Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods 299 (2022) 114333

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

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

Chloe Toi-Mei Chan [a,1], Jake Siu-Lun Leung [a,1], Lam-Kwong Lee [a], Hazel Wing-Hei Lo [a], Evelyn Yin-Kwan Wong [a], Denise Sze-Hang Wong [a], Timothy Ting-Leung Ng [a], Hiu-Yin Lao [a], Kelvin Keru Lu [a], Stephanie Hoi-Ching Jim [a], Miranda Chong-Yee Yau [b], Jimmy Yiu-Wing Lam [b], Alex Yat-Man Ho [c], Kristine Shik Luk [c], Kam-Tong Yip [d], Tak-Lun Que [d], Kelvin Kai-Wang To [e], Gilman Kit-Hang Siu [a,*]

[a] Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Hong Kong Special Administrative Region
[b] Department of Clinical Pathology, Pamela Youde Nethersole Eastern Hospital, Hong Kong Special Administrative Region
[c] Department of Pathology, Princess Margaret Hospital, Hong Kong Special Administrative Region
[d] Department of Clinical Pathology, Tuen Mun Hospital, Hong Kong Special Administrative Region
[e] Department of Microbiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong Special Administrative Region

ARTICLE INFO

Keywords:
COVID-19
SARS-CoV-2
Variant of concern
N501Y variants
One-step RT-qPCR

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.

The pangolin B.1.1.7 lineage of SARS-CoV-2 that emerged in the United Kingdom back in November 2020 has rapidly become the predominant strain across the country (Volz et al., 2021). Their highly mutated spike glycoprotein, especially the N501Y substitution, has been shown to enhance viral entry into the host cell (Liu et al., 2021; Luan et al., 2021; Hoffmann et al., 2020). The N501Y mutation is also observed in other SARS-CoV-2 variants of concern, including B.1.351 and P.1, where a similar sharp increase in spread and frequency of the virus was noticed in their respective area of origin (Giandhari et al., 2021; Faria et al., 2021). The increasing prevalence of N501Y containing variants has kindled global concern due to their heightened transmissibility (Liu et al., 2021) and their potential to evade host immune responses (Wang et al., 2021; Ho et al., 2021) and cause reinfection (Harrington et al., 2021). Currently, whole genome sequencing (WGS) is the gold standard method to differentiate and identify different emerging variants of SARS-CoV-2. However, the application of genome

* Corresponding author at: Department of Health Technology and Informatics, Hong Kong Polytechnic University, Hong Kong Special Administrative Region.
E-mail address: gilman.siu@polyu.edu.hk (G.K.-H. Siu).
[1] These authors contributed equally.

https://doi.org/10.1016/j.jviromet.2021.114333
Received 21 June 2021; Received in revised form 1 October 2021; Accepted 11 October 2021
Available online 14 October 2021

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.

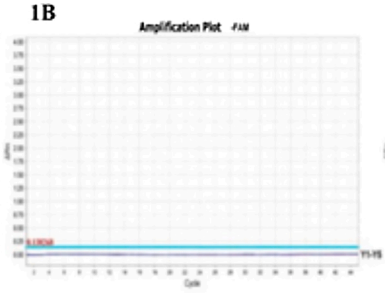
A total of 168 SARS-CoV-2 positive respiratory specimens were collected between January 2020 to June 2021 from five public hospitals in Hong Kong. Total nucleic acid was extracted from supernatant using the MagNA Pure 96 System (Roche Diagnostics, Germany) or NucliSENS® easyMAG® (bioMérieux, The Netherlands) 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-

