High Throughput Nanopore Sequencing of SARS-CoV-2 Viral Genomes from Patient Samples

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ABSTRACT

In late 2019, a novel coronavirus began spreading in Wuhan, China, causing a potentially lethal respiratory viral infection. By early 2020, the novel coronavirus, called SARS-CoV-2, had spread globally, causing the COVID-19 pandemic. The infection and mutation rates of SARS-CoV-2 make it amenable to tracking movement and evolution by viral genome sequencing. Efforts to develop effective public health policies, therapeutics, or vaccines to treat or prevent COVID-19 are also expected to benefit from tracking mutations of the SARS-CoV-2 virus. Here we describe a set of comprehensive working protocols, from viral RNA extraction to analysis using online visualization tools, for high throughput sequencing of SARS-CoV-2 viral genomes using a MinION instrument. This set of protocols should serve as a reliable 'how-to' reference for generating high-quality SARS-CoV-2 genome sequences with ARTIC primer sets and next-generation nanopore sequencing technology. In addition, many of the preparation, quality control, and analysis steps will be generally applicable to other sequencing platforms.

INTRODUCTION

COVID-19, an ongoing global pandemic, has taken the lives of over 400,000 people in the United States (U.S) and approximately 2 million people worldwide in its first year (https://www.worldometers.info/coronavirus/). Effective pandemic response and infection containment have remained challenging (*1*). The ability of viruses to evolve quickly further underscores the need for constant surveillance and monitoring, especially at the genetic level for tracking emergence of novel variants of concern (*2*). Viral genome sequencing can help address these challenges and shed light on the identity and evolution of genetic variants (*3*). Additionally, viral genome sequencing can delineate conserved and mutable regions which would provide valuable insights in the development of effective treatments and vaccines (*4-6*).

Several different approaches exist to sequence SARS-CoV-2 genomes, such as metagenomic sequencing, targeted enrichment sequencing and PCR-amplification based methods (*6*). Metagenomic and target enrichment sequencing are prohibitively expensive to generate large-scale complete genomes and are associated with incomplete coverage and depth (*7*). A solution to address these limitations is a PCR-based amplification which enriches and amplifies the target of interest at the same time (*8*). By utilizing a tiling amplicon scheme with multiplex PCR, it is possible to generate enough coverage and depth to produce complete genome sequences of SARS-CoV-2 in a cost-effective manner (*9*). This approach can even reconstruct complete genomes of samples that have partially degraded viral RNA genomes or low viral load.

Our lab adopted and modified protocols initially developed by the ARTIC Network (*9*) to sequence SARS-CoV-2 viral genomes using the MinION nanopore sequencing platform from Oxford Nanopore Technologies (ONT). We present here an entire validated workflow, including a how-to guide for every step from viral RNA extraction to data visualization. These protocols have been validated and benchmarked through the successful processing of over 1000 samples and sequencing of over 600 high-quality SARS-CoV-2 genomes from the U.S. state of Illinois (*10*), which are available on the GISAID database and publicly viewable on Nextstrain (https://nextstrain.org/groups/illinois-gagnon-public/ncov/gagnon).

RESULTS and DISCUSSION

The entire workflow to generate SARS-CoV-2 genomes (**Figure 1**) is performed in a 96-well plate format. All detailed working protocols are provided in the supplemental material and are referenced throughout. The workflow begins with RNA extraction from viral transport media (VTM) containing patient-derived nasopharyngeal (NP) swabs or other sample sources. Extracted RNA is converted to complementary DNA (cDNA), which is used for approximation of viral load by quantitative PCR (qPCR). Following qPCR, multiplex PCR with two pools of ARTIC primers is performed to specifically amplify the SARS-CoV-2 viral genome. PCR products, or amplicons, can then be optionally visualized by gel electrophoresis. The two separate ARTIC PCR product pools for each sample are pooled and the amplicons isolated through a clean-up step with magnetic Solid Phase Reversible Immobilization (SPRI) beads. The isolated PCR amplicons can be optionally quantified by Qubit fluorescence prior to sequencing library preparation, which entails

an amplicon end-prep step, 96-well sample barcoding, sample pooling and clean-up, and sequencing adapter ligation and clean-up. Sample library is loaded onto a MinION nanopore sequencer (ONT) and sequenced. Analysis involves demultiplexing of barcodes, bioinformatic filtering for amplicon size, and alignment to the SARS-CoV-2 genome. Finally, genomes with sufficient coverage (usually >20X) can be visualized by phylogenetic analyses or clade and variant calling performed using web-based tools like Nextstrain (*11*). An overview of workflow steps is provided and detailed working protocols for each step are provided in supplemental materials.

