Original Research *Highlight article*

"Nano COVID-19": Nanopore sequencing of spike gene to identify SARS-CoV-2 variants of concern

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Impact Statement

In this study, "Nano COVID-19" was developed to amplify the full-length spike gene of all SARS-CoV-2 variant of concerns (VOCs) for long-read nanopore sequencing combined with flexible data analysis options. Therefore, this workflow would be attractive for large-scale, simple, rapid, and cost-effective viral epidemic screening and transmission tracking of SARS-CoV-2 VOCs. Furthermore, "Nano COVID-19" might be applied to detect novel variants and mutations in the future.

Abstract

Coronavirus disease 2019 (COVID-19) is a worldwide pandemic infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). World Health Organization (WHO) has defined the viral variants of concern (VOC) which cause more severe disease, higher transmissibility, and reduced vaccine efficacy. In this study, the "Nano COVID-19" workflow based on Oxford nanopore sequencing of the full-length spike gene combined with flexible data analysis options was developed to identify SARS-CoV-2 VOCs. The primers were designed to cover the full-length spike gene and can amplify all VOC strains. The results of VOC identification based on phylogenetic analysis of the full-length spike gene were comparable to the whole genome sequencing (WGS). Compared to the standard VOC identification pipeline, the fast analysis based on Read Assignment, Mapping, and Phylogenetic Analysis in Real Time (RAMPART) and the user-friendly method based on EPI2ME yielded 89.3% and 97.3% accuracy, respectively. The EPI2ME pipeline is recommended for researchers without bioinformatic skills, whereas RAMPART is more suitable for bioinformaticians. This workflow provides a cost-effective, simplified pipeline with a rapid turnaround time. Furthermore, it is portable to point-of-care SARS-CoV-2 VOC identification and compatible with large-scale analysis. Therefore, "Nano COVID-19" is an alternative viral epidemic screening and transmission tracking workflow.

Keywords: Nano COVID-19, COVID-19, SARS-CoV-2, spike gene, variant of concerns, nanopore sequencing

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Introduction

Coronavirus disease 2019 (COVID-19) is a worldwide pandemic that has spread to all countries. The first COVID-19 case was reported in Wuhan, China, in December 2019. The first case was identified as a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and labeled "Wuhan-Hu-1" (NC_045512.2).[1] Whole genome sequencing (WGS) is a technique that can report and characterize viral genomes. The Global Initiative on Sharing All Influenza Data (GISAID) (https://www.gisaid.org/) assigned the high-quality genome sequence, WIV04 (EPI_ISL_402124), to use as a reference SARS-CoV-2 sequence in several COVID-19 databases.[2,3] Millions of genome sequences were submitted to the database for tracking viral transmission and mutation.

Besides surveillance, the viral genome database is important for developing diagnostic methods, exploring therapeutic drugs, and investigating vaccines. Currently, several SARS-CoV-2 databases have been published online and are updated in real-time. The World Health Organization (WHO) has defined variants as one or more mutations in the viral genome that are used to classify the lineage of the SARS-CoV-2. The Centers for Disease Control and Prevention (CDC) and the WHO have classified variants as Variant Being Monitored (VBM), Variant of Interest (VOI), Variant of Concern (VOC), and Variant of High Consequence (VOHC),

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depending on the nucleotide variation ratios, the predicted effects of the cluster of mutations, the efficacy of medical treatments, the severity of the illness, and the capacity for disease transmission between individuals. VBM represents the variants circulating at low levels, while VOI represent the variants associated with particular bindings between the virus and host cell receptor affecting the disease severity, the vaccine efficiency, and the generated host antibody. Finally, VOC represent the variants that cause more severe disease, higher transmissibility rates, and reduced vaccine effectiveness.

Genomic surveillance plays an important role in detecting, monitoring, and predicting the viral pandemic. Viral WGS is the gold standard technique for presenting nucleotide variants. Several sequencing platforms, including Illumina short-read sequencing and Oxford nanopore long-read sequencing, have been applied to identify and report the sequences as rapidly as possible. While the sequences acquired from short-read sequencing represent the highest accuracy in investigating the nucleotide variants, the genome assembly is relatively cumbersome due to the nature of short reads. In contrast, the data obtained from long-read sequencing generate longer reads, significantly facilitating genome assembly.

