Part No.: H-940-002628-00

High-throughput Methylation Sequencing Set

DNBSEQ-T7RS

Instructions for use

Version: 1.0

Leading Life Science Innovation

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About the user manual

This user manual is applicable to DNBSEQ-T7RS High-throughput Methylation Sequencing Set and DNBSEQ-T7RS Methylation DNB Make and Load Reagent Set. The manual version is 1.0.

Revision history

Version	Date	Description
1.0	December 2024	Initial release

Sequencing set

Cat. No.	Name	Model	Version
940-002625-00	DNBSEQ-T7RS High-throughput Methylation Sequencing Set	T7 Methylation FCL PE150	V1.0
940-002628-00	DNBSEQ-T7RS Methylation DNB Make and Load Reagent Set	T7 Methylation FCL PE150	V1.0
1000005033	Standard Library Reagent	V3.0	/
1000020834	CPAS Barcode Primer 3 Reagent Kit	/	V2.0
1000020832	High-throughput Pair-End Sequencing Primer Kit (App-A)	/	V2.0
1000014047	High-throughput Barcode Primer 3 Reagent Kit (App-A)	/	V1.0
1000028550	High-throughput Pair-End Sequencing Primer Kit (App-D)	/	V2.0

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Contents

Chapter 1 Itroduction

This instructions for use explains how to perform sequencing by using the DNBSEQ-T7RS High-throughput Methylation Sequencing Set & DNBSEQ-T7RS Methylation DNB Make and Load Reagent Set and includes instructions on sample preparation, Flow Cell preparation, sequencing kit storage, the sequencing protocol and device maintenance.

1.1 Applications

This product is a specialized kit used for determining pure methylation libraries or for mixed sequencing of methylation libraries with other non-methylation libraries (such as WGS, RNAseq, etc.) sequencing on DNBSEQ-T7RS.

Tips This sequencing set is intended to be used for scientific research only and cannot be used for clinical diagnosis.

1.2 Sequencing principle

This sequencing set utilizes DNBSEQ technology. A sequencing run starts with the hybridization of a DNA anchor, then a fluorescent probe is attached to the DNA Nanoball (DNB) using combinatorial probe anchor sequencing (cPAS) chemistry. Finally, the high-resolution imaging system captures the fluorescent signal. After digital processing of the optical signal, the sequencer generates high quality and highly accurate sequencing information.

1.3 Sample requirements

This reagent kit , when used with a primer kit, can be used to sequence MGI libraries or App libraries . The MGI library is the library prepared by MGI Library Prep Kits. After being converted from the third-party library by the MGIEasy Universal Library Conversion Kit (Cat. No.: 1000004155) or other MGI Library Conversion Kits, the App library (including TruSeq and Nextera adapter) is applicable to the MGI sequencing platforms.

1.4 Data analysis

During the sequencing run, the control software automatically operates basecalling analysis software and delivers raw sequencing data outputs for secondary analysis.

1.5 Sequencing read length

PE150 cycle run performs reads of 150 cycles (2× 150) for a total of 300 cycles. At the end of the insert sequencing run, one or two extra 10 cycles of barcode read can be performed, if required.

Tips Both read 1 and read 2 need an extra calibration cycle. Barcode does not need calibration. The calibration cycle is generated automatically in the system based on the sequencing read length without the need for specific settings.

Table 1 Sequencing cycle

Sequencing read length	Read1 read length	Read2 read length	Barcode read length	Dual barcode read length	Total read length	Maximum cycles
FCL PE150	150	150	10	10	302+10+10	322

1.6 Sequencing time

Tips
- Sequencing run time for both single flow cell and four flow cells only refer to the time elapsing from the “start” to the “finish” of the sequencing run. The time used for DNB preparation, DNB loading and Write FQ is not included. Write FQ for a single flow cell will take about 1.5 hours.
- Two flow cells can be loaded with DNB concurrently using one MGIDL-T7RS instrument. Total time is about 2 hours.
- Sequencing run time is based on the DNBSEQ-T7RS instrument with standard model, actual sequencing run time could vary among different instruments.

Table 2 Theoretical sequencing time

Sequencing read length	Single flow cell (hours)	Four flow cells (hours)	DNB preparation (hours)	DNB loading (hours)
FCL PE150	21.0~ 23.0	23.0~28.0	1	2

1.7 Precautions and warnings

- This product is for research use only. Please read the instructions for use carefully before use.
- Ensure that you are familiar with the SOP&Attention of all the laboratory apparatus to be used.
- Avoid direct skin and eye contact with any samples or reagents. Don't swallow. Please wash with plenty of water immediately and go to the hospital if this happens.
- All the samples and waste materials should be disposed of according to relevant laws and regulations.
- This product is for one sequencing run only and cannot be reused.
- The components and packages are batched separately. Keep the components in the packages until use and do not remove them. Mixed use of reagent components from different batches is not recommended.
- Do not use expired products.

Chapter 2 List of sequencing set components and User-supplied equipment and consumables

2.1 List of sequencing set components

Tips
- App Make DNB Buffer can be used for the preparation of DNBs for both MGI libraries and App libraries.
- Mixed use of reagent components from different batches is not recommended.

Table 3 DNBSEQ-T7RS High-throughput Methylation Sequencing Set V1.0 Catalog number: 940-002625-00

Component	Cap color	Spec& Quantity	Storage temperature	Transportation temperature	Expiry date
DNBSEQ-T7RS Methylation Sequencing Flow Cell Catalog number: 940-002697-00					
Sequencing Flow Cell (T7-2 FCL)	/	1 EA	2 ℃ ~8 ℃	2 ℃ ~8 ℃	10 months
DNBSEQ-T7RS Methylation DNB Make Reagent Kit V1.0 Catalog number: 940-002444-00					

Component	Cap color	Spec& Quantity	Storage temperature	Transportation temperature	Expiry date
Low TE Buffer		960 μL×1 tube	-25 ℃ ~ -15 ℃	-80 ℃ ~ -15 ℃	10 months
Methylation Make DNB Buffer		240 μL×1 tube			
App Make DNB Buffer		400 μL×1 tube			
Methylation Make DNB Enzyme Mix I		480 μL×1 tube			
Make DNB Enzyme Mix I		800 μL×1 tube			
Make DNB Enzyme Mix II (LC)		80 μL×1 tube			
Stop DNB Reaction Buffer		400 μL×1 tube			
DNB Load Buffer V		360 μL×1 tube			
DNBSEQ-T7RS Methylation DNB Load Reagent Kit V1.0 Catalog number: 940-002696-00					
Micro Tube 0.5 mL (Empty)		1 tube	-25 ℃ ~ -15 ℃	-80 ℃ ~ -15 ℃	12 months
Methylation Post Load Plat	/	1EA			
DNBSEQ-T7RS High-throughput Methylation Sequencing Kit V1.0 Catalog number: 940-002698-00					

Component	Cap color	Spec& Quantity	Storage temperature	Transportation temperature	Expiry date
dNTPs Mix II		5.61 mL ×2 tubes	-25 ℃ ~ -15 ℃	-80 ℃ ~ -15 ℃	12 months
dNTPs Mix V		3.74 mL ×1 tube			
Sequencing Enzyme Mix		7.48 mL ×1 tube			
MDA Reagent		4.20 mL ×1 tube			
MDA Enzyme Mix		0.60 mL ×1 tube			
MethylationSequencing Reagent Cartridge	/	1 EA			
Transparent Sealing film	/	2 sheets			
DNBSEQ-T7RS Methylation Cleaning Reagent Kit V1.0 Catalog number: 940-002693-00					
Methylation Washing Cartridge	/	1 EA	0 ℃ ~ 30 ℃	Below 40℃	12 months

Table 4 DNBSEQ-T7RS Methylation DNB Make and Load Reagent Set V1.0 Catalog number: 940-002628-00

Component	Cap color	Spec& Quantity	Storage temperature	Transportation temperature	Expiry date
DNBSEQ-T7RS Methylation DNB Make Reagent Kit V1.0 Catalog number: 940-002444-00					

Component	Cap color	Spec& Quantity	Storage temperature	Transportation temperature	Expiry date
Low TE Buffer		960 μL×1 tube	-25 ℃ ~ -15 ℃	-80 ℃ ~ -15 ℃	10 months
Methylation Make DNB Buffer		240 μL×1 tube			
App Make DNB Buffer		400 μL×1 tube			
Methylation Make DNB Enzyme Mix I		480 μL×1 tube			
Make DNB Enzyme Mix I		800 μL×1 tube			
Make DNB Enzyme Mix II (LC)		80 μL×1 tube			
Stop DNB Reaction Buffer		400 μL×1 tube			
DNB Load Buffer V		360 μL×1 tube			
DNBSEQ-T7RS Methylation DNB Load Reagent Kit V1.0 Catalog number: 940-002696-00					
Micro Tube 0.5 mL (Empty)		1 tube	-25 ℃ ~ -15 ℃	-80 ℃ ~ -15 ℃	12 months
Methylation Post Load Plat	/	1 EA			

