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Amplifying RT-PCR manually

Sample requirements

This package is applicable to the total RNA extracted from multiple sample types, including influenza strains, swabs and so on.

It is recommended to use the high-quality total RNA sample with its Ct value being no greater than 32, and set the initial input to 10 μL.

Consumables information

- 0.2 mL PCR tube
- Ice box
- Pipettes and applicable filtered tips

Performing the RT-PCR amplification process

1. Take out the following reagents from the MGIEasy Respiratory Microorganisms Genome Amplification Kit. Prepare RT-PCR reaction mixture on ice, mix it thoroughly and transfer it to a new 0.2 mL PCR tube.

RT-PCR reaction mixture	
Reagent	**Volume (μL)**
Flu A/B Primer Pool	2
Flu Control Primer	2
RT-PCR buffer	25
RT-PCR Enzyme Mix	2.5
Nuclease-Free water	8.5
Total volume	40

2. Transfer 10 μL of RNA sample into the PCR tube and the total volume is 50 μL.
3. Mix the mixture by pipetting for 10 times and centrifuge it briefly to collect the solution to the bottom of the tube.

Tips

- Do not vortex the mixture.
- It is recommended to add a negative control sample to each batch of experiments. Add 10 μL of nuclease-free water into a single-tube reaction to replace RNA sample. The negative control can be involved in the whole process to evaluate whether there is a risk of cross-contamination during each experiment. If the negative control in the analysis report is negative for the influenza virus, the risk of cross-contamination is low. Otherwise, the risk of cross-contamination is high.

4. Place the PCR tube into the thermal cycler for next reaction according to the following conditions.

Temperature	Time	Cycle
Heated lid (105 °C)	On	1
45 °C	30 min	
55 °C	15 min	
95 °C	3 min	
95 °C	30 s	5
55 °C	30 s	
68 °C	3 min	
95 °C	30 s	38
64 °C	30 s	
68 °C	3 min	
68 °C	5 min	1
12 °C	Hold	

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Tips

- Set the number of amplification cycles according to the Ct value of sample. If the Ct value of sample is greater than 20 but less than or equal to 32, it is recommended to use 38 cycles.
- Perform RT-PCR amplification reaction in the post-PCR area.

Purifying the RT-PCR product

Preparing the device

Tips

- It is recommended to perform automation with MGISP-100RS of V1.0 and above.
- Before using the device, ensure that the application scripts and PCR program have been imported.

1. Start the computer and MGISP-100RS. Double-click the icon of MGISP-100RS on the desktop to run the software.
2. Select **Real**, click **Create**, and click **User Entry** to enter the initialization interface.
3. Click **Initialize** to start initialization.

   After successful initialization, a prompt will appear on the interface.
4. Perform a pre-clean according to the following steps:
   ① Click **Pre-post clean** > **Pre-clean** > **Start**.
   ② Follow the prompts to complete operations. Click **Continue**.

Preparing the consumables

Prepare the following consumables and reagents for automation before the experiment:

- 250 µL automated filtered tips
- 1.3 mL 96-well deep-well plate
- Break-away PCR 8-strip tubes and the caps (hereafter referred to as 8-strip tubes)
- 2 mL SC micro tube, PCR-PT
- MGIEasy DNA Clean Beads
- Absolute ethanol
- Milli-Q water

Preparing the samples

Transfer 50 µL of RT-PCR product into a new 8-strip tube. Ensure that no bubbles exist at the bottom of the tube, and no liquid remains on the tube wall. Place the product on ice until use.

Tips

- One 8-strip tube is needed for 8 samples and two tubes for 16 samples.
- If the number of samples is less than 8 or 16, fill the empty wells with Milli-Q water and prepare reagents and consumables for 8 or 16 samples.

