A novel, room temperature-stable, multiplexed RT-qPCR assay to distinguish lineage B.1.1.7 from the remaining SARS-CoV-2 lineages

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Abstract

The recent emergence of a novel variant of SARS-CoV-2 called lineage B.1.1.7 has sparked global alarm due to evidence of increased transmissibility, mortality, and uncertainty about vaccine efficacy, thus accelerating worldwide efforts to detect and track the variant. Current approaches to detect the B.1.1.7 variant include sequencing and molecular tests that contain a target assay that fails or results in reduced sensitivity towards the B.1.1.7 variant. Since many countries lack a robust genomic surveillance program and the failed target assays detect multiple unrelated variants that contain similar mutations as B.1.1.7, there is an urgent need to develop molecular tests that can accurately and rapidly identify the B.1.1.7 variant. We have developed a room temperature-stable, multiplexed RT-qPCR test that readily differentiates the B.1.1.7 variant from the most common SARS-CoV-2 variants. The test consists of two assays that target either the common SARS-CoV-2 spike gene or the two deletions in the spike gene (ΔH69/ΔV70 and ΔY144) that are present in the B.1.1.7 variant. Moreover, a simple relative comparison of the Ct values of the two assays permits not only identification of the B.1.1.7 variant but also its differentiation from other variants that harbor only the ΔH69/ΔV70 deletion. Each assay is multiplexed with a human RNase P internal control to assess RNA extraction and assay performance. This test can easily be implemented in diagnostic labs to rapidly scale B.1.1.7 surveillance efforts and is particularly useful in countries with high prevalence of variants possessing only the ΔH69/ΔV70 deletion because current strategies using target failure assays incorrectly identify these as putative B.1.1.7 variants.

Introduction

In Dec 2020, Rambaut et al. [1] reported the genomic characterization of a distinct phylogenetic cluster named lineage B.1.1.7, also referred to as 20I/501Y.V1 by Nextstrain (https://nextstrain.org/sars-cov-2/) or Variant of Concern (VOC) 202012/01, briskly spreading over the past four weeks in the United Kingdom. The new lineage has 23 mutations: 13 non-synonymous mutations, 4 deletions, and 6 synonymous mutations. The spike protein contains ten mutations at the amino-acid level (ΔH69/ΔV70 and ΔY144 deletions, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H) that could potentially change binding affinity of the virus [2–4] and consequently virus-host interaction. Indeed, emerging evidence suggests lineage B.1.1.7 has enhanced transmissibility [3,5–8], results in higher viral loads [9,10], and causes increased mortality [11]. These data highlight the need for tools to facilitate enhanced surveillance of lineage B.1.1.7 as well as other variants that may harbor spike gene mutations that alter viral dynamics.

The dominant approach used to putatively detect lineage B.1.1.7 involves conducting multigene RT-qPCR tests that result in positive detection of SARS-CoV-2 in one or more gene sets together with a so-called S gene target failure (SGTF), which is used as a proxy for the B.1.1.7 variant. This approach permits widespread, rapid screening, but is limited by the fact that other variants, in addition to the lineage B.1.1.7, produce SGTFs. Thus, the SGTF screening method depends on the presence of other variants in a region and how they vary over time. The gold-standard for detection of the B.1.1.7 variant is through whole genome sequencing. This provides direct confirmation of the variant and identification of emerging variants; however, it is expensive, time consuming, low throughput, and many countries lack a robust genomic surveillance program, making this approach unwieldy to adopt for mitigating the spread of the B.1.1.7 variant.

Here, we report for the first time, the development of a novel, room temperature-stable, and multiplexed RT-qPCR test for differentiating lineage B.1.1.7 from all other SARS-CoV-2 lineages. The assay has been validated on clinical samples verified by sequencing to be positive for lineage B.1.1.7. Unlike other tests that rely on indirect detection via SGTF, this test contains primers that target the ΔH69/ΔV70 and ΔY144 deletions in the spike gene that permit the direct detection of lineage B.1.1.7. This RT-qPCR assay will provide a useful tool for countries to rapidly identify hot spots of this new B.1.1.7 variant and implement test, trace, and isolate strategies to prevent this variant from becoming widespread. This test would be particularly useful in countries currently experiencing extensive circulation of variants carrying

only the ΔH69/ΔV70 deletion as these would be via SGTF falsely identified as the B.1.1.7 variant.

