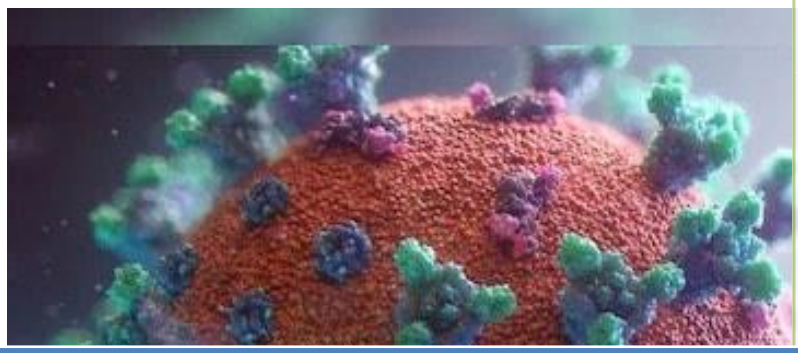
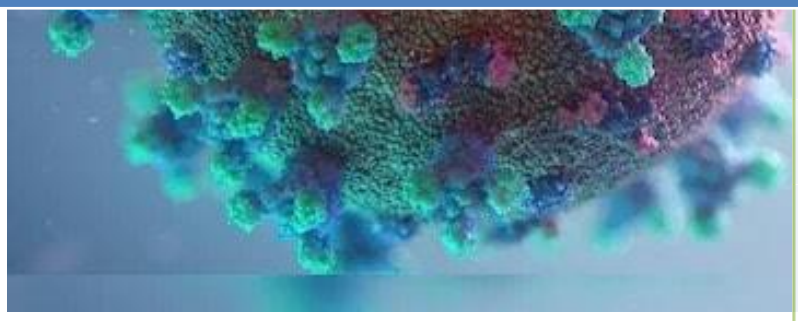


SOP

Sample collection, processing and Whole Genome Sequencing (WGS) of SARS-CoV-2 from Clinical Specimens



Pasteur Institute Shillong

March 2024

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INTRODUCTION

Oxford Nanopore Technologies' nanopore sequencing is widely used for Whole Genome Sequencing (WGS) of SARS-CoV-2. Currently, there are two methods available for nanopore sequencing SARS-CoV-2: (i) Midnight protocol, and (ii) ARTIC Classic protocol. Both methods employ a PCR tiling approach in which the viral genome is amplified in overlapping sections, maximising coverage across the full genome. This protocol describes the use of Midnight protocol for SARS-CoV-2 WGS of clinical samples.

Midnight protocol is a simple, rapid method of sequencing SARS-CoV-2 genomes at low cost per sample. The approach is highly flexible, allowing the on-demand sequencing of small numbers of samples or scaling up to high-throughput sequencing needs. Hands-on time is also minimal, facilitating automation. In the Midnight protocol, the SARS-CoV-2 genome is amplified in ~1,200 bp overlapping segments, making it more resilient to drop-out caused by mutations in the viral genome.

SARS-CoV-2 clinical specimens are first collected and run for RTPCR. Only RTPCR positive samples are subjected to WGS. In general, specimens with a cycle-threshold (Ct) value of ≤25 are considered appropriate for obtaining good quality whole genome sequences. Samples with Ct>30 may not give good whole genome sequence results but may still be appropriate for determining SARS-CoV-2 lineages or variants.

