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Fast-S: Single tube amplification and PCR barcoding of SARS-CoV-2 S gene 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 a fast and cost-effective screening of COVID-19 positive samples for lineage/clade assignment and mutational surveillance of the spike gene.

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).
- [Optional] TriTrack DNA Loading Dye (6X)

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

The primers used in this protocol are from the V3 scheme from the ARTIC Network group (https://github.com/artic-network/primer-schemes/blob/master/nCoV-2019/V3/nCoV-2019.tsv) for SARS-CoV-2 whole genome sequencing. The selection of primers was based in the Oxford Nanopore protocol "PCR tiling of SARS-CoV-2 spike protein gene with rapid barcoding and Spike Seq RT PCR Expansion (SQK-RBK110.96 and EXP-SRT001), Version: SRT-9128_v110_revB_14Jul2021" (https://nanoporetech.com/). These primers span the S gene region of the SARS-CoV-2 genome from approximately the position 21076 to 26315 of the Wuhan reference (WIV04) and have the ONT universal

sequence tags for compatibility with the Ligation sequencing amplicons - PCR barcoding (SQK-LSK109 with EXP-PBC096) protocol (**https://nanoporetech.com/)**.

Forward: 5′ TTTCTGTTGGTGCTGATATTGC-[PRIMER] 3′
Reverse: 5′ ACTTGCCTGTCGCTCTATCTTC-[PRIMER] 3′

	Name	Secuencia 5'-3'	ARTIC Network V3 primers
	ONT_Sseq_1_LEFT	TTTCTGTTGGTGCTGATATTGC ACAAAAGAAAATGACTCTAAAGAGGGTTT	nCoV-2019_70_LEFT
	ONT_Sseq_1_RIGHT	ACTTGCCTGTCGCTCTATCTTC ACTCTGAACTCACTTTCCATCCAAC	nCoV-2019_72_RIGHT
	ONT_Sseq_3_LEFT	TTTCTGTTGGTGCTGATATTGC AGAGTCCAACCAACAGAATCTATTGT	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	nCoV-2019_80_RIGHT
	ONT_Sseq_7_LEFT	TTTCTGTTGGTGCTGATATTGC GGGCTATCATCTTATGTCCTTCCCT	nCoV-2019_82_LEFT
	ONT_Sseq_7_RIGHT	ACTTGCCTGTCGCTCTATCTTC AGGTGTGAGTAAACTGTTACAAACAAC	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 TGTGTACAAAAACTGCCATATTGCA	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 sequence TAG for PCR barcoding using ONT sequencing platforms

Odd and pair primers are equimolar aliquoted separately to a final concentration of 100 µM.

Troubleshooting

Safety warnings

Follow standard health and safety guidelines when manipulating COVID-19 patient samples.

Barcode immobilization

1h 20m

1 Place PCR tubes with caps in a 96 sample rack for pool A and pool B amplification step. 15m

2 Label a set of PCR tube caps for amplification using the pool A and pool B according to the Oxford Nanopore PCR Barcoding Expansion 1-96 (stock 10 µM). 5m

Note

You will need two sets of PCR tubes with the same barcode identification.
PCR strips caps are more easy to handle.

	-	1A/1B	2A/2B	3A/3B	4A/4B	5A/5B	6A/6B	7A/7B	8A/8B	9A/9B	10A/10B	11A/11B	12A/12B
	A	BC01	BC02	BC03	BC04	BC05	BC06	BC07	BC08	BC09	BC10	BC11	BC12
	B	BC13	BC14	BC15	BC16	BC17	BC18	BC19	BC20	BC21	BC22	BC23	BC24
	C	BC25	BC26	BC27	BC28	BC29	BC30	BC31	BC32	BC33	BC34	BC35	**BC36**
	D	BC37	BC38	BC39	BC40	BC41	BC42	BC43	BC44	BC45	BC46	BC47	**BC48**
	E	BC49	BC50	BC51	BC52	BC53	BC54	BC55	BC56	BC57	BC58	BC59	**BC60**
	F	BC61	BC62	BC63	BC64	BC65	BC66	BC67	BC68	BC69	BC70	BC71	**BC72**
	G	BC73	BC74	BC75	BC76	BC77	BC78	BC79	BC80	BC81	BC82	BC83	**BC84**
	H	BC85	BC86	BC87	BC88	BC89	BC90	BC91	BC92	BC93	BC94	BC95	**BC96**

