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VIROLOGY

Single-Amplicon Multiplex Real-Time Reverse Transcription-PCR with Tiled Probes To Detect SARS-CoV-2 *spike* Mutations Associated with Variants of Concern

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ABSTRACT To provide an accessible and inexpensive method to surveil for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) mutations, we developed a multiplex real-time reverse transcription-PCR (rRT-PCR) assay, the Spike single-nucleotide polymorphism (SNP) assay, to detect specific mutations in the *spike* receptor binding domain. A single primer pair was designed to amplify a 348-bp region of *spike*, and probes were initially designed to detect K417, E484K, and N501Y. The assay was evaluated using characterized variant sample pools and residual nasopharyngeal samples. Variant calls were confirmed by SARS-CoV-2 genome sequencing in a subset of samples. Subsequently, a fourth probe was designed to detect L452R. The lower limit of 95% detection was 2.46 to 2.48 $\log_{10}$ genome equivalents (GE)/ml for the three initial targets (~1 to 2 GE/reaction). Among 253 residual nasopharyngeal swabs with detectable SARS-CoV-2 RNA, the Spike SNP assay was positive in 238 (94.1%) samples. All 220 samples with threshold cycle (C_T) values of <30 for the SARS-CoV-2 N2 target were detected, whereas 18/33 samples with N2 C_T values of ≥30 were detected. Spike SNP results were confirmed by sequencing in 50/50 samples (100%). Addition of the 452R probe did not affect performance for the original targets. The Spike SNP assay accurately identifies SARS-CoV-2 mutations in the receptor binding domain, and it can be quickly modified to detect new mutations that emerge.

KEYWORDS COVID-19, diagnostics, molecular epidemiology, variants

Viral mutations are a natural phenomenon and a by-product of replication (1). Like other RNA viruses, coronaviruses rely on an error-prone RNA-dependent RNA polymerase (RdRp) for replication (2). Coronaviruses also encode a 3′-to-5′ exoribonuclease that provides a proofreading function and reduces error rate, resulting in a comparatively low rate of viral mutation (approximately 1 or 2 nucleotides per month) and low sequence diversity (3). While many mutations are inconsequential, over time, mutations that confer a fitness advantage with respect to viral replication, transmission, or escape from immunity

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will be positively selected (1). Since its identification in China in late 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused more than 167 million infections and more than 3.4 million deaths worldwide (1). With high transmission rates globally, there has been a dramatic increase in the emergence of variants with concerning mutations and phenotypic traits (2, 3). These lineages, many of which share common mutations in the spike protein (e.g., N501Y, E484K and L452R), have been declared variants of concern (VOC) by public health authorities (https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html). Since their emergence, studies have confirmed increased transmissibility and decreased susceptibility to antibody neutralization of strains bearing these mutations compared to those of ancestorial lineages (2, 4–7).

Global genomic surveillance and rapid, open-source sharing of viral genome sequences have facilitated the detection and tracking of VOC. SARS-CoV-2 genome sequencing remains the gold standard to identify circulating variants. However, given the limited availability, technical requirements, and turnaround time for viral genome sequencing, broadly accessible and inexpensive assays would be advantageous to track specific mutations around the world, analogous to the initial use of the S gene target failure to track and monitor the emergence of B.1.1.7 (8, 9). To this end, we designed and validated a single-reaction multiplex real-time reverse transcription-PCR (rRT-PCR) assay to detect specific mutations associated with VOC, here referred to as the Spike SNP assay.

MATERIALS AND METHODS

Study design. Spike single-nucleotide polymorphism (SNP) primers and probes were initially designed based on available whole-genome sequences of VOC. The assay was optimized and evaluated using characterized variant sample pools. Finally, clinical evaluation was performed by testing a convenience set of clinical samples in the Spike SNP assay and a comparator SARS-CoV-2 rRT-PCR assay. Mutation calls were evaluated by SARS-CoV-2 genome sequencing in a subset of samples.

Spike SNP design and optimization. The Spike SNP assay was designed from an alignment of all whole-genome sequences of B.1.351 and P.1 variants that were uploaded into the GISAID database between 1 September 2020, and 4 February 2021. Sequences were downloaded into Geneious Prime software (version 2021.1.1) and aligned to the reference strain hCoV-19/USA/WA1/2020 (GenBank accession number MN985325.1). Target regions for primer design in the *spike* gene were selected manually by identifying fully conserved regions 5′ to codon 417 (forward primer) and 3′ to codon 501 (reverse primer). Primers and probes were designed in Primer3 (version 4.1.0; https://bioinfo.ut.ee/primer3/). Probes were designed for SNPs that confer mutations at amino acids 417, 484, and 501 in spike, with the goal of positioning the SNP in the middle of the probe. Probe sequences from Primer3 were entered into OligoAnalyzer (Integrated DNA Technologies, Coralville, IA) and altered to (i) include 3 consecutive locked nucleic acids (LNAs) centered on the SNP and (ii) shorten probe length to maintain the predicted melting temperature. An unmodified probe for 452R was later designed using similar methodology. Candidate probes were manually redesigned based on Primer3 results to have melting temperatures similar to those of the LNA probes.