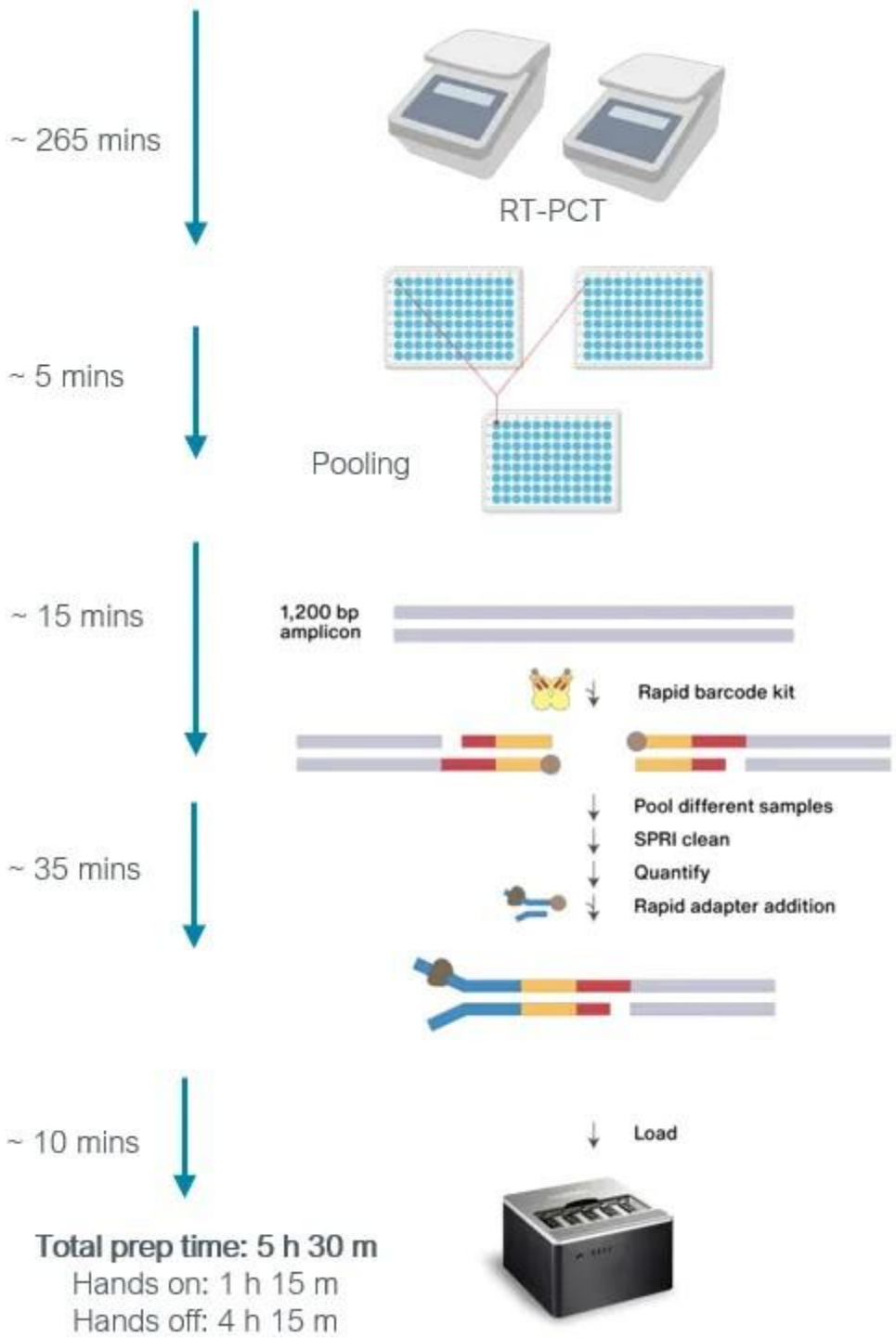


Figure 1: Illustration of the Midnight Protocol for SARS-CoV-2 WGS

1. MATERIALS REQUIRED FOR SPECIMEN COLLECTION

- Dacron or rayon flexible nasopharyngeal swab
- Dacron or rayon flexible oropharyngeal swab
- Viral transport media (test tube with screw cap, plastic preferred)
- Gloves (suggested gloves are powder-free)
- Mask for covering nose and mouth of health worker (N95 respirator)
- Facial tissues (for patient use)
- Eye protection/goggles
- Gown

2. PROCEDURE FOR COLLECTION OF SPECIMEN

A. NASOPHARYNGEAL SPECIMEN (NP) COLLECTION

1. Follow recommended infection control precautions including putting on N95 respirator, goggles, gown and gloves before proceeding.
2. Tilt patient's head back 70 degrees.
3. Gently and slowly insert a mini tip swab with a flexible shaft (wire or plastic) through the nostril parallel to the palate (not upwards) until resistance is encountered or the distance is equivalent to that from the ear to the nostril of the patient, indicating contact with the nasopharynx.
4. Gently rub and roll the swab.
5. Leave swab in place for several seconds to absorb secretions.
6. Slowly remove swab while rotating it. Specimens can be collected from both sides using the same swab, but it is not necessary to collect specimens from both sides if the mini tip is saturated with fluid from the first collection.
7. If a deviated septum or blockage creates difficulty in obtaining the specimen from one nostril, use the same swab to obtain the specimen from the other nostril.
8. Place swab; tip first, into the transport tube provided.

i. OROPHARYNGEAL SPECIMEN (OS) COLLECTION

1. Follow recommended infection control precautions including putting on N95 respirator, goggles, gown and gloves before proceeding.
2. Insert swab into the posterior pharynx and tonsillar areas.
3. Rub swab over both tonsillar pillars and posterior oropharynx and avoid touching the tongue, teeth, and gums.
4. Place swab; tip first, into the transport tube provided.