Table 5 CPAS Barcode Primer 3 Reagent Kit Catalog number:1000020834

Component	Cap color	Spec& Quantity	Storage temperature	Transportation temperature	Expiry date
Primer for dual barcode sequencing (Pair End Sequencing use only)					
1 μM AD153 Barcode Primer 3		3.50 mL×1 tube	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃	12 months

Table 6 High-Throughput Pair-End Sequencing Primer Kit (App-A) Catalog number: 1000020832

Component	Cap color	Spec& Quantity	Storage temperature	Transportation temperature	Expiry date
Primer for single/dual barcode sequencing (Pair End Sequencing use only)					
App-A Make DNB Buffer		400 μL× 1 tube	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃	12 months
1 μM App-A Insert Primer 1		2.20 mL× 1 tube			
1 μM App-A Insert Primer 2		4.20 mL× 1 tube			
1 μM App-A MDA Primer		4.20 mL× 1 tube			
1 μM App-A Barcode Primer 2		3.50 mL× 1 tube			

Table 7 High-throughput Barcode Primer 3 Reagent Kit (App-A) Catalog number: 1000014047

Component	Cap color	Spec& Quantity	Storage temperature	Transportation temperature	Expiry date
Primer for dual barcode sequencing (Pair End Sequencing use only)					
1 μM App-A Barcode Primer 3		3.50 mL× 1 tube	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃	12 months

Table 8 High-Throughput Paired-End Sequencing Primer Reagent Kit (App-D) Catalog number: 1000028550

Component	Cap color	Spec& Quantity	Storage temperature	Transportation temperature	Expiry date
Primer for dual barcode sequencing					

Component	Cap color	Spec& Quantity	Storage temperature	Transportation temperature	Expiry date
1 μM App-D Insert Primer 1		2.20 mL×1 tube	-25 ℃ to -15 ℃	-80 ℃ to -15 ℃	12 months
1 μM App-D MDA Primer		4.20 mL×1 tube			
1 μM App-D Insert Primer 2		4.20 mL×1 tube			
1 μM App-D Barcode Primer 2		3.50 mL× 1 tube			
1 μM App-D Barcode Primer 3		3.50 mL× 1 tube			
App Make DNB Buffer		400 μL× 1 tube			

2.2 User-supplied equipment and consumables

Table 9 Self-prepared equipment and consumables

Equipment and consumables	Recommended brand	Catalog number
Qubit 4 Fluorometer	Thermo Fisher	Q33226
Thermal cycler	Bio-Rad	/
MPC2000 96-well plate centrifuge	/	/
Pipette	Eppendorf	/
Electronic pipette	Labnet	FASTPETTEV-2
Mini centrifuge	/	/
Vortex mixer	/	/
2 ℃ to 8 ℃ Refrigerator	/	/
-25 ℃ to -15 ℃ Freezer	/	/
ssDNA assay kit	YESEN	12645ES60 / 12645ES76

Equipment and consumables	Recommended brand	Catalog number
75% Ethanol	/	/
100% Tween-20	BBI	A600560 - 0500
5 M NaCl solution	SIGMA	S5150 - 4 L
2 M NaOH solution	Aladdin	S128511 - 1 L
Qubit Assay Tubes	Thermo Fisher	Q32856
200 µL Wide-bore pipette tips(non-filter)	AXYGEN	T - 205 - WB - C
200 µL Wide-bore pipette tips(non-filter)	MGI	091-000355-00
Power dust remover	MATIN	M - 6318
1.5 mL microcentrifuge tube	AXYGEN	MCT - 150 - C
Microfiber clean wiper	/	/
Lint-free paper	/	/
15 mL Sterile tube	SARSTEDT	60.732.001
100 mL Serological pipet	CORNING	4491
25 mL Serological pipet	CORNING	4489
10 mL Serological pipet	CORNING	4488
5 mL Transport tubes	AXYGEN	/
Ice box	AXYGEN	/
Ice machine	/	/
Ziplock bag	/	/

Chapter 3 Sequencing workflow

Tips If the 3'end starting base of the pure methylation library is a fixed sequence (e.g., single-stranded library preparation methods from companies like IDT, Vazyme, etc.), it is necessary to contact an MGI engineer for special settings in the sequencing software before running the sequencing.

The sequencing workflow is as follows:

1. **Preparation before sequencing:** check the integrity of the reagent cartridge and thaw the Sequencing Reagent Cartridge (4-24 hours), prepare the wash reagents and fill the pure water container, and check the available capacity of waste liquid container and storage space.
2. **Making DNB:** make DNB using reagents from DNB Make Reagent Kit and DNA library.
3. **Loading DNB:** load DNB onto the flow cell using reagents from DNB Load Reagent Kit at MGIDL-T7RS loader.
4. **Preparing Sequencing Reagent Cartridge:** Add the reagents in tubes from the Sequencing Kit into the Sequencing Reagent Cartridge and mix well.
5. Click **Start**, and the machine starts self-check.
6. **Sequencing**
7. **Data analysis**

Chapter 4 Preparation before sequencing

4.1 Thawing the Sequencing Reagent Cartridge

Perform the steps below:

1. Take out the Methylation Sequencing Reagent Cartridge from the High-Throughput Methylation Sequencing Reagent Kit.
2. Thaw in a water bath at room temperature until completely thawed (or thaw in a 2 ℃ to 8 ℃ refrigerator 1 days in advance). The approximate time to thaw is listed in the following table. Store in a 2 ℃ to 8 ℃ refrigerator until use.

Table 10 Approximate thaw times for various sequencing kits

Model	Method		
	Water bath at room temperature (h)	Refrigerate at 2 ℃ to 8 ℃ overnight, then water bath at room temperature (h)	Refrigerate at 2 ℃ to 8 ℃ (h)
T7 Methylation FCL PE150	3.0	2.0	24.0

4.2 Preparing wash reagents

Tips The following cleaning reagents must be stored at 2°C to 8°C and have a shelf life of 28 days.

- Prepare the Wash Reagent I (1 M NaCl+0.05% Tween-20) following the table below:

Table 11 Wash reagent I: 1 M NaCl+0.05% Tween

Reagent	Volume
5 M NaCl	200 mL
100% Tween-20	0.5 mL
Laboratory-grade water	799.5 mL

- Prepare the Wash Reagent II (0.1 M NaOH) following the table below:

Table 12 Wash reagent II: 0.1 M NaOH

Reagent	Volume
2M NaOH	50 mL
Laboratory-grade water	950 mL

4.3 Filling the pure water container

Fill the pure water container with laboratory-grade water according to the table below:

Tips

- Check whether the water in the pure water container is sufficient. If it is insufficient, the sequencing will fail. Replenish pure water in time, and pay attention to opening the air vent of the pure water container.
- Prepare pure water before sequencing. If you need to add water during the sequencing process, gently pour it along the wall of the water container to avoid bubbles entering the pump water pipeline, which could affect sequencing quality.
- The pure water will be used in sequencing so it must be kept clean. Renew the pure water in the pure water container on a weekly basis.
- Before refilling the pure water container, empty the container and spray 75% ethanol on the inner surface of the container lid and the surface of the pure water tube. Wipe and clean the surfaces with new microfiber clean wipers. Rinse the container with fresh pure water 3 times.
- Refer to *H-020-000157-00 DNBSEQ-T7RS Genetic Sequencer User Manual* for the preparation of the water container.

Table 13 Pure water consumption (L)

Product Model	1 flow cell	2 flow cell	3 flow cell	4 flow cell
T7 Methylation FCL PE150	4.5	9.0	13.5	18.0

4.4 Performing pre-run checks

Before each sequencing run, perform the following checks:

- Check the disk space. For 1 flow cell (PE150), if it includes FASTQ files, ensure that the remaining space is no less than 4.7 TB. If the disk space is insufficient, clear historical data.
- Check the waste liquid container. For 1 flow cell (PE150), the available capacity for waste liquid should be ≥7L. If the capacity is insufficient, empty the waste liquid container before sequencing.
- If any issues other than those mentioned above occur, restart the control software.