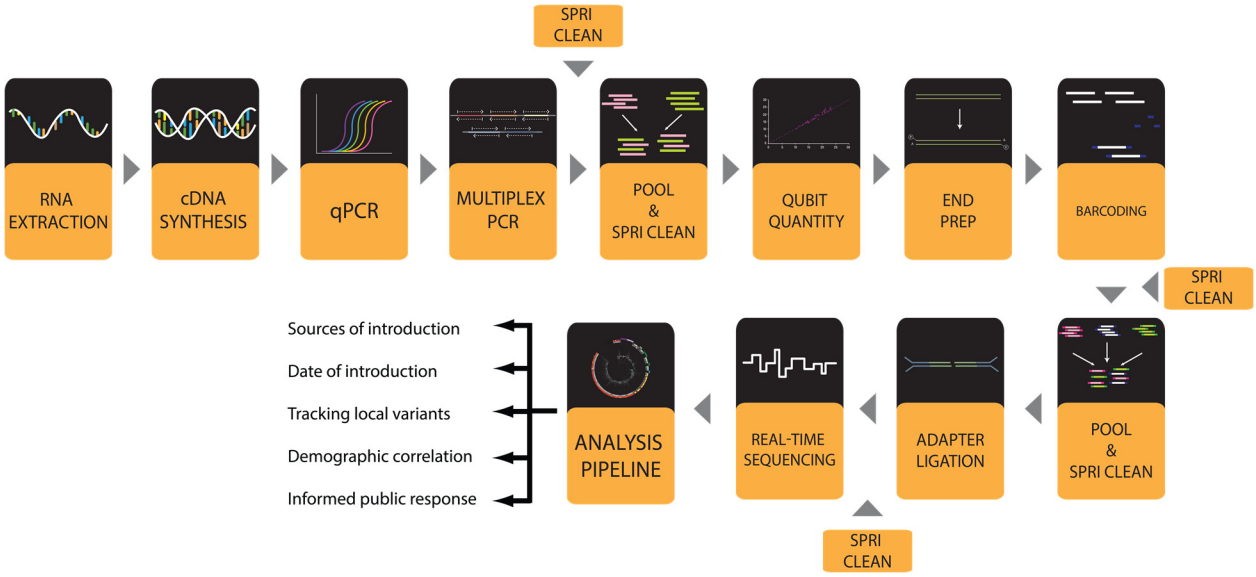

Figure 1. Workflow for SARS-CoV-2 viral genome sequencing with a MinION instrument.

Extraction of Viral RNA Genomes

The viral RNA genome is typically extracted from various VTM solutions of clinical COVID-19 samples. Samples should be stored at -80 °C, thawed on ice, and kept cold to maintain viral genome integrity. To increase safety and compliance, our laboratory only receives samples from clinics or public health laboratories that have first been treated with an inactivation solution (*12*) that is compatible with our RNA extraction protocol (see *Working Protocol S1: Viral RNA Extraction*). The RNA extraction procedure is carried out in a BSL-2 class biosafety cabinet. A magnetic bead-based commercial kit is used in 96-well format following the manufacturer's recommended protocol with some minor modifications. These include the use of home-made lysis (inactivation) solution (see Working Protocol S1) in place of, or mixed 1:1 with, the manufacturer's lysis solution. Additionally, elution is performed in a buffer containing EDTA and an RNase inhibitor to help preserve genome integrity. This protocol requires a 96-well magnetic stand. Once RNA extraction is complete, RNA samples are kept on ice and immediately converted to cDNA. Extracted RNA is stored in heat-sealed plates at -80°C.

During the RNA extraction it is always recommended to include a negative control containing phosphate buffered saline (PBS) or clean VTM in place of a patient sample in at least one well of each 96-well plate. Additionally, a positive commercially available sample can be used to validate the workflow at early stages if needed. These controls can help identify general cross-contamination issues and ensure efficient extraction. When setting up the workflow for the first time, extracted RNA can be quantified and resolved on denaturing agarose gels to evaluate efficiency and RNA integrity. We do not provide protocols for these optional steps.

Conversion of Viral RNA to Complementary DNA (cDNA)

Following extraction, RNA is converted to cDNA as soon as possible, preferably without a freeze-thaw cycle, to prevent possible RNA degradation. We have typically used the affordable ABI Hi-Capacity cDNA Synthesis Kit in 96-well format and, in addition to the manufacturer's recommended protocol, included a small addition of $MgCl_2$ (see *Working Protocol S2: cDNA Synthesis*). Other reverse transcription kits have not been systematically tested but are expected to be suitable. A constant volume of RNA, half the total cDNA reaction volume, is used as template in all cDNA reactions. Although each sample will have varying concentrations of viral RNA, we have found that this is sufficient to recover genomes from samples with sufficient viral load and does not result in any significant reaction inhibition. Our protocol includes a moderate heating and snap-cooling step in the presence of random primers to improve primer binding (see Working Protocol S2*)*. After synthesis, cDNA should be stored temporarily at 4°C while awaiting qPCR and multiplex ARTIC PCR. For long-term stability, store at -20°C or -80°C after heat-sealing.

