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We use this protocol and it's working

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Standard-S: PCR barcoding of SARS-CoV-2 S gen amplicons for Nanopore sequencing

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ABSTRACT

Most of the defining mutations of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) variants of concern (VOCs) have been identified in the S gene sequence. For this reason, S-based lineage assignment is possible using the current nomenclature system. We have developed a protocol for overlapping amplification of the S gene sequence using previously reported primer sequences (V3 primers of ARTIC Network) in combination with a PCR barcoding approach of the samples for Nanopore sequencing plataforms. This protocol allows the screening of multiple COVID-19 positive samples for lineage/clade assignment and mutational surveillance of the spike gene. Additionally, this protocol can be is easily adapted for dual barcoding using the "Ligation sequencing amplicons - dual barcoding (SQK-LSK109 with EXP-NBD104, EXP-NBD114, and EXP-PBC096)" Nanopore protocol to upscale the number of samples per run.

MATERIALS

Reagents:

- LunaScript® RT SuperMix
- Nuclease free water
- Absolute ethanol
- Q5® Hot Start High-Fidelity 2X Master Mix
- PCR Barcoding Expansion Pack 1-96 (EXP-PBC096)
- Ligation Sequencing Kit (SQK-LSK109)
- Agencourt AMPure XP beads (Beckman Coulter™, A63881)
- NEBNext FFPE Repair Mix (M6630)
- NEBNext Ultra II End repair/dA-tailing Module (E7546)
- NEBNext Quick Ligation Module (E6056)
- Sequencing Auxiliary Vials (EXP-AUX002).

- Flow Cell Priming Kit (EXP-FLP002)
- Qubit™ 1X dsDNA High Sensitivity (HS) and Broad Range (BR) Assay (Q33266)

Plastics:

- 0.2 ml thin-walled PCR tubes, PCR strips with caps, PCR plate
- 1.5 ml Eppendorf DNA LoBind tubes
- Filter tips P2, P10, P20, P100, P200, P1000
- Qubit™ Assay Tubes (Q32856)

Equipment:

- Pipettes and pipette tips P2, P10, P20, P100, P200, P1000
- Thermal cycler
- Magnetic separator, suitable for 0.2 ml tubes and 1.5 ml tubes.
- Microfuge
- Vortex mixer
- Ice bucket with ice
- PCR cooler
- Qubit fluorometer

Primers sequences

This primers used in this protocol are from the V3 scheme of the ARTIC primers for whole genome sequences. This primers span the S gene region of the SARS-CoV-2 genome and have the ONT tag at the 5' end for compatibility with the PCR Barcoding Expansion Pack 1-96 (EXP-PBC096).

	Name	Secuencia 5'-3'	ARTIC Network V3 primers
	ONT_Sseq_1_LEFT	TTTCTGTTGGTGCTGATATTGC ACAAAAGAAAATGACTCTAAAGA GGGTTT	nCoV-2019_70_LEFT
	ONT_Sseq_1_RIGHT	ACTTGCCTGTCGCTCTATCTTC ACTCTGAACTCACTTTCCATCCAA C	nCoV-2019_72_RIGHT
	ONT_Sseq_3_LEFT	TTTCTGTTGGTGCTGATATTGC AGAGTCCAACCAACAGAATCTATT GT	nCoV-2019_75_LEFT
	ONT_Sseq_3_RIGHT	ACTTGCCTGTCGCTCTATCTTC ACCTGTGCCTGTTAAACCATTGA	nCoV-2019_76_RIGHT_alt0
	ONT_Sseq_5_LEFT	TTTCTGTTGGTGCTGATATTGC CAACTTACTCCTACTTGGCGTGT	nCoV-2019_78_LEFT
	ONT_Sseq_5_RIGHT	ACTTGCCTGTCGCTCTATCTTC TGGAGCTAAGTTGTTTAACAAGCG	TGGAGCTAAGTTGTTTAACAA GCG
	ONT_Sseq_7_LEFT	TTTCTGTTGGTGCTGATATTGC GGGCTATCATCTTATGTCCTTCCC T	nCoV-2019_82_LEFT

	Name	Secuencia 5'-3'	ARTIC Network V3 primers
	ONT_Sseq_7_RIGHT	ACTTGCCTGTCGCTCTATCTTC AGGTGTGAGTAAACTGTTACAAAC AAC	nCoV-2019_84_RIGHT
	ONT_Sseq_2_LEFT	TTTCTGTTGGTGCTGATATTGC ACACGTGGTGTTTATTACCCTGAC	nCoV-2019_72_LEFT
	ONT_Sseq_2_RIGHT	ACTTGCCTGTCGCTCTATCTTC GCAACACAGTTGCTGATTCTCTTC	nCoV-2019_74_RIGHT
	ONT_Sseq_4_LEFT	TTTCTGTTGGTGCTGATATTGC CCAGCAACTGTTTGTGGACCTA	nCoV-2019_77_LEFT
	ONT_Sseq_4_RIGHT	ACTTGCCTGTCGCTCTATCTTC TGTGTACAAAAACTGCCATATTGC A	nCoV-2019_78_RIGHT
	ONT_Sseq_6_LEFT	TTTCTGTTGGTGCTGATATTGC TTGCCTTGGTGATATTGCTGCT	nCoV-2019_80_LEFT
	ONT_Sseq_6_RIGHT	ACTTGCCTGTCGCTCTATCTTC TGCCAGAGATGTCACCTAAATCAA	nCoV-2019_82_RIGHT
	ONT_Sseq_8_LEFT	TTTCTGTTGGTGCTGATATTGC TGCTGTAGTTGTCTCAAGGGCT	nCoV-2019_84_LEFT
	ONT_Sseq_8_RIGHT	ACTTGCCTGTCGCTCTATCTTC ACGAAAGCAAGAAAAAGAAGTAC GC	nCoV-2019_86_RIGHT

