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Rapid Identification of SARS-CoV-2 Variants of Concern Using a Portable *peak*PCR Platform

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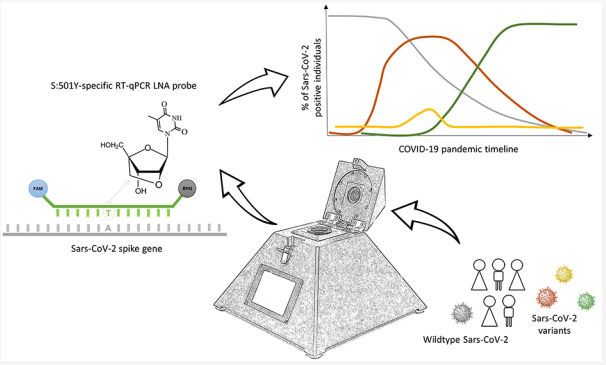
ABSTRACT: The need for tools that facilitate rapid detection and continuous monitoring of SARS-CoV-2 variants of concern (VOCs) is greater than ever, as these variants are more transmissible and therefore increase the pressure of COVID-19 on healthcare systems. To address this demand, we aimed at developing and evaluating a robust and fast diagnostic approach for the identification of SARS-CoV-2 VOC-associated spike gene mutations. Our diagnostic assays detect the E484K and N501Y single-nucleotide polymorphisms (SNPs) as well as a spike gene deletion (HV69/70) and can be run on standard laboratory equipment or on the portable rapid diagnostic technology platform *peak*PCR. The assays achieved excellent diagnostic performance when tested with RNA extracted from culture-derived SARS-CoV-2 VOC lineages and clinical samples collected in Equatorial Guinea, Central-West Africa. Simplicity of usage and the relatively low cost are advantages that make our approach well suitable for decentralized and rapid testing, especially in resource-limited settings.



INTRODUCTION

More than a year after the World Health Organization (WHO) declared the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) outbreak a Public Health Emergency of International Concern, coronavirus disease 2019 (COVID-19) has caused more than 3.7 million deaths.[1] Globally, public health systems are severely impacted and are further challenged by the emergence of SARS-CoV-2 variants carrying mutations that are of concern (VOC).[2] Molecular diagnostic tools, particularly reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for viral RNA detection and next-generation sequencing (NGS) for molecular monitoring SARS-CoV-2 genetic diversity at the whole genome level, have proven critical for public health decision-making.[3] Investigating SARS-CoV-2 genomes by NGS to track transmission chains, understand transmission dynamics, and rapidly identify mutations that potentially have an impact on transmissibility, morbidity, and mortality, as well as potential escape of diagnostic tools or vaccine-induced immunity have become an integral part of public health measures during this pandemic.[4]

Since the first whole genome sequence (WGS) analysis of SARS-CoV-2 has been published in January 2020,[5] the virus has been continuously sequenced, characterized, and data made publicly available through global initiatives such as Global Initiative on Sharing All Influenza Data (GISAID). More than 1.8 million SARS-CoV-2 sequences have been publicly shared via GISAID, and numerous mutations in the gene encoding the spike protein have been identified.[6] For example, the D614G variant has been shown to increase the viral load of infected patients and has replaced the original variant since June 2020 around the globe.[7] More recently, SARS-CoV-2 lineages characterized by a combination of multiple mutations in the spike gene have emerged independently in different regions of the world. The SARS-CoV-2 lineages B.1.1.7 (also known as the Alpha variant, VOC 202012/01 or 501Y.V1), B.1.351 (also known as the Beta variant or 501Y.V2), and P.1 (also known as the Gamma variant, B.1.1.28.1 or 501Y.V3) were the first VOCs identified.[8] The Alpha variant (lineage B.1.1.7) was first described in mid-December 2020 in the United Kingdom, and the mutation appears to have substantially increased transmissibility and has

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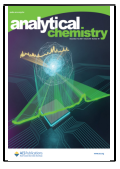

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Table 1. Primer and Probe Combinations Developed for SARS-CoV-2 VOC Identification and Discrimination

assay	primer name	oligo sequence (5′-3′)	modifications
HV69/70 assay: 21765−21770[a] deletion in alpha VOC	HV69/70_F	TCA ACT CAG GAC TTG TTC TTA CCT	
	HV69/70_R	TGG TAG GAC AGG GTT ATC AAA C	
	Δ69/70 (mut)	TCC ATG CTA TCT CTG GGA CCA	FAM - BHQ1
	HV69/70 (wt)	ACA TGT CTC TGG GAC CAA TGG	YYE[b] - BHQ1
N501Y assay: A23063T[a] SNP in alpha, beta and gamma VOCs	spike_gene_ LNA_F	C+TA TCA GGC +CGG TAG CAC +AC	
	spike_gene_ LNA_R	+AGT ACT ACT ACT CTG TAT +GGT TGG +T	
	501Y_LNA (mut)	C+CC A+CT +t+AT G+GT +G	FAM - BHQ1
	N501_LNA (wt)	C+CC A+CT +A+AT G+GT +G	YYE[b] - BHQ1
E484K assay: G23011A[a] SNP in beta and gamma VOCs	Spike_gene_ LNA_F	C+TA TCA GGC +CGG TAG CAC +AC	
	Spike_gene_ LNA_R	+AGT ACT ACT ACT CTG TAT +GGT TGG +T	
	484K_LNA (mut)	TGG +T+GT TaA A+GG T	FAM - BHQ1
	E484_LNA (wt)	TGG +T+GT TGA A+GG T	YYE[b] - BHQ1

[a]Genome position according to MN908947.3 (SARS-CoV-2 isolate Wuhan Hu-1). LNA nucleotides are indicated with + in front of the nucleotide. The SNP associated with VOCs is indicated as lower case and bold nucleotides. [b]YYE = Yakima Yellow (VIC and HEX dye alternative).

quickly developed into the dominant variant circulating in the UK and beyond.[9] The Beta variant (lineage B.1.351) was identified in December 2020, which emerged most likely in South Africa and is also associated with higher transmissibility.[10] The Gamma variant (lineage P.1) was identified in January 2021 in Manaus, the largest city in the Amazon region of Brazil.[11] In January 2021, this region experienced a resurgence of COVID-19 despite the reported high seroprevalence of antibodies against SARS-CoV-2 in this population.[12,13] During the preparation and revision of this manuscript, the WHO had designated the emerging lineage B.1.617.2 as the Delta VOC.

