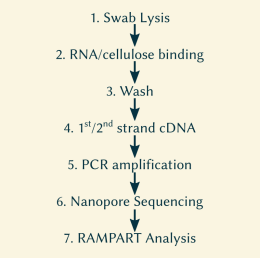
Mar 24, 2020 Version 1

Viral Sequencing, from Gunk to Graph V.1



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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.

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

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 [M] 2 micromolar (µM) reverse primers
- 1 µL x [M] 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 [M] 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 [M] 70 % volume ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

23 Repeat the previous step: wash with 200 µL [M] 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)