Nanopore sequencing from Oxford Nanopore Technologies (ONT) is becoming popular because it has simple library preparation for DNA and RNA, uses portable sequencers, and provides data in real-time. The principle of the ONT sequencing platform is based on ionic current alteration. A single DNA or RNA molecule passing through a nanopore embedded in a flow cell creates a unique current disruption. Sequencing machines have numerous models, including a stand-alone sequencer (MinION Mk1C) and a computer-controlled sequencer (MinION Mk1B). Subsequently, the current profile can be converted to nucleotide sequences in real-time using a user-friendly machine learning–based program called MinKNOW. ONT is compatible with point-of-care (POC) diagnostic techniques, which are powerful for rapid microbial identification. For SARS-CoV-2 genome sequencing, ONT first developed the Midnight RT PCR Expansion (EXP-MRT001; Oxford Nanopore Technologies, UK). However, this has now been changed to COVID Mini-Maxi combined with ARTIC Network to improve primer binding efficiency for emerging SARS-CoV-2 strains. The Midnight protocol (EXP-MRT001; Oxford Nanopore Technologies) contains 29 pairs of primers to generate overlapping amplicons (approximately 1.2 kb) covering the viral genome. Combined with the ARTIC network,[4] this pipeline provided real-time molecular epidemiology tracking for outbreak response. Briefly, the ARTIC network compared the sequencing data with the database to track the distance or similarity of the sequences. The results were visualized as reads coverage in the viral genome and classified as clade based on Nextclade or lineage based on Pango lineage.[5]

SARS-CoV-2 is a positive-sense single-stranded RNA (+ss-RNA) virus classified in the *Coronaviridae* family and *Betacoronavirus* genus. The viral genome (approximately 29.9 kb) consists of 14 open reading frames (ORFs) which encode for 16 non-structural polyproteins (nsp 1–16) and 4 structural proteins, including envelope (E), membrane (M), nucleoprotein (N), and spike (S). The nucleotide variants can occur in several genes; interestingly, major mutations have been observed in the spike gene, affecting viral infectivity and host immune response. The spike protein plays an important role in binding with the angiotensin-converting enzyme 2 (ACE2) receptor on the host cell surface, which is the main point for virus adaptation.[6,7] Moreover, the spike gene is the main region to classify the variants of concern that represent the origin of the pandemic, virus transmission, and disease severity. This study aims to develop a "Nano COVID-19" workflow that provides a cost-effective, simplified pipeline, with a rapid turnaround time and which is portable to the POC identification of SARS-CoV-2 VOC, based on the Oxford nanopore sequencing of the spike gene.

Materials and methods

Sample collection and processing

Nasal pharyngeal swabs (NP swabs) of patients with SARS-CoV-2 suspected cases from the Institute for Urban Disease Control and Prevention (IUDC), Thailand, were collected in VTM media (MP Biomedicals, USA). RNA was extracted from 200 µL of NP swab using a magLEAD 12gC instrument with a magLEAD Consumable Kit (Precision System Science, Japan), following the manufacturer's instructions. RNA samples were confirmed for SARS-CoV-2 infection by Allplex™ 2019-nCoV Assay (Seegene, Korea) based on quantitative reverse transcription polymerase chain reaction (qRT-PCR). The 11.5 µL of RNA extracted from SARS-CoV-2 positive samples were incubated with 5 µM random hexamers at 65°C for 5 min. The reverse transcription mixtures consisting of 1× reaction buffer, 1 mM deoxynucleoside triphosphates (dNTPs), 20 U RiboLock RNase inhibitor (Thermo Fisher Scientific, USA), and 100 U RevertAid RT (Thermo Fisher Scientific, USA) were added into the premix extracted RNA and primer. The reactions were incubated at 25°C for 10 min, 42°C for 60 min, and then heat-inactivated at 70°C for 10 min.

Primer design and synthesis

The genome sequences of SARS-CoV-2 VOI and VOC, including Wuhan, Alpha, Beta, Delta, Epsilon, Eta, Gamma, Iota, Kappa, Lambda, Mu, Omicron (BA.1, BA.2, BA.2.12.1, BA.2.75, BA.3, BA.4, BA.5), and Zeta were classified based on the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) (https://www.bv-brc.org/) and retrieved from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). The 20 representative sequences (Supplementary Table 1) were multiple aligned by BioEdit version 7.0.5.3. Primers for the generation of overlapping amplicons (at least 250 bp) were chosen from the conserved regions of alignment and covered the spike gene of the SARS-CoV-2 genome (Supplementary Figure 1). The primers were tailed with a barcode adapter, as summarized in Table 1 and Supplementary Figure 2.

Spike gene amplification

The polymerase chain reaction (PCR) reaction for spike gene employed a mixture containing 1 µL of complementary DNA (cDNA), 1× Phusion Plus Buffer, 200 µM dNTPs mixed, 1 U

Table 1. The primer sequences for the SARS-CoV-2 full-length spike gene amplification.

Primer name	Nucleotide position[a]	Nucleotide sequence (5'→3')	Product size (bp)		
Nano_S_F1	21418–21441	TTTCTGTTGGTGCTGATATTGTATCTCTTCTTAGTAAAGGTAGAC	1194	2083	4077
Nano_S_R1	22589–22611	ACTTGCCTGTCGCTCTATCTTTAGGACAGAATAATCAGCAACAC			
Nano_S_F2	22354–22375	TTTCTGTTGGTGCTGATATTGGGAACCATTACAGATGCTGTAG	1147		
Nano_S_R2	23478–23500	ACTTGCCTGTCGCTCTATCTTGTATGTCACACTCATATGAGTTG			
Nano_S_F3	23219–23239	TTTCTGTTGGTGCTGATATTGACACTACTGATGCTGTCCGTG	1293	2276	
Nano_S_R3	24491–24511	ACTTGCCTGTCGCTCTATCTTGAAGTCTGCCTGTGATCAACC			
Nano_S_F4	24238–24259	TTTCTGTTGGTGCTGATATTGGTTACACAGAATGTTCTCTATG	1257		
Nano_S_R4	25473–25494	ACTTGCCTGTCGCTCTATCTTGAAGTGCAACGCCAACAATAAG			