Chapter 5 Prepare library

5.1 Insert size recommendation

For general purpose, library refers to single stranded circular DNA (ssDNA). For the best sequencing quality, it is recommended that the insert size of the library should be between 250 bp and 500 bp, and the main band is centered within ± 100 bp.

Tips
- The insert size and required data output should be considered when selecting sequencing kits.
- Average data output will vary with different library type and applications.
- If there is any special requirement or specification from the library preparation kit, then the requirement of the kit should be followed.

Table 14 Recommended insert size and theoretical throughput for each flow cell

Model	Suggested insert distribution (bp)	Sequencing Protocol	Applications	Data Output (M)	Data Output (Gb)
T7 Methylation FCL PE150	250-500	Methylation library as major equencing (0~30% balanced library, lalanced library data not usable)	Methylation Library, MGI Standard Library(V3), and other balanced libraries	≥ 4500	≥ 1350
		Methylation library mixed sequencing (recommended non-methylation library proportion ≥ 80%)	Methylation Library, WGS, RNAseq, and other non-methylation libraries	≥ 5800	≥ 1740

Chapter 6 Making DNB

6.1 Calculating the required amount of ssDNA library

Tips
- Reagent kits from different batches must not be mixed.
- If mixing DNB, use wide-bore tips without filters.

For T7 Methylation FCL PE150 Sequence Set, 270 µL of DNB is required to load one flow cell. Based on the data volume required for sequencing each sample and the library pooling method, decide whether the DNB preparation system should be 100 µL or 50 µL. Typically, the barcode splitting uniformity of the DNB pooling method is better than other methods (e.g., PCR pooling or ssCir pooling).

The required ssDNA library volume to make one DNB reaction are shown in the table below.

Tips C refers to the concentration of the ssDNA library (fmol/µL).

Table 15 Methylation FCL PE150 required ssDNA volume

Sample type	Required ssDNA Library Volume V (µL)	
	100 µL DNB reaction	50 µL DNB reaction
Methylation Library	V = 160 fmol / C	V = 80 fmol / C
Non-methylation Library	V = 60 fmol / C	V = 30 fmol / C
Non-methylation PCR-free Library	V = 75 fmol / C	V = 37.5 fmol / C

- For a given sample A. If it requires “a” million base data output and the total theoretical expected data output for this flow cell is “b” million bases, then the required DNB volume (V) in the pooling for sample A is as follows: V=a/b×270 (µL).
- The theoretical volume required for sample A is: V = a / b × 270 µL. The number of DNB preparations for sample A is V / 90, rounded up to the nearest whole number + 1.

6.2 Preparing reagents for making DNBs

Perform the steps below:

1. Place the library on ice until use.
2. Remove the TE buffer, Make DNB Buffer, and Stop DNB Reaction Buffer from the Methylation DNB Make Reagent Kit packaging and thaw reagents at room temperature.
   - For non-methylation library sequencing: Remove the App Make DNB Buffer packaging and thaw reagents at room temperature.
3. Remove the Methylation Make DNB Enzyme Mix I on ice for approximately 30 minutes.
   - For non-methylation library sequencing: Remove the DNB Enzyme Mix I on ice for approximately 30 minutes.
4. After thawing, mix the reagents by using a vortex mixer for 5 seconds. Centrifuge them briefly, and place them on ice until use.

Tips At this time, do not remove the DNB Polymerase Mix II (LC).

6.3 Making DNBs

This section includes DNB Making protocols for different library types.

- For performing methylation library pure test pooling or methylation library pure test pooling with standard library reagents (V3.0), refer to *"6.3.1 Methylation Library and Standard Library Reagents (V3.0) DNB Making" on page 15*
- For performing mixed pooling of methylation libraries, in addition to the above methylation DNB making, the following steps are also required: *"6.3.2 Non-methylated Library DNB Making" on page 18*

6.3.1 Methylation Library and Standard Library Reagents (V3.0) DNB Making

Perform the steps below:

1. Take out a 0.2 mL 8-strip tube or PCR tubes, and prepare the Make DNB Reaction Mixture 1 on ice according to the different library types:

   Tips
   - The table below shows one DNB Reaction.
   - The amount of input ssDNA and the required number of Make DNB Reactions are determined by the actual application as described in *"6.1 Calculating the required amount of ssDNA library" on page 14*.

- Retain the Low TE buffer after use here, as it will also be needed for the DNB Loading.

Table 16 Make DNB reaction mixture 1

Component	Cap color	Volume Added for 100 µL (µL)	Volume Added for 50 µL (µL)
Low TE buffer		20 - V	10 - V
Methylation Make DNB Buffer		20	10
ssDNA	/	V	V
Total Volume		40	20

2. Mix the Make DNB Reaction Mixture 1 thoroughly by using a vortex mixer for 5 seconds. Centrifuge it briefly and place it on ice until use.
3. Place the mixture into a thermal cycler, and start the primer hybridization reaction according to the following table:

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

Table 17 Primer hybridization reaction conditions

Temperature	Time
Heated lid (105 ℃)	On
95 ℃	1 min
65 ℃	1 min
40 ℃	1 min
4 ℃	Hold

4. Take the Make DNB Enzyme Mix II (LC) out of freezer. Centrifuge it briefly for 5 seconds, and place it on ice until use.

Tips
- Do not place the DNB Polymerase Mix II (LC) at room temperature
- Avoid holding the tube for a prolonged time.

5. Take the PCR tube out of the thermal cycler when the temperature reaches 4 ℃ . Centrifuge briefly for five seconds, place the tube on ice.

Prepare Make DNB Reaction Mixture 2 according to the table below:

Table 18 Make DNB reaction mixture 2

Component	Cap color	Volume Added for 100 µL (µL)	Volume Added for 50 µL (µL)
Methylation Make DNB Enzyme Mix I		40	20
Make DNB Enzyme Mix II (LC)		1.6	0.8

6. Add all the Make DNB Reaction mixture 2 into Make DNB Reaction Mixture 1. Mix the reaction mixture thoroughly by using a vortex mixer. Centrifuge it for five seconds and place it on ice until use.
7. Place the tubes into a thermal cycler for the next reaction. The condition is shown in the table below:

Tips
- Some brands of PCR instruments have slow heating lid temperature ramp rates. For this type of PCR instrument, preheat or cool the heating lid in advance to ensure that the heating lid is at the working temperature during the DNB reaction.
- It is recommended to set the heating lid temperature to 35°C, or as close to 35°C as possible.

Table 19 RCR (Rolling Circle Replication) conditions

Temperature	Time
Heated lid (35 ℃)	On
30 ℃	40 min
4 ℃	Hold

8. Immediately add Stop DNB Reaction Buffer to the tube when the temperature reaches 4 ℃ . Mix the tube gently by pipetting 5 to 8 times by using a wide-bore, non-filtered pipette tip.

Table 20 Stop DNB Reaction Buffer Addition Volume

Component	Cap color	Volume Added for 100 µL (µL)	Volume Added for 50 µL (µL)
Stop DNB Reaction Buffer		15	7.5

Tips
- Keep DNBs on ice during the entire operation to prevent DNBs from performing secondary replication.

- It is very important to mix DNBs gently by using a wide-bore, non-filtered pipette tip. Do not centrifuge, vortex, pipette vigorously or shake the tube
- Store the DNBs at 2°C ~ 8°C and perform sequencing within 8 hours. To ensure sequencing quality, it is recommended that you pool and load DNBs for FCL PE150 as soon as possible.
- This is not a STOP point, immediately go to the next step: *"6.3.3 Quantifying DNBs" on page 21*

6.3.2 Non-methylated Library DNB Making

Perform the steps below:

1. Take out a 0.2 mL 8-strip tube or PCR tubes, and prepare the Make DNB Reaction Mixture 1 on ice according to the different library types:

   Tips
   - The table below shows one DNB Reaction.
   - The amount of input ssDNA and the required number of Make DNB Reactions are determined by the actual application as described in *"6.1 Calculating the required amount of ssDNA library" on page 14*.
   - Retain the Low TE buffer after use here, as it will also be needed for the DNB Loading.

Table 21 Make DNB reaction mixture 1

Component	Cap color	Volume Added for 100 µL (µL)	Volume Added for 50 µL (µL)
Low TE buffer		20 - V	10 - V
App Make DNB Buffer		20	10
ssDNA	/	V	V
Total Volume	40	20	

2. Mix the Make DNB Reaction Mixture 1 thoroughly by using a vortex mixer for 5 seconds. Centrifuge it briefly and place it on ice until use.

