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

Forked from Viral Sequencing, from Gunk to Graph

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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.

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

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).

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 Milimolar (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

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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)

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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 / Extend	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 **[M]70 % volume ethanol** without disturbing the pellet. Remove the ethanol using a pipette and discard.

19 Repeat the previous step: wash with **500 µl** **[M]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).

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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)

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