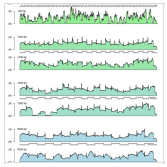

Version 2

May 13, 2020

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

Forked from nCoV-2019 sequencing protocol v2

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In Development dx.doi.org/10.17504/protocols.io.bgc8jszw

Coronavirus Method Development Community

Nikki Freed

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.org/, described here https://www.nature.com/articles/nprot.2017.066.

Primer sequences are here:
https://docs.google.com/spreadsheets/d/1M5I_C56ZC8_2Ycgm9EFieVlVNqxsP7dXAnGoBZy3nDo/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.

STEPS MATERIALS

NAME	CATALOG #	VENDOR
SQK-RBK004 Rapid Barcoding Kit	SQK-RBK004	Oxford Nanopore Technologies

MATERIALS TEXT

- Primers 25nm, desalted, ideally LabReady formulation from IDT:
  https://docs.google.com/spreadsheets/d/1M5I_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

Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw

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

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**

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.

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**

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

Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw

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**

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

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.

7.1 Primers used to generate 1200 bp amplicons are here:

Primer Name	Sequence	Pool	Length	Tm	GC%	Start
SARSCoV_1200_1_LEFT	ACCAACCAACTTTCGATCTCTTGT	1	24	60.69	41.67	30
SARSCoV_1200_1_RIGHT	GGTTGCATTCATTTGGTGACGC	1	22	61.49	50	1205
SARSCoV_1200_3_LEFT	GGCTTGAAGAGAAGTTTAAGGAAGGT	1	26	61.19	42.31	2153

Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw

SARSCoV_1200_3_RIGHT	GATTGTCCTCACTGCCGTCTTG	1	22	61.5	54.55	3257
SARSCoV_1200_5_LEFT	ACCTACTAAAAAGGCTGGTGGC	1	22	60.55	50	4167
SARSCoV_1200_5_RIGHT	AGCATCTTGTAGAGCAGGTGGA	1	22	61.16	50	5359
SARSCoV_1200_7_LEFT	ACCTGGTGTATACGTTGTCTTTGG	1	24	60.8	45.83	6283
SARSCoV_1200_7_RIGHT	GCTGAAATCGGGGCCATTTGTA	1	22	61.53	50	7401
SARSCoV_1200_9_LEFT	AGAAGTTACTGGCGATAGTTGTAATAACT	1	29	60.59	34.48	8253
SARSCoV_1200_9_RIGHT	TGCTGATATGTCCAAAGCACCA	1	22	60.29	45.45	9400
SARSCoV_1200_11_LEFT	AGACACCTAAGTATAAGTTTGTTCGCA	1	27	60.74	37.04	10343
SARSCoV_1200_11_RIGHT	GCCCACATGGAAATGGCTTGAT	1	22	61.8	50	11469
SARSCoV_1200_13_LEFT	ACCTCTTACAACAGCAGCCAAAC	1	23	61.55	47.83	12450
SARSCoV_1200_13_RIGHT	CGTCCTTTTCTTGGAAGCGACA	1	22	61.38	50	13621
SARSCoV_1200_15_LEFT	TTTTAAGGAATTACTTGTGTATGCTGCT	1	28	60.06	32.14	14540
SARSCoV_1200_15_RIGHT	ACACACAACAGCATCGTCAGAG	1	22	61.12	50	15735
SARSCoV_1200_17_LEFT	TCAAGCTTTTTGCAGCAGAAACG	1	23	61.28	43.48	16624
SARSCoV_1200_17_RIGHT	CCAAGCAGGGTTACGTGTAAGG	1	22	61.19	54.55	17754
SARSCoV_1200_19_LEFT	GGCACATGGCTTTGAGTTGACA	1	22	61.91	50	18596
SARSCoV_1200_19_RIGHT	CCTGTTGTCCATCAAAGTGTCCC	1	23	61.62	52.17	19678
SARSCoV_1200_21_LEFT	TCTGTAGTTTCTAAGGTTGTCAAAGTGA	1	28	60.58	35.71	20553
SARSCoV_1200_21_RIGHT	GCAGGGGGTAATTGAGTTCTGG	1	22	60.95	54.55	21642
SARSCoV_1200_23_LEFT	ACTTTAGAGTCCAACCAACAGAATCT	1	26	60.18	38.46	22511
SARSCoV_1200_23_RIGHT	TGACTAGCTACACTACGTGCCC	1	22	61.52	54.55	23631
SARSCoV_1200_25_LEFT	TGCTGCTACTAAAATGTCAGAGTGT	1	25	60.51	40	24633
SARSCoV_1200_25_RIGHT	CATTTCCAGCAAAGCCAAAGCC	1	22	61.45	50	25790
SARSCoV_1200_27_LEFT	TGGATCACCGGTGGAATTGCTA	1	22	61.75	50	26744
SARSCoV_1200_27_RIGHT	TGTTCGTTTAGGCGTGACAAGT	1	22	60.74	45.45	27894
SARSCoV_1200_29_LEFT	TGAGGGAGCCTTGAATACACCA	1	22	61.1	50	28677
SARSCoV_1200_29_RIGHT	TAGGCAGCTCTCCCTAGCATTG	1	22	61.61	54.55	29790