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.
[a]Nucleotide position based on NC_045512.2; underlined sequence represents the target gene binding sites.

of Phusion Plus DNA polymerase (Thermo Fisher Scientific, USA), 0.25 µM of each primer (Bionics, Korea), and diethylpyrocarbonate (DEPC)-treated water to a final volume of 20 µL. Amplification was performed with the following PCR conditions: initial denaturation at 98°C for 30 s, 40 cycles of amplification (98°C for 10 s, 60°C for 10 s, 72°C for 2 min), and final extension at 72°C for 5 min. The PCR barcoding reaction employed a mixture containing 5 µL of amplified products, 1× Phusion Plus Buffer, 200 µM dNTPs mixed, 1 U of Phusion Plus DNA polymerase (Thermo Fisher Scientific, USA), 0.125 µM of each barcode primer from PCR Barcoding Expansion 1–96 (EXP-PBC096) kit (Oxford Nanopore Technologies, UK), and DEPC-treated water to a final volume of 100 µL. PCR barcoding was performed with the following PCR conditions: initial denaturation at 98°C for 30 s, five cycles of amplification (98°C for 10 s, 60°C for 10 s, 72°C for 2 min), and final extension at 72°C for 5 min. The PCR product was verified on 1% agarose gel electrophoresis in Tris-boric-ethylenediaminetetraacetic acid (TBE) buffer to confirm the size of the amplicons (Supplementary Figure 3) and then purified by the QIAquick PCR Purification kit (Qiagen, Germany), following the manufacturer's protocol. DNA libraries with different barcodes were quantified using the Qubit dsDNA HS Assay Kit and Qubit 4 fluorometer (Thermo Fisher Scientific, USA).

Nanopore sequencing

All DNA libraries were pooled in equimolar concentration and purified using a 0.5× AMPure XP bead (Beckman Coulter, USA). The ONT libraries pool was eluted in 50 µL of elution buffer (EB; Qiagen, Germany) and quantified using the Qubit dsDNA HS Assay Kit and Qubit 4 fluorometer (Thermo Fisher Scientific, USA). According to the manufacturer's instruction, the ONT libraries were end-repaired using NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (New England BioLabs, USA). Briefly, a total of 1 µg ONT libraries were incubated with 3.5 µL of NEBNext FFPE DNA Repair Buffer, 2 µL of NEBNext FFPE DNA Repair Mix, 3.5 µL of Ultra II End-prep reaction buffer, and 3 µL of Ultra II End-prep enzyme mix. The end-repaired reaction was incubated at 20°C for 5 min and 65°C for 5 min in a thermal cycler. Next, the end-repaired library was cleaned up with 1× AMPure XP bead (Beckman Coulter, USA) and eluted in 61 µL of nuclease-free water and quantified using the Qubit dsDNA HS Assay Kit and Qubit 4 fluorometer (Thermo Fisher Scientific, USA).

Adapter ligation was performed using the Ligation Sequencing Kit (Q20+) (SQK-LSK112; Oxford Nanopore Technologies, UK), following the manufacturer's instructions. Briefly, 60 µL of the end-repaired library was mixed with 25 µL of Ligation Buffer (LNB), 10 µL of NEBNext Quick T4 DNA Ligase (New England BioLabs, USA), and 5 µL of Adapter Mix H (AMX H) and incubated at room temperature for 10 min. Then, the adapter-ligated library was cleaned up with 0.4× AMPure XP bead (Beckman Coulter, USA) and washed twice by Short Fragment Buffer (SFB). The adapter-ligated library was eluted in 15 µL of EB and quantified using the Qubit dsDNA HS Assay Kit and the Qubit 4 fluorometer (Thermo Fisher Scientific, USA).

Before sequencing, the flow cell was primed with 800 µL of the priming mix in the priming port (30 µL of Flush Tether in 1170 µL of Flush Buffer) and incubated at room temperature for 5 min. After that, the flow cell was flushed with 200 µL of the priming mix in the priming port while the SpotON port was open. Finally, the loading library was prepared by mixing 37.5 µL of the Sequencing Buffer II (SBII) and Loading Bead II (LBII) with 12 µL of DNA Library, which was then loaded into the SpotON port of the flow cell R10.4 version (FLO-MIN112). The MinION Mk1C machine and MinKNOW version 5.0.5 (Oxford Nanopore Technologies, UK) were used for sequencing with real-time base calling.

