Aug 08, 2024

RSV Nanopore Whole Genome Sequencing

This protocol is a draft, published without a DOI.

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Protocol Citation: Rebecca Dewar, Martin McHugh, Seb Cotton, Goncalo Fernandes, Daniel Maloney, Kate Templeton 2024. RSV Nanopore Whole Genome Sequencing. **protocols.io https://protocols.io/view/rsv-nanopore-whole-genome-sequencing-dhp835rw**

Protocol status: Working
We use this protocol and it's working

Created: July 23, 2024

Last Modified: August 08, 2024

Protocol Integer ID: 103904

Keywords: RSV, WGS, ARTIC, Nanopore, Amplicon, Sequencing

Abstract

This is the Nanopore sequencing protocol used with ARTIC RSV primers to sequence RSV samples in the Royal Infirmary of Edinburgh, which has been utilised in this virological post (https://virological.org/t/preliminary-results-from-two-novel-artic-style-amplicon-based-sequencing-approaches-for-rsv-a-and-rsv-b/918). Primer scheme details can be found on the ARTIC RSV github page (https://github.com/artic-network/artic-rsv).

This protocol is used for routine RSV sequencing here, but these RSV primers have been developed to slot in to any existing amplicon sequencing protocols used in your lab, and have been used successfully in a range of different labs (publication in preparation).

Materials

Equipment:

GridION or MinION sequencer (Oxford Nanopore Technologies)
Magnet rack DynaMag-2 Magnet (Thermo Fisher)
Qubit 3.0 Fluorometer (Thermo Fisher)
Veriti 96-well thermocycler (Thermo Fisher)
Bio-rad thermocyclers (Bio-rad, C1000) Benchtop microfuge (Mikro200; Hettich)
Benchtop centrifuge (Thermo CL10)
Vortex
P10, P20, P200, P1000 pipette (ErgoOne, Starlab)
8-channel multi-channel pipette (0.5-10 µl) (ErgoOne, Starlab)
8-channel multi-channel pipette (20-200 µl) (Rainin)
Electronic multi-dispense pipette (10-100 µl) (Rainin)
10 µl, 20 µl, 200 µl and 1000 µl filtered pipette tips (Rainin, Cleaver, TipOne)
Invitrogen E-Gel Power Snap System (Thermo Fisher)

Consumables & Reagents:

LoBind 1.5ml tubes (Fisher scientific)
LoBind 2ml tubes (Fisher Scientific)
96-well PCR plates (Fisher Scientific)
Hard-Shell 96 well Low Skirted plates (Bio-rad)
Microseal adhesive seals (Bio-rad)
8 strip domed PCR caps (Fisher Scientific)
Qubit dsDNA High Sensitivity Kit (Thermo Fisher)
ARTIC V1 RSV Primer Pool 1
ARTIC V1 RSV Primer Pool 2
E-Gel EX 1% Agarose (Thermo Fisher)
100bp DNA Ladder (Promega)
70% Ethanol
R9.4.1 Flow cell (Oxford Nanopore Technologies, FLO-MIN106D)

NEBNext ® ARTIC SARS-CoV-2 Companion Kit (Oxford Nanopore Technologies):

- LunaScript RT SuperMix
- Q5 Hot Start High-Fidelity 2X Master Mix
- NEBNext Ultra II End Prep Enzyme Mix
- NEBNext Ultra II End Prep Buffer
- Blunt/TA Ligase Master Mix
- NEBNext Quick T4 DNA Ligase
- NEBNext Quick Ligation Reaction Buffer (5x)
- Nuclease-free water
- NEBNext Sample Purification Beads

NEBNext Ultra II Ligation Module:

- NEBNext Ultra II Ligation Master Mix

- NEBNext Ligation Enhancer

Native Barcoding Expansion 96 (Oxford Nanopore Technologies, EXP-NBD196);
- Adapter Mix (AMII)

SFB expansion (Oxford Nanopore Technologies, EXP-SFB001)
- Short Fragment Buffer (SFB)

Flow cell priming kit (Oxford Nanopore Technologies, EXP-FLP002)
- Flush Tether (FLT)
- Flush Buffer (FB)

Sequencing Auxiliary Vials (Oxford Nanopore Technologies, EXP-AUX001)
- Elution buffer (EB)
- Sequencing Buffer (SQB)
- Loading Beads (LB)

Before start

This protocol requires nucleic acid extracts from RSV positive specimens. Nucleic acid extracts tested have been extracted using either the Biomerieux easyMAG or eMAG automated extractors.

