Commentary

Rapid and accurate identification of SARS-CoV-2 variants containing E484 mutation

Yuan Zhang,[1,6] Yanqiu Wei,[1,6] Siyuan Yang,[3,6] Yunlong Li,[1,6] Jing Wang,[4] Zhaoyan Nie,[5] Yuhai Bi,[1,2] Wenjun Liu,[1,2] Linghang Wang,[3,*] and Limin Yang[1,*]

[1]CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Center for Influenza Research and Early Warning (CASCIRE), CAS-TWAS Center of Excellence for Emerging Infectious Diseases (CEEID), Chinese Academy of Sciences, Beijing 100101, China

[2]University of Chinese Academy of Sciences, Beijing 101408, China

[3]Laboratory of Infectious Diseases Center, Emergency Department of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing 100015, China

[4]National Institute of Metrology, Beijing 102200, China

[5]The Fourth Hospital of Hebei Medical University, Shijiazhuang 050011, China

[6]These authors contributed equally

*Correspondence: lmyang@im.ac.cn (L.Y.); linghang.wang@ccmu.edu.cn (L.W.)

Received: June 29, 2021; Accepted: November 4, 2021; Published Online: November 10, 2021; https://doi.org/10.1016/j.xinn.2021.100183

Citation: Zhang Y., Wei Y., Yang S., Li Y., Wang J., Nie Z., Bi Y., Liu W., Wang L., and Yang L. (2022). Rapid and accurate identification of SARS-CoV-2 variants containing E484 mutation. The Innovation **3(1)**, 100183.

The ongoing coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has led to over 240 million confirmed infections and severe economic burdens worldwide. Multiple emerging SARS-CoV-2 variants of concern (VOCs) and variants of interest (VOIs) have recently been identified and are now spreading internationally, including B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.617 (Delta and Kappa), C.37 (Lambda), and B.1.621 (Mu), all of which appear to be more easily transmitted (WHO, COVID-19 Weekly Epidemiological Update). Beta and Gamma VOCs can seriously impair the protective efficacy of existing vaccines, potentially precluding the possibility of attaining "herd immunity."[1] Further studies have shown that a single mutation in the viral spike (S) protein, E484 (shared by the Beta and Gamma VOCs and Mu VOI), can play a pivotal role in evading vaccine immunity; variants harboring the E484 mutation have shown resistance to neutralizing antibodies induced by previous infection,[2] resulting in a second or third wave of infections in several countries.[3] Therefore, rapid screening for E484-carrying strains is highly relevant to successful vaccination and variant monitoring efforts.[4]

At present, SARS-CoV-2 variants are primarily identified through viral whole-genome sequencing. Due to the advanced training requirements and limited availability of this technology, variant strains cannot be tracked quickly through local surveillance, rendering many epidemic prevention strategies ineffective. PCR-based screening is currently the most widely used platform for nucleic acid detection, with many diagnostic laboratories that perform PCR-based assays running at capacity throughout the pandemic. The wide availability of this technology suggests that a reliable PCR-based assay for variant strain detection could serve as a supplement to sequencing methods. However, routine TaqMan real-time PCR technology relies on the 5′–3′ exonuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence, releasing a fluorophore to generate the fluorescence signal. Even if there are a few nucleotide mutations in the complementary target sequence, TaqMan probes can still function, meaning that they are not able to distinguish between single-nucleotide differences. Thus, because the standard real-time PCR technology has shown limited ability to detect single-nucleotide mutations, there is an urgent need for development of a rapid PCR-based screen for mutant strains in global SARS-CoV-2 variant surveillance.

Early studies have shown that guanine can quench the fluorescence signal of a fluorophore via oxidative electron transfer from guanine,[5] meaning that fluorophores cannot be labeled on guanine nucleotides when designing TaqMan probes. Surprisingly, we found that, when guanine paired with cytosine, the fluorophore-quenching effect was substantially reduced. Based on this finding, we designed a self-quenching probe with the fluorophore placed adjacent to a guanine nucleotide. Because guanine will automatically quench the fluorescence, the fluorophore produced no signal, i.e., the guanine functioned similarly to the quencher of a TaqMan probe. However, when the probe was hybridized with its complementary sequence, the guanine lost its quenching ability, and the probe generated a fluorescence signal. Importantly, if there was a single-base mutation in the complementary sequence that prevented G-C base pair formation, the probe would not fluoresce due to quenching by the unpaired guanine (Figure 1A). This probe can therefore be used for identification of a single-nucleotide mutation.

