Viral Sequencing, from Gunk to Graph (Two-step, strand-switching) V.2

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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.

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KEYWORDS
SARS-CoV-2, COVID-19, nanopore, sequencing

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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:

   - 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

C DNA Synthesis

5. Transfer disc to a new 200 µl PCR tube using a pipette tip

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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 [n]2 Micromolar (µM) reverse primers
- 1 µl x [n]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']

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

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

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

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

In a separate tube, mix together in an 8 µl reaction:

- 4 µl 5X RT Buffer
- 1 µl RNaseOUT
- 1 µl Nuclease-free water
- 2 µl x [n]10 Micromolar (µM) ONT Strand-switching primer (SSP)

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

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

Incubate at 42 °C for 00:02:00

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

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

15. Incubate using the following protocol:

<table>
<thead>
<tr>
<th>Cycle step</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcription and strand-switching</td>
<td>42 °C</td>
<td>90 mins</td>
<td>1</td>
</tr>
<tr>
<td>Heat inactivation</td>
<td>85 °C</td>
<td>5 mins</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4 °C</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

Thermal cycler settings for reverse transcription and strand switching

**PCR amplification**

16. 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)

17. Amplify using the following cycling conditions:

<table>
<thead>
<tr>
<th>Cycle step</th>
<th>Temperature</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95 °C</td>
<td>30 secs</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>15 secs</td>
<td>10-40*</td>
</tr>
<tr>
<td>Annealing</td>
<td>62 °C</td>
<td>15 secs</td>
<td>10-40*</td>
</tr>
<tr>
<td>Extension</td>
<td>65 °C</td>
<td>50 secs per kb</td>
<td>10-40*</td>
</tr>
<tr>
<td>Final extension</td>
<td>65 °C</td>
<td>6 mins</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4 °C</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

Thermal cycler settings for PCR amplification

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

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

19. Incubate the reaction at **37 °C** for **00:15:00**, followed by **80 °C** for **00:15:00**
Run 1 μl 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

1. Add 160 μl of resuspended AMPure XP beads to the 1.5 mL tube and mix by pipetting.

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

3. Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

4. 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.

5. Repeat the previous step: wash with 200 μl 70% volume ethanol, and discard the ethanol / wash liquid.

6. Spin down 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).

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

8. Incubate at Room temperature for 00:10:00.

9. Pellet beads on magnet 00:05:00 until the eluate is clear and colourless.

10. While still on the magnet, carefully remove and retain 12 μl of eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.

11. Quantify 1 μl of the amplified cDNA library using the Quantus Fluorometer using the ONE dsDNA assay (see nccov 2019 sequencing protocol, step 16).

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Adapter Addition

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

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

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

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

Nanopore Sequencing

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

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

RAMPART Analysis

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