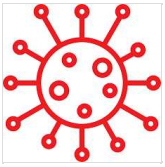

Version 3

Mar 03, 2021

Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb V.3

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.bsybnfsn

Coronavirus Method Development Community

Valeriia Kaptelova

SUBMIT TO PLOS ONE

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.bsybnfsn

EXTERNAL LINK

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

PROTOCOL CITATION

Valeriia Kaptelova, Speranskaya AS, et.al 2021. Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. **protocols.io**
https://dx.doi.org/10.17504/protocols.io.bsybnfsn
Version created by Valeriia Kaptelova

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

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 (03/03/2021). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. https://dx.doi.org/10.17504/protocols.io.bsybnfsn

CREATED

Mar 03, 2021

LAST MODIFIED

Mar 03, 2021

PROTOCOL INTEGER ID

47843

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:

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (03/03/2021). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. https://dx.doi.org/10.17504/protocols.io.bsybnfsn

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.**
Table 1. Primers for whole-genome sequencing of the SARS-CoV-2.

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (03/03/2021). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. https://dx.doi.org/10.17504/protocols.io.bsybnfsn

A	B	C	D	E	F	G
PrimerName	**PrimerSequence 5'-3'**	**Start***	**End***	**Overlap**	**Pool**	**ProductLength**
CV_1_F	gtttataccttcccaggtaacaaacc	8	33	155	5	2054
CV_1_R	actgaacaacaccacctgtaatgtag	2087	2062			
CV_2_F	ttctcccgcactcttgaaactg	1907	1928	134	3	1808
CV_2_R	ggtcagcaccaaaaataccagct	3737	3715			
CV_3_F	agcggacacaatcttgctaaaca	3581	3603	132	4	1946
CV_3_R	ggttgtctgctgttgtccacaa	5548	5527			
CV_4_F	ttgtgcacttatcttagcctactgt	5395	5419	141	5	1839
CV_4_R	tgccaaaaaccactctgcaact	7255	7234			
CV_5_F	cactattgcaacctactgtactggt	7093	7117	126	2	2009
CV_5_R	cgtgtgtcagggcgtaaacttt	9123	9102			
CV_6_F	gtacactgactttgcaacatcagc	8977	9000	197	3	1851
CV_6_R	gcacacatatctaaaacggcaattc	10851	10827			
CV_7_F	agcagctggtacggacacaac	10630	10650	144	1	2028
CV_7_R	tttgacagcagaattggccctt	12679	12658			
CV_8_F	gtgtgatggtacaacatttacttatgc	12514	12540	130	2	1793
CV_8_R	tgggtggtatgtctgatcccaa	14328	14307			
CV_9_F	ccttgaccagggctttaactgc	14177	14198	132	1	1783
CV_9_R	ttacgatatcatctacaaaacagccg	15985	15960			
CV_10_F	agcaaaatgttggactgagactga	15828	15851	162	3	1906
CV_10_R	ccaagcagggttacgtgtaagg	17754	17733			
CV_11_F	gctgaaattgttgacactgtgagt	17572	17595	188	1	1892
CV_11_R	agcaccacctaaattgcaacgt	19485	19464			
CV_12_F	ggttgtgatggtggcagtttgt	19276	19297	74	2	1944
CV_12_R	taacaaaggctgtccaccatgc	21241	21220			
CV_13_F	ttggaggttccgtggctataaaga	21146	21169	28	4	1733
CV_13_R	ccaccaaccttagaatcaagattg	22902	22879			
CV_14_F	caggctgcgttatagcttggaa	22851	22872	242	2	1999
CV_14_R	aaccagtgtgtgccatttgaa	24870	24850			
CV_15_F	gcagaaatcagagcttctgctaatc	24608	24632	237	3	1959
CV_15_R	actaggttccattgttcaaggagc	26590	26567			
CV_16_F	ttacactagccatccttactgcg	26330	26352	457	1	2000
CV_16_R	actgccagttgaatctgagggt	28351	28330			
CV_17_F	acttgtcacgcctaaacgaaca	27873	27894	-	5	1896
CV_17_R	taggcagctctccctagcattg	29790	29769			

***Comparing to the reference genome in GenBank, accession MT121215.1**

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (03/03/2021). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. https://dx.doi.org/10.17504/protocols.io.bsybnfsn

A	B	C	D
Five multiplexed primer pools each sample			**cDNA**
	pM	**µl**	**µl**
pool 1	12,5	0,5	5
pool 2	12,5	0,4	5
pool 3	12.5	0,4	5
pool 4	10	0,6	5
pool 5	16	0,35	5

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

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

A	B
Component	**Volume per reaction (µl)**
Q5 master mix	12,5
primer pool (1-5)*	**variable (0,35-0,6) ***
H2O	**Variable to 25**
cDNA	5
Total volume:	25

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

Amplify samples using the following PCR conditions:

A	B	C
°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 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

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (03/03/2021). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. https://dx.doi.org/10.17504/protocols.io.bsybnfsn

with primer pools. 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)

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

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (03/03/2021). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. https://dx.doi.org/10.17504/protocols.io.bsybnfsn

5m

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

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.
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- 5,5%, cycles per burst-200, treatment Time (s) - 52, temperature (°C)-20, sample volume (µl)- 50.

8. Clean up

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

See point 5.2-5.12

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

1h

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

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

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

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (03/03/2021). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. https://dx.doi.org/10.17504/protocols.io.bsybnfsn

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**.

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.

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (03/03/2021). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. https://dx.doi.org/10.17504/protocols.io.bsybnfsn

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

13. Indexing PCR

13 **Indexing PCR**

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.

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (03/03/2021). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. https://dx.doi.org/10.17504/protocols.io.bsybnfsn

14. Clean up

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

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.

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.

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (03/03/2021). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. https://dx.doi.org/10.17504/protocols.io.bsybnfsn

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.

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

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

16.15 Transfer supernatant into the new tubes.

17. Agilent 2100 Bioanalyzer.

17 **Agilent 2100 Bioanalyzer.**

See point 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

Citation: Valeriia Kaptelova, Speranskaya AS, et.al (03/03/2021). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. https://dx.doi.org/10.17504/protocols.io.bsybnfsn

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 (03/03/2021). Protocol for SCV-2000bp: a primer panel for SARS-CoV-2 full-genome sequencing.v3.Neb. https://dx.doi.org/10.17504/protocols.io.bsybnfsn