VERSION 4

JUL 02, 2020

🌐 nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon) V.4

🔗 Version 1 is forked from [nCoV-2019 sequencing protocol v2 \(GunIt\)](#)

Nikki Freed¹, Olin Silander¹

¹Massey University

Coronavirus Method Development Community

Nikki Freed

OPEN ACCESS

DOI:

dx.doi.org/10.17504/protocols.io.bh7hj9j6

Protocol Citation: Nikki Freed, Olin Silander 2020. nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon). **protocols.io** <https://dx.doi.org/10.17504/protocols.io.bh7hj9j6>

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working
We use this protocol and it's working

Created: Jul 02, 2020

Last Modified: Jul 02, 2020

PROTOCOL integer ID:
38857

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/1M5l_C56ZC8_2Ycgm9EFieVIVNqxsP7dXAnGoBZy3nDo/edit?usp=sharing

The primer scheme .bed and .tsv files necessary for the ARTIC variant calling pipeline are at Zenodo: <https://zenodo.org/record/3897530#.Xv5EFpMzadY>

Version history:

V4: updated .bed and .tsv file link to point to Zenodo (and not google drive).

V1-V3: included primers sequences in the protocol, fixed step 17.12 from elute in "molecular grade water or Elution buffer" to elute in "10 mM Tris-HCl pH 8.0 with 50 mM NaCl", as suggested on the Oxford Nanopore protocol, changed images from ARTIC protocol image to our own.

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/1M5I_C56ZC8_2Ycgm9EFieVIVNqxsP7dXAnGoBZy3nDo/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

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 1200bp amplicons. Primer names and dilutions are listed here:

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

We have tested multiplexing 1500 nt and 2000 nt amplicons as well, all work. These are included in the link. Here we will discuss just the protocol for 1200 nt amplicons as they worked best in our hands.

You can order these as an oligo pool from IDT: <https://sg.idtdna.com/site/order/poolentry/>

7.1 Primers used to generate 1200 bp amplicons are here:

Primer Name	Sequence	Pool	Length	Tm	Start
SARSCoV_1200_1_LEF T	ACCAACCAACTTTTCGATCTCTTGT	1	24	60.69	30
SARSCoV_1200_1_RIG HT	GGTTGCATTCATTTGGTGACGC	1	22	61.49	1205
SARSCoV_1200_3_LEF T	GGCTTGAAGAGAAGTTTAAGGAAG GT	1	26	61.19	2153
SARSCoV_1200_3_RIG HT	GATTGTCCTCACTGCCGTCTTG	1	22	61.5	3257
SARSCoV_1200_5_LEF T	ACCTACTAAAAAGGCTGGTGGC	1	22	60.55	4167
SARSCoV_1200_5_RIG HT	AGCATCTTGTAGAGCAGGTGGA	1	22	61.16	5359
SARSCoV_1200_7_LEF T	ACCTGGTGTATACGTTGTCTTTGG	1	24	60.8	6283
SARSCoV_1200_7_RIG HT	GCTGAAATCGGGGCCATTTGTA	1	22	61.53	7401
SARSCoV_1200_9_LEF T	AGAAGTTACTGGCGATAGTTGTAA TAACT	1	29	60.59	8253
SARSCoV_1200_9_RIG HT	TGCTGATATGTCCAAAGCACCA	1	22	60.29	9400
SARSCoV_1200_11_LE FT	AGACACCTAAGTATAAGTTTGTTC GCA	1	27	60.74	1034 3
SARSCoV_1200_11_RI GHT	GCCCACATGGAAATGGCTTGAT	1	22	61.8	1146 9
SARSCoV_1200_13_LE FT	ACCTCTTACAACAGCAGCCAAAC	1	23	61.55	1245 0
SARSCoV_1200_13_RI GHT	CGTCCTTTTCTTGGAAGCGACA	1	22	61.38	1362 1
SARSCoV_1200_15_LE FT	TTTTAAGGAATTACTTGTGTATGC TGCT	1	28	60.06	1454 0
SARSCoV_1200_15_RI GHT	ACACACAACAGCATCGTCAGAG	1	22	61.12	1573 5
SARSCoV_1200_17_LE FT	TCAAGCTTTTTGCAGCAGAAACG	1	23	61.28	1662 4
SARSCoV_1200_17_RI GHT	CCAAGCAGGGTTACGTGTAAGG	1	22	61.19	1775 4
SARSCoV_1200_19_LE FT	GGCACATGGCTTTGAGTTGACA	1	22	61.91	1859 6
SARSCoV_1200_19_RI GHT	CCTGTTGTCCATCAAAGTGTCCC	1	23	61.62	1967 8
SARSCoV_1200_21_LE FT	TCTGTAGTTTCTAAGGTTGTCAAA GTGA	1	28	60.58	2055 3
SARSCoV_1200_21_RI GHT	GCAGGGGGTAATTGAGTTCTGG	1	22	60.95	2164 2
SARSCoV_1200_23_LE FT	ACTTTAGAGTCCAACCAACAGAAT CT	1	26	60.18	2251 1
SARSCoV_1200_23_RI GHT	TGACTAGCTACACTACGTGCCC	1	22	61.52	2363 1