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Preparing the reagents

1. Take out the following reagents from the MGIEasy DNA Clean Beads Kit. Vortex and mix them thoroughly.

Reagent	Volume (μL)	
	8 RXN	16 RXN
DNA Clean Beads	380	660
TE Buffer	500	900

Tips

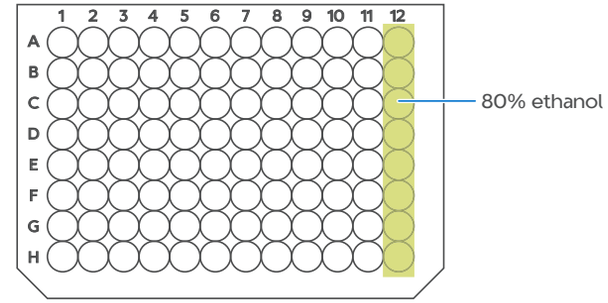
Take out and place the DNA Clean Beads at room temperature for 30 minutes in advance. Vortex and mix it thoroughly before use.

3. Transfer DNA beads and TE Buffer into two different 2 mL SC micro tubes according to the table above. Then close the tube caps and mark them as "DNA Beads1" and "TE" respectively.
4. Use absolute ethanol and Milli-Q water to prepare 25 mL of 80% ethanol.

Tips

Use the prepared 80% ethanol immediately after preparation.

5. Take out a 96-well deep-well plate. Add reagents according to the following figure.
   - 8 RXN: 450 μL/well
   - 16 RXN: 750 μL/well



Performing the RT-PCR purification process

1. Configure **Solution** and **Script** in the Run Wizard interface of MGISP-100RS according to the following table:

Solution	JB-A06-087 MGI Respiratory Microorganisms Genome Sequencing Package_RV1.0_SV1.0
Script	1.ATOPlex_RNA_RespMulti_PCR_Purification_step1.py

Tips

Before using the device, ensure that the application scripts and PCR program have been imported.

2. Thoroughly mix the DNA Clean Beads with a vortex mixer and centrifuge them briefly before placing reagents and consumables.

Tips

Ensure that no bubbles exist at the bottom of the tube, no liquid remains on the tube wall, and that all tube caps are open.

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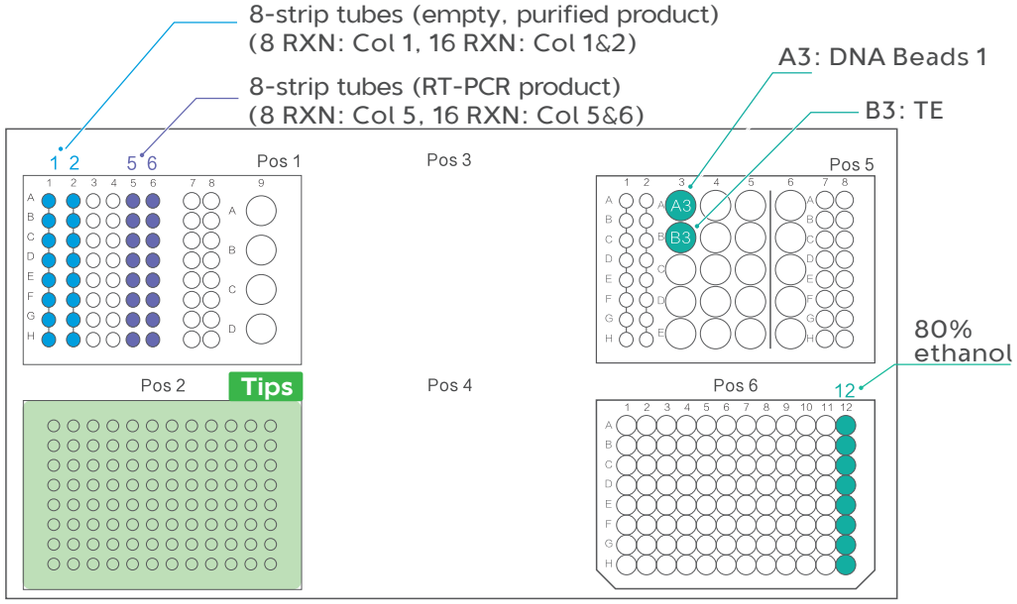
3. Place the consumables according to the figure below.



