Rapid Detection of SARS-CoV-2 Variants by Molecular Clamping Technology Based RT-qPCR

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Abstract

Given the challenges that fast-changing SARS-CoV-2 variants have caused in terms of rapid spread and reduced vaccine efficacy, a rapid and cost-effective assay that can detect new and emerging variants is greatly needed worldwide. We have successfully applied the xenonucleic acid-based molecular-clamping technology to develop a multiplex RT-qPCR assay for SARS-CoV-2 multivariant detection. The assay was tested on 649 nasopharyngeal swab samples that were collected from California and Ohio. The assay was able to correctly identify all 36 Delta variant samples as it accurately detected D614G, T478K and L452R mutations. In addition, the assay was able to correctly identify all 34 Omicron samples by detecting K417N, T478K, N501Y and D614G mutations. This technique reliably detects a variety of variants and has an analytical sensitivity of 100 copies/mL. In conclusion, this novel assay can serve as a rapid and cost-effective tool to facilitate large-scale detection of SARS-CoV-2 variants.

Introduction

Two years after its initial emergence, the SARS-CoV-2 fueled COVID-19 pandemic continues to spread globally with 523 million infections and 6.2 million deaths to date (May 24, 2022)[1]. In the United States alone, there have been 83 million documented cases, and the death toll has passed 999,785 (May 24, 2022) [2]. Despite increasing vaccination levels and a rising number of COVID-19 recovered people who have acquired some degree of natural immunity, the pandemic has continued. This is largely due to the appearance of more transmissible and partially vaccine-resistant novel variants of the virus. Since the inception of the pandemic, five variants of concern (VOC) and seven variants of interest (VOI) have emerged worldwide [3–8]. These include the Alpha B.1.1.7 (501Y V1)[3], Beta B.1.351 (501Y.V2)[4], Gamma P.1 (501Y.V3)[5], Epsilon CAL.20C (20C/S:452R; /B.1.429)[6–8], Delta B.1.617.2, and Omicron (B.1.1.529) [9].

The Alpha B.1.1.7 strain, also known as 20I/501Y.V1 and VOC 20DEC-01 (VOC-20DEC-01, previously written as VOC-202012/01), was found initially in the southeast of England in early October 2020 and became prevalent in both Europe and the United States shortly thereafter [10–13]. It is estimated to be 40–80% more transmissible than the original SARS-CoV-2 strain[14,15].

While the main variant from April to June 2021 in the United States was the Alpha variant, its decline from 70% of cases (May 8th - Jun.19th) to 9.0% of cases (July 17th 2021) corresponded with an increase in the prevalence of the novel Delta variant, which rose from 0.6% (April 24th 2021) to 82.8% (July 17th, 2021)[16–20]. The Delta B.1.617.2 variant was first identified in India[21–22] and is characterized by 13 mutations [23]. In addition, a Delta plus variant, which has the Delta mutations plus an additional K417N mutation, has also been detected. Compared to Alpha, these variants are more contagious and are associated with a high degree of mortality. The Delta variants grow more rapidly in the human respiratory tract and have 1000 times higher viral loads than the original strain[24]. In addition, large numbers of breakthrough cases associated with the Delta variants have been documented in previously vaccinated individuals.

Recently, a new SARS-CoV-2 variant, B.1.1.529, was discovered in Botswana and South Africa, respectively on Nov. 11 and 14, 2021, and reported to WHO on Nov. 24, 2021[9]. This new Omicron variant became the prevalent variant in the United States in Jan 2022 and is classified as one of the five variants of concern (VoC) based on its known epidemiology [9]. The Omicron variant contains more than 30 changes to the spike protein including 15 mutations located in the receptor binding domain (RBD) (residues 319–541)[25, 26]. While this variant demonstrated increased transmissibility as compared to previous VOC, it appears to be associated with a lower level of morbidity and mortality. Currently, Omicron BA.2 (VUL-22Jan-01) with a spike gene deletion at position 69–70, demonstrates about 75% greater relative growth compared with the Omicron BA.1 (B.1.1.529, or VOC-21NOV-01). Even more concerning are the Delta/Omicron BA.1 combination (XD) and the Omicron BA.1 and BA.2 recombinant (XE) [27].

Each one of these main VOCs has a signature set of spike protein mutations that allows them to be uniquely identified. While each VOC has a unique set of spike protein mutations, the D614G mutation is shared by all five VOC. The D614G mutation involves an amino acid change from aspartic acid to glycine, which is caused by an A-to-G nucleotide transition at position 23403 of the viral genome. This change stabilizes the spike protein and enhances its fitness and infectivity[17] and is associated with increased viral infectivity and transmissibility [18–19]. Another key mutation, N501Y, is found in all the VOCs except for Delta. The N501Y mutation is located within the RBD of the spike protein and is associated with stronger binding to ACE2 receptor and higher viral infectivity. Of greater concern though is the fact that the efficacy of vaccine-mediated immunity against strains containing the N501Y mutation seems to be reduced [20]. These two mutations were the two earliest mutations observed and remain the most prominent mutations detected in the SARS-Cov-2 virus at this point in the COVID-19 pandemic.

To date, next-generation sequencing (NGS) has been the standard method used for SARS-CoV-2 variants detection [28]. Although the NGS-based assays facilitate variant detection and confirmation, they are expensive, time consuming, require technical expertise, and are not readily available particularly in low-income countries. These factors limit their utility in large-scale testing and monitoring of SARS-CoV-2 variants.

Given their importance in fighting the pandemic, the MIT Technology Review magazine predicted that “COVID Variant Tracking” will be one of the ten breakthrough technologies in 2022 [29].

