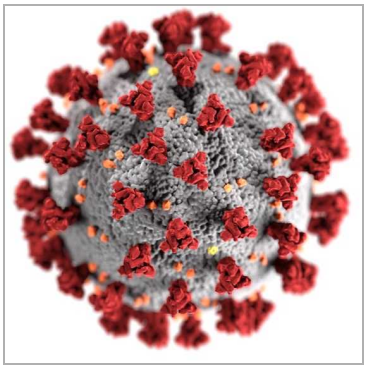

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We use this protocol and it's working

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SARS-CoV-2 S-gene Sanger Sequencing

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ABSTRACT

The presented protocol describes the analysis of fragments of the SARS-CoV-2 S-gene by the Sanger sequencing. Although whole-genome sequencing is the best method for identifying variants, this tool is not available for all laboratories. In addition Sanger sequencing retains its relevance in the face of increasing morbidity, a large number of samples, or a lack of reagents.
For analysis, we selected sequence fragments where mutations of various VOC and VOI variants are most common. Depending on the task, different pairs of primers can be used.

GUIDELINES

It is preferable to use samples (nasopharengial swabs) with a Ct less than 25.

MATERIALS

- Thermal cycler
- PCR tubes 0.2mL
- Filter pipette tips: 1-10µL+ 10-100µL
- Micropipettes: 1-10µL+ 10-100µL
- Riboprep (NA extraction kit, Amplisense, Moscow, Russia)
- Reverta L (cDNA synthesis kit, Amplisense, Moscow, Russia)
- Primers
- Nuclease free water
- SARS-CoV-2 positive samples with low ct values
- Agarose
- TAE buffer (Tris-acetate-EDTA)
- Loading Dye
- Horizontal Electrophoresis cube
- UV Transilluminator
- Nanodrop spectrophotometer

RNA Extraction

1 This section is carried out in accordance with the manufacturer's instructions for the Riboprep 10m

Nucleic Acid Isolation Kit, Amplisens, Moscow, Russia.

1. Introduce 300 µl of lysis solution into the pure tubes.
2. Add 100 µl each of samples and positive controls.
3. Mix gently and place in thermostat for 00:05:00 at 65°C.
4. Add 400 µl of precipitation solution. Mix, centrifuge for 00:05:00 at 13,000 rpm.
5. Remove the supernatant without touching the sediment. Add 500 µl of wash solution 3, wash the precipitate by inverting the tube 3-5 times. Centrifuge 1 min at 13,000 rpm.
6. Remove the supernatant without touching the sediment. Dry the precipitate in a thermostat with an open lid for 5 min at 65°C.
7. Add 50 µl of RNA buffer. Stir, in a thermostat for 5 min at 65°C. Then mix again.
8. Centrifuge 1 min at 13,000 rpm.

The supernatant contains purified RNA and DNA.
Shelf life of purified RNA/DNA at 2-8°C - 24 hours, at -16°C - one year.

cDNA synthesis

2 The cDNA was prepared according to the manufacturer's instructions: «PEBEPTA-L» kit AmpliSens, Central Research Institute of Epidemiology of Rospotrebnadzor, Catalog #K3-4-100.

3 1. In vials for state and control samples:

A	B
Component	**Value**
reaction premix	10 µl
Template RNA	10 µl
Mix on a vortex, precipitate drops	

