



Automated Library Construction and Analysis for High-Throughput Nanopore Sequencing of SARS-CoV-2

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Background: To support the implementation of high-throughput pipelines suitable for SARS-CoV-2 sequencing and analysis in a clinical laboratory, we developed an automated sample preparation and analysis workflow.

Methods: We used the established ARTIC protocol with approximately 400 bp amplicons sequenced on Oxford Nanopore's MinION. Sequences were analyzed using Nextclade, assigning both a clade and quality score to each sample.

Results: A total of 2179 samples on twenty-five 96-well plates were sequenced. Plates of purified RNA were processed within 12 h, sequencing required up to 24 h, and analysis of each pooled plate required 1 h. The use of samples with known threshold cycle (Ct) values enabled normalization, acted as a quality control check, and revealed a strong correlation between sample Ct values and successful analysis, with 85% of samples with Ct < 30 achieving a "good" Nextclade score. Less abundant samples responded to enrichment with the fraction of Ct > 30 samples achieving a "good" classification rising by 60% after addition of a post-ARTIC PCR normalization. Serial dilutions of 3 variant of concern samples, diluted from approximately Ct = 16 to approximately Ct = 50, demonstrated successful sequencing to Ct = 37. The sample set contained a median of 24 mutations per sample and a total of 1281 unique mutations with reduced sequence read coverage noted in some regions of some samples. A total of 10 separate strains were observed in the sample set, including 3 variants of concern prevalent in British Columbia in the spring of 2021.

Conclusions: We demonstrated a robust automated sequencing pipeline that takes advantage of input Ct values to improve reliability.

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Received November 8, 2021; accepted May 23, 2022.

<https://doi.org/10.1093/jalm/jfac054>

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IMPACT STATEMENT

This work will benefit public health and infection control, as viral sequencing is increasingly used to investigate outbreaks of diseases such as SARS-CoV-2, by establishing linkages in disease clusters and helping to elucidate mechanisms of transmission. Here we show that an automated high-throughput sequencing pipeline using ARTIC amplicons on MinION is robust in characterizing variants of concern. We also show that sample normalizations improve performance in low viral load samples and are straightforward to automate but would ideally be based on threshold cycle results from diagnostic quantitative PCR. This is an argument for integrating diagnostic and sequencing workflows and informatics.