Singleplex assays were initially evaluated using SARS-CoV-2 RNA and each primer/probe set with 400 nM each primer and 200 nM probe in the final reaction mixture. PCR cycling conditions were optimized to improve signal differentiation between variant and nonvariant strains. Probes were then combined sequentially, and each multiplex assay was compared side-by-side with the component singleplex assays (see Fig. S1 in the supplemental material). As no change in threshold cycle (C_T) value or fluorescence was observed in the multiplex design during optimization, the Spike SNP assay was further evaluated only as a multiplex test, with probes for the targeted mutations labeled with the following fluorophores: for K417, fluorescein (FAM); 484K, cyanine 5 (Cy5); 501Y, 6-carboxy-2,4,4,5,7,7-hexachlorofluorescein (HEX); and for 452R, Cal Fluor red 610 (Table 1).

rRT-PCR performance. Clinical samples were extracted on either an EMAG (bioMérieux, Durham, NC) or an Abbott m2000sp (Abbott, Chicago, IL) instrument. All variant sample pools were extracted on the EMAG instrument. Nucleic acids were extracted from 500 μl of nasopharyngeal (NP) swabs or from 100 μl of variant sample pools and eluted in 50 μl. All rRT-PCRs were performed on a Rotor-Gene Q instrument (Qiagen, Germantown, MD) using 20-μl reaction mixtures of the Luna probe one-step reverse transcription-quantitative PCR (RT-qPCR) kit (New England Biolabs, Ipswich, MA) and 5 μl of nucleic acid eluate. Samples were tested with a duplex reaction for the SARS-CoV-2 N2 target and RNase P (referred to as N2-RP) and with the Spike SNP assay. N2-RP was performed using the primer and probe concentrations described previously (10). The Spike SNP assay was performed on a separate run with the following conditions: 52°C for 15 min, 94°C for 2 min, and 45 cycles of 94°C for 15 s and 60°C for 60 s. Fluorescent signal was acquired in all channels at 60°C. Thresholds were established for each channel during assay optimization, as described previously (11), and used for analysis of all subsequent runs. During Spike SNP analysis, outlier removal was performed to remove any signal that did not reach 10% (K417) or 20% (484K and 501Y) of the maximum fluorescence in a given channel.

TABLE 1 Spike SNP assay primers and probes

Primer or probe name	Sequence[a]	Concn (nM)[b]	Location (5′–3′)[c]
Primers			
SpikeSNP Ext	TGAAGTCAGACAAATCGCTCC	400	22777–22797
SpikeSNP R1	TGGTGCATGTAGAAGTTCAAAAG	400	23103–23125
LNA probes			
K417	5′-FAM-CAAACTGGA+A+**A**+**G**+ATTGCTG-IABkFQ-3′	200	22802–22820
484K	5′-Cy5-ACCTTGTAATGGTGT+T+**A**+AAGGTTTT-IAbRQSp-3′	200	22996–23020
501Y	5′-HEX-CCCAC+T+**T**+ATGGTGTTGG-IABkFQ-3′	200	23057–23073
Unmodified probes			
452R	5′-CFR610-ATAATTACC**G**GTATAGATTGTTTAGGAAGT-BHQ-2-3′	200	22908–22937

[a]Probe sequences are listed in the following order: 5′-fluorophore-sequence-quencher-3′. A plus sign (+) before a base indicates a locked nucleic acid; underlined and boldfaced bases indicate targeted single-nucleotide change. BHQ, black hole quencher; HEX, 6-carboxy-2,4,4,5,7,7-hexachlorofluorescein; CFR610, Cal Fluor red 610; Cy5, cyanine 5; FAM, fluorescein; IABkFQ and IAbRQSp, Iowa Black quenchers.
[b]Concentration in the final reaction mixture.
[c]Nucleotide location in the Wuhan-Hu-1 complete genome sequence (NCBI reference sequence accession number NC_045512.2).

Reference strains and variant sample pools. Extracted SARS-CoV-2 RNA from cultured strains of B.1.1.7 and B.1.351 were used for assay evaluation and optimization. Following 1 or 2 passages in cell culture, variants were confirmed by sequencing prior to testing in the Spike SNP assay. Inactivated variant sample pools were obtained through the NIH/RADx Variant Task Force for the following variants: B.1.1.7, B.1.2, B.1.351, B.1.375, B.1.427, B.1.429, B.1.525, B.1.526, P.1, and P.2 (Table 2). Briefly, inactivated samples in saline from sequence-confirmed SARS-CoV-2 variants were provided by Helix (San Mateo, CA). Samples were matched based on lineage and specific mutations in *spike*, and pools were made from a combination of samples, followed by dilution and aliquoting. Initial pools were extracted and tested in the N2-RP rRT-PCR assay. Dilution series (5- or 10-fold, based on initial C_T) were prepared, coded, and tested in a blind fashion in the N2-RP and Spike SNP rRT-PCR assays. Dilutions of BEI 52286 were included as a wild-type control.