3. SAMPLE PROCESSING

A. RNA EXTRACTION:

Extraction of SARS-CoV-2 can be done using commercially available kits. This procedure described here is that for RNA extraction using the TRUPCR® viral RNA extraction kit (version 1.0).

Kit Contents

- TRUPCR® Silica Membrane Binding Columns
- Collection Tubes
- Buffer BAVL1* (Lysis Buffer)
- BAW1* (Concentrate) (Wash Buffer-I)
- BAW2 (Concentrate) (Wash Buffer-II)
- Buffer BRE (Elution Buffer)
- Carrier RNA (Poly A)ǂ
- Protocol insert

Storage

1. Kit components except Carrier RNA should be stored at room temperature (15 - 25 °C).
2. Carrier RNA should be stored at -20 °C.
3. TRUPCR® Silica Membrane Binding Columns should be stored dry at room temperature (15– 25°C); storage at higher temperatures should be avoided.

Procedure

1. Pipette 560 µl of prepared Buffer BAVL1 containing carrier RNA into a 1.5 ml micro centrifuge tube.

2. Add 200 µl of sample into the buffer BAVL1-carrier RNA containing micro centrifuge tube. Note: (It is essential that the sample is mixed thoroughly with the Lysis Buffer BAVL1 to yield a homogenous solution for efficient lysis of the sample).

3. Incubate the mixture at room temperature (15-25°C) for 10 minutes.

4. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.

5. Add 560 µl of molecular biology grade ethanol (96–100%) to the sample, close the cap and mix thoroughly by pulse-vortexing for 15 seconds. Incubate the lysate with the ethanol for 5 minutes at room temperature (15–25°C).

Note: If ambient temperature exceeds 25°C, ethanol should be cooled on ice before adding to the lysate.

6. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.

7. Carefully apply 630 µl of the lysate from step 5 onto the TRUPCR® silica membrane binding column without wetting the rim. Close the cap and centrifuge at 10,000 rpm for 1 minute. Place the column in collection tube, if the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the column is empty.

8. Discard the lysate from the collection tube. Carefully open the Spin column and repeat step 7.

9. Place the column into same collection tube carefully, open the column, and add 500 µl of Buffer BAW1 without wetting the rim. Close the cap and centrifuge at 10,000 rpm for 1 minute and discard the filtrate.

10. Place the column into same collection tube carefully, open the column, and add 500 µl of Buffer BAW2 without wetting the rim. Close the cap and centrifuge at full speed (≈20,000 x g; 14,000 rpm) for 3 minutes and discard the filtrate with collection tube.

11. ***Recommended***: Place the column in a new 2 ml collection tube. Centrifuge at full speed (≈20,000 x g; 14,000 rpm) for 1 minute to dry the membrane completely.

12. Place the column in a clean 1.5 ml micro-centrifuge tube (not provided) and discard the collection tube. Carefully open the lid of the column and apply 30-40 µl of Buffer BRE or RNase-free water directly to the centre of the membrane. Close the lid and incubate at room temperature for 1 minute. Centrifuge at 10,000 rpm for 1 minute.

Important:

Ensure that the elution buffer is equilibrated to room temperature. If elution is done in small volumes (<30 µl) the elution buffer must be dispensed onto the centre of the membrane for complete elution of bound RNA.

B. DETECTION

Detection of SARS-CoV-2 is done using standard RTPCR detection kits. Here, the used of the TRUPCR® SARS-CoV-2 RT qPCR kit (V 3.2) is described in detail.

