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• Version: 1.0

Overview

The quick operation guide is used to instruct the quick use of the Microbiome Metabarcoding Sequencing Package Sequencing Package. For details, refer to the relevant reagent kit user manuals and relevant instructions.

Kit type	Name	Specification
Library preparation	ATOPlex 16S V3V4 rDNA Panel (96 RXN)	940-001260-00, 96 RXN
	ATOPlex 16S V3V4 rDNA Panel (576 RXN)	940-000724-00, 576 RXN
	ATOPlex 16SV4 rDNA Panel (96 RXN)	940-002492-00, 96 RXN
	ATOPlex 16SV4 rDNA Panel (576 RXN)	940-002490-00, 576 RXN
	ATOPlex ITS1 rDNA Panel (96 RXN)	940-001536-00, 96 RXN
	ATOPlex ITS1 rDNA Panel (576 RXN)	940-001532-00, 576 RXN
	ATOPlex ITS2 rDNA Panel (96 RXN)	940-002489-00, 96RXN
	ATOPlex ITS2 rDNA Panel (276 RXN)	940-002491-00, 576 RXN
	ATOPlex HiFi PCR Amplification Module(96 RXN)	950-000138-00
	ATOPlex HiFi PCR Amplification Module(576 RXN)	950-000137-00
	MGIEasy Dual Barcode Circularization Kit	1000020570, 16 RXN
	ATOPlex E450 Dual Barcode Balanced Library Reagent	940-000637-00, 40 ng/ tube
DNB making	DNBSEQ OneStep DNB Make Reagent Kit	1000026466, 4 RXN

Library preparation

Preparing samples

The DNA input amount affects the abundance of the species in the target group and the recommended input amount is shown in the table below:

Sequencing primer	Sample type	Input amount range	Recommended input amount
16SV3V4, 16SV4	High bacterial content sample: human-sourced samples, such as stool and saliva.	10-100 ng	10 ng
	Low bacterial content sample: • Human-sourced samples, such as skin and tongue coating swabs. • Fungal fermentation samples, such as lees. • Agriculture samples, such as leave surface.	/	10 µL
ITS1, ITS2	High fungal content sample: fungal fermentation samples, such as lees.	10-100 ng	10 ng
	Low fungal content sample: • Human-sourced samples, such as stool, saliva, skin, and tongue coating swabs. • Agriculture samples, such as leave surface.	/	10 µL

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First PCR amplification

1. Take out ATOPlex HiFi PCR Amplification Module and ATOPlex Metabarcoding Library Prep Primer Pool (16SV3V4/16SV4/ITS1/ITS2) until use.
2. Take out PCR Clean Enzyme and PCR HiFi Enzyme Mix, centrifuge them briefly, and place them on the ice box until use. Thaw other components at room temperature. After thawing, mix the reagents thoroughly, centrifuge them briefly, and place them on the ice box until use.
3. Take out a new 96-well PCR plate, place it on the ice box, and prepare the first PCR amplification reaction mixture in the PCR plate according to the table below:

Component	Volume/reaction
DNA	V
PCR HiFi Enzyme Mix	12.5 μL
16SV3V4/16SV4/ITS1/ITS2 PCR Primer Pool	2 μL
PCR Clean Enzyme	0.5 μL
TE Buffer	10 μL-V
Total volume	25 μL

4. Seal the PCR plate by using a plastic sealing film, mix it by using the vortex mixer, and centrifuge it briefly by using the plate centrifuge to collect the mixture at the bottom of the plate.
5. Place the PCR plate with the first PCR amplification reaction mixture (25 μL/well) into the thermal cycler and start the reaction according to conditions listed in the table below:

Temperature	Time	Cycle
Heated lid (104 ℃)	On	/
37 ℃	5 min	1 cycle
95 ℃	10 min	
95 ℃	20 s	20 to 35 cycles
55 ℃ ()	30 s	
72 ℃	30 s	
12 ℃	Hold	/

⚠ CAUTION

Optimize the number of PCR cycles based on the sample to achieve the desired PCR product concentration. The recommended PCR cycles for different samples and primers are described in the following table:

Primer	Sample type	Recommended PCR cycle
16SV3V4, 16SV4	High bacterial content sample: human-sourced samples, such as stool and saliva.	20
	Low bacterial content sample: ▪ Human-sourced samples, such as skin and tongue coating swabs. ▪ Fungal fermentation samples, such as lees. ▪ Agriculture samples, such as leave surface.	30

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Primer	Sample type	Recommended PCR cycle
ITS1, ITS2	High fungal content sample: agriculture samples, such as leaves.	25
	Low fungal content sample: ▪ Human-sourced samples, such as stool, saliva, skin, and tongue coating swabs. ▪ Agriculture samples such as leave surface.	35

6. After the amplification is finished, take out the PCR plate and centrifuge the plate briefly by using the plate centrifuge to collect the mixture at the bottom of the plate. The mixture at the bottom of the plate is the first PCR product.

⚠ CAUTION

Since different primers require different annealing temperature and cycles, it is recommended to place different PCR amplification reaction mixtures into different 96-well plates or PCR 8-strip tubes and transfer the reaction products into the same PCR plate for purification after the first PCR amplification.

Second PCR amplification reaction mixture preparation and first PCR product purification

Preparing the second PCR amplification reaction mixture

1. Preparing reagents and consumables:
   1) Take out DNA Clean Beads and TE Buffer from MGIEasy DNA Clean Beads Kit 30 minutes in advance and mix the reagents until use.
   2) Prepare fresh 80% ethanol solution until use.
   3) Prepare four new 1.3 mL 96-well U deep plates for use, and mark them as *Clean Beads*, *TE Buffer*, *80% Ethanol*, and *Waste plate*.
   4) Prepare a new hard-shell thin-wall 96-well skirted PCR plate for use and mark it as *Second PCR amplification reaction mixture*.
2. Add reagents according to the table below for use and seal the three deep-well plates by using films.

Name marked on the plate	Reagent	Volume
Clean Beads	DNA Clean Beads	80 µL/well
TE Buffer	TE Buffer	40 µL/well
80% Ethanol	80% ethanol	750 µL/well

3. Prepare the second PCR amplification reaction mixture in the hard-shell thin-wall 96-well skirted PCR plate marked as *Second PCR amplification reaction mixture* according to the table below:

Component	Volume/reaction
PCR HiFi Enzyme Mix	12.5 µL
Barcode 1	2 µL
Barcode 2	2 µL
PCR Clean Enzyme	0.5 µL
PCR Additive	0.5 µL
16SV3V4/16SV4/ITS1/ITS2 PCR Block	1 µL
Total volume	18.5 µL

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CAUTION

If you use ATOPlex Dual Barcode Primer Module (01-96) V1.0, add 4 μL PCR Dual Barcode Primer Mix. If you use ATOPlex Dual Barcode Primer Mix (48 × 96), add 2 μL Barcode 1 (01-96) and 2 μL Barcode 1 (01-48).

