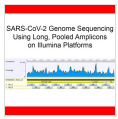

Apr 04, 2020

SARS-CoV-2 Genome Sequencing Using Long Pooled Amplicons on Illumina Platforms

John-Sebastian Eden[1,2], Eby Sim[2,3,4]

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1 Works for me dx.doi.org/10.17504/protocols.io.befyjbpw

Coronavirus Method Development Community

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ABSTRACT

This protocol describes methods to sequence SARS-CoV-2 with pooled amplicons (14 x 2.5kb) using Illumina Platforms.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

https://www.biorxiv.org/content/10.1101/2020.03.15.992818v1

ATTACHMENTS

SARS-CoV-2_amplicon_WGS_protocol.pdf

MATERIALS TEXT

ITEM	SUPPLIER	CATALOGUE
General consumables, chemicals & equipment		
Ethanol, 200 proof, for molecular biology (500 mL)	Sigma-Aldrich	E7023-500ML
UltraPure DNase/RNase-free distilled water (500 mL)	Invitrogen	10977015
β-Mercaptoethanol (10 mL)	Sigma-Aldrich	M6250-10ML
0.2 mL strip of 8 tubes, flat cap, natural (120 pack)	SSIbio	3245-00
DNA LoBind tubes, 1.5 mL (250 tubes)	Eppendorf	0030108051
Conical Tubes, 50 mL (500 tubes)	Eppendorf	0030122178
PCR plate 96 LoBind, semi-skirted (25 plates)	Eppendorf	0030129504
PCR Film, self-adhesive (100 pieces)	Eppendorf	0030127781
SimpliAmp Thermal Cycler	Applied Biosystems	A24811
Mother E-Base device	Invitrogen	EBM03
12 channel VOYAGER pipette (5 – 125 µl)	Integra	4732
Single pipette charging stand	Integra	4210
Part 1 - Viral RNA extraction		
Quick-RNA viral kit (50 preps)	Zymo	R1034
Collection tubes (500 pack)	Zymo	C1001-500
Part 2 - cDNA synthesis		

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SuperScript IV VILO master mix (50 reactions)	Invitrogen	11756050
Part 3 - Genome amplification		
Platinum SuperFi Green PCR master mix (500 reactions)	Invitrogen	12359050
Custom DNA oligos (100 µM)	IDT	~
1 Kb Plus DNA ladder (250 µg)	Invitrogen	10787018
E-Gel 48 Agarose gels, 1%	Invitrogen	G800801
Part 4 - PCR pooling and cleanup		
Qubit 1X dsDNA HS assay kit (500 assays)	Invitrogen	Q33231
Qubit dsDNA BR Assay Kit (500 assays)	Invitrogen	Q32853
Agencourt AMPure XP (60 mL)	Beckman Coulter	A63881
Buffer EB (250 mL)	Qiagen	19086
Part 5 - DNA library preparation and sequencing		
Nextera XT DNA Library Preparation Kit (96 samples)	Illumina	FC-131-1096
Nextera XT Index Kit v2 Set B (96 indexes, 384 samples)	Illumina	FC-131-2002
High Sensitivity D5000 ScreenTape	Agilent	5067-5592
High Sensitivity D5000 Reagents	Agilent	5067-5593
High Sensitivity D5000 Ladder	Agilent	5067-5594
iSeq 100 i1 Reagent (4 pack)	Illumina	20021534
iSeq 100 System	Illumina	20021532

SARS-CoV-2 amplicon WGS materials

SARS-CoV-2 amplicon WGS materials.pdf

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

BEFORE STARTING

The basic protocol uses Illumina sequencing but an addendum is added at the end to take sample amplicons and sequence them using Oxford Nanopore Technology.

Viral RNA extraction

1

Here we provide an example low throughput viral RNA extraction protocol; however, any platform that extracts viral or pathogen RNA (or total nucleic acid) should be suitable. We typically use the residual viral extracts from clinical samples following diagnostic RT-PCRs, which include extracts off both Qiagen BioRobot EZ1 and Roche MagnaPure 96 platforms. Please ensure appropriate PPE and containment to avoid exposure to infectious samples, noting that following the addition of the DNA/RNA Shield, the virus will be inactivated.

