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Sub-ARTIC Illumina SARS-CoV-2 Spike sequencing protocol (LoCost) V3.2

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1

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This protocol describes a procedure for sequencing the Spike region of SARS-CoV-2 using short amplicons (146-208bp). The method has proved to successful with both clinical RNA samples and degraded wastewater samples. The primers are unique to this 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.

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SARS-CoV-2, COVID, variant, sequencing, Illumina, ARTIC , SubARTIC, amplicon

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Primers are listed in the attached **SubArtic primers v3-2 091121.csv**

LunaScript® RT SuperMix Kit, New England BioLabs, Cat# E3010L

Q5® Hot Start High-Fidelity 2X Master Mix, New England BioLabs, Cat# M0494L

NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®, New England BioLabs, Cat# E7645L (contains NEBNext Ultra II End Prep Mix and buffer, Ultra II Q5 Master Mix, Ligation Enhancer, Ultra II Ligation Master Mix)

NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers Set 1), NewEngland BioLabs, Cat# E7600S (contains i5/i7 indexes, Adaptor for Illumina and User enzyme)

NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers Set 2), NewEngland BioLabs, Cat# E7780S (contains i5/i7 indexes, Adaptor for Illumina and User enzyme)

AMPure XP® paramagnetic beads for PCR purification, Beckman Coulter, Cat# A63881

Kapa Library Quantification Kit for Illumina®, Complete Kit (ROX Low), Roche, Cat# KK4873 (for Quantstudio 12k)

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Before starting, generate the "odd" and "even" primer pools as follows:

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) given overleaf to the pooled 1.5-ml tube (between 7.5 - 15). Repeat with the odds. Vortex and spin down. These are your pooled stocks.
4. Dilute the pools one in ten across several aliquots with molecular grade water. Vortex and spin down. These are your working primer pools.

SubArtic primers v3-2 091121.csv

cDNA prep 1h 25m

1 In a freshly bleached Pre-PCR hood (ideally in an isolated clean room), that has been subjected to UV irradiation for **00:40:00** , add LunaScript and RNA sample to a tube/well as follows (making sure to include negative controls): 1h

A	B
Component	**Vol/Rxn**
LunaScript Super Mix	2 µl
RNA sample	8 µl

1.1 Mix thoroughly by pipetting up and down several times, seal plate, and centrifuge briefly. 10m

1.2 Incubate the reaction as follows (with heated lid): 15m

A	B
Temperature	**Time**
25°C	2 min
55°C	10 min
95°C	1 min
4°C	Hold

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Multiplex PCR 4h 10m

2 30m Primers are separated into two pools, odd and even, depending on where they sit across the Spike region. See the guidelines section for further details. In a clean pre-PCR hood set up two PCR reactions, one per pool as follows:

A	B	C
Component	**Rxn 1**	**Rxn 2**
Pool Even	0 µl	1.75 µl
Pool Odd	1.75 µl	0 µl
Q5 Hot Start Hi-Fi 2x Master Mix	6.25 µl	6.25 µl
Total	**8 µl**	**8 µl**

2.1 10m Add **4.5 µL** of cDNA to respective wells to give a total volume of **12.5 µL** . Include negative controls. Mix by pipetting, seal plate and centrifuge briefly.

2.2 3h 30m Perform PCR using the following program (with heated lid):

A	B	C	D
Step	**Temperature**	**Time**	**Cycles**
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	15 s	35
Annealing & Extension	60°C	5 min	
Hold	4°C	Hold	

Fewer PCR cycles can be used for samples of higher concentration.

PCR pooling 30m

2.3 20m In a freshly bleached post-PCR hood that has been subjected to UV for **00:40:00** , combine Pool Even and Pool Odd to give **20 µL** in each tube/well. Add **20 µL** nuclease-free water to dilute products 1 in 2.

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At least a 1 in 5 dilution is advised when using highly concentrated clincial samples subjected to a 35x cycle PCR.