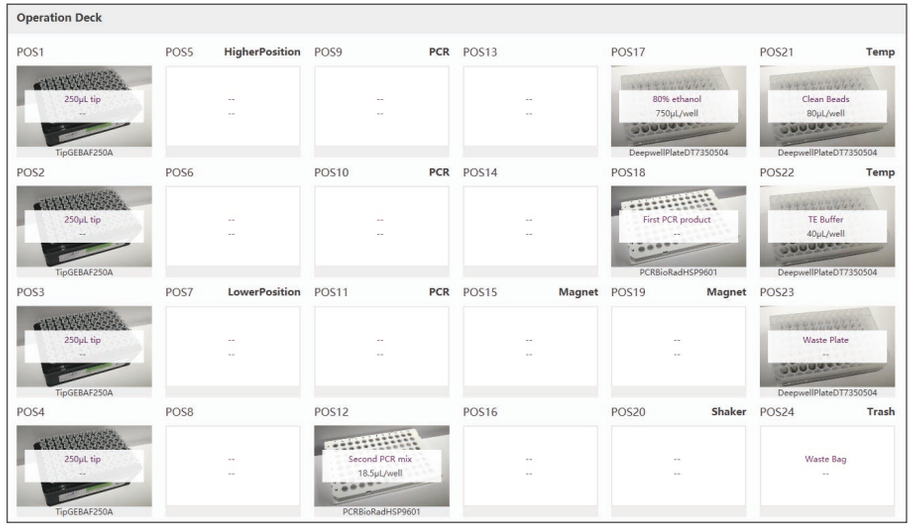
4. Seal the PCR plate prepared in step 3 by using a film, mix it briefly by using the vortex mixer, and centrifuge it briefly to collect the mixture at the bottom of the plate.

First PCR purification

Tips

- Perform the steps in this section on High-throughput Automated Sample Preparation System- Custom Configuration 9-V7.
- For detailed operations, refer to *MGISP-100 & MGISP-960 Application Script Installation Instructions* .

1. Double-click the control software icon on the desktop of the device, select **Real** , and then click **Login** . The home page is displayed.
2. Click ⋮ in the upper-right corner and select **WDesigner** to open the WDesigner.
3. Click **Import Application** and import the applications **JB-A09-145 ATOPlex Metabarcoding Library Prep_step1first purification_RV1.0_SV1.0.wfex** and **JB-A09-145 ATOPlex Metabarcoding Library Prep_step2second purification_RV1.0_SV1.0.wfex** .
4. Click the MGISP-960 icon to open the control software.
5. Click **Initialize** on the home page of the control software. It takes about 2 minutes to initialize the device. When the prompt **Initialization Successfully** appears, the device is ready for use and you can proceed to the next step.
6. Click the left navigation bar on the left and select **Run Wizard** .
7. Click the drop-down list on the right of the **Solution** box in the **Run Wizard** interface and select **JB-A09-145 ATOPlex Metabarcoding Library Prep_ RV1.0_SV1.0** .
8. Click the drop-down list on the right of the **Script** box and select **ATOPlex_Metabarcoding_Library_Prep_step1 first purification.py** . The corresponding layout is displayed in the **Operation Deck** area in the lower section.
9. Place the prepared samples, reagents, consumables onto the deck according to the figure below, and close the device door after rechecking the position of the materials.



Name	Consumable	Position
250 μL automatic tip with filter	250 μL automatic tip with filter	Pos1 to Pos4
Second PCR amplification reaction mixture	Hard-shell Thin-wall 96-well Skirted PCR Plates	Pos12
80% Ethanol	1.3 mL U-bottom deep-well plate	Pos17
First PCR product	Hard-shell Thin-wall 96-well Skirted PCR Plates	Pos18
Clean Beads	1.3 mL U-bottom deep-well plate	Pos21
TE Buffer	1.3 mL U-bottom deep-well plate	Pos22
Waste plate	1.3 mL U-bottom deep-well plate	Pos23

⚠ CAUTION

Manually tear off the film of the filmed consumables before operation.

10. Click **Start** and click **Start** in the pop-up window to run the experiment workflow. The workflow is expected to run about 25 minutes.
11. After the workflow is completed, a notification window is displayed. Click **Continue** and take out the PCR product from Pos16. Seal the PCR product by using a film, centrifuge it briefly, and perform the second PCR amplification manually.
12. Take out and discard the consumables and reagent plates from Pos1 to Pos4 and Pos 12. Seal the **80% Ethanol** plate in Pos17, **Clean Beads** plate in Pos21, **TE Buffer** plate in Pos22, and **Waste plate** in Pos22 and use them for second PCR product purification.

Second PCR amplification

1. Place the first PCR product plate prepared in step 10 of *First PCR purification on page 4* into the thermal cycler and start the second PCR amplification according to the table below:

Temperature	Time	Cycle
Heated lid (104 °C)	On	/
37 °C	5 min	1 cycle
95 °C	10 min	
95 °C	20 s	8 cycles
55 °C	30 s	
72 °C	30 s	
12 °C	hold	/

2. After amplification, take out the PCR plate, centrifuge it to collect the mixture at the bottom of the plate, and conduct the next step immediately.

Tips

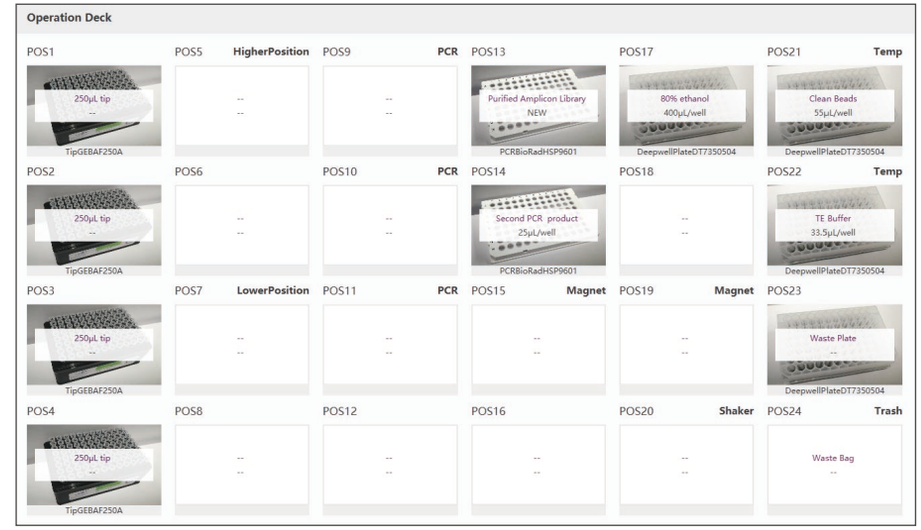
The PCR product should be placed at 12 ℃ within 30 minutes.

Second PCR product purification, library quantitation and quality control

1. Click the drop-down list on the right of the **Solution** box in the **Run Wizard** interface, and select **JB-A09-145 ATOPlex Metabarcoding Library Prep_RV1.0_SV1.0** .
2. Click the drop-down list on the right of **Script** box and select **ATOPlex_Metabarcoding_Library_Prep_step2 first purification.py** . The corresponding layout is displayed in the **Operation Deck** area in the lower section.
3. Place the prepared samples, reagents, and consumables onto the deck according to the figure below, and close the device door after rechecking the position of the materials.

