SARS-CoV-2 COMPLETE GENOME SEQUENCING PROTOCOL

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ABSTRACT

Genome sequencing of SARS-CoV-2 the causative agent of the current COVID-19 pandemic in Nigeria and other African countries is important for rapid detection of emerging SARS-CoV-2 lineages and strains responsible for COVID-19 pandemic and their associated mutations in the continent coupled with phylogenetic relationships with strains from other parts of the world. As a way of building genome sequencing capacity for quality diagnosis, surveillance and research across Africa, the present document represents protocols for complete genome sequencing of SARS-CoV-2 from viral RNA samples extracted from upper and lower respiratory tract samples (nasal, nasopharyngeal, oropharyngeal, sputum, bronchio alveolar lavage) and whole blood using the novel DNA nanoball (DNB) sequencing technology on DNBSEQ-50 sequencing platform . The platform uses combinatorial probe anchor sequencing technology with reagents provided in the ATOPlex Universal Library Preparation and Sequencing Set Kits developed by MGI. The kits works with different flow cell configurations and can accommodate between 50 -500 read length as inserts in libraries. The DNBSEQ-50 runs between 22 – 68 h depending on the sequencing format (single or paired), flow cell read length capacity and size. The present protocol will enhance genome sequencing capacity of Laboratories and improve understanding of an a novel PCR-based sequencing approach for unraveling the genome structure of SARS-CoV-2 circulating in Africa. It will also offer the opportunity for performance evaluation via comparison with other sequencing platforms currently in use for SARS-CoV-2 complete genome sequencing in the continent.

INTRODUCTION

The SARS-CoV-2 sequencing protocol described in this document was a novel DNA nanoball sequencing technology originally developed by MGI Tech Co. Ltd (China). This protocol is unique because it uses combinatorial probe anchor chemistry and of its downstream utilization of single stranded circularization followed by a rolling-circle PCR amplification steps to make DNA nanoballs. The nanoballs are then loaded into flow cells (single ended or pair ended) followed by sequencing on DNBSEQ platforms. However, this protocol shares earlier steps within the workflow of SARS-CoV-2 genome sequencing with other sequencing platforms such as ILUMINA and Nanopore sequencing platforms, which use ARTIC protocol. The shared steps include cDNA synthesis from extracted RNA, PCR-based cDNA amplification and cDNA amplicon barcoding steps to generate a unique size library (150 – 400 bp). Unlike the ARTIC protocol, the protocol employed for single stranded DNA circularization followed by two PCR amplification steps to make DNA nanoballs. The reagents for these reactions are provided as a kit called the ATOPlex RNA Universal Library Preparation module developed by MGI Co. Ltd (China). The ATOPlex kit contains reagents that have passed the stringent quality control and functional validation tests for a good sequencing performance with reproducibility and stability values. The SARS-CoV-2 total RNA utilized by this module could be extracted from whole blood, swabs, faeces and cell lines (e.g. HeLa, Vero E6, hSLAM etc). The module include RNA reverse transcription reagent, PCR reagents [for cDNA amplicon synthesis, end repairs, barcode ligation, splint oligo ligation for circularization and rolling circle (i.e nanoball synthesis)] and DNA clean beads (for the various purification steps within the workflow).

The cDNA lbbraries constructed using the ATOPlex kit are compatible with MGIEasy Dual Barcode Circularization kit(PN: 1000020570) and PE100+10+10sequencing on: MGISEQ-200RS/ DNBSEQ-G50RS; MGISEQ-2000RS/ DNBSEQ-G400RS and DNBSEQ-T7RS.

Apart from the sequencing room, three working areas have been recommended for the production of the cDNA library. This is to reduce the risk of contamination of the cDNA library synthesized. This protocol has been adopted from the originally developed by

MGI Tech Company Limited (China). We thank the organization for making this protocol available for research use only.

The goal of this protocol is to guide the implementation quality and high throughput sequencing of SAR-CoV-2 complete genome from different biological samples in an African laboratory for laboratory strengthening, surveillance, research, epidemiological purposes and human capacity building.