3. Place the mixture into a thermal cycler, and start the primer hybridization reaction according to the following table:

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

Table 22 Primer hybridization reaction conditions

Temperature	Time
Heated lid (105 ℃)	On
95 ℃	1 min
65 ℃	1 min
40 ℃	1 min
4 ℃	Hold

4. Take the Make DNB Enzyme Mix II (LC) out of freezer. Centrifuge it briefly for 5 seconds, and place it on ice until use.

   Tips
   - Do not place the DNB Polymerase Mix II (LC) at room temperature
   - Avoid holding the tube for a prolonged time.

5. Take the PCR tube out of the thermal cycler when the temperature reaches 4 ℃ . Centrifuge briefly for five seconds, place the tube on ice.

   Prepare Make DNB Reaction Mixture 2 according to the table below:

Table 23 Make DNB reaction mixture 2

Component	Cap color	Volume Added for 100 µL (µL)	Volume Added for 50 µL (µL)
Make DNB Enzyme Mix I		40	20
Make DNB Enzyme Mix II (LC)		4	2

6. Add all the Make DNB Reaction mixture 2 into Make DNB Reaction Mixture 1. Mix the reaction mixture thoroughly by using a vortex mixer. Centrifuge it for five seconds and place it on ice until use.

7. Place the tubes into a thermal cycler for the next reaction. The condition is shown in the table below:

Tips
- Some brands of PCR instruments have slow heating lid temperature ramp rates. For this type of PCR instrument, preheat or cool the heating lid in advance to ensure that the heating lid is at the working temperature during the DNB reaction.
- It is recommended to set the heating lid temperature to 35°C, or as close to 35°C as possible.

Table 24 RCR (Rolling Circle Replication) conditions

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

8. Immediately add Stop DNB Reaction Buffer to the tube when the temperature reaches 4 ℃ . Mix the tube gently by pipetting 5 to 8 times by using a wide-bore, non-filtered pipette tip.

Table 25 Stop DNB Reaction Buffer Addition Volume

Component	Cap color	Volume Added for 100 µL (µL)	Volume Added for 50 µL (µL)
Stop DNB Reaction Buffer		20	10

Tips
- Keep DNBs on ice during the entire operation to prevent DNBs from performing secondary replication.
- It is very important to mix DNBs gently by using a wide-bore, non-filtered pipette tip. Do not centrifuge, vortex, pipette vigorously or shake the tube
- Store the DNBs at 2°C ~ 8°C and perform sequencing within 8 hours. To ensure sequencing quality, it is recommended that you pool and load DNBs for FCL PE150 as soon as possible.
- This is not a STOP point, immediately go to the next step:

6.3.3 Quantifying DNBs

When DNB making is completed, take out 2 μL DNBs, and use Qubit ssDNA Assay Kit and Qubit Fluorometer to quantify the DNBs. For details, refer to *"Appendix 1 Instructions for using Qubit to quantify the DNBs" on page 38*

Tips
- If the DNB concentration is not qualified, and inaccurate DNB quantification is ruled out, it needs to be re-prepared.
- If there are too many samples in a single test, it is recommended to quantify in batches to avoid inaccurate DNB quantification due to fluorescence quenching.

Table 26 DNB concentration standard

Model	DNB concentration
Methylation Library and Standard Library Reagents (V3.0)	≥ 5 ng / μL
Non-methylation Library	≥ 8 ng / μL

Tips
- If the concentration exceeds 40 ng/μL, the DNBs need to be diluted to 20 ng/μL by Low TE Buffer before loading.
- If dilution is required, dilute it right before use.

6.3.4 DNB pooling

6.3.4.1 Methylation Library Pure Test Pooling

Tips
- Methylation libraries according to DNB pooling. Convert the DNB concentration (ng/μL) quantified by the Qubit 4.0 Fluorometer to fmol/μL using the following equation:

  Concentration (fmol/μL) = 3030 × Concentration (ng/μL) / N

  N represents the number of nucleotides (total library length including the adapters).
- Use a pointed tip to slowly sample the DNB. After sampling all the DNBs from the samples, mix them using a wide-bore tip without a filter.

When the samples to be pooled are for the same application or have similar insert fragments, calculate the DNB pooling volume for each sample based on the required data volume and DNB concentration.

6.3.4.1.1 Calculating the relative amount for each library

The relative amount of A library (A1) = data output required for library A/the concentration of DNB for library A.

The relative amount of B library (B1) = data output required fors library B/the concentration of DNB for library B.

…...

The relative amount of B library (H1) = data output required for library H/the concentration of DNB for library H.

6.3.4.1.2 Calculating the total relative amount (V) for all library

V = A1 + B1 + …… + H1

6.3.4.1.3 Calculating the DNB volume needed for each library

For each FCL flow cell used for Methylation FCL PE150 requiring 270 μL DNB, the DNB volume for pooling is calculated as follows:

Methylation DNB volume for library A: A2=270×A1/V

Methylation DNB volume for library B: B2=270×B1/V

……

Methylation DNB volume for library H: H2=270×H1/V

6.3.4.2 Methylation Library Mixed Test Pooling

Tips Methylation libraries and non-methylation libraries are pooled using DNB pooling. When there are multiple methylation samples, DNB pooling is required among the methylation samples. When pooling non-methylation samples for the same application, sample pooling can be performed among those samples. DNB pooling is required between samples of different applications. In mixed sequencing, the data volume required for methylation libraries should be ≤ 20% of the total data volume. For a mixed sequencing theoretical total data volume of 1740 Gb, the data volume for methylation libraries should be ≤ 348 Gb.

Convert the DNB concentration (ng/μL) quantified by the Qubit fluorometer to (fmol/μL) using the following formula:

Concentration (fmol/μL) = 3030 × Concentration (ng/μL) / N

'N' represents the average number of nucleotides (total fragment length of the library, including adapter sequences).

Use a pointed tip to slowly sample the DNB. After sampling all the DNBs from the samples, mix them using a wide-bore tip without a filter.

When the pooling samples are for the same application or have similar insert fragments, calculate the DNB pooling volume for each sample based on the required data volume and DNB concentration.

6.3.4.2.1 Calculating the relative amount for each library

The relative amount of methylation A library (A1) =data output required for library A/the concentration of DNB for library A(fmol/μL).

The relative amount of methylation B library (B1) =data output required for library B/the concentration of DNB for library B(fmol/μL)

……

The relative amount of non-methylation AA library (e.g., WGS) (AA1) = data volume required for library AA / DNB concentration of library AA (fmol/μL)

The relative amount of non-methylation AB library (e.g., RNA) (AB1) = data volume required for library AB / DNB concentration of library AB (fmol/μL)

……

6.3.4.2.2 Calculating the total relative amount (V) for all library

V=A1+B1+…+AA1+AB1+…

6.3.4.2.3 Calculating the DNB volume needed for each library

For each FCL flow cell requiring 270 μL DNB, the DNB volume for pooling is calculated as follows:

DNB volume for methylation library A: A2=270×A1/V

DNB volume for methylation library B: B2=270×B1/V

DNB volume for non-methylation library AA (e.g., WGS): AA2 = 270 * AA1 / V

DNB volume for non-methylation library AB (e.g., RNA): AB2 = 270 * AB1 / V

…

6.3.4.3 Methylation Library Pure Test Pooling Standard Library Reagents (V3.0)

Tips Methylation libraries and standard library reagents (V3.0) sequencing use DNB pooling. When there are multiple methylation samples, DNB pooling is required among the methylation samples. The pooling ratio for standard library reagents (V3.0) can be explored based on the fragment size of the methylation library. For pure test theoretical total data volume of 1350 Gb, if the data volume for standard library reagents (V3.0) samples is ≤ 20%, then the data volume for standard library reagents (V3.0) samples should be ≤ 270 Gb.Convert the DNB concentration (ng/μL) quantified by the Qubit fluorometer to (fmol/μL) using the following formula:

Concentration (fmol/μL) = 3030 × Concentration (ng/μL) / N

'N' represents the average number of nucleotides (total fragment length of the library, including adapter sequences).

Use a pointed tip to slowly sample the DNB. After sampling all the DNBs from the samples, mix them using a wide-bore tip without a filter.

6.3.4.3.1 Calculating the relative amount for each library

The relative amount of methylation A library (A1) = data volume required for library A / DNB concentration of library A (fmol/μL)

The relative amount of methylation A library (B1) = data volume required for library B / DNB concentration of library B (fmol/μL)

…..