4. Confirm the placement and close the door. Click **Run**, select sample number **8** or **16** by following the prompt, and click **Continue**.
5. After purification, follow the prompts to take out the purified RT-PCR products from Pos1. The volume of the product is 30 μL.
6. Click **Continue** to end the process.
7. Quantify the products according to the instructions of Qubit® dsDNA HS Assay Kit or Quant-iT™ PicoGreen™ dsDNA Assay Kit. The required concentration should be no less than 5 ng/μL.

Tips

This concentration requirement is not applicable to the purified product of the negative control sample.

Stop point

The purified RT-PCR products can be stored at -20 ℃ and should be used within 2 weeks.

8. Dispose of the used sample tubes, PCR plates, deep-well plates, and waste bags to the designated waste area.
9. (Optional) If no experiment is to be conducted on the day, use laboratory-grade water and 75% ethanol to clean the surface of the device and perform a post-clean.

Preparing Fast PCR-FREE library

Preparing the reagent kits

Prepare the reagent kits according to the following table:

Reagent kit	Component
MGIEasy Fast PCR-FREE FS Library Prep Module	20× Elute Enhancer
	Fast FS Buffer
	Fast FS Enzyme
	Ligation Enhancer
	Fast Ligation Buffer
	Ad Ligase
MGIEasy DNA Clean Beads	TE Buffer
	DNA Clean Beads
MGIEasy UDB PF Adapter Kit	UDB Adapters

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Preparing the samples

1. The recommended initial library preparation input for each sample is 215 ng. Add TE Buffer to bring the final volume to 48 μL.
   - If the volume of purified product ranges from 150 ng to 215 ng, use all the product for library preparation and add TE buffer for a total volume of 48 μL.
   - If the purified product volume in the negative control sample is less than 150 ng, use all the product and add TE buffer for a total volume of 48 μL.
2. Respectively transfer 48 μL of each sample into the prepared 8-strip tubes. Ensure that no bubbles exist at the bottom of the tube, and no liquid remains on the tube wall. Place them on ice for further use.

Tips

- One 8-strip tube is needed for 8 samples and two tubes for 16 samples.
- If the number of samples is less than 8 or 16, fill the empty wells with Milli-Q water and prepare reagents and consumables for 8 or 16 samples.

Preparing the reagents

1. Prepare 1× Elute Enhancer, En-TE and EN-Beads. All reagents can be used within 7 days.

Reagent	Component	Volume (μL)	Storage condition
1× Elute Enhancer	20× Elute Enhancer	1.5	Room temperature
	Nuclease-Free Water	28.5	
	Total volume	30	
En-TE	1× Elute Enhancer	5.4	4 ℃
	TE Buffer	2694.6	
	Total volume	2700	
En-Beads	1× Elute Enhancer	20	4 ℃
	DNA Clean Beads	1980	
	Total volume	2000	

2. Mix the EN-Beads thoroughly. Transfer it into a 2.0 mL SC micro tube. Close the tube cap and mark it as "DNA Beads2".

Reagent	Volume (μL)	
	8 RXN	16 RXN
En-Beads	950	1800

3. Take out the following reagents, thaw them and mix the reagents thoroughly. Centrifuge shortly and place them on ice until use. Take out five (8 RXN) or seven (16 or 96 RXN) 0.5 mL SC micro tubes and dispense library prep reagents into them.

Reagent	Volume (μL)	
	8 RXN	16 RXN
Fast FS Buffer	102	200
Fast FS Enzyme	47.5	110
Ligation Enhancer	25	48
Fast Ligation Buffer	220	430
Ad Ligase	45	105

Tips

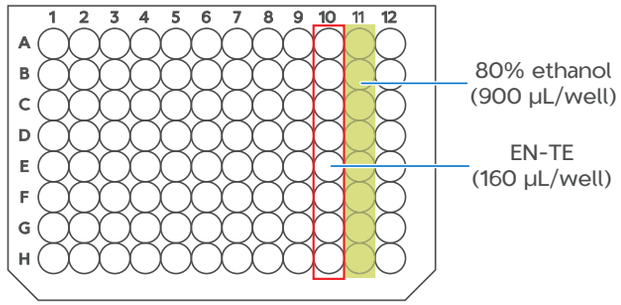
- If you directly purchase the MGIEasy Fast PCR-FREE FS Library Prep Set (16 RXN) for 16 RXN library preparation, you can thaw, mix and centrifuge the reagents in the original tube, and directly use them on the MGISP-100RS without dispensing independently. But 2 additional 0.5 mL SC micro tubes are needed for future use.
- When using 96 RXN reagent kit to perform 16 RXN experiment, 2 tubes are not dispensed with reagents.