Quantitative PCR (qPCR) to Approximate Viral Load

To quantify approximate viral load in each sample, qPCR is performed using cDNA as template. To reduce costs and maintain consistency, we typically use only one primer-probe set, commercially available from Integrated DNA Technologies (IDT). This primer-probe set, N2, detects the nucleocapsid (N) gene and is identical to one of the primer-probe sets recommended early in the pandemic for RT-qPCR-based patient diagnosis of probable COVID-19 infection (11). We typically use the PrimeTime Gene Expression Master Mix (IDT) and manufacturer's recommended protocol for qPCR reactions (see *Working Protocol S3: qPCR*). To help standardize quantitation across plates, the same threshold value is set for each run to consistently call cycle threshold (C_t) values (**Figure 2A**). In our experience, the C_t values determined for any given sample are higher than those usually reported (when such data is available) by the facilities that provided patient samples. We suspect this reduction in detectable viral genome is at least partially due to freeze-thaw cycles and handling of the samples between initial testing and later sequencing. Our recommendation is to avoid freeze-thaw of purified RNA prior to cDNA synthesis to help reduce further loss of viral genome integrity.

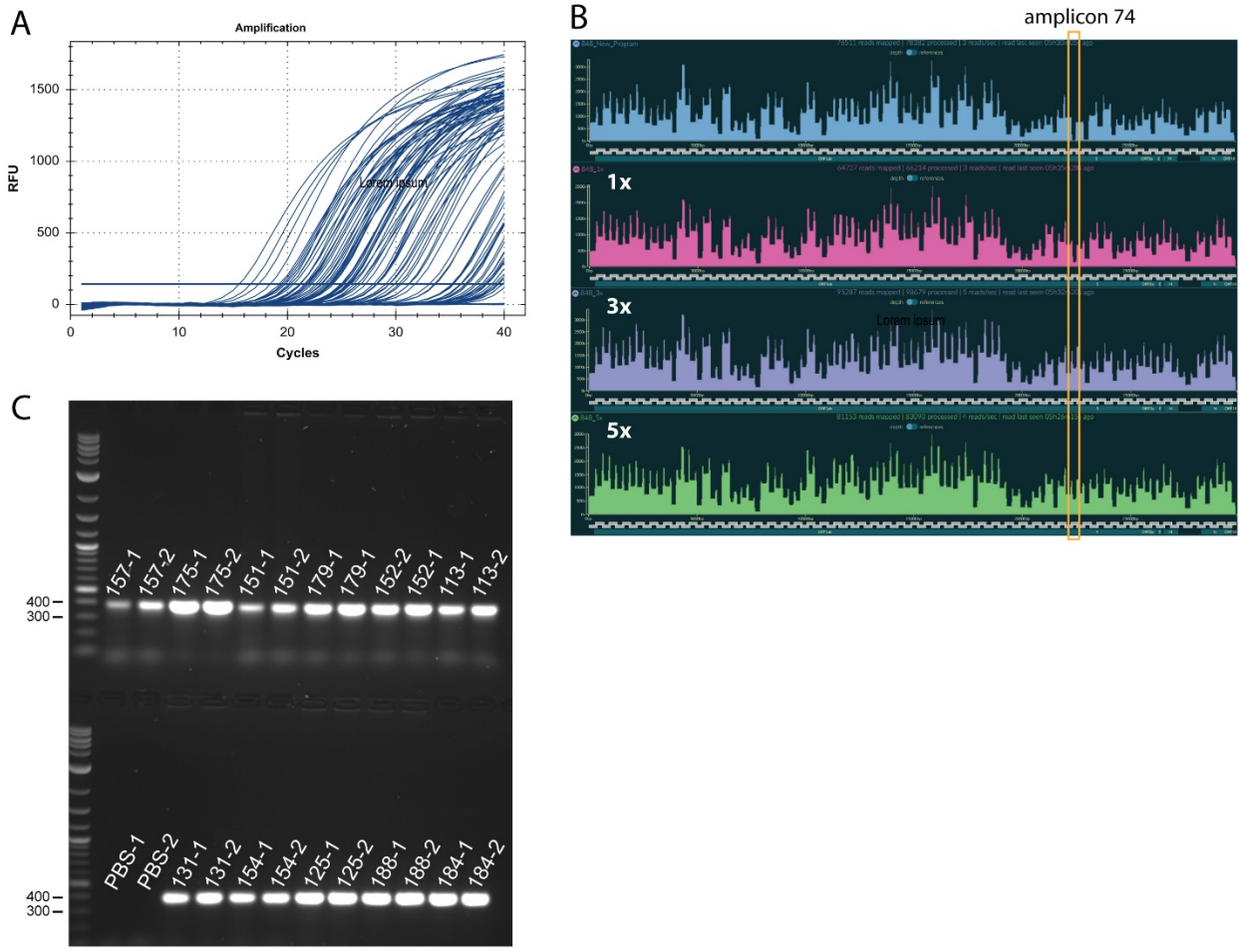

Figure 2. Representative quantitative and ARTIC PCR results. (A) Amplification curves from SARS-CoV-2 qPCR assay and IDT 2019-nCoV CDC approved N2 primers and probes. Baseline adjustment was manually set to 200. **(B)** Coverage plot by Rampart with amplicon 74 dropout using ARTIC V3 Primers (top panel/blue plot). Spike-in plots for 1x (15 nM) (second panel/pink plot), 3x (45 nM) (third panel/purple plot) and 5x (75 nM) (bottom panel/green plot) 74_Right and 74_Left are shown for the same sample (C_t = 20.68). These experiments were conducted at the same time using otherwise identical reagents and protocols. Amplicon 74 region is indicated with an orange box. (**C**) Representative agarose gel electrophoresis of ~400 kb PCR products amplified using ARTIC nCoV V3 Primers (IDT). Reaction pool 1 (labels with suffixes -1) and pool 2 (labels with suffixes -2) were run side by side with 5 uL of sample on the gel. PBS (no template) extraction controls for pool 1 and 2 show no cross-contamination.

Multiplex ARTIC PCR to Amplify Viral Genome Sequences

Once qPCR is performed, viral genome cDNA is amplified in 96-well format to reduce contaminating patient genomic and transcriptomic sequences and to increase the copy number of viral genomes. We use two pools of primers designed by the ARTIC Network (*8, 9*), which are commercially available from IDT as the ARTIC v.3 primer set. These require two pools of PCR reactions for every sample, with pool 1 having 110 primer sets and pool 2 having 108 primer sets. These pooled multiplex PCR reactions create overlapping amplicons that reduce interference