The relative amount of standard library reagent (V3.0) AA library (AA1) = data volume required for library AA / DNB concentration of library AA (fmol/μL)

6.3.4.3.2 Calculating the total relative amount (V) for all library

V=A1+B1+…+AA1

6.3.4.3.3 Calculating the DNB volume needed for each library

For each FCL flow cell requiring 270 μL DNB, the DNB volume for pooling is calculated as follows:

The pooling volume for methylation A library (A2) = 270 * A1 / V

The pooling volume for methylation B library (B2) = 270 * B1 / V

……

The pooling volume for standard library reagent (V3.0) AA library (AA2) = 270 * AA1 / V

Chapter 7 Loading DNB

7.1 Preparing the post load plate and buffers

7.1.1 Preparing the methylation post load plate and buffer

7.1.1.1 Thaw the Methylation DNB Load Plate

Perform the steps below:

1. Prepare the reagents by taking out the Methylation DNB Load Plate from the DNBSEQ-T7RS Methylation DNB Load Reagent Kit.
2. Thaw the reagents according to the following methods:
   - Room temperature thawing: Place in a room temperature water bath for 2 hours.
   - 2°C ~ 8°C refrigerator thawing: Place in a 2°C ~ 8°C refrigerator one day in advance to thaw for later use.
3. After completely thawing, store in a 2°C ~ 8°C refrigerator for later use.
4. Before use, gently invert the reagent plate 5 times to mix, and centrifuge for 1 minute.

7.1.1.2 Thaw the DNB Load Buffer

Perform the steps below:

1. Take out the DNB Load Buffer V from the DNBSEQ-T7RS Methylation DNB Load Reagent Kit.
2. Perform the following steps according to different situations:
   - For App-A libraries sequencing: take the DNB Load Buffer V out of the DNBSEQ-T7RS Methylation DNB Make Reagent Kit. Take the App-A Insert Primer 1 out of the High-throughput Pair-End Sequencing Primer Kit (App-A).
   - For App-A libraries sequencing: take the DNB Load Buffer V out of the DNBSEQ-T7RS Methylation DNB Make Reagent Kit. Take the App-D Insert Primer 1 out of the High-throughput Single-End/Pair-End Sequencing Primer Kit (App-D).
3. Thaw reagents at room temperature for approximately 30 minutes.
4. After thawing, mix the reagents by using a vortex mixer for 5 seconds. Centrifuge them briefly and place them on ice until use.

7.1.1.3 Prepare 0.1 M NaOH Reagent

Prepare 0.1 M NaOH according to the procedure described in *"4.2 Preparing wash reagents" on page 11*. Each Post Load plate requires at least 4 mL of 0.1 M NaOH.

7.1.2 Preparing the sequencing flow cell

Perform the steps below:

1. Take the flow cell out of the DNBSEQ-T7RS Methylation Sequencing Flow Cell packing.

   Tips Do not open the outer plastic package yet.

2. Place the flow cell at room temperature for 0.5 hours to 24 hours.
3. Unwrap the outer package before use.

   Tips
   - If the flow cell is taken out of the refrigerator and placed at room temperature but cannot be used within 24 hours, and the vacuum packaging bag remains intact, it can be returned to storage at 2°C ~ 8°C. However, the transition between 2°C ~ 8°C and room temperature should not exceed 3 times.
   - If the outer plastic package has been opened but the flow cell can not be used immediately. Store the flow cell at room temperature and use it within 24 hours. If exceed 24 hours, it is not recommended to use the flow cell.

4. Take the flow cell out of the inner package and inspect it to ensure that the flow cell is intact.
5. Clean the back of the flow cell by using a canned air duster.

7.1.3 Loading DNBs

Perform the steps below:

1. When starting the MGIDL-T7RS, the compartment doors need tobe closed.
2. Start the MGIDL-T7RS program. Enter the user name **research** and password **Admin123** or the user name **user** and password **Password123** , tap **Log in** to enter the main interface.

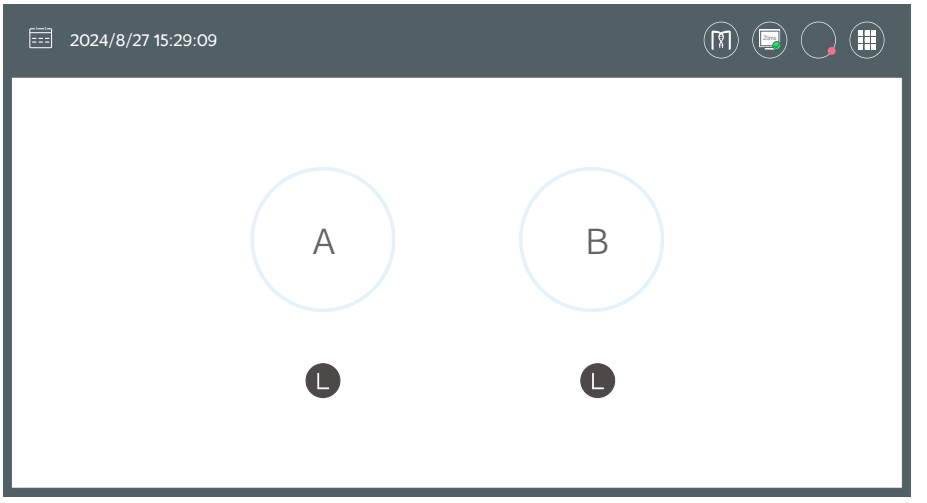

Figure 1 MGIDL-T7RS main interface

3. Tap on A or B to continue the operation, see the figure below:

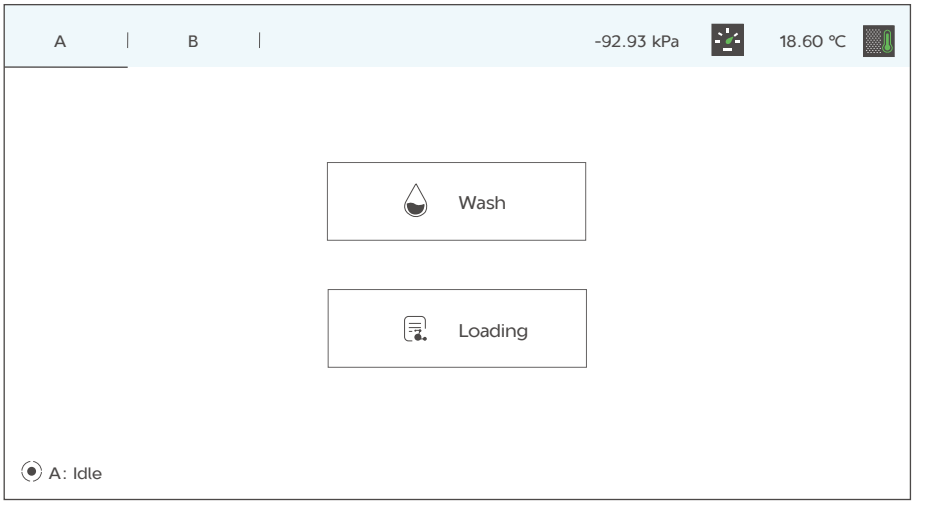

Figure 2 MGIDL-T7RS selection interface

4. Tap on **Loading** and enter the information input interface, see the figure below:

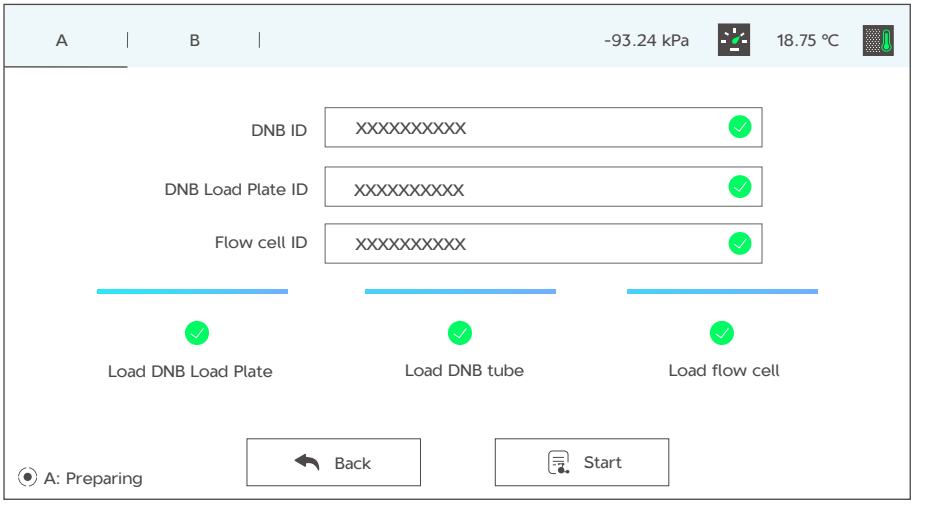

Figure 3 MGIDL-T7RS information input interface

5. Open the loading compartment door.
6. Take out the thawed methylation post load plate. Align the post load plate to the RFID scanning area and the ID information will appear in the text box.