2.4 Check the quality and concentration of negatives and a selection of samples using a fluorometer and/or Agilent TapeStation. 10m

Samples can be normalised at this point if even barcode representation is required, but at the cost of time.

NEBNext Ultra II End Prep 50m

3 Prepare a master mix of the reagents as below by multiplying volumes by the number of samples; add 10% to allow for pipetting error. 5m

A	B
Component	**Vol/PCR Rxn**
NEBNext Ultra II End Prep Enzyme Mix	0.6 µl
NEBNext Ultra II End Prep Reaction Buffer	1.4 µl
Total	**2 µl**

3.1 Mix well by pipetting and centrifuge briefly. 5m

3.2 Combine **2 µL** of End Prep master mix with **10 µL** of amplified cDNA and mix by pipetting. Spin down, seal, place in a thermocycler and run the following program: 40m

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A	B
Temperature	**Time**
20°C	15 min
65°C	15 min
4°C	Hold

Adapter Ligation 45m

4 Prepare adapter ligation master mix by adding volumes as detailed below for each sample, adding 10% to allow for pipetting error. 10m

A	B
Component	**Vol/PCR rxn**
NEBNext Ultra II Ligation Master Mix	6 µl
NEBNext Ligation Enhancer	0.2 µl
NEBNext Adapter	0.5 µl
Total	**6.7 µl**

4.1 Add **6.7 µL** ligation mix to each **12 µL** amplified cDNA/mastermix and mix by pipetting. Incubate at **20 °C** for **00:15:00** . 25m

4.2 Add **1 µL** of USER enzyme to the ligation mixture. Mix by pipetting, centrifuge briefly and incubate at **37 °C** for **00:15:00** . 10m

Magnetic Bead clean up 1h 15m

5 Increase volume of sample from **19.7 µL** to **25 µL** by the addition of nuclease-free water. 5m

5.1 Perform a 0.9x Ampure XP bead clean by adding **22.5 µL** of Ampure XP beads and mix by pepetting. Incubate for **00:05:00** at **Room temperature** . 10m

5.2 Transfer tube/plate to magnet and allow beads to clump for **00:05:00** . Remove supernatant taking care not to disturb pellet, and discard. 15m

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5.3 Wash the beads with **100 µL** 80% ethanol for **00:00:30** then remove ethanol by pipetting. Repeat one more time making sure to remove any residual ethanol. 10m

5.4 Allow beads to air dry for around **00:05:00** ; ensure that all the ethanol has evaporated. Do not let beads dry to the point of cracking, as this could affect the amount of DNA recovered. 5m

5.5 Add **17 µL** of nuclease-free water to each well and mix by pipetting. Allow to stand for **00:05:00** then transfer to magnet and allow beads to clump for **00:05:00** . Remove **15 µL** into a fresh low-bind tube. 20m

5.6 Perform Qubit assay to determine concentration. 10m

Addition of NEBNext indexes 1h 5m

6 Add the following components to separate wells of a PCR plate. Make sure to use the indexes in appropriate wells so that each sample has unique i5 and i7 indexes. If using less than 12 samples, the i5 primer can be replaced by the Universal PCR primer. 20m

A	B
Component	**Vol/Rxn**
Adapter ligated cDNA fragments	7.5 µl
NEBNext Ultra II Q5 Master Mix	12.5 µl
Index Primer i7	2.5 µl
Index Primer i5*	2.5 µl
Total	**25 µl**

* if using 12 samples or fewer replace index primer i5 with the Universal primer.