CAUTION

- Manually tear off the film of the filmed consumables before operation.
- The reagents used in the first PCR product purification in Pos17, Pos21, Pos22, and Pos23 continue to be used to purify the second PCR products. You do not need to change the positions of the four plates or add reagents to the plates.



Name	Category	Position
250 μL automatic tip with filter	250 μL automatic tip with filter	Pos1 to Pos4
Purified Amplicon Library	Hard-shell Thin-wall 96-well Skirted PCR Plates	Pos13
Second PCR product	Hard-shell Thin-wall 96-well Skirted PCR Plates	Pos14
80% Ethanol	1.3 mL U-bottom deep-well plate	Pos17
Clean Beads	1.3 mL U-bottom deep-well plate	Pos21
TE Buffer	1.3 mL U-bottom deep-well plate	Pos22
Waste Plate	1.3 mL U-bottom deep-well plate	Pos23

4. Click **Start** and click **Start** in the pop-up window to run the experiment workflow. The workflow is expected to run about 30 minutes.

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5. After the workflow is completed, a notification window is displayed. Take out the product from Pos 13 with an approximate volume of 23 µL per well. Seal and centrifuge the product. Click **Continue** to complete the workflow.
6. Discard the used deep-well plates, PCR plates, and waste bags into the designated area.

   If no more experiments are to be conducted on the same day, clean the deck of the device by using the pure water and 75% ethanol and conduct a post-clean according to *MGISP-100/MGISP-960 Cleaning Instructions* .
7. Use Qubit dsDNA HS Assay Kit or Quant-iT PicoGreen® dsDNA Assay Kit to quantify the second PCR product according to the instructions of the kit. The library concentration after purification should be greater than or equal to 5 ng/µL.
8. Use Bioanalyzer, Tapestation (Agilent Technologies), and other instruments based on the principle of electrophoretic separation to detect the fragment distribution of the second PCR purification product. The main peak sizes of the PCR purification products prepared by using different primer pools are shown in the table below:

Primer pool	Main peak size of the PCR purification product
16S V3V4	550 bp~650 bp
ITS1	300 bp~600 bp
ITS2	300 bp~600 bp
16SV4	400 bp~500 bp

Library pooling

After the library meets the requirement of the quantitative QC, select the corresponding library pooling scheme based on the used primer pool and sequencing strategy.

Primer pool	Sequencer	Recommended read length
16S V3V4, ITS1, ITS2	DNBSEQ-G99	PE300
16SV4	DNBSEQ-G99	PE150
16SV4	DNBSEQ-E25	PE150

Library pooling and circulation for 16S V3V4, ITS1, and ITS2 primer pools

After the libraries meet the requirement of the quantitative QC, measure the libraries according to the ratio of sequencing data amount and mix them. It is recommended that the total mass after pooling should be greater than or equal to 500 ng and the total volume should be less than or equal to 48 µL.

1. Take out MGI Easy Dual Barcode Circularization Kit for use. Add 500 ng pooling library into a 0.2 mL PCR tube and add TE Buffer until the total volume reaches 48 µL.
2. Place the PCR tube into the thermal cycler and start the reaction according to conditions listed in the table below:

Temperature	Time
Heated lid (104 °C)	On
95 °C	3 min

3. After reaction, immediately place the tube on the ice box for 2 minutes and centrifuge it briefly.

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4. Prepare the single strand circularization reaction mixture on the ice box according to the table below:

Component	Volume
Dual Barcode Splint Buffer	11.6 µL
DNA Rapid Ligase	0.5 µL
Total volume	12.1 µL

5. Transfer 12.1 µL single strand circularization mixture to the PCR tube prepared in step 3. Mix the mixture 6 times (3 seconds each) by using the vortex mixer and centrifuge it briefly to collect the mixture at the bottom of the tube.
6. Place the tube into the thermal cycler and start the reaction according to conditions listed in the table below:

Temperature	Time
Heated lid (104 °C)	On
37 °C	30 min
4 °C	Hold

7. After reaction, immediately take out the tube and prepare the enzymatic digestion reaction mixture on the ice box according to the table below:

Component	Volume
Digestion Buffer	1.4 µL
Digestion Enzyme	2.6 µL
Total volume	4 µL

8. Transfer 4 µL enzymatic digestion mixture into the PCR tube prepared in step 6. Mix the mixture 6 times (3 seconds each) by using the vortex mixer and centrifuge it briefly to collect the solution at the bottom of the tube.
9. Place the tube into the thermal cycler and start the reaction according to conditions listed in the table below:

Temperature	Time
Heated lid (104 °C)	On
37 °C	30 min
4 °C	Hold

10. After reaction, add 7.5 µL of Digestion Stop Buffer to the PCR tube. Mix the mixture 6 times (3 seconds each) by using the vortex mixer and centrifuge it briefly to collect the mixture at the bottom of the tube. Transfer all the mixture to a new 1.5 mL EP tube.
11. Take out DNA Clean Beads 30 minutes in advance and place it at room temperature. Shake and mix it thoroughly before use.
12. Transfer 170 µL DNA Clean Beads to the enzymatic digestion product in step 10. Gently pipette at least 10 times to mix it thoroughly. Ensure that all mixture and beads are dispensed into the EP tube. Incubate the tube at room temperature for 10 minutes.
13. Centrifuge it briefly and place the 1.5 mL EP tube on the magnetic rack for 2 to 5 minutes until the mixture becomes clear. Carefully remove and discard the supernatant by using a pipette.
14. Keep the 1.5 mL EP tube on the magnetic rack and add 500 µL freshly prepared 80% ethanol to the tube. Incubate the tube for 30 seconds. Carefully remove and discard the supernatant.
15. Repeat step 14. Remove all the supernatant as much as possible.

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16. Keep the 1.5 mL EP tube on the magnetic rack with the lid opened. Let the beads air-dry until the surface of the beads has no reflection or cracking.
17. Remove the 1.5 mL EP tube from the magnetic rack and add 22 µL TE Buffer to elute DNA. Gently pipette the mixture up and down 10 times to mix it thoroughly. Incubate the tube at room temperature for 10 minutes.
18. Centrifuge the tube briefly and place it on the magnetic rack for 2 to 5 minutes until the mixture becomes clear. Transfer 20 µL supernatant to a new 1.5 mL EP tube.

Stoppoint

The purified circularization products can be stored at -20 ℃ for up to one month.

19. Quantify the purified circularization product by using Qubit ssDNA Assay Kit. The final yield should be greater than or equal to 10 ng (enough for two sequencing runs).

Library pooling for 16SV4 primer pool

Take the appropriate volume of libraries based on the ratio of the required sequencing data amount and mix the libraries. The total mass after mixing should be greater than or equal to 25 ng and the total volume (≤ 20 µL) should be enough for making DNBs at least one time.

CAUTION

When the library is prepared by using 16SV4 Primer Pool, circularization is not required after pooling.