To rapidly detect and continuously monitor the appearance, introduction, and spread of (novel) VOCs, the level of molecular surveillance needs to be increased globally. The gold standard of genomic surveillance, NGS, allows for unbiased identification of mutations, but is limited by its relatively slow sample-to-result turnaround time and level of laboratory infrastructure and scientific expertise required. Furthermore, the relatively high costs of NGS increase the financial burden on establishing a widespread VOC-tracking strategy, a limiting factor particularly for resource-limited settings. Therefore, mutation-specific PCR-based approaches that are more cost-efficient and allow for high-throughput screening of a significant proportion of SARS-CoV-2-positive individuals were developed.[14,15] To identify the transmission dynamics of VOCs in settings with limited sequencing capabilities, we have designed rapid and cost-efficient RT-qPCR assays detecting relevant mutations in the spike protein of SARS-CoV-2. The N501Y mutation is found in the Alpha, Beta, and Gamma VOCs, while the E484K mutation is restricted to Beta and Gamma variants. The Alpha variant is characterized by an additional spike gene deletion (ΔHV69/70). To further simplify, decentralize, and speed up the process of VOC identification, we transformed our assay to a portable and inexpensive qPCR device, named *peak*PCR.[16] The device can complete up to 20 RT-qPCR reactions in less than 40 min. Important characteristics of *peak*PCR are the relatively low cost of production and the simplicity of usage. By using cartridges that are preloaded with lyophilized RT-qPCR reagents, the user interaction is reduced to loading the sample onto the cartridge. Furthermore, the preloaded *peak*PCR cartridges can easily be shipped and stored at room temperature, making cold chains superfluous. Here, we report the development of a new approach for rapid, robust, and decentralized identification of SARS-CoV-2 VOCs that can be both run on standard laboratory RT-qPCR equipment and the portable and rapid diagnostic technology platform *peak*PCR.

MATERIALS AND METHODS

SARS-CoV-2 Cell Culture Supernatants and Clinical Samples for Assay Evaluation. The cultivation of SARS-CoV-2 was carried out in a Biosafety level 3 laboratory and conducted under appropriate safety conditions. Three different VOC lineages of SARS-CoV-2, namely, B.1.1.7, B.1.351, and P.1 provided from the University Hospital of Geneva, Laboratory of Virology, were grown on VeroE6/TMPRSS2 cells obtained from the Centre for AIDS Reagents (National Institute for Biological Standards and Control).[17,18] The day before infection, VeroE6/TMPRSS2 cells were seeded at 2×10^6 cells per T75 flask in Dulbecco's modified Eagle medium (DMEM) (Seraglob, Switzerland) supplemented with 10% fetal bovine serum (FBS) (Merck, Germany) and 2% SEeticin (Seraglob, Switzerland). On the day of infection, the cells reached about 70−90% confluency. The growth medium was removed and replaced with 5 mL of infection medium (DMEM + 2% FBS + 2% SEeticin). Cells were inoculated with 70 μL of SARS-CoV-2 swab material and incubated for 1 h at 37 °C, 5% CO2, and >85% humidity. After adsorption, 10 mL of the infection medium was added to each flask. Cells were observed for cytopathic effects (CPE) for 3−6 days using an EVOSTM FL digital inverted microscope. When CPE reached 40−100%, the supernatant was collected, cleared from cell debris by centrifugation (10 min at 500 g), and samples were aliquoted and frozen. TCID50 was determined on VeroE6/TMPRSS2 cells. Virus was inactivated with Qiazol, and RNA was extracted with RNeasy Plus Universal Mini Kit (Qiagen, Germany).

As a positive control and for initial assay evaluation, a 1869 bp long synthetic SARS-CoV-2 spike gene fragment (genome position 21,557−23,434 bp), based on the sequence of B.1.1.7, was synthesized (Eurofin Genomics, Ebersberg, Germany; the sequence is provided in Figure S1). Using a serial dilution of the synthetic spike gene, a calibration curve ranging from 0.05 to 50,000,000 copies/μL was prepared (data provided in Figure S2). The initial viral copy number per μL (cp/μL) of the cell culture-derived RNA from SARS-CoV-2 was estimated using the calibration curve's *y*-intercept and its slope. Serial dilutions of the RNA extracted from the culture supernatants of SARS-CoV-2 isolates Wuhan Hu-1, B.1.1.7, B.1.351, and

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P.1, ranging from 0.1 to 1,000,000 cp/μL, was prepared and used to evaluate the assays' performance on both RT-qPCR platforms. Additionally, a SARS-CoV-2 RT-qPCR diagnostic assay, targeting the envelope (E) gene, published by the Institute of Virology at Charité (Berlin, Germany), was used as a positive control for viral RNA on both platforms.[19]

Designing HV69/70-Deletion-, E484K-, and N501Y-Specific RT-qPCR Assays. We developed assays targeting the HV69/70-deletion, the E484K-, and N501Y-single-nucleotide polymorphisms (SNPs). For standard RT-qPCR platforms like the Bio-Rad CFX96 device, multiplex assays were developed. The multiplex assays are able to detect both sequence variations, the wildtype and mutated, in a single RT-qPCR reaction. For the rapid identification of mutations of interest for mobile and rapid RT-qPCR platforms, such as the *peak*PCR device, only the mutated sequence variation is detected, and no multiplex amplification is performed. SNP discrimination was enhanced by using primers and probes containing locked nucleic acids (LNAs). Sequence analysis and primer design were performed using the Geneious Prime 2021.0.3 software. All oligos, including the LNAs, were synthesized at Microsynth AG (Balgach, Switzerland), and details are provided in Table 1.