4. Use absolute ethanol and Milli-Q water to prepare 25 mL of 80% ethanol.

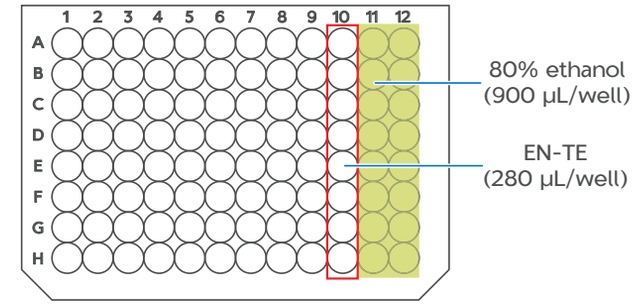
Tips

Use the 80% ethanol immediately after preparation.

5. Take out a 96-well deep-well plate. Add reagents according to the following figure.
   - 8 RXN



   - 16 RXN



6. Take out the adapters from MGIEasy UDB PF Adapter Kit. Mix and centrifuge them thoroughly, then transfer the adapters (10 µL/well) into the 8-strip tube.

Preparing Fast PCR-FREE library

1. Configure the Run Wizard interface of MGISP-100RS according to the following table.

Solution	JB-A06-087 MGI Respiratory Microorganisms Genome Sequencing Package_RV1.0_SV1.0
Script	1.ATOPlex_RNA_RespMulti_PCR_Purification_step1.py

Tips

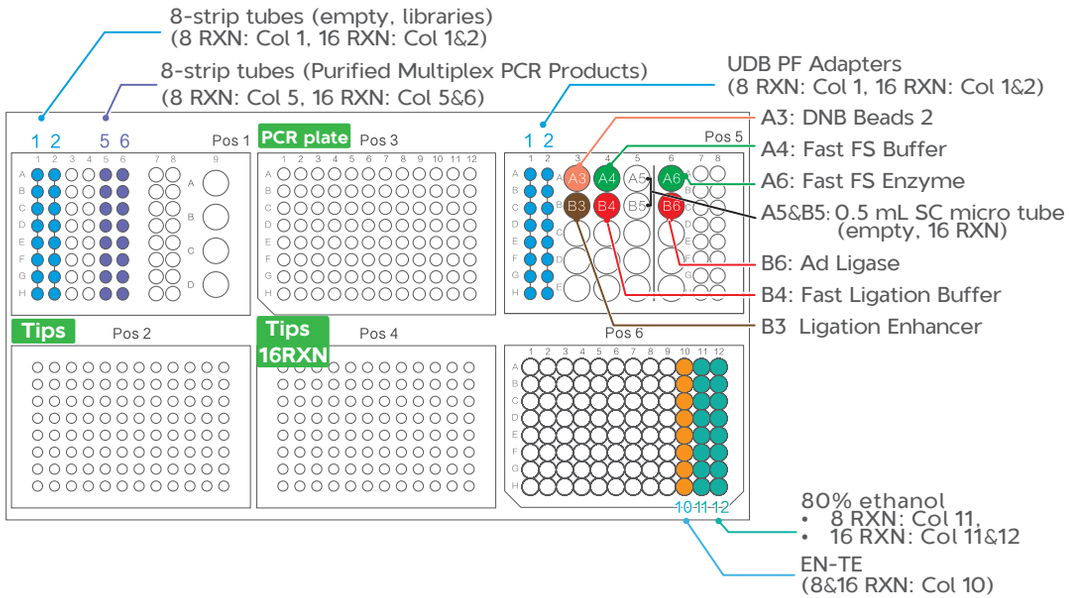
Before using the device, ensure that the application scripts and PCR program have been imported.

2. Thoroughly mix the DNA clean beads with a vortex mixer and centrifuge them briefly before placing reagents and consumables.

