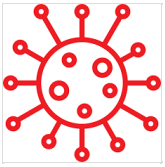

Oct 30, 2020

Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing

Valeriia Kaptelova[1], Speranskaya AS[1], et.al[1]

[1]Central Research Institute of Epidemiology of the Federal Service on Customers' Rights Protection and Human Well-being Surveillance, Moscow, Russia.

1 Works for me dx.doi.org/10.17504/protocols.io.bn77mhrn

Coronavirus Method Development Community

Valeriia Kaptelova

ABSTRACT

Here we present a new primer panel that allows amplifying the complete genome of SARS-CoV-2 (the causative virus of COVID-19) using 17 primer pairs (in four pools). Our results demonstrate that our method allows producing full genomes when we use RNA extract from SARS-CoV-2 positive clinical samples which have a cycle threshold (Ct) in the range of 13 to 26. The resulting primer set exhibits the coverage of the entire viral genome except for only 8 bp on 5'- and 80 bp on 3'- ends in comparison with the reference genome in GenBank (accession number MT121215.1).

EXTERNAL LINK

https://www.biorxiv.org/content/10.1101/2020.08.04.234880v1

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

bioRxiv 2020.08.04.234880; doi: https://doi.org/10.1101/2020.08.04.234880

DOI

dx.doi.org/10.17504/protocols.io.bn77mhrn

EXTERNAL LINK

https://www.biorxiv.org/content/10.1101/2020.08.04.234880v1

PROTOCOL CITATION

Valeriia Kaptelova, Speranskaya AS, et.al 2020. Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bn77mhrn

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

bioRxiv 2020.08.04.234880; doi: https://doi.org/10.1101/2020.08.04.234880

EXTERNAL LINK

https://www.biorxiv.org/content/10.1101/2020.08.04.234880v1

KEYWORDS

Sars-COV-2, NGS, primer panel, Whole-Genome Sequencing, COVID-19, Coronavirus Method, amplicon sequencing, Illumina library construction, coronavirus

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (10/30/2020). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing. https://dx.doi.org/10.17504/protocols.io.bn77mhrn

CREATED

Oct 29, 2020

LAST MODIFIED

Oct 30, 2020

PROTOCOL INTEGER ID

43999

GUIDELINES

the protocol is for RESEARCH ONLY.

MATERIALS TEXT

Protocol for SCV-2000bp a primer panel for SARS-CoV-2 full-genome sequencing.pdf

Types of equipment:
- BioRad T100 thermal cycler
- Covaris M220 Ultrasonicator
- QuantStudio™ 5 Real-Time PCR System

Bioanalyzer
Bioanalyzer

Agilent **G2991AA**

Any bioanalyzer will suffice.

- Illumina MiSeq
- Illumina HiSeq 1500

Reagents:

NEBNext End repair / dA-tailing Module (E7546) **Contributed by users**

NEBNext Ultra II Ligation Module - 96 rxns **New England Biolabs Catalog #E7595L**

SAFETY WARNINGS

RNA samples should be stored at -80 °C and thawed on ice.

DISCLAIMER:

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (10/30/2020). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing. https://dx.doi.org/10.17504/protocols.io.bn77mhrn

information contained in or linked to this protocol or any of our Sites/Apps and Services.

ABSTRACT

Here we present a new primer panel that allows amplifying the complete genome of SARS-CoV-2 (the causative virus of COVID-19) using 17 primer pairs (in four pools). Our results demonstrate that our method allows producing full genomes when we use RNA extract from SARS-CoV-2 positive clinical samples which have a cycle threshold (Ct) in the range of 13 to 26. The resulting primer set exhibits the coverage of the entire viral genome except for only 8 bp on 5'- and 80 bp on 3'- ends in comparison with the reference genome in GenBank (accession number MT121215.1).

1. cDNA

1 RNA samples should be stored at -80 °C and thawed on ice.

NGS library preparation and sequencing:

cDNA
Reverse transcription reaction was performed using 10 µL of the RNA samples, random hexanucleotide primers, and Reverta-L kit (AmpliSens, Russia) according to the manufacturer's instructions.

