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Viral Sequencing, from Gunk to Graph (Two-step, strand-switching) V.3

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Coronavirus Method Development Community

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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.

An alternative protocol using both forward and reverse primers can be found here.

GUIDELINES

When testing, make sure to include at least one positive control (e.g. synthetic plasmid including partial viral sequence) and at least one negative control (e.g. nuclease-free water).

MATERIALS

NAME	CATALOG #	VENDOR
Q5 Hot Start High-Fidelity 2X Master Mix - 500 rxns	M0494L	New England Biolabs
MinION sequencer	View	Oxford Nanopore Technologies
ONT MinION Flow Cell R9.4.1	FLO-MIN106D	Oxford Nanopore Technologies

MATERIALS TEXT

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

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1.1 Add **500 µl** lysis / RNAse inactivation buffer (Twitter reference) to 1.5ml centrifuge tube:

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

OR **500 µl** extraction buffer #2 (see paper):

- **800 Milimolar (mM) guanidine hydrochloride**
- **50 Milimolar (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/Polystryene Handle, 30mm Breakpoint).

3 Break swab and place into the prepared **1.5 ml** tube with lysis buffer to remove the outer viral shell and release RNA.

4 Vortex tube for **00:00:30** , then incubate tube at **60 °C** for **00:10:00 [10-30 mins]** to inactivate viral proteins.

RNA Wash

5 Transfer disc to a new **1.5 ml** tube containing **200 µl** wash buffer using a pipette tip to remove contaminants:

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

6 Incubate tube at **Room temperature** for **00:01:00**

cDNA Synthesis

7 Transfer disc to a new **200 µl** PCR tube using a pipette tip

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8 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 **2 Micromolar (µM) reverse primers**
- **1 µl** x **10 Milimolar (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']

A potential primer pool are the reverse/right ARCTIC primers (and amplification control) with barcodes and ONT anchor sequences from here.

An alternative protocol using both forward and reverse primers can be found here.

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

10 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**

11 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 **10 Micromolar (µM)** ONT Strand-switching primer (SSP)

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

13 Add the strand-switching buffer to the snap-cooled, annealed RNA, mix by ***flicking the tube*** and spin down

14 Incubate at **42 °C** for **00:02:00**

15 Add **1 µl** of Maxima H Minus Reverse Transcriptase, to a total volume of **20 µl**

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

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17 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

18 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)

19 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	10-40*
Annealing	62 °C	15 secs	10-40*
Extension	65 °C	50 secs per kb	10-40*
Final extension	65 °C	6 mins	1
Hold	4 °C	∞	

Thermal cycler settings for PCR amplification

* Starting from viral RNA, the recommended starting point is 20 cycles - adjust this depending on experimental needs.

20 Add **1 µl** of NEB Exonuclease 1 (20 units) directly to each PCR tube to remove unextended primers. Mix by ***pipetting***.

21 Incubate the reaction at **37 °C** for **00:15:00** , followed by **80 °C** for **00:15:00**

22 Run 1µl of amplified product on a gel (or similar length-based QC device) to verify that amplified products exist at the expected length. Because this is a strand-switch protocol, there may be a smear of template DNA rather than specific bands.

Bead Cleanup

23 Add 160 µl of resuspended AMPure XP beads to the **1.5 ml** tube and mix by ***pipetting***

24 Incubate on a gentle agitator (e.g. hula mixer or rotator mixer) for **00:05:00** at **Room temperature**

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25 Spin down **00:00:05** the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

26 Keep the tube on the magnet and wash the beads with **200 µl** of freshly-prepared **[M]70 % volume ethanol** without disturbing the pellet. Remove the ethanol using a pipette and discard.

27 Repeat the previous step: wash with **200 µl** **[M]70 % volume ethanol** , and discard the ethanol / wash liquid.

28 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).

29 Remove the tube from the magnetic rack and resuspend pellet in **12 µl** of Elution Buffer (EB).

30 Incubate at **Room temperature** for **00:10:00**

31 Pellet beads on magnet **00:05:00** until the eluate is clear and colourless

32 While still on the magnet, carefully remove and retain **12 µl** of eluate into a clean **1.5 ml** Eppendorf DNA LoBind tube

33 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

34 Add **1 µl** of Rapid Adapter (RAP) to the amplified cDNA library

35 Mix by ***pipetting*** and spin down **00:00:05**

36 Incubate the reaction for **00:05:00** at **Room temperature**

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

Nanopore Sequencing

38 Load **20 ng** sequencing library onto a MinION flow cell (see ncov 2019 sequencing protocol, step 21)

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

RAMPART Analysis

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

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