   Tips If it does not display, you can manually enter the ID information as prompted.

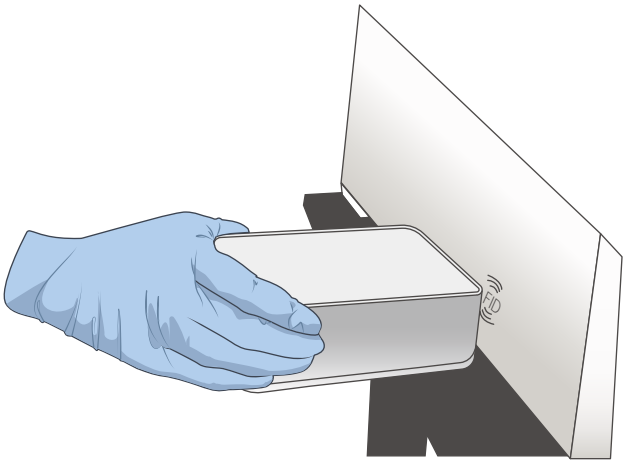

Figure 4 The RFID scanning area of post load plate

7. Gently invert the post load plate 5 times to mix, then centrifuge for 1 minute or gently tap the sealing film of the loading plate and let it stand for 2 to 3 minutes. Remove the seal of the load plate and add 4 mL of 0.1 M NaOH into well No.11.

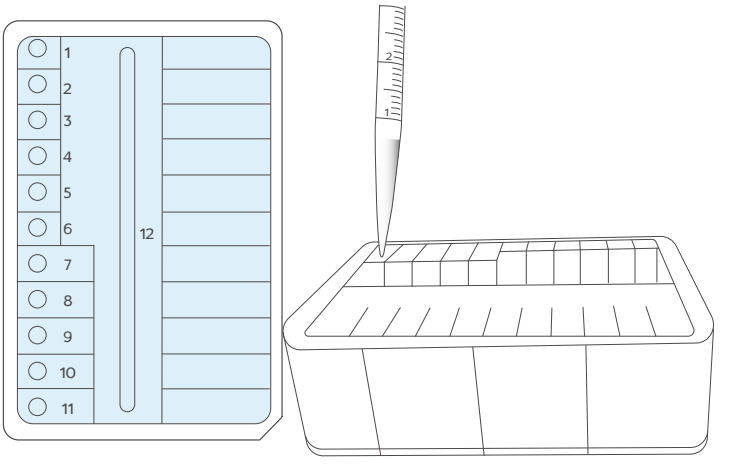

Figure 5 Adding 4 mL of 0.1 M NaOH into well No.11

8. Perform the following steps according to different situations:
   - For App-A libraries sequencing: Use a pipette to completely remove all the reagent in well No.1 of Post Load Plate/Rapid Post Load Plate, then add 2 mL of App-A Insert primer 1 from High-throughput Pair-End Sequencing Primer Kit (App-A).
   - For App-D libraries sequencing: Use a pipette to completely remove all the reagent in well No.1 of Post Load Plate/Rapid Post Load Plate, then add 2 mL of App-D Insert primer 1 from High-throughput Single-End/Pair-End Sequencing Primer Kit (App-D).
9. Place the prepared post load plate on the plate tray of MGIDL-T7RS.The screen will prompt that the post load plate is loaded.

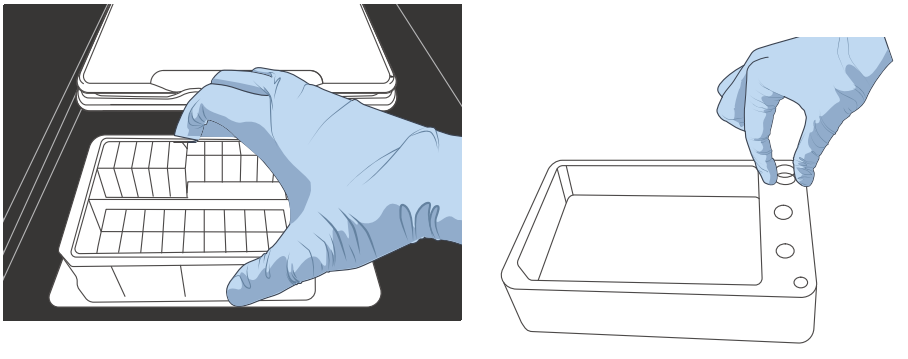

Figure 6 The Post-loading Plate placement diagram

10. Align the flow cell to the RFID scanning area and ID information willappear in the text box.

Tips If ID information does not appears, please enter it manually according to the prompts.

Figure 7 Scaning the Flow cell ID

11. Hold both sides of the flow cell, upwardly align the locating bulge on the flow cell to the locating groove on the flow cell stage. See the figure below:

Tips Ensure that all the four rubber sealing rings are on the four corners of the flow cell.

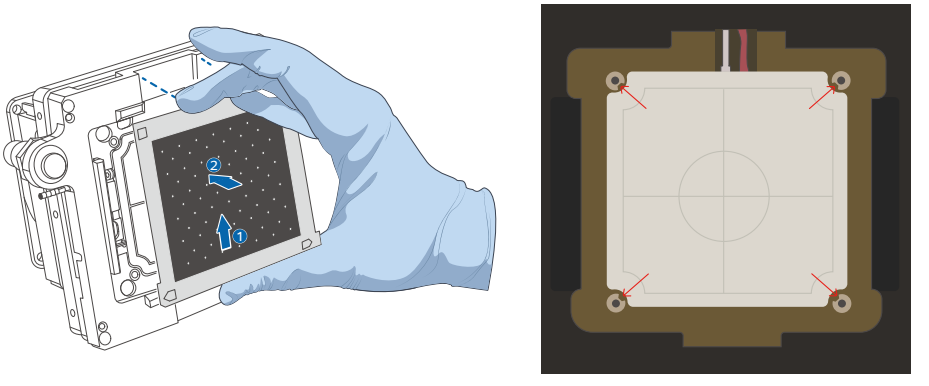

Figure 8 Flow cell locating

12. Press the flow cell attachment button on the flow cell stage and gently press down the edges of the flow cell to ensure that the flow cell is securely seated and held on the stage. The green light of the flow cell attachment button will be lit and the screen will prompt that the flow cell is loaded.

Tips
- Remove the dust on both sides of the flow cell with a canned air dust.
- Do not press or touch the glass cover of the flow cell toavoid flow cell damage or fingerprints and impurities left onthe glass surface.

- Do not move the flow cell after installing the flow cell ontothe stage, or it may cause the sealing gaskets to misalignwith holes of the fluidics line.
- If flow cell attachment fails, gently wipe the back of the flow cell and flow cell stage with microfiber clean wiper moistened with 75% ethanol, then clean with a gas canned air dust.

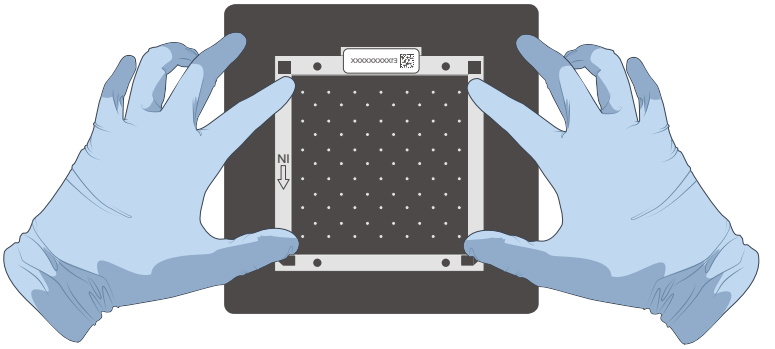

Figure 9 Flow cell loaded

13. Take out a new 0.5 mL microfuge tube from the Load Reagent Kit and add reagents following the table below, then gently pipette the DNB loading mixture 5 to 8 times by using a wide-bore, non-filtered tip.

Table 27 DNB loading mixture

Component	Cap Color	Volume（μL）
DNB	/	270
DNB Load Buffer V		90
Total volume		360

Tips
- DNB in the above table refers to the pooled DNB in *"6.3.4 DNB pooling" on page 21*.
- Do not centrifuge, vortex, vigorously pipette or shake the tube.
- Prepare a fresh DNB loading mixture immediately on ice before the loading run, and use it as soon as possible once prepared.
- For PE150, used the DNB loading mixture within 30 minutes.