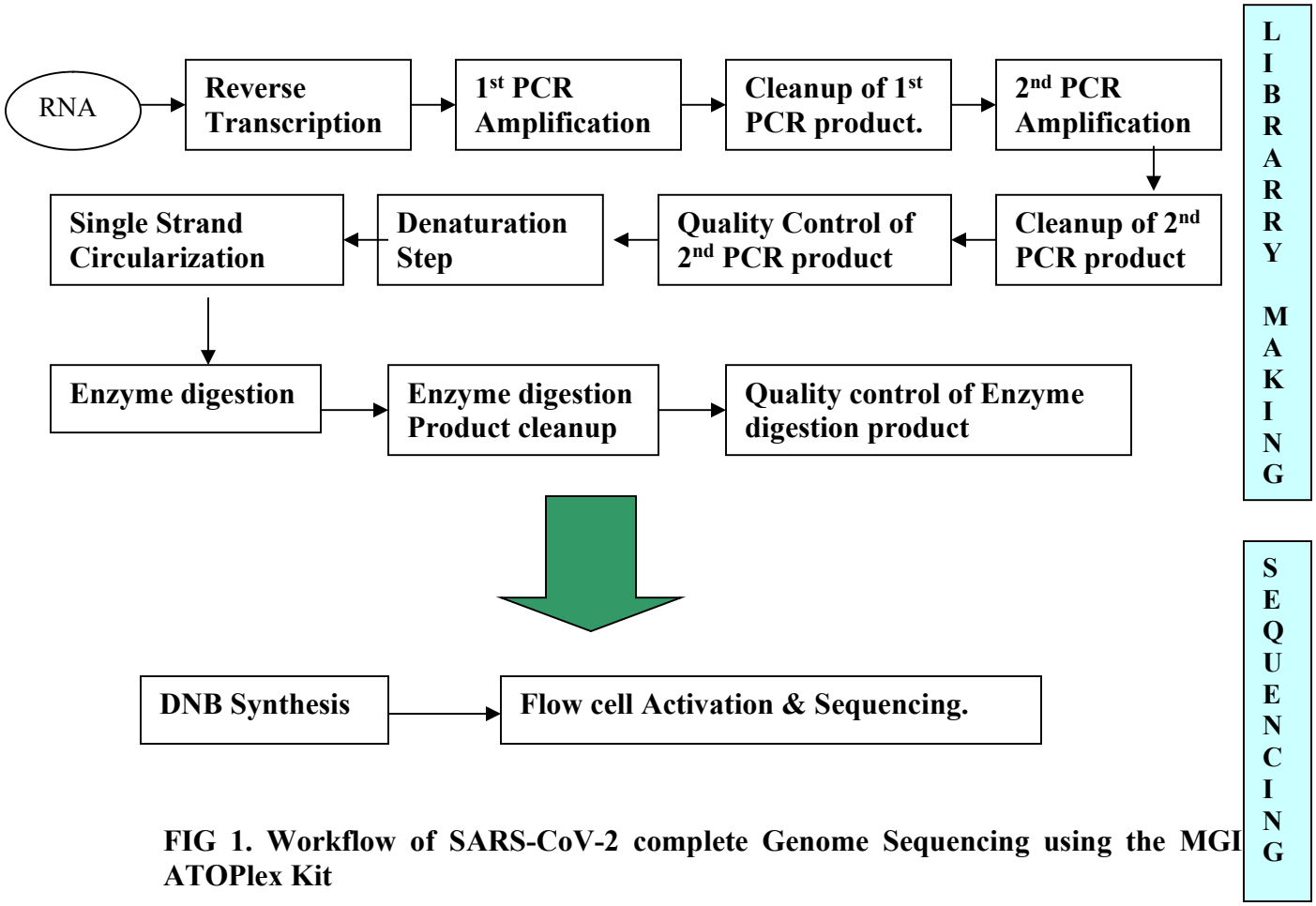

FIG 1. Workflow of SARS-CoV-2 complete Genome Sequencing using the MGI ATOPlex Kit

Reverse Transcription for cDNA Preparation

1.0. Prepare between 1 and 47 samples plus 1 negative control using nuclease free water per library. The RNA sample template should be removed from -20^{0}C to -70^{o}C, allowed to thaw, mixed by vortexing and spin briefly before use. All RNA samples to be sequenced must be kept on ice before use.

Note:
That lesser number of samples can be sequenced provided a QC of 20 ng circular SSDNA in 20 uL total volume can be produced as input for DNB synthesis.

A positive control can also be included, which may be a synthetic SAS-CoV-2 RNA construct or high viral load sample (Ct < 15)

Samples from which RNA was extracted should not be kept for more than 1 week at-20^{0}C or for 6 months at -700C. The extracted RNA samples should be stored at -70^{0}C.

1.1. Transfer 10 µL RNA sample to a new 0.2 mL PCR tube or plate

1.2. Mix the following components in a PCR strip, tubes or plate. Mix by pipetting gently and pulse spin the tube to collect liquid at the bottom of the tube.

Table 1. Reverse Transcription reaction mixture

Constituent	Volume (µL)
NG Buffer	4
RT Buffer	5
RT Enzyme Mix	1
Total	10

Note:
That the PCR Primer pool is a customized tilling primer set developed by MGI. It should be mixed thoroughly before use. Vortex 5 times, 5 s each time. This step is completed in the first working area

1.3. Transfer 10 µL of the reverse transcriptase reaction mixture to the PCR tube step 1.1. Mix by pipetting up and down 10 times and pulse spin to collect the solution at the bottom of the tube/plate. **Do not vortex please.**

1.4. Place the PCR tube from step 1.3 into the thermal cycler and run the program in Table 2.

Table 2. Reverse Transcription reaction condition

Temperature	Time
Heated Lid	On
25^{0}C	10 min
42^{0}C	30 min
70^{0}C	15 min
4^{0}C	Hold

1.5. Put the tube/plate on ice when the reaction is complete. Centrifuge briefly to collect the cDNA product at the bottom of the tube/plate.

1st PCR amplification to make cDNA Amplicon

1.6. transfer 20 uL cDNA from step 1.5 of nuclease free water to a new 0.2 mL PCR tube or plate. Place the tube/plate on ice please.