during PCR and provide better coverage during sequencing when recombined later. We also use the Q5 High-Fidelity DNA Polymerase from New England Biolabs (NEB) for PCR reactions (see *Working Protocol S4: Multiplex ARTIC PCR*).

We have optimized the PCR cycling conditions to generate higher and more consistent coverage from higher C_t samples than can typically be achieved with previously described ARTIC PCR protocols (*8, 9*). Despite optimized cycling conditions, one amplicon is universally known for dropping out: amplicon 74 in primer pool 2. To address this issue, we spiked-in 1-, 3-, or 5-fold more of the primer set for amplicon 74 (which was purchased separately from IDT) and observed improved coverage at 3- and 5-fold molar excess (**Figure 2B**). None of these conditions appeared to reduce coverage of other amplicons from separate samples tested. In fact, coverage of amplicon 76, also often low, seemed to improve as well. Thus, we propose using 3-fold molar excess (60 nM final) of amplicon 74 primers over the amount typically present. The details of our optimized ARTIC PCR protocol are found in *Working Protocol S4*.

In principle, quantitation of viral genomes by qPCR should enable precise addition of specific amounts of cDNA for enrichment and amplification in multiplexed ARTIC PCR. However, in practice we have found that this does not improve quality or yield and dramatically increases hands-on time and PCR preparation. We have determined that a single defined amount of cDNA volume per ARTIC PCR reaction, up to ¼ of the reaction volume, will provide full genomes up to a C_t value of 35 in our hands. Thus, we use qPCR to triage samples that are unlikely to yield full genomes. Samples with a C_t value over 35 using our protocols are usually not processed further. ARTIC PCR reactions can be stored at -20°C or -80°C, but are usually kept at 4°C while awaiting clean-up, quantification, and end prep reactions described below.

Pooling and Bead-Based Enrichment of ARTIC PCR Products

Prior to preparation of the sequencing library, pool 1 and pool 2 ARTIC PCR reactions are combined for every sample and the ~400 base-pair (bp) amplicons are purified from contaminating PCR reactants using SPRI AMPure XP beads (Beckman-Coulter). When first performing this workflow, it is recommended that 1/5 volume of each pool's PCR reaction be separately resolved on an agarose gel to visualize amplicon quantity and quality (**Figure 2C**). The band intensity can be compared to C_t values from qPCR to determine the efficiency of ARTIC PCR and adjust cutoff values for processing. The remaining pool 1 and pool 2 for every sample are combined and then purified with magnetic SPRI beads in a 96-well format following the manufacturer's recommended protocol (see *Working Protocol S5: SPRI Clean-up of ARTIC PCR*). This protocol requires a 96-well magnetic stand. After this step, DNA can be frozen at -20°C or -80°C but is most often kept at 4°C temporarily until Qubit quantification and end prep reactions can be performed.