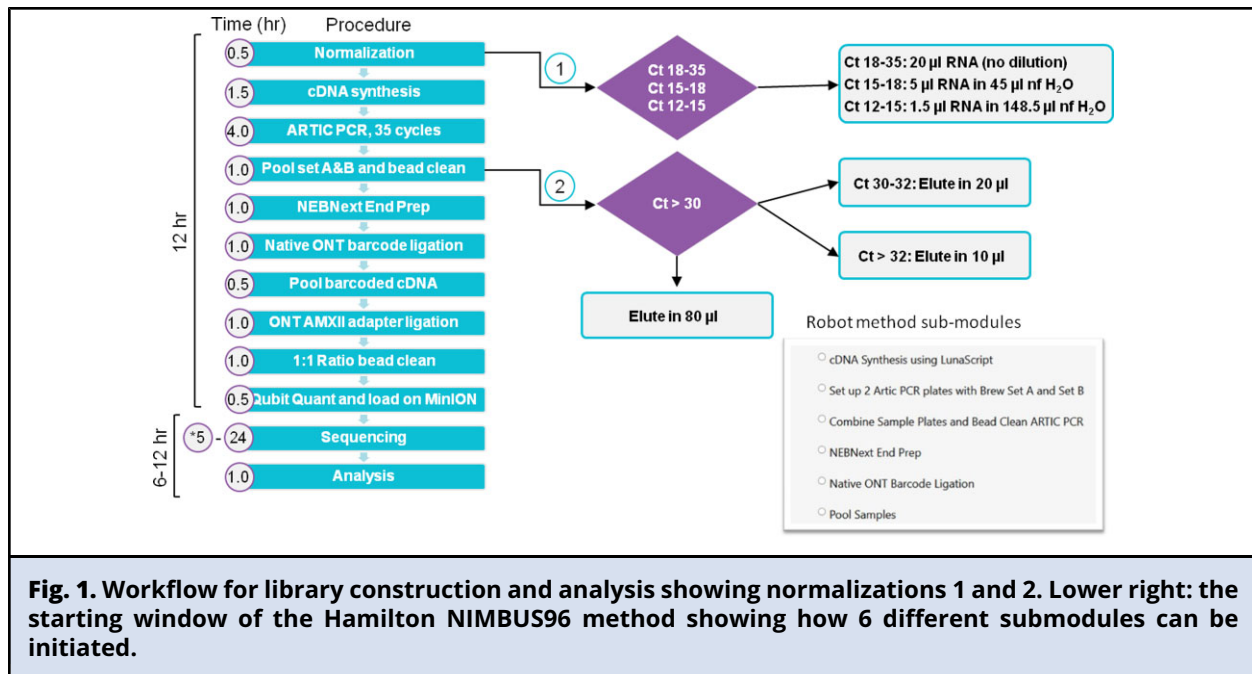
INTRODUCTION

SARS-CoV-2 genome sequencing has elucidated pathways of infection (1) and identified variants of concern (VOCs), which have potentially greater transmissibility (2), disease severity (3–5), and reduced response to therapeutics (6–9). Monitoring such strains is important for informing public health responses, such as vaccine deployment (10) and travel restrictions (11). Mutation detection with single nucleotide polymorphism (SNP) PCR methods has been successfully performed in Matic et al. (12) but whole-genome sequencing (WGS) is required to detect new mutations and explore lineages in more detail. New mutations may not be detected by existing SNP PCR assays, requiring modification of PCR primers. At least in principle, SARS-CoV-2 genome sequencing can capture new mutations, although amplicon-based sequencing approaches may require modification in the event a mutation disrupts a primer annealing site.

Timely access to sequencing results is currently limited by laboratory methods and bioinformatics capacity and expertise, as well as limitations in sensitivity for samples with low viral loads (13). Logistics, such as transport, accessioning, and liquid handling also play a role, but informatics and lab automation can help with all but the first. To support improved clinical deployment of SARS-CoV-2 sequencing, we combined a clinical virology lab's upstream viral

sample processing expertise with a high-throughput genome sequencing center's liquid handling, sequencing, and bioinformatics analysis resources. The goal was to develop a rapid, reliable, and sensitive SARS-CoV-2 sequencing pipeline that could be deployed with common laboratory equipment. To this end, we deployed the ARTIC protocol (14) with approximately 400 bp amplicons split across 2 pools; performed normalization, PCR set-up, and library construction on automated liquid handlers; and used Oxford Nanopore's MinION for sequencing (15–20). The endpoint of the analysis was to classify samples into clades using Nextclade (21). Here, we used the Nextclade clade classification quality scores as a proxy for sequencing data quality.

In addition to supporting the clinical laboratory with an efficient and scalable sequencing workflow, the goals of this proof-of-concept study were to investigate how quickly 96-well plates of samples could be processed in this pipeline and to determine sensitivity limits. Normalization strategies for balancing pools of PCR products for efficient sequencing, based on sample threshold cycle (Ct) values, were also assessed for acquisition of sufficient reads for analysis and sensitivity to samples with Ct values > 30, which can be challenging to completely sequence (15). This study was reviewed and deemed exempt from full ethics review by the Providence Health Care Research Ethics Board.



MATERIALS AND METHODS

Nucleic Acids Extraction and Normalization

Clinical samples from individuals under investigation for COVID-19 infection underwent reverse transcription-PCR for detection of SARS-CoV-2 (TIB Molbiol LightMix® SarbecoV E-gene or Roche Molecular Diagnostics cobas® SARS-CoV-2 Test). Twenty-five plates of SARS-CoV-2 positive samples from the upper respiratory tract (nasopharyngeal swabs in universal transport media or saline mouth rinses) underwent RNA extraction using the MagNA Pure 96 DNA and Viral NA SV Kit, (Roche Diagnostics) eluted in 50 µL in 96-well plates. Screening for VOCs was performed using variant-specific PCR assays, described in detail in Matic et al. (12). The 96-well plate of extracted RNA subsequently underwent reverse transcription, ARTIC multiplexed PCR amplification, and WGS. Ct values for the envelope (E) gene of SARS-CoV-2 were used for a 2-step normalization as shown in the workflow in Fig. 1 (SOP in supplement). The first normalization is recommended in the ARTIC

protocol to prevent cDNA failure from excessive amounts of nucleic acid. In this step, performed on a Perkin-Elmer Janus with the 8-channel Varispan head, samples with Cts from 12 to 15 were diluted 1:100, Cts from 15 to 18 were diluted 1:10, and Cts > 18 were not diluted. Normalization volumes were calculated in an Excel worksheet from the sample submission sheet containing Ct values and output as a .csv file to the Janus. A second, post-ARTIC PCR normalization was added after plate 8 of 25. For these plates, samples with Cts of 30 to 32 were eluted in 20 µL buffer, samples with Cts > 32 were eluted in 10 µL, and the remaining Ct < 30 samples were eluted in 80 µL. To do this, the Janus robot was used to adjust individual well volumes of the post-PCR cleanup elution plate, according to each sample's Ct, and then samples eluted from post-PCR cleanup were transferred to that elution plate prior to pooling.

Library Construction and Sequencing

cDNA synthesis and subsequent library preparation steps were conducted on NIMBUS96 liquid

handlers (Hamilton Company) using an in-house program incorporating liquid transfers and SPRI bead purification from existing sample preparation methods at Canada's Michael Smith Genome Sciences Centre (GSC). [Figure 1](#), lower right, shows the program starting window, which lets the user initiate each submodule of the method. Incubations were performed on offline thermal cyclers. Both Nimbus and Janus methods and related data are available at https://svn.bcgsc.ca/bitbucket/projects/RCORBETT/repos/artic_nextflow/browse.