Reverse Transcription (RT) using LunaScript RT SuperMix

1 Retrieve extracts based on run worksheet.

2 Invert tubes and pulse spin to 3000 rpm to remove drops from lids.

3 Switch on thermal cycler.

4 In clean room, label a PCR plate with RSV, date, run number, and “RT”. Also mark top right-hand corner of plate for orientation.

5 Place RT plate on cold block and add 2 µL RT Supermix to each well.

6 Seal the plate with 8-cap domed PCR lids or plate seal.

7 Add 8 µL of extract to each well of the RT plate (use cold block), based on the plate layout in the run worksheet including NTC (nuclease-free water) and positive control.

8 Seal the plate with 8-cap domed PCR lids, invert to mix, and pulse centrifuge to remove droplets from lids.

9 Load plate into thermal cycler using the cycling conditions below:

Step	Temp	Time	Cycles
Primer Annealing	25 oC	2 min	1
cDNA Synthesis	55 oC	20 min	
Heat Inactivation	95 oC	1 min	
Hold	4 oC	Hold	

SAFE STOP - Samples can be stored at -20 °C if not used immediately.

Tiled Whole Genome PCR Amplification using Q5 Polymerase and Artic primers

10 In clean room, thaw PCR reagents at room temperature. Keep Q5 polymerase in freezer until ready for use.

11 Invert tubes and pulse spin to 3000 rpm to remove drops from lids.

12 Label a PCR plate with RSV, date, run number, and “PCR”. Also mark on the plate wells for pool A and pool B and the mark top right-hand corner of plate for orientation. Place plate on cold block. N.B. For example, for 48 samples 96-well PCR plate pool A (columns 1-6) and B (columns 6-12).

13 Label 2 x 1.5 ml Eppendorf tubes; one ‘Mix 1’ and one ‘Mix 2’.

14 Make two PCR mixes – one using primer mix 1 and one using primer mix 2.

No. of samples	1	12	24	48	96
Compnent	x1 (ul)	x14 (ul)	x26 (ul)	x50 (ul)	x98 (ul)
Q5 Hot Start High-Fidelity 2x Master Mix	6.25	87.5	162.5	312.5	612.5
ARTIC RSV Primer Mix set 1 or 2	2	28	52	100	196
Nuclease free water	0.25	3.5	6.5	12.5	24.5

15 Add 8.5 µL of the ‘Mix 1’ PCR mix into half the columns and ‘Mix 2’ PCR mix into the other half of the columns on the PCR plate.

16 Seal the plate with 8-cap domed PCR lids.

17 Using a multichannel pipette, add 4 µL of RT product to the PCR plate for both ‘Mix 1’ and ‘Mix 2’

18 Seal the plate with 8-cap domed PCR lids or plate seals, invert to mix, and centrifuge to remove droplets from lids.

19 Transfer to the thermocycler and place the PCR plate on a thermocycler using the cycling conditions below.

Step	Temp	Time	Cycles
Heat Activation	98 oC	30 sec	1
Denaturation	95 oC	15 sec	35
Annealing	63 oC	5 min	
Hold	4 oC	Hold	

SAFE STOP - Samples can be stored at -20 °C if not used immediately.

Pooling and diluting of PCR product

2m 6s

20 Label 96-well plate(s) with RSV, date, run number and mark top right corner of plate.

21 Aliquot 45 µL of nuclease-free water into each well.

22 Using a multichannel pipette, aliquot 2.5 µL of Mix 1 PCR product and 2.5 µL of Mix 2 PCR products into the same well.

Component	Volume (ul)
Mix 1 PCR reaction	2.5
Mix 2 PCR reaction	2.5
Nuclease free water	45

23 Re-seal the plate with 8-cap strip lids, invert to mix, and centrifuge to remove droplets from lids.

24 Quantify the positive control (P), the negative control (N), the negative extraction control(s) (EX) and one sample at random (R) using, for example, the Qubit dsDNA HS kit. For full user manuals refer to EXT-995 Qubit 3 Fluorometer and EXT-555 Qubit dsDNA HS assay

24.1 Set up the required number of 0.5 ml Qubit tubes for standards and samples. Qubit assay required 2 standards (S1 and S2).

24.2 Label Qubit assay tube lids as follows (P, N, (E1/E2), R, S1 and S2). N.B. not all PCR products will be checked but two further Qubit reactions will be performed later in the protocol.

24.3 Prepare Qubit working solution in a 5 ml Universal tube as follows:

	Component	x1 (ul)	x10 (ul)
	Qubit dsDNA HS Reagent	1	10
	Qubit dsDNA HS Buffer	199	1990

24.4 Add 190 µL of Qubit working solution to tube S1 and S2.

24.5 Vortex each Qubit standard then add 10 µL to appropriate tube and mix by vortexing for 00:00:03 . 3s

24.6 Add 199 µL of Qubit working solution to tubes P, N and R.

24.7 Add 1 µL of sample to appropriate tube and mix by vortexing for 00:00:03 . 3s

24.8 Allow all tubes to incubate at room temp for 00:02:00 . 2m

24.9 On the home screen of the Qubit 3.0 Fluorometer press DNA and select dsDNA High Sensitivity as assay type. Press Read Standards

24.10 Insert S1 tube into sample chamber, close lid and press read standard. Note S1 reading in run worksheet next to ‘S1 reading’.

24.11 Perform as above for S2.

24.12 Press Run samples, select sample volume as ‘1µl’ and units as ‘ng/µl’.

24.13 Insert positive control tube into sample chamber and read tube.

24.14 Repeat as above for negative and random control tube.

25 For the negative controls expect 0.5-2 ng/µl. For the positive control expect >6 ng/µl.

26 Record qubit conc. (ng/µl) on run worksheet.

End repair and dA-tailing of DNA using NEBNext Ultra II End Prep

27 Prepare the following End Prep mastermix in 1.5 ml Eppendorf tube.

No. of samples	1	12	24	48	96
Compnent	x1 (ul)	x14 (ul)	x26 (ul)	x50 (ul)	x98 (ul)
Ultra II End Prep Reaction Buffer	1.75	24.5	45.5	87.5	171.5
Ultra II End Prep Enzyme Mix	0.75	10.5	19.5	37.5	73.5
Nuclease free w ater	0.75	10.5	19.5	37.5	73.5

28 Place PCR plate on cold block and aliquot 10 µL of the End Prep mastermix to each well.

29 Using a multichannel pipette, aliquot 5 µL of PCR dilution from previous step into End Prep mastermix.

30 Re-seal the plate with 8-cap strip lids or plate seal, invert to mix, and centrifuge to remove droplets from lids.

31 Transfer to the thermocycler using the cycling conditions below:

Temperature	Time
20oC	10 min

Temperature	Time
65oC	10 min
4oC	hold

Attachment of native barcodes using NEBNext Ultra II Ligation

32 Thaw EXP-NBD196 (1-96) barcoding kit at room temperature then centrifuge at 2000 rpm to remove drops from lids.

33 Prepare the following Ligation mastermix in 1.5 ml Eppendorf tube.

No. of samples	1	12	24	48	96
Compnent	x1 (ul)	x14 (ul)	x26 (ul)	x50 (ul)	x98 (ul)
Ultra II Ligation Mastermix	5.0	70	130	250	490
Ligation Enhancer	0.2	2.8	5.2	10	19.6
Nuclease free water	2.1	29.4	54.6	105	205.8

34 Place PCR plate on cold block and aliquot 7.3 µL of the Ligation mastermix to each well.

35 Using a multichannel pipette, add 1.2 µL of NBXX barcode per well. Refer to run worksheet as to which EXP-NBD196 (1-96) columns to use.

36 Using a multichannel pipette, aliquot 1.5 µL of End Prep reaction mixture from previous step into plate containing Ligation mastermix and barcodes.

37 Re-seal the plate with 8-cap strip lids, invert to mix, and centrifuge to remove droplets from lids.

38 Transfer to the thermocycler using the cycling settings below:

Temperature	Time
20oC	40 min
65oC	10 min
4oC	hold

SAFE STOP - Samples can be stored at -20 °C if not used immediately.

DNA purification using NEBNext Sample Purification beads (SFB and ethanol) 32m

39 Mix the NEBNext beads thoroughly before use.

40 Label 2 ml LowBind PCR tube and pool each barcoding reaction into the one tube (see table below for volume of barcoding reaction).

41 Add 0.4x volume of beads (see table below) to tube with barcoded samples and mix gently by flicking.

No. of samples	1	12	24	48	96
Volume of each barcoding reaction to pool	10	10	10	10	5
Total pooled volume	10	120	240	480	480
0.4x SPRI beads	4	48	96	192	192

42 Pulse spin to 3000 rpm to remove drops from lids.

43 Incubate tube for 00:05:00 at room temperature. 5m

44 Place back of tube (lid opening facing forward) on magnetic rack and incubate for at least 00:05:00 until beads have pelleted and supernatant is completely clear. 5m

45 Using P200, slowly remove and discard the supernatant without touching the pellet.

46 Add 250 µL SFB to tube without touching pellet and resuspend beads completely by gently flicking tube and pipette mixing.

47 Pulse spin to 3000 rpm to remove drops from lids.

48 Place back of tube (lid opening facing forward) on magnetic rack and incubate for at least 00:05:00 until beads have pelleted and supernatant completely clear. 5m

49 Using P200, slowly remove and discard supernatant without touching the pellet.

50 Repeat steps 46-49 to perform a second SFB wash.

51 Pulse spin to 3000 rpm to remove drops from lids.

52 Place back on magnet and using P10, remove any residual SFB at bottom of tube.

53 Keeping tube on the magnet, slowly add 200 µL of fresh 70% ethanol over pellet to bathe (without touching pellet with tip).

54 Slowly remove ethanol with P200.

55 Pulse spin to 3000 rpm to remove drops from lids.

56 Place back of tube (lid opening facing forward) on magnetic rack.

57 Using P10, remove any residual ethanol at bottom of tube.

58 Open lid of tube to allow pellet to air dry at room temperature for at least 00:05:00 . NB pellet should it lose it shine but do not allow pellet to completely dry and crack. 5m

59 Add 30 µL Elution Buffer (EB) to tube without touching pellet and resuspend beads completely by gently flicking tube and pipette mixing.

60 Pulse spin to 3000 rpm to remove drops from lids.

61 Incubate tube for 00:05:00 at room temperature. 5m

62 Place back of tube (lid opening facing forward) on magnetic rack and incubate for up to 00:10:00 until beads have pelleted and supernatant completely clear. 10m

63 Transfer supernatant to 0.3 ml PCR tube without touching or disrupting the beads. N.B. ensure no beads are transferred and supernatant is completely clear.

64 Quantify 1 µL of barcoded amplicon pool using the Qubit fluorometer protocol as above (Step 24).

65 Record qubit conc. (ng/ul) on run worksheet. N.B. Expected DNA quantification; at least 1-4 ng/µl for every 24 samples to proceed to next step.

Attachment of sequencing adapters using NEBNext Quick Ligation Reaction Buffer and Quick T4 DNA Ligase 20m

66 In the 0.3 ml PCR tube with the barcoded amplicon pool, add the following components:

Component	Volume (ul)
NEBNext Quick Ligation Reaction Buffer (5x)	10
Adapter Mix (AMII)	5
Quick T4 DNA Ligase	5

67 Transfer to a thermocycler at 25 °C for 00:20:00 . 20m

Checking the number of pores on flow cell before sequencing run 15m

68 Retrieve a new flow cell and insert into either a MinION or GridION sequencer.

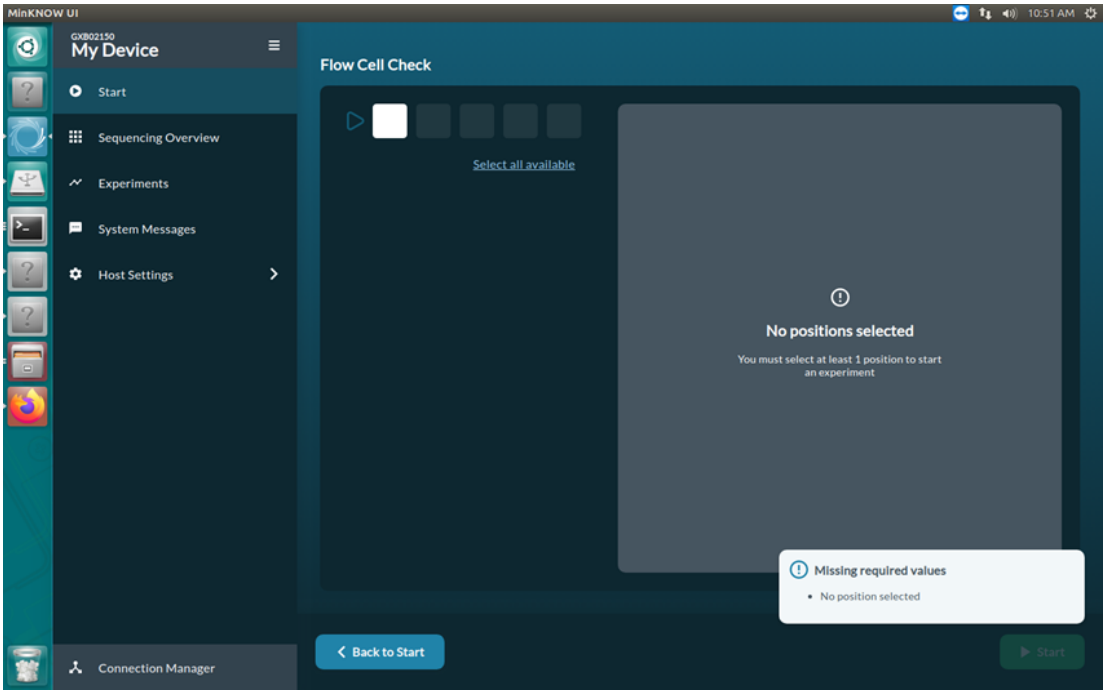
69 Slide open GridION window and place MinION flow cell into one of the five GridION ports (named X1-X5) (N.B rotate the port location for each run) push one end of flow cell under clip and push gently to clip other end of flow cell.

70 Log on to computer connected to GridION.

71 Click on MinKNOW software icon to open:

72 Select the ‘Start’ tab on left-hand side and click on ‘Flow Cell Check’ box.

73 Available flow cells should appear as white squares. Select flow cells to check the number of pores (should change to a blue square). Click ‘Start’ button in right corner of screen. N.B Flow cell check can take 00:15:00 . 15m



74 To check the flow cell run click 'Sequencing Overview' tab on left-hand side. The number of pores available for sequencing will appear above flow cell image.

75 Place flow cell record sticker on flow cell packet and record the date and number of pores. N.B. For a new flow cell there should be a minimum of 800 pores. For a used flow cell, a minimum of 700 pores is required for a run of 48 samples. One flow cell can perform approx. 2 x 48-BC runs or 1 x 96-BC run.

DNA purification using NEBNext Sample Purification beads (SFB only) 36m

76 Vortex NEBNext beads thoroughly before use and add 50 µL NEBNext beads (1:1 ratio) to tube with barcoded samples and mix gently by flicking.

77 Pulse spin to 3000 rpm to remove drops from lid.

78 Incubate reaction for 00:05:00 at room temperature. 5m

79 Place back of tube (lid opening facing forward) on magnetic rack and incubate for at least 00:05:00 until beads have pelleted and supernatant completely clear. 5m

80 Using P200, slowly remove and discard supernatant without touching the pellet.

81 Add 250 µL SFB to tube without touching pellet and resuspend beads completely by gently flicking tube and pipette mixing.

82 Pulse spin to 3000 rpm to remove drops from lid.

83 Place back of tube (lid opening facing forward) on magnetic rack and incubate for at least 00:05:00 until beads have pelleted and supernatant completely clear. 5m

84 Using P200, slowly remove and discard supernatant without touching the pellet.

85 Repeat steps 81-84 to perform a second SFB wash.

86 Pulse spin to 3000 rpm to remove drops from lids.

87 Using P10, remove any residual SFB at bottom of tube.

88 Add 15 µL Elution Buffer (EB) to tube without touching pellet and resuspend beads completely by gently flicking tube and pipette mixing.

89 Pulse spin to 3000 rpm to remove drops from lid.

90 Incubate for 00:05:00 at room temperature. 5m

91 Place back of tube (lid opening facing forward) on magnetic rack and incubate for for 00:05:00 to 00:10:00 until beads have pelleted and supernatant completely clear. 15m

92 Transfer supernatant to new 1.5ml LoBind Eppendorf tube without touching or disrupting the beads. N.B. ensure no beads are transferred and supernatant is completely clear.

93 Quantify 1 µL of barcoded amplicon pool using the Qubit fluorometer as above (step 24).

94 Expected DNA quantification; 2-6 ng/µl (24 samples).

95 Record final qubit conc. (ng/µl) on run worksheet alongside volume for loading. Require 20 ng for sequencing in volume of 12 µL. For example:

	Final Qubit conc. (ng/ul)	Volume of DNA for loading (ul)	Volume of H2O for loading (ul)
	x	20/x = y	12 - y = z

x = final qubit concentration of your barcoded amplicon pool from step 93. y = the volume of this pool in ul to add to make the final library. z = the volume of nuclease free water to add to the final library to get a total volume of 12ul with 20ng of DN

95.1 N.B. If the barcoded amplicon pool concentration is less than [M] 1.6 ng/ul load 12 µL of undiluted library in the next section.

Priming the flow cell and preparing/loading the sequencing library

5m

96 Thaw the following reagents and place on ice:

- Sequencing buffer (SQB)
- Loading beads (LB)
- Flush buffer (FB)
- Flush tether (FLT)

97 Add 30 µL FLT to a new unopened FB tube and vortex thoroughly.

98 Rotate inlet port cover clockwise 90° to expose priming port.

99 Using a P1000, set volume to 800 µl and insert tip vertically in inlet port. Ensure tight seal between tip and inlet port and slowly turn volume dial anti-clockwise. Once a small amount of volume appears in tip (20 µL - 30 µL), withdraw tip vertically.

100 Using a P1000, slowly dispense 800 µL of FLB (with FLT; as prepared above) into inlet port. To avoid introduction of air bubbles, ensure there is no air space at end of tip before inserting into port and remove tip before fully dispensing all liquid from tip.

101 Incubate for 00:05:00 .

5m

102 During incubation, prepare sequencing library; in a 1.5 ml Eppendorf tube, add the following components:

	Component	Volume (ul)
	SQB	37.5
	Loading Beads	25.5
	Final Library*	12

* Calculate 20ng as described in step 95.

103 Gently lift the SpotON cover to reveal SpotON port.

104 Using a P1000, slowly dispense 200 µL of FLB (with FLT) (as prepared above) into the priming port. Avoid introduction of air bubbles as above.

105 Immediately before loading, gently mix library by pipette mixing. Dispense 75 µL of loading library onto the SpotON sample port in a drop-wise manner. N.B. ensure each drop of library is drawn into the port before adding the next (see diagram below).

106 Gently close SpotON sample port cover and close inlet port. Slide GridION window closed.

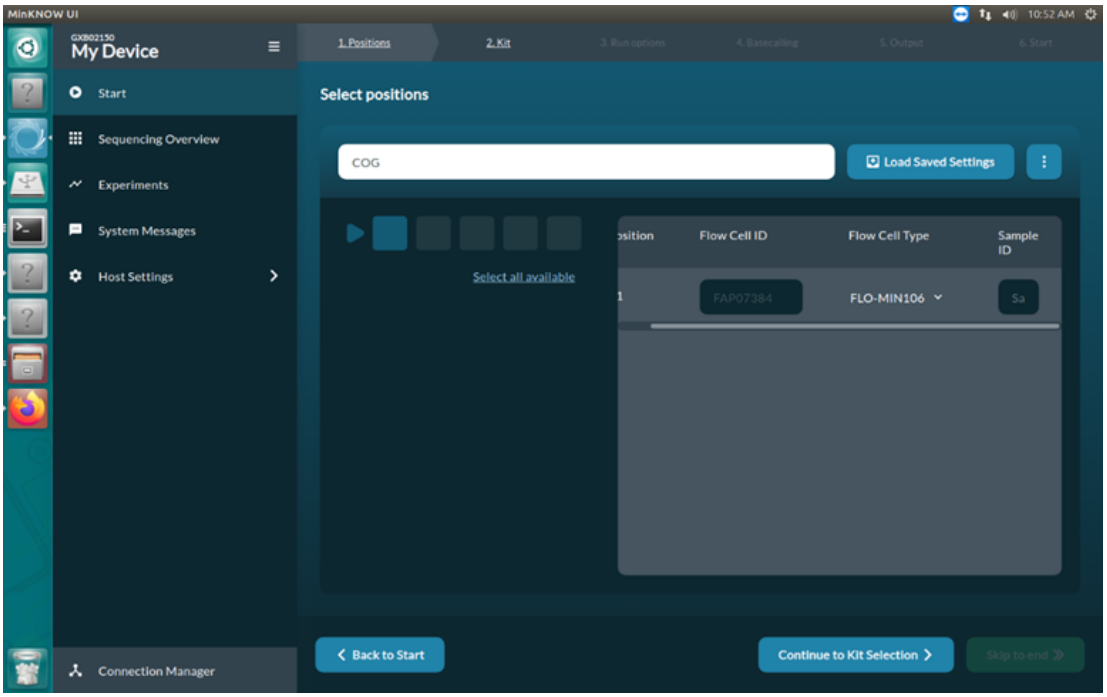
Starting the Sequencing Run

107 Log on to the GridION.

108 Click on MinKNOW icon to open software.

109 Select the ‘Start’ tab on left-hand side and click on ‘Start Sequencing’ box.

110 Available flow cells should appear as white squares. Select flow cells to check number of pores (should change to blue square).



111 Type ‘**RSV**’ in ‘Experiment name’ box.

112 Scroll along to ‘Sample ID’ and type the run date and run number in sample box in the following format: **‘YYYY-MM-DD_RunXXX’**.

113 Click on ‘Continue to Kit Selection’. Under the ‘Kit Selection’ heading select ‘**SQK-LSK109**’ and under the ‘Barcoding Expansion Packs’ heading select ‘**EXP-NBD196**’.

114 Click on ‘Continue to Run Output’. For sequencing 48 samples, type ‘**12**’ under the ‘Run Length (Hours)’ heading. N.B. Run length should be adjusted based on number of samples:

	No. of samples	Run length (hrs)
	12	6
	24	8
	48	12
	96	18

115 Click ‘Continue to Basecalling’. Under ‘Barcoding’ heading click ‘options’ and turn “**Barcode both end**’ selector to **ON**. Change threshold to **60** then ‘save’.

116 Click ‘Continue to Output’. Keep default settings: Output Location should be ‘/data/’ Output formats as follows: FAST5 selected, ‘Raw Signal’ and ‘FASTQ Record’ ticked, ‘Compression’ ‘zlib’ selected and ‘Reads per File’ should be 4000. FASTQ selected, ‘Compression’ ‘off’ selected and ‘Reads per File’ should be 4000.

117 Then click ‘**Start**’.

118 To view sequencing run, click ‘Experiment’ tab then on run. Scroll through (arrow on right) to view run statistics.

Protocol references

This method used the ARTIC loCost V3 protocol as a starting point: **dx.doi.org/10.17504/protocols.io.bp2l6n26rgqe/v3**