Considering the rapid emergence of novel variants, testing platforms capable of rapid detection of specific variants in a cost-effective manner are urgently needed. Toward this end, target-specific RT-PCR screens for specific mutations within the spike protein had been developed [30–31]. In this study, we have developed a multiplex reverse-transcription quantitative real-time polymerase (RT-qPCR) assay that can rapidly and reliably detect known and emerging SARS-CoV-2 variants, based on their mutation profiles (Fig. 1). The assay is based on molecular-clamping technology and uses xenonucleic acids (XNA) as molecular clamping probes. XNAs are artificial genetic polymers that retain the Watson-Crick base-pairing capability and exhibit high chemical and biological stability. In practical applications for disease diagnosis and treatment, XNAs have been used as a source of nuclease-resistant affinity reagents (aptamers) and catalysts (xenozymes). More notably, they can be employed as molecular clamps in RT-qPCR or as highly specific molecular probes for detecting nucleic acid target sequences due to the stronger hybridizing/binding capability seen in XNA/DNA duplexes than DNA/DNA duplexes[32]. Furthermore, even a single base-pair mismatch near the center of the sequence of an XNA/DNA duplex can result in a big drop of 10–18°C in melting temperature (TM) [33]. This appealing feature allows for the highly specific clamping of the XNA molecule onto the targeted sequence (usually the wildtype, WT) to block WT amplification, thus minimizing WT background in RT-qPCR and selectively enhancing the signal of the mutant. Robust XNA based assays have been extensively used for *in vitro* diagnostic assays for detecting rare cancer-associated gene mutations [34]. Based on their molecular properties and prior utility in cancer mutation detection, XNAs represent an ideal tool for developing highly sensitive RT-qPCR assays for detecting SARS-CoV-2 mutations.

In this report, we demonstrated for the first time the feasibility, efficiency, reliability and applicability of using an XNA-based RT-qPCR method to detect various SARS-CoV-2 variants. This assay provides a rapid, reliable and cost-effective testing platform for rapid detection and monitoring of known and emerging SARS-CoV-2 variants.

Methods

Study design and ethics

Deidentified patient nasopharyngeal swab (NPS) were collected and tested from early 2021 to early January 2022 at San Francisco VA Medical Center clinical laboratory and DiaCarta clinical laboratory for clinical diagnostic or screening purposes. This portion of the study was approved by the institutional review board (IRB) at UCSF (UCSF IRB #11-05207) as a no-subject contact study with waiver of consent and as exempt under category 4. The second set of deidentified NPS samples were collected and tested from June 2021 to early January 2022 at the COVID-19 screening lab at Franciscan University, Ohio. This portion of the study was approved by the IRB at Franciscan University (FUS IRB #2021-07). All experiments were performed in accordance with relevant guidelines and regulations.

Sample collection and RNA extraction

From early 2021 to early January 2022, patient NPSs were collected in virus transportation medium (VTM), tested for SARS-CoV-2 infection, and then stored at − 80°C before being used for analysis in this study. An automatic RNA/DNA extraction instrument MGISP-960 (MGI Tech Co., Ltd.) and the MGI Easy Nucleic Acid Extraction Kit (Cat# 1000020261) were used to extract SARS-CoV-2 viral RNA according to the manufacturer’s instructions. The extraction output was RNA in 30–40 µL RNase-free water, 5.5 µL of which was then used for a single RT-qPCR reaction. The typical turnaround time from sample RNA extraction was about 90 min for 384 samples (Fig. 1) [35].

Multiplex primer and probe design

In order to design primers and probes to detect all the SARS-CoV-2 variants of concern, the conserved regions of ORF1ab gene [31] and areas adjacent to the N501Y, D614G, T478K, L452R, K417T and K417N mutations in S protein RBD were targeted (Fig. 1A). Unique combinations of these mutants can be used to identify each specific VOC (Table 1).

Table 1
List of SARS-CoV-2 Variants of Concern Targeted Mutations Detected by QuantiVirus™ SARS-CoV-2 Variants Detection Kit

S Gene Mutants	Alpha (B.1.1.7)	Beta (B.1.351)	Gamma (P.1)	Delta (B.1.617.2)	Delta Plus	Epsilon /Kappa (B.1.427/B.1.429)/(B.1.617.1)	Omicron (B.1.1.529)
D614G	√	√	√	√	√	√	√
N501Y	√	√	√				√
T478K				√	√		√
L452R				√	√	√	
K417T			√				
K417N		√			√		√

Gene sequences were retrieved from GenBank and GISAID databases for primer and probe design to ensure coverage of all SARS-CoV-2 variant strains. Multiple alignments of the collected sequences were performed using Qiagen CLC Main Workbench 20.0.4., and conserved regions in each target gene were identified using BioEditor 7.2.5. Primers and probes were designed by using Primer3plus software. All primers were designed with a Tm of approximately 60°C and the probes were designed with a Tm of about 65°C. The amplicon sizes were kept within the range of 70 bp to 150 bp for each primer pair in order to achieve high amplification efficiency and detection sensitivity. All designed primers and probes were ordered from Integrated DNA Technologies, Inc. (Coralville, IA) and LGC Biosearch Technologies (Novato, CA), respectively.

XNA design, synthesis & analysis and optimization

Five groups of XNAs, each specific to one of five SARS-COV-2 mutations (D614G, N501Y, T478K, L452R, K417T/K417N), were designed to be exact matches with the wild type (WT) sequence in order to facilitate selective blocking of qPCR amplification of WT targets. For each mutation, a number of XNAs of different lengths were synthesized in our chemistry laboratory (Supplementary Table 2). Each XNA was also designed to partially overlap with and be of the same strand/sense as the corresponding qPCR probe. The best XNA probe sequences were selected based on iterative adjustment of multiple major physicochemical factors: probe sequence length, GC content, purine content and arrangement, self-complementarity and melting temperature. Five groups of XNAs were synthesized and each group was tested using qPCR to find the optimal size and concentration of XNA for each group. Figure 2 shown a MALDI-TOF mass spectra of XNA.

Real-time reverse-transcription PCR

Two different detection kits assays were developed using this methodology. The QuantiVirus™ SARS-CoV-2 Variants Detection Kit assay consisted of three multiplex PCR tubes: tube A tested for the presence of D614G and L452R, tube B tested for the presence of K417 T, N501Y, and the human Rp gene as internal control, and tube C tested for the presence of T478K, K417 N, and ORF1ab as wildtype target. The QuantiVirus™ SARS-CoV-2 Delta + Detection Kit assay consisted of only one 4-plex PCR tube that tested for the presence of T478K, K417N, ORF1ab, and Rp.

