

A Simple Reverse Transcriptase PCR Melting-Temperature Assay To Rapidly Screen for Widely Circulating SARS-CoV-2 Variants

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ABSTRACT The increased transmission of SARS-CoV-2 variants of concern (VOC), which originated in the United Kingdom (B.1.1.7/alpha), South Africa (B.1.351/beta), Brazil (P.1/gamma), the United States (B.1.427/429 or epsilon), and India (B.1.617.2/delta), requires a vigorous public health response, including real-time strain surveillance on a global scale. Although genome sequencing is the gold standard for identifying these VOCs, it is time-consuming and expensive. Here, we describe a simple, rapid, and high-throughput reverse transcriptase PCR (RT-PCR) melting-temperature (T_m) screening assay that identifies the first three major VOCs. RT-PCR primers and four sloppy molecular beacon (SMB) probes were designed to amplify and detect the SARS-CoV-2 N501Y (A23063T) and E484K (G23012A) mutations and their corresponding wild-type sequences. After RT-PCR, the VOCs were identified by a characteristic T_m of each SMB. Assay optimization and testing was performed with RNA from SARS-CoV-2 USA WA1/2020 (wild type [WT]), B.1.1.7, and B.1.351 variant strains. The assay was then validated using clinical samples. The limit of detection for both the WT and variants was 4 and 10 genomic copies/reaction for the 501- and 484-codon assays, respectively. The assay was 100% sensitive and 100% specific for identifying the N501Y and E484K mutations in cultured virus and in clinical samples, as confirmed by Sanger sequencing. We have developed an RT-PCR melt screening test for the major VOCs that can be used to rapidly screen large numbers of patient samples, providing an early warning for the emergence of these variants and a simple way to track their spread.

KEYWORDS SARS-CoV-2, variants, N501Y, E484K, melting temperature, screening test, sloppy molecular beacon

In December 2020, public health officials in the United Kingdom observed a surge of COVID-19 cases in Kent, England, that appeared to be largely due to a specific variant of SARS-CoV-2 (1, 2). The new variant was named a variant of concern (VOC 202012/1) or B.1.1.7 based on its phylogenetic lineage. Early reports suggested that B.1.1.7 was more transmissible and more virulent than previous SARS-CoV-2 strains (3, 4), although the evidence did not suggest that B.1.1.7 caused a decrease in vaccine efficacy (5–8). The B.1.1.7 variant has a number of mutations in the spike protein, including single-nucleotide polymorphisms (SNPs) resulting in N501Y, A570D, D614G, and P681H mutations and deletions at amino acids 69 and 70 and 144Y (6, 9). The N501Y (A23063T) mutation has been identified as an important contributor to the worrisome phenotype (2, 4, 10, 11). Other SARS-CoV-2 strain variants have been implicated in large outbreaks in South Africa (known as 20H/501Y.V2 or B.1.351 lineage) and Brazil (20J/501Y.V3 or P.1 lineage) (12). These variants appear to have decreased the efficiency of some

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COVID-19 vaccines (2, 13, 14). All three variants, B.1.1.7, B.1.351, and P.1, contain the N501Y mutation, while the South African and Brazilian variants additionally contain mutations in E484K and K417N (1, 12). Thus, the N501Y mutation appears to be an excellent marker for all three strains, while the E484K mutation can be used to differentiate the other two strains from B.1.1.7. New SARS-CoV-2 variants have also been recently reported in the United States (5, 15). Most of the new variants also have mutations at the 501 and/or 484 position, although additional mutations, such as L452R, have also been reported in more recent variants (15, 16); however, the epidemiological and clinical relevance of these new variants are not well understood.

All three of the major SARS-CoV-2 N501Y variants are circulating around the world (1). However, the distribution, extent, and spread of these variants are poorly understood in countries such as the United States, where only a small fraction (0.04 to 3.5%) of COVID-19 cases are analyzed by viral genomic sequencing per the CDC's national genomic surveillance dashboard (17). Viral genome sequencing is currently the only method available to reliably detect rapidly emerging SARS-CoV-2 variants. Genomic sequencing has the advantage of providing a detailed map of new mutations, which supports new variant discovery as well as monitoring the exact type of the variant. However, genomic sequencing is expensive and difficult to perform in real time. In contrast, reverse transcriptase PCR (RT-PCR) testing for SARS-CoV-2 has become widespread. This diagnostic approach is easy to perform in a high-throughput manner, and rapid turnaround times are possible. However, routine RT-PCR tests do not differentiate among SARS-CoV-2 variants or do so only by producing negative assay results (18), which still require sequence confirmation (19). Furthermore, the potential for additional mutations to appear near key variant-defining alleles may complicate the development of RT-PCR assays for SARS-CoV-2 variants (20). We have previously demonstrated that sloppy molecular beacons (SMBs) combined with melting temperature (T_m) code analysis may be used to specifically detect mutations in short genomic regions where a variety of mutations can exist (21).