Prepare a ready-to-use reagent mix for 12 reactions.
1.1. Add 5 µl of RT-G-mix-1 to the tube containing RT-mix,
1.2. Add 6 µl of Revertase (MMlv) into the tube with reagent mix,
1.3. Mix well
1.4. Dispense 10 µl of ready-to-use reagent mix into each prepared test tube (0.2 ml).
1.5. Add 10 µl RNA-sample to the appropriate test tube with a ready-to-use reagent mix.

Place the test tubes into the thermocycler and incubate at 37 °C for 30 minutes.

1.6. Dilute each cDNA sample in the ratio 1:1 with DNA-buffer. To do that, add 20 µl DNAbuffer to each test tube. Carefully mix, using the pipette (10 times).

2. Amplification

2 The cDNA was immediately used as a template for the amplification of genome fragments.

The primer panel you can find here: **see Table 1.**
https://www.biorxiv.org/content/10.1101/2020.08.04.234880v1.full

Four multiplexed primer pools			cDNA
	pM	µl	µl
pool 1	8,3	3	5
pool 2	10	2	8
pool 3	12.5	1	5
pool 4	10	2	5

Set up the following four PCR master mixes, one for each of the four multiplexed primer pools (multiply below volumes by the number of reactions plus desired overage):

Q5 High-Fidelity DNA Polymerase was used according to the manufacturer's instructions (New England BioLabs, NEB).

Component	Volume per reaction (µl)
Q5 master mix	12,5

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (10/30/2020). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing. https://dx.doi.org/10.17504/protocols.io.bn77mhrn

primer pool (1-4)*	variable (1-3) *
H2O	7,5
cDNA*	**variable (5-8)***
Total volume:	25

***- It depends on the primer pool.**

Amplify samples using the following PCR conditions:

°C	Time	Cycles
98	0:30 s	1
98	0:10 s	35
64	0:30 s	
72	2:30	
72	3:00	1

-20 °C SAFE STOPPING POINT

3. 1,7% agarose electrophoresis.

3 We used the following approach: at first we amplified only pool 2 because this pool works worst of all. Then products of the amplification reaction were analyzed with electrophoresis using 1,7% agarose gel stained with SYBR Green. The samples in which visible PCR products of the expected size were found were used for PCR reactions with other primer pools (1, 3, and 4). Then products of PCR were visualized using 1,7% agarose electrophoresis stained with SYBR Green.

4. mix

4 Amplified fragments **were mixed** in equimolar amounts according to the visual estimation of concentration.

5. Clean up 25m 30s

5 **Clean up (**in the ratio **0,7x).**

Clean up PCR products of the expected size (1700-2100 bp) from the reaction mixture and to remove the nonspecific short fragments obtained during the amplification step.

5.1 Premixed amplicons were cleaned in the ratio 0,7x using Agencourt AMPure XP (Beckman Coulter, Danvers, MA, USA)

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (10/30/2020). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing. https://dx.doi.org/10.17504/protocols.io.bn77mhrn

5.2 Incubate at Room temperature for **00:05:00** 5m

5.3 Place the tubes on a magnet: allow beads to separate for **00:05:00** 5m

5.4 Remove supernatant.

5.5 Add **200 µl 80% fresh ethanol** to wash the beads, incubate for **00:00:30** 30s

5.6 Remove **200 µl** 80% EtOH.

5.7 **Repeat steps** 5.5-5.6.

5.8 Briefly spin the tubes and remove additional ethanol. Ensure any visible quantities of ethanol are removed.

5.9 Leave the beads in open tubes for **00:05:00** 5m

5.10 Add low TE and incubate for **00:05:00** . 5m

5.11 Place the tubes on a magnet: allow beads to separate for **00:05:00** 5m

5.12 Transfer supernatant into the new tubes.

SAFE STOPPING POINT!Store at -20 °C.

