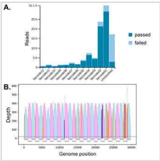

Version 2

Jul 08, 2021

nanopore nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon, combined RT-PCR) V.2

Version 1 is forked from nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon)

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Coronavirus Method Development Community

Anton Pembaur

ABSTRACT

We established a protocol for fast, cost efficient Sars-CoV-2 sequencing with little as possible hands-on time (around 3h in total, excluding RNA extraction). The whole Sequencing can be done in one working day, including the bioinformatic pipeline. The cost per sample accumulates at around 40$, with already isolated RNA.

We adapted and simplified existing workflows using the ‘midnight’ 1,200 bp amplicon split primer sets for PCR, which produce tiled overlapping amplicons covering almost all of the SARS-CoV-2 genome. Subsequently, we applied the Oxford Nanopore Rapid barcoding protocol and the portable MinION Mk1C sequencer in combination with the ARTIC bioinformatics pipeline. We tested the simplified and less time-consuming workflow on confirmed SARS-CoV-2-positive specimens from clinical routine and identified pre-analytical parameters, which may help to decrease the rate of sequencing failures. Duration of the complete pipeline was approx. 7 hrs for one specimen and approx. 11 hrs for 12 multiplexed barcoded specimens.

This protocol is a modified version of Nikki Freed and Olin Silanders protocol. To get information such as Primers, visit their protocol.

Nikki Freed, Olin Silander 2020. nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon).doi: 10.1093/biomethods/bpaa014

DOI

dx.doi.org/10.17504/protocols.io.bwe8pbhw

PROTOCOL CITATION

Anton Pembaur, Erwan Sallard, Patrick Weil, Jennifer Ortelt, Parviz Ahmad-Nejad, Jan Postberg 2021. nanopore nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon, combined RT-PCR). **protocols.io**
https://dx.doi.org/10.17504/protocols.io.bwe8pbhw
Version created by Anton Pembaur

FORK NOTE

FORK FROM

Forked from nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon), Nikki Freed

KEYWORDS

nanopore sequencing, whole genome sequencing, midnight protocol, Sars-CoV-2, Rapid Barcoding, combined RT-PCR, Fast Corona Sequencing, Cheap whole genome Sequencing

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MATERIALS TEXT

STEP MATERIALS

- Primers 25nM, desalted, ideally LabReady formulation from IDT: https://docs.google.com/spreadsheets/d/1M5I_C56ZC8_2Ycgm9EFieVlVNqxsP7dXAnGoBZy3nDo/edit#gid=755704891

- RNA Extraction kit

- Luna®Universal Probe One-Step RT-qPCR Kit NEB eg E3007
- Agencourt AMPure XP Beckman Coulter A63880
- Rapid Barcoding Kit 1-12 Nanopore SQK-RBK004
- R9.4.1 flow cell Nanopore FLO-MIN106

BIOINFORMATIC REQUIREMENTS

- Linux environment with ARTIC and interARTIC pipeline. Installation - InterARTIC Documentation (psy-fer.github.io)

SAFETY WARNINGS

Please follow standard health and safety guidelines when working with COVID-19 patient samples.

Sample selection, RNA isolation

1 For whole genome sequencing using combined RT-PCR, samples with a Ct <20 are suitable. Higher Ct values ≤26 can be suitable as well, but we strongly recommend verification of the amplicon quality utilizing gelelectrophresis and/or microcapillary electrophoresis (Agilent) after combined RT-PCR.
Isolate RNA using any suitable protocol. We tried magnetic Bead based (Nimbus/TanBead), spin collum based (QIAamp Viral RNA, Qiagen) and trizol/chloroforme extraction, with no significant differences.