The E484 mutation in the SARS-CoV-2 S protein corresponds to a nucleic acid coding sequence substitution from guanine (in the original Wuhan-Hu-1 isolate, wild type) to adenine (Beta and Gamma VOCs) or cytosine (Kappa former VOI). We designed a self-quenching probe for this site, which emits fluorescence when fully bound to the target sequence in wild-type strains but remains quenched when bound to variants carrying the E484 mutation. We combined this probe with PCR to produce a self-quenching real-time PCR assay that generates positive Ct values when the probe anneals to wild-type viral amplicons and negative Ct values for variants. To incorporate functions for both viral nucleic acid detection and mutation site identification, we added another universal probe labeled with a different fluorophore complementary to a region between the primers that is conserved among wild-type and variant strains. This universal probe could detect all SARS-CoV-2 strains (Figure 1B). Thus, we developed a dual self-quenching probe real-time PCR (DSQP-qPCR) assay, consisting of a primer pair and two probes (one universal and one specific, universal probe: ATTACCACCAACCTTAGAATCAAGATT(FAM)G; specific probe: GGTAGCAC AC CTTGTAATGGTGTT(TAMRA)G) (Figure 1C). The assay was performed using standard procedures for real-time PCR.

RNA standards based on the wild-type virus (GenBank: MN908947.3) and the Beta VOC (GISAID EPI_ISL_1250476) were obtained by *in vitro* transcription and quantified by digital PCR. We evaluated the analytical sensitivity of the DSQP-qPCR assay using these RNA standards while a TaqMan real-time PCR assay was performed in parallel. The results showed that the limit of detection for the DSQP-qPCR for both the wild-type and Beta VOC was as low as two copies/reaction, which demonstrated high analytical sensitivity and was consistent with that of the TaqMan PCR assay. Moreover, this assay could effectively distinguish between wild-type and variant viral RNA (Figure 1D). We then prepared simulated specimens by spiking different concentrations of inactivated SARS-CoV-2 isolate strain 20SF107 and recombinant lentiviral vector pseudovirus bearing the S gene of the Beta VOC into saliva sampled from healthy individuals. After extracting viral RNA, we used the DSQP-qPCR assay to detect RNA from authentic virus and pseudovirus samples. The results showed that the limit of detection for the virus and pseudovirus reached 200 genome copies/mL, which was consistent with that of the TaqMan PCR assay. We further evaluated the specificity of this assay using a series of respiratory pathogens, including influenza A viruses H1N1 and H3N2, influenza B viruses Yamagata and Victoria, respiratory syncytial virus A/B, parainfluenza virus 1/2/3, adenovirus 3/7, rhinovirus A, and coronaviruses NL63 and HKU1. The results showed that the DSQP-qPCR had no cross-reaction with other viral pathogens and showed good specificity.

Finally, we validated the DSQP-qPCR assay using 100 clinical samples, including nasopharyngeal swabs, feces, and sputum from 12 patients infected by SARS-CoV-2 (median age, 43 years; range, 28–62 years; four females and eight males). The DSQP-qPCR showed comparable sensitivity (98.84%; 95% CI: 96.67%–99.98%) and specificity (100%) with a commercial TaqMan-qPCR kit (BioGerm, China); McNemar's test showed no significant difference between the two assays. One sample was judged positive by the commercial kit and negative by DSQP-qPCR. The TaqMan-qPCR Ct value of this sample was 39.66, which is very close to the cutoff value (40); in other words, the amount of RNA in this sample was very low. The false-negative result may be due to RNA degradation

