used in the following conditions: ① Do not use. ② Use Spike-in control PCR Primer Pool as positive control, then input 1 μL Spike-in control PCR Block.

3. Add 14.5 μL of 2nd PCR mixture to each sample tube (from step 1). Mix quickly by pipetting 5-8 times or vortexing 3 times (3 sec each). Centrifuge briefly to collect the liquid to the bottom of the tube.
4. Place the PCR tube(s) into the thermocycler. Run the program with the following conditions.

Table 17 Second PCR reaction conditions (Volume: 25 μL)

Temperature	Time	Cycle
105 ℃ Heated lid	On	/
37 ℃	5 min	1
95 ℃	10 min	1
95 ℃	20 sec	10
55 ℃	30 sec	
72 ℃	30 sec	
12 ℃	Hold	/

5. After the reaction, centrifuge briefly to collect the solution at the bottom of the tube for second purification.

3.4 Cleanup of 2nd PCR product

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

3.4.1 Preparation

Table 18 Preparing the reagents

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

3.4.2 Cleanup of 2nd PCR product

CAUTION If 1.5 mL centrifuge tube and adaptive magnetic rack are used for purification, transfer all the reaction liquid of each sample to a new 1.5 mL centrifuge tube first.

1. Mix the DNA Clean Beads thoroughly. Add 25 µL of DNA Clean Beads to each sample tube (from step 5 in section 3.3.2). Pipette at least 10 times to mix thoroughly. Ensure that all of the solution and beads in the pipette are transferred into the tube after mixing.
2. Incubate at room temperature for 5 min.
3. Centrifuge the tube briefly and place on a magnetic rack for 2 to 5 min until the liquid is clear. Carefully remove and discard the supernatant.
4. While keeping the tube or 96-well plate on the magnetic rack, add 160 µL of 80% ethanol to wash the beads and tube wall. After slowly pipetting 3 to 5 times, wait for 30 sec. Carefully remove and discard the supernatant.
5. Repeat step 4. Remove all the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom of the tube, separate the beads magnetically and then remove any remaining liquid using a low-volume pipette.
6. Keep the tube or 96-well plate 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 or 96-well plate from the magnetic rack and add 25 µL of TE Buffer to elute the DNA. Pipette at least 10 times until all beads are suspended.
8. Incubate at room temperature for 5 min.

9. Centrifuge briefly and place on the magnetic rack for 2-5 min until the liquid is clear. Transfer 23 μL of supernatant to a new tube or 96-well plate.

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

3.5 QC of 2nd PCR product

Quantify the purified 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 PCR products is ≥ 5 ng/μL.

It is recommended to assess the fragment size distribution of purified PCR products with electrophoresis based equipment such as Bioanalyzer, Tapestation (Agilent Technologies), LabChip GX, GXII, GX Touch (PerkinElmer), Fragment Analyzer (Advanced Analytical).

The main peak fragment sizes of PCR purified products from different primer pools are shown in following Table.

Panel	PCR purified product fragment size range
16S V3V4	550-650 bp
18SV4	400-600 bp
ITS1	300-600 bp
COI	400-600 bp
Ac12S	400-600 bp
MiFish	300-400 bp

3.6 Libraries pooling

After the libraries (purified 2nd PCR products) meet the requirement of the quantitative QC, select the appropriate library pooling scheme based on the primer pool and sequencing strategy.

Panel	Sequencer	Read length
16S V3V4, 18SV4, ITS1, COI, Ac12S	DNBSEQ-G99	PE300
MiFish	DNBSEQ-G99	PE150
MiFish	DNBSEQ-E25	PE150

For libraries prepared with the 16S V3V4, 18SV4, COI, ITS1, Ac12S primer pool, pool and mix the single primer pool libraries according to the required proportion of sequencing data. The total yield after pooling should be greater than or equal to 500 ng, with the total volume less than 48 μL. To prepare the ssDNA (single-stranded circular DNA) library, uses the MGIEasy Dual Barcode Circularization Kit (Cat. No: 1000020570), ensuring that the ssDNA library yield is at least 10 ng. For detailed instructions, refers to *MGIEasy Dual Barcode Circularization Kit User Manual*.

⚠ CAUTION When multiple primer pools are utilized for library preparation, it is recommended to prepare the ssDNA libraries separately.

For libraries prepared with the MiFish primer pool, pool and mix the libraries according to the required proportion of sequencing data. The total yield after pooling should be greater than or equal to 25 ng, with the total volume less than 20 µL. It is recommended to use the one-step method to prepare DNB for sequencing. For detailed information, refer to Section 4.