Tips

Ensure that no bubbles exist at the bottom of the tube, no liquid remains on the tube wall, and that all tube caps are open.

3. Place the consumables according to the figure below.



4. Confirm the placement, close the door, and click **Run**.
5. After the library preparation, follow the prompts to take out the libraries from Pos1. The volume of the products is 25 μL.
6. Click **Continue** to end the process.
7. Quantify the products according to the instructions of Qubit dsDNA HS Assay Kit or Quant-iT PicoGreen dsDNA Assay Kit. The required concentration should be no less than 0.8 ng/μL.

Tips

This concentration requirement is not applicable to the purified product of the negative control sample.

Stop point

Store the libraries in a -20 ℃ freezer.

8. Dispose of the used sample tubes, PCR plates, deep-well plates, and waste bags to the designated waste area.
9. (Optional) If you will not conduct any experiments on the day, use laboratory-grade water and 75% ethanol to clean the surface of the device and perform a post-clean.

Making DNBs

Tips

- Do not use filtered pipette tips for making and loading DNBs.
- Each kit is sufficient to make DNBs for 4 sequencing runs.

1. Prepare the DNBSEQ-E25RS High-throughput Sequencing Set (FCL PE150) according to the following table.

Reagent kit	Component
DNBSEQ-E25RS High-throughput Sequencing Set (FCL PE150)	Low TE buffer
	Make DNB Buffer (OS-V2.0-DB)
	Make DNB Buffer (OS-V2.0-SB)
	Make DNB Enzyme Mix I (OS)
	Make DNB Enzyme Mix II (OS)
	Stop DNB Reaction Buffer
	DNB Load Buffer II
	Signal Protein 1
	Signal Protein 2
	Signal Protein Buffer

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Reagent kit	Component
DNBSEQ-E25RS High-throughput Sequencing Set (FCL PE150)	MDA T-Reagent
	MDA Enzyme Mix
	Sequencing Reagent Cartridge
	Waste container
	Funnel
DNBSEQ-E25RS Sequencing Flow Cell	Flow Cell

2. Take out Low TE Buffer, Make DNB Buffer (OS-V2.0-DB), Make DNB Enzyme Mix I (OS) and Stop DNB Reaction Buffer from DNBSEQ-E25RS High-throughput Sequencing Set (FCL PE150) and thaw them on ice for 30 min.

Tips

Do not to take Make DNB Buffer (OS-V2.0-SB) by mistake.

3. After thawing, use a vortex mixer to vortex them for 5 s. Centrifuge briefly and place them on ice until use.
4. Take out a 0.2 mL 8-strip tube or PCR tube and prepare the Make DNB reaction mixture 1 according to the following table.

Component	Volume (μL)
Low TE Buffer	20-V
dsDNA library	V
Make DNB Buffer (OS-V2.0-DB)	20
Total volume	40

Tips

dsDNA library input V(μL)=15 ng / mixed library concentration (ng/μL)

5. Mix Make DNB reaction mixture 1 thoroughly by vortexing, centrifuge it for 5 s, and place the tube into a thermal cycler to start reaction according to the following condition.

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

6. Place Make DNB Enzyme Mix II (OS) on ice, centrifuge briefly for 5 s and place it on ice until use.

Tips

- Do not place Make DNB Enzyme Mix II (OS) at room temperature.
- Avoid holding the tube for a long time.

7. Take the tube out of the thermal cycler when the temperature reaches 4 ℃. Centrifuge briefly for 5 s and prepare the Make DNB reaction mixture 2 on ice according to the following table.

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

8. Mix the reagent gently by vortexing, centrifuge for 5 s, place the tube into a thermal cycler, and start the reaction according to the following condition.

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

9. Take the tube out of the thermal cycler when the temperature reaches 4 ℃, add 20 μL of Stop DNB Reaction Buffer to the tube, and use a non-filtered wide-bore tip to pipette up and down 8 times to mix the reagent gently.

Tips

- Do not centrifuge, vortex or pipette vigorously.
- Store DNBs at 2 to 8 ℃ and perform sequencing within 48 hours.