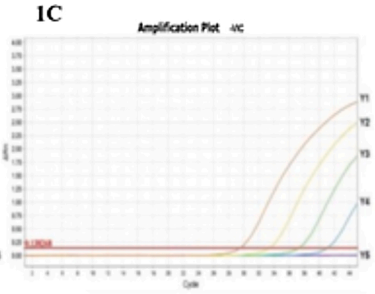
1A

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

1B



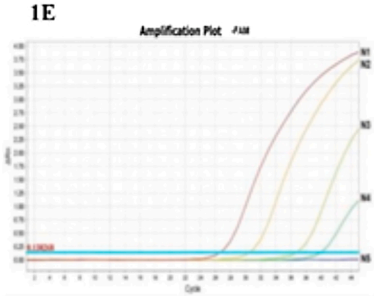
1C



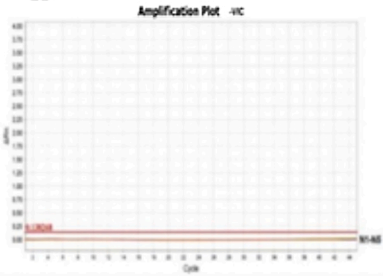
1D

Serial dilution of B.1.36.27 sample (Non- N501Y)	Concentration (copies/μL)	Average CT values
N1	1500	26.6
N2	150	30.5
N3	15	36.7
N4	1.5	39.9
N5	0.15	N/A

1E



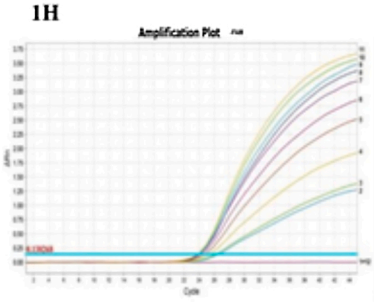
1F



1G

N501Y : non-N501Y RNA ratio (B.1.1.7 : B.1.36.27)	
1	10:0
2	9:1
3	8:2
4	7:3
5	6:4
6	5:5
7	4:6
8	3:7
9	2:8
10	1:9
11	0:10
12	NTC

1H



1I

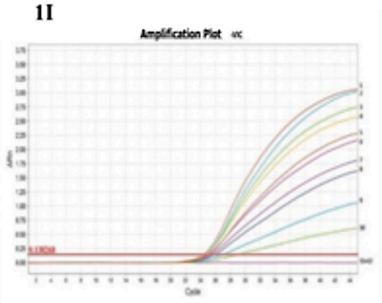


Fig. 1. Determination of limit of detection (LoD) of the N501Y RT-qPCR and detection of low-frequency N501Y variants. (**1A**) The concentration (copies/μL) of serial dilutions of B.1.1.7 sample. (**1B**) Amplification curves of N501 (wildtype allele) for ten-fold serial dilutions of B.1.1.7. **(1C)** 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. **(1 F)** 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. **(1 G)** 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.

Time PCR System (Thermo Fisher Scientific, US), LightCycler® 480 System (Roche Life Science, Germany), Rotor-Gene Q (Qiagen, Germany) and CFX96 Real-Time PCR Detection (BIO-RAD, US). The cycling conditions and the working protocol of each platform were described in Supplemental Material. For assessment of diagnostic accuracy, the tests were run on ViiA 7 Real-Time PCR System only.

All 168 specimens were confirmed to be positive for SARS-CoV-2 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 I 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

Chloe Toi-Mei CHAN: Conceptualization, Methodology, Investigation, Validation, Writing - Original Draft, Writing - Review & Editing.

Jake Siu-Lun LEUNG: Conceptualization, Methodology, Investigation, Validation, Writing - Original Draft, Writing - Review & Editing.

Lam-Kwong LEE: Methodology, Validation, Investigation, Writing - Review & Editing.

Hazel Wing-Hei LO: Methodology, Validation, Investigation, Writing - Review & Editing.

Evelyn Yin-Kwan WONG: Methodology, Validation, Writing - Review & Editing.

Denise Sze-Hang WONG: Validation, Writing - Review & Editing.

Timothy Ting-Leung NG: Conceptualization, Data Curation, Writing - Review & Editing.

Hiu-Yin LAO: Validation, Investigation, Writing - Review & Editing.

Kelvin Keru LU: Formal analysis, Writing - Review & Editing.

