Sanger sequencing of SARS-CoV-2 Spike protein

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The SARS-CoV-2 responsible for the ongoing COVID pandemic reveals particular evolutionary dynamics and an extensive polymorphism, mainly in Spike protein. Monitoring the S protein mutations is crucial for successful controlling measures and detect variants that can evade vaccine immunity. Even after the costs reduction imposed by the pandemic, the new generation sequencing methodologies remain unavailable to many scientific groups. Therefore, to support the urgent surveillance of SARS-CoV-2 S protein, this work describes a protocol for complete nucleotide sequencing of the S protein using the Sanger technique. Thus, any laboratory with experience in sequencing can adopt this protocol.

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protocol

Salles TS, Cavalcanti AC, da Costa FB, Dias VZ, de Souza LM, et al. (2022) Genomic surveillance of SARS-CoV-2 Spike gene by sanger sequencing. PLOS ONE 17(1): e0262170. https://doi.org/10.1371/journal.pone.0262170

Sanger sequencing, SARS-CoV-2, Spike protein

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This protocol works well with recent extracted RNA samples, ideally with ct values lower than 20.

- Thermal cycler
- PCR tubes 0.2mL
- Filter pipette tips: 1-10µL+ 10-100µL
- Micropipettes: 1-10µL+ 10-100µL
- Superscript III one-step RT-PCR kit (Invitrogen, Carlsbad, CA, USA)
- Primers
- Nuclease free water
- Extracted RNA from SARS-CoV-2 positive samples with low ct values
- Agarose
- TAE buffer (Tris-acetate-EDTA)
- LGC biotecnologia - Blue Green loading Dye I
- Horizontal Electrophoresis cube
- UV Transilluminator
- Nanodrop spectrophotometer

1 RT-PCR

- Program the thermal cycler before setting up the reaction. The thermal cycler should be preheated to 45–60°C.
- Keep all components, reaction mixes, and samples on ice. After preparation of the samples, transfer them to the preheated thermal cycler and immediately start the RT–PCR program.
- Reaction mix should be prepared according to table 1.

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A	B	C
Component	Volume	Final concentration
2X Reaction Mix	12.5 µL	1x
Sense primer (10 µM)	1.75 µL	0.7 µM
Anti-sense primer (10 µM)	1.75 µL	0.7 µM
SuperScript™ III RT/Platinum™ Taq High Fidelity Enzyme Mix[1]	0.5 µL	
Template RNA	5 µL	
Nuclease free water	to 25 µL	

Table 1 - RT-PCR master mix

- **RT-PCR cycle is described below**:

60°C 1 min |
50°C 45 min | Reverse Transcription | 1 cycle
94°C 2 min |

95 °C 15 s Denaturation |
53 °C 30 s Annealing | 40 cycles
68 °C 60 s Extension |

68 °C 7 min Final extention | 1 cycle
4 °C ∞

- **Primers used**: **Position Melting temperature Size**

P1 forward GTTTGTTTTTCTTGTTTTATT (21551-21574) 43.5 ºC
923bp
P1 reverse ACAGTGAAGGATTTCAACGTACAC (22450-22474) 55.3 ºC

P2 forward CGTGATCTCCCTCAGGGTTTT (22190-22211) 56.8 ºC
620bp
P2 reverse TCAGCAATCTTTCCAGTTTGCC (22810-22832) 56.1 ºC

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P3	forward	GTAATTAGAGGTGATGAAGTCAGA	(22751-22775)	51.8 ºC
892bp				
P3	reverse	ACATAGTGTAGGCAATGATGGA	(23621-23643)	53.6 ºC
P4	forward	CTTGGCGTGTTTATTCTACAG	(23445-23466)	51.4 ºC
979bp				
P4	reverse	GCTTGTGCATTTTGGTTGACC	(24403-24424)	55.6 ºC
P5	forward	AGACTCACTTTCTTCCACAGCA	(24355-24377)	56.1 ºC
342bp				
P5	reverse	AGATGATAGCCCTTTCCACA	(24699-24719)	53.3 ºC
P6	forward	TTCTGCTAATCTTGCTGCTACT	(24610-24632)	54.0 ºC
766bp				
P6	reverse	GTTTATGTGTAATGTAATTTGACTCC	(25348-25372)	50.7 ºC
P7	forward	TAGAGAAAACAACAGAGTT	(21492-21511)	45.6 ºC
712bp				
P7	reverse	TGAGGGAGATCACGCACTAA	(22184-22204)	55.4 ºC
P8	forward	TTCTGCTAATCTTGCTGCTACT	(24610-24632)	54.0 ºC
825bp				
P8	reverse	CCTTGCTTCAAAGTTACAGTTCCA	(25409-25433)	55.6 ºC

2 Agarose gel Electrophoresis

- Dissolve agarose in 1.X TAE Buffer (40 mM Tris-acetate, 1 mM EDTA) to a final concentration of 1.5% agarose.
- Heat the solution in a microwave and let it cool at room temperature.
- Apply the agarose gel to the casting tray with the appropriate well comb and let it solidify.
- Mix 5 µL of PCR product with 5µL of loading buffer and 1µL Blue Green Loading dye I (LGC Biotecnologia), apply it to the gel, and run the electrophoresis at 120V.
- Visualize the gel with UV Transilluminator

3 Preparing Samples for sequencing

- Sequencing procedures are performed according to BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) guidelines.
- Measure DNA Concentration with a nanodrop spectrophotometer
- Dilute template to 200 ng/µl with nuclease-free water
- Dilute primers to 1 µM with nuclease-free water. Only one primer is used for each

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sequencing reaction, leading to two reactions per sample. Each reaction will need 1µl of diluted primer.
- Label and ship the samples and primers according to the sequencing service provider guidelines.

sequencing reaction:
- BigDye™ Terminator 3.1 Ready Reaction Mix - 8 µL
- primer (1 µM) - 6.4 µL
- Template (200ng) - 2 µL
- Deionized water (RNase/DNase-free) to - 20 µL

sequencing cycle:

96°C	1 min	Incubation	1 cycle
96 °C	10 s	Denaturation	
50 °C	05 s	Annealing	25 cycles
60 °C	4 min	Extension	
4 °C	∞		1 cycle

4 Sequence analysis

- Upon receiving the electropherograms, edit them with proper programs like chromas or Bioedit.
- For better editing accuracy, align the forward sequence with the reverse complement of the reverse sequence.
- Use BLAST search to confirm if the sequenced product corresponds to the desired target.
- Create contigs by aligning the planned overlaps between each target fragment and form one consensus sequence covering the full ORF of SARS-CoV-2 Spike protein
- Edited sequences can now be analyzed with the CoVsurver mutations app, provided by GISAID, to trace the mutation patterns of each sample and study their effects on protein structure.
- Sequences can also be deposited in the GISAID database of SARS-CoV-2.

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