Table 1: Oxford Nanopore PCR Barcoding Expansion 1-96 tube labeling for pool A and pool B amplification step

3 Spin down briefly the PCR Barcoding Expansion 1-96 tubes and open each carefully avoiding spray generation. Transfer 1 µL of each barcode using a multichannel pipette to the PCR tube cap and incubate 1hr at 37 ºC or at room temperature until the drop has dried. 1h

Note

Optional: add 1 µL of a dilution of 1:10 of a dye, such as TriTrack DNA Loading Dye (6X) to visualize the drop in the PCR tube cap.

Store in a dry place until use.

Reverse transcription

30m

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

5 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. 10m

Note

Add negative and positive controls for results validation.

6 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. 5m

7 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 2: SARS-CoV-2 RT-PCR thermal profile.

S gene tiled amplification

2h

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

5m

	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
	LongAmp® Taq 2X Master Mix	625 µL	625 µL
	Final volume	**1050 µL**	**1050 µL**

Table 3: S gene PCR multiplex amplification master mixes for pool A and B.

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

5m

10 Using a multichannel pipette, transfer 2 µL of reversed transcribed product from the "Reverse Transcription" section to the corresponding Pool A and Pool B PCR strip tubes. Carry over the negative and positive controls.

10m

Note

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

11 Spin down briefly and replace the PCR strip caps with the PCR tube caps containing the immobilized Barcoding Expansion 1-96 barcodes as described in the "Barcode immobilization" section.

Note

Make sure to match the sample and barcode ID correctly for both pool A and B.

12 Incubate in the thermal cycler with the following program:

	Step	Temperature (ºC)	Time	Cycles
	Initial denaturation	94	30 s	1
	Denaturation	94	15 s	20
	Annealing and extension	63	3 m	
	Hold	10	--	--

Table 4: S gene PCR amplification using the LongAmp™ *Taq*2X polymerase.

Single tube PCR barcoding of S gene amplicons

1h

13

Remove the PCR strips off the thermal cycler and invert the tubes at least 10 times until the immobilized PCR barcodes in the PCR strip caps are dissolved in the PCR master mix from the amplification step. Spin down and return the tubes into the thermal cycler and proceed to the barcoding step:

	Step	Temperature (ºC)	Time	Cycles
	Initial denaturation	95	3 m	1
	Denaturation	95	15 s	15
	Annealing	62	15 s	
	Extension	65	50 s	
	Final extension	65	10 m	1
	Hold	10	--	--

Table 5: S gene PCR barcoding of S gene amplified material using the amplification PCR master mix from the amplification step

Note

We use a 4X molar excess of barcodes for the PCR barcoding step.

Pooling and clean-up

30m

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

15 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 5 minutes.

Note

Make sure AMPure XP beads have reached room temperature before use.

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

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

18 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.

19 Recover the supernatant.

20 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

30m

21 Prepare the end prep mix as follows:

	Sample/Reagent	Volume (µL)
	NEBNext FFPE DNA Repair Mix	2

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

Table 6: End prep reaction mix.

Note

(Optional) DNA CS can be added for internal control and to increase DNA in the final library.

22 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

Table 7: End-prep thermal incubation

23 [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. Discard the supernatant by aspiration, taking care not to disturb the pellet beads. Let the pellet air dry for ~30 seconds and add 61 µL of nuclease free water and incubate at room temperature for 2 minutes.Spin down and rest the tubes in the magnetic rack for 5 minutes and recover the supernatant. 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

24 Prepare the following adapter ligation mix:

	Reagent	Volume (µL)
	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.

25 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.

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

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

28 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.

29 Repeat the previous step.

30 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.

31 Quantify the final library using a fluorometric assay.

Flow cell priming and loading

15m

32 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

33 Once the flow cell is correctly 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.

	A	B
	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.

Basic data analysis

1h

34 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

Run the sequencing experiment until reaching at least 300X of average sequencing depth and or more than 90% of S gene completeness for optimal results.