SARS-CoV-2 WGS

The cDNA samples were amplified as 6 amplicons (approximately 5 kb/amplicon) along with the SARS-CoV-2 genome and used as a template for secondary PCR to generate 12 amplicons (approximately 2.3–2.7 kb/amplicon).[8] All 12 amplicons per sample were purified using the QIAquick PCR Purification kit (Qiagen, Germany) and pooled as a final 5 µg. The samples were quantified, and the DNA concentration was adjusted to 3 µg in a final volume of 48 µL. According to the manufacturer's instructions, the ONT libraries were end-repaired using NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (New England BioLabs, USA). Briefly, a total of 3 µg of ONT libraries were incubated with 3.5 µL of NEBNext FFPE DNA Repair Buffer, 2 µL of NEBNext FFPE DNA Repair Mix, 3.5 µL of Ultra II End-prep reaction buffer, and 3 µL of Ultra II End-prep enzyme mix. The end-repaired reaction was incubated at 20°C for 5 min, and then 65°C for 5 min, in a thermal cycler. Next, the end-repaired library was cleaned up with 1× AMPure XP bead (Beckman Coulter, USA), eluted in 25 µL of nuclease-free

water, and quantified using the Qubit dsDNA HS Assay Kit and Qubit 4 fluorometer (Thermo Fisher Scientific, USA).

Native barcode ligation was performed by Native Barcoding Expansion 1–12 (EXP-NBD104) and 13–24 (EXP-NBD114) (Oxford Nanopore Technologies, UK), following the manufacturer's instructions. First, the end-repaired libraries were adjusted to 750 ng/sample with DEPC-treated water to a final volume of 22.5 µL and then mixed with 25 µL of Blunt/TA Ligase Master Mix (New England BioLabs, USA) and 2.5 µL of the different barcodes. The mixtures were incubated at room temperature for 10 min, purified with 1× AMPure XP bead (Beckman Coulter, USA) and eluted in 26 µL of nuclease-free water. Each barcoded sample was quantified and then pooled in equimolar amounts.

Following the manufacturer's instructions, an adapter ligation was performed by Ligation Sequencing Kit (SQK-LSK109; Oxford Nanopore Technologies, UK). Briefly, the 65 µL of the pooled library were mixed with 20 µL of NEBNext Quick Ligation Reaction Buffer, 10 µL of NEBNext Quick T4 DNA Ligase, and 5 µL of Adapter Mix II (AMII) and incubated at room temperature for 10 min. Then, the adapter-ligated library was cleaned up by 0.5× AMPure XP bead (Beckman Coulter, USA) and washed twice by SFB. Finally, the ONT libraries were eluted with 15 µL of EB and quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA).

Data analysis

Fast analysis (Read Assignment, Mapping, and Phylogenetic Analysis in Real Time) During the ONT sequencing, the FAST5 files from MinION Mk1C were converted to FASTQ files using MinKNOW (Oxford Nanopore Technologies) version 5.0.5 with a FAST model and *Q*-score ⩾7. The real-time analysis of nanopore sequencing data was performed and visualized as coverage of reads across the samples using RAMPART (Read Assignment, Mapping, and Phylogenetic Analysis in Real Time) software (https://artic.network/rampart). After demultiplexing, the passed filter reads were mapped with the reference spike gene of the SARS-CoV-2 Wuhan-Hu-1 strain (NC_045512.2.) and used for matching against a custom database consisting of full-length spike genes of the 20 representative VOC reference sequences.

User-friendly analysis (EPI2ME) All 20 full-length spike genes of representative SARS-CoV-2 VOC reference sequences were uploaded into EPI2MEAgent version 3.5.7 and made the FASTA reference sequence. Next, the FASTQ files were uploaded, demultiplexed with PBC096 barcode with *Q*-score ⩾7, and custom aligned with the reference sequences. The mapped reads were identified and then counted to summarize the dominant strain of each sample.

Standard pipeline and phylogenetic analysis The raw data (FAST5 format) were base called by Guppy base caller v6.0.1 (Oxford Nanopore Technologies) with a super-accuracy (SUP) model to generate the FASTQ sequences. The FASTQ reads were quality-examined using MinIONQC.[9] Then, the reads were demultiplexed and adapter-trimmed using Porechop v0.2.4 (https://github.com/rrwick/Porechop). The statistics of demultiplexed reads was checked using seqstats (https://github.com/clwgg/seqstats). The filtered reads were subsequently mapped to the spike gene reference sequence of SARS-CoV-2 isolate Wuhan-Hu-1 (NC_045512.2) using Minimap2.[10] The assembled contigs were created using bcftools, mpileup, and vcf2fq utilities integrated within Samtools.[11] Then, the FASTQ draft sequences were converted to FASTA consensus sequences using seqtk (https://github.com/lh3/seqtk). The full-length spike gene of samples was polished using Medaka v1.6.0 (https://github.com/nanoporetech/medaka) and multiple aligned with the 20 representative VOC reference sequences (Supplementary Figure 2) using MAFFT version 7 (https://mafft.cbrc.jp/alignment/software/).[12,13] The phylogenetic analysis was performed with the maximum likelihood method (1000 bootstrapping replicates) and Tamura 3-parameter model (Gamma distributed with Invariant sites (G + I) rates among sites) using MEGA X.[14]

