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A low-cost TaqMan minor groove binder probe-based one-step RT-qPCR assay for rapid identification of N501Y variants of SARS-CoV-2

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ABSTRACT

The increasing prevalence of N501Y variants of SARS-CoV-2 has kindled global concern due to their enhanced transmissibility. Genome sequencing is the gold standard method to identify the emerging variants of concern. But it is time-consuming and expensive, limiting the widespread deployment of genome surveillance in some countries. Health authorities surge the development of alternative assay to expand screening capacity with reduced time and cost. In this study, we developed an in-house TaqMan minor groove binder (MGB) probe-based one-step RT-qPCR assay to detect the presence of N501Y mutation in SARS-CoV-2.

A total of 168 SARS-CoV-2 positive respiratory specimens were collected to determine diagnostic accuracy of the RT-qPCR assay. As a reference standard, PANGO lineages and the mutation patterns of all samples were characterised by whole-genome sequencing. The analytical sensitivity and the ability of the assay to detect low frequency of N501Y variants were also evaluated.

A total of 31 PANGO lineages were identified from 168 SARS-CoV-2 positive cases, in which 34 samples belonged to N501Y variants, including B.1.1.7 (n=20), B.1.351 (n=12) and P.3 (n=2). The N501Y RT-qPCR correctly identified all 34 samples as N501Y-positive and the other 134 samples as wildtype. The limit-of-detection of the assay consistently achieved 1.5 copies/μL on four different qPCR platforms. N501Y mutation was successfully detected at an allele frequency as low as 10% in a sample with mixed SARS-CoV-2 lineage.

The N501Y RT-qPCR is simple and inexpensive (US$1.6 per sample). It enables robust high-throughput screening for surveillance of SARS-CoV-2 variants of concern harbouring N501Y mutation.
surveillance might not be widely deployed in some countries because it is a relatively time-consuming and expensive approach to screen all SARS-CoV-2 positive samples to detect relevant mutation, where result delivery will take at least 3–4 days. As N501Y has started to dominate the current circulating viral population in Asia (GISAID, 2020), health authorities surge the development of alternative assay to expand screening capacity with reduced time and cost. In this study, we present an in-house developed TaqMan MGB (minor groove binder) probe-based one-step RT-qPCR assay to detect the presence of N501Y mutation in SARS-CoV-2 positive specimens, providing analytical sensitivity as low as 1.5 copies/μL and result delivery within 40 min. Unlike other commercial assays, which are only compatible with certain qPCR platforms, our assay can be conducted on a wide range of qPCR instruments with identical analytical performance.

A total of 168 SARS-CoV-2 positive respiratory specimens were collected between January 2020 to June 2021 from five public hospitals in Hong Kong. Total nucleic acid was extracted from supernatant using the MagNA Pure 96 System (Roche Diagnostics, Germany) or NucliSENS easyMAG® (bioMérieux, The Netherlands) according to manufacturers’ instructions. The PANGO lineages, nucleotide mutation and amino acid substitution patterns of SARS-CoV-2 were characterised by whole genome sequencing using Nanopore MinION as described previously (Leung et al., 2021; Siu et al., 2020). All sequences are available on the NCBI Genbank under the accession numbers MZ266365–MZ267539.

The primers were designed based on the conserved regions of the Spike (S) gene of SARS-CoV-2 genomes available in the GISAID database as of March 2021. Two MGB TaqMan probes, with FAM and VIC-labelled respectively, were used to differentiate the nucleotide A from T at position 23063, where the N501Y mutation is located (Fig. 1). The RT-qPCR was conducted by adding 2 μL of extracted total nucleic acid to the reaction mixture consisting of Luna® Universal One-Step RT-qPCR reagents, 0.8 μM of forward and reverse primers, and 0.4 μM of N501 and Y501 probes. For evaluation of analytical sensitivity, the RT-qPCR was performed on four real-time PCR systems, namely ViiA 7 Real-

| Serial dilution of B.1.1.7 sample (N501Y) | Concentration (copies/μL) | Average CT values |
|------------------------------------------|---------------------------|------------------|
| Y1                                       | 1500                      | 29.5             |
| Y2                                       | 150                       | 33.4             |
| Y3                                       | 15                        | 37.3             |
| Y4                                       | 1.5                       | 41.1             |
| Y5                                       | 0.15                      | N/A              |

| Serial dilution of B.1.36.27 sample (Non-N501Y) | Concentration (copies/μL) | Average CT values |
|-------------------------------------------------|---------------------------|------------------|
| N1                                              | 150                       | 26.6             |
| N2                                              | 15                        | 30.5             |
| N3                                              | 15                        | 36.7             |
| N4                                              | 1.5                       | 39.9             |
| N5                                              | 0.15                      | N/A              |