XNA-based RT-qPCR reactions with a total volume of 10 mL were developed using the following reagents: 5.5 µL of viral RNA, 1.0 µL of primer and probe mixture (final concentration of 0.2 µM and 0.1 µM, respectively), 1.0 µL 10x XNAs mixes, and 2.5 µL of 4x One-step qRT-PCR Master Mix (Catalog# A28526, Thermos Fisher, MA).

The qPCR was performed at 25°C for 2 min with uracil-N- glycosylase (UNG) incubation to remove potential carryover, then 53°C for 10 min for reverse transcription, followed by 95°C for 2 min. Then 45 cycles of 95°C for 3 sec and 60°C for 30 sec were used for amplification. A Bio-Rad CFX384 (Bio-Rad, CA) was used for RT-qPCR amplification and detection. [35–37]. The results were interpretated according to the Supplementary Table 1.

Analysis of assay sensitivity and cross-reactivity test

Twist SARS-CoV-2 RNA controls 16, 17, 23 and 48 (Twist Bioscience, CA) were used as references to test the assay sensitivity. We used a two-fold dilution series from 800 copies/mL down to 25 copies/mL of the templates in triplicates and identified the lowest concentration that was detectable with 95% confidence to determine the analytical sensitivity limit of detection (LoD).

MERS- coronavirus and SARS-CoV coronavirus samples were ordered from ATCC (Manassas, Virginia) and the ZeptoMetrix NATtrol Respiratory Validation Panel was ordered from ZeptoMetrix (cat# NATRVP-3, Buffalo, NY). RNA/DNA was extracted from high titer stocks of these potentially cross-reacting microorganisms (estimated 10^5 units/mL) using the Thermo Fisher's PureLink™ Viral RNA/DNA Mini Kit (cat# 12280050) and the Qiagen QIAamp DNA Mini Kit (Cat#. 51304). The extracted sample RNA/DNA was eluted by sterile RNase-free water to

give a 100 µL solution. 5.5 µLs of each of the purified RNA/DNA samples was tested in triplicate with QuantiVirus™ SARS-CoV-2 Variants Detection Kit.

Clinical evaluation of samples

All the clinical samples were evaluated using the QuantiVirus SARS-CoV-2 Variants Detection kit. In addition, the presence/absence of SARS-CoV-2 was confirmed for all samples using the FDA EUA approved QuantiVirus ^{TM} SARS-CoV-2 multiplex Kit at the DiaCarta CLIA-certified clinical laboratory or at the Franciscan University Covid screening lab.

Sanger sequencing & next generation sequencing verification

All samples that were identified as variant positive by this assay were sent out for Sanger Sequencing to confirm their mutational status (SequeTech, CA), and all sequences were analyzed via the UCSC SARS-CoV-2 Genome Browser.[38]

The Next Generation Sequencing (NGS) library was prepared with amplicon methodology using the CleanPlex® SARS-CoV-2 FLEX Research and Surveillance Panel (Cat#. 918010, Paragon Genomics, CA) or Illumina COVIDSeq Test (FDA EUA approved, cat# 20049393), and sequenced on the MiSeq instrument (Illumina, CA). All the raw data were analyzed by ARTIC workflow on Galaxy (https://github.com/galaxyproject/SARS-CoV-2), using a reference sequence from the NCBI database (NC_045512.2) [39–41]. All tools were used with default parameters.

Results

XNA enhances the ability to distinguish viral variants from wild type in RT-qPCR

In order to test whether XNA clamping of the wild type sequences enhances mutant detection, we compared the RT-qPCR results with and without XNA. An amplification curve of the D614G mutant versus wild type without XNA showed that it is difficult to distinguish the mutant and the wild-type virus (Fig. 3A&C). However, with XNA, the differentiation of the mutant from wild type SARS-CoV-2 (Fig. 3B&D) was clear (Δ Ct ~ 13–20). To optimize the assay, we tested different concentrations of XNA. Representatively, the optimization results of D614G and N501Y XNAs are shown in Supplementary Tables 3 and 4, respectively. The XNA that generated the better delta-Ct between wild type and the mutant was selected for use in RT-qPCR. For example, 8uM D614G XNA001 displayed a better Δ-Ct 15.9 and 0.25 mM N510Y XNA003 has delta Ct 32.7. Through the optimization process, we were able to select D614 XNA001, N501 XNA003, T478 XNA001, L452 XNA003, and K417 XNA001 as the optimal XNAs (Fig. 2).

Analytical sensitivity and cross-reactivity evaluations

In order to determine the analytical sensitivity of the assay, we performed the QuantiVirus ^{TM} SARS-CoV-2 variant detection test on a Bio-Rad CFX384. We diluted SARS-CoV-2 RNA control 16 which contains the N501Y and D614G mutants (Twist Bioscience, CA) from 800 copies/mL down to 25 copies/mL and tested each level dilution in triplicate. The data show that the N501Y and D614G mutants were detected with Ct values of 34.07 and 33.78 at a viral RNA concentration of 100 copies/mL (Table 2). The analytical sensitivity, namely the limit of detection (LoD), was determined to be 100 copies/mL.

Table 2
Limit of Detection (LoD) of the Multiplex RT-qPCR for SARS-CoV-2 Variant Detection

Estimated Viral Concentration (Copy/mL)	N501Y		D614G		ORF1ab		RP gene	
	Ct	Ct Mean	Ct	Ct Mean	Ct	Ct Mean	Ct	Ct Mean
800 Copies/ml	31.48	31.31	29.16	29.3	30.57	30.43	31.97	31.64
	31.26		29.34		30.35		31.27	
	31.2		29.39		30.36		31.67	
400 Copies/ml	32.88	32.51	30.57	30.46	31.55	31.47	32.86	32.65
	32.44		30.37		31.58		32.72	
	32.22		30.45		31.29		32.36	
200 Copies/ml	33.19	33.24	32.59	31.93	32.63	32.8	33.79	33.65
	33.36		31.44		33.16		33.69	
	33.17		31.75		32.6		33.47	
100 Copies/ml	34.32	34.07	32.64	33.78	33.5	33.4	34.25	34.36
	34.45		36.18		33.4		34.27	
	33.43		32.5		33.29		34.55	
50 Copies/ml	36.52	36.44	40.3	36.75	34.89	34.51	37.14	36.31
	35.94		34.59		35.18		36.21	
	36.84		35.35		33.44		35.59	
25 Copies/ml	36.76	36.1	39.68	38.53	35.03	34.88	37.11	36.56
	> 40		37.47		34.65		35.64	
	35.44		38.44		34.95		36.93	

We evaluated the cross-reactivity of the assay to a variety of other pathogens. The results are summarized in Supplementary Table 5 and indicate that there is no cross-reactivity between the SARS-CoV-2 variants XNA-based qPCR assay and any of the organisms tested.

Use of XNA-based RT-qPCR to confirm rapid surge of Alpha variant in San Francisco Bay Area in early 2021

In order to determine the ability of the Quantivirus ^{TM} SARS-CoV-2 Variants Detection kit to detect the Alpha variant, 374 confirmed positive samples that were collected between January and March 2021 in the San Francisco Bay Area were analyzed. Among the 139 positive specimens sampled from mid-January 2021, 58 (41.7%) were positive for the D614G mutation but not the N501Y (Supplementary Table 6). None of the samples were positive for both N501Y and D614G mutations, a defining characteristic of the Alpha variant (B1.1.7). However, of the 139 positive specimens sampled from late February and the 96 positive specimens collected in March 2021, there were 7 (5.04%) and 10 (10.42%) specimens, respectively, that tested positive for both mutations. The data indicated an increase in the Alpha variant frequency in Northern California, from 0% in January to above 10% in March 2021. Additionally, the multiplex RT-qPCR test showed high specificity, i.e., amplification in non-N501Y samples and negative controls was completely blocked by the XNA in the RT-qPCR, while amplification was present in all N501Y positive clinical samples and positive controls (Fig. 4A).

To verify that the D614G and the D614G/N501Y mutations in these mutant samples were detected correctly, the amplicons from these qPCR samples were also analyzed by Sanger Sequencing. All 160 D614G positive samples and all 17 D614G/N501Y double positive samples and all 197 wildtype samples, as detected by the Quantivirus ^{TM} SARS-CoV-2 Variants Detection kit, were confirmed by Sanger Sequencing indicating that the specificity of the test was 100%. The Sanger sequencing peaks showed the target mutations in the viral cDNA, T < C in the case of D614G (Fig. 4B), and A < T in the case of N501Y (Fig. 4C).

As summarized in Table 3, the XNA-based RT-qPCR has a Positive Predictive Value (PPV) of 100.0% (95% CI:0.99-1.00) and Negative Predictive Value (NPV) of 100% (95% CI: 0.96-1.00) for detecting the Alpha variant.

Table 3
Clinical Samples Evaluation with Multiplex qPCR Test in Early 2021

SARS-CoV-2	Patients Samples (N)	Quantivirus SARS-COV-2 Variant Detection			Variant Identification			Sensitivity (95% CI)	Specificity (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
		Mutant Detection	Positive	Negative	Alpha	early mutation/ wildtype	Negative				
Positive	374	N501Y	17	357	17	160/197	102	100% (0.77-1.00)	100% (0.99-1.00)	100% (0.77-1.00)	100% (0.99-1.00)
		D614G	177	197				100% (0.97-1.00)	100% (0.98-1.00)	100% (0.97-1.00)	100% (0.98-1.00)
		ORF1ab	374	0				100% (0.99-1.00)	100% (0.96-1.00)	100% (0.99-1.00)	100% (0.96-1.00)
Negative	102	Rp	476	0							

* Bio-Rad CFX384 qPCR instrument was used for this test; all of 374 positive samples were detected ORF1ab gene (wildtype) positive; all variants data were confirmed with Sanger Sequencing

Use of XNA-based RT-qPCR to detect breakthrough COVID-19 cases caused by Delta variant and Omicron variant

Despite widespread vaccination, a significant number of breakthrough cases were seen in late 2021 and early 2022 due to emergence of two new SARS-CoV-2 variants, Delta and Omicron. To examine the ability of the Quantivirus ^{TM} SARS-CoV-2 Delta Plus Detection kit to detect these variants, we analyzed 39 nasopharyngeal swabs (NPS) that were initially collected from symptomatic COVID-19 positive individuals from Franciscan University in Ohio (Fall 2021) who had been vaccinated ("breakthrough" infection). In addition, 6 NPS samples collected from healthy donors were analyzed as well. All the breakthrough patient samples tested 36 positive for tne SARS-CoV-2 Delta variant as they displayed the T478K mutation with the wild-type ORF1ab and 3 Delta plus variant as K417N and T478K mutant detectable, whereas all 6 samples collected from healthy donors tested negative for SARS-CoV-2 (Supplementary Table 7). As a control, we also tested an additional 44 negative samples and determined that the assay's PPV for Delta detection was 100% (95% CI: 0.89-1.0) and its NPV was 100% (95% CI: 0.91-1.0) (Table 4).