SARSCoV_1200_25_LEFT	TGCTGCTACTAAAATGTCAGAGTG T	1	25	60.51	2463 3
SARSCoV_1200_25_RIGHT	CATTTCCAGCAAAGCCAAAGCC	1	22	61.45	2579 0
SARSCoV_1200_27_LEFT	TGGATCACCGGTGGAATTGCTA	1	22	61.75	2674 4
SARSCoV_1200_27_RIGHT	TGTTCGTTTAGGCGTGACAAGT	1	22	60.74	2789 4
SARSCoV_1200_29_LEFT	TGAGGGAGCCTTGAATACACCA	1	22	61.1	2867 7
SARSCoV_1200_29_RIGHT	TAGGCAGCTCTCCCTAGCATTG	1	22	61.61	2979 0

Primers for **Pool 1**

Primer Name	Sequence	Pool	Length	Tm	Start
SARSCoV_1200_2_LEFT	CCATAATCAAGACTATTCAACCAAGGGT	2	28	61.27	1100
SARSCoV_1200_2_RIGHT	ACAGGTGACAATTTGTCCACCG	2	22	61.33	2266
SARSCoV_1200_4_LEFT	GGAATTTGGTGCCACTTCTGCT	2	22	61.66	3144
SARSCoV_1200_4_RIGHT	CCTGACCCGGGTAAGTGGTTAT	2	22	61.49	4262
SARSCoV_1200_6_LEFT	ACTTCTATTAAATGGGCAGATAACA AACTG	2	29	60.18	5257
SARSCoV_1200_6_RIGHT	GATTATCCATTCCCTGCGCGTC	2	22	61.75	6380
SARSCoV_1200_8_LEFT	CAATCATGCAATTGTTTTTCAGCTA TTTTG	2	30	60.39	7298
SARSCoV_1200_8_RIGHT	TGACTTTTTGCTACCTGCGCAT	2	22	61.39	8385
SARSCoV_1200_10_LEFT	TTTACCAGGAGTTTTCTGTGGTGT	2	24	60.32	9303
SARSCoV_1200_10_RIGHT	TGGGCCTCATAGCACATTGGTA	2	22	61.5	1045 1
SARSCoV_1200_12_LEFT	ATGGTGCTAGGAGAGTGTGGAC	2	22	61.48	1137 2
SARSCoV_1200_12_RIGHT	GGATTTCCACAATGCTGATGC	2	22	60.48	1256 0
SARSCoV_1200_14_LEFT	ACAGGCACTAGTACTGATGTCGT	2	23	61.12	1350 9
SARSCoV_1200_14_RIGHT	GTGCAGCTACTGAAAAGCACGT	2	22	61.94	1464 1
SARSCoV_1200_16_LEFT	ACAACACAGACTTTATGAGTGTCT CT	2	26	60.18	1560 8
SARSCoV_1200_16_RIGHT	CTCTGTCAGACAGCACTTCACG	2	22	61.17	1672 0