Library construction was performed using the NEBNext ARTIC SARS-CoV-2 companion kit for Oxford Nanopore. PCR products were indexed using the ONT native indexing kit EXP-NBD-196. Each plate, containing between 35 and 94 samples, was pooled and prepared for sequencing using Oxford Nanopore's MinION with R9 flow cells. Available Fast5 files were collected for base calling after 5-h run time and again at 24 h when the run was ended, and both data sets were run through the ARTIC analysis workflow. Representative samples of 3 VOCs of relatively high concentration, B.1.351 (Beta) Ct = 16.3, P1. (Gamma) Ct = 17.11, and B.1.1.7 (Alpha) Ct = 19.03, which had been validated by SNP PCR, were used as controls. At least 1 sample of these was present in each plate that was run.

Sequence Analysis

Whole genome sequencing reads were first base called using Guppy 4.0.11 with the recommended (<https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.html>) “-require_barcode_both_ends” parameter. This approach is recommended to minimize the chance of index miss-assignment during barcode splitting. In testing on non-ARTIC samples, we estimated the routine barcode misassignment to be 0.06% when not using this parameter, but undetectable down to 10^{-6} when using it. Use of this parameter came at the expense of a 40%

loss in total usable reads, which is significant, but the rate of read acquisition is still adequate vs the risk of misassigned reads. The guppyplex and minion commands were combined with Nextclade assignment and quality control (21) in a nextflow 0.1.1 (22) workflow to allow easy parallelization on our network. Our analysis workflow is available for download at https://svn.bcgsc.ca/bitbucket/projects/RCORBETT/repos/artic_nextflow/browse?at=refs%2Ftags%2F0.1.1.

Additional Experiments

A Quant-iT double-stranded DNA assay (Thermo Fisher) was performed using 188 samples of ARTIC PCR amplification products to characterize the relationship between PCR yield, Ct, and sequencing yields. A limit of detection experiment was also performed. The 3 high-concentration Alpha, Beta, and Gamma variant samples as previously above were serially diluted 1 in 32 (Δ Ct 5) through 8 iterations and in triplicate for a total of 72 samples. cDNA synthesis, library construction, and pooling were performed as previously described, with no normalization, and the pool was sequenced on 1 MinION R9 flow cell.

RESULTS

Workflow and Turnaround Time

To streamline the workflow between the clinical lab and the GSC, we created a project-specific sample submission (Excel) sheet for each submitted plate (see [Supplementary Materials](#) in the online Data Supplement). The clinical lab could then add patient sample IDs and Ct values and, optionally, a comment about whether the case was part of a suspected outbreak. Sheets were then automatically uploaded to the laboratory information system at the GSC, which tracked samples through library construction, sequencing, and analysis to automated upload of results.

A total of 2179 SARS-CoV-2 samples were sequenced in 25 runs (data at <https://www.ncbi.nlm.nih.gov/bioproject/810429>). The workflow is shown in Fig. 1 with times for processing each plate of up to 92 samples. Laboratory sample processing time was approximately 12 h starting from the receipt of extracted RNA with sample data already uploaded to the GSC laboratory information system. In practice, ARTIC PCR was set up to complete overnight with library construction and sequencing on day 2, but this timeline could be compressed. Available data from the MinION runs were captured and analyzed after 5 h and after 24 h. Sequence analysis, described in the following discussion, required between 20 and 60 min per pooled plate, depending on number of samples in a pool, to achieve clade classification and quality assignment.

