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 We are still developing and optimizing this protocol

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Viral Sequencing, from Gunk to Graph V.1

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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.

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.

SAFETY WARNINGS

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 μ L wash buffer using a pipette tip to remove contaminants:
 - 10 millimolar (mM) Tris [pH 8.0]

- 1M 0.1 % volume Tween-20

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

cDNA Synthesis

5 Add the following additional components into the 200 µL PCR tube (see the [Nanopore protocol for Sequence-specific cDNA-PCR Sequencing \(SQK-PCS109\)](#)) in a 11 µL reaction:

- 1 µL x 1M 2 micromolar (µM) reverse primers
- 1 µL x 1M 10 millimolar (mM) dNTPs
- 9 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']

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

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

8 In a separate tube, mix together the following in an 8 µL reaction:

- 4 µL 5X RT Buffer
- 1 µL RNaseOUT
- 1 µL Nuclease-free water
- 2 µL x 1M 10 micromolar (µM) Strand-switching primer (SSP)

Note: It might be possible to instead carry out only the first-strand synthesis (i.e. excluding SSP), then use a forward primer, tailed with sample-specific barcode sequences (if used) and the ONT forward anchor sequence, i.e. [5' - TTTCTGTTGGTGCTGATATTGC - [barcode] - [sequence-specific] - 3']. One-Step RT-PCR sequencing kits may help with this (e.g. [OneTaq One-Step RT-PCR Kit](#)). For more details about the reverse anchor sequence and four-primer amplicon sequencing, see the Nanopore protocol for [Four-primer PCR \(SQK-PSK004 or SQK-PBK004\)](#).

- 9 Mix gently **by flicking the tube** and spin down 00:00:05
- 10 Add the strand-switching buffer to the snap-cooled, annealed RNA, mix by **flicking the tube** and spin down
- 11 Incubate at 42 °C for 00:02:00
- 12 Add 1 µL of Maxima H Minus Reverse Transcriptase, to a total volume of 20 µL
- 13 Mix gently by **flicking the tube** and spin down 00:00:05
- 14 Incubate using the following protocol:

Cycle step	Temperature	Time	No. of cycles

Reverse transcription and strand-switching	42° C	90 mins	1
Heat inactivation	85° C	5 mins	1
Hold	4° C	∞	

Thermal cycler settings for reverse transcription and strand switching

PCR amplification

- 15** 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)

- 16** Amplify using the following cycling conditions:

Cycle step	Temperature	Time	No. of cycles
Initial denaturation	95 °C	30 secs	1
Denaturation	95 °C	15 secs	10-18*
Annealing	62 °C	15 secs	10-18*
Extension	65 °C	50 secs per kb	10-18*
Final extension	65 °C	6 mins	1
Hold	4 °C	∞	

Thermal cycler settings for PCR amplification

* The recommended starting point is 14 cycles - adjust this depending on experimental needs.

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

Bead Cleanup

- 19 Add 160 μL of resuspended AMPure XP beads to the 1.5 mL tube and mix by ***pipetting***
- 20 Incubate on a gentle agitator (e.g. hula mixer or rotator mixer) for 00:05:00 at Room temperature
- 21 Spin down 00:00:05 the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
- 22 Keep the tube on the magnet and wash the beads with 200 μL of freshly-prepared 70 % volume ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
- 23 Repeat the previous step: wash with 200 μL 70 % volume ethanol , and discard the ethanol / wash liquid.

- 24 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).
- 25 Remove the tube from the magnetic rack and resuspend pellet in 12 μL of Elution Buffer (EB).
- 26 Incubate at Room temperature for 00:10:00
- 27 Pellet beads on magnet 00:05:00 until the eluate is clear and colourless
- 28 While still on the magnet, carefully remove and retain 12 μL of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube
- 29 Quantify 1 μL of the amplified cDNA library using the Quantus Fluorometer using the ONE dsDNA assay (see [ncov 2019 sequencing protocol, step 16](#))

Adapter Addition

- 30 Add 1 μL of Rapid Adapter (RAP) to the amplified cDNA library
- 31 Mix by ***pipetting*** and spin down 00:00:05

32 Incubate the reaction for 00:05:00 at Room temperature

33 Store the prepared library On ice until ready to load onto a flow cell.

Nanopore Sequencing

34 Load 20 ng sequencing library onto a MinION flow cell (see [ncov 2019 sequencing protocol, step 21](#))

35 Start the sequencing run using MinKNOW, using SQK-PCS109 as the sample preparation protocol (see [ncov 2019 sequencing protocol, step 22](#))

RAMPART Analysis

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