Here, we apply this same T_m -based approach to detect and differentiate variant strains of SARS-CoV-2 with high sensitivity and specificity. This approach is flexible and can be used in a high-throughput manner for screening and detection of SARS-CoV-2 variants, easily allowing the addition of new mutation-detecting assays as needed to identify and track new variants as they emerge. Furthermore, this approach can be performed on a wide range of real-time PCR instrumentation as long as they have the capacity to run melting curve analysis. The wide availability of such instruments can allow quick adoption of this assay around the world, increasing access to real-time monitoring of SARS-CoV-2 variant spread.

MATERIALS AND METHODS

Ethical considerations. The use of deidentified clinical samples from confirmed COVID-19-positive and -negative patients for PCR testing and sequencing was approved by the Rutgers Institutional Review Board under protocol numbers 20170001218 and 2020001541.

Viral cultures and RNA. Genomic RNA from SARS-CoV-2 USA WA1/2020 (wild type [WT]) and viral culture stocks of SARS-CoV-2 hCoV-19/England/204820464/2020 (B.1.1.7 variant, 501-MT [mutant]), SARS-CoV-2 isolate hCoV-19/South Africa/KRISP-K005325/2020 (B.1.351 variant, 484-MT), and SARS-CoV-2 isolate hCoV-19/Japan/TY7-503/2021 (P.1, 484-MT) were obtained from BEI Resources, NIAID (Manassas, VA). RNA was isolated from both variant strains in a biosafety level 3 (BSL3) laboratory, using an RNAdvance viral RNA extraction kit (Beckman Coulter, Indianapolis, IN). RNA extracted from all three variant strains was quantified against a standard curve generated with the N1 gene-specific real-time RT-PCR assay (22, 23), and the concentration was determined to be 4×10^5 GE/ μ l for B.1.1.7, 7×10^6 GE/ μ l for B.1.351, and 8.5×10^6 GE/ μ l for the P.1 variant strains.

Genome sequence analysis for assay design. For initial analysis of the mutations in SARS-CoV-2, a total of 330,132 high-quality viral genome sequences deposited in GISAID (24) as of 12 January 2021, were analyzed. Publicly available data sets were analyzed in this study. These data can be found at <https://www.gisaid.org/>. A 250-nucleotide region around the N501Y (A23063T) and E484K (G23012A) positions in the reference strain (GenBank accession number MN908947) was selected and used to identify the corresponding regions in the GISAID data set using BLAST (25). These matching sequences were condensed into a set of unique sequences and aligned using a multiple-sequence alignment program, MAFFT (26). Candidate amplification primers and probes were identified on the basis of sequence conservation and predicted T_m using the algorithm of SantaLucia (27), and a final set of primers was

TABLE 1 Primers and probes^a

Assay	Primer/probe	5' End	Sequence	3' End	Amplicon size (bp)
SMB-501	501-F		ggttttaattgttacttcttcttaca		89
	501-R		gaaagtactactactctgtatggttgg		
	501-WT	Quasar 570	CCGCgtt[<i>pdU</i>]ccatcccactaatgctg[<i>pdU</i>]tggttaccaacGCGG	BHQ-2	
	501-MT	Quasar 670	CGCGgtt[<i>pdu</i>]ccatcccactatgctg[<i>pdU</i>]tggttaccaacCGCG	BHQ-2	
SMB-484	484-F		ctatcaggccggtagcacac		76
	484-R		gaaacctatgattgtaaaggaaag		
	484-WT	Quasar 570	CCGCGccttgaatggtgtgaaggtttaattgttacGCGCGG	BHQ-2	
	484-MT	Quasar 670	CCGCGccttgaatggtgttaaggtttaattgttacGCGCGG	BHQ-2	

^aThe lowercase letters indicate the SMB probe region, and the uppercase letters indicate the SMB stem region. [*pdU*], C5 propynyl-deoxyuridine. BHQ, black hole quencher.

designed with the help of the Primer3 program (28). SMB probe design was performed using the web servers DNA mfold (<http://www.unafold.org/mfold/applications/dna-folding-form.php>) and DINAmelt (<http://www.unafold.org/hybrid2.php>) to predict the probe folding structures and probe-target hybrid T_m values, respectively.

Primers and probes. The list of primers and probes used for the 501 variant (SMB-501) and the 484 variant (SMB-484) assays are shown in Table 1. Both assays were run as two separate reactions. For the SMB-501 assay, an 89-bp region surrounding the 23063 position was amplified using an asymmetric PCR. For SMB-484 assay, a 76-bp region surrounding the 23012 position was amplified using an asymmetric PCR. SMB were designed targeting both the wild-type 501N/484E (23063A/23012A) and the mutant 501Y/484K (23063T/23012G) sequences (SMB-501-MT). Primers were obtained from Millipore Sigma (The Woodlands, TX), and SMBs were synthesized by LGC Biosearch Technologies (Petaluma, CA). An internal control (IC) assay developed by the CDC (22, 23), targeting the human RNaseP gene, was simultaneously performed for each extracted RNA specimen as a separate reaction in a separate well, using the TaqMan real-time PCR assay probe tagged with 6-carboxyfluorescein (FAM) at the 5' end and Dabcyl quencher at the 3' end.

