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nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon) V.1

Forked from nCoV-2019 sequencing protocol v2 (GunIt)

DOI
https://dx.doi.org/10.17504/protocols.io.bfwnjpde

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Coronavirus Method De...

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Protocol status: In development
We are still developing and optimizing this protocol

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Abstract

To enable faster, easier sequencing of SARS-COV2 genomes with fewer steps than current methods, we use multiplexed 1200 base pair PCR amplicons with the Oxford Nanopore RAPID barcoding kit (RBK004).

This is a modification of the ARTIC amplicon V3 sequencing protocol for MinION for nCoV-2019 developed by Josh Quick, which produces 400 base pair amplicons and uses the Oxford Nanopore Ligation barcoding kit (LSK-109).

We have increased the size of the amplicons to 1200bp and use the RAPID barcode kit (RBK004), which enables requires less time and fewer reagents than the LSK-109 protocol. The amplicons produced in this protocol could also be used for Illumina sequencing.

Primers were all designed using Primal Scheme: **http://primal.zibraproject.or**g/, described here https://www.nature.com/articles/nprot.2017.066.

Primer sequences are here: https://docs.google.com/spreadsheets/d/1M5I_C56ZC8_2Ycgm9EFieVIVNqxsP7dXAnGoBZy3nDo/edit?usp=sharing

We can ship a small amount of pooled primers to interested labs for further testing, email freednikki@gmail.com or olinsilander@gmail.com

Guidelines

This has so far been testing using only five SARS-CoV2 patient positive samples, with Cq values ranging from 20 to 31. Further testing might be needed to test the method on low viral load samples/high Cq samples.

Materials

STEP MATERIALS

-

- Primers 25nm, desalted, ideally LabReady formulation from IDT: https://docs.google.com/spreadsheets/d/1M5l_C56ZC8_2Ycgm9EFieVlVNqxsP7dXAnGoBZy3nDo/edit#gid=755704891

- Extraction kits; Zymo Quick-RNA Viral Kit Zymo R1034

OR

- i.e. QIAamp Viral RNA Mini Qiagen 52904

- SuperScript IV (50 rxn) Thermo 18090050
- dNTP mix (10 mM each) Thermo R0192

- Random Hexamers (50 µM) Thermo N8080127

OR

- Random Primer Mix (60 µM) NEB S1330S

- RNase OUT (125 rxn) Thermo 10777019
- Q5 Hot Start HF Polymerase NEB M0493S
- **Agencourt AMPure XP** **Beckman Coulter** **A63880**
- Rapid Barcoding Kit 1-12 Nanopore SQK-RBK004
- R9.4.1 flow cell Nanopore FLO-MIN106

Protocol materials

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

Safety warnings

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

cDNA preparation

5m

1 Mix the following components in an 0.2mL 8-strip tube; 5m

Component	Volume
50µM random hexamers	1 µL
10mM dNTPs mix (10mM each)	1 µL
Template RNA	11 µL
Total	13 µL

Note

Viral RNA input from a clinical sample should be between Ct 18-35. If Ct is between 12-15, then dilute the sample 100-fold in water, if between 15-18 then dilute 10-fold in water. This will reduce the likelihood of PCR-inhibition. It is good practice to carry a negative control (e.g. water) through the entire process from cDNA preparation to sequencing.

Note

A mastermix should be made up in the **mastermix cabinet** and aliquoted into PCR strip tubes. Tubes should be wiped down when entering and leaving the mastermix cabinet.

2 Gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

3 Incubate the reaction as follows: 6m

65 °C for 00:05:00

Snap cool in a prechilled metal rack or on ice 00:01:00

Note

A quick cooling step using a PCR cooling block or ice helps to inhibit secondary structure formation and can decrease variation in overall coverage.

4 Add the following to the annealed template RNA : 5m

Component	Volume
SSIV Buffer	4 µL
100mM DTT	1 µL
RNaseOUT RNase Inhibitor	1 µL
SSIV Reverse Transcriptase	1 µL
Total	20 µL

Note

A mastermix should be made up in the **mastermix cabinet** and added to the denatured RNA in the **extraction and sample addition cabinet**. Tubes should be wiped down when entering and leaving the mastermix cabinet.