⚠ CAUTION

- If there are N libraries to be mixed and each library requires an equal amount of sequencing data, then all libraries should be mixed in equal mass. The pooling mass of a library (ng) = M ng/N (M is the total amount of mixed libraries required for a single reaction, choose 500 ng or 25 ng according to the above description), the pooling volume of a library (µL) = the pooling mass of a library (ng) / the concentration of a library (ng/µL).
- When the pooling volume of each library is very small and to minimize pipetting errors, it is recommended to prepare the mixed libraries at X times the volume. Then, take 1/X of the total volume of the mixed libraries for ssDNA preparation or one-step DNB preparation.

4 DNB preparation and sequencing

4.1 16S V3V4, 18SV4, ITS1, COI, Ac12S libraries

The following steps describe the DNB preparation and sequencing process of 16S V3V4, 18SV4, ITS1, COI, Ac12S libraries based on DNBSEQ-G99 sequencing platform. For specific DNB preparation and sequencing procedures, please refer to the corresponding instruction manual.

4.1.1 DNBSEQ-G99 High-throughput Sequencing Platform

4.1.1.1 DNB preparation

The sample ssDNA library prepared in "Libraries pooling" on page 18, should be used with ATOPlex E450 Dual Barcode Balanced Library Reagent (Cat. No: 940-000637-00) for sequencing balance. To make DNB, both the ssDNA sample library and the standard library use DNB preparation reagents from the DNBSEQ-G99RS High-throughput Sequencing Set (G99 SM FCL PE300) (Cat. No: 940-000415-00). The steps are as follows. For detailed information, refers to *DNBSEQ-G99RS High-throughput Sequencing Set User Manual*.

4.1.1.1.3 ssDNA library

- If one primer pool is used, take out 6 ng of the ssDNA library to make DNB, and then supplement with TE Buffer to reach a total volume of 10 μL.
- If multiple primer pools are used, it is recommended to pool and mix multiple ssDNA libraries according to the required proportion of sequencing data to make DNB. After mixing ssDNA libraries from multiple primer pools, and then supplement the mixture with TE Buffer to reach a total volume of 10 μL.

CAUTION For example, if N ssDNA libraries are mixed with equal mass, the pooling mass for each primer ssDNA library = 6 ng/N, the pooling volume for each primer ssDNA library (μL) = the pooling mass for each ssDNA library (ng) / the concentration of ssDNA library (ng/μL).

4.1.1.1.2 Standard library

- Use 5 ng of ATOPlex E450 Dual Barcode Balanced Library Reagent to make DNB, and then supplement with TE Buffer to reach a total volume of 10 μL.

4.1.1.1.1 DNB pooling

- Sample library DNB and standard library DNB are mixed according to a 4:1 mass ratio.

4.1.1.2 Sequencing

We recommend customer to use sequencing set of DNBSEQ-G99RS high-throughput platform to perform dual barcode sequencing, select the sequencing type of PE300+10+10 for operation:

DNBSEQ-G99RS High-throughput Sequencing Set (G99 SM FCL PE300) (Cat. No: 940-000415-00)

CAUTION Before sequencing, please carefully read the corresponding instructions and strictly follow the instructions for operation.

CAUTION Other MGI sequencers with PE300 sequencing strategies, such as DNBSEQ-G400 sequencer, can also be used, please refer to the corresponding instructions for operation.

4.2 MiFish libraries

The following steps describe the DNB preparation and sequencing process of MiFish libraries based on DNBSEQ-G99 and DNBSEQ-E25 sequencing platforms. For specific DNB preparation and sequencing procedures, please refer to the corresponding instruction manual.

4.2.1 DNBSEQ-G99 High-throughput Sequencing Platform

4.2.1.1 DNB preparation

MiFish libraries prepared in “Libraries pooling” on page 18, should be used with ATOPlex E450 Dual Barcode Balanced Library Reagent (Cat. No: 940-000637-00) for sequencing balance. DNB preparation according to the following strategies.

CAUTION The mixed MiFish libraries are composed of purified 2nd PCR products, and the standard library is ssDNA. Therefore, different DNB preparation methods will be used for both.