DNB making

Making DNBs with 16S V3V4, ITS1, or ITS2 library

1. Take out Low TE Buffer, Make DNB Buffer, Make DNB High-efficiency Enzyme Mix V and Stop DNB Reaction Buffer from the storage of DNBSEQ-G99RS High-throughput Sequencing Set (G99 App-D FCL PE300) and thaw them on the ice box for approximately 30 minutes. After thawing, mix reagents thoroughly by using the vortex mixer for 5 seconds, centrifuge them briefly, and place them on the ice box until use.
2. Take out the circularized and purified ssDNA, take out Balanced Library from the storage of ATOPlex E450 Dual Barcode Balanced Library Reagent and place them on the ice box until use.
3. Take out two new 0.2 mL PCR tubes, separately add 6 ng ssDNA and 6 ng Balanced Library into the two tubes, and then add Low TE Buffer into the two tubes until the total volume of the solution in the two tubes reaches 10 µL.

CAUTION

Make DNBs separately by using the sample library and Balanced Library. Do not add the two libraries into the same PCR tube.

4. Take out a 0.2 mL PCR tube. Prepare Make DNB reaction mixture 1 on the ice box according to the table below:

Component	Volume
Make DNB Buffer	10 µL
Sample library or Balanced Library	10 µL

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5. Mix Make DNB reaction mixture 1 thoroughly by using the vortex mixer and centrifuge it by using the mini centrifuge for 5 seconds. Place it into the thermal cycler and start the reaction according to conditions listed in the table below:

Temperature	Time
Heated lid (104 ℃)	On
95 ℃	1 min
65 ℃	1 min
40 ℃	1 min
4 ℃	Hold

6. Take out Make DNB Enzyme Mix II (LC) from the storage, centrifuge it briefly for 5 seconds, and place it on the ice box.

CAUTION

Neither place Make DNB Enzyme Mix II (LC) at room temperature, nor touch the tube wall for a long time.

7. Take out the PCR tube out of the thermal cycler when the temperature reaches 4 ℃. Centrifuge it briefly by using the mini centrifuge for 5 seconds, place the tube on the ice box, and add the solution according to the table below into the tube:

Component	Volume
Make DNB Enzyme Mix V	20 μL
Make DNB Enzyme Mix II (LC)	0.8 μL

8. Mix the reaction mixture thoroughly by using the vortex mixer and centrifuge it briefly by using the mini centrifuge for 5 seconds. Place the tube (with a total volume of 41 μL) into the thermal cycler and start the reaction according to conditions listed in the table below:

Temperature	Time
Heated lid (35 ℃)	On
30 ℃	30 min
4 ℃	Hold

CAUTION

When conducting the reaction, some sample blocks of thermal cyclers may remain at ambient temperature while the lid is being heated or cooled. For these types of thermal cyclers, pre-heating of the heated lid is required to ensure that the heated lid is at operating temperature (35 ℃) during the DNB reaction.

9. Immediately add 10 μL Stop DNB Reaction Buffer when the temperature reaches 4 ℃ . Mix the solution gently by pipetting 5 to 8 times using a wide-bore tip.

CAUTION

Use the wide-bore tip to gently pipette the DNBs. Do not centrifuge, vortex, pipette vigorously, or shake the tube.

10. Quantify the DNBs.

    Take 2 μL DNBs and use the Qubit ssDNA Assay Kit and Qubit 3.0 Fluorometer to quantify the DNBs. The concentration should be greater than or equal to 12 ng/μL. If the concentration is more than 40 ng/μL, dilute the DNBs with Low TE Buffer until its concentration reaches 30 ng/μL. Store the qualified DNBs at 4 ℃ and use the DNBs within 48 hours.

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Making DNBs with 16SV4 library

The process of making DNBs using the DNBSEQ-G99ARS genetic sequencer is different from that using the DNBSEQ-E25RS genetic sequencer. Select one of the following process according to the sequencer you used.

Using DNBSEQ-G99ARS

1. Take out the sample library (ensure that the sample library has been mixed according to *Library pooling for 16SV4 primer pool on page 9*), take out ATOPlex E450 Dual Barcode Balanced Library Reagent, and take out Low TE Buffer, Make DNB Buffer, Make DNB Enzyme Mix I (OS), and Stop DNB Reaction Buffer from the storage of DNBSEQ OneStep DNB Make Reagent Kit (OS-DB) V1.0, and thaw them for approximately 30 minutes on the ice box.
2. After thawing, mix the reagents thoroughly by using the vortex mixer for 5 seconds, centrifuge them briefly, and place them on the ice box until use.
3. Take out two new 0.2 mL PCR tube, separately add 13 ng sample library and 6 ng Balanced Library into the two tubes, and then add Low TE Buffer into the two tubes until the total volume of the mixture in the two tubes reaches 10 µL.
4. Take out a new 0.2 mL PCR tube, place it on the ice box, and add the solution according to the table below into the tube to prepare Make DNB reaction mixture 1:

Component	Volume
Make DNB Buffer (OS)	10 µL
ssDNA libraries or Balanced Library	10 µL

5. Mix Make DNB reaction mixture 1 thoroughly by using the vortex mixer and centrifuge it by using the mini centrifuge for 5 seconds. Place it into the thermal cycler and start the reaction according to conditions listed in the table below:

Temperature	Time
Heated lid (104 °C)	On
95 °C	3 min
40 °C	3 min
4 °C	Hold

6. Take out Make DNB Enzyme Mix II (OS) from the storage, centrifuge it briefly for 5 seconds, and place it on the ice box until use.

⚠ CAUTION

Neither place Make DNB Enzyme Mix II (OS) at room temperature, nor touch the tube wall for a long time.

7. Take the PCR tube out of the thermal cycler when the temperature reaches 4 °C. Centrifuge the tube briefly by using the mini centrifuge for 5 seconds, place it on the ice box, and add the solution according to the table below into the tube (with a total volume of 42 µL):

Component	Volume
Make DNB Enzyme Mix I (OS)	20 µL
Make DNB Enzyme Mix II (OS)	2 µL

8. Mix the reaction mixture thoroughly by using the vortex mixer and centrifuge it by using the mini centrifuge for 5 seconds. Place it into the thermal cycler and start the reaction according to conditions listed in the table below:

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

CAUTION

When conducting the reaction, some sample blocks of thermal cyclers may remain at ambient temperatures while the lid is being heated or cooled. For these types of thermal cyclers, pre-heating of the heated lid is required to ensure that the heated lid is at operating temperature (35 ℃) during the DNB reaction.

9. Immediately add 10 µL Stop DNB Reaction Buffer when the temperature reaches 4 ℃ . Mix the mixture gently by pipetting it 8 times with a wide-bore tip. Store DNBs at 4 ℃ and perform sequencing within 48 hours.

CAUTION

Use the wide-bore tip to gently pipette the DNBs. Do not centrifuge, vortex, pipette vigorously, or shake the tube.

10. Quantify the DNBs.

    Take 2 µL DNBs, and use the Qubit ssDNA Assay Kit and Qubit 3.0 Fluorometer to quantify the DNBs. The concentration should be greater than or equal to 8 ng/µL. If the concentration is higher than 40 ng/µL, dilute the DNBs with Low TE Buffer until its concentration reaches 20 ng/µL. Store the qualified DNBs at 4 ℃ and use the DNBs within 48 hours.