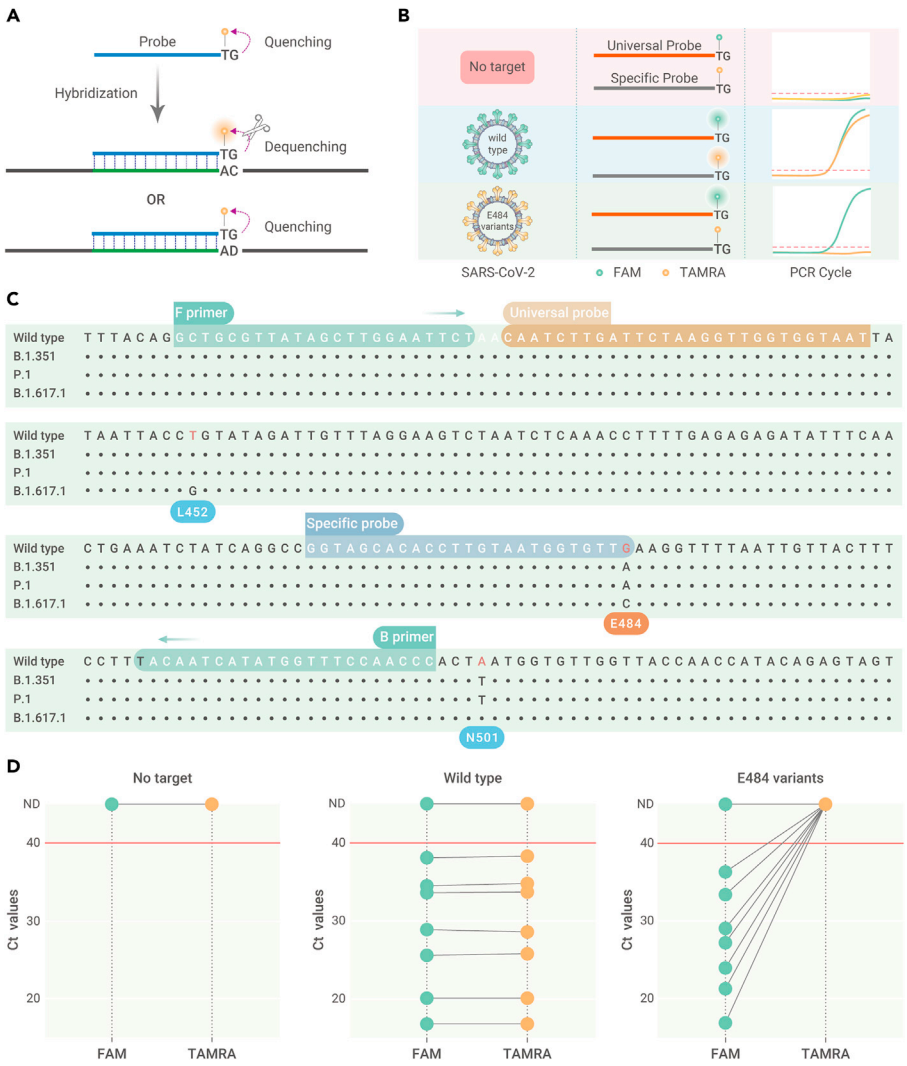

Figure 1. Single-nucleotide differential diagnosis of SARS-CoV-2 variants (A) Schematic illustration of the detection of single-base differences using a self-quenching probe. The probe is labeled with a fluorophore at the thymine base adjacent to the guanine 5′ end. D is A/T/G. (B) Detection and identification of wild-type and variant virus strains by dual-self-quenching probe real-time PCR (DSQP-qPCR) assay. (C) Sequence alignment of wild-type SARS-CoV-2 and variants carrying the E484 mutation. (D) Serially diluted RNA standards of the wild-type and (B) 1.351 variant viruses (2×10^2–4×10^6 copies/mL, i.e., 1–2×10^4 copies/reaction) were detected by DSQP-qPCR assays.

caused by prolonged preservation. Another 200 confirmed negative nasopharyngeal swab samples were also tested, and no false-positive results were found. Collectively, these results demonstrate that DSQP-qPCR exhibits high detection efficiency for clinical specimens.

Unlike TaqMan probe chemistry, this novel probe does not require cleavage by a 5′-exonuclease to release the fluorophore. Theoretically, the DNA polymerase used for DSQP-qPCR is therefore not limited to Taq polymerase. To test this versatility, we tried other DNA polymerases, including modified Taq without 5′-exonuclease, and both Pfu and KOD without 5′-exonuclease but with 3′-exonuclease. The results using the modified Taq were consistent with the results using standard Taq, proving that DSQP-qPCR does not require the assistance of a 5′-exonuclease. However, although Pfu and KOD could also generate Ct values, they could not effectively distinguish single-nucleotide mutations, such as Taq polymerase, due to the potential interference of their 3′-exonuclease activity. We found that the quenching ability of guanine was weaker than that of the TaqMan probe quencher. The relative fluorescence unit change of this self-quenching probe is therefore smaller than that of the TaqMan probe, but the final result is unaffected. A limitation of DSQP-qPCR is that only guanine or cytosine mutations can be detected by this assay. Nevertheless, the probability of a mutation meeting this requirement exceeds 80%, giving this technique a broad application scope. Compared with the TaqMan-based PCR assays, our technology circumvents the application limitations of traditional real-time PCR to allow accurate identification of single nucleotides, thus expanding the functionality of traditional PCR. Compared with sequencing technology, this method has lower equipment and technical requirements, and is therefore more deployable. Our assay combines the advantages of PCR and sequencing technology; it cannot only identify mutant strains as accurately as sequencing technology, but also achieve rapid detection like PCR.

In summary, we have developed a novel DSQP-qPCR technique that enables PCR-based detection of single-nucleotide mutations, expanding the potential range of applications for traditional PCR. Using this strategy, we established a rapid nucleic acid detection assay for screening SARS-CoV-2 variants containing the E484 mutation. This assay shows high sensitivity and specificity, and when tested with clinical samples, it showed the same effectiveness in virus detection as TaqMan-based assays. Moreover, the wide availability of standard real-time PCR equipment suggests that the DSQP-qPCR assay can be used as a supplement to viral genome sequencing for first-line SARS-CoV-2 variant screening.

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DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest.