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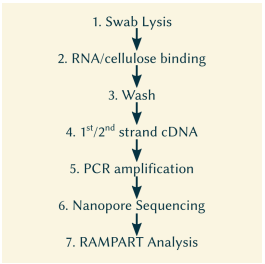
Viral Sequencing, from Gunk to Graph (One-Step four-primer PCR alternative)



Forked from Viral Sequencing, from Gunk to Graph

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Coronavirus Method De...

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Protocol status: In development
We are still developing and optimizing this protocol

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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

MATERIALS

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 µL lysis / RNAse inactivation buffer (**Twitter reference**) to 1.5ml centrifuge tube:

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

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

- [M] 800 millimolar (mM) guanidine hydrochloride
- [M] 50 millimolar (mM) Tris [pH 8]
- [M] 0.5 % volume Triton X100
- [M] 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:

- [M] 10 millimolar (mM) Tris [pH 8.0]
- [M] 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 [M] 2 micromolar (µM) reverse primers
- 1 µL x [M] 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 / 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).

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)