6. Qubit

6 **Qubit dsDNA HS Assay Kit.**

Prepare the Qubit® working solution by diluting the Qubit® dsDNA HS Reagent 1:200 in Qubit® dsDNA HS Buffer. Use a clean plastic tube each time you prepare a Qubit® working solution.
Add Qubit® working solution to individual assay tubes so that the final volume in each tube after adding a sample is 200 µL.

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (10/30/2020). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing. https://dx.doi.org/10.17504/protocols.io.bn77mhrn

Note: Your sample can be anywhere from 1–20 µL (We use 1µL). Add a corresponding volume of Qubit® working solution to each assay tube: anywhere from 180–199 µL.

7. Covaris.

7 **Covaris.**
PCR products (50 µl)were sheared in microTUBE-50 AFA Fiber Screw-Cap (PN 520166) using Covaris M220 (Covaris, Woburn, MA) using the following settings: peak incident power (W)- 75, duty factor- 5%, cycles per burst-200, treatment Time (s) - 50, temperature (°C)-20, sample volume (µl)- 50.

8. Clean up

8 **Clean up (**in the ratio **1,5x**)

See point 5.2-5.12 **go to step #5.2**

9. Qubit

9 **Qubit dsDNA HS Assay Kit.**
See point 6.

10. End repair with NEBNext® Ultra™ II End Repair/dA-Tailing Module (E7546) 1h

10 **End repair with NEBNext® Ultra™ II End Repair/dA-Tailing Module (E7546)** 1h

Starting Material: **11-100 ng** in **total reaction.**

Mix the following components in a sterile, nuclease-free tube:

COMPONENT VOLUME	VOLUME, µL, PER REACTION
(green) NEBNext Ultra II End Prep Enzyme Mix	3
(green) NEBNext Ultra II End Prep Reaction Buffer	7
Fragmented DNA (11-100 ng in total reaction)	**variable (1-5 µL)**
nuclease free water	**variable (45- 49 µL)**
Total Volume	60

Set a 100 µl or 200 µl pipette to 50 µl and then gently pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.
Note: It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

Place in a thermocycler, with the heated lid set to ≥ 75°C, and run the following program:

20 °C for **00:30:00**

65 °C for **00:30:00**

Hold at **4 °C**

Proceed directly to NEBNext Ultra II Ligation Module NEB **E7595**.

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (10/30/2020). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing. https://dx.doi.org/10.17504/protocols.io.bn77mhrn

11. Ligation with NEBNext Ultra II Ligation Module (E7595) 15m

11 **Ligation with NEBNext Ultra II Ligation Module (E7595) and Y-shaped adapters compatible with Nextera XT Index Kit.** 15m

need to dilute adaptor to **3 pM** (for DNA input was **11-100 ng in total** reaction) (0,6 pM for 1-10 ng in total reaction)

Add the following components directly **to the End Prep reaction mixture (60 µl)** and mix well:

COMPONENT VOLUME	VOLUME, µL, PER REACTION
Y-shaped adapters compatible with Nextera XT Index Kit (dilution adapter)	2,5
(red) NEBNext Ligation Enhancer	1
(red) NEBNext Ultra II Ligation Master Mix	30
Total Volume	93,5

Note: The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours at 4°C. We do not recommend premixing the Ligation Master Mix, Ligation Enhancer and adaptor prior to use in the Adaptor Ligation Step.

Set a 100 µl or 200 µl pipette to 80 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

(Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance).

Incubate **at** **20 °C** **for** **00:15:00** in a thermocycler with the heated **lid off.**

12. Clean up

12 **Clean up (**in the ratio **1,5x**)

See point 5.2-5.12 **go to step #5.2**

13. Indexing PCR

13 **Indexing PCR**

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (10/30/2020). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing. https://dx.doi.org/10.17504/protocols.io.bn77mhrn

COMPONENT VOLUME	VOLUME, µL, PER REACTION
DNA	8,5
Q5 Master Mix	12,5
Index /i5 Primer (5pM)	2
Index /i7 Primer (5pM)	2
Total (mix)	25

* for Real-time PCR used 1µL EvaGreen (20x).