SMB assay formulation and procedure. TaqPath 1-step RT-quantitative PCR (qPCR) master mix CG (ThermoFisher Scientific, Waltham, MA) was used for the RT-PCR. Each one-step reaction mix was supplemented with 0.2 μ M the forward primer 501-F/484-F and 2 μ M/4 μ M the reverse primer 501-R/484-R, 0.4 μ M each of the SMB probes (SMB-501-WT and SMB-501-MT/SMB-484-WT and SMB-484-MT), and 1 μ M of the template RNA (note that the concentration of the forward primers did not match the concentration of the reverse primers so as to create an asymmetric PCR). The internal control contained primers and a probe specific for human RNaseP as described previously (22, 23). Each reaction was run in replicates of 4 in 384-well plates in a Roche LightCycler 480 (Roche, Indianapolis, IN). The one-step RT-PCR amplification was performed with the following thermocycling conditions: uracil DNA glycosylase incubation for 2 min at 37°C and reverse transcription (RT) for 15 min at 50°C, followed by asymmetric PCR for 45 cycles (denaturation at 95°C for 1 s, annealing/extension at 55°C for 30 s). The post-PCR melt was performed with the following conditions: denaturation at 95°C for 30 s, followed by cooling down to 45°C and gradual heating to 85°C, with continuous monitoring of fluorescence at a rate of 2 acquisitions per degree Celsius. The total assay time was 77 min. Automated T_m calls were performed by the LC480 T_m detection software at the end of the PCR. The resulting T_m for each probe was identified and matched with the T_m signature code defined for the wild type or the mutant variants.

Analytical sensitivity. The prequantitated genomic RNA from the SARS-CoV-2 USA WA1/2020 (WT), B.1.1.7 (alpha/UK variant), and B.1.351 (beta/SA variant), obtained from BEI Resources, were diluted in Tris-EDTA (TE) buffer. Total RNA was extracted from a SARS-CoV-2 negative nasopharyngeal (NP) specimen (confirmed negative by Xpert Xpress SARS-CoV-2 test). The diluted SARS-CoV-2 RNA from the WT and the variants was spiked into the extracted matrix at final concentrations of 1,000, 500, 100, 50, 10, 5, and 1 GE/ μ l and mixed well. A 1- μ l aliquot of the RNA spiked into the NP matrix was added to 19 μ l of the one-step RT-PCR mix containing the primers and probes and was evaluated in the SMB-501 assay or the SMB-484 assay; 1,000 GE/ μ l of the quantified RNA from P.1 was also validated with both assays (Table 2).

Validation with patient samples. Deidentified NP swabs obtained from patients undergoing routine COVID-19 clinical testing using the Xpert Xpress SARS-CoV-2 or Xpert Xpress SARS-CoV-2/Flu/RSV (4-plex) test (Cepheid, Sunnyvale CA) assay in the CLIA- and CAP-certified laboratory at the Public Health

TABLE 2 T_m profile for SARS-CoV-2 VOC tested by SMB-501 and SMB-484 assays

SARS-CoV-2 strain	SMB-501 assay		SMB-484 assay	
	501-WT probe (°C)	501-MT probe (°C)	484-WT probe (°C)	484-MT probe (°C)
WA1 USA/2020 (WT strain)	59.8 ± 0.4	58.2 ± 1	64 ± 0.1	58.7 ± 0.4
B.1.1.7 (alpha, MT strain)	55.2 ± 0.4	62.2 ± 0.6	64.5 ± 0.05	59.2 ± 0.03
B.1.351 (beta, MT strain)	56.2 ± 0.01	63.1 ± 0.01	59.7 ± 0.5	63 ± 0.6
P.1 (gamma, MT strain)	56.3 ± 0.07	63 ± 0.16	60.7 ± 0.03	63.5 ± 0.05

Research Institute (PHRI), Newark, NJ, were selected for this study. NP swabs were collected in 3 ml viral transport medium (VTM) from Hardy Diagnostics (Santa Maria, CA) or Labscoop (Little Rock, AR) and were banked under either refrigerated conditions (for specimens first tested within the previous 2 weeks) or frozen (for specimens stored longer than 2 weeks). The samples consisted of 46 randomly selected COVID-19-positive samples of unknown genotype and an RT-PCR cycle threshold (C_T) of <42 with either Xpert Xpress SARS-CoV-2 or Xpert Xpress CoV-2/Flu/RSV test. Nine of the samples had been banked between October 2020 and 31 December 2020, 16 in the month of January 2021, 15 in February 2021, and 6 in March 2021. Thirty COVID-19-negative specimens were randomly selected from samples banked between October 2020 and February 2021. RNA was extracted from each specimen using a QiaAmp viral RNA isolation kit by following the manufacturer's instructions (Qiagen, Valencia, CA) in a BSL2 laboratory. A 5- μ l volume of RNA was added to the one-step RT-PCR mix containing the primers and probes. A subset of these clinical samples consisting of 26/46 of the COVID-19-positive samples and 19/30 of the COVID-19-negative samples used the SMB-484 assay, mainly based on the continued availability of the sample for testing. The SMB-501, SMB-484, and IC assays were tested in separate wells in replicates of two. The samples that failed the IC assay were repeated starting with the RNA extraction. A subset of samples that tested positive either for 501N/484E wild type or 501Y/484K mutant was confirmed by Sanger sequencing using the primer pair F, 5'CTATCAGGCCGGTAGCACAC3', and R, 5'CTTTCTTTGAACTTCTACATG3', which amplifies a 143-bp segment of the S-gene, inclusive of amino acid positions 484 and 501. Sequencing chromatograms were analyzed using Ugene (ver 37) compared to the known WT and MT sequences using MegAlign Pro software (DNASStar, ver16).