Table 4
Summary of Delta Variant Detection for Breakthrough Patient Samples during Oct-Nov 2021

qPCR Kit	Patient Sample (N)	Mutant Target	Positive	Negative	Variant Identification			PPV (%)	NPV (%)
					delta	**delta +**	**negative**		
Quantivirus SARS-CoV-2 delta plus	89	K417N	3	86	36	3	50	100% (95%CI: 0.888-1.0)	100% (95% CI:0.911-1.0)
		T478K	39	50					
		Orf1ab	39	50					
		RP	89	0					

*all variants data were confirmed with Sanger Sequencing

To assess the ability of Quantivirus ^{TM} SARS-CoV-2 Variants Detection kit to detect the Omicron variant, we tested an additional 37 NPS samples, 34 of which were collected from vaccinated symptomatic patients in early January 2022. All 34 samples collected from vaccinated symptomatic patients tested positive, while the other three samples tested negative for SARS-CoV-2 by the FDA EUA approved kit (Quantivirus ^{TM} SARS-COV-2 Multiplex Test). All 34 positive samples further tested positive for the K417N, T478K, N501Y and D614G mutations, characteristic of the Omicron variant, using the XNA-based RT-qPCR assay in this kit (Supplementary Table 8). As a control, we also tested 50 negative confirmed samples and determined that the assay's PPV for Omicron detection was 100% (95% CI: 0.87-1.00) its NPV was 100% (95% CI: 0.92-1.00) (Table 5).

Table 5
Summary of Omicron Variant Detection for Breakthrough Patients during early 2022

qPCR Kit	Patient Sample (N)	Mutant Target	Positive	Negative	Variant Identification		PPV (%)	NPV (%)
					Omicron	Negative		
Quantivirus SARS-CoV-2 Variant Detection	87	D614G	34	53	34	53	100% (95% CI: 0.87-1.0)	100% (95% CI: 0.92-1.0)
		N501Y						
		K417N						
		T478K						
		Orf1ab						
		RP	87	0				

* all variants data were confirmed by Sanger Sequence.

Summary of XNA-based RT-qPCR assay in detecting SARS-CoV-2 variants and Comparing with NGS

In total, 649 samples were screened using the kit, including 447 SARS-CoV-2 positive samples and 202 negative samples (Table 6). Among the 447 positive samples, 17 cases of the Alpha variant, 5 cases of the Beta variant, 36 cases of the Delta variant, 3 cases of the Delta Plus variant (AY.4.2) and 34 cases of the Omicron variant were detected. Notably, there were 160 cases which were only positive for D614G and 192 cases of “wildtype” (no mutation), mostly from early 2021. In summary, 21.3% (95/447) of the samples were identified to be SARS-CoV-2 variants and their prevelance over time followed the sequential emerging of the variants in North America. At least 57% (255/447) of the positive samples had at least one mutation. All these data were confirmed by Sanger Sequencing.

Table 6
Summary of SARS-CoV-2 Multivariant Detection in January 2021-January 2022

Sample Total (N)	Status (n) pos/neg	Variant (n)	ORF1ab	D614G	N501Y	T478K	L452R	K417T	K417N	Variant Identify
649	447 (positive)	34	x	x	x	x			x	Omicron
		36	x	x		x	x			Delta
		3	x	x		x	x		x	Delta Plus
		5	x	x	x				x	Beta
		17	x	x	x					Alpha
		160	x	x						Early Mutation
		192	x							Wildtype
	202 (negative)	202								Negative

To further validate the XNA-based RT-qPCR assay in detecting SARS-CoV-2 variants, a random subset of eight positive samples and two SARS-CoV-2 negative samples from early 2021 were tested by this assay and NGS. All eight tested N501Y/D614G positive, while the 2 SARS-CoV-2 negative samples tested negative using the kit. All ten samples were then sequenced by NGS using the CleanPlex® SARS-CoV-2 FLEX kit and all 8 positive samples were confirmed as Alpha variants, and the 2 negative samples were confirmed as negative (Supplementary Table 9). We additionally sequenced and analyzed 13 positive samples (vaccinated breakthrough cases) and 2 negative samples collected in early 2022 using both the QuantiVirus™ SARS-CoV-2 Variants Detection Kit and the Illumina COVIDSeq Test. Again, the results were all congruent (Supplementary Table 9). The data demonstrated that our RT-qPCR platform gave the same result as the Illumina COVIDSeq Test with 100% concordance.

Discussion

We have described the successful development of a multiplex RT-qPCR testing platform for the rapid detection of SARS-CoV-2 variant strains using XNA-based molecular clamping technology. In order to develop a sensitive and specific molecular clamping assay, it is not only

necessary to select a suitable set of primers and probe for a given mutant gene target, but it is also imperative to choose an XNA with appropriate sequences and desired performance characteristics. Our XNA selection process included a two-fold approach. First, during the sequence design, we excluded those improper sequences with problematic features (e.g., sequences that were too long or too short, had too high or too low Tm values, high purine content, long purine stretch, or unwanted self-complementarity within or between XNA molecules). Secondly, for each mutation assay we designed and synthesized multiple XNAs and compared their qPCR clamping robustness and clamping specificity (higher delta Ct difference between wild-type gene and mutation gene amplification). Notably, across the XNA groups, the XNA with a Tm of nearly 80°C stood out in the selection process, which is likely related to the established RT-qPCR temperature-cycling conditions[42].

Using this assay, we were able to track the changes in SARS-CoV-2 variants over time. Due to its higher transmissibility compared to the original SARS-CoV-2 strain, the D614G mutant of SARS-CoV-2 became the dominant variant in the beginning of 2021 in the United States. Its sub-clade, the N501Y mutation independently emerged in the UK and South Africa and subsequently spread to North America during the first half of 2021. Our data indicated that the spread of this Alpha variant in northern California likely started in February 2021 because patient samples collected in January 2021 were all negative for the N501Y mutation, whereas the N501Y mutation was detectable in up to 5% of the samples collected in late February 2021. Although the U.S. CDC predicted that, according to an epidemiology model [43] at that time, the B.1.1.7 or Alpha variant would quickly dominate soon, in actuality the Alpha variant was soon largely replaced around the middle of 2021 by the even more potent and infectious Delta variant,. The Delta variant in turn was soon replaced by the more contagious Omicron variants in late 2021 to early 2022. Our RT-qPCR data, confirmed by Sanger or NGS sequencing, were consistent with the sequential surges of COVID-19 cases caused by the D614G mutant and the Alpha, Delta and Omicron variants in the United Stated from late 2020 to early 2022.