SARS-CoV-2 HV69/70-, E484K-, and N501Y-Specific RT-qPCR Assays. The HV69/70-, E484K-, and N501Y-specific assays were performed using the Bio-Rad CFX96 real-time PCR System (Bio-Rad Laboratories, California, USA). A RT-qPCR run was completed within 1 h and 10 min using the following thermal profile: reverse transcription step at 50 °C for 5 min; polymerase activation at 95 °C for 20 s; and 45 cycles of 3 s at 95 °C and 30 s at 61 °C. Each reaction consisted of 2 μL of RNA and 8 μL of reaction master mix containing 1× TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific, Leiden, The Netherlands) and the corresponding 1× primer/probe mixture consisting of 0.4 μM primers and 0.2 μM probes. All RT-qPCR assays were run in duplicates with appropriate controls. The mutated sequences were detected by Fluorescein (FAM)-labeled probes and the wildtype sequences by Yakima yellow (YYE)-labeled probes in multiplex reactions. Data analysis of the RT-qPCR data was conducted using the CFX Maestro Software (Bio-Rad Laboratories, California, USA). RT-qPCR amplification efficiencies were calculated based on the slope of the standard curve, as described elsewhere.[20]

The HV69/70-, E484K-, and N501Y-specific RT-qPCR assays were transferred to the *peak*PCR platform (Diaxxo AG, Zurich, Switzerland) on which FAM-labeled probes detected the mutated sequence variations only. In order to simplify the testing procedure for the user, the *peak*PCR aluminum sample holders (herein referred to as cartridges) were preloaded with all necessary reagents in the freeze-dried form. Lyophilized cartridges were loaded with 4.4 μL of sample, sealed off with 1.2 mL of paraffin oil (Sigma-Aldrich, Germany), and run on the *peak*PCR device using the following program: reverse transcription step at 50 °C for 5 min; initial denaturation at 95 °C for 60 s; and 45 cycles of 6 s at 95 °C and 30 s at 62 °C. The total runtime of a *peak*PCR experiment was 37 min. *Peak*PCR data were analyzed using the *peak*PCR *data*Analysis 1.0 software (Diaxxo AG, Zurich, Switzerland). No drop in performance was observed when lyophilized reagents were used compared to nonlyophilized standard RT-qPCR reagents (Figure S3).

Evaluation of Diagnostic Performance with Clinical Samples. Clinical evaluation was conducted using RNA extracted from SARS-CoV-2-positive samples collected in Equatorial Guinea. Sample collection and analysis was done as part of a research collaboration with the Equato-Guinean Ministry of Health and Social Welfare and was enabled by several presidential emergency decrees. All patient data were fully anonymized, and publication was approved by the National Technical Committee for the Response and Monitoring of the Novel Coronavirus (Comité Técnico Nacional de Respuesta y Vigilancia del Nuevo Coronavirus), which is charged with preventing, containing, controlling, tracking, and evaluating the development and evolution of COVID-19 in Equatorial Guinea.

MinION SARS-CoV-2 Whole Genome Sequencing. A total of 59 SARS-CoV-2-positive samples from Equatorial Guinea were selected for reconfirmation using WGS by MinION (Oxford Nanopore Technologies, Oxford, UK) according to the open-source ARTIC protocol (https://artic.network/ncov-2019). Sample preparation for MinION sequencing was based on the ARTIC Network nCoV-2019 sequencing protocol v2[21] and v3.[22] The RNA samples were diluted in nuclease-free water according to their cycle threshold value in the diagnostic RT-qPCR. (Cq <15: 1:100 dilution, Cq 15−18: 1:10 dilution) for cDNA synthesis, for which either SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, USA) or LunaScript RT SuperMix (New England BioLabs, USA) was used with random hexamer primers. A total of 218 primer pairs covering the whole virus genome were used for PCR amplification.[23] The ligation sequencing kit (Oxford Nanopore Technologies, UK) was used for library preparation. Sequencing was conducted on a FLO-MIN106 (R9.4.1) flow cell. Base calling was performed in real time on a MinION Mk1c using MinKNOW version 20.10.6. The ARTIC Network bioinformatics protocol was followed for data analysis.[24] Consensus sequences were generated with the Wuhan Hu-1 isolate (GenBank accession number MN908947.3) as a reference sequence. Variants were called using Nanopolish and Medaka. Lineage assignment was done using the pangolin tool.[25] All sequences are deposited in GISAID.