4.2.1.1.3 MiFish libraries

Use 13 ng of mixed MiFish libraries to make DNB, and then supplement with TE Buffer to reach a total volume of 10 μL. To make DNB, use the DNBSEQ OneStep DNB Make Reagent Kit (Cat. No: 1000026466), and refer to the *DNBSEQ DNB Make Reagent Kit User Manual*for detailed steps. It is worth mentioning that the "Make DNB buffer (OS)" in "Make DNB reaction mixture 1" and the "Make DNB enzyme Mix I", as well as "Make DNB enzyme Mix II (OS)" in "Make DNB reaction mixture 2", should have their volumes reduced by half. However, the "Primer hybridization reaction" and "Rolling circle amplification conditions" should remain unchanged.

4.2.1.1.2 Standard library

Use 5 ng of ATOPlex E450 Dual Barcode Balanced Library Reagent to make DNB, and then supplement with TE Buffer to reach a total volume of 10 μL. To make DNB, use the DNB Make reagent from the DNBSEQ-G99RS High-throughput Sequencing Set (G99 SM FCL PE150) (Cat. No: 940-000410-00). For detailed information, refers to *DNBSEQ-G99RS High-throughput Sequencing Set User Manual*.

4.2.1.1.1 DNB pooling

MiFish library DNB and balanced library DNB are mixed according to a 4:1 mass ratio.

4.2.1.2 Sequencing

We recommend customer to use sequencing set of DNBSEQ-G99ARS high-throughput platform to perform dual barcode sequencing, select the sequencing type of PE150+10+10 for operation:

DNBSEQ-G99RS High-throughput Sequencing Set (G99 SM FCL PE150) (Cat. No: 940-000410-00)

CAUTION Before sequencing, please carefully read the corresponding instructions and strictly follow the instructions for operation.

4.2.2 DNBSEQ-E25 High-throughput Sequencing Platform

MiFish libraries prepared in "Libraries pooling" on page 18, should be used with Standard Library Reagent (PCR Product) V4.0 (Cat. No: 1000027585) for sequencing balance. To make DNB, both the mixed MiFish libraries and the standard library use DNB preparation reagents from the DNBSEQ-E25RS High-throughput Sequencing Set (FCL PE150) (Cat. No: 940-000567-00). The steps are as follows. For detailed information refers to *DNBSEQ-E25RS High-throughput Sequencing Set User Manual*.

4.2.2.1 DNB preparation

4.2.2.1.1 MiFish libraries

Use 25 ng of mixed MiFish libraries to make DNB, and then supplement with TE Buffer to reach a total volume of 20 μL.

4.2.2.1.2 Standard library

Use 25 ng of standard Library Reagent to make DNB, and then supplement with TE Buffer to reach a total volume of 20 μL.

4.2.2.1.3 DNB pooling

MiFish library DNB and standard library DNB are mixed according to a 2:1 mass ratio.

4.2.2.2 Sequencing

We recommend customer to use sequencing set of DNBSEQ-E25RS high-throughput platform to perform dual barcode sequencing, select the sequencing type of PE150+10+10 for operation:

DNBSEQ-E25RS High-throughput Sequencing Set (FCL PE150) (Cat. No: 940-000567-00)

CAUTION Before sequencing, please carefully read the corresponding instructions and strictly follow the instructions for operation.

CAUTION Other MGI sequencers with PE150 sequencing strategies, such as DNBSEQ-G400 sequencer, can also be used, please refer to the corresponding instructions for operation.

5 Appendix

5.1 ATOPlex Dual Barcode Primer Module usage strategies

5.1.1 ATOPlex PCR Dual Barcode Primer Module (01-96)

- This module is for 96 RXN dual barcode module, which contains one 96-well plate with 96 Dual Barcode Primer Mix in each well.
- 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 read the usage rules carefully when using.
- Do not store above room temperature to avoid structural changes such as degradation, which might affect performance.
- Before use, centrifuge at 2000 rpm for 2 min to collect the liquid at the bottom of the plate and carefully tear off the sealing film. Pay attention to replace the tips during use to avoid contamination. After use, use a plastic seal film to seal the plate (be careful not to use heat seal film), and store at -20 ℃.