ARTIC Network primers with a universal tag for PCR barcoding using ONT sequencing plataforms

Odd and pair primers are equimolar aliquoted to a final concentration of 100 µM, separately in a pool A and pool B scheme, as described for whole genome sequencing. The working primer pool stock for the odd and pair pool used in this protocol were at a concentration of 30 µM.

Reverse transcription

1h

1 Keeping the SARS-CoV-2 extracted RNA samples on ice all the time and spin down the tubes.

2 Set up the RT-PCR reaction tubes in a clean pre-PCR cabinet by adding 2 µL of LunaScript® RT SuperMix to each PCR tube. Include a RT-PCR negative control by replacing RNA sample with nuclease free water.

Note

Add positive and negative controls if available for results validation.

3 Add 8 uL of sample to the tube containing the LunaScript® RT SuperMix and mix gently. The final volume of the reaction is 10 µL.

4 Incubate in a thermal cycler using the the following instructions:

	Step	Temperature (ºC)	Time	Cycles
	Primer annealing	25	2 min	1
	cDNA Synthesis	55	10 min	
	Heat inactivation	95	1 min	
	Hold	10	-	

Table 1: SARS-CoV-2 RT-PCR thermal profile.

S gene tiled amplification

2h

5 Set up the first round PCR reaction in a pre-PCR cabinet for primer pool A and primer pool B

	Reagent	PCR master mix pool A	PCR master mix pool B
	Nuclease free water	400 µL	400 µL
	Primer pool A (30 µM)	25 µL	--
	Primer pool B (30 µM)		25 µL
	Q5® Hot Start High-Fidelity 2X Master Mix	625 µL	625 µL
	Final volume	1050 µL	1050 µL

Table 2: S gene PCR amplification master mixes pool A an B.

Note

Keep the reversed transcribed sample on a PCR cooler or ice.

6 Transfer 10,5 µL of the PCR Master mix Pool A to the PCR tube set A and 10,5 µL of the PCR Master mix Pool B to the PCR tube set B, respectively.

7 Using a multichannel pipette, transfer 2 µL of reversed transcribed product from the Reverse Transcription step to the corresponding Pool A and Pool B PCR tube. Carry over the negative and positive controls.

Note

Store at -20 ºC the remaining reverse transcribed sample for further characterization, if needed.

8 Spin down the PCR tubes and incubate in the thermal cycler with the following program:

	Step	Temperature (ºC)	Time	Cycles
	Initial denaturation	98	30 sec	1
	Denaturation	98	15 sec	20
	Annealing and extension	63	3 min	
	Hold	10	--	--

Table 3: S gene PCR amplification using the Q5® Hot Start High-Fidelity polymerase.

9 Remove the tubes from the thermal cycler and spin down briefly. Mix the volume of pool A and pool B in the PCR tube set B.

Note

Up to this point it is highly recommended to check pool A and pool B amplification of the positive and negative controls and some random samples in a 1% agarose gel electrophoresis. Amplicons are expected to have ~1 Kb.

10 Prepare a set of PCR tubes with 9 µL of nuclease free water for a 1:10 dilution of the first round PCR amplification.

PCR barcoding of S gene amplicons

1h 15m

11 Set up the second round PCR reaction in a pre-PCR cabinet for primer pool A and primer pool B as follows:

	Reagent	Volume (µL)
	Q5® Hot Start High-Fidelity 2X Master Mix	6.00
	PCR Barcodes (BC01-BC96)	1.00
	Final volume	6.75

Table 4: S gene PCR barcoding mix using the Q5® Hot Start High-Fidelity polymerase.

12 Using a multichannel pipette, add 5 µL of the dilution 1:10 of the first round amplification of each sample. Spin down and incubate in the thermal cycler as follows:

	Step	Temperature (ºC)	Time	Cycles
	Initial denaturation	98	30 s	1
	Denaturation	98	15 s	15
	Annealing	62	15 s	
	Extension	72	1m	
	Final extension	72	10 m	1
	Hold	4	--	--

Table 5: PCR barcoding thermal cycling profile

Pooling and clean-up

50m

13 Spin down the tubes and pool all samples in a 1.5 mL LoBind tube.

> Note
>
> If barcode balance is desired, barcoded samples should be individually purified using AMPure XP beads 0.5X and then equimolar pooled.

14 Add 0.5X volume of Ampure XP beads. Incubate 5 minutes in a rotator mixer. Spin down and rest the tubes in a magnetic rack for PCR tubes for 5 minutes.