RESULTS

Design of RT-qPCR Assays for the Rapid Identification of SARS-CoV-2 VOCs. Three mutation-specific RT-qPCR assays based on TaqMan chemistry were designed. The first assay targets the 6 bp deletion in the spike gene, leading to the loss of two amino acids at positions 69 and 70 within the spike protein (HV69/70 assay). This deletion is found in the Alpha VOC, but not in Beta or Gamma VOCs. Universal primers amplify a 102-bp (wildtype) or 96-bp (mutant) amplicon. Based on the presence or absence of the deletion, either a FAM-labeled probe or YYE-labeled probe binds, and the resulting fluorescence is detected. The second assay targets a nonsynonymous SNP in the spike gene (A23011G), leading to an amino-acid exchange at positions 484 (E484K). The E484K mutation is only present in Beta and Gamma VOCs. In a multiplex reaction, a YYE-labeled probe detects the wildtype and a FAM-labeled probe the mutated sequence. The third assay targets a nonsynonymous SNP in the spike gene (A23063T), leading to an amino-acid exchange at positions 501 (N501Y). The N501Y mutation is present in Alpha, Beta, and Gamma VOCs. Similar to the E484K assay, a YYE-labeled

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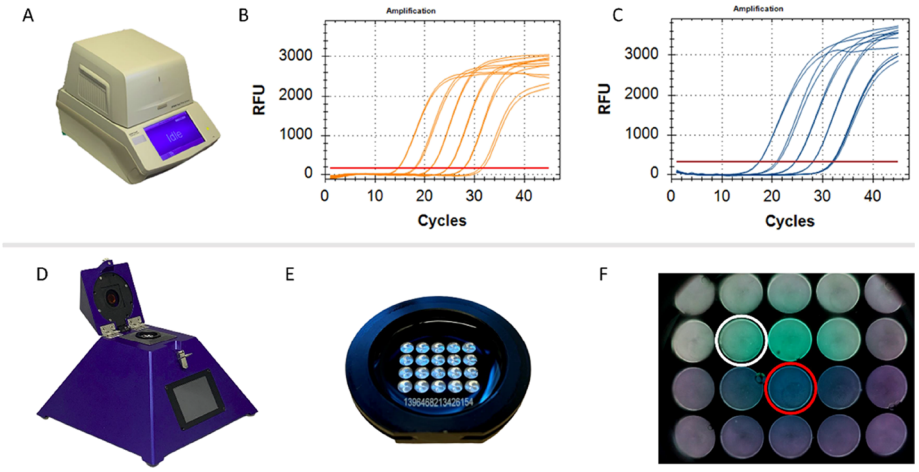

Figure 1. RT-qPCR platforms for SARS-CoV-2 VOC identification. (A) Standard RT-qPCR device Bio-Rad CFX96. (B) Detection of N501Y-wt in serial dilution of Wuhan Hu-1 lineage, ranging from 1 to 10,000 cp/μL using the YYE channel of the Bio-Rad CFX96 instrument. (C) Detection of N501Y-mut in serial dilution of P.1 lineage, ranging from 1 to 1,000,000 cp/μL using the FAM channel of the Bio-Rad CFX96 instrument. (D) Portable and rapid diagnostic platform *peak*PCR. (E) Ready-to-use cartridges with preloaded lyophilized RT-qPCR reagents. (F) Photograph after cycle 45, detecting fluorescence in each well with a CCD sensor. The depicted well marked with a white circle contains a positive signal after RT-qPCR amplification, while the negative sample, marked with a red circle, did not display amplification of a PCR product.

probe detects the wildtype and a FAM-labeled probe the mutated sequence. A summary of the oligonucleotide sequences used is provided in Table 1. Wildtype sequences are defined as the nucleotide sequences of the original Wuhan Hu-1 isolate published.[26]

The novel assays were run on two different RT-qPCR platforms in parallel. On the Bio-Rad CFX96 platform (Figure 1A), three sequence-discriminatory assays were run as duplex assays, detecting the wildtype sequence in the YYE channel (Figure 1B) and the mutated sequence in the FAM channel (Figure 1C). As a second technology platform, the *peak*PCR device was selected (Figure 1D), which is a portable and rapid diagnostic technology platform running the RT-qPCR reaction on ready-to-use cartridges (Figure 1E). Fluorescence is detected using the Raspberry Pi Camera Module V2 as an inexpensive charge-coupled detector (CCD) sensor (Figure 1F). For the *peak*PCR device, the multiplex assays were reduced to mutation-specific assays, capable of detecting the mutated sequences only.

Analytical Performance of HV69/70, E484K, and N501Y Assays Using Well-Characterized RNA from SARS-CoV-2 VOCs. Four SARS-CoV-2 lineages, namely, Wuhan Hu-1 (wildtype, non VOC), B.1.1.7 (Alpha VOC), B.1.351 (Beta VOC), and P.1 (Gamma VOC), were used to assess the RT-qPCR efficiency, specificity, and sensitivity of novel mutation-specific assays. We used serial dilutions, ranging from 0.1 to 1,000,000 cp/μL, of cell culture-derived viral RNA for assay characterization. The presence and quantity of RNA molecules in these serial dilutions were confirmed by monitoring the pan-Sarbecovirus E-gene amplification (Figure 2A). Mutation-specific assays for HV69/70 (Figure 2B), E484K (Figure 2C), and N501Y (Figure 2D) were run on both platforms, while wildtype-specific assays were solely run on the Bio-Rad CFX96 platform.