5.1.1.1 Usage rules of PCR Dual Barcode Primer Mix (01-96)

The layout of PCR Dual Barcode Primer Mix (01-96) is showed in the figure below:

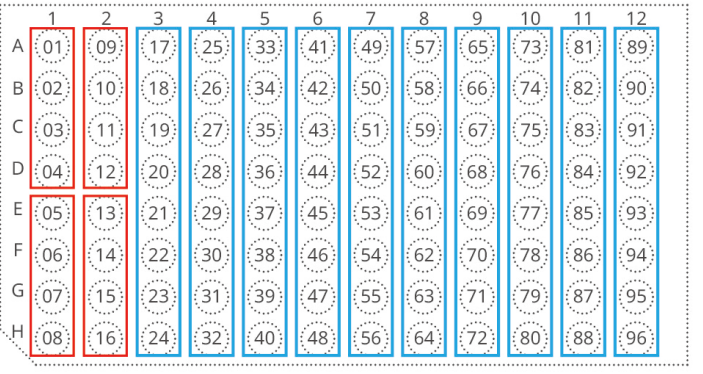

Figure 1 The layout of PCR Dual Barcode Primer Mix (01-96)

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

- 4 groups of 4 PCR Dual Barcode Primer Mix: 01-04, 05-09, 09-12, and 13-14 (Red box in above).
- 12 groups of 8 PCR Dual Barcode Primer Mix: 01-08, 09-16, 17-24, 25-32, 33-40 and 41-48, 49-56, 57-64, 65-72, 73-80, 81-89, and 89-96, which means 8 Barcodes in one column.

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

- When the sample number is 1-4, add 1 μL from each of 1 group of 4 PCR Dual Barcode Primer Mix (Red box in above) to one sample, every sample add 4 μL Barcode Primer Mix totally.
- When the sample number is 5-96, add 4 μL from 1 PCR Dual Barcode Primer Mix to each sample, and the sample code corresponding to the number in above. For example, Barcode 01 is added to sample1, Barcode 02 is added to sample 2, and so on.

5.1.2 ATOPlex PCR Dual Barcode Primer Module (48X96)

- This module is for 576 RXN dual barcode module, which contains one 96-well plate with Barcode 1 (01-96) and another 96-well plate with Barcode 2 (01-48). 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 read the usage rules carefully when using.
- Do not store above room temperature to avoid structural changes such as degradation, which might affect performance.
- Before use, centrifuge at 2000 rpm for 2 min to collect the liquid at the bottom of the plate and wipe the surface of the aluminum film clean with absorbent paper. Pay attention to replace the tips during use to avoid contamination. After use, use a plastic seal film to seal the plate (be careful not to use heat seal film), and store at -20 ℃.

5.1.2.1 Usage rules of PCR Dual Barcode Primer (48X96)

The layout of PCR Dual Barcode Primer F and PCR Dual Barcode Primer R are shown in the following figures. Based on the principle of base balance, PCR Dual Barcode Primer F and PCR Dual Barcode Primer R must be used in specific groups.

- 6 groups of 8 PCR Dual Barcode Primer F: 01-08, 09-16, 17-24, 25-32, 33-40, 41-48.

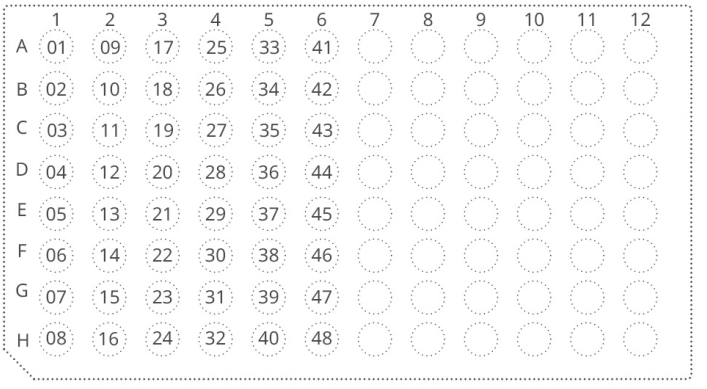

Figure 2 Layout of PCR Dual Barcode Primer F

- 12 groups of 8 PCR Dual Barcode Primer R: 01-08, 09-16,17-24, 25-32, 33-40, 41-48, 49-56, 57-64, 65-72, 73-80, 81-88, 89-96.

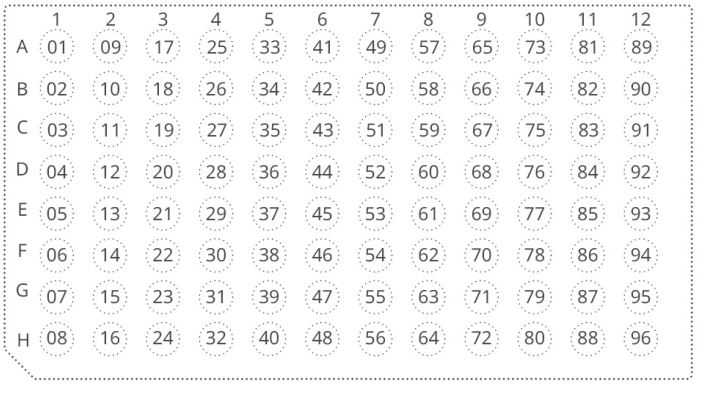

Figure 3 Layout of PCR Dual Barcode Primer R

- When the data requirements of each sample are the same, different sample numbers can refer to the recommended Dual Barcode combination scheme as below.

CAUTION

- To ensure optimal sequencing quality, it is recommended that at least 8 Barcodes combination per lane, less than 8 Barcodes combination may result in a lower split rate due to base imbalance.
- PCR Dual Barcode Primer F in each row must be used in conjunction with the PCR Dual Barcode Primer R of the same row (For example: Any PCR Dual Barcode Primer F in row A of PCR Dual Barcode Primer F can only be used with any PCR Dual Barcode Primer R in row A of PCR Dual Barcode Primer R), otherwise it could not decode the Barcode and output the sample data.

5.1.2.1.1 Senario I: 8 samples/ lane

Perform the following steps:

1. Take a group of 8 PCR Dual Barcode Primer F and each sample add 1 PCR Dual Barcode Primer F in 2 μL.

   For example, F01-F08 are added to samples 1-8 respectively.

2. Take a group of 8 PCR Dual Barcode Primer R, and each sample add 2 μL of 1 PCR Dual Barcode Primer R, which is in the corresponding row of PCR Dual Barcode Primer F.

   For example, R09-R16 are added to samples 1-8 respectively. The table below is an exmple for 8 samples.

Table 19 Example of Barcode F and R combination for 8 samples

Sample	Barcode F	Barcode R
1	F01	R09
2	F02	R10
3	F03	R11
4	F04	R12
5	F05	R13
6	F06	R14
7	F07	R15
8	F08	R16

Tips When sample number is less than 8, 8 groups of Barcode combination can be randomly allocated to each sample, and mixed Barcode Primer according to the allocation results, then added to the sample.

For example, when sample number is 5, F01-F02, F03-F04, F05-F06, R01-R02, R03-R04, R05-R06 can be mixed equally in pairs. Then, 2 μL F01-F02 + 2 μL R01-R02, 2 μL F03-F04 + 2 μL R03-R04, 2 μL F05-F06 + 2 μL R05-R06 are added to sample 1, 2 and 3, respectively. Then 2 μL F07 + 2 μL R07, 2 μL F08 + 2 μL R08 are added to sample 4 and sample 5.

5.1.2.1.2 Senario II: 9-96 samples/ lane

When the sample number is 8n+x (1≤n<11, 1≤x≤8, total: 9-96 samples) , every 8 samples are divided into one group, and x is the number of ungrouped samples.

1. Eight PCR Dual Barcode Primer F in the same column are added into each group of samples. (For example, PCR Dual Barcode Primer F01-F08 are added to samples 1-8 respectively, and PCR Dual Barcode Primer F01-F08 are also added to samples 9-16 respectively, and so on until samples 89-96). According to the number of x, a single PCR Dual Barcode Primer F from the same PCR Dual Barcode Primer F group is added to the ungrouped samples.
2. Add 1 PCR Dual Barcode Primer R to each sample and the PCR Dual Barcode Primer R added to each sample should be different. Based on the principle of base balance, it still need to follow the method of Senario I (For example, PCR Dual Barcode Primer R01-R08 are added

to samples 1-8, Primer R09-R16 are added to samples 9-16, and so on). According to the number of x, a single PCR Dual Barcode Primer R from the PCR Dual Barcode Primer R set is added to each sample.

Take library preparation for 70 samples as an example. The PCR Dual Barcode combination of each sample is shown in the table below:

Table 20 Example of Barcode F and R combination for 70 samples

70 samples	1	2	3	4	5	6	7	8	9
A	R01-F01	R09-F01	R17-F01	R25-F01	R33-F01	R41-F01	R49-F01	R57-F01	R65-F01
B	R02-F02	R10-F02	R18-F02	R26-F02	R34-F02	R42-F02	R50-F02	R58-F02	R66-F02
C	R03-F03	R11-F03	R19-F03	R27-F03	R35-F03	R43-F03	R51-F03	R59-F03	R67-F03
D	R04-F04	R12-F04	R20-F04	R28-F04	R36-F04	R44-F04	R52-F04	R60-F04	R68-F04
E	R05-F05	R13-F05	R21-F05	R29-F05	R37-F05	R45-F05	R53-F05	R61-F05	R69-F05
F	R06-F06	R14-F06	R22-F06	R30-F06	R38-F06	R46-F06	R54-F06	R62-F06	R70-F06
G	R07-F07	R15-F07	R23-F07	R31-F07	R39-F07	R47-F07	R55-F07	R63-F07	
H	R08-F08	R16-F08	R24-F08	R32-F08	R40-F08	R48-F08	R56-F08	R64-F08	

5.1.2.1.3 Senario III: 96-576 samples/ lane

When the sample number is between 96 to 576, which is N*96-well plate $^{(N=1-6)}$, use the above method of 9-96 samples/lane to add PCR Dual Barcode Primer F and PCR Dual Barcode Primer R.

CAUTION PCR Dual Barcode Primer F with different columns should be added for different sample plates.

Take library preparation of 2 plate samples as an example, the PCR Dual Barcode combination of each sample is shown in the table below:

Table 21 PCR Dual Barcode combination example of the first plate

Plate1	1	2	3	4	5	6	7	8	9	10	11	12
A	R01-F01	R09-F01	R17-F01	R25-F01	R33-F01	R41-F01	R49-F01	R57-F01	R65-F01	R73-F01	R81-F01	R89-F01
B	R02-F02	R10-F02	R18-F02	R26-F02	R34-F02	R42-F02	R50-F02	R58-F02	R66-F02	R74-F02	R82-F02	R90-F02
C	R03-F03	R11-F03	R19-F03	R27-F03	R35-F03	R43-F03	R51-F03	R59-F03	R67-F03	R75-F03	R83-F03	R91-F03
D	R04-F04	R12-F04	R20-F04	R28-F04	R36-F04	R44-F04	R52-F04	R60-F04	R68-F04	R76-F04	R84-F04	R92-F04
E	R05-F05	R13-F05	R21-F05	R29-F05	R37-F05	R45-F05	R53-F05	R61-F05	R69-F05	R77-F05	R85-F05	R93-F05
F	R06-F06	R14-F06	R22-F06	R30-F06	R38-F06	R46-F06	R54-F06	R62-F06	R70-F06	R78-F06	R86-F06	R94-F06
G	R07-F07	R15-F07	R23-F07	R31-F07	R39-F07	R47-F07	R55-F07	R63-F07	R71-F07	R79-F07	R87-F07	R95-F07
H	R08-F08	R16-F08	R24-F08	R32-F08	R40-F08	R48-F08	R56-F08	R64-F08	R72-F08	R80-F08	R88-F08	R96-F08

Table 22 PCR Dual Barcode combination example of the second plate

Plate2	1	2	3	4	5	6	7	8	9	10	11	12
A	R01-F09	R09-F09	R17-F09	R25-F09	R33-F09	R41-F09	R49-F09	R57-F09	R65-F09	R73-F09	R81-F09	R89-F09
B	R02-F10	R10-F10	R18-F10	R26-F10	R34-F10	R42-F10	R50-F10	R58-F10	R66-F10	R74-F10	R82-F10	R90-F10
C	R03-F11	R11-F11	R19-F11	R27-F11	R35-F11	R43-F11	R51-F11	R59-F11	R67-F11	R75-F11	R83-F11	R91-F11
D	R04-F12	R12-F12	R20-F12	R28-F12	R36-F12	R44-F12	R52-F12	R60-F12	R68-F12	R76-F12	R84-F12	R92-F12
E	R05-F13	R13-F13	R21-F13	R29-F13	R37-F13	R45-F13	R53-F13	R61-F13	R69-F13	R77-F13	R85-F13	R93-F13
F	R06-F14	R14-F14	R22-F14	R30-F14	R38-F14	R46-F14	R54-F14	R62-F14	R70-F14	R78-F14	R86-F14	R94-F14
G	R07-F15	R15-F15	R23-F15	R31-F15	R39-F15	R47-F15	R55-F15	R63-F15	R71-F15	R79-F15	R87-F15	R95-F15
H	R08-F16	R16-F16	R24-F16	R32-F16	R40-F16	R48-F16	R56-F16	R64-F16	R72-F16	R80-F16	R88-F16	R96-F16

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