Sequence Results

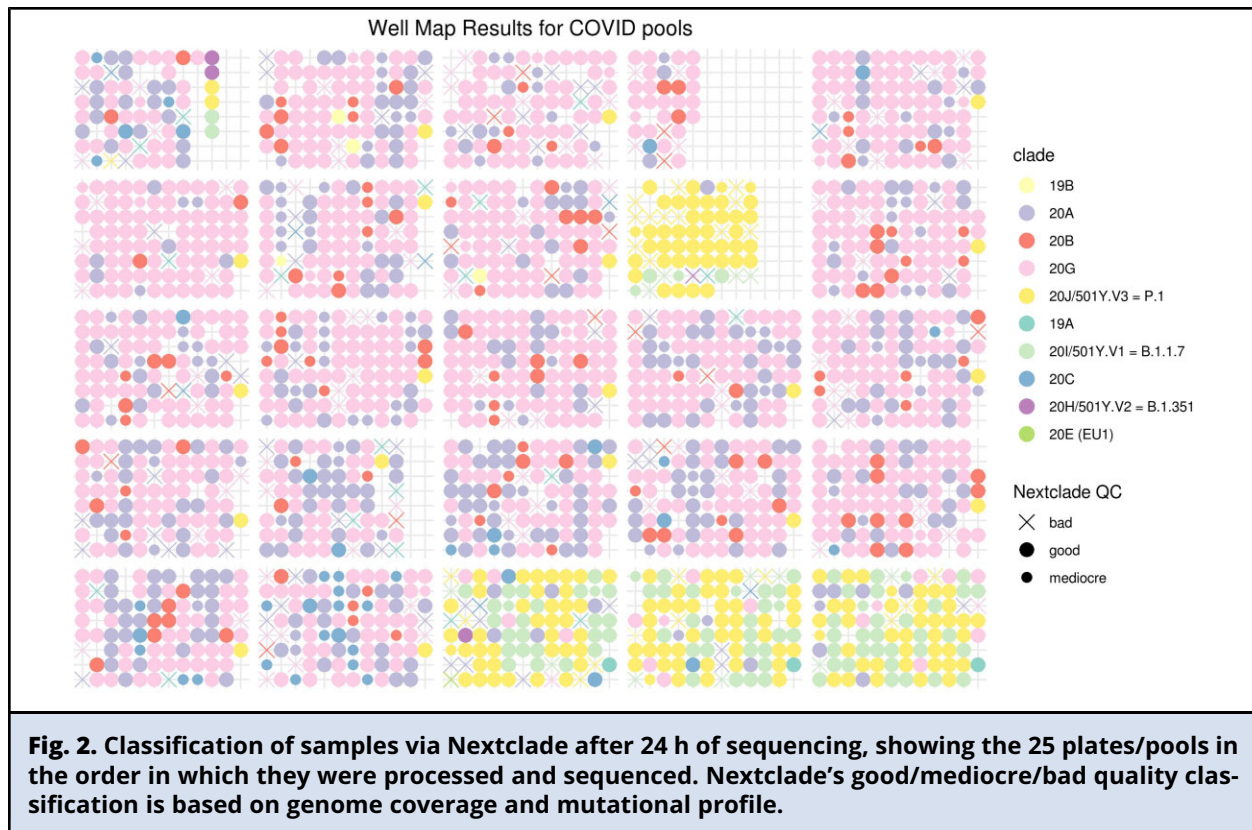
Nextclade, part of the ARTIC analysis pipeline (<https://clades.nextstrain.org/>), classifies samples into clades and assigns a quality score of Good, Mediocre, or Bad based on several factors, including the fraction of unambiguously sequence-covered genome and the number of mutations compared to currently known clades. In nanopore sequencing, some less well-covered regions can achieve sufficient read depth if sufficient depth of sequence read redundancy is obtained. Supplementary Table 1 shows the change in Nextclade's quality classification for reads obtained at 5 h (lower redundancy) and 24 h (higher redundancy) of sequencing. Improvements were generally observed with longer run times and higher redundancy of sequencing depth, but at the plate level (Supplementary Fig. 1), there was substantial variation, with many plates exhibiting sufficient read depths at 5 h, while other plates required the full 24 h, going from 90% Bad samples to 90% Good samples between the 2 time points.

Because an objective of this study was to investigate the reliability of the method on real patient

samples, plates of extracted positive samples were sent from the clinical lab for sequencing without further curation. Approximately 80 of these samples had less than the expected volume of 41 μL (9 μL of the 50 μL purification elution volume is required for 1 SNP PCR test). These generally correlated with high Cts, as these samples may have undergone multiple SNP PCR tests to get a result, and in some cases, this left less than the 8 μL required for the GSC protocol. Some of these samples did produce usable data, but most did not, and these may comprise the bulk of the 56 N/A samples in Supplementary Table 1. The individual fates of these samples were not exhaustively analyzed due to not initially recording these variable input volumes but once the issue came to light, it was straightforward to identify low input samples.

Sample clade and quality classifications for 24-h MinION runs are shown in Fig. 2 (see online Supplementary Materials for data). The grouping of clades, particularly the 20J/501Y.V3 clade in certain plates, reflects collection of samples from outbreaks suspected to be from a common source. Further epidemiological analysis of these data will be the subject of future work. Sample quality across the whole set correlated strongly with starting Ct values as shown in Fig. 3. Of the samples with a Ct ≥ 30 , 59% were classified as Mediocre or Bad, whereas this fraction dropped to 15% for Ct < 30 (1530 of 1802 of samples with Ct < 30 were classified as Good).

A median of 24 mutations were found in each sample, and a total of 1281 unique mutations, the total count of different base changes across all locations, were found across the 2179 samples. Variability in sequence coverage, shown in Fig. 4, occasionally resulted in underrepresented amplicons, although the genomes could still be assigned a clade using Nextclade. Supplementary Table 2 shows ranges of amplicon coverage post filtering, where the maximum number of reads used by the ARTIC analysis is 400 per amplicon. Of the 98 amplicons, 89 had 400 reads for all high-



quality samples, and 9 had fewer, with the lowest having a median coverage of 169 reads.

Nucleic Acids Yield vs Ct and Quality

Figure 3, A presents results from 2 representative 96-well plates that underwent mass quantification, illustrating the relationship in yield from ARTIC PCR between the sample's starting Ct and Nextclade quality classification. Lower Ct values resulted in higher PCR yield, suggesting these samples had more successful cDNA synthesis.