Stephanie Hoi-Ching JIM: Validation, Writing - Review & Editing.

Miranda Chong-Yee YAU: Data Curation, Resources, Formal analysis.

Jimmy Yiu-Wing LAM: Supervision, Data Curation, Resources.

Alex Yat-Man HO: Data Curation, Resources, Formal analysis.

Kristine Shik LUK: Supervision, Data Curation, Resources.

Kam-Tong YIP: Data Curation, Resources, Formal analysis.

Tak-Lun QUE: Supervision, Data Curation, Resources.

Kelvin Kai-Wang TO: Supervision, Data Curation, Resources.

Gilman Kit-Hang SIU: Conceptualization, Methodology, Validation, Formal analysis, Project administration, Funding acquisition, Writing - Original Draft, Writing - Review & Editing.

Declaration of Competing Interest

We declare no competing interests.

Acknowledgments

This work was support by the Health and Medical Research Fund (HMRF) Commissioned Research on COVID-19 (COVID190204) and the Innovation and Technology Fund - Innovation and Technology Support Programme - Public Sector Trial Scheme (SST/050/20GP).

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.

References

Faria, N.R., Mellan, T.A., Whittaker, C., Claro, I.M., Candido, D.D.S., Mishra, S., et al., 2021. Genomics and epidemiology of the P.1 SARS-CoV-2 lineage in Manaus, Brazil. Science 372 (6544), 815–821.

Giandhari, J., Pillay, S., Wilkinson, E., Tegally, H., Sinayskiy, I., Schuld, M., et al., 2021. Early transmission of SARS-CoV-2 in South Africa: an epidemiological and phylogenetic report. Int. J. Infect. Dis. 103, 234–241.
GISAID, 2020. Clade and Lineage Nomenclature Aids in Genomic Epidemiology Studies of Active hCoV-19 Viruses.
Harrington, D., Kele, B., Pereira, S., Couto-Parada, X., Riddell, A., Forbes, S., et al., 2021. Confirmed reinfection with SARS-CoV-2 variant VOC-202012/01. Clin. Infect. Dis.
Ho, D., Wang, P., Liu, L., Iketani, S., Luo, Y., Guo, Y., et al., 2021. Increased resistance of SARS-CoV-2 variants B.1.351 and B.1.1.7 to antibody neutralization. Res Sq.
Hoffmann, M., Kleine-Weber, H., Schroeder, S., Kruger, N., Herrler, T., Erichsen, S., et al., 2020. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell. 181, 271–80 e8.
Leung, K.S.-S., Ng, T.T.-L., AK-L, Wu, MC-Y, Yau, Lao, H.-Y., Choi, M.-P., et al., 2021. A territory-wide study of early coronavirus disease outbreak, Hong Kong, China. Emerging Infectious Disease J. 27.
Liu, Y., Liu, J., Plante, K.S., Plante, J.A., Xie, X., Zhang, X., et al., 2021. The N501Y spike substitution enhances SARS-CoV-2 transmission. bioRxiv.
Luan, B., Wang, H., Huynh, T., 2021. Enhanced binding of the N501Y-mutated SARS-CoV-2 spike protein to the human ACE2 receptor: insights from molecular dynamics simulations. FEBS Lett.
Siu, G.K., Lee, L.K., Leung, K.S., Leung, J.S., Ng, T.T., Chan, C.T., et al., 2020. Will a new clade of SARS-CoV-2 imported into the community spark a fourth wave of the COVID-19 outbreak in Hong Kong? Emerg. Microbes Infect. 9, 2497–2500.
Volz, E., Mishra, S., Chand, M., Barrett, J.C., Johnson, R., Geidelberg, L., et al., 2021. Assessing transmissibility of SARS-CoV-2 lineage B.1.1.7 in England. Nature.
Wang, P., Casner, R.G., Nair, M.S., Wang, M., Yu, J., Cerutti, G., et al., 2021. Increased resistance of SARS-CoV-2 variant P.1 to antibody neutralization. Cell Host Microbe.