> Note
>
> Make sure AMPure XP beads reached room temperature before use.

15 Discard the supernatant by aspiration, taking care not to disturb the pellet beads.

16 Wash the beads with Ethanol 70%. Repeat this step.

17 Let the pellet air dry for ~30 seconds and add 50 uL of nuclease free water. Incubate for 2 minutes at room temperature, spin down and place the PCR tubes in the magnetic rack for 5 minutes. Recover the supernatant.

18 Quantify the clean barcoded pool using a suitable fluorometric assay.

19 Prepare 1 µg of clean barcoded pool in 48 µL.

Note

Take the barcoded pool the repair and end-prep step or store the DNA at 4 ºC overnight or -20 ºC for long term storage.

End prep and clean-up

50m

20 Prepare the end prep mix as follows:

	Sample/Reagent	Volume (µL)
	Clean barcoded pool (1 µg)	48
	NEBNext FFPE DNA Repair Buffer	3.5
	NEBNext FFPE DNA Repair Mix	2
	Ultra II End-prep reaction buffer	3.5
	Ultra II End-prep enzyme mix	3
	Final volume	60

Table 6: End prep reaction mix.

21 Incubate the End-prep mix in the thermal cycler as follows:

	Step	Temperature (ºC)	Time
	Enzymes incubation	20	5 min
	Enzymes inactivation	65	5 min
	Hold	4	--

Table 7: End prep and DNA repair thermal incubation

22 (Optional) Add 1X volume of AMPure XP beads to the reaction and incubate in the rotator mixer for 5 minutes. Spin down and rest the tubes in the magnetic rack for 5 minutes.

23 Discard the supernatant by aspiration, taking care not to disturb the pellet beads.

24 Wash the pellet with Ethanol 70% twice.

25 Let the pellet air dry for ~30 seconds and add 61 µLof nuclease free water and incubate at room temperature for 2 minutes.

26 Spin down and rest the tubes in the magnetic rack for 5 minutes and recover the supernatant.

Note

Take forward the end-prepped DNA into the adapter ligation step or store the sample at 4 ºC overnight

ONT adapter ligation and final clean-up

1h 20m

27 Prepare the following adapter ligation mix:

	Reagent	Volume (uL)
	Clean end-prepped DNA	60
	Ligation Buffer (LNB)	25
	NEBNext Quick T4 DNA Ligase	10
	AMX adapter	5
	Final volume	100

Table 8: Adapter (AMX) ligation mix.

28 Incubate the reaction for 10 minutes at room temperature.

Note

Do not incubate the reaction for longer than 10 minutes if the AMPure XP beads purification was omitted.

29 Spin down the tubes and add AMPure XP beads 0.4X volume. Incubate in the rotator mixer for 5 minutes at room temperature.

30 Spin down the tubes and rest the tubes in the magnet rack for 5 minutes. Pipette off the supernatant.

31 Add 200 µL of Short Fragment Buffer (SFB) and mix gently. Spin down and place the tube in the magnet rack for 5 minutes. Discard the supernatant.

32 Repeat the previous step.

33 Spin down the tube and place it in the magnet rack. Remove any residual SFB and let it air dry for ~30 seconds.

34 Remove the tubes from the magnet and add 15 µL of Elution Buffer (EB). Flick the tube, spin down briefly and incubate the tube for 10 minutes at 37 ºC.

Note

The final library can be stored for up to 3 days at 4 ºC.

35 Quantify the final library using a fluorometric assay.

Flow cell priming and loading

15m

36 Use the Nanopore standard procedure for priming the FLO-MIN106D or FLO-FLG001 flow cells. https://community.nanoporetech.com/nanopore_learning/lessons/priming-and-loading-your-flow-cell

37 Once the flow cell is correclty primer proceed to load the library mixing the following:

	Sample/Reagent	Volume (uL)
	Sequencing Buffer (SQB)	37.5
	Loading Beads (LB)	25.5
	DNA library (~300 ng)	12
	Final volume	75

Table 9: DNA library to load in a FLO-MIN106D.

	Sample/Reagent	Volume (uL)
	Sequencing Buffer (SQB)	15
	Loading Beads (LB)	10
	DNA library (~200 ng)	5
	Final Volume	30

Table 9: DNA library to load in a FLO-FLG001.

Note

We recommend to load initially 300 ng for the FLO-MIN106D and 200 ng for the FLO-FLG001 and monitor the pore occupancy over the first 20 minutes and reload if the pore occupancy is lower than 60%. Additionally, a refuel after 12 hours of the sequencing run is recommended.
Note that the amount of DNA library is significantly higher than the amount recommended by Oxford Nanopore (20-50 fmol). If not sure, begin from this number and upscale accordingly to reach a satisfactory pore occupancy.

Data analysis

1h

38 Use epi2me-labs/wf-artic V1 scheme for consensus generation

Software

wf-artic NAME

hedgehog for lineage set assignment using maximum ambiguity

Software

hedgehog NAME

Use president for S gene completeness

Software

president NAME

and samtools for average sequencing depth

Software

NAME

samtools