Our rationale for both sample input normalization and use of approximately 400 bp amplicons was to have the best possible balance in number of reads across each sample and across samples in each pool. This would, in turn, reduce the number of samples with insufficient reads for analysis, thereby reducing sequencing time for each pool. The elevated read counts in Ct > 30 samples,

visible in Fig. 5 where the read counts jump up on the right of the Ct=30 line, are from the second, post-ARTIC PCR normalization. This was added for the eighth plate that was run to ascertain whether increasing reads could improve quality scores, and it was performed on the 17% of the subsequent samples that had Ct > 30. Figure 3, B shows the results of 12 previously sequenced samples that were resequenced in a separate pool after this normalization. Read counts did increase, in proportion to Ct value, and 2 of the 12 showed improved quality scores. Surveying the first 5 plates processed prior to the second normalization showed 11 of 42 (26%) of samples with Ct > 30 receiving a Good quality score. After application of the second normalization, the subsequent pools had a Good quality rate of 129/304 (42%) for Ct > 30 samples (Fisher exact P value = 0.046)

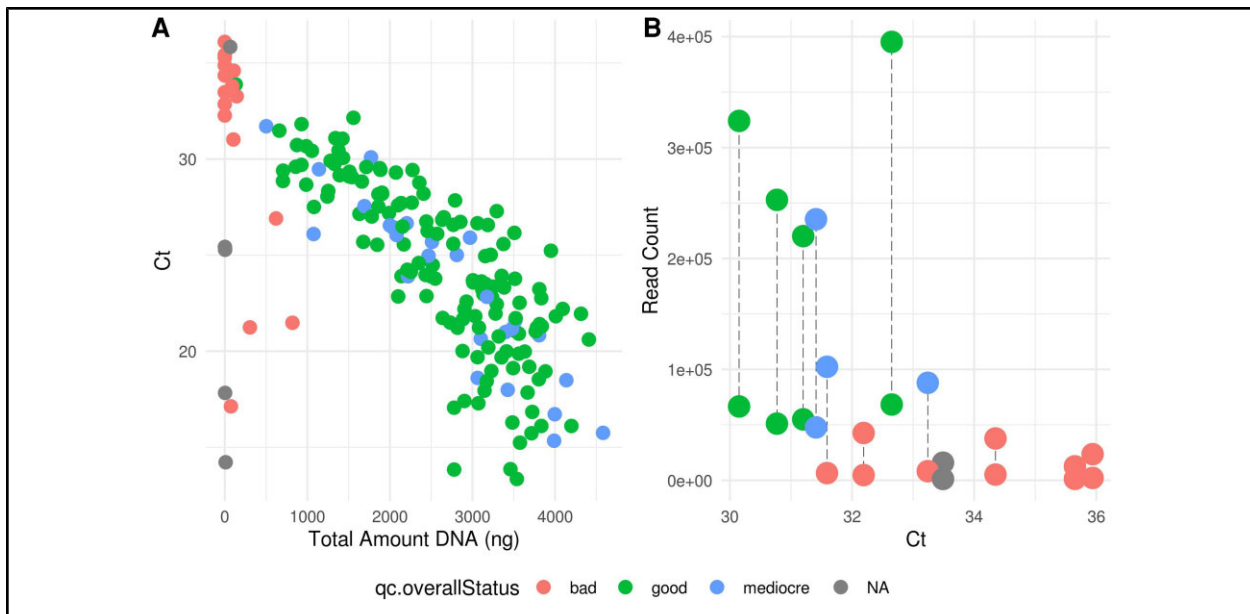
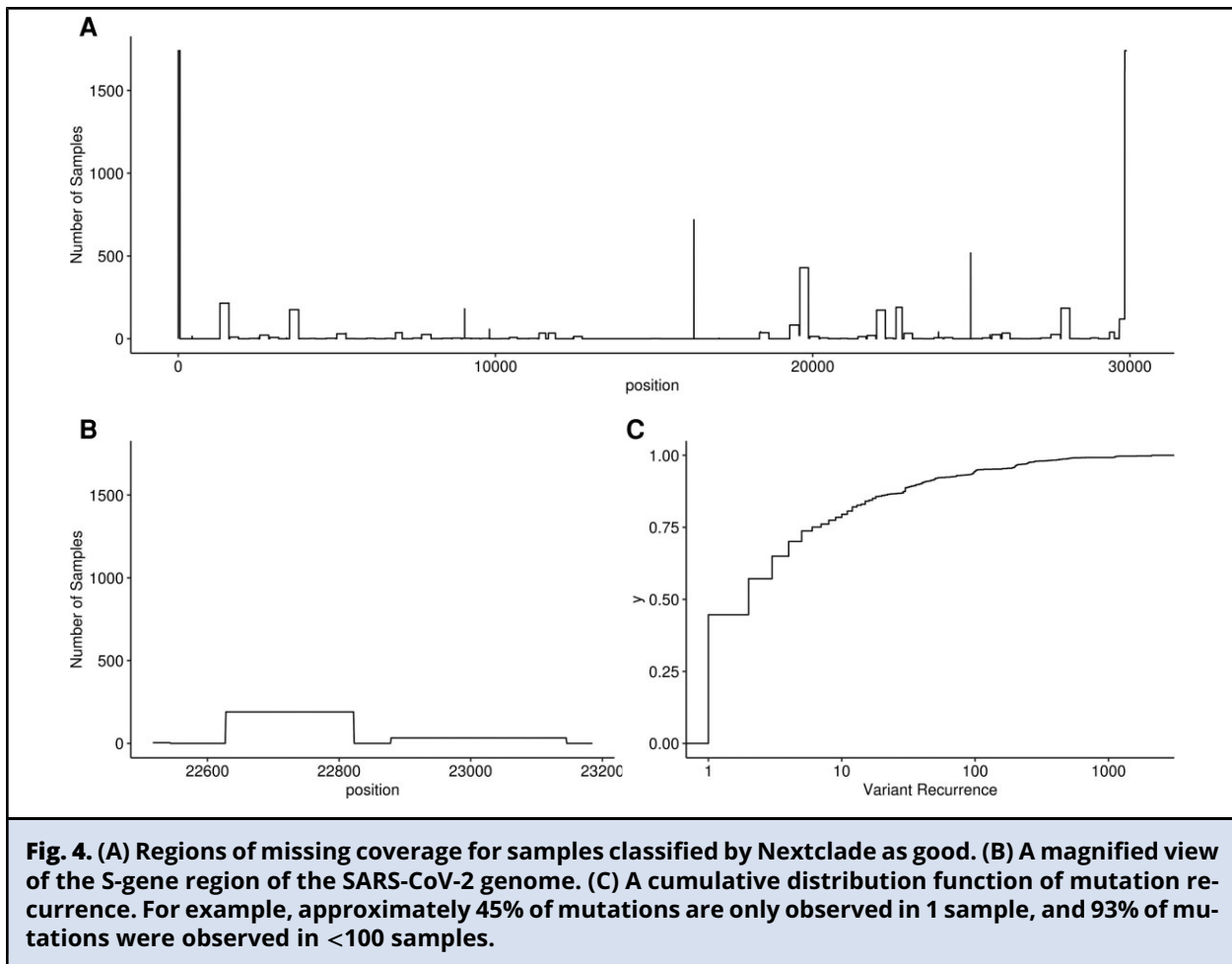