Fluorescence-Based Qubit Quantification of Enriched ARTIC PCR Products

The purified PCR pools are quantified using a Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit following the manufacturer's recommended protocol (see *Working Protocol S6: Qubit Quantification*). This method is specific to double-stranded DNA and is suitable for detection of low levels of DNA. When first performing this workflow, we recommend quantifying all samples

with Qubit to determine the level of recovery with respect to C_t values from qPCR. However, to reduce time and cost, we routinely only quantitate a representative group of 10-20 samples to ensure expected results are being obtained. We have found that our multiplex ARTIC PCR protocol provides very similar recovery, ~100 ng/µL, after SPRI clean-up for samples with C_t values up to 35.

Preparation of DNA Ends for Library Construction

Following the approximation of pooled PCR product concentration by Qubit, 60 ng of PCR amplicons from SPRI clean-up are added to reactions designed to prepare the ends of the amplicons for ligation of oligonucleotide barcodes. These are referred to as end prep reactions. We use the Ultra II End-Prep kit from NEB following the manufacturer's recommended protocol with minor modifications (see *Working Protocol S7: Library End Prep*). If the concentration of the product from Qubit is over 100 ng/µL, we use this as the approximate concentration for use in reactions. Samples are kept at 4°C while awaiting barcoding. It is not recommended to freeze samples at any of the remaining steps of the workflow but to store temporarily at 4°C, typically no longer than overnight.

DNA Library Sample Barcoding and Adapter Ligation

To sequence a full 96-well plate of samples, each sample must be uniquely barcoded. To achieve this, we use the Native Barcoding Expansion 96 (EXP-NBD196) kit from ONT. We use the NEBNext Ultra II Ligation Master Mix from NEB to ligate barcodes to the DNA library (see *Working Protocol S8: Sample Barcoding*). After individual barcoding reactions are performed in a 96-well plate, all samples are pooled and cleaned-up with SPRI beads similar to that described above. This step requires a micro centrifuge tube magnetic stand. Barcoded samples are stored temporarily at 4°C while awaiting adapter ligation.

After barcoding and clean-up, sequencing adapters are then ligated to each amplicon end for the pooled libraries in a single reaction. This reaction sequentially uses Adapter Mix II (AMII) from ONT, NEBNext Quick Ligation Reaction Buffer, and Quick T4 DNA Ligase (see *Working Protocol S9: Adapter Ligation*). The final reaction is then cleaned up using SPRI beads and eluted. After elution, the final library is stored at 4°C until Qubit quantification and preparation for loading onto a nanopore sequencing instrument, such as the MinION from ONT.

Loading and Running the MinION

For optimal performance on a MinION instrument, 20 ng of adapter ligated library should be loaded onto the flow cell. Overloading the flow cell results in lower throughput. Therefore, a final Qubit quantification is performed similar to that described above. The DNA sequencing library is then prepared by (ONT) following recommended protocols (see *Working Protocol S10: MinION Loading and Running*). The flow cell is then primed using the Flow Cell Priming Kit (ONT) (**Figure 3A**). Priming and loading is described in detail in *Working Protocol S10*. To start the sequencing run ONT's MinKNOW software is used which can be downloaded and installed from the ONT's website (https://community.nanoporetech.com/downloads). Once the sequencing run

is complete, the flow cell should be washed and stored appropriately following *Working Protocol S11: Flow Cell Wash and Store*.