Statistical analysis. Standard statistical analyses (averages and standard deviations [SD]) and graphing were performed using Microsoft Excel (ver 2102) and GraphPad Prism 8.4.3 for Windows.

RESULTS

Limit of detection. The one-step RT mix containing asymmetric PCR primers and both wild-type and MT-specific probes was added with either WT RNA or MT RNA at different concentrations, ranging from 1,000 to 1 GE/reaction for both SMB-501 and SMB-484 assays. After T_m analysis was performed, the T_m peak heights were highest in the assays with the largest added number of GE, and the peak heights progressively decreased as the number of GEs present in the reactions decreased. However, T_m peak heights produced by both SMB 501-WT/SMB 484-WT and SMB 501-MT/SMB 484-MT could still be reproducibly detected at concentrations down to 10 GE/reaction when tested against both WT and N501Y mutant strains, defining the assay limit of detection as ≤ 10 GE per reaction for both the SMB-501 and SMB-484 assays (Fig. 1; see also Fig. S1 in the supplemental material).

T_m code definition. The T_m values produced by both SMBs against the reference WT and the MT SARS-CoV-2 strains are listed in Table 2. The means and standard deviations shown were derived from at least 4 replicates. When reference RNA (1,000 GE/reaction) was tested with the SMB 501-WT probe, WT-RNA produced a T_m of $59.8 \pm 0.4^\circ\text{C}$, while MT N501Y RNA from B.1.1.7/B.1.351/P.1 variants produced T_m values of $55.2 \pm 0.4^\circ\text{C}$, $56.2 \pm 0.01^\circ\text{C}$, and $56.3 \pm 0.07^\circ\text{C}$, respectively. Similarly, when RNA was tested with the SMB 501-MT probe, WT-RNA produced a T_m of $58.2 \pm 1^\circ\text{C}$, while MT N501Y RNA from B.1.1.7/B.1.351/P.1 variants produced T_m values of $62.2 \pm 0.6^\circ\text{C}/63.1 \pm 0.01^\circ\text{C}/63 \pm 0.16^\circ\text{C}$, respectively. Given that T_m values can vary slightly between clinical samples, we specified a two-temperature T_m code that identified either 501N or 501Y alleles within a T_m range of approximately 1.5 to 4 times the experimentally verified \pm SD values for each probe T_m . Thus, the 501N (WT) T_m code was defined as an SMB-501-WT T_m of $59.8 \pm 1.5^\circ\text{C}$ and an SMB-501-MT T_m of $58.2 \pm 1.5^\circ\text{C}$, and a 501Y (MT) T_m code was defined as an SMB-501-WT T_m of $55.2 \pm 1.5^\circ\text{C}$ and an SMB-501-MT T_m of $62.2 \pm 1.5^\circ\text{C}$. Similarly, for SMB-484 assay, the reference T_m code for 484E (WT) was 64 ± 0.1 (with SMB-484-WT probe) and 58.7 ± 0.4 (with SMB-484-MT probe), and the reference T_m code for 484K (MT, B.1.351 and P.1) was 59.7 ± 0.5 (WT probe) and 63 ± 0.6 (MT probe). Any samples that failed to produce a T_m value for either of the SMBs or produced T_m values outside the range defined for the T_m codes would have been defined as indeterminate and the assay repeated. Using this code definition, we retested our reference RNA samples 20 times, and the correct 501/484 allele was detected in each case, achieving an analytic sensitivity and specificity of 100%. These results clearly indicate that the combination of both SMB 501-WT and SMB 501-MT probes can specifically detect and differentiate the N501Y variants from the wild-type strains with high confidence.