The data provided in Figure 2 were used to obtain the RT-qPCR amplification efficiencies, sensitivities, and specificities shown in Figure 3. High amplification efficiencies (>90%) were obtained for the E-gene, the HV69/70 and E484K assays on the Bio-Rad CFX96 platform, and for the E-gene and E484K assays on the *peak*PCR device. All other assays achieved amplification efficiencies >80%, which is considered moderate (Figure 3A). The analytical sensitivity of the assays was defined as the lowest viral RNA concentration at which mutations are identified in >80% of replicates. We used the detection rate among all four SARS-CoV-2 lineages to identify the limit of detection (LOD) (Figure 3B). For the E-gene, HV69/70, and E484K assay, a detection rate of 100% was achieved at a viral RNA concentration as low as 10 cp/μL. At the same concentration for the N501Y assay, 5 out of 6 replicates (83%) were amplified. At the LOD of 10 cp/μL (dashed line in Figure 3B), no difference between the two RT-qPCR platforms in terms of sensitivity was observed. Viral RNA concentrations below 10 cp/μL cannot be detected, with the exception of the HV69/70 assay run on the Bio-Rad CFX96 device, where 1 cp/μL is still reliably detected.

The specificity of all three assays and their ability to distinguish between mutated and wildtype sequences were assessed by testing the assays with RNA from SARS-CoV-2 cell culture supernatants. On both platforms, no signal was observed at any viral RNA concentration if there was not a perfect sequence match of the oligos to the nucleotide sequence to be detected, resulting in a 100% analytical specificity. At a viral RNA concentration of 10,000 cp/μL, the HV69/70, E484K, and N501Y genotypes were all correctly identified among wildtype, Alpha, Beta, and Gamma SARS-CoV-2 strains (Figure 3C). The mutation-specific probe of the HV69/70 assay gave a signal only when run with RNA of the Alpha VOC carrying the mutation. The E484K-mutation assay did not result in amplification when run on RNA from wildtype non-VOC Wuhan Hu-1 lineage and Alpha VOC. The N501Y-mutation assay correctly detected all VOCs but not the wildtype non-VOC Wuhan Hu-1 lineage. In summary, the three assays correctly identify lineage-associated mutations with moderate to high RT-qPCR efficiencies in samples with more than 10 cp/μL of SARS-CoV-2 RNA. We also demonstrated that these assays can be successfully conducted on the rapid diagnostic platform *peak*PCR, and the performance in terms of sensitivity and specificity does not significantly differ between these two RT-qPCR platforms.

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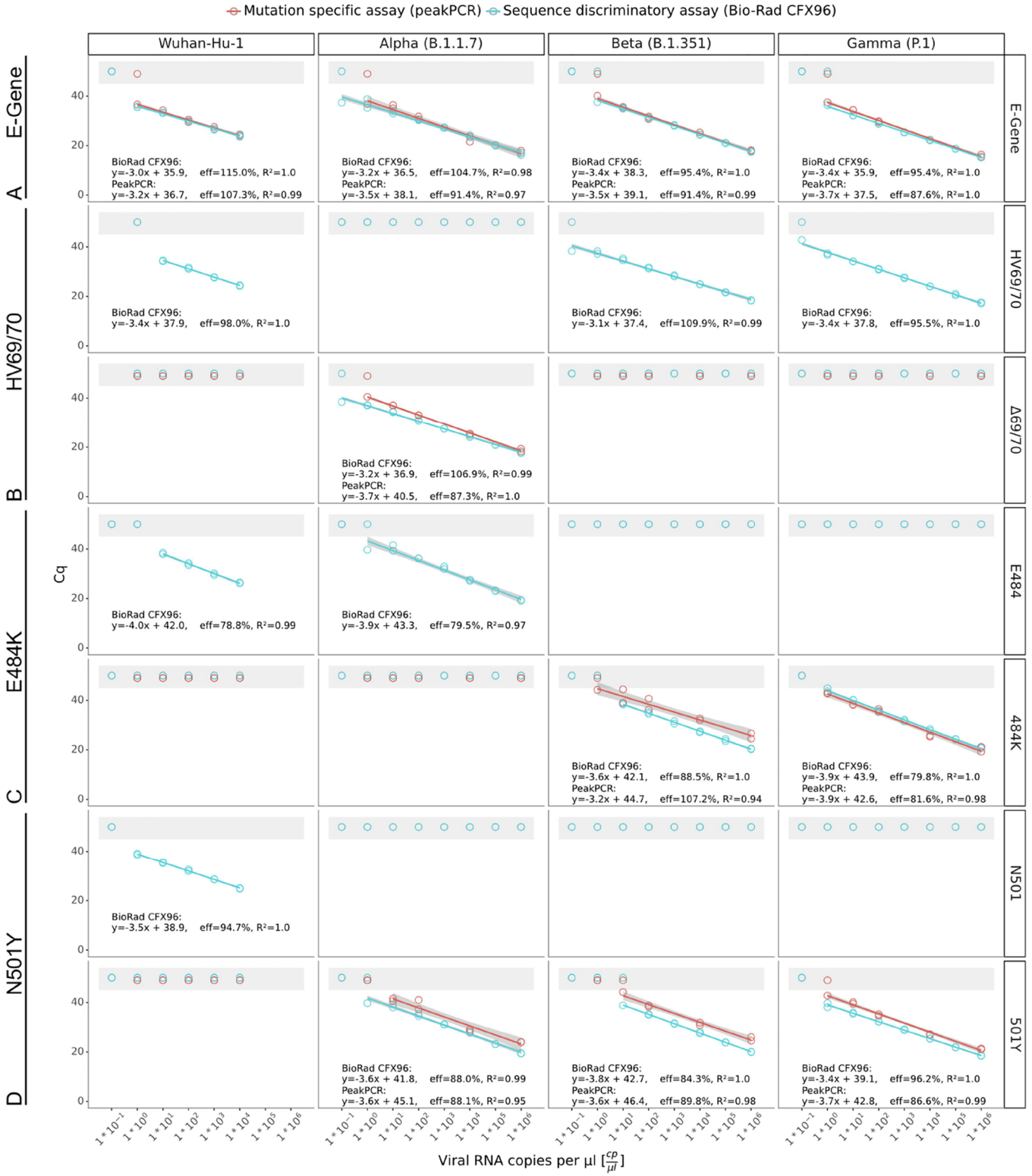