Kit Contents

Reagent	Description
Master Mix	• Hot-start DNA polymerase • Reaction Buffer • dNTPs (dATP, dCTP, dGTP, dTTP) • MgCl2 and stabilizers
Enzyme Mix	• Enzyme Mix for RT
Primer Probe Mix	Primer Probe mix for E gene, RdRP gene & N gene and human RNaseP gene detection
Negative Control	Sterilized water
Positive Control	Positive control

Storage and Handling:

All the components of TRUPCR® SARS-CoV-2 RT qPCR kit should be stored at -20˚C and are stable until the date of expiry stated. Do not use reagents past their expiration date. The reagents can be aliquoted and stored at -20˚C in order to maintain the stability and sensitivity.

Materials required:

- TRUPCR® Viral RNA Extraction Kit (3B213V/3B214V)
- Adjustable pipettes with sterile filter or positive displacement tips
- Disposable powder-free gloves
- Sterile bi-distilled water
- Sterile 1.5 ml and 2 ml micro centrifuge tubes
- 50 ml conical tubes
- Vortex mixer
- Real time PCR
- Laminar airflow cabinet
- PCR vials (0.2 ml, thin-walled)
- 96 – 100% ethanol
- Personal protection equipment (lab coat, gloves, goggles)

I. Reaction Preparation:

Prepare the reaction mix as follows:

Name of the Reagent	For 1 reaction
Master Mix	10.0 µl
Enzyme Mix	0.35 µl
Primer Probe Mix	4.65 µl
Total reaction	**15.0µl**

1. Transfer 15 µL of the above prepared Reaction mix into a 0.2 mL PCR tube and close the tube.
2. Add 10 µL of RNA sample, positive control, or negative control to make a final volume of 25 µL.

II. Program Setup:

Define the following setting for temperature profile

Step	Temperature, ˚C	Time	Dye acquisition	Cycles
1	50	15 Minutes	-	1
2	95	05 Minutes	-	1
3	95	05 Seconds	-	38
4	60	40 Seconds	Yes	
5	72	15 Seconds	-	

III. Channel Selection

Detector Name	Reporter	Quencher
***E* gene**	Texas Red/Orange/ROX	None
***RNaseP* gene**	HEX/Yellow/VIC	None
***RdRp* & *N* gene**	FAM/Green	None

IV. Result Analysis

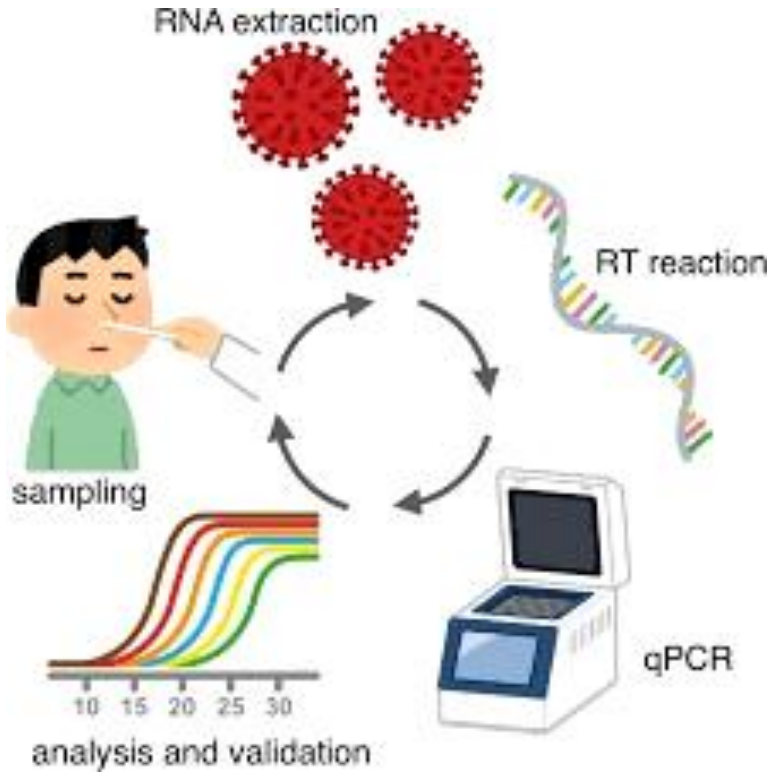
1. The negative control reactions for probe/primer sets should not exhibit fluorescence growth curves that cross the threshold line. If a false positive occurs with one or more of the primers and probe Non Template Control (NTC) reactions, sample contamination may have occurred.
2. The positive control reactions for each probe/primer reactions should give following Ct values:

Serial Number	Channels	Positive Control	Expected Ct Values
1.	Texas Red/Orange/ROX	*E gene*	22±5
2.	FAM/Green	*RdRp + N gene*	22±5
3.	HEX/VIC/Yellow	*RNaseP gene*	22±5

3. All clinical samples should exhibit RNaseP reaction curves that cross the threshold line at or before 35 cycles, thus indicating the presence of sufficient RNA from human RNaseP gene indicating the specimen is of acceptable quality. However, it is possible that some samples may fail to give positive reactions due to low cell numbers in the original clinical sample. Failure to detect RNaseP in any of the clinical samples may indicate:
    a. Improper extraction of nucleic acid from clinical materials resulting in loss of RNA or carry-over of RT-PCR inhibitors from clinical specimens
    b. Absence of enough human cellular material in sample to enable detection
    c. Improper assay set up and execution
    d. Reagent or equipment malfunction
4. **Cutoff-** This assay runs for 38 cycles however for easy interpretation, threshold cutoff cycle Ct is 35.
5. When all controls meat stated requirements, a specimen is considered with following interpretations:

Case	Amplification Signals in			Results	Interpretation
	RNaseP (HEX/Yellow/VIC)	*E Gene* (Texas Red/Orange/ROX)	*RdRp/N gene* (FAM/Green)		
1	+/-	+	+	SARS-Cov-2 Positive	Sarbecovirus (E gene) and SARS-CoV-2 (N or/and RdRp) specific RNA detected
2	+/-	-	+	SARS-Cov-2 Positive	SARS-CoV-2 (N or/and RdRp) specific RNA detected. Test sample is Positive for SARS-Co-2
3	+/-	+	-	Sarbecovirus Positive	Test sample is Presumptive Positive for Sarbecovirus. For samples with the same result on a repeat test, additional confirmatory testing may be conducted, if it is necessary to differentiate between SARS-CoV-2 and SARS-CoV-1 or other Sarbecovirus

					currently unknown to infect human, for epidemiological purposes or clinical or clinical management.
4	+	-	-	Negative	Test Sample is Negative for SARS-Co-2
5	-	-	-	Invalid	Results are invalid. Repeat test. If the result is still invalid, a new specimen should be obtained.



4. RAPID BARCODING SEQUENCING

This protocol describes how to carry out rapid barcoding of genomic DNA using the Rapid Barcoding Sequencing Kit (SQK-RBK109).

A. MATERIALS REQUIRED

- Flow Cell Priming Kit (EXP-FLP002)
- Loading Beads (LB)
- Sequencing Buffer (SQB)

B. CONSUMABLES

- 1.5 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937)

C. EQUIPMENT

- MinION Mk1B or Mk1C
- SpotON Flow Cell
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

D. REVERSE TRANSCRIPTION:

1. In a clean pre-PCR hood, using a stepper pipette, or a multiple channel pipette, add 4 µl of LunaScript ^{TM} RT SuperMix to a fresh 96-well plate (RT Plate).
2. To each well containing LunaScript reagent of the RT plate, add 16 µl of sample and gently mix by pipetting. If adding less than 16 µl, make up the rest to the volume with Nuclease-free water.
3. Seal the RT plate and spin down. Return the plate to ice.
4. Preheat the thermal cycler to 25^0C.
5. Incubate the samples in the thermal cycler using the following program:

Temperature	Time
25^{0}C	2 minutes
55^{0}C	10 minutes
95^{0}C	1 minutes
4^{0}C	Hold

E. PCR:

a. In the pre-PCR hood, prepare the following master mixes in Eppendorf DNA LoBind tubes and mix thoroughly as follows:

Reagent	Pool A	Pool B
Rnase free Water	7 µl	7 µl
Primer Pool A (10pmole)	3 µl	-
Primer Pool B (10pmole)	-	3 µl
Q5 Hot Start HF 2x Master Mix	12.5 µl	12.5 µl

b. Using a stepper pipette or a multichannel pipette, aliquot 22.5 µl of Pool A and Pool B into a clean 96-well plate(s).

c. Using a multichannel pipette, transfer 2.5 µl of each RT reaction from the RT plate to the corresponding well for both Pool A and Pool B of the PCR plate(s). Mix by pipetting the contents of each well up and down.