14. Tap on the text box next to DNB ID, enter the DNB information into the text

Tips Use only numbers or letters or a combination of numbers and letters for DNB ID.

15. Place the 0.5 mL micro tube containing DNB loading mixture into the DNB tube hole, the screen will prompt that the DNB tube is loaded.

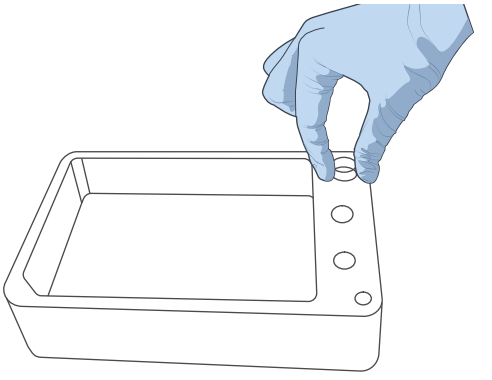

Figure 10 Place the DNB Tube

16. Close the loading compartment door.
17. Tap the**Start** button and select**Yes** as shown in the in the figure below.

Tips This section shows “Methylation FCL Post Load Plate”

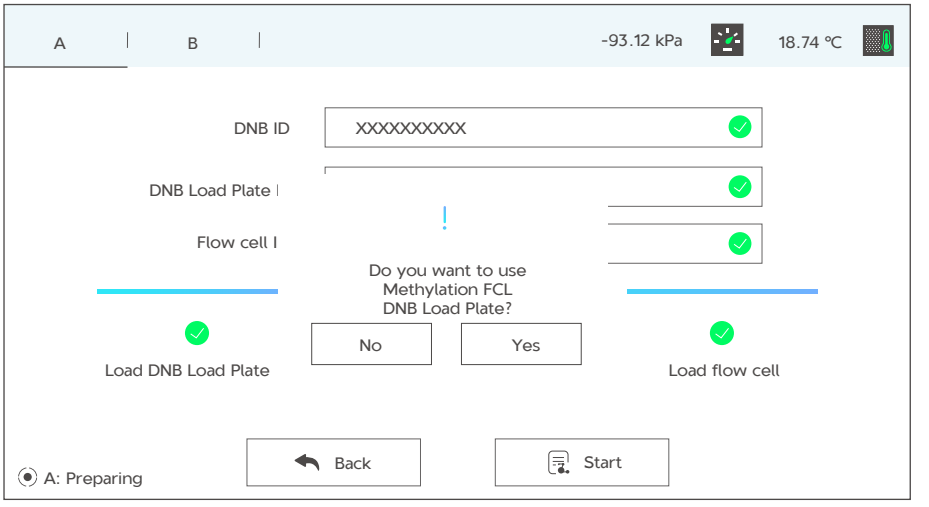

Figure 11 MGIDL-T7RS loading confirmation dialog box

18. Flow cell loading starts as shown in the figure below.

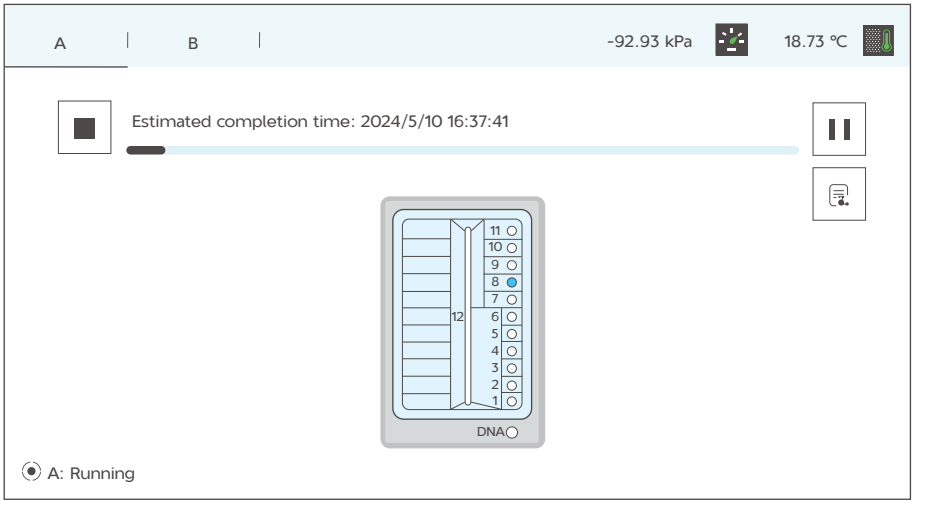

Figure 12 MGIDL-T7RS flow cell loading interface

19. The process take around 2 hours. When the screen is shown as inthe figure below, the flow cell loading is completed.

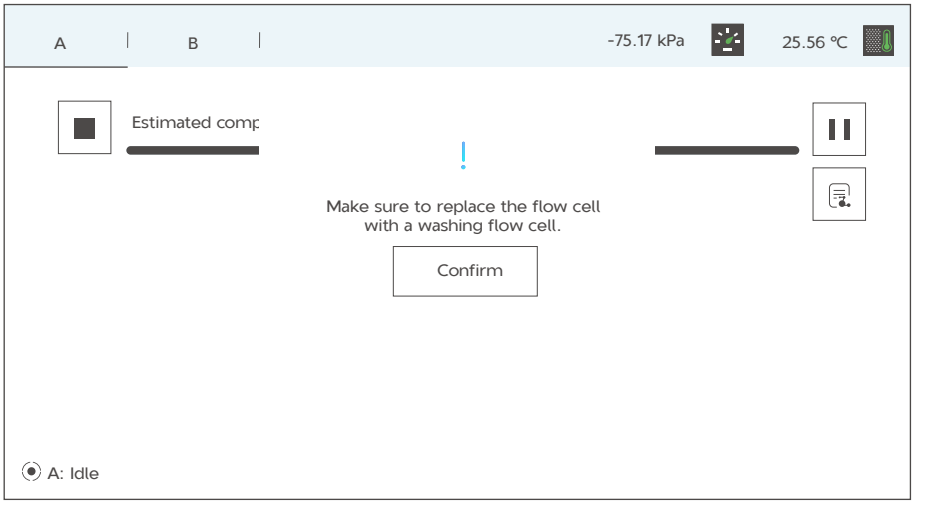

Figure 13 MGIDL-T7RS flow cell loading complete status window

20. Press the flow cell attachment button and remove the loaded flow cell from the stage. The flow cell is now ready for sequencing.

    Tips
    - If sequencing cannot be performed immediately, put the loaded flow cell in a clean zip bag and store it at 2 ℃ to 8 ℃ until use.
    - The maximum storage time for loaded flow cell is 48 hours.

21. Tap **Confirm**, install the washing flow cell ontothe flow cell stage and press the flow cell attachment button.
22. Tap **Post-Wash**.

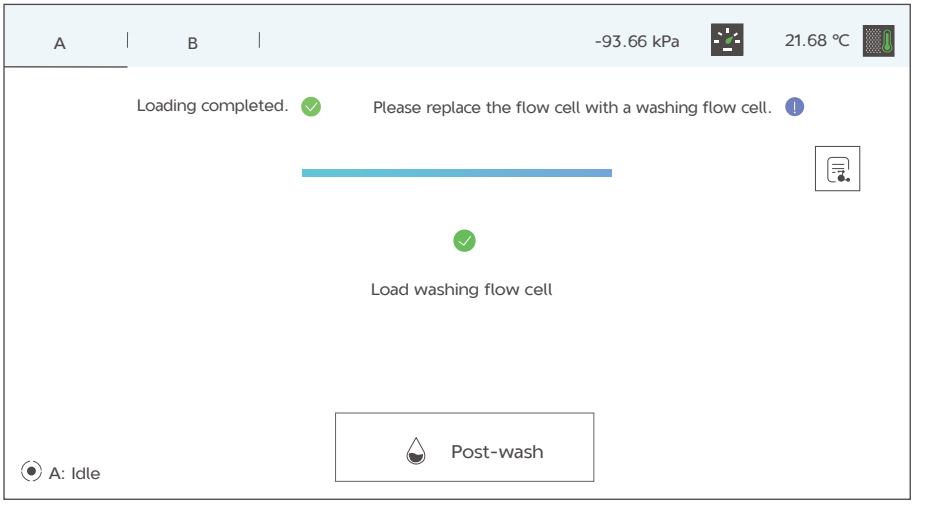

Figure 14 MGIDL-T7RS post-wash interface

23. and select**Yes** to start MGIDL-T7RS wash (see the figures below), which will take around 20 minutes.