1.7. Prepare 1st PCR amplification reaction mixture on ice as indicated in Table 3 Below.

Table 3. 1st PCR Amnpllification mixture

Constituent	Volume µL
PCR Enzyme mix	25
PCR Clean Enzyme	0.5
PCR Primer Pool	4.0
Total	29.5

1.8. Transfer 29.5 µL of the 1stPCR amplification mixture to the PCR tube from step 1.7 Vortex three times (3s each) and centrifuge briefly to collect the solution at the bottom of the tube.

Note: This step is completed in the first working area.

Table 4. 1st PCR amplification reaction condition

Temperature	Time	Cycles
Heated Lid	On	1 cycle
37^0C	5 min	
95^0C	10 min	
95^0C	15 s	15 cycles
64^0C	1 min	
60^0C	1 min	
72^0C	30 s	
4^0C	Hold	

1.9. Centrifuge briefly to collect the cDNA amplicon at the bottom of the tube

Note: The first amplification step from Table 4 is completed in the second working area.

2.0. Cleanup of the 1st PCR product

2.1. Take out DNA Clean Beads from refrigerator and allow 30 minutes for the solution to come to room temperature. Vortex and mix thoroughly before use.

2.2. Transfer 60 µLDNA Clean Beads to the centrifuge tube from step 3.2.4. Pipette up and down at least 10 times to mix thoroughly. Ensure that all of the liquid and beads are fully dispensed from the pipette tip into the tube before proceeding.

2.3. Incubate at room temperature

2.4. Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant.

2.5. Keep the tube on the Magnetic Separation Rack and add 150µLfreshly prepared 80%

ethanol to wash the beads and the walls of the tube. Incubate for 30 seconds, then carefully remove and discard the supernatant.

2.6. Repeat step 2.5 once, remove all of the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom of the centrifuge tube, separate the beads magnetically and then remove any remaining liquid using a small volume pipette.

2.7 Keep the centrifuge tube on the Magnetic Separation Rack with the lid open, and air-dry beads at room temperature until no wetness (reflectiveness) is observed. Do not over-dry the beads (cracks can be observed on pellet).

2.8 Remove the centrifuge tube from the Magnetic Separation Rack and add 14µL of Elution Buffer to elute the DNA. Make sure that the Elution Buffer fully wets the magnetic beads to prevent the magnetic beads from drying out and causing loss of PCR products.

2.9. Incubate at room temperature for 5 minutes.

Note: The next step is carried out with magnetic beads. Do not place the centrifuge tube back into the magnetic rack or transfer the supernatant to a new tube.

After cleanup, purified PCR product can be stored at -20°C.

3.0. 2nd PCR amplification

3.1 Take out ATOPlex PCR Dual Barcode Primer Module (01-96).Add 8µLPCR Dual Barcode Primer Mix (01-96)to the corresponding position in the PCR tube from step 2.8

3.2. Prepare 2nd PCR amplification mixture on ice as indicated in Table 5 below.

Table 5. 2nd Amplification reaction mixture

Constituent	Volume µL
PCR Enzyme Mix	25
PCR Clean Enzyme	0.5
PCR Additive	1.0
PCr Block	2.0
Total	28.5

Note:

That the PCR Block is a component of the customized primer pool developed by MGI. It should be mixed thoroughly before use. Vortex 5 times, 5 s each time. This step is completed in the second working area

3.3. Transfer 28.5 µLof the 2nd PCR amplification mixture to the PCR tube from step 3.2.

3.4. Vortex three times (3s each) and centrifuge briefly to collect the solution at the bottom of the tube.

3.5 Place the PCR tube from step 3.3 into the thermocycler and run the program in Table 6

Table 6. 2nd PCR amplification reaction condition

Temperature	Time	Cycles
Heated Lid	On	1
37ºC	5 min	
95ºC	10 min	
95^0C	15 s	27 cycles
64^0C	1 min	
60^0C	1 min	
72^0C	30 s	
4^0C	Hold	

3.6. Centrifuge briefly to collect the cDNA amplicon at the bottom of the tube

Note: The second amplification step from Table 6 is completed in third working area.

4.0. Cleanup of the 2nd PCR product

4.1. Take out DNA Clean Beads from refrigerator and allow 30 minutes for the solution to come to room temperature. Vortex and mix thoroughly before use.

4.2. Transfer 60 µLDNA Clean Beads to the centrifuge tube from step 3.5. Pipette up and down at least 10 times to mix thoroughly. Ensure that all of the liquid and beads are fully dispensed from the pipette tip into the tube before proceeding.

4.3. Incubate at room temperature

4.4. Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant.

4.5. Keep the tube on the Magnetic Separation Rack and add 150µLfreshly prepared 80%

ethanol to wash the beads and the walls of the tube. Incubate for 30 seconds, then carefully remove and discard the supernatant.

4.6. Repeat step 4.5 once, remove all of the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom of the centrifuge tube, separate the beads magnetically and then remove any remaining liquid using a small volume pipette.

4.7 Keep the centrifuge tube on the Magnetic Separation Rack with the lid open, and air-dry beads at room temperature until no wetness (reflectiveness) is observed. Do not over-dry the beads (cracks can be observed on pellet).