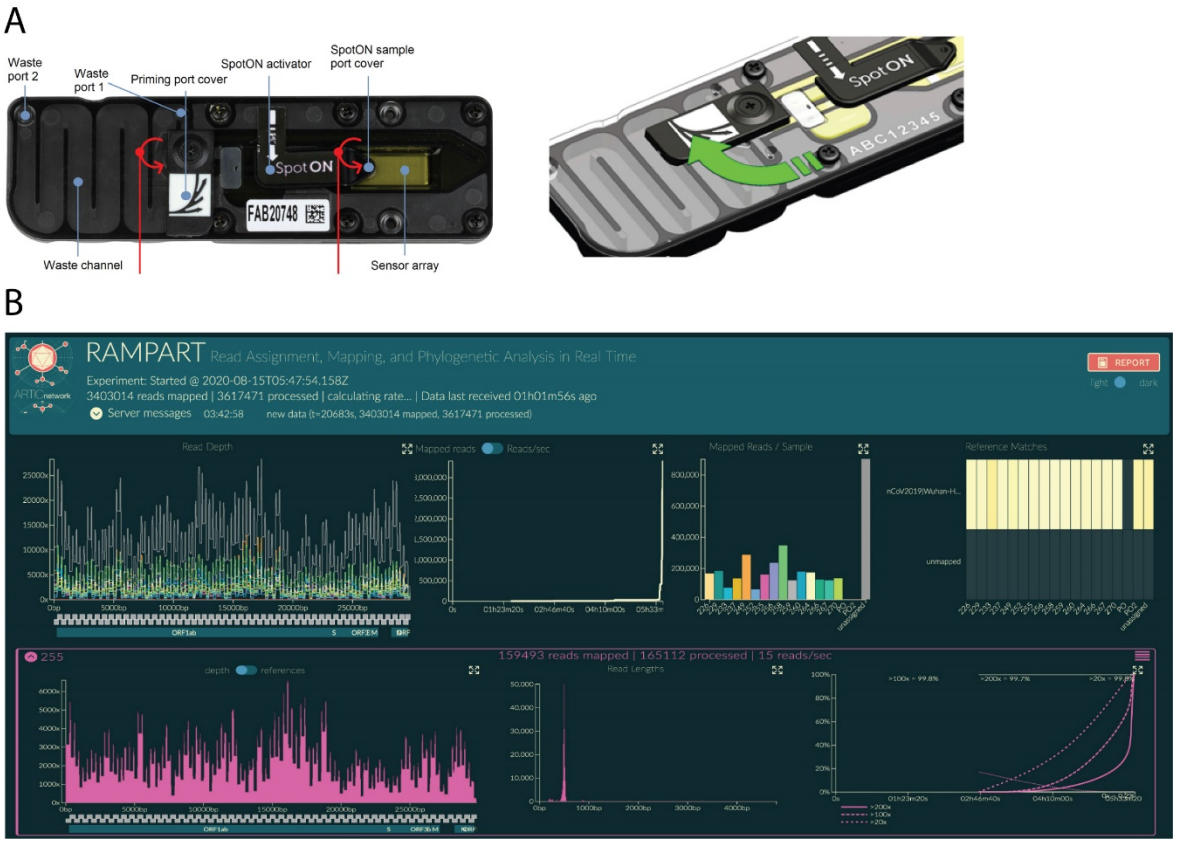

Figure 3. Loading and running the MinION for SARS-CoV-2 genome sequencing. (**A**) Image of the R9.4.1 flow cell displaying the different components of the device (left panel). An illustration demonstrating the priming port opening to primer port cover turning clockwise to expose the opening of the priming port (right panel). (**B**) Example of RAMPART display. (Top from left to right): 1) Top right shows coverage across the genomes for all samples. 2) Number of mapped reads through time. 3) Number of reads mapped for each sample. 4) Heatmap showing reads mapped to nCoV2019| Wuhan-Hu-1 (accession MN908947). (Bottom from left to right) 5) The coverage across the genome for an individual sample. 6) Read length distribution for an individual barcoded sample showing the expected peak of ~400 bp. 7) Percent >20x. >100x and >200x over time.

Sequencing, Real-Time Visualization, and Data Analysis

Recommendations for desktop requirements and software generally follow the recommendations from ONT. These include a minimal SSD storage of 1 TB, 16 GB of RAM and Intel i7 or Xeon with 4+ cores (https://nanoporetech.com/community/lab-it-requirements). We also recommend using a graphics card from NVIDIA GPU to increase basecalling speed if high accuracy basecalling via Guppy is used.

We recommend implementing Rampart (**Figure 3B**) in order to visualize coverage for each barcoded sample and gather qualitative insight during sequencing runs

(https://artic.network/rampart). Rampart can be run concurrently with MinKNOW and be used to determine whether sufficient coverage and depth has been achieved. Since real-time visualization requires basecalling in near real-time, it is recommended that MinKNOW is set to fast basecalling to quickly generate FASTQ files and gather sequencing insight using Rampart in real time.