d. Seal the plate(s) and spin down

e. Incubate using the following program, with the heated lid set to 105 ^{0}C:

Step	Temperature	Time	Cycles
Initial denaturation	98^{0}C	30 sec	1
Denaturation	98^{0}C	15 sec	35
Annealing and Extension	65^{0}C	5 min	
Hold	4^{0}C	∞	

F. ADDITION OF RAPID BARCODES:

1. Spin down the Rapid Barcode plate and PCR reactions prior to opening to collect material in the bottoms of the wells.
2. Using a multichannel pipette, transfer 2.5 µl from the Rapid Barcode plate to the corresponding well of the Barcode Attachment Plate.
3. Using a multichannel pipette, transfer 25 µl of each well of PCR Pool B to the corresponding well of PCR Pool A and mix by pipetting.
4. Using a multichannel pipette, transfer 5 µl from each well of PCR Pool A (now containing pooled PCR products) to the corresponding well of the Barcode Attachment Plate and mix by pipetting.
5. Using a stepper pipette or a multichannel pipette, add 2.5 µl of nuclease-free water to the wells in a clean 96-well plate (Barcode Attachment Plate).
6. Seal the Barcode Attachment Plate and spin down.
7. ***Incubate the plate in a thermal cycler at 30^{0}C for 2 minutes and then at 80^{0}C for 2 minutes.***

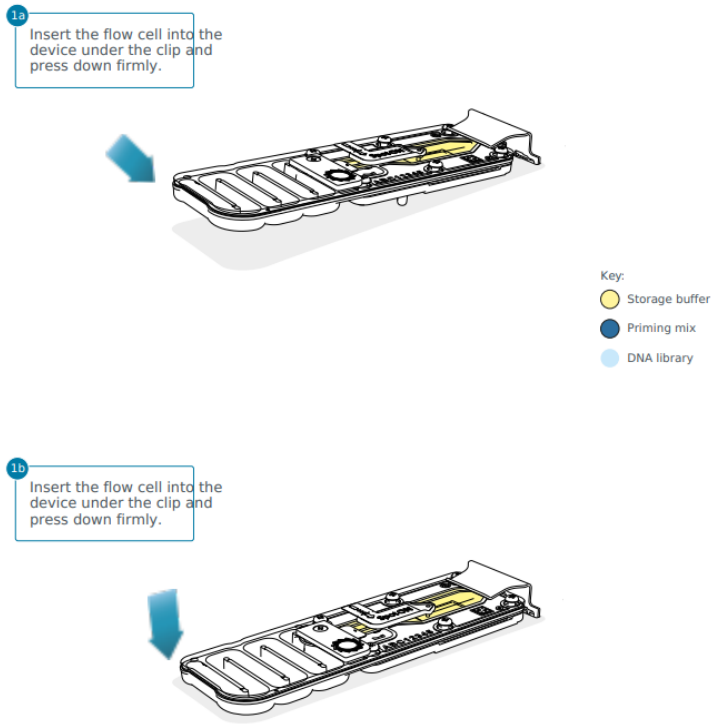
G. POOLING SAMPLES AND CLEAN-UP:

1. Briefly spin down the Barcode Attachment Plate to collect the liquid at the bottom of the wells prior to opening.
2. Pool the barcode samples in 2 ml Eppendorf DNA LoBind tube.
3. Resuspend the SPRI beads by vortexing.
4. To the entire pool barcoded sample, add an equal volume of resuspended SPRI beads and mix by flicking the tube.

5. Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.
6. Prepare atleast 3 ml of fresh 80% ethanol in nuclease free water.
7. Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
8. Keep the tube on the magnet and wash the beads with 1.5 ml of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
9. Repeat the previous step.
10. Briefly spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 30 seconds, but do not dry the pellet to the point of cracking.
11. Remove the tube from the magnetic rack and resuspend the pellet by pipetting in 30 µl elution buffer (EB). Incubate for 10 minutes at room temperature.
12. Pellet the beads on a magnet until the eluate is clear and colourless.
13. Remove and retain 30 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.
14. Dispose the pellet beads.
15. Take forward 600-800ng of library and make up the volume to 11 µl with EB.
16. Add 1 µl of Rapid Adapter F (RAP-F) to 11 µl of barcoded DNA.
17. Incubate at room temperature for 5 minutes.