Multiplex RT-PCR 3h 10m

2 For Primer dilution and setup, visit Nikki Freed and Olin Silanders protocol.

2.1 Prepare RT-PCR reaction in a new PCR tube 10m

Pipette Scheme for Luna Universal Probe One-Step RT-qPCR Kit (2X)
(Luna Probe One-Step RT-qPCR 4X Mix with UDG works too, adjust the volumes accordingly)

Luna Universal Probe One-Step Reaction Mix (2X)	10 µl	
Luna Enzyme Mix (20X)	1 µl	
Primer Pool1 or Pool2	1 µl	
Sample	up to 8 µl	(depending on Ct Value and concentration)
Nuclease free H_2O	fill up to 20 µl	

0,75-1$ *2 per sample

2.2 Set-up the following program on the thermal cycler: 3h

Step	Temperature	Time	Cycles
Reverse Transcription	55 °C	00:30:00	1
Heat Activation	95 °C	00:01:00	1
Denaturation	95 °C	00:00:20	34 for Pool 1, 30 for Pool 2
Annealing and Extension	60 °C	00:03:30	34 for Pool 1, 30 for Pool 2
Final Extension	65 °C	00:05:00	
Hold	4 °C	Indefinite	1

Final concentrations of PCR products can range from ~20-150ng/µl.
We dont see unexpected products while using more than sufficient input material.

Pooling and PCR quantification/normalisation 25m

3

At this stage, care should be taken with amplified PCR products. Only open tubes in a designated post-PCR workspace with equipment that is separate from areas where primers and mastermixes are handled.

After combining the two pools of amplified DNA, the PCR products can be used for Oxford Nanopore Sequencing, using the RAPID barcode kit RBK004, as described in this protocol.

Alternatively, these amplicons can be used for Oxford Nanopore Sequencing, following Josh Quick's ligation based protocol (CoV-2019 sequencing protocol v2, dx.doi.org/10.17504/protocols.io.bdp7i5rn, at step 15) using the SQK-LSK109 kit.

Alternatively, these amplicons can also be used for Illumina sequencing, such as found here: x.doi.org/10.17504/protocols.io.betejeje

Silander et al stated that performing an Ampure XP bead clean up at this stage does not improve performance. Therefore, it is not necessary to clean up the PCR reaction at this step.

3.1 Quantify each PCR reaction using a Qubit or other method. Quantification using Nanodrop is not 15m

Citation: Anton Pembaur, Erwan Sallard, Patrick Weil, Jennifer Ortelt, Parviz Ahmad-Nejad, Jan Postberg (07/08/2021). nanopore nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon, combined RT-PCR). https://dx.doi.org/10.17504/protocols.io.bwe8pbhw

recommended.

DNA quantification using the Qubit fluorometer
by Nikki Freed
PREVIEW RUN

0.35$ *2 per sample

3.1.1 Prepare a mastermix of Qubit™ working solution for the required number of samples and standards. The Qubit dsDNA kit requires 2 standards for calibration (see note below).

Per sample:

Qubit® dsDNA HS Reagent **1 µl**

Qubit® dsDNA HS Buffer **199 µl**

If you have already performed a calibration on the Qubit machine for the selected assay you can use the previous calibration stored on the machine. We recommend performing a new calibration for every sample batch but a same-day calibration would be fine to use for multiple batches.

To avoid any cross-contamination, we recommend that you remove the total amount of working solution required for your samples and standards from the working solution bottle and then add the required volume to the appropriate tubes instead of pipetting directly from the bottle to each tube.

3.1.2 Label the tube lids. Do not label the side of the tube as this could interfere with the sample reading.

Use only thin-wall, clear, 0.5mL PCR tubes. Acceptable tubes include Qubit™ assay tubes (Cat. No. Q32856)

3.1.3 Aliquot Qubit™ working solution to each tube:
- standard tubes requires 190µL of Qubit™ working solution
- sample tubes require anywhere from 180–199µL (depending how much sample you wish to add).

The final volume in each tube must be 200µL once sample/standard has been added.

3.1.4 Add 10µL of standard to the appropriate tube.

3.1.5 Add 1–20µL of each user sample to the appropriate tube.

If you are adding 1–2µL of sample, use a P-2 pipette for best results.

3.1.6 Mix each tube vigorously by vortexing for 3–5 seconds.

3.1.7 Allow all tubes to incubate at room temperature for 2 minutes, then proceed to "Read standards and samples".

3.1.8 On the Home screen of the Qubit™ 3 Fluorometer, press DNA, then select 1X dsDNA HS as the assay type. The Read standards screen is displayed. Press Read Standards to proceed.