Figure 2. RT-qPCR performance of novel SARS-CoV-2 mutation-specific assays. (A) SARS-CoV-2 E-gene reference assay, (B) HV69/70 assay, (C) E484K assay, and (D) N501Y assay. Each circle represents a technical replicate. Mutation-specific assays were run on both platforms, while wildtype-specific assays were only run on the Bio-Rad CFX96 platform. For the Wuhan Hu-1 lineage, the two highest RNA concentrations of 1,000,000 and 100,000 cp/μL were not available. Tests for performance on the *peak*PCR device used the 1,000,000, 10,000, 100, 10, and 1 cp/μL concentrations. The Cq values for samples without amplification are set arbitrarily to 46 for the *peak*PCR and to 47 for Bio-Rad CFX96 devices. Data points within the gray area are considered negative (Cq values >45).

Clinical Performance of HV69/70, E484K, and N501Y Detecting RT-qPCR Assays. We used 59 clinical samples positive for SARS-CoV-2 collected in Equatorial Guinea between November 2020 and March 2021 for further evaluation of all three mutation-specific RT-qPCR assays. The outcome of the RT-qPCR assays was compared to

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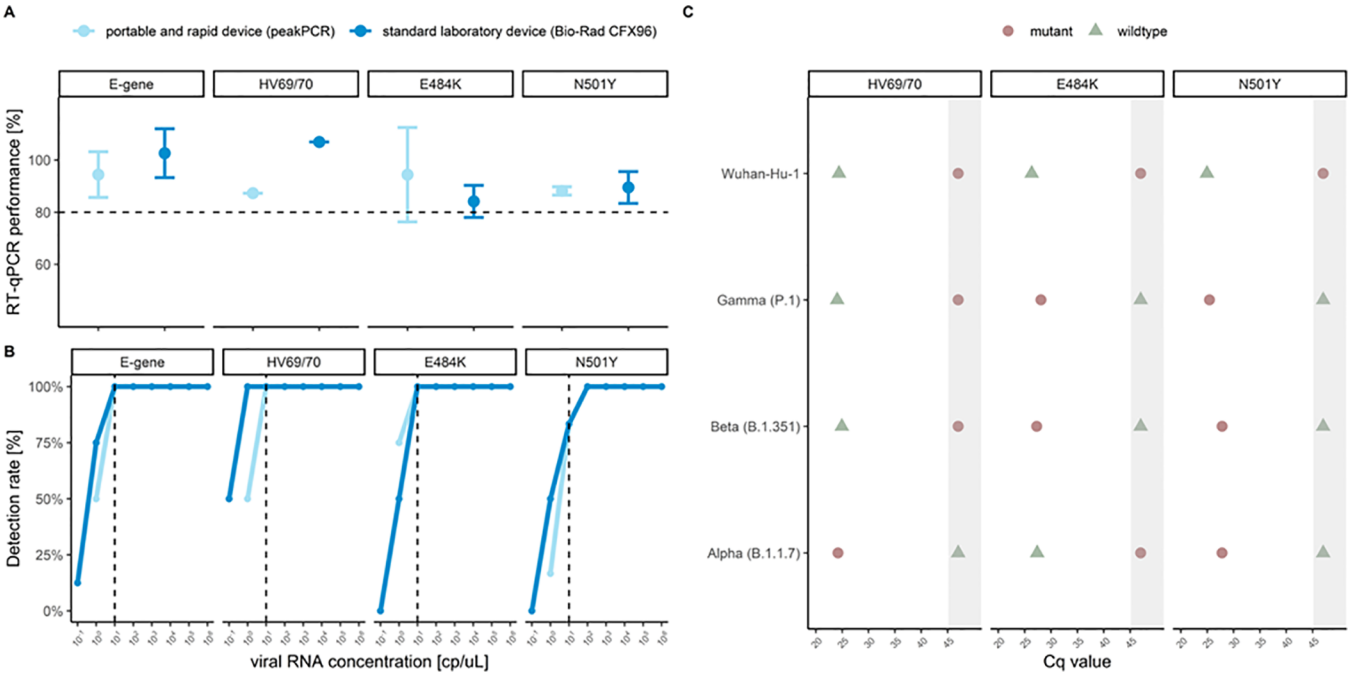

Figure 3. Analytical performance of HV69/70, E484K, and N501Y detecting RT-qPCR assays. (A) RT-qPCR amplification efficiency of the E-gene, HV69/70, E484K, and N501Y assays as determined by serial dilutions of RNA derived from four cell culture supernatant SARS-CoV-2 lineages. Amplification efficiencies >80% are considered moderate (dashed lines). (B) Analytical sensitivity represented by detection rates calculated from all replicates for each viral RNA concentration. The LOD was defined as the lowest concentration at which >80% of replicates were amplified. The dashed line represents the LOD of 10 copies per μL. (C) Analytical specificity for the multiplex sequence-discrimination assays run on the Bio-Rad CFX96 device. The data shown are based on RT-qPCR amplification for viral RNA concentrations of 10,000 cp/μL. Data points within the gray area are considered negative (Cq values >45).