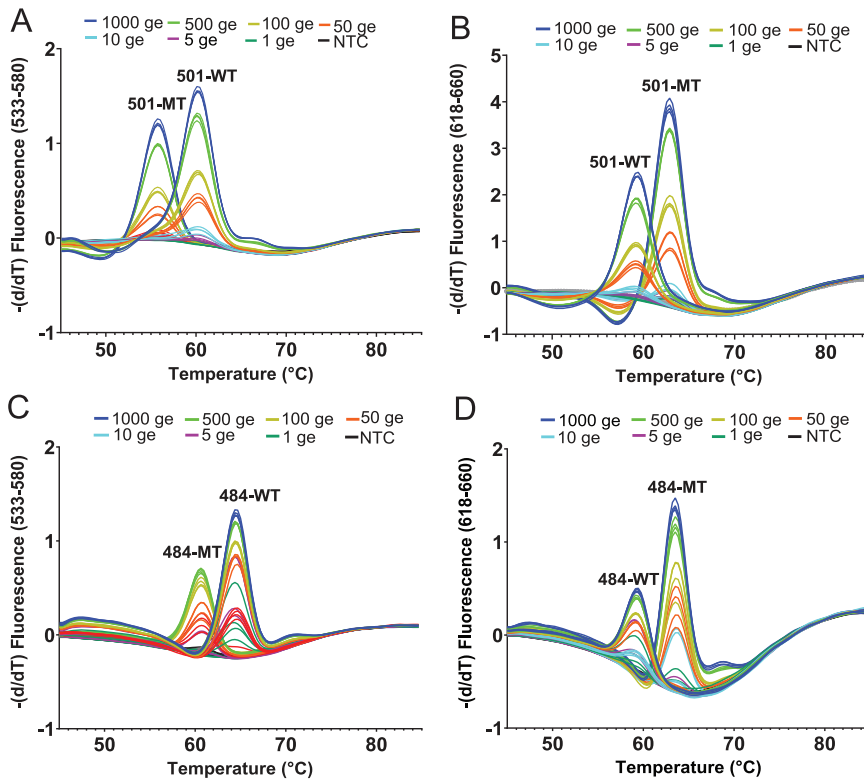


FIG 1 Analytical limit of detection and T_m values generated by the SMB-501 (A and B) and SMB-484 (C and D) assays tested against SARS-CoV-2 RNA in the presence of the nasopharyngeal (NP) matrix. Shown are SARS-CoV-2 wild type (WT) RNA (A and C), B.1.1.7 mutant (MT) RNA (B), and B.351 mutant (MT) RNA (D) at the indicated number of genomic equivalents (GEs).

Validation with patient samples. A total of 76 patient samples, 46 confirmed COVID-19 positive and 30 confirmed COVID-19 negative, were tested using the SMB-501 assay. A subset of 45 of these same samples (26 COVID-19 positive and 19 COVID negative) were additionally tested with the SMB-484 assay. None of the COVID-19-negative samples produced any measurable T_m values from either of the assays, yielding a specificity of 100%. Positive samples were selected based on a wide range of N2-C₇ values between 16 and 41 based on the Xpert Xpress SARS-CoV-2 assay. As shown in Table 3, 40/45 (89%) of the COVID-19-positive samples produced measurable T_m values for both SMB probes in the SMB-501 assay in at least one of the two replicates. Two samples were identified as indeterminate based on the inability of either the WT or MT probe to generate a measurable T_m and/or due to the presence of a failed internal control. Five clinical samples that had previously tested positive for SARS-CoV-2 generated negative results in our variant assay, in that neither probe produced a measurable T_m . All of the samples with negative assay results had previously shown initial Xpert C₇ values of ≥ 35 . Although many of the samples with Xpert C₇ values as late as 39 could still be detected by our assay, we found that the samples with a C₇ of ≥ 35 using the Xpert assay yielded relatively stunted melting peak heights of ≤ 0.3 with WT-SMB and ≤ 0.1 with MT-SMB, similar to that observed when testing < 10 GE in our limit-of-detection studies, which is indicative of very low viral loads in these samples. However, the T_m could still be identified for at least one of the 501 SMB probes in all samples, except the negatives. Overall, 27/45 (60%) of the COVID-19-positive samples had SMB 501-WT and SMB 501-MT T_m values consistent with the WT N501 allele, and 11/45 (24.4%) of the samples had T_m values consistent with the mutant Y501 allele within ± 1 standard deviation of our reference standards (Table 3, Fig. 2). Twelve representative WT and mutant samples, as predicted by the SMB assay, underwent Sanger sequencing of the PCR products for confirmation of the PCR results. In all cases, the WT or mutant sequences identified by the SMB N501Y assay were confirmed by the sequencing

TABLE 3 Assay and Sanger sequencing results from COVID-positive clinical samples^a