Analytical evaluation. Linearity and lower limit of 95% detection (95% LLOD) of the Spike SNP assay were determined for each target using quantified genomic RNA from B.1.1.7 and B.1.526 variant sample pools. RNA was quantified in genome equivalents per milliliter on a single run of the N2-RP assay with a standard curve of quantitative synthetic RNA (catalog no. NR-52358; BEI Resources, Manassas, VA). Linearity and 95% LLOD were then evaluated on a single run of the Spike SNP assay by testing 5 serial 10-fold dilutions of each strain in quadruplicate, ensuring that <4/4 replicates were detected for at least one dilution. The 95% LLOD was calculated by probit analysis.

Clinical evaluation. Residual SARS-CoV-2 RNA-positive NP samples were obtained from the Emory Hospital Laboratory. Samples included in the current analysis were collected from adults in the Emory Healthcare System between 1 January and 31 March 2021. NP swabs had originally been collected into saline or viral transport medium. Once collected, samples were deidentified, aliquoted, and stored at −80°C until nucleic acid extraction. The study protocol was reviewed and approved by the Emory Institutional Review Board (STUDY00000260).

Sequence analysis. Genome sequencing of RNA from clinical samples was performed as previously described (12). Genomes were aligned to a reference genome using Geneious Prime software (version 2021.1.1). Spike SNP calls were compared to the aligned whole-genome sequences for confirmation.

Statistical analysis. Basic statistical analyses were performed using Prism version 9.0.2 (GraphPad Software, San Diego, CA). The 95% LLOD was calculated by probit analysis using MedCalc version 19.8 (MedCalc Software Ltd., Ostend, Belgium).

RESULTS

Spike SNP assay target *in silico* evaluation. The Spike SNP assay amplifies a 348-bp region of *spike* using a single primer set and probes for specific mutations tiled across the amplicon, which encompasses codons 413 to 514 between the primer sequences. This region contains the receptor-binding motif of spike protein found at the distal aspect of the receptor-binding domain (see Fig. S2 in the supplemental material). Probes using locked nucleic acids (LNAs) centered on specific mutations to selectively detect sequences encoding K417 (reference), E484K (GAA→AAA), and N501Y (AAU→UAU). The nonvariant 417 sequence was targeted to yield (i) signal dropout for B.1.351 (AAG→AAU) and P.1 (AAG→ACG) variants, which harbor distinct mutations in this codon, and (ii) positive-control signals for strains bearing no mutations in targeted codons. Representative results from testing specific SARS-CoV-2 variants, along with result interpretation, are shown in Fig. 1.

An *in silico* evaluation of Spike SNP primers and probes was performed using a set of 382 complete genome sequences from the state of Georgia. In total, 380 sequences

TABLE 2 C_T values for dilution series of pooled variant samples in the N2RP and Spike SNP assays, with expected Spike SNP results shown for each variant

Variant	Concn (log_{10} genome equivalents/ml)[a]	C_T value for:			
			Spike SNP assay		
		N2	K417	484K	501Y
B.1.1.7 (K417, 501Y)	6.5	21.24	25.95		25.18
	5.5	25.69	30.03		29.27
	4.5	29.59	35.45		34.58
	3.5	33.03	38.27		37.51
	2.5	35.23			
	1.5				
B.1.2 (K417)	4.5	28.59	33.40		
	4.2	29.69	34.13		
	3.5	32.12	38.07		
	2.8	34.89			
	2.0	37.53			
B.1.351 (484K, 501Y)	4.7	27.77		32.86	31.50
	4.3	29.38		34.31	32.95
	3.6	31.88		38.47	37.41
	2.7	35.15			
	1.7				
B.1.375 (K417)	4.5	28.59	34.70		
	3.5	31.72	36.04		
	2.5	35.50	41.48		
	1.5		40.31		
B.1.427 (K417)	7.1	18.76	23.44		
	6.1	22.69	27.58		
	5.1	27.03	32.45		
	4.1	30.87	36.43		
	3.1	35.45	39.40		
	2.1				
B.1.429 (K417)	5.8	23.85	28.58		
	4.5	28.31	33.35		
	3.1	33.46	37.10		
	2.1				
B.1.525 (K417, 484K)	6.2	22.39	27.00	27.43	
	5.2	25.98	30.81	31.41	
	4.2	30.71	36.18	36.60	
	3.2	34.52	40.43	40.84	
	2.2		40.50	40.97	
	1.2				
B.1.526 (K417, 484K)	6.3	21.77	26.26	29.37	
	5.3	26.54	31.22	33.96	
	4.3	29.83	35.47	38.69	
	3.3	33.23	39.27	41.38	
	2.3	37.65			
P.1 (484K, 501Y)	5.8	23.71		28.70	27.21
	4.8	27.67		32.78	31.40
	3.8	32.70		37.90	36.61
	2.8	34.67			
	1.8				
P.2 (K417, 484K)	5.8	23.67	27.67	28.34	
	4.8	27.85	31.98	32.55	
	3.8	31.58	36.00	36.48	
	2.8	35.19	39.75	40.24	
	1.8				

(Continued on next page)

TABLE 2 (Continued)