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2 

Combine **100 µl 2X DNA/RNA Shield** with 100 µl respiratory sample (sputum, aspirate or swab medium), then mix well by gentle vortexing.

3 

Add **400 µl Viral RNA Buffer** to each 200 µl sample and mix well by gentle vortexing.

4 

Transfer the mixture into a Zymo-Spin IC column placed in a collection tube. Centrifuge at **12000 x g** for **00:02:00** to bind viral RNA to matrix.

5 Transfer the column into a new collection tube.

6 

Add **500 µl Viral Wash Buffer** to the column, centrifuge at **12.000 x g** for **00:00:30** .

7 Transfer the column into a new collection tube.

8 Repeat steps 6 & 7:

8.1 

Add **500 µl Viral Wash Buffer** to the column, centrifuge at **12.000 x g** for **00:00:30** .

8.2 Transfer the column into a new collection tube.

9 

Add **500 µl Ethanol (95-100%)** to the column and centrifuge at **12.000 x g** for **00:02:00** to ensure complete removal of the wash buffer.

10 Transfer the column into a clean elution tube (2 ml Eppendorf PCR clean, DNA LoBind tube).

11 

Add **25 µl DNase/RNase-Free Water** directly to the column matrix, incubate for **00:05:00** and then centrifuge at **12.000 x g** for **00:00:30** to elute the viral RNA.

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12

The eluted viral RNA can be used immediately or stored frozen at **-80 °C** .

cDNA synthesis

13

cDNA is prepared from viral RNA using SuperScript IV VILO Master Mix, which combines both random and oligo-dT priming for first strand synthesis. The oligo-dT priming is essential as the reverse primer for the final amplicon sits close to the viral poly-A tail in the 3' UTR. When using individual components to set up cDNA synthesis instead of the VILO mastermix, use the recommended protocol for a random hexamer primed reaction but supplement the reaction with oligo-dT so the final random hexamer/oligo-dT ratio is 3:1.

14

Setup the following reaction mix for each sample and a no template control (NTC):

REAGENT	VOLUME (µl)
SuperScript IV VILO MasterMix (5X)	5
Nuclease free water	15
Viral RNA	5
TOTAL	25

15

Incubate the reaction as follows:

STEP	TEMP (°C)	TIME (mm:ss)
Priming	25	10:00
Extension	50	20:00
Denature RT	85	05:00
Hold	4	∞

16

Keep the cDNA **On ice** if being used immediately or store frozen at **-20 °C** .

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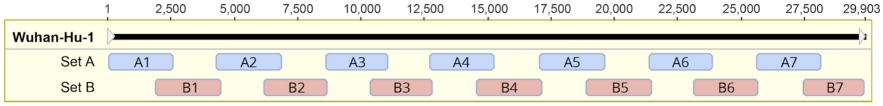
Genome Amplification

17

Next we take the cDNA prepared from viral RNA and amplify 14 regions (~2.5kb each) that tile across the viral genome. The PCRs are performed in parallel and is NOT a multiplexed reaction. The general approach is demonstrated by the following schematic:



18 The amplicon primers are as follows:

PCR	PRIMER	SEQUENCE (5' - 3')	LENGTH (nt)
A1	SARS2_A1F_31	ACCAACCAACTTTCGATCTCTTGT	2562
	SARS2_A1R_2569	GCTTCAACAGCTTCACTAGTAGGT	
A2	SARS2_A2F_4295	ACAGTGCTTAAAAAGTGTAAAAGTGCC	2579
	SARS2_A2R_6847	ACAGTATTCTTTGCTATAGTAGTCGGC	
A3	SARS2_A3F_8596	ACTTGTGTTCCTTTTTGTTGCTGC	2479
	SARS2_A3R_11049	GAACAAAGACCATTGAGTACTCTGGA	
A4	SARS2_A4F_12711	TACGACAGATGTCTTGTGCTGC	2536
	SARS2_A4R_15225	TAACATGTTGTGCCAACCACCA	
A5	SARS2_A5F_16847	ACTATGGTGATGCTGTTGTTTACCG	2432
	SARS2_A5R_19254	ACCAGGCAAGTTAAGGTTAGATAGC	
A6	SARS2_A6F_21358	ACAAATCCAATTCAGTTGTCTTCCTATTC	2490
	SARS2_A6R_23823	TGTGTACAAAAACTGCCATATTGCA	
A7	SARS2_A7F_25602	ACTAGCACTCTCCAAGGGTGTT	2571
	SARS2_A7R_28146	AGGTTCCTGGCAATTAATTGTAAAAGG	
B1	SARS2_B1F_1876	ATCAGAGGCTGCTCGTGTTGTA	2575
	SARS2_B1R_4429	AGTTTCCACACAGACAGGCATT	
B2	SARS2_B2F_6287	TGGTGTATACGTTGTCTTTGGAGC	2565
	SARS2_B2R_8828	CACTTCTCTTGTTATGACTGCAGC	
B3	SARS2_B3F_10363	TGTTCGCATTCAACCAGGACAG	2440
	SARS2_B3R_12780	CCTACCTCCCTTTGTTGTGTTGT	
B4	SARS2_B4F_14546	AGGAATTACTTGTGTATGCTGCTGA	2607
	SARS2_B4R_17131	ACACTATGCGAGCAGAAGGGTA	
B5	SARS2_B5F_18897	TGTTAAGCGTGTTGACTGGACT	2559
	SARS2_B5R_21428	TGACCTTCTTTTAAAGACATAACAGCAG	
B6	SARS2_B6F_23123	CCAGCAACTGTTTGTGGACCTA	2551
	SARS2_B6R_25647	AGGTGTGAGTAAACTGTTACAAACAAC	
B7	SARS2_B7F_27447	TCACTACCAAGAGTGTGTTAGAGGT	2420
	SARS2_B7R_29837	TTCTCCTAAGAAGCTATTAAAATCACATGG	

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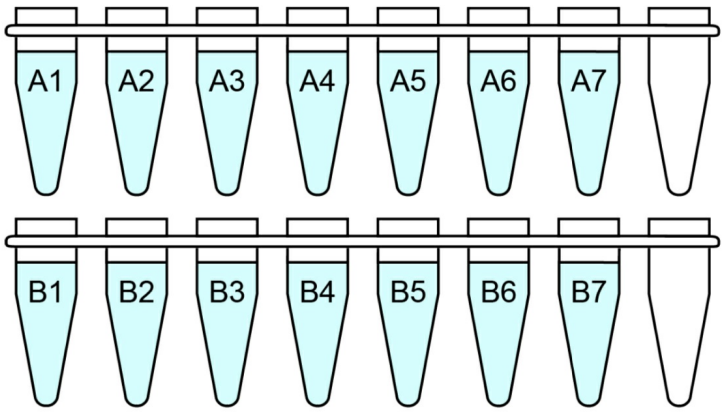
19

Prepare the primers for each amplicon set by combining **4.5 µl forward primer (100 µM)** , **4.5 µl reverse primer (100 µM)** and **171 µl nuclease free water** into clean PCR strips, as below:



20 One standard RT-PCR is shown below, but each sample requires 14 reactions in total, each containing the different amplicon primers A1-A7 & B1-B7.

REAGENT	VOLUME (µl)
Platinum SuperFi Green Mastermix (2X)	10
Nuclease free water	6.5
Primer pool (5 µM)	2
Viral cDNA	1.5
TOTAL	20

21

Setup the following reaction mix for each sample and the no template control (NTC) according to requirements (one sample has 14.5 reactions, 14 primers + 0.5 for pipetting error):

REAGENT	VOLUME (µl), s=samples			
	S=1	S=3	S=6	S=12
Platinum SuperFi Green Mastermix (2X)	145	435	870	1740
Nuclease free water	94.25	282.75	565.5	1131

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22

Dispense **16.5 µl reaction mix** into each well as required, a suggested plate setup is provided below for a 6 sample plate:

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	NTC	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	NTC
	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A1	A1	A1	A1	A1	B1	B1	B1	B1	B1	B1
B	A2	A2	A2	A2	A2	A2	B2	B2	B2	B2	B2	B2
C	A3	A3	A3	A3	A3	A3	B3	B3	B3	B3	B3	B3
D	A4	A4	A4	A4	A4	A4	B4	B4	B4	B4	B4	B4
E	A5	A5	A5	A5	A5	A5	B5	B5	B5	B5	B5	B5
F	A6	A6	A6	A6	A6	A6	B6	B6	B6	B6	B6	B6
G	A7	A7	A7	A7	A7	A7	B7	B7	B7	B7	B7	B7
H	*Empty*	*Empty*	*Empty*	*Empty*	*Empty*	*Empty*	*Empty*	*Empty*	*Empty*	*Empty*	*Empty*	*Empty*
	Set A primers						Set B primers					

23

Using a 8-channel multipipettor, add **2 µl of the diluted and premixed primer sets** into each well according to the plate layout.