If you have already performed a calibration for the selected assay, the instrument prompts you to choose between reading new standards and running samples using the previous calibration. **If you want to use the previous calibration, skip to step 12**. Otherwise, continue with step 9.

3.1.9 Insert the tube containing Standard #1 into the sample chamber, close the lid, then press Read standard. When the reading is complete (~3 seconds), remove Standard #1.

1.10 Insert the tube containing Standard #2 into the sample chamber, close the lid, then press Read standard. When the reading is complete, remove Standard #2.

1.11 The instrument displays the results on the Read standard screen. For information on interpreting the calibration results, refer to the Qubit™ Fluorometer User Guide, available for download at thermofisher.com/qubit.

1.12 Press Run samples.

1.13 On the assay screen, select the sample volume and units:

- Press the + or – buttons on the wheel, or anywhere on the wheel itself, to select the sample volume added to the assay tube (from 1–20µL).
- From the unit dropdown menu, select the units for the output sample concentration (in this case choose ng/µL).

1.14 Insert a sample tube into the sample chamber, close the lid, then press Read tube. When the reading is complete (~3 seconds), remove the sample tube.

1.15 **The top value (in large font) is the concentration of the original sample and the bottom value is the dilution concentration**. For information on interpreting the sample results, refer to the Qubit™ Fluorometer User Guide.

1.16 Repeat step 14 until all samples have been read.

Citation: Anton Pembaur, Erwan Sallard, Patrick Weil, Jennifer Ortelt, Parviz Ahmad-Nejad, Jan Postberg (07/08/2021). nanopore nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon, combined RT-PCR). https://dx.doi.org/10.17504/protocols.io.bwe8pbhw

1.17 Carefully **record all results** and store run file from the Qubit on a memory stick.

1.18 All negative controls should ideally be ‘too low’ to read on the Qubit machine, but MUST be < 1ng per ul. If your negative controls >1ng per ul, considerable contamination has occurred and you must redo previous steps.

3.2 Pool the two PCR reactions for each sample in a new PCR tube using 200 ng for each sample to a total amount of **400 ng** . Add nuclease free water to a total volume of **7.5 µl** . 10m

Rapid barocoding using the SQK RBK004 30m

4 Multiple samples can be run on the same flow cell by barcoding. Up to 12 samples can be multiplexed in this approach. Amplicons from each sample will be individually barcoded in the following steps. These follow the RBK004 protocol from Oxford Nanopore. Tip: aliquot the Rapid barcodes into a PCR strip to enable multichannelling.

SQK-RBK004 Rapid Barcoding Kit **Oxford Nanopore Technologies Catalog #SQK-RBK004**

4.1 Set up the following reaction for each sample: 4m

Component	**Volume**
DNA amplicons from step 15 (100ng total)	**7.5 µl**
Fragmentation Mix RB01-12 (one for each sample, included in kit)	**2.5 µl**
Total	**10 µl**

8.12$ per sample For higher number of samples it may be more efficient, to use SQK-RBK110.96 with 96 Barcodes, to come to a cost of **3$ per sample** .

4.2 Mix gently by flicking the tube, and spin down.

4.3 Incubate the reaction in a PCR machine: 5m

30 °C for **00:01:00**

80 °C for **00:01:00**

4 °C for **00:00:30**

Pool all barcoded samples, noting the total volume.

Citation: Anton Pembaur, Erwan Sallard, Patrick Weil, Jennifer Ortelt, Parviz Ahmad-Nejad, Jan Postberg (07/08/2021). nanopore nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon, combined RT-PCR). https://dx.doi.org/10.17504/protocols.io.bwe8pbhw

4.4

5 Ampure XP Bead Cleanup. Use a 1:1 ratio of sample to beads. 15m

Amplicon clean-up using SPRI beads for RAPID nanopore kit RBK004
by Nikki Freed

PREVIEW RUN

5.1 Vortex SPRI beads thoroughly to ensure they are well resuspended, the solution should be a homogenous brown colour.

Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

5.2 Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. For example add **50 µl** room temperature SPRI beads to a **50 µl** reaction.

5.3 Pulse centrifuge to collect all liquid at the bottom of the tube.

5.4 Incubate for **00:05:00** at room temperature.

5.5 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear.