Variant	Concn (log_{10} genome equivalents/ml)[a]	C_T value for:			
			Spike SNP assay		
		N2	K417	484K	501Y
BEI 52286 (K417)	7.1	18.93	22.94		
	6.1	24.11	28.24		
	5.1	28.00	31.99		
	4.1	31.83	36.34		
	3.1	36.60	39.39		
	2.1	36.44	39.31		
	1.1				

[a]Calculated from a standard curve of quantitative synthetic RNA (catalog no. NR-52358; BEI).

completely matched the forward primer sequence (99.48%), and 381 completely matched the reverse primer (99.74%). Two and one sequences harbored a single base pair difference compared to the forward and reverse primers, respectively. No mutations were identified in probe target regions that would alter the identification of targeted SNPs.

Analytical evaluation and testing of variant sample pools. Linearity and the lower limit of 95% detection of the Spike SNP assay were evaluated with dilution series of quantified genomic RNA from variant pools of B.1.1.7 and B.1.526 (Fig. 2A to F). The linear range of the assay extended from 3.3 to 6.3 log_{10} genome equivalents (GE)/ml for the K417 and 501Y targets (B.1.1.7 pool) and from 3.2 to 6.2 log_{10} GE/ml for the K417 and 484K targets (B.1.526 pool). The highest concentrations tested for B.1.1.7 and B.1.526 in this analysis were 6.3 and 6.2 GE/ml, respectively. The lower limit of 95% detection in log_{10} GE/ml was estimated by probit analysis to be 2.46, 2.54, and 2.48 for the K417, 484K, and 501Y targets, respectively. These values correlate to ~1 to 2 GE/reaction.

Sample pools were prepared for the following 10 SARS-CoV-2 variants: B.1.1.7, B.1.2, B.1.351, B.1.375, B.1.427, B.1.429, B.1.525, B.1.526, P.1, and P.2 (Table 2). Dilution series

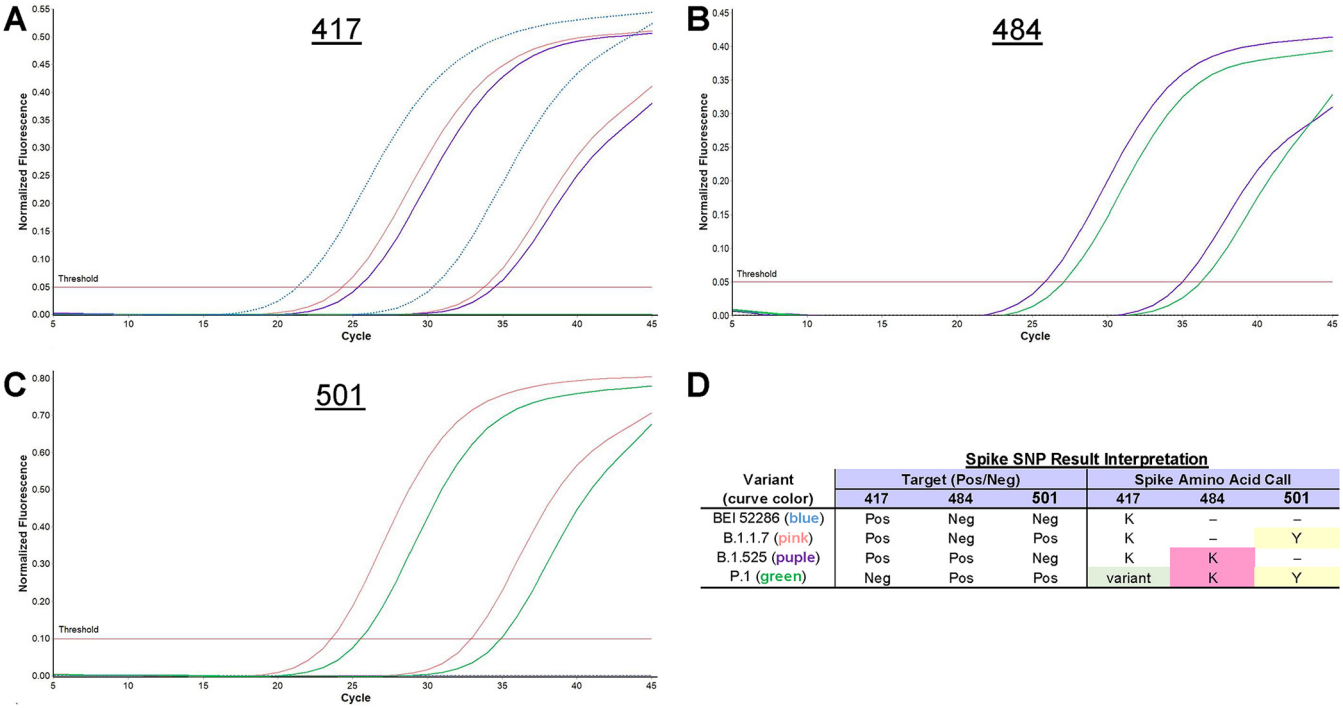


FIG 1 Spike SNP assay distinguishes mutations occurring in different lineages. (A to C) Representative results of variant detection from a single Spike SNP run are shown for mutations in the codons for K417 (A) and mutations that encode 484K (B) and 501Y (C). Curves show dilutions of the following variants: blue, BEI 52286 (wild type); pink, B.1.1.7; purple, B.1.525; green, P.1. Variant pools were used for B.1.1.7, B.1.525, and P.1 strains. Curves are displayed for a given dilution in each channel. (D) Result interpretation.