Availability of rapid and accurate testing platforms is critical for tackling the challenge of emerging SARS-CoV-2 variants. Amongst the many detection methods, a qPCR-based platform could serve as a rapid, cost-effective and practical testing tool for monitoring SARS-CoV-2 evolution.

A few biotechnology companies and academic institutions have been developing or have reported variant detection methods using qPCR. These methods can be categorized into one of the following: (1) mutant gene-specific or allele-specific primers and probe; (2) spike gene target failure (SGTF); (3) E gene target failure, and (4) ORF gene deletion [44–47]. Some of the methods still require NGS to confirm the results, whereas other platforms require pre-testing by regular SARS-CoV-2 RT-qPCR before carrying out the variant assay. While these assays can be useful in certain circumstances [48] also emphasized the cost-effectiveness, quick and scalability, they are limited by the effectiveness and accuracy of the primers and/or probes used. This issue can limit the use of these variant testing methodologies. For example, the allele-specific PCR (AS-PCR) method quickly attracted attention for mutation detection by some test developers [49]. However, AS-PCR has two inherent shortcomings: (1) due to the fixed 3' end of the allele-specific primer, it is not always feasible to choose optimal primers for PCR amplification; and (2). high purity DNA/RNA is essential as low-quality or crude DNA/RNA samples are prone to providing inconclusive results [50].

However, XNA-based qPCR can overcome these aforementioned shortcomings because XNA sequences can be designed with more flexibility compared to restrictive primer sequence design [51], and XNA-based qPCR works better with samples of low-concentration DNA/RNA and samples with high-background mutant gene targets. Compared to AS-PCR, as demonstrated in our studies, the XNA-based RT-qPCR assay can achieve a lower LOD, about 100 copies/mL for variant detection.

The molecular clamping technology used in this study increases the sensitivity and specificity of conventional qPCR as wild-type background amplification is minimized by this method. This XNA clamping-based RT-qPCR assay can also improve multiplexing of targets, as we were able to differentiate the wild-type SARS-CoV-2 (ORF1ab gene for wild-type detection) and its variants in a single run. In addition to detecting the existing SARS-CoV-2 mutations D614G and N501Y, the assay described here also detected L452R, T478K, K417N and K417T. The presence or absence of these major mutations can be used to identify all five VOCs based on their unique mutation profiles VOCs [52] (Supplementary Table 1). As a result, this assay covers almost all SARS-CoV-2 variants (both VOC and VOI), including Delta, “Delta Plus” and Omicron, all of which have the potential to breakthrough vaccine-induced protective immunity [52]. This strategy can provide an effective, rapid and easily adoptable testing platform for known and emerging SARS-CoV-2 variants in the future, and this technology can be easily adopted by any clinical laboratories that perform routine SARS-CoV-2 RT-qPCR testing. Given the past stages of the pandemic, future new variant(s) with increased transmissibility could possibly lead to a potential exponential increase in cases and/or deaths [10]. Therefore, timely and convenient detection of the concerning variants is of high importance.

There are 15 mutations in the RBD of Omicron variant, including Q393R, K417N, T478K, S477N, E484A, G496S, Q498R, Y505H, G446S, N501Y, N440K, S75F, S373P, S371L and G339D [26]. This posts a significant challenge for RT-qPCR based assays as this high number of mutations can render some primers dysfunctional. Interestingly, the N501Y primer, which works well for Alpha, Beta and Delta variant detection, had a lower signal for N501Y detection in Omicron variants due to the fact that the original N501Y primer was located in Omicron high mutant region. To

avoid this issue in the future, we have re-designed new set of “degenerate” primers and probe to better fit the mutant area in Omicron, while still covering the original N501Y region in Alpha, Beta and Delta variants. Evaluation of this revised N501Y assay design showed that it is effective in distinguishing these variants effectively. This illustrates how this method can be rapidly adapted to test new and emerging variants while still successfully detecting older variants.

Despite this issue, our XNA-based RT-qPCR assay kit was able to identify the Omicron variant because of the presence of three mutations D614G/T478K/K417N and the absence of L452R mutation, a combination unique to the Omicron variant. Indeed, we were able to detect and differentiate Omicron and Delta variants from successfully detected the Omicron variant in patients that had breakthrough SARS-CoV-2 infections in early January 2022.

In summary, we have developed a multiplex RT-qPCR testing platform for the rapid detection of SARS-CoV-2 variants using the XNA-based molecular clamping technology. This testing platform can be easily adopted by laboratories that perform routine SARS-CoV-2 PCR testing, providing a rapid and cost-effective method in lieu of NGS-based assays, for detecting, differentiation and monitoring SARS-CoV-2 variants. In addition, this assay is easily scalable to any new variant(s) should it emerge.

Declarations

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Author Contributions

Data collection, analysis and interpretation (S.S., M. J., A.F., J. L. L.P. C.Z., M.S., C.M.L.M.R. M.K. D. K. J.N. I. A.); chemical synthesis, purification and analysis (A.F., D.C.); clinical sample processing (J. L. and S. S. L.P. J.P.); writing of original draft (S.S., M. J., A.F., M.S.); revision and editing (A.F., M.S., S.S., D.K., J.P. C.M.L); conceptualization (M.S., M. P., A.F., Q.S); project planning and administration (M.S., A. Z.).

Competing interest

The authors declare no competing interests.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data generated and analyzed during this study are available from the corresponding author upon reasonable request.