Table 2. Performance Evaluation of HV69/70, E484K, and N501Y Detecting RT-qPCR Assays Using Clinical Samples

SARS-CoV-2 lineage	Mutation profile[a]	*n*	HV69/70	Δ69/70	E484	484K	N501	501Y
wild type[b]	HV69/70, E484, N501	14	14/14	0/14	14/14	0/14	14/14	0/14
alpha (B.1.1.7)	Δ69/70, E484, 501Y	1	0/1	1/1	1/1	0/1	0/1	1/1
beta (B.1.351)	HV69/70, 484K, 501Y	43	43/43	0/43	0/43	43/43	0/43	43/43
B.1.620	Δ69/70, 484K, N501	1	0/1	1/1	0/1	1/1	0/1	0/1

[a]Based on SARS-CoV-2 WGS. [b]Includes the following SARS-CoV-2 lineages: B.1, B.1.1, B.1.177, B.1.192, B.1.36.10, B.1.535, B.1.596, B.1.623.

Nanopore MinION-based SARS-CoV-2 WGS data obtained from the same clinical samples (Table 2). The HV69/70 assay identified the spike gene deletion Δ69/70 correctly in 2 out of 2 samples, while for all other samples, in accordance with WGS, the wildtype HV69/70 genotype was found. The E484K assay accurately identified the 484K SNP in 43 samples with the Beta VOC and in one sample assigned to the B.1.620 lineage. The N501Y assay correctly identified the 501Y mutation in one confirmed Alpha VOC sample and 43 confirmed Beta VOC samples. In summary, the evaluation with clinical samples resulted in a 100% agreement between the novel mutation-specific RT-qPCR assays and SARS-CoV-2 WGS.

Investigating the Introduction and Spread of SARS-CoV-2 Beta VOC in Equatorial Guinea Using Mutation-Specific RT-qPCR Assays. In total, we analyzed 184 SARS-CoV-2-positive samples from Equatorial Guinea using all three mutation-specific RT-qPCR assays collected from November 2020 to March 2021 (Figure 4A). While between November and December 2020, all samples were wildtype for the three spike gene mutations associated with SARS-CoV-2 VOCs, starting from January 2021, more than 85% (102/119) of samples carried the 484K + 501Y mutant combination. The WGS analysis of a subset of these samples revealed an expansion of the SARS-CoV-2 Beta VOC (lineage B.1.351) in Equatorial Guinea (Figure 4B). Other combinations of mutations of interest were also found, the sample with Δ69/70 + 501Y was identified as the Alpha variant (B.1.1.7 lineage), and the sample with Δ69/70 + 484K was identified as the B.1.620 lineage. In summary, these RT-qPCR-based assays enable rapid and cost-effective genotyping of larger numbers of clinical samples, resulting in a more accurate reflection of SARS-CoV-2 epidemiology and their local transmission dynamics.

DISCUSSION

The COVID-19 pandemic is a continuous, unprecedented, global public health crisis with severe economic and social consequences.[27] More than 1 year into the pandemic, the emergence of VOCs starts to pose again a serious threat to contain the virus. Rapid and reliable identification of SARS-CoV-2 variants is a critical component of public health interventions to mitigate the further spread of VOCs that might undermine the performance of diagnostic tests and vaccine-induced immunity against this virus.[28]

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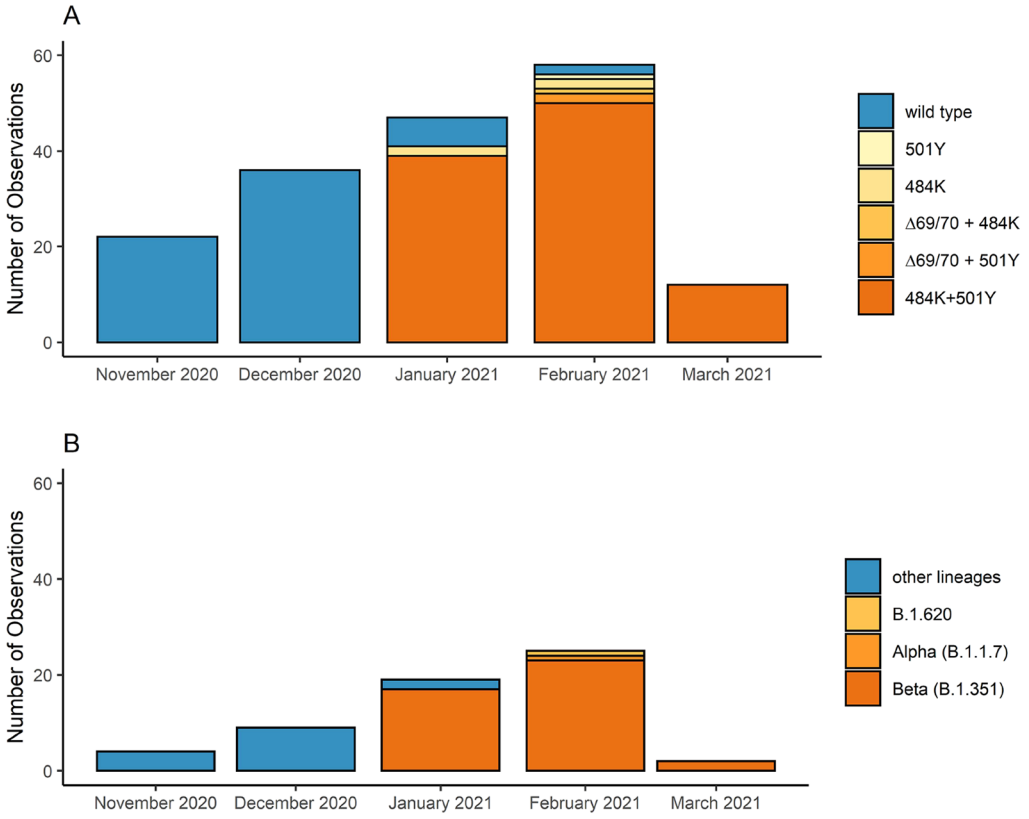

Figure 4. Rapid detection of SARS-CoV-2 VOC-associated mutations using HV69/70, E484K, and N501Y RT-qPCR assays. (A) Identification of spike gene mutations using HV69/70, E484K, and N501Y RT-qPCR assays in 184 clinical samples collected in Equatorial Guinea from November 2020 to March 2021. (B) Identification of SARS-CoV-2 lineages using Nanopore MinION SARS-CoV-2 WGS in 59 clinical samples collected in Equatorial Guinea from November 2020 to March 2021.