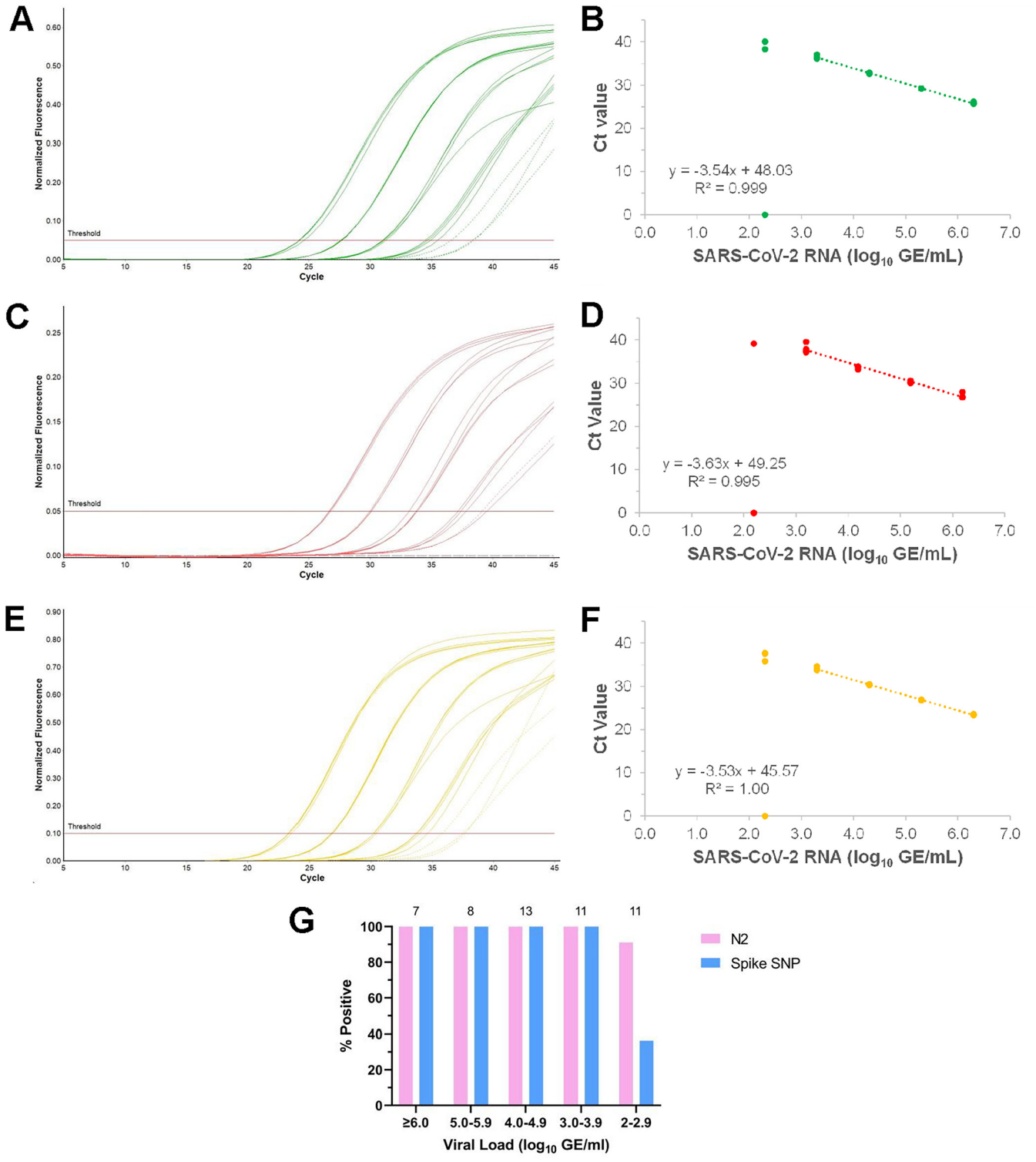
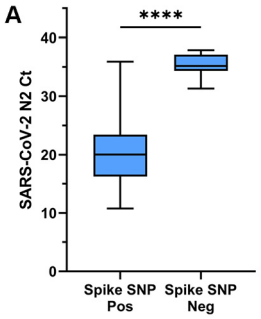


FIG 2 Spike SNP assay linearity and limit of detection. Linearity and limit of detection of K417 (A and B), 484K (C and D), and 501Y (E and F) in the Spike SNP assay were established using 10-fold dilutions of quantified pools of B.1.1.7 (K417 and 501Y) and B.1.526 (K417 and 484K). Amplification curves (A, C, and E) and graphs of threshold cycle (C_T) values versus concentration (B, D, and F) are displayed for each probe. (G) Sensitivity of SARS-CoV-2 detection with the Spike SNP assay compared to detection of the N2 target using dilution series of 11 pools of variant and wild-type strains. Results are expressed as the percentage of pools detected within a given concentration range. Numbers above the graph represent the number of pools tested. Viral loads were calculated from N2 C_T values and a standard curve of BEI quantitative synthetic RNA.