4.8 Remove the centrifuge tube from the Magnetic Separation Rack and add 14μL of Elution Buffer to elute the DNA. Make sure that the Elution Buffer fully wets the magnetic beads to prevent the magnetic beads from drying out and causing loss of PCR products.

4.9. Incubate at room temperature for 5 minutes.

Note: The next step is carried out with magnetic beads. Do not place the centrifuge tube back into the magnetic rack or transfer the supernatant to a new tube.

After cleanup, the purified second PCR product can be stored at -20°C.

5.0. Quality control of the purified 2nd PCR Product

5.1. Quantify the purified 2ndPCR product with dsDNA Fluorescence Assay Kits such as Qubit®dsDNA HS Assay Kit or Quant-iT™PicoGreen®dsDNA Assay Kit. The required concentration of 2ndPCR products is ≥4ng/μL.

5.1 Determine he fragment size distribution of purified 2ndPCR product with phoresis-basedequipment such as Bioanalyzer, Tapestation (Agilent Technologies), LabChip®GX, GXII, GX Touch (PerkinElmer), or Fragment Analyzer™(Advanced Analytical). The final size distribution of purified 2nd PCR products should correspond with the size of the customized panel.

5.2 After the library passes the quality control metrics, pool the library according to the actual conditions. The total amount of 2nd PCR products after pooling is 400 ng and the total volume is ≤48 μL.

Note:
For example: There are N libraries that need to be mixed, and each sample library needs the same amount of sequencing data, then all libraries are mixed

with the same mass, the pooling massof a library (ng) = 400 ng/N, the pooling volume of a library (µL) = the pooling massof a library (ng)/the concentration of a library (ng/µL).

6.0 Denaturation

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

6.1. Transfer 400 ng of 2nd PCR products to a new 0.2 mL PCR tube. Add TE Buffer for a total volume of 48 µL.

6.2. Place the 0.2 mL PCR tube from step 3.7.1 into the thermocycler and run the program in Table 7 below

Table 7. The reaction condition for denaturation

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

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

7.0. Single Strand Circularization

7.1 Prepare the single strand DNA circularization mixture in a new 0.2 mL PCR tube on ice as indicated in Table 8.

Table 8. Single strand circularization mixture

Constituent	Volume µL
Dual Barcode splint Buffer	11.5
DNA Rapid Ligase	0.5
Total	12.0

7.2. Transfer 12 µL single strand DNA circularization mixture to the 0.2 mL PCR tube from step 6.3 on ice. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube.

7.3. Place the PCR tube into the thermocycler and run the program in Table 9.

Table 9. Single Stranded DNA Circularization Reaction condition

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

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

8.0. Enzymatic Digestion

8.1 Prepare the following enzymatic digestion mixture in Table 10 below in a new 0.2 mL PCR tube on ice during the reaction in step 7.3.

Table 10. Enzyme Digestion Mixture

Constituent	Volume µL
Digestion Buffer	1.4
Digestion Enzyme	2.6
Total	4.0

8.2. Centrifuge briefly to collect the solution at the bottom of the tube.

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

9.0. Enzyme Digestion Product cleanup

9.1. Take out DNA Clean Beads from the refrigerator and allow 30 minutes for the solution to come to room temperature. Vortex and mix thoroughly before use.

9.2 Transfer 170 µL of DNA Clean Beads to the Enzymatic Digestion product from step 8.3. Gently pipette atleast 10 times to mix thoroughly. Ensure that all of the solution and beads are fully dispensed from the tip into the tube.

9.3. Incubate at room temperature for 10 minutes.

9.4. Centrifuge briefly and place the 1.5 mL tube on the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant using a pipette.

9.5. With the 1.5 mL tube on the Magnetic Separation Rack, add 200 µL of freshly prepared 80% ethanol to the tube without disturbing the beads. Incubate for 30 seconds. Carefully remove and discard the supernatant.

9.6. Repeat step 9.5 once. Remove all of the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom, separate the beads magnetically, then remove any remaining liquid using a small volume pipette.

9.7. Keep the 1.5 mL centrifuge tube on the Magnetic Separation Rack with the lid open, and air-dry beads at room temperature until no wetness (reflectiveness) is observed. Do not over-dry the beads (cracks can be observed on pellet).

9.8. Remove the 1.5 mL centrifuge tube from the Magnetic Separation Rack and add 22µL of Elution Buffer to elute the DNA. Gently pipette up and down at least 10 times to mix thoroughly or until the beads are fully suspended.

9.9. Incubate at room temperature for 10 minutes.

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

Note:
After cleanup, the purified enzyme digestion product can be stored at -20°C.

10.0. Quality control of Enzymatic Digestion Product

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

11.0. Making of DNB

The constructed library need be sequenced with ATOPlex Dual Barcoded Balance Library Reagent.

11.1. Prepare the Make DNB reaction mixture 1 in a new 0.2 mL PCR tube as indicated in Table 11 below using the enzymatic digestion product in step 9.10

Table 11. Make DNB Reaction Mixture 1

Constituent	Volume µL
ssDNA Library (i.e. Enzymaric digestion Product)	V
Low TE buffer	20 – V
Make DNB Buffer	20
Total	40

Note: The volume of ssDNA library is determined by the required library amount. This should be the volume equivalent of 10 ng as obtained in step 10.0

11.2. Mix gently by vortexing and centrifuge for 5 s. Place the mix into the thermocycler and start the primer hybridization reaction using the program set in Table 12 below.