Results

Primer design

The representative sequences of all VOCs classified based on the BV-BRC were downloaded from the NCBI database. Then, the sequences were multiple aligned by BioEdit version 7.0.5. All primer sequences (Table 1) were designed to complement the conserved region and cover the full-length spike gene of the SARS-CoV-2 genome (Supplementary Figure 1). All primers have the same melting temperature (approximately 64°C) and can be used under the same PCR conditions. Hence, any pair of forward and reverse primers can be used to perform singleplex PCR to generate various amplicon sizes (approximately 1.2, 2, and 4 kb). The alignments within the primer binding sites showed that the primers were conserved against representative SARS-CoV-2 strains, indicating that these primer sets should effectively amplify any strains of SARS-CoV-2 (Supplementary Figure 2).

Spike gene amplification

This study examined the oligonucleotide primers to amplify the full-length spike gene of SARS-CoV-2 and validated the rapid workflow for VOC identification using the Nanopore sequencing platform (Figure 1). In the development phase, the full-length spike gene amplification was validated in three systems. Option 1: full-length spike gene (4077 bp) was amplified using Nano_S_F1/R4. Option 2: the two overlapping amplicons (2083 bp and 2276 bp) were amplified using Nano_S_F1/R2 and Nano_S_F3/R4, respectively. In the last option, four pairs of primers were designed for amplifying overlapping PCR products (approximately 1.2 kb) from clinical samples with low viral titers (Table 1 and Supplementary Figure 3). Then, 48 RNA extracted from SARS-CoV-2 positive nasopharyngeal swab specimens were tested with the optimized conditions to evaluate the efficiency of primers. First, a 66.67% success rate (32/48 samples) was obtained from option 1 to amplify the largest fragment. After that, two amplicons were efficiently amplified based on option 2 with a 91.67% success rate (44/48 samples). The last option

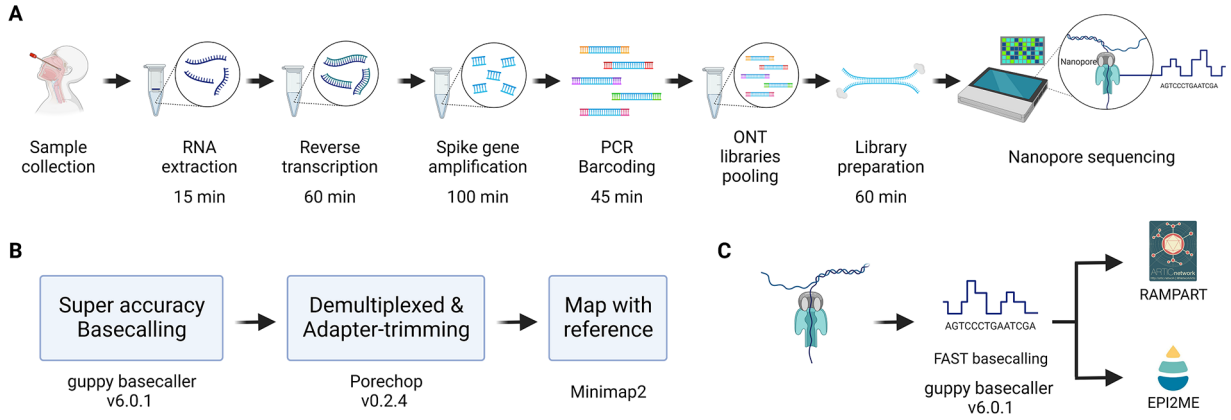

Figure 1. Nano COVID-19 workflow. (A) Process from clinical sample collection to nanopore sequencing. (B) Super-accuracy base calling and standard pipeline. (C) FAST base calling followed by RAMPART and EPI2ME.

Table 2. Summary of nanopore sequencing results.

Parameters	RUN1	RUN2	RUN3	RUN4
Flow cell number	FAR84516	FAR83945	FAR84122	FAR84097
Starting pores	1121	1594	1438	1385
Samples/run	48	47	46	32
Reads count	94.59 K	183.58 K	186.3 K	783.35 K
Average reads/sample	772	1239	1252	1706
Average bases/sample	1,812,382	2,685,103	2,572,475	2,777,573
Average length/sample	2909	2190	2055	1633

yielded a 97.92% success rate (47/48 samples) in generating four amplicons (Supplementary Figure 3). Therefore, option 2 was selected and used for further validation due to long amplicon production with an acceptable success rate.