10. Use Qubit ssDNA Assay Kit and Qubit Fluorometer to quantify 2 μL of DNBs. The concentration of DNBs should range from 4 to 40 ng/μL. Otherwise, you need to make DNBs again.

Preparing the reagent cartridge and flow cell

Preparing the reagent cartridge

1. Place the reagent cartridge upright (with label facing upwards), thaw it in a refrigerator at 2 ℃ to 8 ℃ for 12 hours or at 15 ℃ to 25 ℃ for 4.5 to 5 hours.

Tips

Do not thaw the reagent cartridge in a water bath.

2. Thaw Signal Protein Buffer by placing them on ice.
3. Thaw Signal Protein 1 and Signal Protein 2 on ice for about 10 min.
4. After thawing, shake the cartridge to check whether there is ice in it. If there is sound of cracked ice, place the reagent cartridge at room temperature until no ice exists.
5. 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.
6. Hold the reagent cartridge upright and swing downward 10 times and remove the outer packaging.
7. Vortex the Signal Protein 1 and Signal Protein 2 for 5 s. Centrifuge them briefly for 4 s to 5 s and place them on ice until use.
8. Add an appropriate amount of Signal Protein 1 and Signal Protein 2 to the Signal Protein Buffer tube according to the following table.

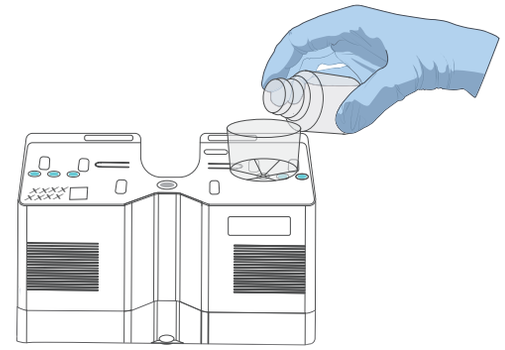
Signal protein mixture	
Component	Volume
Signal Protein 1	31.5 μL
Signal Protein 2	21 μL
Signal Protein Buffer	21 mL

9. Screw the cap of the Signal Protein Mixture tube and invert the tube 10 to 15 times to mix thoroughly.

Tips

To avoid bubble formation, do not vortex the mixture vigorously.

10. Place the reagent cartridge on a flat surface as the following figure. Place the funnel over the MSP well and add the Signal Protein Mixture into the MSP well.



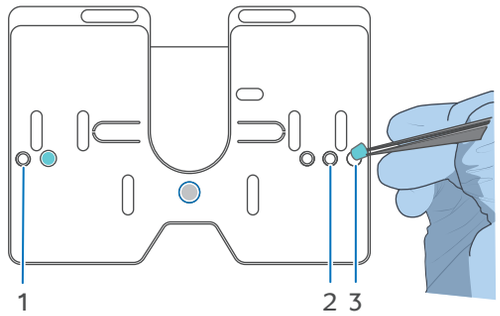
11. Take out MDA T-Reagent and MDA Enzyme Mix. Invert the MDA Enzyme Mix to mix it and then centrifuge it briefly. Transfer 50 μL of MDA Enzyme Mix to the MDA T-Reagent tube and invert it 6 times.

12. Use a clean tip to pierce the MDA well and transfer the MDA mixture into the MDA well.

Tips

The reagent cartridge with the MDA mixture should be loaded within 20 min. Failure to do so might affect the sequencing quality.

13. Use the pointed-tip tweezers to remove the stoppers in wells No. 1, No. 2, and No. 3. Place these stoppers into designated container.



Preparing the flow cell

Take the flow cell box out of storage and remove the flow cell from the box. Unwrap the outer plastic package before use and use it within 24 hours.

Performing sequencing

Inputting samples

Tips

It is recommended to perform sequencing with ZLIMS-Lite V2.1 and above on DNBSEQ-E25 V1.0 and above.