Table 12. Primer Hybridization Reaction condition.

Temperature	Time
Heated Lid (105ºC)	On
95ºC	1 min
65ºC	1 min
40ºC	1 min
4ºC	Hold

11.3. Remove the Make DNB Enzyme Mix II (LC) from storage and place on ice. Centrifuge briefly for 5 s and hold on ice.

Note: Do not place Make DNB Enzyme Mix II (LC) at room temperature and avoid holding the tube for a prolonged time.

11.4. Take the PCR tube out of the thermocycler, centrifuge briefly for 5 s and place the tube on ice. Prepare the Make DNB reaction mix 2 as indicated in Table 13 below.

Table 13. Make DNB reaction Mixture 2

Constituent	Volume µL
Make DNB primer hybridization mix (i.e. Make DNB reaction mix I)	40
Make DNB Enzyme mix II (LC)	4
Total	44

11.5. Add all the Make DNB reaction mix II into the Make DNB reaction mix I. Mix gently by vortexing and centrifuge briefly for 5 s. Place the PCR tube/plate into the thermocycler and start the reaction using the program set in Table 14.

Table 14. DNB Reaction condition 2

Temperature	Time
Heated Lid (35ºC)	On
30C	25 min
4C	Hold

11.6. Immediately add 20 µL stop DNB Reaction Buffer once the temperature in the thermocycler reaches 4ºC. Mix gently by pipetting 8 times using a wide bore tip.

Note:
Do not vortex, shake the tube or pipette vigorously. It is very important to mix DNB gently using a wide bore tip.

Store DNB at 4ºC and perform sequencing within 48 h

12.0 Quantify DNB

When the make DNB is completed. Take 2 uL DNB used Qubit ssDNA assay kit and Qubit fluorometer to quantify the DNB. Sequencing requires a minimum of 8 ng/uL

(expected range 8 – 40 ng/uL) if the concentration is lower than 8 ng/uL, make a new DNB preparation.
Note

a. Because DNB is viscous, it recommended to take 2 uL for quantification. If the number of samples is large, it is recommended to quantify in batches to avoid inaccurate DNB quantification due to fluorescence quenching.
b. If the concentration exceeds 40 ng/uL, the DNB needs to be diluted to 20 ng/uL with DNB load buffer 1 for loading.
c. Store DNB at 4^0C and perform sequencing with 48 h.

12.1: Make balanced DNB for sequencing

The constructed library needs to be sequenced with ATOPlex dual barcoded balanced library reagent. Both the sample library and balanced library are performed "make DNB" operation by the reagent in the sequencing kit.

a. Make DNB of ATOPlex dual barcoded according to the concentration of the dual barcoded balanced library, transfer 6 ng library for 'Make DNB operation" as indicated Tables 11 – 14. Add TE buffer to a final volume of 20 uL.
b. Mix the DNBs of sample from step 12.0 and DNB of the balanced library from step 12.1a with 3 : 1 in mass for subsequent sequencing. The mass of sample DNB (ng) : The mass of standard library (ng) 3 : 1

13.0: Sequencing

The constructed libraries march with PE100+10+10 sequencing DNBSEQ G-50RS for RNA full length genome sequencing.
The sequencing kits include
cPAS barcode primer 3 reagent kit (PN:1000020834) for paired-end sequencing.
DNBSEQ G-50RS High-throughput sequencing set (FCLPE100) or
DNBSEQ G-50RS High-throughput rapid sequencing set (FCSPE100)
Note:
cPAS barcode primer 3 reagent kit (PN:1000020834) for PE sequencing.
Please follow the protocols from 14.0

14.0: DNB Loading

14.1. Remove DNB Load Bufffer I and DNB Load Buffer II from storage and thaw reagent on ice for about 30 min
14.2. After thawing mix reagent by vortexing, centrifuge briefly for 5 s and place on ice.
14.3 Take 0.5 mL microfuge tube and add reagents as indicated in Table 15 below.

Table 15. DNB Loading mixture preparation.

Constituent	Volume µL
DNA Load Buffer I	50
DNA Load buffer II	50
Make DNA enzyme mix II (LC)	1
DNB	100
Total	201

14.4. Mix by gentle pipetting 6 times using a wide bore tip. Place the mixture at 4°C until use

Note:
Prepare a fresh DNA loading mixture before the sequencing run. It is recommended to prepare the DNB loading mixture after preparing the sequencing cartridge in step 13.

15.0. Preparation of the sequencing cartridge

15.1. Remove the sequencing catridge from storage and thaw in a room temperatire water bath until thawed. Store catridge at 2 – 8°C storage until use (or thaw catridge in 2-8°C fridge one day in advance). Invert catrigde 3 times to mix before use.

Note:
Make sure that no visible layer can be seen in the catridge especially for reagents in well No. 17 and No. 18.

If dark green crystals appear in well No. 18, it is precipitation of raw materials of the reagent in well No. 18. this is a normal phenomenon. When the cartridge is thawed, mix the reagents in the catridge well and the crystals will dissolve. Sequencing quality will not be affected.