A general SARS-CoV-2 targeted amplification bioinformatics pipeline will include basecalling, demultiplexing, trimming, alignment, variant identification and consensus building. In order to reduce false variant calls, a fully validated pipeline should be used (see *Working Protocol S12: Sequencing Data Analysis*). We have implemented the ARTIC bioinformatics pipeline due to its wide use and ONT support (https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.html). Once fast5 files are generated it is highly recommended that high accuracy basecalling is used in order to generate more accurate results of single nucleotide polymorphisms (SNPs). High accuracy basecalling results in better single molecule accuracy but is considerably slower. The basecalled data is then demultiplexed using Guppy with strict parameters to ensure that both barcodes are at both ends of the read. This increases the number of reads binned into the "unclassified" reads but reduces barcode mismatches due to the presence of in silico chimera reads. To remove additional chimeras length filtering is performed using ARTIC guppyplex and reads between 400-700 bp are retained and inputed into the ARTIC bioinformatics pipeline. There are two workflows developed by ARTIC network to generate consensus sequences and identify variants. Nanopolish uses fast5 signal data while the other, Medaka, does not. In our experience both workflows tend to give consistent results with high variant and SNV detection accuracy (*13*). We have preferentially used Medaka due to its speed and GPU compatibility. By default, the pipeline minimum coverage is set to 20x to call sites. Otherwise, a masking model applies ambiguous bases (Ns) to sites with lower than the minimum required coverage. After the pipeline has run, a consensus genome sequence is generated which can be concatenated with other consensus genome sequences and further analyzed.

If variants are called from high C_t samples or partial genomes, the genome sequence should be carefully evaluated and visualized in a graphical viewer. Very few copies of starting genomes may lead to artefactual error and call false variants. To reduce artefactual error, it is also recommended to use high fidelity enzymes during cDNA synthesis and PCR. If contamination is observed in the negative control, mutation sites should only be called if depth greatly exceeds the number of SARS-CoV-2 reads observed in the negative control. To assess amplicon dropouts, we recommend using CoV-GLUE (*14*) which identifies mismatches in sequencing primers/probe sets.

Phylogenetic Analysis, Variant Calling, and Database Deposition of SARS-CoV-2 Genomes

To process sequenced samples, they must first be properly prepared and formatted (see *Working Protocol S13: Visualizing data in Nextclade and Nextstrain*). A convenient way to observe your sequences and determine the quality is to use the NextClade website (https://clades.nextstrain.org/). Dragging and dropping your sequence FASTA file onto the site will initiate an evaluation process that returns the number of mutations compared to the source SARS-CoV-2 strain, as well as the number of ambiguous nucleotides (Ns) and what 'clade' each sequence corresponds with, which are the phylogenetic categories the Nextstrain pipeline separates sequences into. This application also allows searching of the aligned sequences for specific

nucleotide or amino-acid mutations (the 'filter' button), which is useful for quickly identifying unusual sequences for more careful analysis.

If planning to submit sequences to a global database, such as NCBI or the GISAID Initiative, certain requirements must be met. GISAID requires metadata for each sequence, including collection date and location, the passage history of the sample, and the sequencing technology used. To submit samples in a batch format, more than one at a time, they must be provided with a FASTA file containing all assembled sequences, along with a comma-separated values (.csv) file containing all the metadata. Upon registering and requesting permission for batch upload (https://www.epicov.org/epi3/frontend), a template metadata file is provided explaining the correct formatting. The upload process will do a quality-control pass, alerting users to missing metadata, missing FASTA sequences, or other missing informaiton. Additionally, GISAID is currently checking to ensure that any frameshift mutations present in submitted sequences are correct, and not simply the result of sequencing errors. Submitted sequences with frameshifts must be accompanied with a confirmation email or they will be rejected.

NCBI (https://submit.ncbi.nlm.nih.gov/sarscov2/) has similar requirements. A date and location of collection must be provided. Submitted samples will be processed to trim ambiguous ends, and low-quality sequences (those with too many ambiguous nucleotides, or unacceptably short or long sequences) will be removed.

Finally, if the sequences are going to be placed into a phylogenetic context using the Nextstrain pipeline (https://nextstrain.org/sars-cov-2/), they must also meet requirements there as well. Nextstrain rejects any sample that lacks metadata (ie. time and location of collection, length of sequence) or is too ambiguous. If more than 10% of the sequence is comprised of ambiguous nucleotides, the software will automatically discard it. Nextstrain also estimates the mutation rate of SARS-CoV-2. If samples contain an unlikely number of mutations based on their collection date, these samples will also be excluded from the analysis.