2. Incubate the reaction as follows:

Time	**Temperature**
30 min	37°C
Hold at	4°C

3. Dilute the resulting cDNA 2-fold:

A	B
Component	Value
DNA buffer	20 µl

A	B
Mix on a vortex, precipitate drops	

Primers sequences

4 Primer sets targeting the several Spike fragments and residue binding domain (RBD).

A	B	C	D
Primer set	**Flanked region**	**Amplicon size**	**Covered mutations**
Name			
CacV 513 F2	21530 – 22115	586 bp	L18F, T19R, T20N, P62S, delLPP25-26, A67V, delHV69-70, D80A, V83A, T95I, D138Y, G142D, delY144, delY145, delGVY143-145, H146Q, W152C, E154K, delQFR156-158
CacV 513 R			
CacV 512 F	21663 – 22158	496 bp	A67V, delHV69-70, D80A, V83A, T95I, D138Y, G142D, delY144, delY145, delGVY143-145, H146Q, W152C, E154K, delQFR156-158
CacV 512 R			
CacV 55 F	22407 –22991	585 bp	F306L, G339D, G339H, R346K/S/T, L368Y, S371L, S373P, S375F, T367T, K417N, N440K, V445P, G446S, L452R
CacV 55 R			
CacV 55 F	22407 – 23281	875 bp	F306L, G339D, G339H, R346K/S/T, L368Y, S371L, S373P, S375F, T367T, K417N, N440K, V445P, G446S, L452R, N460K, S477N, T478K, E484A/K/Q, Q493K, S494P, G496S, Q498R, N501Y, Y505H, A522S, T547K
CacV 7 R			
CacV 61 F	22517 – 23131	615 bp	R346K/S/T, L368Y, S371L, S373P, S375F, T367T, K417N, N440K, V445P, G446S, L452R, N460K, S477N, T478K, E484A/K/Q, Q493K, S494P, G496S, Q498R, N501Y, Y505H
CacV 73 R			
CacV 72 F	22752 –23335	584 bp	R346K/S/T, L368Y, S371L, S373P, S375F, T367T, K417N, N440K, V445P, G446S, L452R, N460K, S477N, T478K, E484A/K/Q, Q493K, S494P, G496S, Q498R, N501Y, Y505H, A522S, T547K
CacV 72 R			

These fragments represent overlapping regions of amplification. We recommend amplifying all fragments, and choosing a combination of fragments for the sequence depending on the tasks. In some cases, different pairs of primers work with different efficiency.

PCR amplification

5 Mix the following components in an 0.2mL 8-strip tube or 96 well PCR plate;

Component **Value**

10x Buffer	2.5 µL
MgCl2	0.5 µL
dNTP (10 mM)	1.0 µL
Forward primer (10uM)	0.5 µL
Reverse primer (10uM)	0.5 µL
Taq Polymerase	0.25 µL
H2O	18.25 µL
cDNA input	1.5 µL
Total	**25 µL**

6 11m 25s

Step	Time	Temperature	Cycle
Initial denaturation	00:05:00	**98 °C**	1x
Denaturation	00:00:00	**98 °C**	35x
Annealing	00:00:35	**59 °C**	35x
Extension	00:00:50	**72 °C**	35x
Final extension	00:05:00	**72 °C**	1x
Hold	Indefinite	**4 °C**	

Electrophoresis and amplicon purification

7 Agarose gel was prepared in 1.5 g/ml and stained with ethidium bromide.
PCR products were purified from agarose gel according to Cleanup Mini kit instructions, Evrogene, Catalog # BC023S.

1. Cut out and weigh the gel fragment containing the DNA. Put it in test tube 2 ml.
2. Add 3 volumes of "Binding Solution" to the tube with gel, but at least 350 µl.
3. Incubate mixture at 50-55°C until complete dissolution gel. To speed up the dissolution, it is recommended to stir the solution shaking the tube.
4. Place the spin column in a collection tube.
5. Transfer the sample prepared according to paragraphs 2.1-2.3 to the column andcentrifuge 30 seconds. Remove the filtrate from the collection tube.
6. Add 700 µl of Wash Solution to the column, centrifuge for 30 seconds. Remove the filtrate from the collection tube.
7. Centrifuge the empty column for 1 minute to completely remove the Wash Solution.
8. Transfer the column to a new 1.5- or 2.0-ml tube. Apply to the center of the column 15 µl of "Eluent Solution".
9. Centrifuge 1 minute to collect purified DNA.

Preparing Samples for sequencing

8 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 sequencing reaction, leading to two reactions per sample. Each reaction will need 1µl of diluted primer.
Sequencing reaction is performed with BigDye Terminator v3.1 (Applied Biosystems) and run in capillary electrophoresis (ABI 3500, Applied Biosystems), according to the manufacturer's instructions.