Using DNBSEQ-E25RS

1. Take out the sample library (ensure that the sample library has been mixed according to *Library pooling for 16SV4 primer pool on page 9*), and take out Balanced Library from the storage of Standard Library Reagent (PCR product) V4.0.
2. Take out Low TE Buffer, Make DNB Buffer (OS-V2.0-DB), DNB Enzyme Mix I (OS), and Stop DNB Reaction Buffer from the storage of DNBSEQ-E25RS High-throughput Sequencing kit (FCL PE150) and place them on the ice box for 30 minutes until use.
3. Thaw the reagents and mix them thoroughly by using the vortex mixer for 5 seconds, centrifuge them briefly, and place them on the ice box until use.
4. Take out two new PCR tube, separately add 25 ng dsDNA library and 25 ng Balanced Library into the two tubes, and then add Low TE Buffer into the two tubes until the total volume of the mixture in the two tubes reaches 20 µL.

CAUTION

Make DNBs separately by using the sample library and Balanced Library. Do not add the two libraries into the same PCR tube.

5. Prepare the Make DNB reaction mixture 1 on the ice box according to the following table:

Component	Volume
Make DNB Buffer (OS-V2.0-DB)	20 µL
Sample library or Balanced Library	20 µL

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6. Mix Make DNB reaction mixture 1 thoroughly by using the vortex mixer and centrifuge it by using the mini centrifuge for 5 seconds. Place it into the thermal cycler, and start the primer hybridization reaction according to conditions listed in the table below:

Temperature	Time
Heated lid (104 ℃)	On
95 ℃	3 min
57 ℃	3 min
4 ℃	Hold

7. Take out Make DNB Enzyme Mix II (OS) from storage, centrifuge it briefly for 5 seconds, and place it on the ice box until use.

⚠ CAUTION

Neither place Make DNB Enzyme Mix II (OS) at room temperature, nor touch the tube wall for a long time.

8. Take the PCR tube out of the thermal cycler when the temperature reaches 4 ℃. Centrifuge it briefly by using the mini centrifuge for 5 seconds, place it on the ice box, and add the solution according to the table below into the tube (with a total volume of 84 μL):

Component	Volume
Make DNB Enzyme Mix I (OS)	40 μL
Make DNB Enzyme Mix II (OS)	4 μL

9. Mix the reaction mixture by using the vortex and centrifuge it by using the mini centrifuge for 5 seconds. Place it into the thermal cycler and start the reaction according to conditions listed in the table below:

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

⚠ CAUTION

When conducting the reaction, some sample blocks of thermal cyclers may remain at ambient temperatures while the lid is being heated or cooled. For these types of thermal cyclers, pre-heating of the heated lid is required to ensure that the heated lid is at operating temperature (35 ℃) during the DNB reactions.

10. Add 20 μL Stop DNB Reaction Buffer to the tube when the temperature reaches 4 ℃ , and use a wide-bore tip to pipette up and down 5 to 8 times to mix the reagent gently.

⚠ CAUTION

Use the wide-bore tip to gently pipette the DNBs. Do not centrifuge, vortex, pipette vigorously, or shake the tube.

11. Quantify the DNBs.

Take 2 μL DNBs, and use the Qubit ssDNA Assay Kit and Qubit 3.0 Fluorometer to quantify the DNBs. The concentration should be 4 ng/μL to 40 ng/μL. If it is less than 4 ng/μL or more than 40 ng/μL, remake the DNBs. Store the qualified DNBs at 4 ℃ and use the DNBs within 48 hours.

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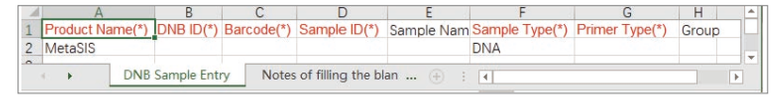
Sequencing and bioinformatics analysis

Creating a sequencing + analysis task and importing the DNB ID

Tips

- If you use the bioinformatics server for automated analysis, ensure that the sequencer is connected to the bioinformatics server before starting a sequencing run.
- If you use the DNBSEQ-E25RS genetic sequencer for sequencing, start the analysis server before you log in to MGI ZLIMS. For details, refer to *DNBSEQ-E25RS High-throughput Sequencing Set Instructions for Use* .
- When using the DNBSEQ-G99ARS genetic sequencer for sequencing, open ZLIMS Lite by tapping ZLIMS in the main interface of the control software. For detailed operations, refer to the relevant instructions.

1. Launch the Chrome browser, type the following IP address in the address bar, and press **Enter** :
   127.0.0.1
2. Input the authorized username *lite* and password *lite123456* , and click **Login** to open the ZLIMS Lite Home page.
3. Click **Sequencing + Analysis** on the Home page. The New Sequencing + Analysis page is displayed.
4. Select **MetaSIS** as the analysis product. Choose **Import the Sample ID** , and click **New** . The Import Sequencing + Analysis dialog box is displayed.
5. Click **Excel template** or **CSV template** to download the DNB sample template in *.xlsx* or *.csv* format.
6. Fill in the DNB sample template.
   The template is shown below:



   Tips for filling in the template are as follows:

   - A field with a red asterisk (*) is required, and other fields are optional.
   - Cells in the Excel template cannot be merged, and no spaces or special characters should be input in each cell.
   - Some fields include drop-down options, and other fields require manual input. The inputting method varies among the fields.
   - **DNB ID** : The DNB ID should include both letters and numbers. It cannot be the same as the DNB ID that is already in ZLIMS Lite.
   - **Barcode** : When one sample ID corresponds to multiple barcodes, the multiple barcodes are separated by half-width commas (,). For multiple consecutive barcodes, a tilde (~) can be used.
   - **Sample ID** : The sample ID should include both letters and numbers. It should be unique.
   - **Sample Name** : The sample name should also be unique.
   - **Sample Type** : Only **DNA** can be selected.

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- **Primer Type** : The primer type should be one or a combination of the following: 16SV3V4, 16SV4, ITS, and ITS2. It is case-insensitive but should be correct. For example, ITS starts with the letter I/i, not the letter L/l or the number 1. Multiple primer types can be separated by half-width semicolons (;) in any order, but should not be repeated. For example, you can fill in **16SV3V4;ITS** , but cannot fill in **16SV3V4;ITS;16SV3V4** . The primer type and the corresponding reagent is shown as follows:

Name	Primer type
ATOPlex 16SV3V4 rDNA Panel	16SV3V4
ATOPlex 16SV4 rDNA Panel	16SV4
ATOPlex ITS1 rDNA Panel	ITS
ATOPlex ITS2 rDNA Panel	ITS2

- **Group** : Set the group information as need. Same information should be input for the samples in the same group (with a minimum of 3 samples per group). For example, if a batch is divided into three groups, you can fill in **A,B,C** . If there is no grouping, leave it blank or input **NA** .

7. Return to the Import Sequencing + Analysis dialog box, click **Choose File** , and select the completed DNB sample template.
8. Click **Upload** . The prompt **Import successfully** indicates that the multiple parts of sample information have been successfully imported into ZLIMS Lite.
9. After inputting the DNB sample information is completed, the New Sequencing + Analysis page returns to view. Click **Save** in the lower-right corner. The New Task page is displayed, and click **OK** .