Sample	Date of 1st test	SMB-501 assay				SMB-484 assay				Confirmation by sequencing ^c			
		PCR C _T ^b	T _m (°C)		ID	T _m (°C)	T _m (°C)		ID	Wild type	N501Y mutant (AAT-TAT)	E484K mutant (GAA-AAA)	
			WT probe (Cy3)	MT probe (Cy5)			WT probe (Cy3)	MT probe (Cy5)					
WT-reference			59.8 ± 0.4	58.2 ± 1	WT	64 ± 0.1	58.7 ± 0.4	WT	Yes	No	No		
MT-reference			55.2 ± 0.4	62.2 ± 0.6	MT	59.7 ± 0.5	63 ± 0.6	MT	No	Yes	No		
SMBP-1	Oct-Nov 2020	29.1	59.9	58.4	WT	63.9	58.2	WT	Yes	No	No		
SMBP-2		34.8	NP	NP	Neg								
SMBP-3		34.5	NP	NP	Neg	64.0	58.7	WT					
SMBP-4		35.4	NP	NP	Neg								
SMBP-5		39.2	59.5	58.2	WT								
SMBP-6		36.0	59.1	58.2	WT				Yes	No	No		
SMBP-7		33.7	58.9	58.1	WT	64.0	NP	Ind					
SMBP-8		36.8	59.5	58.2	WT								
SMBP-9		27.9	59.4	58.3	WT								
SMBP-10	Jan 2021	29.4	59.3	58.3	WT	63.8	58.2	WT	Yes	No	No		
SMBP-11		39.1	59.9	58.6	WT								
SMBP-12		33.8	59.0	58.2	WT	64.3	58.8	WT	Yes	No	No		
SMBP-13		17.7	59.3	58.5	WT	64.2	58.9	WT	Yes	No	No		
SMBP-14		21.3	59.4	58.5	WT	64.2	58.8	WT	Yes	No	No		
SMBP-15		17.5	59.1	58.3	WT	64.0	58.7	WT	Yes	No	No		
SMBP-16		30.0	55.5	62.6	MT	64.2	58.5	WT	No	Yes	No		
SMBP-17		41.2	NP	NP	Neg								
SMBP-18		23.2	59.8	58.9	WT	64.0	58.7	WT					
SMBP-19		28.3	59.3	58.1	WT	63.8	58.2	WT	Yes	No	No		
SMBP-20		17.1	59.2	58.3	WT	64.0	58.8	WT	Yes	No	No		
SMBP-21		16.4	59.2	58.4	WT	64.2	58.9	WT	Yes	No	No		
SMBP-22		18.2	58.9	58.1	WT				Yes	No	No		
SMBP-23		21.1	58.9	58.1	WT	64.1	58.6	WT	Yes	No	No		
SMBP-24		39.0	59.5	58.2	WT								
SMBP-25		28.4	54.5	61.6	MT	64.1	NP	Ind	No	Yes	No		
SMBP-26	Feb 2021	27.8	54.5	61.6	MT	63.9	58.3	NP	No	Yes	No		
SMBP-27		33.5	59.3	58.9	WT	60.1	63.0	MT	Yes	No	Yes		
SMBP-28		38.3	59.7	58.5	WT	64.1	NP	Ind	ND	ND	ND		
SMBP-29		18.1	58.9	58.1	WT	64.1	58.8	WT	Yes	No	No		
SMBP-30		27.8	59.2	58.2	WT								
SMBP-31 ^a		17.6	55.1	61.9	MT	60.4	63.3	MT	No	Yes	Yes		
SMBP-32 ^a		17.4	55.1	61.9	MT	64.0	58.7	WT	No	Yes	No		
SMBP-33		32.8	55.0	62.1	MT								
SMBP-34		25.2	55.5	62.7	MT	60.2	63.3	MT	No	Yes	Yes		
SMBP-35		18.4	55.6	62.5	MT	60.5	63.4	MT	No	Yes	Yes		
SMBP-36		18.8	54.9	61.9	MT				No	Yes	No		
SMBP-37	28.4	54.9	62.2	MT									

(Continued on next page)

TABLE 3 (Continued)

Sample	Date of 1st test	PCR C _T ^b	SMB-501 assay			SMB-484 assay			Confirmation by sequencing ^c		
			T _m (°C)			T _m (°C)			Wild type	N501Y mutant (AAT-TAT)	E484K mutant (GAA-AAA)
			WT probe (Cy3)	MT probe (Cy5)	ID	WT probe (Cy3)	MT probe (Cy5)	ID			
SMBP-38		16.4	59.8	58.5	WT	64.1	58.6	WT			
SMBP-39		32.2	NP	NP	Neg						
SMBP-40		32.3	58.9	57.8	WT				Yes	No	No
SMBP-41	Mar 2021	32.6	55.4	62.6	MT	63.9	58.3	WT	No	Yes	No
SMBP-42		36.8	59.7	58.4	WT						
SMBP-43		30.1	59.8	58.9	WT				Yes	No	No
SMBP-44		33.3	NP	NP	Inv						
SMBP-45		34.6	NP	61.3	Ind						
SMBP-46		23.3	59.2	58.285	WT	64.0	58.6	WT	Yes	No	No

^aIC, internal control; NP, no T_m peak; Neg, negative; ND, not determined; Inv, invalid; Ind, indeterminate. Gray cells indicate corresponding samples that were not tested by the SMB-484 assay or by sequencing due to insufficient clinical sample remaining.

^bXpert Xpress SARS-CoV-2 or Xpert Xpress SARS-CoV-2/Flu/RSV tests.

^cRepresentative strains were sequenced. Sequencing was repeated twice to confirm the identified mutations.