6.1 Mix thoroughly by pipetting and centrifuge briefly. 5m

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6.2 Place on thermocycler and perform PCR using the following program: 40m

A	B	C	D
Cycle Step	**Temperature**	**Time**	**Cycles**
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	3 - 15*
Annealing/Extension	65°C	75 s	
Final Extension	65°C	5 mins	1
Hold	4°C	Hold	

*Refer to NEBNext protocol for number of cycles required. (Examples: 100 ng = ~3 cycles, 50 ng = ~3–4 cycles, 10 ng = ~6–7 cycles)

Pool 1h 15m

7 Pool **5 µL** of each sample together in a fresh low-binding microfuge tube and mix. This will give a final volume of **60 µL** for twelve samples. If larger numbers of samples are used then pool **5 µL** of each and mix. Split into aliquots of about **120 µL** . 10m

7.1 Perform a 0.7x bead clean by adding **42 µL** or **84 µL** magnetic beads, depending on sample quantity. Leave for **00:05:00** to allow beads to bind DNA. 10m

7.2 Place on a magnet and allow beads to clump for **00:05:00** . Remove supernatant and discard. 10m

7.3 Wash pellet with **100 µL** 80% ethanol for **00:00:30** and remove. Repeat one more time. Allow the ethanol to dry off for about **00:05:00** , making sure the pelleted beads do not dry out, which can reduce DNA recovery. 10m

7.4 Add **30 µL** of nuclease-free water and mix by pipetting. Leave for **00:05:00** at **Room temperature** to allow DNA to elute from the beads. 10m

7.5 Place the tube/plate on the magnet and let the beads clump for **00:05:00** . Elute DNA into a fresh low-binding tube. 10m

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7.6 Perform TapeStation assay to determine if primer dimers are present. If present, perform another 0.7x bead clean and check again. 15m

qPCR 2h

8 Perform qPCR by using a Kapa Library Quant kit for Illumina from Roche. Reactions are scaled down to **10 µL** . Use the appropriate Kapa kit for the type of qPCR machine used, see https://pim-eservices.roche.com/eLD/api/downloads/ca670ceb-fb38-eb11-0291-005056a71a5d?countryIsoCode=pi 1h 30m

8.1 Enter the values obtained from qPCR into the KAPA Library Quantification Data Analysis Template. 15m

8.2 Dilute sample to **4 Nanomolar (nM)** . 15m

Library dilution 14m

9 Take **5 µL** of **4 Nanomolar (nM)** dilution of library and add **5 µL** of **0.1 Molarity (M)** NaOH to denature the DNA and incubate at **Room temperature** for **00:05:00** . 5m

9.1 Neutralise with **5 µL** of **200 Milimolar (mM)** Tris-HCl **pH 7.0** for **00:01:00** . 5m

9.2 Add **985 µL** cold HT1 (hybridisation buffer) to give **20 Picomolar (pM)** denatured library 2m

9.3 Take **35 µL** of **20 Picomolar (pM)** sample and add **465 µL** of HT1. This will give **500 µL** of **1.4 Picomolar (pM)** sequencing mix. 2m

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PhiX control 18m

10 Thaw a tube of [M]**10 Nanomolar (nM)** PhiX stock . 5m

10.1 Combine the following volumes in a microcentrifuge tube: 5m
[M]**10 Nanomolar (nM)** PhiX (10 µl) and RSB (Resuspension Buffer) (15 µl).
The total volume is 25 µl at [M]**4 Nanomolar (nM)** .
Vortex briefly and then pulse centrifuge. The [M]**4 Nanomolar (nM)** PhiX can be stored frozen for 3 months.

10.2 Combine **5 µL** of [M]**4 Nanomolar (nM)** PhiX with 5 µl of 5m
[M]**0.1 Molarity (M)** NaOH. Vortex briefly and pulse centrifuge. Incubate at **Room temperature** for **00:05:00**

10.3 Add **5 µL** of [M]**200 Nanomolar (nM)** Tris-HCl (pH**7.0**), vortex briefly, 1m
pulse centrifuge and incubate at **Room temperature** for **00:01:00**

10.4 Add **985 µL** of HT1 (hybridisation buffer) to give a concentration of 1m
[M]**20 Picomolar (pM)** . Vortex briefly and pulse centrifuge.

10.5 PhiX control at [M]**20 Picomolar (pM)** can be frozen at -20°C for up to two 1m
weeks. After this time cluster numbers tend to decrease.

MiniSeq Sequencing 5m

11 Combine your library with PhiX control to give a final dilution of PhiX of 5% 5m

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