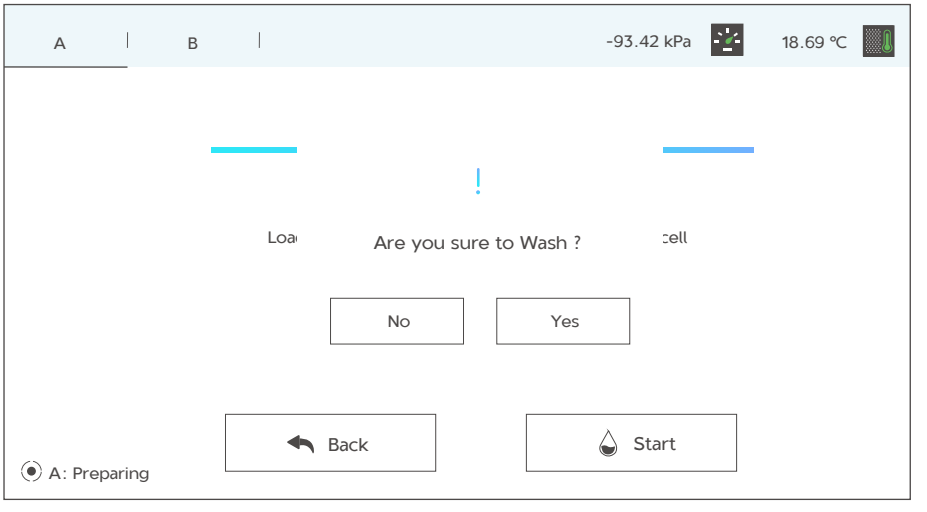

Figure 15 MGIDL-T7RS post-wash confirmation interface

24. MGIDL-T7RS wash starts, see the figure below:

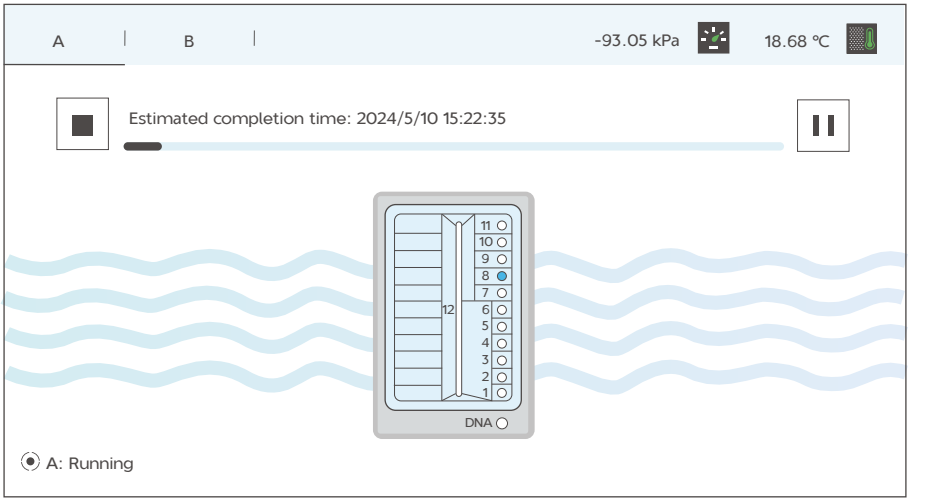

Figure 16 MGIDL-T7RS wash interface

25. When the screen is shown as the figure below, the wash is complete. Tap **Finish** and another flow cell loading can be performed.

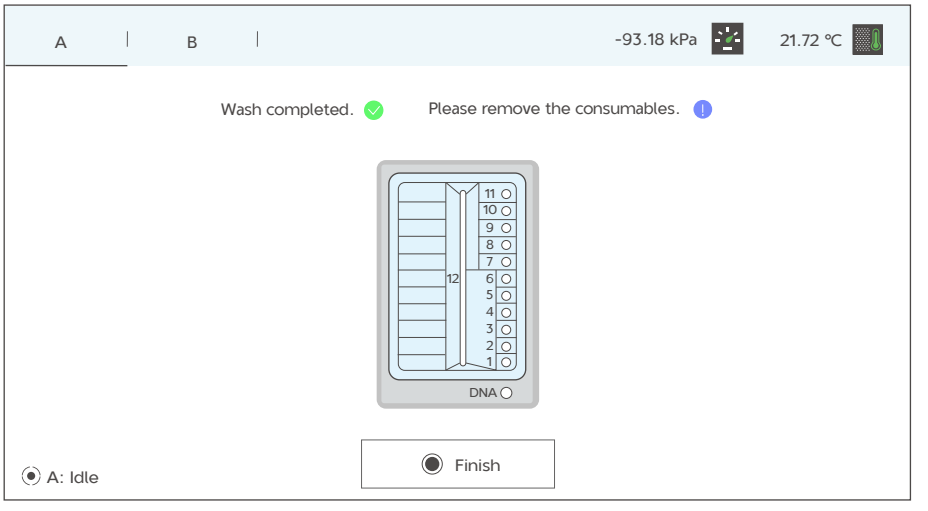

Figure 17 MGIDL-T7RS wash complete status window

Chapter 8 Preparing the sequencing reagent cartridge and the washing cartridge

Please refer to the *Chapter 7: Preparing the sequencing reagent cartridge and the washing cartridge"* in the *DNBSEQ-T7RS High-Throughput Sequencing Reagent Kit User Manual* or the *DNBSEQ-T7RS System Operation Guide* for sequencing steps.

Chapter 9 Sequencing

Please refer to the *Chapter 8: Sequencing"* in the *DNBSEQ-T7RS High-Throughput Sequencing Reagent Kit User Manual* or the *DNBSEQ-T7RS System Operation Guide* for sequencing steps.

Appendix 1 Instructions for using Qubit to quantify the DNBs

Tips
- Working solution should be used within 0.5 hours after preparation.
- Avoid touching the wall of tapered detection tubes.
- Avoid introducing bubbles in detection tubes.

1. Prepare the Qubit working solution by diluting the Qubit ssDNA Reagent 1:199 in Qubit ssDNA Buffer. Use a clean Qubit assay tube each time you prepare Qubit working solution. Do not mix the solution in a glass container.

   Tips The final volume in each tube must be 200 µL. Each standard tube requires 190 µL of Qubit working solution, and each sample tube requires anywhere from 180 to 199 µL of Qubit working solution.

   Prepare sufficient Qubit working solution to accommodate all standards and samples.

   For example: for 8 samples, prepare enough working solution for the samples and 2 standards. ~200 µL per tube in 10 tubes yields a total of 2 mL of working solution (10 µL of Qubit reagent plus 1990 µL of Qubit Buffer).
2. Add 190 µL of Qubit working solution to each of the tubes used for standards.
3. Add 10 µL of each Qubit standard to the appropriate tube and mix by vortexing 3 to 5 seconds. Be careful not to create bubbles.
4. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit ssDNA Assay requires 2 standards.

   Tips
   - Use only thin-wall, clear, 0.5 mL PCR tubes. Acceptable tubes include Qubit assay tubes (Cat. No. Q32856) or Axygen PCR-05-C tubes (Part No. 10011-830).
   - Number of Qubit test tubes needed are the number of samples plus 2 standards tubes. For example, if you have 3 samples, you will need 5 tubes.
5. Label the tube lids. Do not label the side of tube.

/	S1 (µL)	S2 (µL)	D1 (µL)	D2 (µL)	D3 (µL)
working solution	190	190	198	198	198
S1 (0 ng/µL)	10	/	/	/	/
S2 (20 ng/µL)	/	10	/	/	/

/	S1 (μL)	S2 (μL)	D1 (μL)	D2 (μL)	D3 (μL)
Sample	/	/	2	2	2
Tatal	200	200	200	200	200

6. Mix tubes by using a vortex mixer and centrifuge briefly for 5 seconds. Incubate at room temperature for 2 minutes.
7. Proceed instructions in section "Reading standards and samples" of relevant Qubit user guide; follow the procedure appropriate for your instrument.
8. Proceed instructions in section "Reading standards and samples" of relevant Qubit user guide; follow the procedure appropriate for your instrument.

Appendix 2 Manufacturer

Manufacturer	Wuhan MGI Tech Co., Ltd.
Address	Building B13, No.818, Gaoxin Avenue, East Lake High-Tech Development Zone, 430075, Wuhan, P.R.China
	Building 24, Stage 3.1, BioLake Accelerator, No.388, 2nd Gaoxin Road, East Lake High-Tech Development Zone, 430075, Wuhan, P.R.China
E-mail	MGI-service@mgi-tech.com
Website	www.mgi-tech.com