Mar 23, 2020

Viral Sequencing, from Gunk to Graph

David Eccles[1]

[1]Malaghan Institute of Medical Research (NZ)

In Development dx.doi.org/10.17504/protocols.io.bd3yi8pw

Coronavirus Method Development Community

David A. Eccles
Malaghan Institute of Medical Research (NZ)

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

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

Citation: David Eccles (03/23/2020). Viral Sequencing, from Gunk to Graph. https://dx.doi.org/10.17504/protocols.io.bd3yi8pw

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

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 **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']

Citation: David Eccles (03/23/2020). Viral Sequencing, from Gunk to Graph. https://dx.doi.org/10.17504/protocols.io.bd3yi8pw

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 **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

Citation: David Eccles (03/23/2020). Viral Sequencing, from Gunk to Graph. https://dx.doi.org/10.17504/protocols.io.bd3yi8pw

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.

Citation: David Eccles (03/23/2020). Viral Sequencing, from Gunk to Graph. https://dx.doi.org/10.17504/protocols.io.bd3yi8pw

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)

Citation: David Eccles (03/23/2020). Viral Sequencing, from Gunk to Graph. https://dx.doi.org/10.17504/protocols.io.bd3yi8pw