B

SARS-CoV-2 N2 Ct	N	Median (range)	Spike SNP Positive n (%)
< 30	220	19.39 (10.77-29.69)	220 (100)
≥ 30	33	33.57 (30.32-37.88)	18 (54.5)

FIG 3 Clinical evaluation of the Spike SNP assay. (A) SARS-CoV-2 N2 C_T values for clinical samples that tested positive or negative in the Spike SNP assay (****, $P < 0.0001$). (B) Distribution of clinical samples tested and the percentage detected in the Spike SNP assay based on an N2 C_T value cutoff of 30.

of variant pools plus one nonvariant strain (BEI 52286) were tested in a blind fashion in the N2-RP and Spike SNP assays. The Spike SNP assay accurately detected the expected mutations in all strains at concentrations of ≥3.0 $\log_{10}$ GE/ml (Fig. 2G and Table 2). Of 11 dilutions tested at concentrations of 2 to 3 $\log_{10}$ GE/ml, Spike SNP detected 4 (36.4%), compared to 10 (91.1%) detected in the N2-RP assay.

Clinical evaluation. A total of 262 residual NP swabs were tested side by side with the N2-RP and Spike SNP rRT-PCR assays, such that all results were available on the same day as sample processing and no additional freeze-thaw was necessary. Of these, 253/262 samples (96.6%) had detectable SARS-CoV-2 RNA in the N2-RP assay, with a median N2 C_T of 20.3 (range, 10.8 to 37.9). All samples gave the expected signal for the RNase P specimen control. The Spike SNP assay detected a positive signal in at least one channel in 238/253 (94.1%) N2-positive samples and gave a negative result for 15 (5.9%). N2 C_T values were significantly higher in samples that tested negative in the Spike SNP assay (median, 35.2; range, 31.3 to 37.9) compared to those that tested positive (median, 20.0; range, 10.8 to 35.9; $P < 0.0001$; Fig. 3A). All samples with N2 C_T values of <30 (220/220) were detected in the Spike SNP assay, whereas 18/33 samples with N2 C_T values of ≥30 were detected (Fig. 3B).

Results from the Spike SNP assay were consistent with SARS-CoV-2 genome sequencing for 50/50 samples (100%) with complete genome sequences (Table 3). Notably, two samples from one patient had signal for E484K (2/2) and N501Y (1/2), which were not identified in the consensus. Upon review of all reads from these samples, the mutations were found as minor variants in 20 to 45% of reads.

Modification to detect emerging mutations. During the evaluation of the Spike SNP assay, an unmodified probe (without LNAs) was designed in response to the emergence of 452R (CUG→CGG) in the amplified region (Table 1). The probe was designed without modification to hasten production and evaluate assay specificity with standard chemistries. The 452R probe was incorporated into the existing Spike SNP triplex, and variant pools were used to evaluate probe performance over two runs on a single day following receipt of the probe. Addition of the 452R probe had no effect on C_T values for the other three probes in the Spike SNP assay (Fig. 4) and demonstrated specific detection of 452R in B.1.427 and B.1.429 variant pools, as well as in four clinical samples known to carry that mutation.

DISCUSSION

The rapid development and distribution of diagnostics has been an integral component of the SARS-CoV-2 pandemic response to date and will remain essential in the response to emerging variants (13). Here, we developed and validated a single-step multiplex rRT-PCR for the detection of spike SNPs harbored by SARS-CoV-2 VOC. The

TABLE 3 Results of the Spike SNP clinical evaluation based on availability of whole-genome sequence data

Characteristics[a]	Results with:	
	Sequence confirmed	No sequence data
Total no.	50	212
N2 target positive	50 (100)	203 (95.8)
N2 C_T, mean (SD)	18.95 (4.00)	21.66 (6.58)
Spike SNP positive	50 (100)	188 (88.7)
K417	49 (98.0)	188 (100)
484K	6 (12.0)	6 (3.2)
501Y	15 (30.0)	6 (3.2)

[a]Data are displayed as *n* (%) unless otherwise noted.

assay demonstrated an analytical sensitivity correlating to a limit of detection ~1 to 2 GE/reaction, excellent clinical performance, including detection of samples with N2 C_T values of >30, and perfect concordance with SARS-CoV-2 genome sequencing.

Mutations in the spike protein such as N501Y (present in B.1.1.7, B.1.351, and P.1) and E484K (present in B.1.351, B.1.525, B.1.526, B.1.620, B.1.621, P.1, P.2 and P.3), among others, are likely driving worrisome phenotypes seen with emerging variants (https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html). In particular, N501Y has been shown to increase binding affinity to human ACE2, leading to increased infectivity (14), while E484K has been shown to decrease neutralization by monoclonal antibodies, as well as that by convalescent and postvaccine sera (14–17). The Spike SNP assay was designed to amplify a 348-bp region of the spike receptor-binding domain in a single amplicon with two primers located in conserved flanking regions. Probes were then tiled across the amplicon to specifically detect 484K and 501Y, as well as mutations in codon 417.