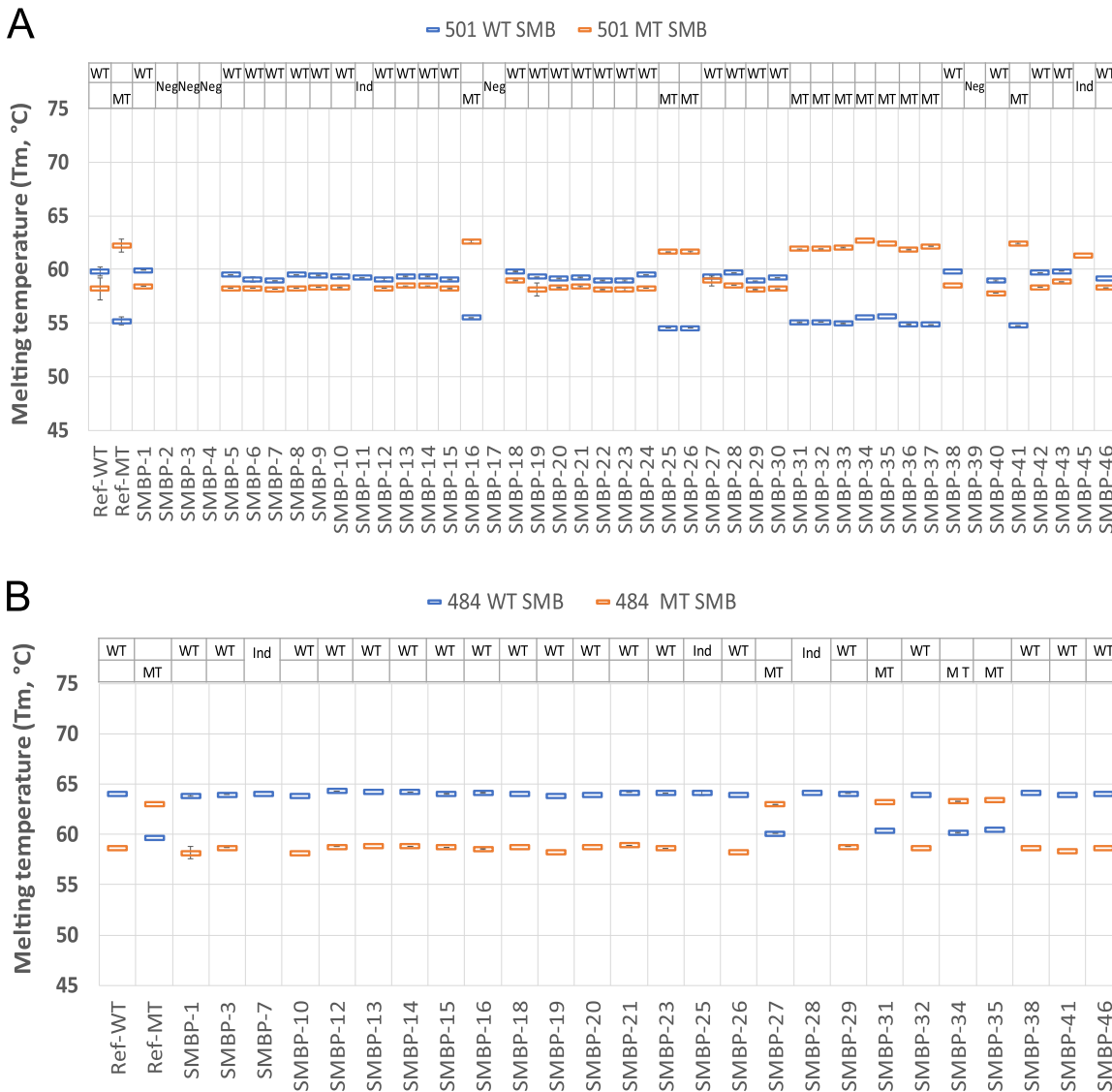


FIG 2 Sloppy molecular beacon (SMB) T_m profile of positive clinical nasopharyngeal (NP) samples tested using the SMB-501 and SMB-484 T_m assay. T_m signatures consisting of the T_m values for both the WT probe (blue) and MT probe (orange) are shown for the SMB-501 assay (A) and the SMB-484 assay (B). Ref WT indicates the T_m profile of the reference WT SARS-CoV-2 strain, and Ref MT indicates the T_m profile of the reference MT SARS-CoV-2 B.1.1.7 strain (A) and B.351 (B). SMBP1 to SMBP46 indicate that T_m profiles of the 46 COVID-positive clinical samples tested in this study with SMB-501 assay and the 26 COVID-positive samples tested by SMB-484 assay. Error bars show \pm one standard deviation.

result. Using the confirmed sequencing results as a gold standard, 4/4 of the wild-type clinical samples were detected as WT by the SMB N501Y assay, and 8/8 N501Y mutant results were detected as mutant by the assay, demonstrating a clinical sensitivity and specificity of 100%. Similarly, with the SMB-484 assay, 19/26 (73%) of the samples tested were wild type and 4/26 (15.3%) were variants. Out of the 4 variants, 4/4 were sequenced and confirmed to harbor the G-to-A mutation in the 484th codon, yielding an assay specificity of 100% compared to sequencing. It should be noted that one of the specimens (SMBP-27) contained an E484K mutation but was found to have a WT sequence at codon 501. Considering that the major three VOCs contain either N501Y alone (B.1.1.7) or both N501Y and E484K mutations (B.1.351 and P.1), the presence of this single 484K mutant indicates that this sample contains the variant of interest, B.1.525/B.1.526, which originated in New York City. Thus, the combination of our SMB-501 and SMB-484 assays should help capture most of the variants in circulation other than those with 452 mutations. We also observed