5 Gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

6 Incubate the reaction in a preheated PCR machine: 1h 5m

42 °C 00:50:00
70 °C 00:10:00
Hold at 5 °C

Primer pool preparation

7 If required, resuspend lyophilised primers at a concentration of 100µM each

Note

Primers for this protocol were designed using **Primal Scheme** and generate overlapping 1200nt amplicons. Primer names and dilutions are listed here: https://docs.google.com/spreadsheets/d/1M5I_C56ZC8_2Ycgm9EFieVlVNqxsP7dXAnGoBZy3nDo/edit?usp=sharing.
We have tested multiplexing 1500 nt and 2000 nt amplicons as well, all work well. These are included in the link. Here we will discuss just the protocol for 1200 nt amplicons as this longer amplicons might be sensitive to RNA degradation.

8 Generate primer pool stocks by adding 5 µL of each odd region primer to a 1.5 mL Eppendorf labelled “Pool 1 (100µM)” and each even region primer to a 1.5 mL Eppendorf labelled “Pool 2 (100µM)”. The pool is also given in the link above. These are your 100µM stocks of each primer pool.

Note

Primers should be diluted and pooled in the **mastermix cabinet** which should be cleaned with decontamination wipes and UV sterilised before and after use.

9 Dilute this primer pool 1:10 in molecular grade water, to generate 10µM primer stocks. It is recommend that multiple aliquots of each primer pool are made to in case of degradation or contamination.

Note

Primers need to be used at a final concentration of 0.015µM per primer. In this case (1200 nt amplicons), pool 1 has 30 primers and pool 2 has 28 primers, so the requirement is 1.13µL for primer pool 1 and 1.05µL for primer pool 2 (10uM) per 25µL reaction. However, as these values are relatively close, we round up and down to 1.1ul for both pools, so the pools can be made in a similar fashion. For other schemes, adjust the volume added appropriately.

Multiplex PCR

10 In the mastermix hood set up the multiplex PCR reactions as follows in 0.2mL 8-strip PCR tubes:

Component	Pool 1	Pool 2
5X Q5 Reaction Buffer	5 µL	5 µL
10 mM dNTPs	0.5 µL	0.5 µL
Q5 Hot Start DNA Polymerase	0.25 µL	0.25 µL
Primer Pool 1 or 2 (10µM)	1.1 µL	1.1 µL
Nuclease-free water	15.9 µL	15.9 µL
Total	22.5 µL	22.5 µL

Note

A PCR mastermix for each pool should be made up in the **mastermix cabinet** and aliquoted into PCR strip tubes. Tubes should be wiped down when entering and leaving the mastermix cabinet.

11 In the **extraction and sample addition cabinet** add 2.5 µL cDNA to each tube and mix well by pipetting.

Note

The **extraction and sample addition cabinet** should should be cleaned with decontamination wipes and UV sterilised before and after use.

12 Pulse centrifuge the tubes to collect the contents at the bottom of the tube.

13 Set-up the following program on the thermal cycler: 2h 40m

Step	Temperature	Time	Cycles
Heat Activation	98 °C	00:00:30	1
Denaturation	98 °C	00:00:15	25-35
Annealing and Extension	65 °C	00:05:00	25-35
Hold	4 °C	Indefinite	1

Note

Cycle number should be 25 for Ct 18-21 up to a maximum of 35 cycles for Ct 35.

Expected result

Final concentrations of PCR products can range from 20- 150ng/ul.

Pooling and PCR quantification

14 Label a 1.5 mL Eppendorf tube for each sample and combine the two pools the PCR reaction as follows:

Component	Volume
Pool 1 PCR reaction	25 µL
Pool 2 PCR reaction	25 µL
Total	50 µL

Note

At this stage the PCR products can be used for Oxford Nanopore Sequencing, using the RAPID barcode kit RBK004, as described below.

