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 We are still developing and optimizing this protocol

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Viral Sequencing, from Gunk to Graph (One-Step four-primer PCR alternative)

Forked from [Viral Sequencing, from Gunk to Graph](#)

David A Eccles¹

¹Malaghan Institute of Medical Research (NZ)

Coronavirus Method Development Community

David A Eccles

Malaghan Institute of Medical Research (NZ)

ABSTRACT

This is a fast "gunk to graph" protocol for analysing viral RNA from nasopharyngeal swabs. The approach involves swab lysis and inactivation at the point of sampling, uses a cellulose binding / wash protocol to reduce extraction cost, incorporates sample-specific barcodes during first-strand synthesis, nanopore rapid-attachment primers during PCR amplification, and nanopore sequencing with parallel RAMPART analysis for fast assembly and phylogenetics.

Note: this is a one-step RT-PCR reaction protocol. For an alternative method that allows more multiplexing flexibility, see the protocol that this has been forked from:

<https://dx.doi.org/10.17504/protocols.io.bd3yi8pw>

MATERIALS

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Q5 Hot Start High-Fidelity 2X Master Mix - 500 rxns [New England Biolabs Catalog](#) #M0494L

MinION sequencer [Oxford Nanopore Technologies](#)

ONT MinION Flow Cell R9.4.1 [Oxford Nanopore Technologies Catalog](#) #FLO-MIN106D

Additional materials TBA.

This protocol is UNTESTED, and is in the early stages of development. Do not trust the protocol; question everything.

Assume samples are potentially infectious during extraction, and make sure to use proper sterile technique to avoid cross-contamination.

Swab Lysis

1 Prepare a 1.5 mL centrifuge tube with heated lysis buffer and a cellulose disc

1.1 Add 500 μ L lysis / RNase inactivation buffer ([Twitter reference](#)) to 1.5ml centrifuge tube:

- 10 millimolar (mM) Tris
- 10 millimolar (mM) EDTA
- 0.5 % volume SDS
- 150 millimolar (mM) NaCl

OR 500 μ L extraction buffer #2 (see [paper](#)):

- 800 millimolar (mM) guanidine hydrochloride
- 50 millimolar (mM) Tris [pH 8]
- 0.5 % volume Triton X100
- 1 % volume Tween-20

1.2 Add a 3 mm diameter punched disc from [Whatman #1 filter paper](#) (see [paper](#))

1.3 Preheat 1.5 mL tube to 60 °C

2 Collect sample using a sterile polystyrene swab with a 30mm breakpoint (e.g. [Puritan 25-3606-U; PurFlock Ultra 6" Sterile Elongated Flock Swab w/Polystyrene Handle, 30mm Breakpoint](#)).

RNA Wash

3 Transfer disc to a new 1.5 mL tube containing 200 µL wash buffer using a pipette tip to remove contaminants:

- 10 millimolar (mM) Tris [pH 8.0]
- 0.1 % volume Tween-20

4 Incubate tube at Room temperature for 00:01:00

cDNA Synthesis setup

5 Transfer disc to a new 200 µL PCR tube using a pipette tip

6 Add the following additional components into the 200 µL PCR tube (see the [TaqMan Fast Virus 1-Step Master Mix Product Sheet](#) and [Nanopore protocol for Sequence-specific cDNA-PCR Sequencing \(SQK-PCS109\)](#)) in a 50 µL reaction:

- 1 µL x 2 micromolar (µM) reverse primers
- 1 µL x 2 micromolar (µM) forward primers

- 1.5 µL cDNA primer (cPRM)
- 12.5 µL TaqMan Fast Virus 1-StepMaster Mix
- 34 µL RNase-free water

Reverse primers should be prefixed with sample-specific barcode sequences (if used) and the ONT reverse anchor sequence, i.e. [5' - ACTTGCCTGTCGCTCTATCTTC - [barcode] - [sequence-specific] - 3']

Forward primers should be prefixed with sample-specific barcode sequences (if used) and the ONT forward anchor sequence, i.e. [5' - TTTCTGTTGGTGCTGATATTGC - [barcode] - [sequence-specific] - 3']