5.6 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

5.7 Add **200 µl** of freshly prepared room-temperature [M] **80 % volume** ethanol to the pellet.

5.8 Keeping the magnetic rack on the benchtop, rotate the bead-containing tube by 180°. Wait for the beads to migrate towards the magnet and re-form a pellet. Remove the ethanol using a pipette and discard.

5.9 and repeat ethanol wash.

5.10 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette.

5.11 With the tube lid open incubate for **00:01:00** or until the pellet loses it's shine (if the pellet dries completely it will crack and become difficult to resuspend).

5.12 Remove the tube from the magnetic rack. Resuspend pellet in **10 µl** 10 mM Tris-HCl pH 8.0 with 50 mM NaCl, mix gently by flicking and incubate at room temperature for **00:02:00** .

5.13 Place on magnet and transfer sample to a clean 1.5mL Eppendorf tube ensuring no beads are transferred into this tube.

6 Add **1 µl** of RAP (from the RBK004 kit) to **10 µl** cleaned, barcoded DNA from previous step . Mix gently by flicking the tube, and spin down. 1m

7 Incubate the reaction for **00:05:00** at room temperature. 5m

8 The prepared library is used for loading into the MinION flow cell according to Oxford Nanopore Rapid Barcoding (RBK004) protocol. Please refer to the Oxford Nanopore Rapid Barcoding RBK004 protocol at this stage. Store the library on ice until ready to load**.** 5m

MinION sequencing 4h 25m

9 Start the sequencing run using MinKNOW. 5m
For real-time surveillance of basecalling and demultiplexing for each of the 12 multiplexed samples per run, enable these options in the MinKNOW software or use RAMPART (https://github.com/artic-network/rampart) on a dedicated LINUX environment.

MinION
Sequencer

Oxford Nanopore Technologies	**MinION 1B / MinION 1C**

Citation: Anton Pembaur, Erwan Sallard, Patrick Weil, Jennifer Ortelt, Parviz Ahmad-Nejad, Jan Postberg (07/08/2021). nanopore nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon, combined RT-PCR). https://dx.doi.org/10.17504/protocols.io.bwe8pbhw

MinION Flow Cell
Sequencer

Oxford Nanopore Technologies	**FLO-MIN106D**

24$ per sample if using the flowcell three times with 12 Barcodes each

10 Depending on the variation in coverage of each amplicon, generally, you will need approx 10,000 to 20,000 reads or 10-20Mb **per sample** to confidently assemble and call variants. This can typically be achieved on a minION flow cell in around four hours when runnning 12 samples. Shorter, if running fewer samples.

11 20m

For a simple bioinformatic pipeline optimized on Sars-CoV-2 genome assembly and analysis, we recommend using the interARTIC tool, wich provides a GUI to ether medaka or nanopolish workflow.
Note: If basecalling and demultiplexing during sequencing was enabled, the passed fastq files needed to be directly in the passed fastq folder, not in subfolders. Simply search for fastq in this folder and subfolders, copy results and paste it in this folder directly.

If needed, for more accurate variant calling, we wrote a python script which combines the consensus sequence from medaka and nanopolish pipeline. After downloading this tool **consensus_merging.py** , direct to the save path of this tool in the command line of the console via cd command. Follow the example below, which, after python command, uses the arguments 'medaka_consesensus_sequence_path', 'nanopolish_consesensus_sequence_path', 'reference_genome_path' and 'output_path':

python merge_consensus.py '/home/documents/interartic_medaka/nCoV_2019_midnight_V1_sample1_medaka_single_medaka/medaka.consensus.fasta' '/home/documents/interartic_nanopolish/nCoV_2019_midnight_V1_sample1_nanopolish_single_nanopolish/param2.consensus.fasta' '/home/documents/sarscov2reference.fasta' '/home/documents/merge_output'

All data from our SEQs is available: https://t1p.de/minion-seqdata

Citation: Anton Pembaur, Erwan Sallard, Patrick Weil, Jennifer Ortelt, Parviz Ahmad-Nejad, Jan Postberg (07/08/2021). nanopore nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon, combined RT-PCR). https://dx.doi.org/10.17504/protocols.io.bwe8pbhw