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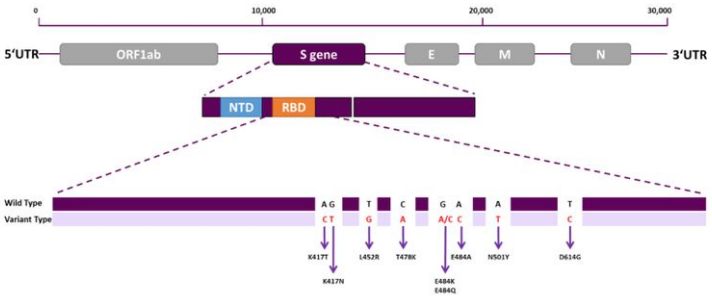
Figures



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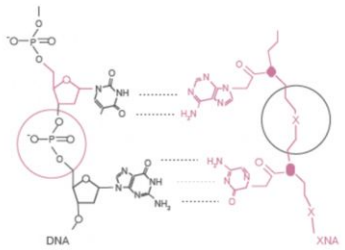

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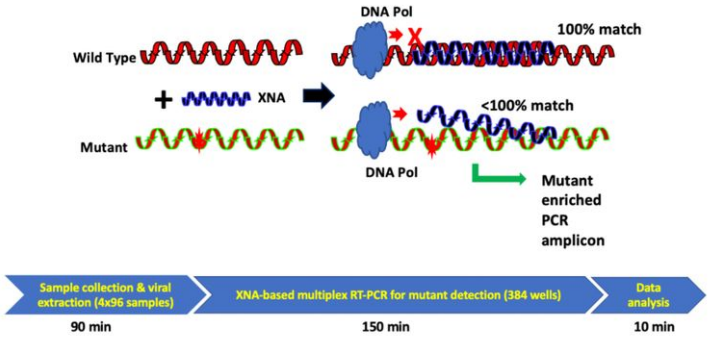


(C)

Figure 1

Principle of SARS-COV-2 Variant Detection RT-qPCR. (A) SARS-CoV-2 genome structure and its spike gene target sites for our multivariant assay. (B) Primary structure of the XNA clamping molecule in a typical XNA/DNA duplex. (C) A high-throughput RT-qPCR workflow for SARS-CoV-2 variant detection, highlighting the mechanism of XNA-based molecular clamping technique capable of distinguishing a targeted mutation from its wildtype.

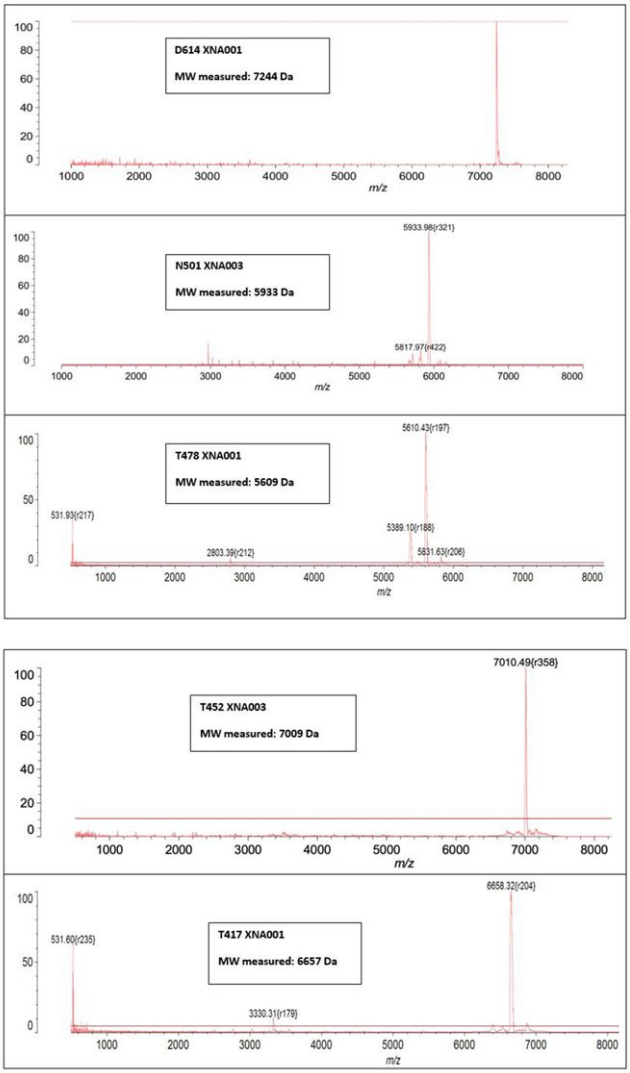


Figure 2

MALDI-TOF mass spectra of 5 representative XNA biomolecules: (a) D614 XNA001; (b) N501 XNA003; (c) T478 XNA001; (d) L452 XNA003; (e) K417 XNA001. Their molecular weights (MW) were measured based on the cationic $(M+H)^+$ peaks for the characteristic singly charged parent ions.