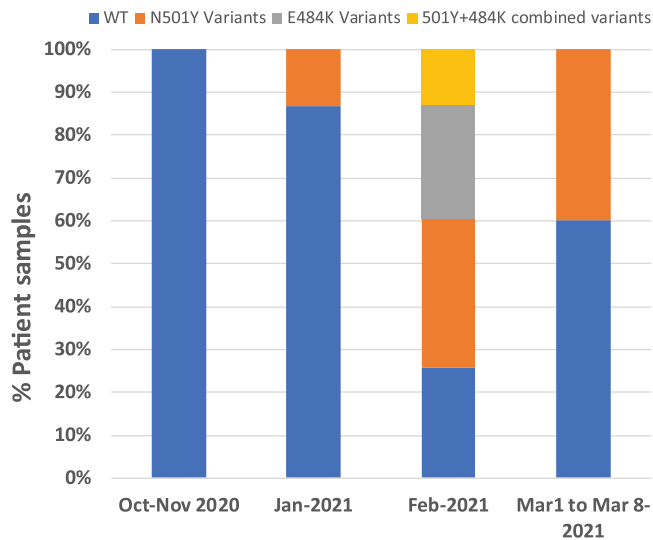


FIG 3 Prevalence of N501Y variant strains among the tested sample set over time. The proportion of N501Y and E484K variants is shown for samples obtained during the periods of October to November ($n=9$ tested with SMB-501/ $n=3$ tested with SMB-484), January ($n=16/n=12$), February ($n=15/n=9$), and the first week of March ($n=6/n=2$). No samples from December were tested in our study.

that the frequency of N501Y and E484K variants increased substantially between the clinical samples obtained in October 2020 and the samples obtained in February and March 2021 (Fig. 3).

DISCUSSION

The emergence of SARS-CoV-2 VOC with the potential for increased transmission, disease severity, and resistance to vaccine-induced immunity is of grave concern (14, 29). A simple screening assay to monitor the emergence and spread of these strains may be helpful for implementing public health strategies to counter these and future strains. Our study demonstrates that our assay is simple, rapid, and sensitive and specific for detecting key variant-identifying mutations using a high-throughput PCR assay platform. Thus, this assay has the potential for relatively inexpensive high-throughput testing for rapid identification of N501Y, E484K variants. In designing this assay, we took advantage of the fact that the N501Y and E484K mutations are common in the major SARS-CoV-2 variants. Since this mutation also appears to be responsible for the increased infectivity and possibly the other adverse manifestations of these strains (4), assays that detect this mutation may also prove useful to detect any future strain that evolves to have increased transmission potential.

Our assay is the first, to our knowledge, that uses post-PCR T_m -based analysis to detect and differentiate SARS-CoV-2 variants using SMB probes demonstrated in clinical samples. This assay format has the benefit of producing a measurable T_m result irrespective of whether the SMB probe is fully complementary to its target nucleic acid sequence. Instead of detecting a mutation by either producing or not producing a signal, SMBs detect mutations by producing a T_m shift. Failure to produce a T_m signal indicates an invalid assay rather than the absence of a mutation. The robustness of our assay is further increased by our use of two different SMBs, one complementary to the WT sequence and one complementary to the mutant sequence. The pattern of T_m values or T_m signature produced by the combined T_m values of each SMB probe provides an unequivocal identification of a WT or MT sequence. We have also shown that T_m signatures can be used to detect mixtures of mutant and WT sequences and to identify numerous mutations present in an assay's target region (29). Thus, we expect that our assay should continue to be able to identify N501Y and E484K variants even if additional mutations develop near this primary mutation within the probe-footprint region once the specific T_m signatures of each new

genotype are characterized. Our assay is meant to be a screening assay that will identify samples likely to contain SARS-CoV-2 variants of concern. Although in this study we have proposed the use of a combination of two probes per assay for increased accuracy, we expect that most SARS-CoV-2 variants can be identified using only the MT probe alone, and future versions of our assay are likely to use a single SMB to identify mutations that are less critical than N501Y and E484K. We suggest that a useful public health strategy would be to screen COVID-19-positive samples in near real time with our assay and then to perform genomic sequencing on a subset of screen-positive samples. This sequencing would confirm the presence of the expected variants and, in some cases, lead to the discovery of new variants. Ongoing sequencing of a subset of all SARS-CoV-2 samples will also be required to identify completely novel variants or to investigate the epidemiology and clinical characteristics of variants such as those recently reported in the United States. Fortunately, our assay is easily extensible, and additional tests for new key mutations can be added in a modular format to a screening panel when new mutations associated with critical new variants are discovered. In fact, it is our intention to continuously update this assay until our work is superseded by a better approach or much more widespread genome sequencing becomes commonplace. At the time of this assay development and manuscript preparation, additional strains originated in United States (epsilon) and India (delta) were not considered VOCs. We are currently optimizing and screening samples for these additional mutations. Key updates will also be posted on a preprint journal. In the meantime, we hope that our current test will help increase surveillance and potentially help control the spread of the new emerging variants of concern.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

SUPPLEMENTAL FILE 2, XLSX file, 11.6 MB.

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