1. Double-click the ZLIMS icon on the desktop, input the username *lite* and password, and select **Login**.
2. Click **Sequencing+Analysis** in the Sample interface, and click **Sample Template** in the pop-up page.
3. Select **MGI_FluTrack** as the analysis product. Select **Import the Sample ID** as the way to input the sample info and click **New**. In the pop-up window, click **Excel template** or **CSV template** to download the DNB sample template in *.xlsx* or *.csv* format.
4. Fill in the work sheet **DNB Sample Entry** in the template file and save the file.

	A	B	C	D	E	F
1	Product Name(*)	DNB ID(*)	Barcode(*)	Sample ID(*)	Sample Name	Sample Type(*)
2	MGI_Flutrack	test2023	UDB-449,UDB-450	sample1		RNA
3	MGI_Flutrack	test2023	UDB-451,UDB-452	sample2		RNA
4	MGI_Flutrack	test2023	UDB-453,UDB-454	sample3		RNA
5	MGI_Flutrack	test2023	UDB-455,UDB-456	sample4		RNA
6						

DNB Sample Entry | Notes of filling the blank

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Tips

- The cells with * are required.
- The cells cannot be merged. Blank spaces or special characters are not allowed.
- Different field types correspond to different entering formats.
- DNB ID generally consists of letter and number, which cannot be repeated with the DNB ID that already exists in the system.
- Sample ID generally consists of letter and number, which can be repeated with the sample ID that already exists in the system.
- If the sample name and sample type are both the same, the data will be merged for analysis.

5. Click **Choose File** to import the prepared file and click **Upload**.
6. Ensure that the uploaded file is correct and click **OK** to save the task.
7. Refresh the Task management interface and view the status of DNB sample.

Starting sequencing

Tips

If you perform sequencing on the DNBSEQ-E25 with this package for the first time, you should import the special TXT Barcode file **MGI_PFAS_PE** in advance, and name it as "PFAS_PE" in the Customization interface.

1. Enter the username and password in the Login interface, and click **Log in**.
2. Click to enter the Customization interface. The compartment door opens automatically and the rack slides out.
3. Click the **Recipe** list and select **PE150**.
4. Select the **Read 1** box and set the read length of read 1 to **100** with the on-screen keyboard.
5. Repeat *Step 4* to configure **Read 2**. Select the **Barcode** list to click **PFAS_PE**.
6. Ensure that the required recipe is selected, and select .
7. Enter flow cell information on the left interface by scanning the QR code on the package of the flow cell or manually with the on-screen keyboard.
8. Open the flow cell package to check whether the flow cell is intact and whether the scanned ID is the same as the ID on the flow cell. After confirming, install the flow cell.
9. Take out the prepared reagent cartridge, and enter reagent cartridge information on the right interface by scanning the QR code on the cartridge or manually with the on-screen keyboard.
10. Remove the bottom cover in the middle of the reagent cartridge, align the reagent cartridge with the positioning columns on the rack, and place it over the flow cell.
11. Ensure that the cover of the waster container is open, place the waste container on the rack, and ensure that it fits into the bent metal clip. Click > **Yes** > .
12. Enter DNB ID information by scanning the QR code on the sample tube or manually with the on-screen keyboard.
13. Prepare the DNB loading mixture:
    ① Vortex to mix DNB Load Buffer II thoroughly for 5 s, centrifuge it briefly, and place it on ice until use.
    ② Prepare DNB loading mixture according to the following table.

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

③ Use a non-filtered, wide-bore tip to pipette up and down 8 times to mix the DNB loading mixture gently.

④ Use a wide-bore tip to transfer all the DNB loading mixture into the DNB loading well. Ensure that no bubbles exist in the tube.

Tips

Use the DNB loading mixture immediately after preparation. Do not centrifuge, vortex or shake the tube.

14. Close the compartment door and click ⟩ to check all the information.
15. If the information is correct, ensure that the computing module is connected. Select Run > **Yes** to start sequencing.

    You can check the sample status in the sample management interface of the ZLIMS system.
16. After sequencing, open the compartment door. Remove the flow cell, the reagent cartridge, and waste container. Click ⟩ to return to the main interface.

Viewing the report

1. In the Task management interface of ZLIMS, click the task name to enter the **Analysis Report** interface.
2. View report details on the report page.

Tips

If you need to download any table or graph in the report, click ☁↓ above the table or graph.