Validation of SARS-CoV-2 VOC identification based on spike gene

The PCR products from 48 samples were pooled and sequenced via MinION Mk1C to investigate the efficiency of identifying SARS-CoV-2 VOC. All 48 samples were successfully sequenced to produce approximately 100 K reads in 1 h (Table 2). The data based on the SUP model were analyzed with a standard pipeline and then visualized by the phylogenetic tree. WGSs were performed by MinION to confirm the results of SARS-CoV-2 VOC based on the full-length spike gene. The WGS and the full-length spike gene from 20 samples were analyzed with the representative reference strains of SARS-CoV-2 and constructed phylogenetic trees. Phylogenetic analysis revealed that 4 samples belonged to the Alpha strain, 1 sample was closely related to the Beta strain, and 15 samples were clustered to Delta strains. The results suggested that VOC identification based on the full-length spike gene analyzed by a standard pipeline combined with a phylogenetic tree was comparable to the WGS data (Figure 2).

Different pipelines for SARS-CoV-2 identification

Next, two data analysis pipelines (RAMPART for rapid identification and EPI2ME for user-friendly software) were applied and validated for VOC identification from spike gene sequencing. Currently, several strains of SARS-CoV-2 have been reported, including Omicron sublineage (BA.1, BA.2, BA.4, and BA.5) that have highly similar sequences. The extracted RNAs from 149 COVID-19 patients (Alpha = 10, Delta = 27, Omicron BA.1 = 56, Omicron BA.2 = 24, Omicron BA.4 = 12, and Omicron BA.5 = 20) were amplified with the full-length spike gene and prepared ONT libraries. The FAST5 files were converted into FASTQ files (fast base called model) immediately after the sequencing run. For rapid identification, RAMPART was performed by inputting the real-time base calling FASTQ files. Then, the data were demultiplexed, mapped with the representative reference sequences (Supplementary Table 1) and the SARS-CoV-2 VOC was visualized as a heat map graph (data not shown). The result of VOC identification based on RAMPART is summarized in Supplementary Table 2

Meanwhile, using the user-friendly "EPI2ME" software, exclusively available for ONT users, the FASTQ files obtained from 149 samples were demultiplexed, custom-aligned, and calculated mapped reads with the representative reference sequences (Supplementary Table 1). The SARS-CoV-2 VOC was determined by the reference strain with the highest number of mapped reads (Supplementary Table 2).

The VOC identification from two different pipelines was compared with the standard phylogenetic analysis to calculate the accuracy of SARS-CoV-2 VOC identification. The results from RAMPART analysis revealed that 133 from 149 samples (89.3% accuracy) were correctly identified, while EPI2ME software properly classified 145 from 149 samples (97.3% accuracy) when compared with the standard phylogenetic tree.

