Metagenomic analysis of SARS-CoV-2 respiratory samples via Sequence-Independent Single Primer Amplification (SISPA) and nanopore sequencing.

Samples: SARS-CoV-2 positive nasopharyngeal swabs + negative controls

1 Prepare

Consider use of a spike-in internal positive control
e.g. Hazara virus virions, as used by Lewandowski *et al.*[1]

Perform depletion of human/bacterial material
e.g. filtration & centrifugation (Moreno & O'Connor[2]) or centrifugation only (Lewandowski *et al.*[1])

Take supernatant forward into nucleic acid extraction

2 Extract nucleic acid

Extract total nucleic acid from supernatant using the QIAamp Viral RNA Mini Kit (Qiagen)

3 Purify RNA

Perform DNase treatment

Purify and concentrate RNA using the RNA Clean & Concentrator-5 kit (Zymo Research)

4 Perform unbiased, full-genome RNA amplification with SISPA

Synthesise and amplify cDNA libraries using SISPA A/B primers[1]

- Perform reverse transcription and second strand synthesis with primer A
- Split reaction into three
- Perform random amplification of cDNA libraries with primer B

5 Clean up and quantify

Clean up amplified cDNA samples with a 1x excess of AMPure XP (Beckman Coulter)

Quantify libraries with the Qubit High Sensitivity dsDNA kit (Thermo Fisher)

6 Prepare barcoded sequencing libraries

Prepare 250 ng cDNA from each of up to 12 samples for sequencing in a single run, using the Ligation Sequencing Kit and Native Barcoding Expansion pack

Ligate a different native barcode to each sequenced in multiplex

7 Sequence

Sequence samples in multiplex on one FLO-MIN106 Flow Cell on the MinION™ Mk1B or GridION™ Mk1 device

8 Basecall and demultiplex reads

Perform basecalling and demultiplexing of reads
using e.g. MinKNOW™ or guppy

9 Perform host depletion and filtering

Bioinformatically deplete unwanted (e.g. host) reads

Filter reads to discard any under 300 bp in length and/or of Q-score ≤7

10 Map reads to SARS-CoV-2 reference and call variants

Map cleaned reads to SARS-CoV-2 reference genome
using e.g. minimap2[3]

Call variants of ≥10% frequency
using e.g. the callvariants.sh script from the BBMap repository[4]

Map reads to reference file containing only coded gene regions
using e.g. minimap2[3]

Call variants in coded gene regions
using e.g. the callvariants.sh script from the BBMap repository[4]

11 Perform metagenomic analysis

Perform metagenomic analysis of data
e.g. via rapid sequence comparison against the GenBank nucleotide database using the sendsketch script from the BBMap repository[4]

Kits & devices

Library preparation

Ligation Sequencing Kit (SQK-LSK109)

Native Barcoding Expansion: 1-12 (EXP-NBD104) or 13-24 (EXP-NBD114)

Sequencing

MinION Mk1B or GridION Mk1 + R9.4.1 Flow Cells

References

Sample & library preparation workflow based on protocol described in 2. Bioinformatics workflow based on pipeline described in 5.

1. Lewandowski, K. *et al.* (2019). Metagenomic Nanopore sequencing of influenza virus direct from clinical respiratory samples. J. Clin Microbiol., 58:e00963-e01019. doi: 10.1128/JCM.00963-19
2. Moreno, G. and O'Connor, D. Protocol: Sequence-Independent, Single-Primer Amplification of RNA viruses V.3. Available at: https://www.protocols.io/view/sequence-independent-single-primer-amplification-o-bckxiuxn [accessed 31Mar20]
3. Li, H. (2018). Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics, 34:3094-3100. doi: 10.1093/bioinformatics/bty191
4. BBMap repository, GitHub. https://github.com/BioInfoTools/BBMap [accessed 31Mar20]
5. Brown, K. & Moreno, G., SARS-CoV-2 parallel sequencing by Illumina and Oxford Nanopore Technologies, https://openresearch.labkey.com/Coven/wiki-page.view?name=SARS-CoV-2%20Deep%20Sequencing [accessed 31Mar20]

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