7 Mix gently **by flicking the tube** and spin down ⌚ 00:00:05

8 Denature RNA and anneal reverse primers at 🔥 65 °C for ⌚ 00:05:00 and then snap cool on a pre-chilled freezer block for ⌚ 00:01:00

[Note: this step may not be needed]

9 Mix gently **by flicking the tube** and spin down ⌚ 00:00:05

RT and PCR amplification

10 In four new 200 µL PCR tubes, prepare the following reaction at 🔥 Room temperature in a 50 µL reaction:

- 25 µL 2X Q5 Hot Start High-Fidelity Master Mix
- 1.5 µL cDNA primer (cPRM)
- 18.5 µL Nuclease-free water
- 5 µL Reverse-transcribed cDNA from the previous step (pool, or single sample)

11 Amplify using the following cycling conditions:

Cycle step	Temperature	Time	No. of cycles
Reverse transcription	50° C	5 mins	1
RT inactivation /initial denaturation	95° C	30 secs	1
Denaturation	95 °C	15 secs	10-40*
Anneal / Extended	62 °C	60 secs	10-40*
Final extension	65 °C	6 mins	1
Hold	4 °C	∞	

Thermal cycler settings for PCR amplification

* The recommended starting point is 20 cycles (i.e. 14 cycles + 6 for direct RNA amplification penalty, see [here](#)) - adjust this depending on experimental needs.

- 12 Add 1 µL of NEB Exonuclease 1 (20 units) directly to each PCR tube to remove unextended primers. Mix by ***pipetting***.
- 13 Incubate the reaction at 37 °C for 00:15:00, followed by 80 °C for 00:15:00 to

Bead Cleanup

- 14 ***Pool liquid from amplified samples*** into 1.5 µL Eppendorf DNA LoBind tubes, with no more than 500 µL in each tube

- 15 Add 2X resuspended AMPure XP beads (e.g. 1000 μL XP beads to 500 μL pooled sample) to the 1.5 mL tube and mix by *pipetting*
- 16 Incubate on a gentle agitator (e.g. hula mixer or rotator mixer) for 00:05:00 at Room temperature
- 17 Spin down 00:00:05 the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
- 18 Keep the tube on the magnet and wash the beads with 500 μL of freshly-prepared 70 % volume ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
- 19 Repeat the previous step: wash with 500 μL 70 % volume ethanol, and discard the ethanol / wash liquid.
- 20 Spin down 00:00:05 and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 00:00:30 [at most] but do not dry the pellet to the point of cracking (the magnetic beads should just start to lose their shiny sheen).
- 21 Remove the tube from the magnetic rack and resuspend pellet in 12 μL of Elution Buffer (EB).
- 22 Incubate at Room temperature for 00:10:00

- 23 Pellet beads on magnet 00:05:00 until the eluate is clear and colourless
- 24 While still on the magnet, quantify 1 µl of the amplified cDNA library using the Quantus Fluorometer using the ONE dsDNA assay (see [ncov 2019 sequencing protocol, step 16](#))
- 25 While still on the magnet, carefully remove and retain 11 µL of eluate *from each pooled sample* into a clean 1.5 mL Eppendorf DNA LoBind tube

Adapter Addition

- 26 Transfer 11 µL from the total pool into a clean 1.5 mL Eppendorf DNA LoBind tube
- 27 Add 1 µL of Rapid Adapter (RAP) to the amplified cDNA library
- 28 Mix by *pipetting* and spin down 00:00:05
- 29 Incubate the reaction for 00:05:00 at Room temperature
- 30 Store the prepared library On ice until ready to load onto a flow cell.

Nanopore Sequencing

- 31 Load 20 ng sequencing library onto a MinION flow cell (see [ncov 2019 sequencing protocol, step 21](#))
- 32 Start the sequencing run using MinKNOW, using SQK-PCS109 as the sample preparation protocol (see [ncov 2019 sequencing protocol, step 22](#))

RAMPART Analysis

- 33 Analyse the run results using RAMPART (see <https://artic.network/ncov-2019/ncov2019-using-rampart.html>)