15.2. Open the cartridge cover and wipe any water condensation with lint-free paper. Well position is shown in Figure 2 below

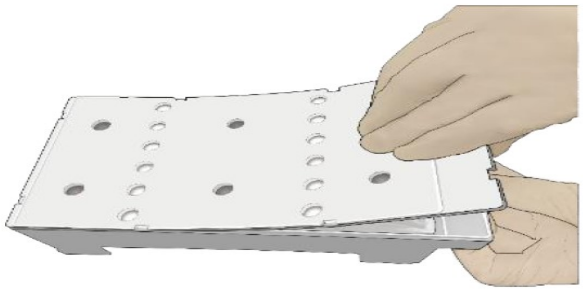

Figure 2. Open and Clean the Cartridge

15.3. Remove dNTPs Mix III and dNTPs Mix II from storage 1h in advance and thaw at room temperature. Store at 4°C until use. Mix the reagent by vortexing and centrifuge briefly for 5 s before use.

15.4. Remove Sequencing Enzyme Mix from -20°C storage and place on ice until use. Invert Sequencing enzyme Mix 6 times before use.

15.5. Pierce the seal at the edge of the well No. 1 and No. 2 to make a hole around 1 cm in diameter using 1 mL sterile tip as shown in Figures 3 & 4 below.

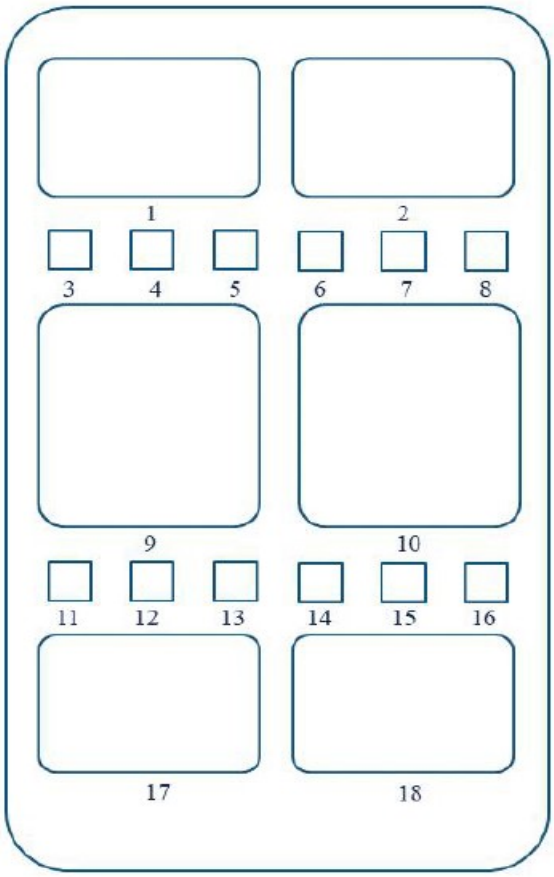

Figure 3. Well Position

Figure 4. Pierce the seal on the catridge.

15.6. Take a pipette with appropriate volume range and add reagents to well No. 1 as indicated in Table 16 below.

Table 16. dNTP Mix III Loading

Sequencing Kit	Reagent name	Loading Volume µL
FCL SE50 / FCS SE 100	dNTP Mix III	320
FCL SE 100	dNTP Mix III	440
FCL PE50 / FCS PE 100	dNTP Mix III	560
FCL PE100 / FCS PE 50	dNTP Mix III	800
FCL PE 150	dNTP Mix III	960

15.7. Take a pipette with appropriate volume range and add reagents to well No. 2 as indicated in Table 17 below.

Table 17. dNTP Mix II Loading

Sequencing Kit	Reagent name	Loading Volume µL
FCL SE50 / FCS SE 100	dNTP Mix II	560
FCL SE 100	dNTP Mix II	760
FCL PE50 / FCS PE 100	dNTP Mix II	920
FCL PE100 / FCS PE 50	dNTP Mix II	1600
FCL PE 150	dNTP Mix II	2040

15.8. Take a pipette with appropriate volume range and add reagents to well No. 1 and No. 2 as indicated in Table 18 below.

Table 18. Sequencing Enzyme Mix Loading

Sequencing Kit	Reagent Name	Well no. 1 Loading Volume µL	Well No. 2 Loading Volume µL
FCL SE50 / FCS SE 100	Sequencing Enzyme Mix	320	280
FCL SE 100	Sequencing Enzyme Mix	440	380
FCL PE50 / FCS PE 100	Sequencing Enzyme Mix	560	460
FCL PE100 / FCS PE 50	Sequencing Enzyme Mix	740	800
FCL PE 150	Sequencing Enzyme Mix	960	1020

15.9. Seal the loading wells with the transparent sealing film. Do not cover the centre of the well to avoid blocking the sampling needle.

15.10. Place the cartridge horizontally on the table, hold both sides of the cartridge with both hands. Shake the cartridge clockwise 10 -20 times and then anticlockwise 10 – 20 times, until the reagent colour in well no. 1 is uniform. Ensure uniform mixing of reagents in the wells.