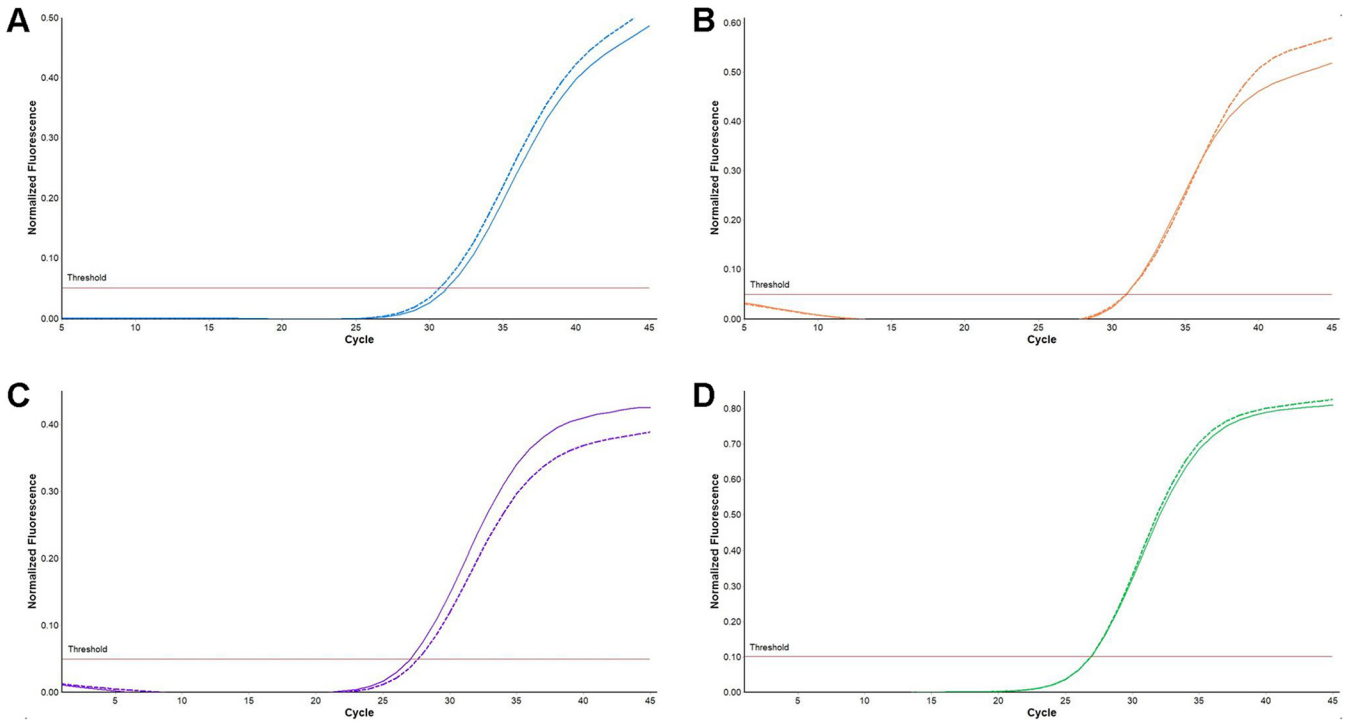


FIG 4 Addition of 452R probe does not affect signal from other Spike SNP probes. (A to D) Curves generated from probes for K417 (A), 452R (B), 484K (C), and 501Y (D) are displayed from a single run that included the Spike SNP assay without the 452R probe (panels A, C, and D; dashed curves), the 452R singleplex assay (panel B; dashed curve), or the Spike SNP assay with the 452R probe (solid curves). Variants tested included BEI 52286 (blue), B.1.427 (orange), B.1.351 (purple), and B.1.1.7 (green).

To provide sensitive discrimination of single-nucleotide difference, we initially designed Spike SNP probes with LNA monomers (18). LNA-DNA binding exhibits increased affinity and specificity compared to DNA-DNA binding, thereby increasing melting temperatures, allowing for shorter probe lengths and preserved discrimination of single-base-pair mismatches (19). Furthermore, LNA-probes can be designed to optimize specific SNP detection with minimal loss of assay sensitivity. As the thermodynamic effects of LNAs are localized within one nucleotide of the LNA modification, discriminatory power is generally highest with three LNAs in sequence and the mismatch target as the middle nucleotide (18). Unmodified probes were also designed to rapidly detect emerging SARS-CoV-2 mutations. These demonstrated similar analytical performance to LNA probes and present an option for future assay customization. New probe designs will therefore need to balance the shorter probe length afforded by LNAs with increased availability of unmodified probes.