Fig. 1. Determination of limit of detection (LoD) of the N501Y RT-qPCR and detection of low-frequency N501Y variants. (1A) The concentration (copies/μL) of serial dilutions of B.1.1.7 sample. (1B) Amplification curves of N501 (wildtype allele) for ten-fold serial dilutions of B.1.1.7. (1C) Amplification curves of Y501 (N501Y allele) for ten-fold serial dilutions of B.1.1.7. (1D) The concentration (copies/μL) of serial dilutions of B.1.36.27 (non-N501Y variant) sample. (1E) Amplification curves of N501 (wildtype allele) for ten-fold serial dilutions of B.1.36.27. (1F) Amplification curves of Y501 (N501Y allele) for ten-fold serial dilutions of B.1.36.27. The LoD of this assay was determined as 1.5 copies/μL for both N501 and Y501 alleles. (1G) Wildtype and N501Y containing SARS-CoV-2 RNA were mixed at ratios from 9:1 (allele frequency = 90 %) to 1:9 (allele frequency = 10 %). (1H, 1I) Amplification curves of mutant: wildtype RNA mixtures. N501Y mutation was successfully detected at an allele frequency as low as 10 % in the RNA mixtures.
samples without N501Y, majority of them were genotyped as B.1.36.27 classified as lineages B.1.1.7, B.1.351 and P.3 respectively. Among 134 identified from 164 SARS-CoV-2 positive cases, 34 samples from 12.4% were confirmed to carry N501Y mutation, in which 20, 12 and 2 were with SARS-CoV-2 Droplet Digital PCR (ddPCR) Kit (BIO-RAD, US) and (N501Y variant) and B.1.36.27 (non-N501Y variant) were quantified using Xpert® Xpress SARS-CoV-2 (Cepheid, US) with Ct values ranged from 12.4–36.7. Based on WGS result, a total of 31 PANGO lineages were identified from 164 SARS-CoV-2 positive cases. Thirty-four samples were confirmed to carry N501Y mutation, in which 20, 12 and 2 were classified as lineages B.1.1.7, B.1.351 and P.3 respectively. Among 134 samples without N501Y, majority of them were genotyped as B.1.36.27 (n = 39, 30%) and B.1.1.63 (n = 23, 17.7%) (Table S1, Supplemental Material).

For determination of analytical sensitivity, one sample of B.1.1.7 (N501Y variant) and B.1.36.27 (non-N501Y variant) were quantified with SARS-CoV-2 Droplet Digital PCR (ddPCR) Kit (BIO-RAD, US) and were normalized to 1500 copies/μL respectively, followed by serially diluted down to 0.15 copies/μL. Both sets of dilutions were tested with the optimized RT-qPCR protocol in 10 replicates on four different qPCR platforms. All platforms could consistently identify N501Y variant at a concentration as low as 1.5 copies/μL, which was therefore considered as the limit of detection (LoD) of the assay (Fig. 1).

In addition, the ability of our assay to identify low-frequency N501Y variants was evaluated by mixing B.1.1.7 SARS-CoV-2 RNA with B.1.36.27 SARS-CoV-2 RNA at ratios from 9:1 (allele frequency = 90%) to 1:9 (allele frequency = 10%). Interestingly, N501Y mutation was successfully detected at an allele frequency as low as 10% in the RNA mixtures (Fig. 1).

Regarding the diagnostic accuracy, the RT-qPCR results were in 100% agreement with the WGS data, where all 34 samples were correctly identified as N501Y-positive, and the other 134 samples were indicated as wildtype. N501Y was accurately discriminated regardless of the lineages of SARS-CoV-2 (Table S1, Supplemental Material).

N501Y mutation is a surrogate marker of SARS-CoV-2 variants of concern, including lineages B.1.1.7, B.1.351 and P.1. Although the utilisation of whole genome surveillance remains a necessity to classify the lineage of SARS-CoV-2, the application of N501Y RT-qPCR screening for early identification of these highly contagious variants may help the health authorities to better plan for enhanced infection control measures. Currently, in Hong Kong, all cases tested positive for N501Y variants on hospital admission would be arranged under single airborne infection isolation room isolation until discharge. Therefore, N501Y RT-qPCR is being used as a reflex test for every sample which was tested positive for SARS-CoV-2, and subsequent WGS analysis is performed to determine the lineages and the phylogenetic relatedness of N501Y-positive samples.

Recently, several N501Y RT-qPCR kits are commercially available, such as Allplex™ SARS-CoV-2 Variants 1 Assay (SeeGene, Republic of Korea) and VirSNiP SARS-CoV-2 Spike N501Y (TIB Molbiol, Germany). However, the reagent costs of these tests are expensive (US$ 11–20 per sample) and are only compatible with specific qPCR platforms, limiting the widespread deployment of these assays to clinical laboratories in resource-constraint regions. Furthermore, our assay uses MGB probes that can offer better sequence specificity, especially when targeting single base mismatches, than standard DNA probes via the formation of stable probe-target duplex during hybridization using shorter probe sequences. In this study, we developed an in-house TaqMan MGB probe-based one-step RT-qPCR assay that can specifically and accurately detect the N501Y containing variants in SARS-CoV-2 positive samples. The reagent cost per sample is around US$ 1.6, which is at least 7-fold less than the commercial assays. The LoD of our assay is as low as 1.5 copies/μL (i.e. 3 copies/reaction) and is capable of detecting minor N501Y variant at 10% frequency. The evaluation study demonstrated that our RT-qPCR achieved diagnostic sensitivity and specificity of 100% in comparison to WGS result as a reference. Our assay also offers compatibility with a wide range of common qPCR platforms.

The major limitation of the assay is its inability to detect the recently expanding SARS-CoV-2 variant of concern, B.1.617.2 lineage, (a.k.a G/452R.V3), which does not harbour N501Y mutation but E484Q and L452R instead. Our laboratory is developing another TaqMan MGB probe-based one-step RT-qPCR assay to detect the presence of L452R mutation, which shall be used in conjunction with N501Y RT-qPCR. Combined use of these two RT-qPCR assays should be able to detect all reported variants of concern.

In conclusion, the N501Y RT-qPCR can be rapidly implemented and widely adopted by clinical laboratories as it is simple and inexpensive to run robust high-throughput screening for surveillance of SARS-CoV-2 variants of concern harbouring N501Y mutation.

Author contribution
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Declaration of Competing Interest
We declare no competing interests.

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Appendix A. Supplementary data
Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2021.114333.

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