24

Using a 8-channel multipipettor, add **1.5 µl viral cDNA** into each well according to the plate layout.

25

Seal the plate, and centrifuge for **00:00:30** in a plate spinner.

26

Incubate the reaction as follows:

STEP	TEMP (°C)	TIME (mm:ss)	CYCLES
Hot start	98	02:00	1X
Denaturation	98	00:15	40X
Annealing	65	00:30	
Extension	72	02:00	
Final extension	72	05:00	1X
Hold	4	∞	

27

Analyse **5 µl each RT-PCR reaction** on a 1% agarose gel with DNA staining dye.

When using Invitrogen E-Gel 48 Agarose precast gels, preload each well with 10 µl of EB buffer. All bands are expected to be ~2.5kb, so run with an appropriate DNA ladder. To aid in pooling visualisation, it is preferable to run all set A and B reactions for one sample on the same row.

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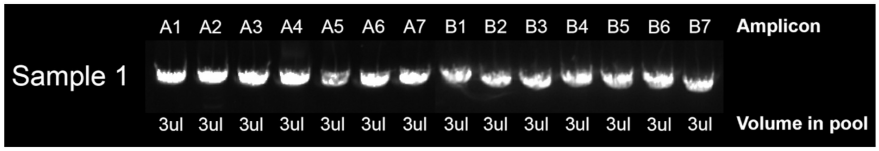
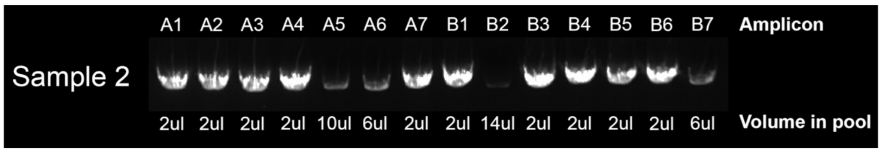
PCR pooling and cleanup

28 An example gel is provided below for two different samples:





29 Pool the individual amplicons for each sample into a single well of a clean plate or PCR strip. The aim here is to roughly balance the amount of DNA from each amplicon to provide even coverage across the genome. Target the final volume in the pool to be ~ **40 µl** (i.e. **3 µl** for all 14 amplicons when yield is even).

30

Adjust the **final volume** of the pooled PCR product to **50 µl** by aliquoting when exceeding or adding an appropriate volume of clean EB buffer when less.

31

Add **40 µl room temperature AMPure XP beads** (0.8X bead ratio). Gently pipette the entire volume up and down 15 times to mix thoroughly.

32

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

33 Place the plate/tubes onto the magnetic stand for at least **00:02:00** , until the solution appears clear. Do not remove from magnetic stand during washing steps, and take care to not disturb beads.

34 Remove and discard the supernatant from each well by pipetting.

35

Add **200 µl freshly prepared 80% EtOH** to each well without disturbing the beads.

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36 

Incubate at **Room temperature** for **00:00:30** .

37 Remove and discard the supernatant from each well by pipetting.

38 Repeat steps 35 to 37 for a total of two EtOH washes:

38.1 

Add **200 µl freshly prepared 80% EtOH** to each well without disturbing the beads.

38.2 

Incubate at **Room temperature** for **00:00:30** .

38.3 Remove and discard the supernatant from each well by pipetting.

39 Allow the beads to air dry for **00:05:00** to **00:15:00** . Visually inspect wells to ensure any small droplets are completely removed by pipetting or evaporation.

40 Remove the plate/tubes from the magnetic stand.

41 

Resuspend the dried bead pellet with **40 µl EB buffer** .

42 

Gently pipette the entire volume up and down 15 times to mix thoroughly.

43 Place the plate/tubes onto the magnetic stand at room temperature for at least **00:02:00** , until the solution appears clear.

44 

Transfer the cleared supernatant containing the purified DNA into a suitable plate, strip or tube. Use immediately or store frozen at **-20 °C** .

45 Quantify all purified DNA using the Qubit dsDNA broad range assay.

46 

For any samples >10ng/µl, roughly dilute using EB buffer so the final concentration is between **1 nanogram per microliter (ng/µL)** to **10 nanogram per microliter (ng/µL)** .

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47 Re-quantify all purified and adjusted DNA using the Qubit dsDNA high sensitivity assay.

48

Dilute to **0.2 nanogram per microliter (ng/µL)** using EB buffer (ensure at least **20 µl total volume**) and proceed to library prep.

DNA library preparation and sequencing

49

The following protocol follows the Illumina Nextera XT DNA library prep kit except for two important changes: i) all reaction volumes are halved to save on reagents and ii) that following the clean up after library amplification, the libraries are manually normalised rather than with the provided normalisation beads.

50

Combine **5 µl Tagment DNA Buffer (TD)** and **2.5 µl pooled, diluted (0.2 ng/µl) amplicons** for each sample into a PCR plate or strip.

51

Mix well by pipetting 10 times.

52

Add **2.5 µl Amplicon Tagment Mix (ATM)** to each well on top of the TD/DNA mix.

53

Mix well by pipetting 10 times. Seal and briefly centrifuge.

54

Incubate the reaction mix as follows:

STEP	TEMP (°C)	TIME (mm:ss)
Tagmentation	55	05:00
Hold	10	∞

55

Following tagmentation, immediately remove the reaction from the thermocycler and add **2.5 µl Neutralize Tagment Buffer (NT)** to stop the reaction.

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56 

Mix well by pipetting 10 times. Seal and briefly centrifuge.

57 

Incubate at **Room temperature** for **00:05:00** .

58 Add indices and Nextera PCR Master Mix (NPM) to the neutralised tagmentation reaction for each sample as below:

REAGENT	VOLUME (µl)
Neutralised tagmentation reaction	12.5
i7 adapter	2.5
i5 adapter	2.5
Nextera PCR Master Mix	7.5
TOTAL	25

59 

Incubate the reaction as follows:

STEP	TEMP (°C)	TIME (mm:ss)	CYCLES
Hot start	72	03:00	1X
Initial denature	95	00:30	1X
Denaturation	95	00:10	12X
Annealing	55	00:30	
Extension	72	00:30	
Final extension	72	05:00	1X
Hold	10	∞	

60 

Add **15 µl room temperature AMPure XP beads** (0.6X bead ratio) to the 25 µl of amplified libraries. Gently pipette the entire volume up and down 15 times to mix thoroughly.

61 

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

62 Place the plate/tubes onto the magnetic stand for at least **00:02:00** , until the solution appears clear. Do not remove from magnetic stand during washing steps, and take care to not disturb beads.

63 Remove and discard the supernatant from each well by pipetting.

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64 

Add **200 µl freshly prepared 80% EtOH** to each well without disturbing the beads.

65 

Incubate at **Room temperature** for **00:00:30** .

66 Remove and discard the supernatant from each well by pipetting.

67 Repeat steps 64 to 66 for a total of two EtOH washes:

67.1 

Add **200 µl freshly prepared 80% EtOH** to each well without disturbing the beads.

67.2 

Incubate at **Room temperature** for **00:00:30** .

67.3 Remove and discard the supernatant from each well by pipetting.

68 Allow the beads to air dry for **00:05:00** to **00:15:00** . Visually inspect wells to ensure any small droplets are completely removed by pipetting or evaporation.

69 Remove the plate/tubes from the magnetic stand.

70 

Resuspend the dried bead pellet with **22.5 µl EB buffer** .

71 

Gently pipette the entire volume up and down 15 times to mix thoroughly.

72 Place the plate/tubes onto the magnetic stand at room temperature for at least **00:02:00** , until the solution appears clear.

73 

Transfer **20 µl cleared supernatant** (containing the purified DNA) into a suitable plate, strip or tube. Use immediately or store frozen at **-20 °C** .

74 Quantify all purified DNA using the Qubit dsDNA high sensitivity assay.

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75 Pool the individual libraries equally in DNA amount based on the Qubit values. Here, we assume the libraries will have similar fragment lengths and distributions.

76 Quantify the final pool of libraries using the Qubit dsDNA high sensitivity assay.

77

Analyse **2 µl final pool of libraries** with Agilent High Sensitivity D5000 Screen Tape ensuring the whole fragment peak is captured.

78

Scale the calculated molarity from the Tapestation to the Qubit DNA concentration using the following formula:

Final molarity = (Tapestation DNA molarity) x (Qubit DNA conc / Tapestation DNA conc)

79

Dilute the final pooled libraries down to **1 Nanomolar (nM)** (at least 50µl) using EB buffer, and add PhiX sequencing control if required (we normally don't).

80

Combine **20 µl of 1nM library pool** and **80 µl of EB buffer** to dilute the final pool of libraries to **0.2 Nanomolar (nM)** for loading.

81

Load **20 µl of 0.2nM library pool** into a defrosted Illumina iSeq cartridge with flow cell, and sequence with at least 75 nt paired end sequencing.

Assuming even pooling, each run can comfortably include 12-18 SARS-CoV-2 genome libraries with >1000x coverage for consensus calling.

82 Finally, assemble your viruses and upload them onto www.gisaid.org. Some more details on basic bioinformatic workflow will be provided soon.

Addendum: Sequencing pooled amplicons with Oxford Nanopore Rapid Barcoding kit (SQK-RBK004)

83

Due to the larger amplicon size generated from this protocol, the application of Nanopore sequencing using the Rapid Barcoding kit was tested. The input for this workflow are four individual pooled samples that have been cleaned and quantified. The output of this process is a “pseudomolecule” with the variant bases of sequenced sample relative to the genome of MN908947.3

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84 Perform Flow cell QC as per Oxford Nanopore's instructions prior to starting library preparation.

85 

For each sample, combine between 100-200 fmol of pooled amplicons with **2.5 µl fragmentation mix** .

86 

Adjust the final volume for each tube to **10 µl** using Nuclease free water. Gently flick to mix and spin down.

87 

Incubate tubes at **30 °C** for **00:01:00** and then at **80 °C** for **00:01:00** in a thermocycler. Place tubes **On ice** to cool.

88 

Pool all samples into a 1.5 ml LoBind tube. Gently flick to mix and spin down.

89 

Aspirate and dispense **10 µl of pooled RBK004 libraries** into a different 1.5 ml LoBind tube and add **1 µl RAP** . Gently flick to mix and spin down.

90 

Incubate the tube at **Room temperature** for **00:05:00** .

91 Store the prepared library **On ice** until required.

92 Perform priming and loading the Spot ON Flow Cell as per Oxford Nanopore's instructions.

93 Perform sequencing for up to **02:00:00** or until enough data is obtained.

94 Once sequencing run is completed, perform base calling, demultiplexing and adaptor trimming using Guppy.

95 Filter each demultiplexed read set using NanoFilt (https://github.com/wdecoster/nanofilt) to keep reads with a minimum quality of 10 and a maximum length of 2,700 bases.

96 Map each filtered read set to onto the reference genome (accession: MN908947.3) separately using Minimap2 (https://github.com/lh3/minimap2) and convert the resulting mapping file into a sorted .bam file using SAMtools (https://github.com/samtools/samtools).

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97 Visualise and inspect the mapping profile over the reference genome.

N.B. Expect no/low coverage (< 10 x coverage) in some bases in 5’ and 3’ ends of the reference genome (approximately the first 40 bases and the last 50 bases of the reference genome).

98 Perform variant calling using medaka_variant (https://github.com/nanoporetech/medaka).

Ensure that you select the correct medaka model based on your Nanopore sequencing platform. In addition, variants with a QUAL score of less than 30 should be interrogated before generating a consensus pseudomolecule.

99 Generate consensus sequence from the resulting .vcf file using BCFtools (https://samtools.github.io/bcftools/).

Citation: John-Sebastian Eden, Eby Sim (04/04/2020). SARS-CoV-2 Genome Sequencing Using Long Pooled Amplicons on Illumina Platforms. https://dx.doi.org/10.17504/protocols.io.befyjbpw