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SubARTIC ONT SARS-CoV-2 Spike sequencing protocol (LoCost) V3.2

nCoV-2019 sequencing protocol v3 (LoCost)

Paul J Parsons[1], Gavin Horsburgh[1], Kathryn Maher[1], Steve Paterson[2], Terry Burke[1]

[1]NERC Environmental Omics Facility, Ecology & Evolutionary Biology, School of Biosciences, University of Sheffield, Sheffield, UK;
[2]NERC Environmental Omics Facility, Department of Evolution, Ecology and Behaviour, Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, UK
Paul J Parsons: https://orcid.org/ 0000-0002-1995-9110;

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Paul Parsons
University of Sheffield

This protocol describes a procedure for sequencing the *Spike* gene of SARS-CoV-2 using short amplicons (146-208bp) with Oxford Nanopore technology (R9.4.1 MinION/GridION Flow Cell). The method has proved to be successful with both clinical RNA samples and degraded wastewater samples. The primers are unique to the SubARTIC method. The library prep procedure has been heavily adapted from the ncov-2019 sequencing v3 (ARTIC) protocol by Josh Quick (https://www.protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-bh42j8ye) and the "low cost" method from the NEOF Liverpool Illumina ARTIC protocol. See this link for the version of the protocol using Illumina sequencing (https://www.protocols.io/view/sub-artic-illumina-sars-cov-2-spike-sequencing-pro-btpjnmkn).

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nCoV-2019 sequencing protocol v3 (LoCost), Josh Quick

SARS-CoV-2, COVID, variant, sequencing, Oxford Nanopore, ARTIC , SubARTIC, short amplicon, MinION, GridION, Flow Cell, Covid-19, wastewater, RNA, Virus, Spike

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This protocol was devised using a R9.4.1 Flow Cell on a GridION, please be aware of any potential amendments needed for other sequencers/flow cell types. Consult the Nanopore community if you are in need of assistance https://nanoporetech.com/community. The primers are unique to this method. See **SubARTIC primers v3-2 091121.csv** and the BEFORE STARTING section for details.

Primers details are given in the attached. SubARTIC primers v3-2 091121.csv

A	B	C
Component	**Supplier**	**Part number**
Primer panel	IDT/SIGMA	
LunaScript RT SuperMix Kit	NEB	E3010
Q5 Hot Start High-Fidelity 2X Master Mix	NEB	M0494
Nuclease-free water (100 mL)	NEB	B1500
NEBNext Ultra II End Repair/dA-tailing module	NEB	E7546
Blunt/TA Ligase Master Mix	NEB	M0367
Native Barcoding Expansion Kit 1-12 and/or	ONT	EXP-NBD104
Native Barcoding Expansion Kit 13-24	ONT	EXP-NBD114
AMPure XP beads	Beckman	A63881
NEBNext Quick Ligation Module	NEB	E6056S
Sequencing Auxiliary Vials	ONT	EXP-AUX001
Short Fragment Buffer Expansion Kit	ONT	EXP-SFB001
Qubit dsDNA HS Assay Kit	Thermo	Q32854
Flow Cell Priming Kit	ONT	EXP-FLP002
Flow Cell Wash Kit (optional)	ONT	EXP-WSH003
R9.4.1 flow cells	ONT	FLO-MIN106
DNA LoBind 1.5ml tubes	Eppendorf	0030108051

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SubARTIC primers v3-2 091121.csv

Before starting, generate the "Odd" and "Even" primer pools as follows:

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1. Fully resuspend lyophilised oligonucleotides in 1x TE to a concentration of 100 micromolar (µM) , vortex thoroughly and spin down.
2. Sort the odd and even primer sets into separate batches and label two 1.5-ml tubes.
3. Starting with the even primer set add the volume (µl) of stock primer given in the attached (above) to the pooled 1.5-ml tube (between 7.5µl - 15µl). Vortex and spin down. Then repeat the pooling with the odd primers. These are your two pooled stocks.
4. Dilute the pool stocks one in ten across several aliquots with ultrapure water. Vortex and spin down. These are your working primer pools used in step 4.