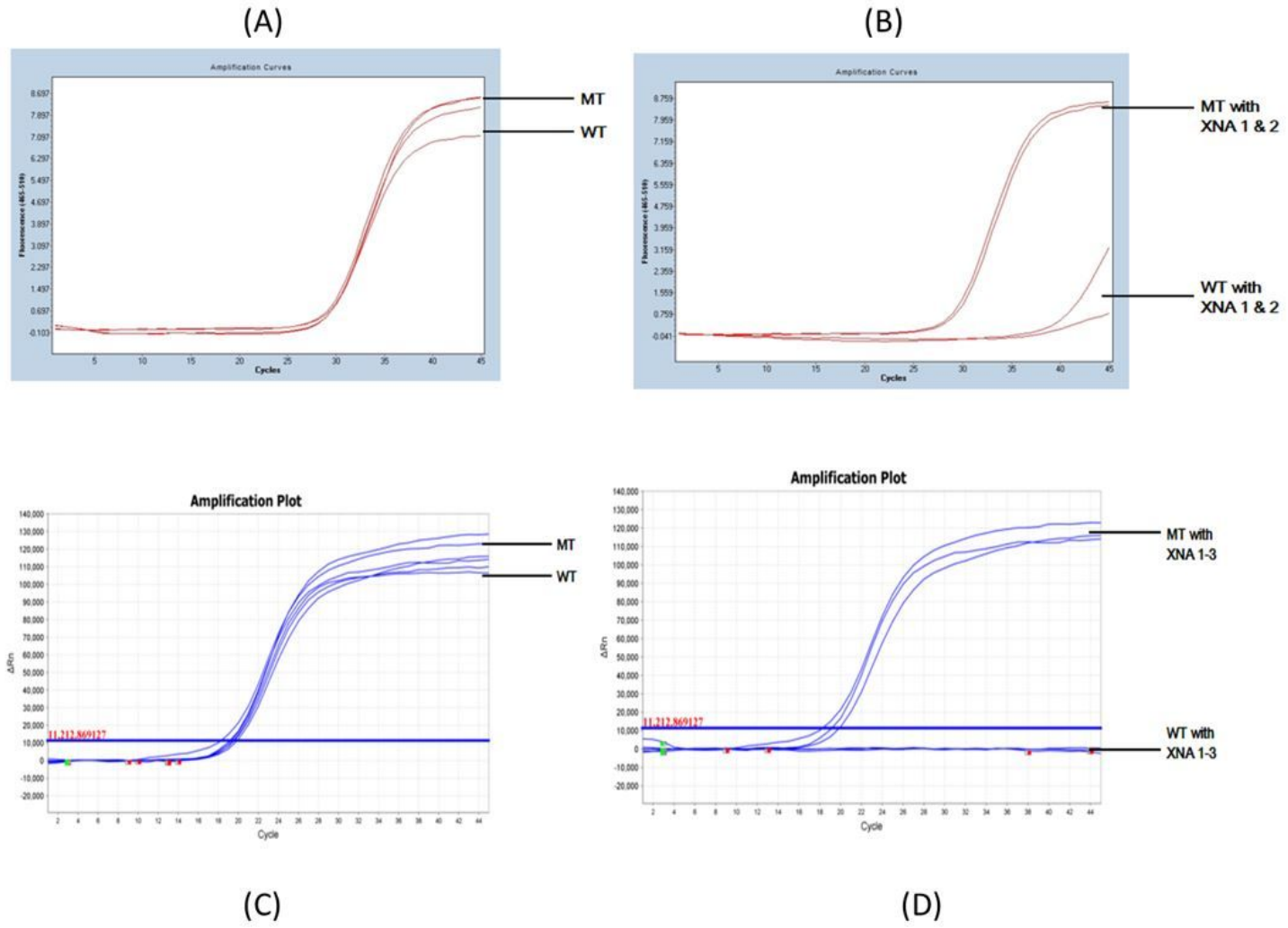
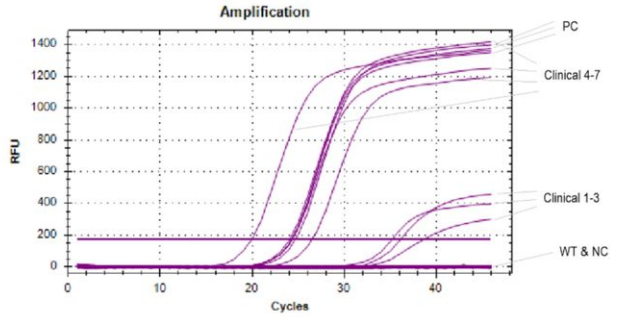
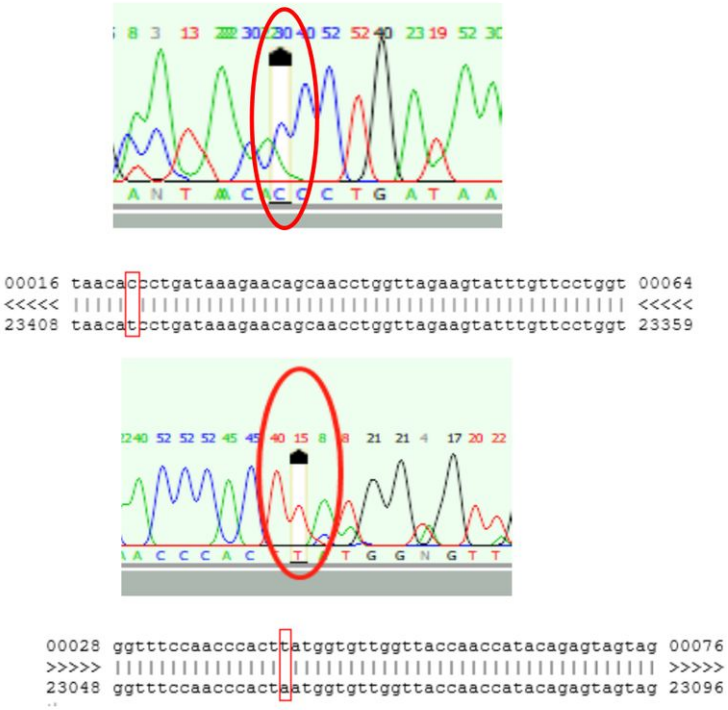


Figure 3

qPCR test with and without XNA. (A) Amplification curve of D614G mutant (MT) vs wild type (WT) without XNA. Both wild-type and mutant had ~30 Ct. It is difficult to distinguish mutant and wild type. (B) Amplification curve of D614G mutant vs wild type with XNA. Wild type amplification was blocked and had high Ct ~40, but mutant was enhanced and had Ct ~27, making the differentiation of WT/MT highly reliable, easy, and accurate. (C) N501Y mutant (MT) vs wild type (WT) without XNA. Both wild-type and mutant had ~21 Ct. It is difficult to distinguish the mutant and the wild type. (D) N501Y mutant vs wild type with XNA. Wild-type amplification was blocked and had high Ct >40, but mutant was enhanced and had Ct ~20, making the distinction apparent and interpretation intuitive.



(A)



(C)

Figure 4

(A) Representative RT-qPCR amplification curves of SARS-CoV-2 variant N501Y. PC, positive control; WT, wild type; NC, negative control. Clinical, clinical samples 1-7. (B-C) Confirmation of D164G and N501Y mutations by Sanger sequencing. (B) Sanger sequencing peaks (C in red circle: D614G mutant). Sequence alignment of D614G, Red squares indicate D164G mutant sequence T<C; (C) Sanger sequencing peaks (T in red circle: N501Y mutant). Sequence alignment of N501Y. Red squares indicate N501Y mutant sequence A<T.

Supplementary Files

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- ShenetalSARSCoV2variantdetectionSupplementary.docx