The simple design of the SNP assay provides consistent amplification of the target region, limits the potential for amplicon dropout from mutations in the primer sequences, and facilitates the addition of new probes to detect emerging mutations identified among SARS-CoV-2 variants (e.g., L452R). Indeed, no difference in assay results were observed with the sequential addition of probes during assay development (see Fig. S1 in the supplemental material) or the later addition of an unmodified probe for 452R detection (Fig. 4). An advantage of our assay and others that screen for SNPs (20, 21), rather than deletions (22, 23), is the specificity of these SNPs relative to the phenotypes of concern. Furthermore, our single-reaction multiplex design, which can be run in parallel or as reflex to a positive RT-PCR, is of importance when considering scalability and implementation into existing workflows, allowing for faster turnaround times and reagent conservation. The Spike SNP and N2RP assays are currently run in parallel in our research laboratory, providing results within 2 h of assay setup. This contrasts with multistep processes and assays described by others (24, 25). One could envision a real-word clinical use scenario where the Spike SNP assay is performed in a reflex manner for all SARS-CoV-2-positive samples, regardless of C_T value. However, if no signal was detected in any of the SNP assay channels (including the positive control, K417) for the SNP assay, results would not be released so as to not falsely assure interpreting clinicians.

Rapid sequencing of SARS-CoV-2 offers the ability to detect new emerging variants in near-real time. Despite concerted national efforts to increase genomic surveillance, most states lag behind the estimated 5% sequencing threshold required to detect emerging strains when they are less than 1% of all strains in a population (26, 27). Moreover, the global use and application of genomic surveillance of SARS-CoV-2 remains highly varied, with many countries having no sequence data uploaded to GISAID (28, 29). This highlights the profound disparities in the ability to sequence genomes and the acute need for an assay which can be run on existing lab infrastructure. Even at centers with access to genomic technologies, recovery of a full genome and variant calling can be restricted by sample viral load (12). This may limit the ability to perform surveillance among cases that present later in their disease course or in those who develop infection after vaccination or natural infection, where partial immune protection may lead to lower viral loads (30). Our assay, along with others (20, 21, 23), represents an important and efficacious step toward an equitable global response to emerging variants.

RT-PCRs for VOC may also serve as an adjunct to enhance sequencing efforts. Although care must be taken to not overly bias genomic surveillance, such assays can be applied as a screening step to select samples for further sequencing and VOC confirmation, as performed in our research laboratory. A screening strategy in which positive samples, in addition to a proportion of negative samples (to allow for detection of novel mutations and VOC), are selected for sequencing would allow centers to enrich samples of interest and capitalize on existing sequencing infrastructure. In California, use of a targeted SNP assay allowed for real-time surveillance of the emergence of

variants harboring the L452R mutation (20). In addition to surveillance efforts, such assays may have a role in clinical decision making. Due to the rise of E484K and the inability of clinical labs to screen for this mutation, the Food and Drug Administration revoked the emergency use authorization of bamlanivimab when administered alone (https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-revokes-emergency-use-authorization-monoclonal-antibody-bamlanivimab). Here, application of a E484K SNP assay could allow for the selection of appropriate monoclonal antibodies, akin to the role of HIV genotyping in antiretroviral regimen selection.

The limitations of this work include targeting one region of the genome for amplification and identification of mutations. While our assay targets important mutations in *spike*, this cannot provide phylogenetic and other comprehensive information gained through sequencing. In addition, sporadic E484K and N501Y mutations may be present and may not necessarily be indicative of VOC; however, these mutations are likely still of phenotypic significance. This is evidenced by repeated convergent evolution of tolerable mutations such as E484K and N501Y. The set of clinically significant mutations in this region continues to increase and highlights the utility of the Spike SNP design, where probe sets can be quickly modified and adapted for relevant emerging SNPs. Multiplexing in this region, however, will be limited by the number of fluorophores that can be detected by most real-time PCR instruments (4 or 5), as well as by the inherent constrains of tiling probes within a given region. These limitations were avoided in the current design, which targets a limited number of key nonoverlapping mutations to be detected by probes of restricted length and similar annealing temperatures. With the emergence of mutations nearer to one another (e.g., 478K and 490S), implementing laboratories may need to select the most informative probe sets for their specific region.

Detection and surveillance of SARS-CoV-2 variants is of great public health importance. While sequencing remains the gold standard, broadly accessible and inexpensive assays are needed to enhance variant surveillance and detection worldwide. Here, we describe the development and validation of a highly sensitive and specific assay for the detection and surveillance of mutations in the spike receptor-binding motif. Centered around a methodology ubiquitous to clinical labs offering SARS-CoV-2 testing, the Spike SNP assay can be adapted and implemented widely to greatly increase the capacity for detection of these mutations.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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W.A.L., J.J.W. Supervision: C.S.K., A.C., R.F.S., G.S.M., A.P., W.A.L., J.J.W. Writing—original draft: A.B., K.I., M.S., J.J.W. Writing—review & editing: A.B., K.I., S.D.S., A.R., L.B., M.S., V.N., J.M.I., H.L.B., M.M., N.S., C.S.K., A.C., R.F.S., G.S.M., A.P., W.A.L., J.J.W.

We declare that we have no conflicts of interest.

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