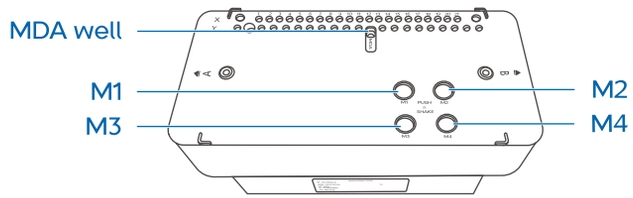
(Optional) Performing sequencing on DNBSEQ-G99ARS sequencer

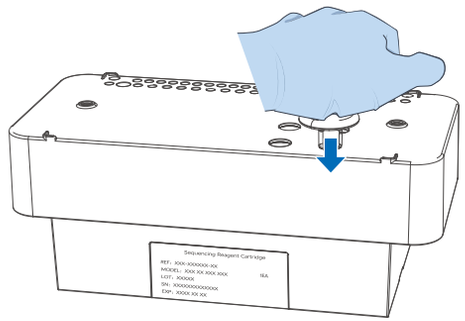
Preparing the flow cell and the sequencing cartridge

1. Take the flow cell box out of the sequencing set stored at -25 ℃ to -15 ℃ refrigerator.
   Do not open the outer plastic package at this moment.
2. Place the flow cell at room temperature for 0.5 to 24 hours.
3. Take the sequencing cartridge out of the storage. Thaw the cartridge in a water bath at room temperature or in a 2 ℃ to 8 ℃ refrigerator a day in advance. Store the thawed cartridge in a 2 ℃ to 8 ℃ refrigerator until use. The thawing time is listed in the following table:

Model	Method		
	Water bath at room temperature (hours)	Refrigerator at 2 ℃ to 8℃ overnight then water bath at room temperature (hours)	Refrigerator at 2 ℃ to 8 ℃ (hours)
G99 SM FCL PE150	3.0	0.5	24.0
G99 SM FCL PE300	4.5	0.5	24.0

4. Shake the cartridge vertically and horizontally for 5 times to mix it before use. Open the package and wipe any water condensation on the cartridge cover and wells with a Kimwipes™ tissue.
5. Use the FATA-Puncher to pierce the M1, M2, M3, and M4 wells of the cartridge with the pre-mixed reagents.





6. Hold the A and B sides of the cartridge with both hands by following the marks A and B on the cartridge, and shake it vertically and horizontally for 10 to 20 times to mix the reagents thoroughly.
7. Pierce the seal of MDA well (marked on the cartridge) by using a clean 1 mL pipette tip.
8. Take out MDA Reagent and MDA Enzyme Mix, and add 125 µL MDA Enzyme Mix to the MDA reagent tube by using a 200 µL pipette.
9. Shake the tube vertically and horizontally for 4 to 6 times to mix the reagents.
10. Add all the mixture to MDA well. The cartridge of PE sequencing now is ready for use.

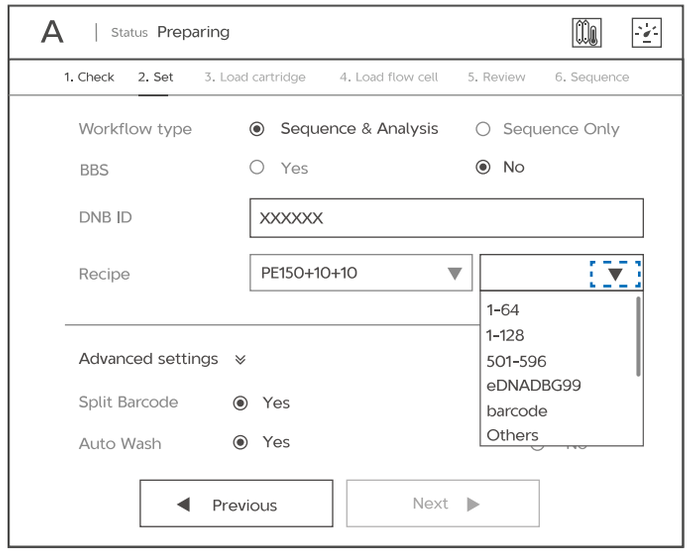
Tips

When adding the MDA mixture, keep the pipette tip close to the concave side of the MDA well, tilt it and avoid generating bubbles. Transfer the mixture carefully to prevent the mixture from spilling out of other wells.

Starting a sequencing run

1. Ensure that the power switch is in the ◯ position. Connect the device to the power supply through the power cord. Turn the power switch of the device to the | position. After you power on the device, the login interface is displayed.
2. Select the user name, after you input the password, the device starts to check. If the check is successful, the main interface is displayed. If the check fails, troubleshoot the failure according to *DNBSEQ-G99RS & DNBSEQ-G99ARS Genetic Sequencer User Manual* .
3. Tap (👤) in the upper-right corner of the main interface. Input the user name *user* and password *123* and tap **Log in** to log in to the control software. The main interface is displayed.
4. Select **Sequence** in operation A or B in the main interface according to your requirements. If both A and B are required, tap **Sequence A&B** .
5. Tap **Sequence** , and perform one of the following operations:
   - If the waste container compartment door opens automatically, place an empty waste container into the compartment according to the on-screen prompts, and gently press the compartment door to close it. The system automatically starts the check before sequencing.
   - If the waste container compartment door does not open, the system automatically starts to check.

6. After the check is completed, tap **Next** to enter the sequencing parameters. Select **Sequence** & **Transmission** as the workflow and **No** in the **BBS** box by default. Input the DNB ID in the **DNB ID** box (Ensure that the DNB ID is consistent with that of Sequencing + Analysis task created on ZLIMS Lite).



7. Select **PE300+10+10** in the drop-down list of **Recipe** for 16S V3V4, ITS1, or ITS2 primer pool. Select **PE150+10+10** in the drop-down list of **Recipe** for 16SV4 primer pool.
8. Tap the drop-down list next to the **Recipe** box and select **eDNADBG99** . For details of importing the barcode, refer to *DNBSEQ-G99 Series Genetic Sequencer Software Guide* . Select **Yes** for **Split Barcode** and **Auto Wash** .
9. Tap **Next** and wait for auto-sliding screen moving up. Push the prepared sequencing reagent cartridge into the reagent compartment. The RFID (Radio Frequency Identification) scanner will automatically identify the sequencing cartridge ID. Enter the cartridge ID after **SN** on the cartridge manually if the RFID scanner fails to identify the ID.
10. Tap **Prime** > **Yes** to start priming. The screen will display priming progress.

Loading DNBs

Tips

- Prepare a fresh DNB loading mixture immediately before the sequencing run.
- Each flow cell requires 10 µL DNB loading mixture.
- Do not centrifuge, vortex, or shake the tube. Use wide bore, non-filtered tip only when mixing the mixture.