H. PRIMING AND LOADING THE SPOTON FLOW CELL

1. Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature before mixing the reagents by vortexing, and spin down at room temperature.
2. To prepare the flow cell priming mix, add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at room temperature.
3. Open the MinION device lid and slide the flow cell under the clip.
   Press down firmly on the flow cell to ensure correct thermal and electrical contact.



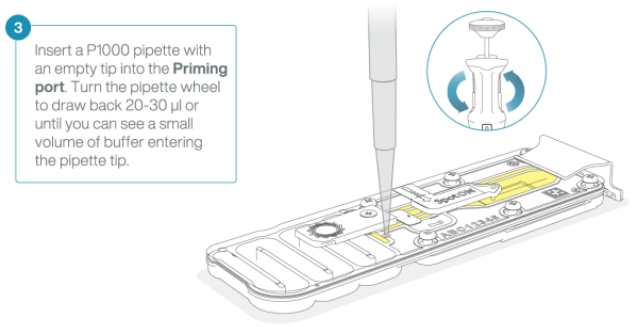
4. Slide the flow cell priming port cover clockwise to open the priming port.

IMPORTANT
Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the arrays of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

5. After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:
   I. Set a P1000 pipette to 200 µl
   II. Insert the tip into the priming port
   III. Turn the wheel until the dial shows 220-230 ul, to draw back 20-30 ul, or until you can see a small volume of buffer entering the pipette tip

Note: Visually check that there is continuous buffer from the priming port across the sensor array.

6. Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes. During this time, prepare the library for loading by following the steps below.



7. Thoroughly mix the contents of the Loading Beads (LB) by pipetting.

IMPORTANT

The Loading Beads (LB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

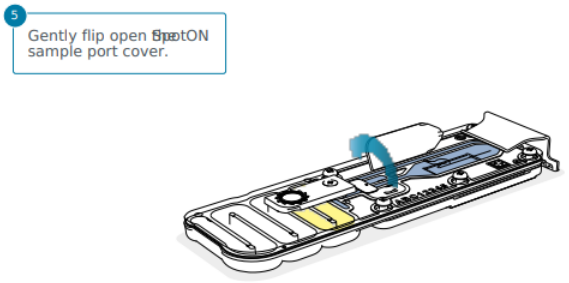
8. In a new tube, prepare the library for loading as follows:

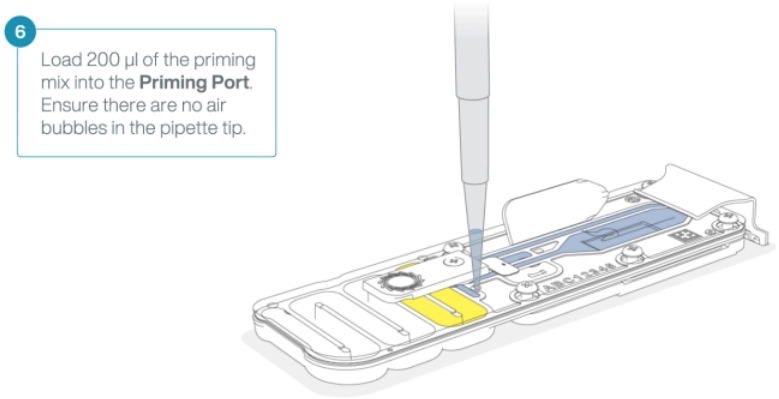
Reagent	**Volume per flow cell**
Sequencing Buffer (SQB)	37.5 µl
Loading Beads (LB), mixed immediately before use	25.5 µl
DNA library	12 µl
Total	75 µl

Note: Load the library onto the flow cell immediately after adding the Sequencing Buffer (SQB) and Loading Beads (LB) because the fuel in the buffer will start to be consumed by the adapter.

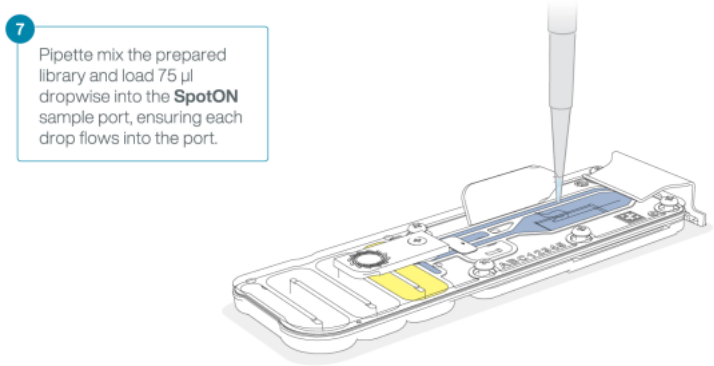
9. Complete the flow cell priming:
    - Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

- Load 200 µl of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.