Primers for **Pool 1**

Primer Name	Sequence	Pool	Length	Tm	GC%	Start
SARSCoV_1200_2_LEFT	CCATAATCAAGACTATTCAACCAAGGGT	2	28	61.27	39.29	1100
SARSCoV_1200_2_RIGHT	ACAGGTGACAATTTGTCCACCG	2	22	61.33	50	2266
SARSCoV_1200_4_LEFT	GGAATTTGGTGCCACTTCTGCT	2	22	61.66	50	3144
SARSCoV_1200_4_RIGHT	CCTGACCCGGGTAAGTGGTTAT	2	22	61.49	54.55	4262
SARSCoV_1200_6_LEFT	ACTTCTATTAAATGGGCAGATAACAACTG	2	29	60.18	34.48	5257
SARSCoV_1200_6_RIGHT	GATTATCCATTCCCTGCGCGTC	2	22	61.75	54.55	6380
SARSCoV_1200_8_LEFT	CAATCATGCAATTGTTTTTCAGCTATTTTG	2	30	60.39	30	7298
SARSCoV_1200_8_RIGHT	TGACTTTTTGCTACCTGCGCAT	2	22	61.39	45.45	8385
SARSCoV_1200_10_LEFT	TTTACCAGGAGTTTTCTGTGGTGT	2	24	60.32	41.67	9303
SARSCoV_1200_10_RIGHT	TGGGCCTCATAGCACATTGGTA	2	22	61.5	50	10451
SARSCoV_1200_12_LEFT	ATGGTGCTAGGAGAGTGTGGAC	2	22	61.48	54.55	11372
SARSCoV_1200_12_RIGHT	GGATTTCCCACAATGCTGATGC	2	22	60.48	50	12560
SARSCoV_1200_14_LEFT	ACAGGCACTAGTACTGATGTCGT	2	23	61.12	47.83	13509
SARSCoV_1200_14_RIGHT	GTGCAGCTACTGAAAAGCACGT	2	22	61.94	50	14641
SARSCoV_1200_16_LEFT	ACAACACAGACTTTATGAGTGTCTCT	2	26	60.18	38.46	15608
SARSCoV_1200_16_RIGHT	CTCTGTCAGACAGCACTTCACG	2	22	61.17	54.55	16720
SARSCoV_1200_18_LEFT	GCACATAAAGACAAATCAGCTCAATGC	2	27	62.03	40.74	17622
SARSCoV_1200_18_RIGHT	TGTCTGAAGCAGTGGAAAAGCA	2	22	60.68	45.45	18706
SARSCoV_1200_20_LEFT	ACAATTTGATACTTATAACCTCTGGAACAC	2	30	60.15	33.33	19574

Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw

SARSCoV_1200_20_RIGHT	GATTAGGCATAGCAACACCCGG	2	22	61.39	54.55	20698
SARSCoV_1200_22_LEFT	GTGATGTTCTTGTTAACAACTAAACGAACA	2	30	61.44	33.33	21532
SARSCoV_1200_22_RIGHT	AACAGATGCAAATCTGGTGGCG	2	22	62.03	50	22612
SARSCoV_1200_24_LEFT	GCTGAACATGTCAACAACTCATATGA	2	26	60.13	38.46	23518
SARSCoV_1200_24_RIGHT	ATGAGGTGCTGACTGAGGGAAG	2	22	61.74	54.55	24736
SARSCoV_1200_26_LEFT	GCCTTGAAGCCCCTTTTCTCTA	2	22	60.29	50	25690
SARSCoV_1200_26_RIGHT	AATGACCACATGGAACGCGTAC	2	22	61.5	50	26857
SARSCoV_1200_28_LEFT	TTTGTGCTTTTTAGCCTTTCTGCT	2	24	60.14	37.5	27784
SARSCoV_1200_28_RIGHT	GTTTGGCCTTGTTGTTGTTGGC	2	22	61.82	50	29007

Primers for **Pool 2**

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.

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.

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**

A PCR mastermix for each pool should be made up in the **mastermix cabinet** and aliquoted into PCR strip

Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw

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.

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

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

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**

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 (below, Steps 15 onward).

Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw

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. Therefore, do not clean up the PCR reaction at this step.

14.1 Quantify DNA using a Qubit or other method. Quantification using Nanodrop is not recommended.

DNA quantification using the Qubit fluorometer
by Nikki Freed
PREVIEW RUN

4.1.1 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.

4.1.2 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)

4.1.3 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.

Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw

4.1.4 Add 10µL of standard to the appropriate tube.

4.1.5 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.

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

4.1.7 Allow all tubes to incubate at room temperature for 2 minutes, then proceed to “Read standards and samples”.

4.1.8 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.

4.1.9 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).

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

Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw

1.14 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.

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.

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
by Oxford Nanopore Technologies
Catalog #: SQK-RBK004

16.1 5m

Add **7.5 µl** of each diluted PCR reaction from step 15 to the labeled PCR tube.
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**

Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw

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

Amplicon clean-up using SPRI beads
by Nikki Freed
PREVIEW RUN

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

Agencourt AMPure XP
by 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.

Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw

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.

7.10 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.

7.11 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).

7.12 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)
by Qiagen
Catalog #: 19086

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

7.14 Quantify **1 µl** product using the Quantus Fluorometer using the ONE dsDNA assay.

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

Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw

Quantus
Fluorometer
Promega E6150

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.

Starting a MinION sequencing run using MinKNOW
by Nikki Freed
PREVIEW RUN

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.

Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw

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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Citation: Nikki Freed, Olin Silander (05/13/2020). nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). https://dx.doi.org/10.17504/protocols.io.bgc8jszw