This method has been run successfully in house using up to 24 Native barcodes per library, it may be feasible however, to use up to 96 barcodes if your coverage requirements are low.

cDNA preparation 30m

1 Prepare between 11 and 23 RNA samples plus a negative control (nuclease-free water) per library. If previously frozen, mix by briefly flicking and pulse spin to collect liquid. Keep samples on ice at all times.

A positive control can also be included here to help monitor run performance. This could be either synthetic RNA or a diluted clinical sample (see below).

Ideally work should be performed in a freshly bleached Pre-PCR hood in an isolated clean room, that has been subjected to UV irradiation for at least **00:40:00** prior to cDNA preparation.

2 Mix the following components in PCR strip-tubes/plate. Gently mix by pipetting and pulse spin the tube to collect liquid. Total volume per well is **10 µL** .

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A	B
Component	**Volume**
LunaScript RT SuperMix (5X)	2 µL
Template RNA	8 µL

Viral RNA input from a clinical sample should be between Ct 18-35. If Ct is between 12-15, then dilute the sample 100-fold in water, if between 15-18 then dilute 10-fold in water. This will reduce the likelihood of PCR-inhibition. Viral RNA input from a wastewater sample is likely to be of high Ct and as such no dilution is warranted.

3 Incubate the reaction as follows:

25 °C for **00:02:00**

55 °C for **00:10:00**

95 °C for **00:01:00**

Hold at **4 °C**

Multiplex PCR 4h

4 Primers are separated into two pools, Odd (O) and Even (E), depending on where they sit across the *Spike* region. Each sample is then amplified with these two pools separately and pooled after PCR. See the BEFORE STARTING section (above) for further details. Set up two master mixes (O and E) to cover your sample number with an excess of around 10%. Do not over-vortex the Q5. Dispense **8 µL** of this master mix in to each well.

A	B	C
Component	Master mix O	Master Mix E
Working primer pool O	1.75 µl	-
Working primer pool E	-	1.75 µl
Q5® Hot Start High-Fidelity 2X Master Mix	6.25 µl	6.25 µl
Add to well	**8µl**	**8 µl**

5 Add **4.5 µL** cDNA to each of the PCR reactions, gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube. Total volume per well is **12.5 µL** .

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6 Run the following program on the thermal cycler; same PCR profile is used for both pools:

Step	Temperature	Time	Cycles
Heat Activation	**98 °C**	**00:00:30**	1
Denaturation	**98 °C**	**00:00:15**	35
Annealing	**60 °C**	**00:05:00**	35
Hold	**4 °C**	Indefinite	1

Cycle number can be reduced for samples of higher concentration.

Pooling of O and E PCR products 15m

7 Move to a cleaned and UV sterilised Post-PCR hood. Combines pool O and E of each sample. Dilute ~1 in 2 by adding **20 µL** **nuclease-free water** and pipette mix.

Dilution of 1 in 2 should be considered a minimum. Further dilution is certainly warranted if using samples of high concentration (<25 ct). Samples can also be quanitifed and normalised if a more even spread of coverage is required.

End prep 45m

8 Create a master mix as listed below - one per pooled sample - make an excess of ~10%. Dispense **6.7 µL** of this master mix to each well.

A	B
Reagent	**Volume**
Nuclease-free water	5 µl
NEB Ultra II End-prep reaction buffer	1.2 µl
NEB Ultra II End-prep enzyme mix	0.5 µl
Add to well	**6.7 µl**

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9 Add **3.3 µL** of each pooled and diluted product (from step 7) to the end-prep reactions, gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

10 Incubate the reaction using the following program (with a heated lid of 75 °C): 35m

Step	Temperature	Time	Cycles
Incubate	**20 °C**	**00:20:00**	1
Denaturation	**65 °C**	**00:15:00**	1
Hold	**4 °C**	Indefinite	1

Now is a good time to thaw Blunt/TA ligase MM and Native barcodes (enough for one barcode per sample) and take Ampure XP beads out of the fridge to reach **Room temperature** .

Native Barcoding 1h

11 Add **1.25 µL** of each barcode to a new PCR strip/plate. One barcode per sample. Place the droplet on the side of the well to confirm it is present and then spin down.

12 Create a master mix as listed below one per pooled sample. Make an excess of ~10%. Dispense **7.75 µL** of this master mix to each well.

A	B
Reagent	**Volume**
NEB Blunt/TA ligase Master Mix	5 µl
Nuclease-free water	2.75 µl
Add to well	**7.75 µl**

To save time Steps 11 and 12 can be done whilst the PCR product is in the process of being end-prepped.

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13 Once the end prep is complete, Add **1 µL** of the end-prepped PCR product to each well. Mix by flicking and spin down. The total in each well will be **10 µL**

14 Incubate the reaction using the following program (with a heated lid at 75 °C): 40m

Step	Temperature	Time	Cycles
Incubate	**20 °C**	**00:30:00**	1
Denaturation	**65 °C**	**00:10:00**	1
Hold	**4 °C**	Indefinite	1

Now is a good time to thaw Short Fragment buffer (SFB).

One-pot Ampure bead clean 1 45m

15 If you have 12-16 samples - then pool **10 µL** of each sample into a 1.5 ml DNA LoBind tube.
or
If you have 17-24 samples - then pool **7.5 µL** of each sample into a 1.5 ml DNA LoBind tube.

16 Resuspend the AMPure XP beads by vortexing. Add **1.0x** volumes of resuspended **AMPure XP beads** to the reaction and mix by pipetting.

17 Incubate for Incubate for **00:10:00** at **Room temperature** 10m

Now is a good time to thaw ONT Elution Buffer (EB), NEBNext Quick Ligation Reaction Buffer (5x), NEBNext Quick T4 DNA ligase and Adapter Mix II (AMII) on ice.

18 Prepare **1 mL** of fresh **80% ethanol** with nuclease-free water.

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19 Spin down the sample and pellet the beads on a magnet for **00:05:00** . Keep the tube on the magnet, and pipette off the supernatant. 5m

20 **Take the sample off the magnet** and add **250 µL** **Short Fragment Buffer (SFB).** Resuspend beads completely by pipette mixing. Return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.

21 Repeat the previous step (**250 µL** wash with **SFB**, pellet, and then discard supernatant).

22 **Keep the tube on the magnet** and wash the beads with **250 µL** of freshly-prepared **80% ethanol** without disturbing the pellet. Remove the ethanol using a pipette and discard.

> You only want to bathe the pellet with ethanol. Only with SFB do you fully resuspend the beads.

23 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to air dry for ~2 min, but do not dry the pellet to the point of cracking.

24 Remove the tube from the magnetic rack and resuspend pellet in **30 µL** **nuclease-free water.** Gently pipette mix.

> You can elute in as low as **18 µL** if you are running 12-16 samples.

25 Incubate for **00:02:00** at **Room temperature** 2m

26 Pellet the beads on a magnet until the eluate is clear and colourless. Remove and retain **28 µL** of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

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Optional - Quantify eluted sample using a fluorometer. Note **15 µL** will be needed for the next steps.

Adapter ligation 25m

27 By adding directly to the pooled and barcoded sample, perform adapter ligation as follows. Make sure to mix by flicking the tube between each sequential addition. Total volume in the tube will be **25 µL** .

A	B
Reagent	**Volume**
Clean Pooled barcoded sample	15 µl
Adapter Mix II (AMII)	2.5 µl
NEBNext Quick Ligation Reaction Buffer (5X)	5 µl
NEB Quick T4 DNA Ligase	2.5 µl

28 Spin down and incubate the reaction for **00:20:00** at **Room temperature** 20m

Now is a good time to take out a Flow Cell from the fridge and leave at **Room temperature**

Final library bead clean 40m

29 Add **25 µL** of well mixed **AMPure XP beads (1.0x)** to the reaction and mix by pipetting. 10m

Incubate for **00:10:00** at **Room temperature**

Now is a good time to load a Flow Cell into the MinION/GridION and run the *Check Flow Cell* command.

Thaw SQB, LB, FLT, FB on ice.

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30 Spin down the sample and pellet the beads on a magnet for **00:05:00** . Keep the tube on the magnet, and pipette off the supernatant. 5m

31 **Take the sample off the magnet** and add **125 µL Short Fragment Buffer (SFB).** Resuspend beads completely by pipette mixing. Return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.

32 Repeat the previous step (**125 µL** wash with **SFB**, pellet, and then discard supernatant).

33 Spin down and place the tube back on the magnet. Pipette off any residual **SFB.** Allow to air dry for ~ **00:02:00** , but do not dry the pellet to the point of cracking. 2m

Do not perform an 80% ethanol wash here.

34 Remove the tube from the magnetic rack and resuspend pellet in **15 µL Elution Buffer (EB).**

This must be ONT EB buffer not any other elution buffer.

35 Incubate for **00:02:00** at **Room temperature** 2m

36 Pellet the beads on a magnet until the eluate is clear and colourless. Remove and retain **14 µL** of eluate into a new clean 1.5 ml DNA LoBind tube.

37 Quantify **1 µL** of eluted sample using a Qubit fluorometer High sensitivity kit. – The sample is expected ~ 1-3 ng/ul range depending on the number of samples used.

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38 Dilute if necessary to be ready to load **~15ng** on the Flow Cell. **12 µL** of the final library will be loaded.

Note this is 15ng total, **not** 15ng/µl.

Gridion Sequencing 20m

39 Add **30 µL** FLT to FB and mix well.

Make sure you are familar with the Oxford Nanopore guidance information before proceeding with loading.

40 Rotate inlet port cover of the Flow Cell clockwise by 90 degrees so the inlet port is visible.

41 Take a P1000 and set the volume to 800 µl. Place the tip in the inlet port, make sure the pipette is held perpendicularly (i.e. not at an angle). Remove any air by turning the dial on the pipette slowly. You should see a small amount of liquid on the end of the tip. Do not remove more than necessary.

42 Take **820 µL** from the FLT/FB mix tube being careful that there are no air bubbles present/liquid goes all the way to the bottom of the tip.

43 Load this into the priming port by dispensing slowly. Save the last few µl in the pipette tip to avoid adding any air.

Only **800 µL** needs to be loaded at this point the excess in the tip is to help with the avoidance of bubbles.

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44 Incubate for **00:05:00** 5m

45 Whilst incubating prepare the library as follows. Total volume in the tube will be **75 µL** .

A	B
Reagent	**Volume**
Final library (Max ~15ng total)	12 µl
SQB	37.5 µl
LB	25.5 µl

Make sure the LB is especially well mixed before adding as it settles quickly.

46 Gently lift the SpotON cover to open the SpotOn port.

47 Slowly load another **210 µL** of the FB/FLT mix into the **Inlet port.** This should initiate a siphon at the **SpotON port**. As previously, leave the last amount of liquid in the end of the tip to avoid any bubbles.

48 Pipette mix the library mixture together just prior to loading, as the loading beads can quickly settle.

49 Load the **75 µL** of mixture to the flow cell via the **SpotON port** in a dropwise fashion.

50 Gently replace the SpotOn port cover, making sure the bung sits in correctly, close the inlet port and close the GridION lid.

Starting the experiment 5m

51 Select Start – Start sequencing.

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52 Name the run and select the correct X position.

53 Choose the flowing options – LSK109 – NBD104 and NBD114.

54 Run for 16-24 hours, align to a *Spike* reference, select high-accuracy base calling, and set the minimum barcoding score to 80.

> These options can be easily customised depending on data requirements.

55 Start the run.

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