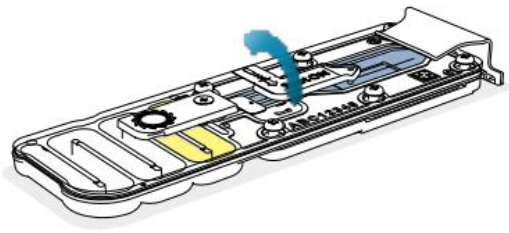


10. Mix the prepared library gently by pipetting up and down just prior to loading.
11. Add 75 µl of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

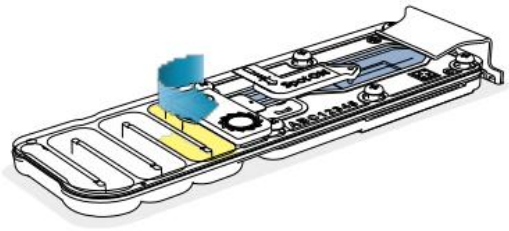


12. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION device lid.

8 Gently replace the **SpotON** sample port cover.



9 Gently close the **Priming port.**



I. SETTING UP THE MINION MK1C AND SEQUENCING

The device described here combines the real-time, rapid, portable sequencing of a MinION Mk1C with a Graphical Processing Unit (GPU) and a high resolution screen. It can be used with MinION Flow Cells, as well as the Flongle Adapter and flow cells. The device is operated by the MinKNOW software that controls the device, experimental scripts, and performs basecalling.

1. Performing a flow cell check.

A flow cell check must be carried out before loading a DNA or RNA library to assess the number of pores available.

- **I.** Open the device and slide your flow cell under the clip. Firmly press down on the flow cell. For Flongle, slide the Flongle Adapter under the clip and insert a Flongle Flow Cell.
- **II.** Navigate to the Start page and click Flow Cell Check.
- **III.** MinKNOW will recognise the MinION Flow Cell type and IDs. Note: For Flongle, fill in the flow cell ID manually.
- **IV.** Click Start and the flow cell check will begin.

2. Setting up a sequencing run.

Prime and load the flow cell with DNA or RNA library by following a library prep protocol and set up a sequencing run, as follows:

- **I.** Navigate to the Start page and click Start Sequencing.
- **II.** In the Positions tab, fill in the experiment name, sample ID, and select the flow cell type. Click Continue to kit selection. Note: For Flongle, fill in the flow cell ID manually.
- **III.** Select the kit and any expansion(s) used to prepare the library. Click Continue to run options.
- **IV.** Specify the sequencing run length and minimum read length or keep the default settings. Click Continue to analysis.
- **V.** Choose your basecaller model and select any available barcoding and alignment options or keep the default settings. Click Continue to output.

VI. Specify your output data location, format and filtering options or keep the default settings. Click Continue to final review.

VII. Click Start.

3. Data acquisition and basecalling

The sequencing device control, data acquisition and real-time basecalling are carried out by the MinKNOW software.

Basecalling models can be selected at two stages in the MinKNOW software:

Real-time basecalling: Basecalling model can be selected when setting up a sequencing run on the "Run options" page of MinKNOW. This is basecall the sequencing run in real-time.

Post-run basecalling: Basecalling can be set up post-sequencing run on MinKNOW. Instructions can be found in the “Post-run basecalling" section of this protocol.

J. DATA ANALYSIS

The native format of the raw data from MinION is the “Fast5” format. The reads in Fast5 can be easily extracted into the FASTQ format using poretools. However, there are numerous software available which do the conversion to standard formats and also generate consensus sequences in FASTA formats. One such example is the Commander Software from Genotypic. Once the FASTA files are obtained, they can be uploaded onto online data bases like Nextclade (https://clades.nextstrain.org/) or Pangolin (https://pangolin.cog-uk.io/) and the SARS-CoV-2 variants or lineages are then obtained.