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**

We have found that performing an Ampure XP bead clean up at this stage does not improve performance. For the sake of time, we, therefore, do not clean up the PCR reaction.

Normalisation

15 Label a 0.2 mL PCR tube for each sample.

15.1 Adjust the amount of DNA in the tube to be 100 ng total per sample in 7.5 µL molecular grade water. For example if your PCR reaction is at 100ng/ul, add 1ul of the PCR reaction to 6.5ul of molecular grade water. Input to the Rapid Barcoding kit will vary depending on the amplicon length but we have determined 50-200 ng works for efficient barcoding of this amplicon length. Use 7.5ul of the negative control, even if there is no detectable DNA in the PCR reaction.

Rapid barocoding using the SQK RBK004

16 Mulitple samples can be run on the same flow cell by barcoding. Up to 12 samples at a time can be run. Amplicons from each sample will be individually barcoded in the following steps. These follow the RBK004 protocol from Oxford Nanopore. It is highly recommended to use their protocol for the following steps.

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

16.1 Add 7.5 µL of each diluted PCR reaction from step 15 to the labeled PCR tube. 5m

Set up the following reaction for each sample:

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

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

16.3 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

16.4 Pool all barcoded samples, noting the total volume.

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

Protocol

NAME

Amplicon clean-up using SPRI beads

CREATED BY

Nikki Freed

Preview

17.1 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

17.2 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.

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

17.4 Incubate for 00:05:00 at room temperature.

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

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

17.7 Add 200 µL of freshly prepared room-temperature [M] 80 % volume ethanol to the pellet.

17.8 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.

17.9 and repeat ethanol wash.

17.10 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.

17.11 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).

17.12 Remove the tube from the magnetic rack. Resuspend pellet in 10 µL molecular grade water or Elution buffer, mix gently by flicking and incubate for 00:02:00 .

Elution Buffer (EB) **Qiagen Catalog #**19086

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

17.14 Quantify 1 µL product using the Quantus Fluorometer using the ONE dsDNA assay.

QuantiFluor(R) ONE dsDNA System, 100rxn **Promega Catalog #**E4871

Equipment	
Quantus	NAME
Fluorometer	TYPE
Promega	BRAND
E6150	SKU
https://www.promega.co.uk/products/microplate-readers-fluorometers-luminometers/fluorometers/quantus-fluorometer	LINK

18 Add 1 µL of RAP (from the RBK004 kit) to 10 µL cleaned, barcoded DNA from step 17 . Mix gently by flicking the tube, and spin down. 1m

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

20 The prepared library is used for loading into the MinION flow cell according to Oxford Nanopore Rapid Barcoding (RBK004) protocol. Store the library on ice until ready to load. 10m

MinION sequencing

21 Start the sequencing run using MinKNOW.

Protocol

NAME

Starting a MinION sequencing run using MinKNOW

CREATED BY

Nikki Freed

Preview

21.1 If required plug the MinION into the computer and wait for the MinION and flowcell to ben detected.

21.2 Choose flow cell 'FLO-MIN106' from the drop-down menu.

21.3 Then select the flowcell so a tick appears.

21.4 Click the 'New Experiment' button in the bottom left of the screen.

21.5 On the New experiment popup screen, select the running parameters for your experiment from the individual tabs:

Experiment: Name the run in the experiment field, leave the sample field blank.

Kit: Selection: Select RBK004

Run Options: Set the run length to 6 hours (you can stop the run once sufficient data has been collected as determined using RAMPART).

Basecalling: Select 'fast basecalling'.

Output: The number of files that MinKNOW will write to a single folder. By default this is set to 4000 but can be reduced to make RAMPART update more frequently.

Click 'Start run'.

21.6 Monitor the progress of the run using the MinKNOW interface.

22 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 under two hours when runnning 12 samples. Shorter, if running fewer samples.

23 The primer scheme .bed and .tsv files necessary for the ARTIC variant calling pipeline are **here**
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