We designed, tested, and validated three mutation-specific RT-qPCR assays, detecting the E484K and N501Y SNPs as well as the 6-bp deletion affecting HV69/70, all located in the SARS-CoV-2 spike gene. All assays can be performed under standard RT-qPCR conditions, simplifying integration into existing laboratory environments, and the assays proved to be highly sensitive, specific, and reproducible. We demonstrated the usefulness of such screening assays to rapidly identify potential VOCs using clinical samples. Using our assays, we were able to observe the introduction and spread of the B.1.351 lineage in Equatorial Guinea. It is noteworthy that it took less than 4 weeks for the B.1.351 lineage to become the dominant lineage in this cohort. Using more than one VOC marker enabled us to identify SARS-CoV-2 variants with an unusual combination of mutations, such as the E484K plus the HV69/70 deletion. This sample was later assigned to the SARS-CoV-2 lineage B.1.620, which is most likely of Central African origin and has recently been described in several countries in Europe.[29]

The approach presented here is well suited for cost-effective, robust, and high-throughput screening of large cohorts. Mutation-specific RT-qPCRs are not intended to replace NGS, but rather complement and extend molecular surveillance programs and focal outbreak monitoring. During the preparation of this manuscript, the WHO had designated the lineage B.1.617.2 as the fourth VOC. The Delta variant (lineage B.1.617.2) was first documented in India, where it has contributed to the surge in cases and has now been detected across the globe.[30] Based on the perfect mismatch discrimination of LNA-based assays and the simplicity of this type of assay in both design and implementation will allow for rapid adaptation of our approach to B.1.617.2 and also to newly emerging VOCs identified in future.

A similar LNA-based approach for the detection of N501Y and HV69/70 has been successfully tested in Canada on 2430 samples.[31] The results of their in-house assay were concordant with the commercial assay VirSNiP SARS-CoV-2 (TIB Molbiol, Berlin, Germany), which is based on melting-curve analysis. This underlines the possibility of using LNA-based RT-qPCR assays for single-nucleotide discrimination as opposed to using melting-curve analysis. Although the SARS-CoV-2 SNP genotyping by melting-curve analysis is widely used,[32] the LNA-based approach has several advantages: it is faster, easier to integrate into existing laboratory workflows, and could be combined with a diagnostic assay, allowing immediate genotyping. Furthermore, LNA-based sequence-discriminatory assays are better suited to identify the presence of more than one lineage of SARS-CoV-2 in a single sample, a phenomenon which was recently observed in Brazil.[33]

To reduce the sample-to-result turnaround time in routine, decentralized testing settings, our strategy included the transfer of these assays to a portable, robust, and rapid diagnostic RT-qPCR platform. Starting from extracted RNA, the *peak*PCR platform completed sample analysis in 37 min, which is half of the time required to run the same assay on a standard RT-qPCR platform, while retaining comparable efficiency, specificity, and sensitivity. For the first time, we show that complex RT-qPCR-based genotyping assays can be transferred to the rapid and portable *peak*PCR platform. Apart from the speed and simplicity of usage, the relatively low costs of

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equipment and reagents make this platform interesting. The *peak*PCR device costs are an estimated $2500, a fraction of the price at which commercially available qPCR devices are being sold, and because of the lower reaction volume used in *peak*PCR experiments (4 μL), the cost per preloaded and freeze-dried 20-well cartridge is kept similar to the reagent costs for a standard RT-qPCR reaction. Future technological developments will focus on simplified sample-preprocessing strategies to replace the RNA extraction step completely. This would allow the placement of this molecular diagnostic platform in peripheral health care settings. Furthermore, the deployment of cartridges preloaded with lyophilized reagents independent from the cold chain for reagent supply is a significant technological advantage that makes this platform well suited for decentralized, rapid molecular testing of infectious diseases, particularly in resource-limited settings.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c02368.

A 1869 bp long synthetic SARS-CoV-2 spike gene fragment based on the sequence of the SARS-CoV-2 Alpha VOC; serial dilution experiments for HV69/70 and N501Y assays using the synthetic gene fragment of the SARS-CoV-2 Alpha VOC; and comparison of lyophilized reagents with nonlyophilized standard RT-qPCR reagents on the *peak*PCR device. (PDF)

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Author Contributions

Conceptualization by S.H., P.B., P.W., and T.S. *peak*PCR platform technology and cartridge design: P.B., A.R.S, M.G., W.J.S. Formal analysis was done by P.W. and T.S. D.S. and O.E. provided the cell culture-derived RNA from SARS-CoV-2 VOCs. Clinical evaluation was conducted by M.P., F.A.O, J.O., and M.O.A. The first draft of the manuscript was written by P.B., C.A.D., and T.S. All authors reviewed and approved the final manuscript. P.B. and P.W. contributed equally to this study.

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Notes

The authors declare the following competing financial interest(s): P.B., M.G., and W.J.S. are co-inventors on a corresponding patent application around the detection platform and share-holders of the ETH spin-off company Diaxxo AG. T.S. has consulted for Diaxxo AG. All other authors declare no competing interests.

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