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

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We use this protocol and it's working

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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 platforms. 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 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 ACAAAAGAAAATGACTCTAAAGAGGGTTT	nCoV-2019_70_LEFT
	ONT_Sseq_1_RIGHT	ACTTGCCTGTCGCTCTATCTTC ACTCTGAACCTCACTTTCCATCCAAC	nCoV-2019_72_RIGHT
	ONT_Sseq_3_LEFT	TTTCTGTTGGTGCTGATATTGC AGAGTCCAACCAACAGAATCTATTGT	nCoV-2019_75_LEFT
	ONT_Sseq_3_RIGHT	ACTTGCCTGTCGCTCTATCTTC ACCTGTGCCTGTTAAACCATTA	nCoV-2019_76_RIGHT_alt0
	ONT_Sseq_5_LEFT	TTTCTGTTGGTGCTGATATTGC CAACTTACTCCTACTTGGCGTGT	nCoV-2019_78_LEFT
	ONT_Sseq_5_RIGHT	ACTTGCCTGTCGCTCTATCTTC TGGAGCTAAGTTGTTTAAACAAGCG	TGGAGCTAAGTTGTTTAAACAAGCG
	ONT_Sseq_7_LEFT	TTTCTGTTGGTGCTGATATTGC GGGCTATCATCTTATGTCCTTCCCT	nCoV-2019_82_LEFT
	ONT_Sseq_7_RIGHT	ACTTGCCTGTCGCTCTATCTTC AGGTGTGAGTAACTGTTACAAACAAC	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 TGTGTACAAAACTGCCATATTGCA	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 ACGAAAGCAAGAAAAAGAAGTACGC	nCoV-2019_86_RIGHT

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

Odd and pair primers are equimolar aliquoted to a final concentration of 100 μ M, 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 μ M.

Troubleshooting

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 μ L 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 μ L of sample to the tube containing the LunaScript® RT SuperMix and mix gently. The final volume of the reaction is 10 μ L.
- 4 Incubate in a thermal cycler using the the following instructions:

	Step	Temperature (°C)	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 and B.

Note

Keep the reverse 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 reverse 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 °C 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 (°C)	Time	Cycles
Initial denaturation	98	30 sec	1

Step	Temperature (°C)	Time	Cycles
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 μ L 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 (°C)	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 °C overnight or -20 °C 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 (°C)	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 µL of 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 °C 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



Reagent	Volume (uL)
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 μ L 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 μ L of Elution Buffer (EB). Flick the tube, spin down briefly and incubate the tube for 10 minutes at 37 °C.

Note

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



- 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 correctly primed 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

samtools

NAME