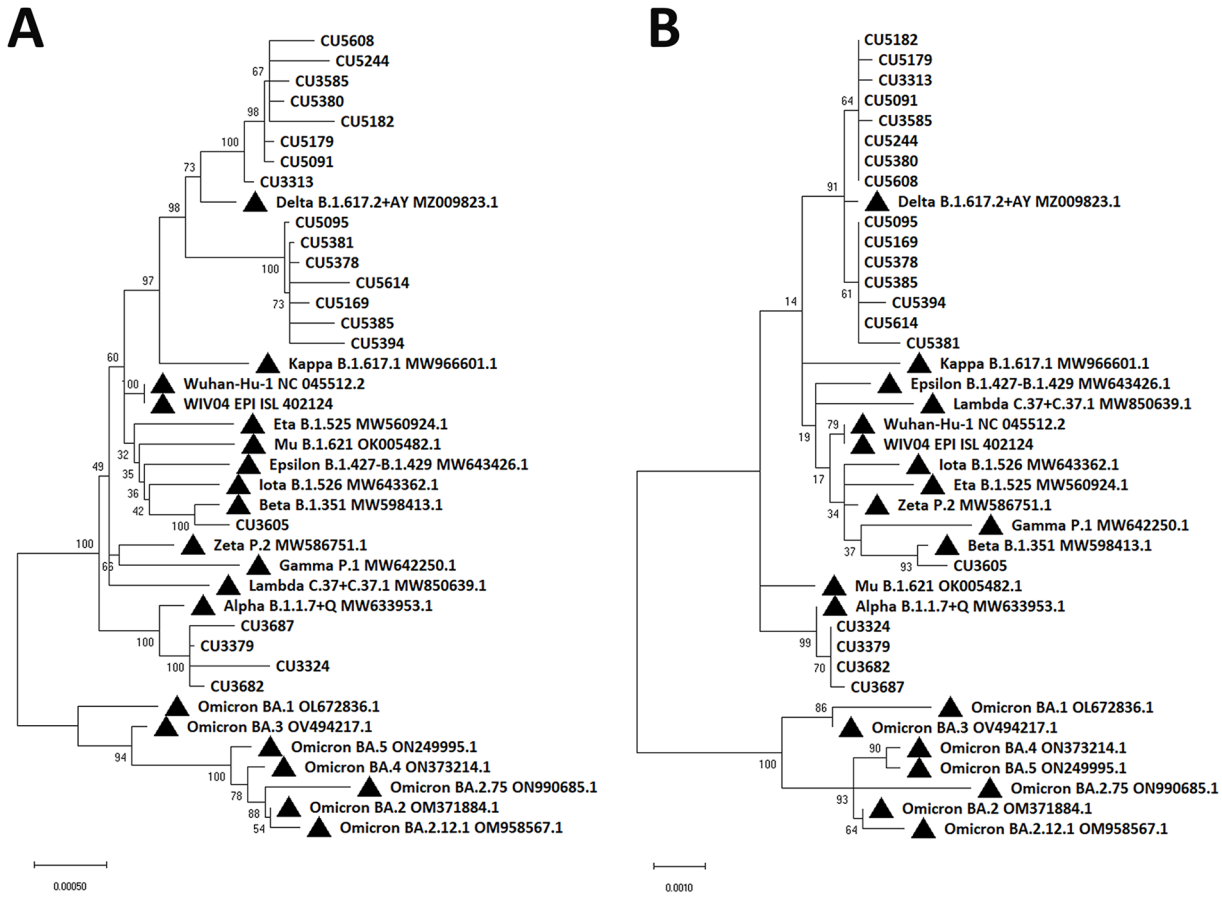

Figure 2. Phylogenetic analysis of SARS-CoV-2 samples compared with 20 representative SARS-CoV-2 VOC sequences (black triangle) based on the maximum likelihood method with 1000 bootstraps. (A) Phylogenetic tree constructed by whole genome sequence data. (B) Phylogenetic tree generated from the full-length spike gene sequences.

Discussion

Currently, the pandemic of COVID-19 is still ongoing around the world. Moreover, new variants are still emerging in several countries. Normally, WGS is the gold standard for characterizing viral mutations. Thus, several SARS-CoV-2 genome sequences have been deposited in numerous databases. Nevertheless, the major mutations have occurred in the spike gene, an important region for VOC classification. This study aims to develop the "Nano COVID-19" to identify the variants in the spike gene that can be applied to classify SARS-CoV-2 strains.

More than 10 variants of SARS-CoV-2 have been reported, including Alpha, Beta, Delta, Epsilon, Eta, Gamma, Iota, Kappa, Lambda, Mu, Omicron, and Zeta. These new variants are still ongoing, with the Omicron sublineages representing the prevalent strains. Therefore, detecting different strains of the virus is necessary to study the current outbreak of the virus. In 2020–2021, Alpha, Beta, Delta, and Gamma were the major strains of attention. Several studies have developed methods to distinguish among different strains of SARS-CoV-2. Isothermal amplification techniques such as Recombinase Polymerase Amplification (RPA),[15,16] Recombinase-Aided Amplification (RAA),[17] and Loop-mediated Isothermal Amplification (LAMP)[18] are rapid and simple methods that can be used for POC detection. On the contrary, there are some limitations of isothermal amplification methods, such as the secondary structure of primers (longer than 30 bp for RPA and RAA), complicated primer design (at least three primer pairs for LAMP), optimized primer concentration, suitable incubation temperature, and downstream detection method. The assay cannot be used for multiplex detection; thus, multiple singleplex reactions may possibly lead to cross-reactivity and contamination.

Moreover, PCR-based amplification is the conventional method of detecting at the molecular level. For the quantitative polymerase chain reaction (qPCR)–based method, the simple interpretation method depends on the specific primers or probes for SARS-CoV-2 detection. The routine qPCR probes specific to the N, E, and Orf1ab genes are the gold standard for SARS-CoV-2 diagnosis, which only report positive or negative results. Variant-specific primers or probes for qPCR assays to screen the SARS-CoV-2 circulating variants have been reported in several studies.[19–22] The efficiency of these methods depends on the complementary primers and targets, which may not be compatible with new emerging strains. Moreover, the qPCR method to classify six major VOC (Alpha, Beta, Gamma, Delta, Mu, and Omicron) of the SARS-CoV-2 strains and six sublineages (BA.1, BA.2, BA.2.275.2, BA.4.6, BA.5, and BQ1.1) of the Omicron strains is commercially available. However, several reactions need to be performed to specify the viral strain.[23]

Furthermore, sequencing technology has been applied to identify SARS-CoV-2 VOC detail at the nucleotide variation level. Ideally, WGS is the best way to characterize the viral genome and the mutations. Currently, mutations of SARS-CoV-2 are rapidly reported in many epidemic VOCs worldwide. WGS is an advanced technique that requires a high-cost instrument, experienced researchers, and complex bioinformatic analysis. Therefore, targeted sequencing is an alternative way to combine with VOC variable region enrichment using conventional PCR. Moreover, the sequencing has been adapted to explore more details that might be misidentified using the PCR or qPCR method.

RNA extracted from clinical samples was used as starting material for Nano COVID-19. Hence, clinical samples such as NP swabs and saliva can be used for Nano COVID-19 testing. From preliminary results tested with a small size of saliva samples (data not shown), the VOCs of SARS-CoV-2 can be identified by Nano COVID-19. However, mucous saliva samples can be difficult to extract. In addition, saliva contains RNases and various reverse transcriptase and polymerase inhibitors, resulting in fragmented RNA and poor amplification in subsequent steps.

The evidence of co-infection between 2 VOCs of SARS-CoV-2 has been reported, such as Alpha/Epsilon,[24] Beta/Delta,[25,26] Gamma/Delta,[27] and Delta/Omicron.[28–30] Previous studies reported the PCR combined with Sanger's sequencing to identify the variations in the viral genes.[31,32] The limitations of first-generation sequencing are a single fragment sequencing with approximately 1 kb in length and the inability to identify co-infections with more than two strains. Based on the ARTIC protocol, 14 pairs of primer were used for amplifying the spike gene of SARS-CoV-2 to generate amplicons (approximately 400 bp), followed by the short-read Illumina sequencing platform.[33,34] Nonetheless, the second-generation sequencing is limited in terms of incorrect assembly in co-infected samples. In contrast, third-generation single-molecule sequencing based on ONT provides multiple long-read nucleotide sequences with simple library preparation, rapid sequencing and real-time analysis. Therefore, the ONT has been applied to viral genome characterizations such as Ebola, [35] Zika,[36] Herpes simplex,[37] and Poliovirus.[38] However, sequence accuracy and base calling in the homopolymers are the main concerns for ONT. The newly released nanopore sequencing Q20+ kit (LSK-112) and flow cell R10.4 (FLO-MIN-112) have significantly improved the sequence accuracy over the previous sequencing kit (LSK-109) and flow cell R9.4.1 (FLO-MIN-106D). The updated flow cell (R10.4) version contains double detectors compared to the single detector in the previous flow cell (R9.4.1) version.[39] The SARS-CoV-2 whole genome study reported that the flow cell R10.4 improved the sequence accuracy to 98.34% and did not require a short-read sequence to confirm the viral genome sequence.[40] In this study, the Q20+ kit and flow cell R10.4 version were used for sequencing to provide highly accurate data to identify SARS-CoV-2 VOC and Omicron sublineages which are different at the nucleotide level. In this study, Nano COVID-19 was developed as a simple workflow approach (Figure 1), both cost-effective and suitable for large-scale analysis (up to 384 barcodes). Furthermore, the primers were incorporated with the conserved region of the viral genome; thus, all strains of SARS-CoV-2 were amplified and identified from the clustering nucleotide reads. Hence, the nucleotide sequences of each strain or co-infected strains can be detected and analyzed by Nano COVID-19 with RAMPART and EPI2ME pipelines as shown in Supplementary Figure 2, and this is particularly attractive for viral transmission tracking and epidemiology studies.

This study provided three alternative analysis pipelines suitable for various bioinformatic knowledge. For general users, Guppy base caller plugged in with the MinKNOW program can be used to convert fast5 to fastq files. Then, the EPI2ME pipeline was recommended for subsequent analysis. For bioinformatic experts, Guppy base caller can be installed in a Linux environment and used for fast (FAST), high (HAC), or super accuracy (SUP) base calling. After that, RAMPART, a command line–based program, can be used to analyze and visualize gene coverage and variants of concern. Alternatively, the standard pipeline offers flexibility for programs used in variance calling and phylogenetic analysis. The system requirements and analysis time from three pipelines were summarized in Supplementary Table 3.

In addition, the base calling model based on machine learning has improved and contains options including FAST, HAC, and SUP base calling models. The base calling model should be selected depending on the scenario. In the case of rapid identification, the FAST base calling model is suitable for real-time sequencing analysis combined with a custom pipeline to identify the VOC of SARS-CoV-2. On the contrary, the SUP base calling model takes longer time but is ideal for mutation analysis.

Several SARS-CoV-2 sublineages of the Omicron strain have been reported worldwide, including XBB.1 (first isolated in Singapore) and BQ.1 (first isolated in the United States). After the viral whole genome data were characterized and submitted in GISAID, the sequences were *in silico* analyzed to evaluate the Nano COVID-19 primer efficiency. The multiple alignments represent that the primers in this study can amplify the recent sublineage of the Omicron strain (data not shown).

In summary, Nano COVID-19 was developed to amplify the full-length spike gene of all SARS-CoV-2 VOCs combined with flexible data analysis options to rapidly identify VOCs or accurately determine mutations. Furthermore, this pipeline is compatible with large-scale screening and is low cost per sample. The long-read full-length spike gene acquired from ONT is suitable for easy VOC identification and nucleotide variant investigation. Therefore, "Nano COVID-19" is an alternative viral epidemic screening and transmission tracking platform.

AUTHORS' CONTRIBUTIONS

SP conceived the study design, coordinated the project, and suggested manuscript preparation. PN conducted the laboratory experiments, interpreted the results, and drafted the manuscript. PN, VS, and PK carried out data analysis and illustrations. JP and YP provided the clinical samples. PN, PK, YP, and SP revised the manuscript. All authors reviewed and approved the final version of this article.

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DECLARATION OF CONFLICTING INTERESTS

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ETHICAL APPROVAL

This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB no. 301/63 and 302/63).

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SUPPLEMENTAL MATERIAL

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