The Nextstrain software can be installed by following their SARS-CoV-2 analysis tutorial (https://nextstrain.github.io/ncov/). Briefly, after the software is installed, all sequence FASTA files must be added in bulk to a sequences.fasta file in the Nextstrain data directory, and the accompanying metadata for those sequences must be added to the metadata.csv file in the same location. Then a profile must be built that directs the software to focus on those sequences. This can be done using the provided example profiles and is tutorialized in the same link above (see *Working Protocol S13*). Once this is completed, running the analysis is as straight-forward as running a single command. It generates a file which contains a phylogenetic tree of the sequences, a map of collection locations, and a graph of the diversity of mutations in the sampled sequences. Results are in the form of a .json file, created in the auspice subdirectory, which can be viewed by dragging-and-dropping the file onto the Auspice website (https://auspice.us/). More permanent hosting for these analyses can be setup via the Nextstrain website (https://docs.nextstrain.org/en/latest/guides/share/nextstrain-groups.html). An example of our laboratory's current Nextstrain build with genome sequences from Illinois is shown in **Figure 4**.

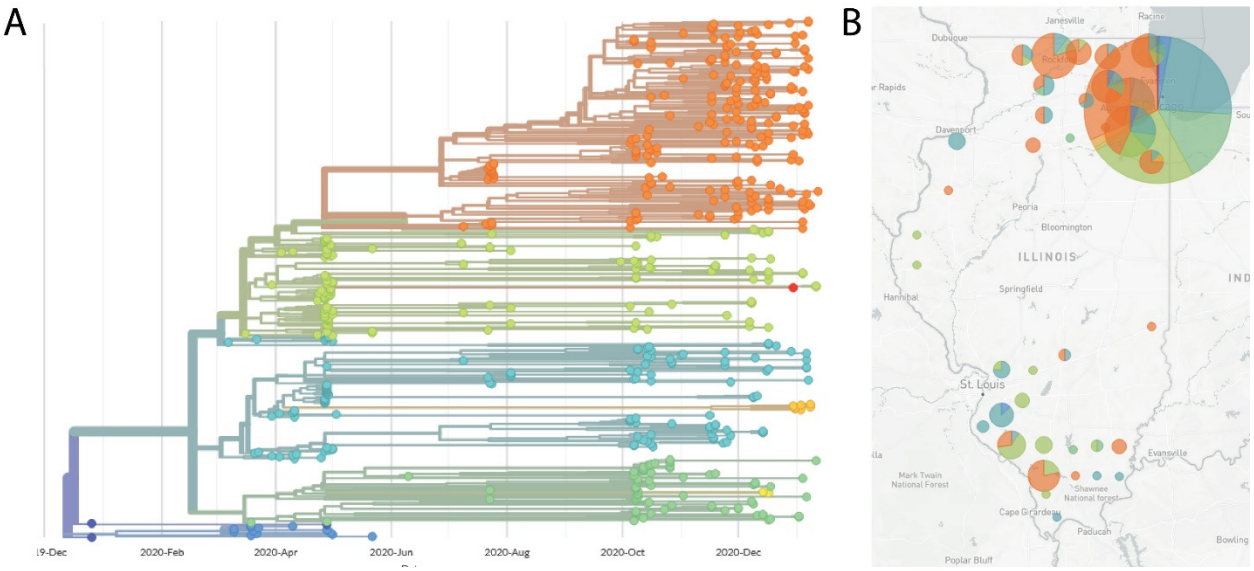

Figure 4. Nextstrain phylogenetic visualization and map view of SARS-CoV-2 genome sequences. (A) Phylogenetic tree generated by the Nextstrain pipeline. Sequences are derived from samples taken in Illinois, between April 2020 and January 2021, and sequenced by our laboratory. Each color represents a different Nextstrain clade. (**B**) Map of Illinois showing sample locations, by county. The size of the circle indicates the relative number of sequences derived from that county. The pie chart indicates the proportional distribution of Nextstrain clades at that location. Clade designation colors for panel A and B are: 19A (dark blue), 19B (blue), 20A (light blue), 20B (green), 20C (yellow-green), 20D (yellow), 20E (yellow-orange), 20G (orange).

CONCLUSIONS

Here we presented a comprehensive and well-validated set of working protocols for sequencing high-quality SARS-CoV-2 genomes in high throughput from patient samples. These protocols implement the popular ARTIC PCR and MinION nanopore sequencing with 96 samples at a time. We have optimized several steps for efficiency and higher recovery, including multiplex ARTIC PCR. Many of the considerations we have covered in these protocols will translate to other sequencing platforms. This workflow and the accompanying supplemental protocols provide a reliable starting point and an invaluable reference for those seeking to generate SARS-CoV-2 genome sequences using nanopore sequencing technology, especially from the cost-effective MinION instrument.

ACKNOWLEDGEMENTS

We thank the Illinois Department of Public Health for access to patient samples. This work was funded by discretionary funds from the Southern Illinois University (SIU) School of Medicine Dean's Office and the Office of the Vice Chancellor for Research at SIU Carbondale. This work

was also funded by the Walder Foundation through a collaborative agreement with the Open Commons Consortium as part of the Chicago CAN initiative.

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