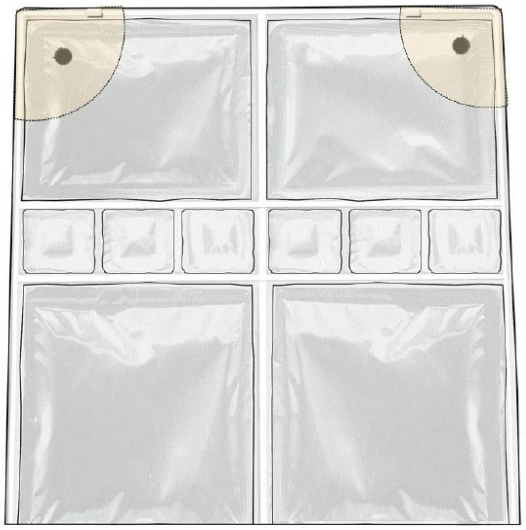

Figure 5. Seal the loading wells.

Figure 6. Mix reagents after loading.

15.11. Add 200 µL of the MDA Enzyme Mix to the MDA Reagent tube with a 200 µL pipette. Invert the tube 6 times to mix the reagent then add the mixture to well No. 15. ensure no bubbles at the bottom of the tube.

Note: when using MDA Enzyme Mix, do not touch the wall of the tube to prevent influencing the enzyme activity.

15.12. For dual barcode sequencing, perform the following steps after preparing the PE cartridge.

15.13. Remove the CPAS Barcode Primer 3 (for Pair End Sequencing only) from the CPAS Barcode Primer 3 Reagent Kit and thaw at room temperature. Mix the CPAS Barcode Primer 3 using a vortex mixer for 5 seconds and centrifuge briefly before use.

15.14. Pierce the seal of well No.12 using a sterile tip, then add 1.30 mL of the CPAS Barcode Primer 3. When adding the reagent, make sure there are no bubbles at the bottom of the tube.

16.0/ Flow Cell Preparation

16.1. Power on the DNBSEQ G-50 sequencing machine

16.2. Remove the sequencing flow cell from storage and unwrap the outer package. Remove the flow cell from the inner package and inspect to make sure that it is intact.

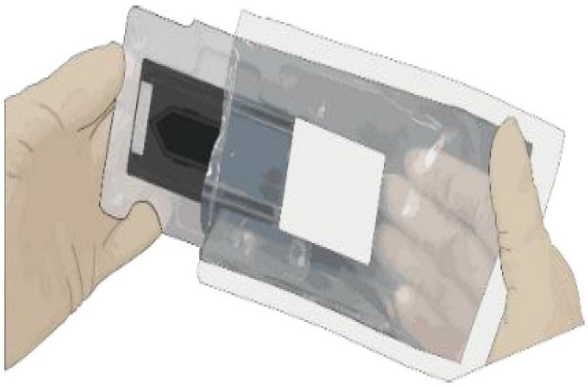

Figure 7. Unwrap the outer package of the flow cell

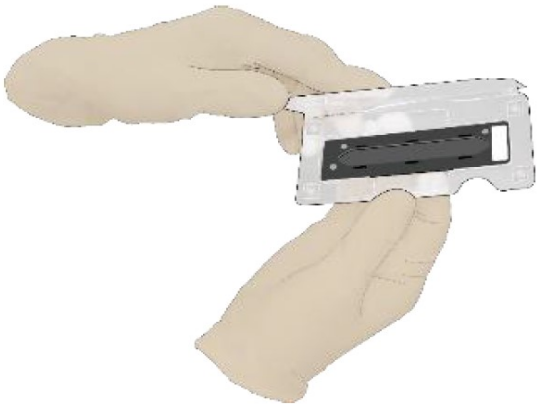

Figure 8. Inspect the flow cell

16.3. Log in into the main interface of the sequencer using the designated user name and password (This will be provided by the Lab Manager)

16.4. Click the sequencing option to load the DNB loading interface by entering the DNB ID or library name

16.5. Open the reagent compartment door, gently lift the sampling needle with one hand, remove the cleaning reagent with the other hand, load the sample tube, then slowly lower the sampling needle until the tip touches the bottom of the tube.

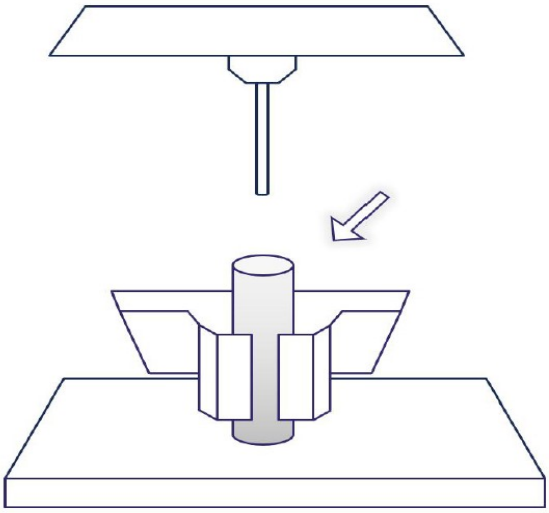

Figure 9. Load the DNB Tube

16.6. Select the sequencing parameters or recipe (E.g. DNBSEQ-G50RS High – throughput Sequencing Set FCLPE150 for PE150 sequencing using a large flow cell).

16.7. Choose the read length and select barcode length / demultiplexing

16.8. Open thereagent compartment, remove cleaning catridge and load the reagent catridge.

16.9. Open the flow cell compartment and use dust remover to remove dust from the flow cell stage.
16.10. Press the flow cell attachment button
16.11. Take out a new flow cell and hold it by the edges with both hands
16.12. Align the holes on the flow cell with the locating pins on the flow cell stage
16.13. Press the right and the left sides of the flow cell to ensure the flow cell is properly seated on the stage.
16.14. Click next the sequencer will automatically enter the flow cell ID. If automatic flow cell entry does not work, move the cursor to the "Flow cell ID", blank and manually enter the ID

Note:
The flow cell is fragile. It should be handled with caution.

When entering manually, the flow cell ID should be entered strictly according to the flow cell number on the label. Different sequencing recipe will be invoked based on the flow cell ID entered. Flow cell ID beginning with S2 is FCL and flow cell ID beginning with K2 is FCS.

A Lab manager will always be in the lab to assist in setting the sequencer up to perform sequencing.

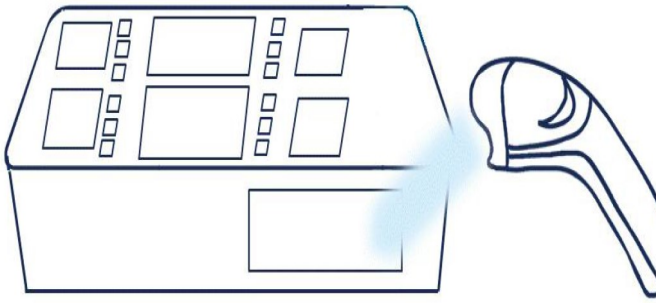

Figure 10. A Reagent Catridge information interface

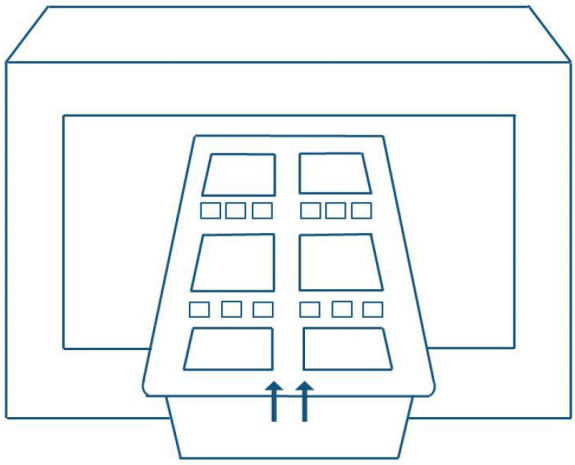

Figure 11. Loading a new Reagent Catridge

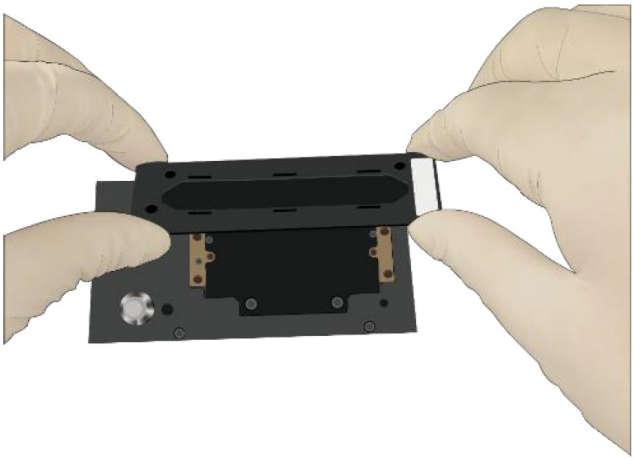

Figure 12. Loading a Flow Cell

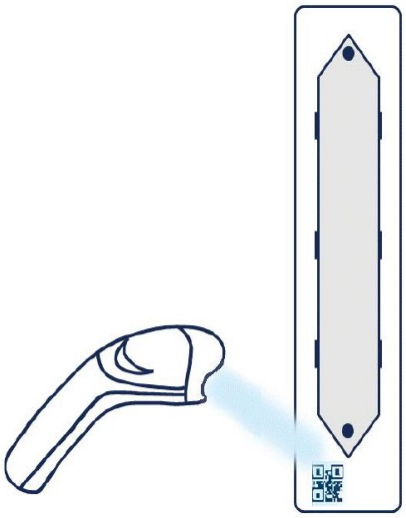

Figure 13. Flow cell information intrface

16.15. Review the sequencing parameter to ensure that all information is correct. . An example is given below in Table 19

16.16. Start sequencing

16.17. Once sequencing has started, immediately open the flow cell compartment door to ensure that DNB (or reagents) are flowing through the cell.

16.18. When sequencing is completed, click wash and change to a cleaning catridge.

Table 19. Reviewed sequencing parameter information

Review	Content
User name	user
DNB ID	123 | 1-128
Sequencing cartridge ID	A0001
Flow cell ID	S200000001
Recipe	PE150_FCL
Cycles	312