Fig. 3. (A) Quant-iT assay on 2 plates (188 samples) after ARTIC PCR showing the relationship between total DNA and Ct. The samples classified as NA with 0 ng yield arrived at the sequencing lab already exhausted from multiple samplings in the clinical lab. These are included as the pipeline is plate-based so all samples are handled together. (B) Nextclade quality score changes showing the effect of post-PCR concentration enhancement for 12 high Ct samples. All the samples consequently showed increased reads, and 2 of the 12 had improved quality scores when additional reads were accumulated.

To evaluate sequence read accumulation as a function of Ct, we serially diluted and sequenced triplicates of 3 samples known to be VOCs and that had Cts of 16 to 19, resulting in a range of concentrations from Cts = 16 to 50. These limit-of-detection samples, along with all patient samples we attempted to sequence, are shown in Fig. 5, with read count plotted against Ct value. Variations in read count are expected as the pools are different sizes and MinION flowcells have variable yields. The serially diluted samples performed comparatively well, showing acceptable results to approximately Ct = 37. The increase in reads from the second normalization for patient samples is visible in the higher read counts for samples just to the right of the Ct = 30 line. As Ct values increased, these patient samples exhibited steeper declines in quality compared to the diluted limit of detection samples.

Overall Classification Concordance vs SNP PCR

Table 1 shows the concordance between the WGS and SNP PCR assays for detecting VOCs. There were a total of 21 samples in which a VOC had been identified using SNP PCR but in which no VOC was detected using sequencing. Conversely, 6 additional sequenced samples identified a VOC that was not identified using SNP PCR. Twenty-four of the 27 discordant samples had Bad quality WGS scores and would not have been used for clinical decision-making in the event this was a clinical assay. When WGS results with Bad quality scores were excluded, the concordance rate with SNP PCR testing for VOC detection was 99.8% (1655/1658). Of the discordant samples with Bad quality WGS scores, 18 (75%) were part of the set with low eluate volume available for WGS at the time of this study. The remaining discordant



samples had Ct values in the range of 30.1 to 36.97 (median 34.1), consistent with low viral loads that may have contributed to sampling bias or poor reproducibility between SNP PCR testing and whole genome sequencing.

DISCUSSION

We have demonstrated a SARS-CoV-2 sequencing pipeline that can be scaled to two to three 96-well plates per day per 96-channel liquid handler. Starting from extracted RNA and a Ct value from a clinical lab, turnaround times were 12 h for sample preparation, up to 24 h for sequencing,

and 1 h for analysis. Fifty percent of samples sequenced could be successfully analyzed after 5 h of sequencing, so a pipeline could conceivably be operated to provide sub-24 h sequencing analysis turnaround, from previously extracted samples, particularly for samples with abundant RNA. Rampart (<https://artic.network/rampart>) for real-time read assignment might be deployed for a fully optimized process to classify samples as soon as possible, but to our knowledge, it currently works with up to 24 samples rather than 96 (20).

Our workflow includes 2 normalization steps that add less than an hour to the process but contributed to sequence reads being generally more evenly distributed across our pooled samples

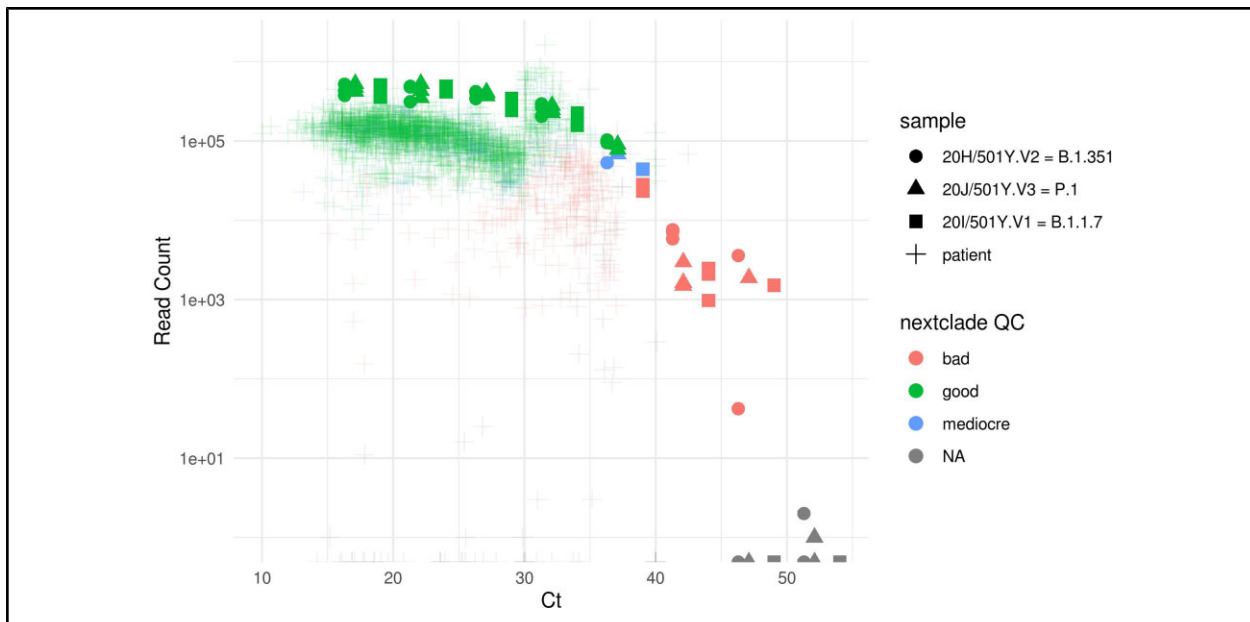


Fig. 5. Plot illustrating the relationship between Ct and read count for all samples for which sequencing was attempted. This includes plates 1 to 7 without and plates 8 to 25 with the second normalization. The improvement in quality from the second normalization can be seen in the bump in the number of good samples to the right of the Ct = 30 line. The limit-of-detection control samples representing 3 VOCs were diluted over a range from approximately Ct 16 to Ct 50. This shows that initially abundant samples can be resolved even when diluted close to Ct 40.

and improved results for low abundance samples. The ability to do these normalizations is critically dependent on the availability of a liquid handler (typically 8 channeled), with pipetting instructions generated from quant data by automated script. The possibility of error in manual pipetting at this scale is too high.

The MinION format fits well with our 96-sample workflow as the desired sequence yield for our pipeline is 100 000 (prefiltering) reads \times approximately 400 bp amplicons \times 96 samples approximately 4 Gb, which is on the same order as the MinION's (maximum theoretical) capacity of 16 Gb/24 h. It is also straightforward in a laboratory to run multiple MinIONs in parallel. Sample success correlated strongly with the Ct value of the input, as shown Fig. 5. Samples with Ct > 30 tended to yield fewer reads and lower quality sequence data. Addition of a second normalization after

ARTIC PCR, however, increased the fraction of samples of Ct > 30 with Good quality scores from 26% to 42%. The limit-of-detection experiment showed dilutions from samples with abundant RNA could be sequenced successfully even with dilutions down to Ct = 37. These observations are compatible with the notion that Ct values themselves are not the sole determinants of success, perhaps pointing to low quality or instability of the nucleic acid as another, untested factor in these experiments. Further optimization of cDNA synthesis may be of value in rescuing such samples, although this remains to be explored. Even so, 76% of the samples (1656/2179) of all starting Ct values were successfully sequenced and assigned a clade, using nucleic acids prepared in a clinical lab for PCR testing, suggesting clinical implementation of our pipeline can be fueled using routinely prepared PCR-grade nucleic acid.

Table 1. Concordance between WGS and SNP PCR in classifying the B.1.1.7, B.1.351, and P.1 VOCs.

All VOCs	Positive by SNP PCR	Negative by SNP PCR	WGS QC ^a score (Nextclade)
Detected by WGS	282	6	All
Not detected by WGS	21	1870	
Detected by WGS	230	2	Good ^b
Not detected by WGS	1	1425	
Detected by WGS	20	0	Mediocre
Not detected by WGS	0	210	
Detected by WGS	32	4	Bad
Not detected by WGS	20	235	

^aQuality control.
^bConcordance is 99.8% (1655/1658) for samples classified as good by Nextclade.

Limitations of the study include the use of Ct values to determine sample processing during normalization, where Ct values are known to vary depending on sample type, assay design, and reagent lots. Related to this, we also did not look at the dependence of sensitivity on whether the sample was from a saline gargle or nasopharyngeal swab. The Ct value ranges used in this study during normalization would need to be validated for laboratories utilizing other molecular platforms. Additionally, the efficiency of the workflow described in this study depends on the availability of deidentified nucleic acid extracts from positive SARS-CoV-2 samples, which would not be routinely available from clinical laboratories using closed-system assays (e.g., Roche cobas, Hologic Panther) without additional pipetting and preparation time. The nature of the samples in this study, where the clinical lab had rearrayed and reextracted positives and performed the SNP PCR test, made it straightforward to show that existing Ct values could be used to enable normalization and act as a quality control check against possible laboratory failures. This could be reproduced, however, in a properly equipped diagnostic laboratory with appropriate informatics and liquid handlers. It would be readily possible to set up “hit-picking”—namely, using independent pipetting channel robots to collect aliquots of diagnostic positive samples from the elution

plates from RNA extraction and achieve results comparable to those reported here.

Another limitation in this analysis is that we used the Nextclade quality score as a metric of sequencing success, because we determined that it best reflects what is of immediate value to the clinical laboratory. Nextclade does reflect typical genome quality metrics in that in that the breadth of unambiguous genome coverage is a key factor in the quality score. However, to assign clades, Nextclade also requires that the number of mutations does not exceed a certain threshold and deducts from the quality score if the mutations count is higher. The quality assessment could therefore be confounded not by sequencing depth but by properties of the virus itself and the state of local epidemiology. Further analysis of these data to understand whether highly mutated samples are biologically significant will be undertaken. We also note that other tools for phylogenetic classification may be readily employed, but Nextclade was sufficient for the purposes of this comparison of WGS data to a SNP PCR assay.

A notable feature of these data is the number of mutations within and across the samples. A median of 24 mutations were found in each sample, and a total of 1281 different mutations were revealed across all samples. As shown in Fig. 4, these are distributed across the genome so have the potential to cause challenges for PCR primer design.

Figure 4 shows regions of reduced coverage including a focus on the S-gene, which is of particular interest for vaccine or therapeutic research. About 10% of samples have reduced coverage in this region. Samples with a small number of regions with reduced sequence coverage are still classifiable by Nextclade, but in cases where there is particular interest in specific regions, extra amplicons or repositioned amplicons may be required.

This study demonstrates an optimized workflow for high-throughput WGS of SARS-CoV-2-positive samples. The turnaround time enables clinical laboratories and public health teams to promptly investigate

potential outbreaks, cases of COVID-19 reinfection, or postvaccination breakthroughs and to monitor for new or emerging VOCs. Strategies for optimizing the sensitivity of WGS, particularly for samples with low viral loads, have also been described and have the potential to be implemented in clinical laboratories with access to comparable resources.

SUPPLEMENTAL MATERIAL

Supplemental material is available at *The Journal of Applied Laboratory Medicine* online.

Nonstandard Abbreviations: VOCs, variants of concern; SNP, single nucleotide polymorphism; WGS, whole-genome sequencing; Ct, threshold cycle; GSC, Michael Smith Genome Sciences Centre.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.

Authors' Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest: **Employment or Leadership:** None declared. **Consultant or Advisory Role:** None declared. **Stock Ownership:** S.D. Chorlton, PathoGene Inc., BugSeq Bioinformatics Inc. **Honoraria:** None declared. **Research Funding:** Researchers from the St. Paul's Hospital Department of Pathology and Laboratory Medicine have received institutional support from the Public Health Agency of Canada's COVID-19 Immunity Task Force Hot Spots Competitive Grant, 2021-HQ-000120 (M.G. Romney, N. Matic, G. Ritchie, C.F. Lowe, M. Young), Genome BC COVID-19 rapid response grant (M.G. Romney, N. Matic, G. Ritchie, C.F. Lowe, M. Young), Genome BC Rapid SARS-CoV-2 Vaccine Research Initiative in BC (M.G. Romney and C.F. Lowe), Providence Health Care Research Institute (N. Matic), and Canada Foundation for Innovation Exceptional Opportunities Fund-COVID-19 (C.F. Lowe). M.A. Marra, S. Jones, D.E. Smailus, R. Corbett, S. Pleasance, S. Chahal, R.E. Carlsen, R.J.N. Coope, P.K. Pandoh, J. Asano, A. Ally, Y. Zhao, and Canada's Michael Smith Genome Sciences Centre, have also received institutional support from Genome BC (COV-088), Genome Canada (262SEQ), and the Canada Foundation for Innovation and BC Knowledge Development Fund (#40991). S.D. Chorlton has received funding from the Open Philanthropy Project. **Expert Testimony:** None declared. **Patents:** None declared.

Other Remuneration: D. Holmes has received travel reimbursements from AACC and MSACL.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, preparation of manuscript, or final approval of manuscript.

Acknowledgments: We thank John Tyson for general guidance, Kirstin Brown for help with this manuscript, Magdalena Glass and Robyn Roscoe for administration of the project, and Young Cheng for data upload. M.A. Marra and S.J.M. Jones are grateful for the support of the Canada Research Chairs Program.

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