SARSCoV_1200_18_LE FT	GCACATAAAGACAAATCAGCTCAA TGC	2	27	62.03	1762 2
SARSCoV_1200_18_RI GHT	TGTCTGAAGCAGTGGAAAAGCA	2	22	60.68	1870 6
SARSCoV_1200_20_LE FT	ACAATTTGATACTTATAACCTCTG GAACAC	2	30	60.15	1957 4
SARSCoV_1200_20_RI GHT	GATTAGGCATAGCAACACCCGG	2	22	61.39	2069 8
SARSCoV_1200_22_LE FT	GTGATGTTCTTGTTAACTAAACGAACA	2	30	61.44	2153 2
SARSCoV_1200_22_RI GHT	AACAGATGCAAATCTGGTGGCG	2	22	62.03	2261 2
SARSCoV_1200_24_LE FT	GCTGAACATGTCAACAACCTCATAT GA	2	26	60.13	2351 8
SARSCoV_1200_24_RI GHT	ATGAGGTGCTGACTGAGGGAAG	2	22	61.74	2473 6
SARSCoV_1200_26_LE FT	GCCTTGAAGCCCCTTTTCTCTA	2	22	60.29	2569 0
SARSCoV_1200_26_RI GHT	AATGACCACATGGAACGCGTAC	2	22	61.5	2685 7
SARSCoV_1200_28_LE FT	TTTGTGCTTTTGTAGCCTTTCTGCT	2	24	60.14	2778 4
SARSCoV_1200_28_RI GHT	GTTTGGCCTTGTTGTTGTTGGC	2	22	61.82	2900 7

Primers for **Pool 2**

- 8 If you have ordered each primer independently and need to generate primer pool stocks: add 5 μL of each primer from Pool 1 to a 1.5 mL Eppendorf labeled “Pool 1 (100 μM)” and each primer from Pool 2 to a 1.5 mL Eppendorf labelled “Pool 2 (100 μM)”. These are your 100 μM 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 10 μM 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 0.015 μ M per primer. In this case (1200 nt amplicons), pool 1 has 30 primers and pool 2 has 28 primers, so the requirement is 1.13 μ L for primer pool 1 and 1.05 μ L for primer pool 2 (10 μ M) per 25 μ L reaction. However, as these values are relatively close, we round up and down to 1.1 μ L 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 μ L	5 μ L
10 mM dNTPs	0.5 μ L	0.5 μ L
Q5 Hot Start DNA Polymerase	0.25 μ L	0.25 μ L
Primer Pool 1 or 2 (10 μ M)	1.1 μ L	1.1 μ L
Nuclease-free water	15.9 μ L	15.9 μ L
Total	22.5 μ L	22.5 μ L

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 μ L cDNA to each tube and mix well by pipetting.

Note

The **extraction and sample addition cabinet** 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. We typically use 30 cycles.

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, 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).

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](https://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](https://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, it is not necessary to clean up the PCR reaction at this step.

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

Protocol

NAME

DNA quantification using the Qubit fluorometer

CREATED BY

Nikki Freed

PREVIEW

- 14.1.1** 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

Note

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.

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

Note

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

- 14.1.3** 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.

- 14.1.4** Add 10µL of standard to the appropriate tube.

- 14.1.5** Add 1–20µL of each user sample to the appropriate tube.

Note

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

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

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

14.1.8 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.

Note

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.

14.1.9 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.

14.1.10 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.

14.1.11 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.

- 14.1.12** Press Run samples.
- 14.1.13** 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).
- 14.1.14** 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.
- 14.1.15** **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.
- 14.1.16** Repeat step 14 until all samples have been read.
- 14.1.17** Carefully **record all results** and store run file from the Qubit on a memory stick.
- 14.1.18** 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 barcoding using the SQK RBK004

- 16** Multiple 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. Tip: aliquot the Rapid barcodes into a PCR strip to enable multichannelling.

SQK-RBK004 Rapid Barcoding Kit **Oxford Nanopore Technologies Catalog #SQK-RBK00**

- 16.1** 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:

5m

Component

DNA amplicons from step 15 (100ng total)

Fragmentation Mix RB01-12 (one for each sample, included in kit)

Total

Volume

7.5 µL

2.5 µL

10 µL

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

- 16.3** Incubate the reaction in a PCR machine:

30 °C	for	00:01:00
80 °C	for	00:01:00
4 °C	for	00:00:30

5m

- 16.4** Pool all barcoded samples, noting the total volume.

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

15m

Protocol

NAME

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

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 μ L of freshly prepared room-temperature 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 its 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 μ L 10 mM Tris-HCl pH 8.0 with 50 mM NaCl, mix gently by flicking and incubate at room temperature for 00:02:00 .

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

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. Please refer to the Oxford Nanopore Rapid Barcoding RBK004 protocol at this stage. Store the library on ice until ready to load.

10m

MinION sequencing

21 Start the sequencing run using MinKNOW.

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 running 12 samples. Shorter, if running fewer samples.

23 The primer scheme .bed and .tsv files necessary for the ARTIC variant calling pipeline are at Zenodo: <https://zenodo.org/record/3897530#.Xv5EFpMzadY>