Amplify samples using the following PCR conditions:

°C	Time	Cycles
98	0:30 s	1
98	0:10 s	8
65 (detect)	1:15 m	

* for low-concentration samples used 9-10 cycles.

14. Clean up

14 **Clean up (**in the ratio **1,2x**)
See point 5.2-5.12 **go to step #5.2**

15. Agilent 2100 Bioanalyzer.

15 **Agilent 2100 Bioanalyzer.**

The quality and fragment length distribution of the obtained libraries were evaluated with Agilent Bioanalyzer 2100 (Agilent Technologies, USA).

16. Size selection (300-600bp). 30m 30s

16 **Size selection (300-600bp).**

The size selection of the final libraries was done using Agencourt SPRISelect Reagent (Beckman Coulter, Danvers, MA, USA).

Selecting for DNA larger than a target size.

16.1 The size selection protocol is based on a **starting volume** of **100 µl.** Adjust the final volume by adding nuclease-free water for a 100 µl total volume.

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (10/30/2020). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing. https://dx.doi.org/10.17504/protocols.io.bn77mhrn

16.2 **Add 60 µl** of resuspended AMPure XP beads to the 100 µl. Mix well by pipetting up and down at least 10 times.

16.3 Incubate for **00:05:00** at **Room temperature** 5m

16.4 Place the tube on an appropriate magnetic stand to separate the beads from the supernatant.After the solution is clear (about 5 minutes), carefully transfer the supernatant containing your DNA to a new tube **(Caution: do not discard the supernatant)**. Discard the beads that contain the unwanted large fragments.

16.5 **SPRIselect Right Workflow** 5m

Selecting for DNA smaller than a target size or selecting for DNA within a target region.

Add 20 µl resuspended AMPure XP beads **to the supernatant**, mix well and incubate for **00:05:00** at **Room temperature**

16.6 Place the tubes on a magnet: allow beads to separate for **00:05:00** 5m

16.7 Remove supernatant.

16.8 Add **200 µl80% fresh** ethanol to wash the beads, incubate for **00:00:30** 30s

16.9 Remove **200 µl** 80% EtOH.

16.10 **Repeat steps** 5.5-5.6. **go to step #5.5**

16.11 Briefly spin the tubes and remove additional ethanol. Ensure any visible quantities of ethanol are removed.

16.12 . Leave the beads in open tubes for **00:05:00** 5m

16.13 Add low TE and incubate for **00:05:00** . 5m

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (10/30/2020). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing. https://dx.doi.org/10.17504/protocols.io.bn77mhrn

5m

16.14 Place the tubes on a magnet: allow beads to separate for **00:05:00**

16.15 Transfer supernatant into the new tubes.

17. Agilent 2100 Bioanalyzer.

17 **Agilent 2100 Bioanalyzer.**

See point 15. **go to step #15**

18. Quantitation of next-generation sequencing (NGS) libraries (NEBNext® Library Quant Kit for Illumina)

18 **Quantitation of next-generation sequencing (NGS) libraries (NEBNext® Library Quant Kit for Illumina)**

The NEBNext® Library Quant Kit has been optimized to provide substantial performance and workflow improvements to qPCR-based library quantitation.

qPCR was performed according to the manufacturer's instructions.

HiSeq or MiSeq

19 **Sequencing** was performed on Illumina HiSeq 1500 with HiSeq PE Rapid Cluster Kit v2 and HiSeq Rapid SBS Kit v2 (500 cycles) or Illumina MiSeqwith the MiSeq Reagent Kit V2 (500 cycles) or V3 (600 cycles) according to the manufacturer's instructions.

For HiSeq Rapid Run v2 dilute the pooled sample to [M]**8 Picomolar (pM)** in HT1, following HiSeq 1500 loading instructions. For Miseq v3 dilute the pooled sample to [M]**12 Picomolar (pM)** in HT1. For Miseq v2 dilute the pooled sample to [M]**8 Picomolar (pM)** in HT1, following MiSeq loading instructions.

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (10/30/2020). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing. https://dx.doi.org/10.17504/protocols.io.bn77mhrn