1. Prepare the DNBs of the sample library and DNBs of Balanced Library.
2. Mix the DNBs of the sample library and DNBs of Balanced Library in a mass ratio of 4:1. Mix the components gently by pipetting 5 to 8 times with a wide-bore pipette tip.
3. Take DNB Load Buffer II out of the storage and thaw the reagents on the ice box for approximately 30 minutes. After thawing, mix the reagents thoroughly by using the vortex mixer for 5 seconds, centrifuge briefly, and place it on the ice box until use.
4. Take out a new 0.2 mL tube from the sequencing set and add the following reagents to prepare the DNB loading mixture:

Component	Volume
DNB Load Buffer II	7.0 µL
Make DNB Enzyme Mix II (LC)	1.0 µL

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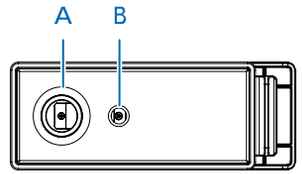
Component	Volume
DNB	21.0 µL
Total volume	29.0 µL

5. Mix the mixture by gently pipetting 5 to 8 times with a wide-bore, nonfiltered pipette tip. Place the mixture at 4 °C until use.
6. Take the flow cell out of the outer package and ensure that the flow cell is intact.
7. Take the loader out of the box, open the loader, press the bottom of the loader to remove the gasket ring, and put the gasket ring in to the box. Hold the loader with one hand and open the cover with the other hand.
8. Place the flow cell into the loader and ensure that the QR code is facing up, and close the cover. Place the loader on the laboratory bench with the back facing up. Aspirate 10 µL of DNB loading mixture by using non-filtered tip, and vertically insert the tip into inlet A as shown in the following figure:



9. Fix the tip with one hand, press the tip ejector on the pipette to unload the tip with the other hand, and observe the liquid level in the tip.
   If the liquid level drops automatically, the DNB loading mixture will automatically flow into the flow cell, and skip to step 11. If the liquid level does not drop, continue to perform step 10.
10. (Optional) If the liquid level does not drop, perform the following steps:
    1) Leave the tip with DNB loading mixture in inlet A.
    2) Attach a new 200 µL non-filtered sharp tip and adjust the aspirate volume to 2 µL.
    3) Hold the new empty tip with one hand and gently insert it into outlet B in step 9 while pressing the button down with the other hand.
    4) Gently release the button and the liquid level of the tip at outlet A drops. When the liquid level in the sharp tip stops dropping, remove the sharp tip at inlet A.
11. Turn the loader upside down, open the cover, remove the flow cell, and transfer it to the sequencer immediately.

Loading the flow cell, reviewing parameters, and performing a sequencing run

1. When priming is finished, tap **Next** , and the auto-sliding screen will move up to expose the flow cell stage. Insert the flow cell prepared in the previous section into the flow cell compartment. The RFID scanner will automatically identify the flow cell ID.
2. Tap **Next** , the auto-sliding screen will move down and show the reviewing parameters interface. Review the information entered.
3. After confirming that the information is correct, tap **Sequence** > **Yes** start sequencing. The auto-sliding screen will move down, and the sequencing interface is displayed.
4. Tap **Finish** after sequencing is completed and the auto-sliding screen will move up. Take out the flow cell, sequencing reagent cartridge, and waste container according to the prompt.

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5. Empty the waste container, put the waste container back into the waste compartment, and close the compartment door. Tap **Return home** .

Tips

The screen of the sequencer displays the sequence phase and step in real-time, or you can monitor and view the phase of the sample from ZLIMS Lite.

If **Auto wash** is selected when setting the sequencing parameters, the device starts an automatic wash after the sequencing is completed. For details, refer to *DNBSEQ-G99RS High-throughput Sequencing Set User Manual* .

(Optional) Performing sequencing on DNBSEQ-E25RS

Preparing the flow cell and the reagent cartridge

1. Take the flow cell box out of the storage and remove the flow cell from the box.
2. Unwrap the outer plastic package before use and use the flow cell within 24 hours.
3. Take out the reagent cartridge from the storage of DNBSEQ-E25RS High-throughput Sequencing Set (FCL PE150) and place it upright with the label facing upwards. Thaw the reagent cartridge at room temperature from 15 ℃ to 25 ℃ for 4.5 hours to 5 hours or in refrigerator at 2 ℃ to 8 ℃ for 10 hours. Take out Signal Protein Buffer from the sequencing set and thaw Signal Protein Buffer in the refrigerator at 2 ℃ to 8 ℃.
4. Thaw Signal Protein 1 and Signal Protein 2 by placing them on the ice box for about 10 minutes. Take out MDA T-Reagent and MDA Enzyme Mix and place them on the ice box until use.
5. After thawing, check whether there is ice in the cartridge by shaking the cartridge. If there is sound of cracked ice, place the reagent cartridge at room temperature until no ice exists and use Kimwipes tissue to remove condensation from the surface of the reagent cartridge.
6. Hold the two sides of the cartridge with two hands. Invert it 20 times and gently tap it on a flat surface 10 times. Invert it 10 times and gently tap it on a flat surface 10 times again. Hold the reagent cartridge upright and swing downward 10 times. Cut the outer packaging and remove it.
7. Use the vortex mixer to mix the Signal Protein 1 and Signal Protein 2 for 5 seconds. Centrifuge them briefly for 5 seconds and place them on the ice box until use. According to the following table, add an appropriate amount of Signal Protein 1 and Signal Protein 2 to the tube containing the Signal Protein Buffer to make the Signal Protein Mixture.

Component	Volume
Signal Protein 1	31.5 µL
Signal Protein 2	21 µL
Signal Protein Buffer	21 mL

8. Screw the cover of the tube containing the Signal Protein Mixture and invert it 10 to 15 times to mix thoroughly. To avoid bubble formation, do not shake the mixture vigorously.
9. Place the funnel over the MSP well and add the Signal Protein Mixture into the MSP well.
10. Take out MDA T-Reagent and MDA Enzyme Mix.
11. Invert the MDA Enzyme Mix to mix it and then centrifuge it briefly.

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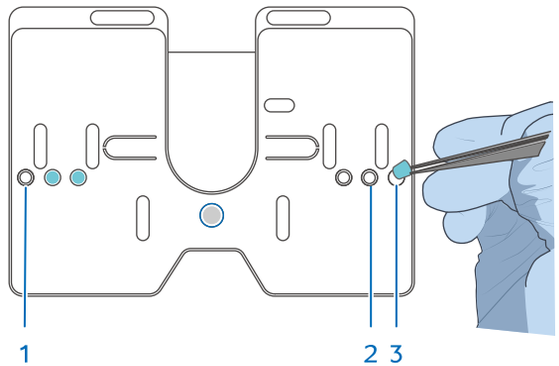
12. Transfer 50 µL MDA Enzyme Mix to the tube containing MDA T-Reagent to make MDA mixture. Pipette the reagent 10 to 15 times to mix it without shaking vigorously to prevent bubble formation.
13. Use a clean tip to pierce the MDA well and transfer the MDA mixture into the MDA well. Do not shake it vigorously in the process to prevent bubble formation.

Tips

The reagent cartridge with the MDA mixture should be loaded as soon as possible. Failure to do so might affect the sequencing quality.

14. Use the pointed-tip tweezers to remove the stoppers in wells No.1, No.2, and No.3.



Starting a sequencing run

1. Connect one end of the power cord to the power port on the device and the other end to the main supply.
2. Connect one end of the power cord to the power port on the computing module and the other end to the main supply.
3. Connect the main unit to the computing module with a network cable.
4. (Optional) If a UPS is prepared, connect one end of the UPS power cord to the device and the other end to the main supply.
5. Turn the power switch of the device to the | position.
6. Power on the computing module. The status indicators light up.
7. Ensure that the main unit and computing module are connected and the network cable between the main unit and the computing module is available.
8. Power on the genetic sequencer, and the login interface is displayed. Enter the username *User* and password *123* , and tap **Log in** .
9. Tap to open the customization interface. When opening the customization interface, the reagent compartment starts initialization. The compartment door opens automatically and the rack slides out.
10. Tap the **Recipe** list and select **PE150** . Tap Barcode list and select **eDNADBE25** . For details of Barcode import, refer to *DNBSEQ-E25RS & DNBSEQ-E25ARS User Manual* .
11. Ensure that the required recipe is selected, and tap to proceed to the next step.

Loading the flow cell, reagent cartridge, and waste container

1. Enter information for the **Flow cell ID** , **Throughput** , and **Expiration date** boxes on the left manually or by scanning the QR code on the plastic package of the sequencing flow cell.
2. Unwrap the package of the flow cell to check whether the flow cell is intact and whether the scanned ID is the same as the ID on the flow cell.

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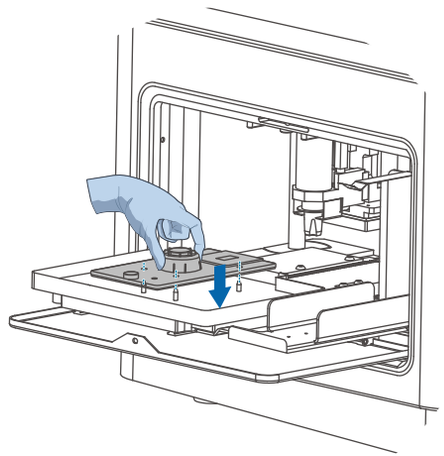
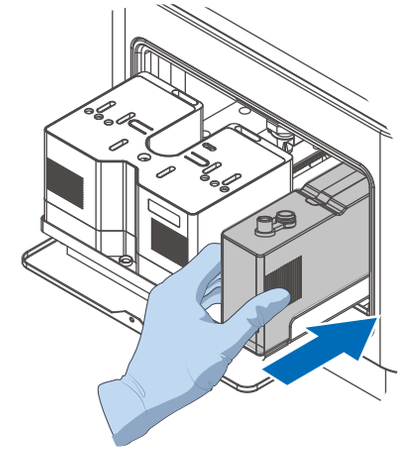
3. After the rack pops up, pinch the rotary valve in the flow cell and align the wells in the flow cell correspondingly with the positioning columns on the rack to install the flow cell onto the rack.



4. Take out the prepared sequencing cartridge. Enter the parameters manually or by scanning the QR code on the cartridge label.
5. Slowly and carefully remove the bottom cover in the middle of the reagent cartridge and ensure that 21 rubber stoppers are present in the wells on the bottom side of the reagent cartridge.
6. Align the reagent cartridge with the positioning columns on the rack and place it over the flow cell. Keep the reagent cartridge horizontal in the process.
7. Ensure that the cover of the waste container is open, place the waste container on the rack according to the direction shown in the figure below, and ensure that it fits into the bent metal clip.



8. After placement, tap > **Yes** . The rack automatically retracts into the compartment and the device presses the components tightly. Tap to proceed to the next step.

Loading DNBs

Tips

- Prepare a fresh DNB loading mixture immediately before the sequencing run.
- Do not centrifuge, vortex, or shake the tube. Use a wide bore, non-filtered tip only when mixing the mixture.

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1. Enter information for the **DNB ID** box by scanning the QR code on the sample tube. Enter the parameters manually by using the on-screen keyboard if the scanner fails to recognize the ID (ensure that the DNB ID is consistent with that of Sequencing + Analysis task created on ZLIMS Lite).
2. Take out DNB Load Buffer II and thaw it on the ice box for approximately 30 minutes.

Tips

If crystal precipitation is visible in DNB Load Buffer II, vigorously mix the reagent for 1 or 2 minutes using the vortex mixer to re-dissolve the precipitation before use.

3. Mix the DNBs of the sample library prepared in the previous section and the DNBs of Balanced Library in a mass ratio of 2:1. Mix the mixture gently by pipetting 5 to 8 times with a wide-bore pipette tip.
4. Take out a new 0.2 mL centrifuge tube and add the following reagents:

Component	Volume
DNB Load Buffer II	34 µL
DNB	102 µL
Total volume	136 µL

5. Use a wide-bore, non-filtered pipette tip to pipette up and down 5 to 8 times to mix the DNB loading mixture gently.
6. Add all the DNB loading mixture into the DNB loading well by using a wide-bore pipette tip. Proceed to the next step when no bubbles exist in the well.
7. Push the compartment door back to close it. Tap ⟩ to proceed to the next step.

Reviewing parameters

1. Check all the information. Ensure that the computing module is connected and the compartment door is closed. Tap Run > **Yes** to start sequencing.

Tips

If the information is incorrect, tap ‹ to return to the previous interface to modify the information. Returning to the customization interface and tapping ‹ to cancel sequencing will cause the loaded reagent cartridge and flow cell to become inoperative.

2. After the sequencing is completed, remove the flow cell, reagent cartridge and waste container, and place them into the designated waste bin.

Tips

- The screen of the sequencer displays the sequence phase and step in real-time, or you can monitor and view the phase of the sample from ZLIMS Lite.
- For details of the removing the flow cell and reagent cartridge, refer to the user manual of the corresponding reagent kit and *Environmental DNA Metabarcoding Sequencing Package User Manual* .

After the sequencing is completed, the device starts to wash automatically.

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Performing an analysis by using MetaSIS

After the sequencing is completed, the MetaSIS software automatically starts the analysis.

You can tap any number in the **Task Status** area or tap **Task** on the left navigation bar on the Home page of ZLIMS to access the Task page and view the status and task progress.

Viewing the analysis report

1. Tap the number under **Report Today** on the Home page. The Analysis Report page is displayed.
   - The number **0** indicates that no report is generated on the current date, and the Analysis Report page displays all reports generated in ZLIMS Lite.
   - A number greater than 0 indicates the number of reports generated on the current date, and the Analysis Report page displays only these reports by default.
2. In the **Query** area, input a query term (for example, enter the sample ID of the target sample), and click to search for the to-be-viewed report.
3. Click in the **Report** column, or click **Analysis** in the **Analysis Type** column, and then click on the pop-up page. The report summary page is displayed.
4. Click **View Report** in the **Report Links** column to view the analysis report corresponding to each primer type.

Downloading the analysis report

1. In the **Query** area of the Analysis Report page, input the sample ID of the target sample, and click to search for the record row of the target sample.
2. Click in the **Result Path** column to go to the analysis result directory.
3. Click **Result** to open the **Result** folder.
4. Click the **.tar.gz* file to download to the local directory the compression package that includes all analysis results of the target sample.

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