Materials and Methods

Analysis of sequences

To identify suitable targets for primer/probe design, we downloaded 1,136 sequences from the GISAID repository filtered during a collection time spanning 1 - 21 December 2020. We focused on the spike gene because lineage B.1.1.7 contains a number of spike gene mutations, including two deletions (ΔH69/ΔV70 and ΔY144) that are ideal for designing a specific assay. We cut the locus encoding the spike protein and used the MAFFT alignment tool (with the parameter - auto)[12] to align all the sequences against the WUHAN reference (NCBI ID: NC_045512.2). Twelve sequences (1.06 %) contained ambiguous signal in the loci of deletions and were not used in the downstream analysis. We separated sequences into two groups: 1) those with the ΔH69/ΔV70 and ΔY144 deletions and 2) those without the deletions (**Table 1**). Using SeaView [13], we called 95% consensus sequences for the ΔH69/ΔV70 and ΔY144 group and the No deletions group that were subsequently used to design primer and probe sets specific to either B.1.1.7 or all other SARS-CoV-2 variants, respectively.

In a separate analysis to determine the prevalence of the ΔH69/ΔV70 and ΔY144 deletions in lineages other than B.1.1.7, we downloaded 416,778 spike protein sequences with the most recent data description file collected from the beginning of the pandemic through 29 January 2021. Using regular expressions (bash pattern matching command grep with the option -P for Perl-compatible regular expression), we searched for loci with both ΔH69/ΔV70 and ΔY144 deletions and for loci without these deletions. In the regular expression, we kept fixed a few amino acids downstream and upstream from the deletions to omit any miscalling of the searched pattern. All commands and scripts will be available here: https://github.com/veveMDX/B117-and-B1351-RT-qPCR-test-design

Primer design and synthesis

We designed primers and probes using the 95% consensus sequences to target both the S gene of the common SARS-CoV-2 and the S gene of SARS-CoV-2 variants containing either the ΔH69/ΔV70 deletion or the ΔY144 deletion, or both deletions. We incorporated

locked nucleic acid (LNA)-modified bases into some primers and probes following general guidelines in order to normalize melting temperatures, increase sensitivity, and enhance specificity [14–16]. Following primer/probe design, we conducted *in silico* analyses using the IDT OligoAnalyzer™ tool (https://www.idtdna.com/pages/tools/oligoanalyzer) to verify melting temperature (Tm), GC content, and potential to form homo-/hetero-dimers as well as the mFold server [17] (http://www.bioinfo.rpi.edu/applications/mfold/) to identify problematic secondary structures or necessary hairpin formation for TaqMan probes. Primers and probes were synthesized at MultiplexDX, Inc. (Bratislava, Slovakia; https://www.multiplexdx.com/). The sequences of primers and probes used in this study are listed in **Tables 2 and 3**.

Positive controls and clinical samples

For primer/probe set optimization, we used the following positive controls: 1) RNA extracted from a patient confirmed positive for a common variant of SARS-CoV-2 that does not contain any deletions in the spike gene (named wild type template), 2) RNA extracted from a patient confirmed positive for a common variant of SARS-CoV-2 that contains the six base pair deletion (bp: 21765-21770) resulting in the deletion of two amino acids at the 69/70 position of the spike protein (named ΔH69/ΔV70 template), and 3) RNA extracted from a patient confirmed positive by whole genome sequencing for the SARS-CoV-2 lineage B.1.1.7 (named B.1.1.7 template). The control samples were confirmed by whole genome sequencing essentially as described by Resende *et al*. (2020) [18].

All clinical specimens used for clinical validation were previously collected for the purpose of primary diagnosis of SARS-CoV-2 and were made unidentifiable for the researchers performing this study. The study has been approved by the Ethics committee of Biomedical Research Center of the Slovak Academy of Sciences, Bratislava, Slovakia (Ethics committee statement No. EK/BmV-02/2020).

RT-qPCR

We optimized RT-qPCR reactions and conducted clinical validations using both an AriaMx (Agilent, CA, USA) and QuantSudio 5 (ThermoFisher Scientific, MA, USA) real-time PCR system. For all the detected genes, we used the SOLIScript® 1-step CoV Kit (Cat. No. 08-65-00250, SOLIS BioDyne, Tartu, Estonia) according to the manufacturer´s recommendations comprised of 4 µl of 5X One-step Probe CoV Mix (ROX), 0.5 µl of 40X One-step SOLIScript® CoV Mix, 2 µl of primers/probe mix, 8.5 µl of PCR water, and 5 µl of sample in a 20 µl total volume. One-step RT-qPCR assays were conducted with the following cycling

conditions: 55 °C for 10 min for reverse transcription, 95 °C for 10 min for initial denaturation, and 45 cycles of 95 °C for 15 s and 60 °C for 30 s. Concentrations for primers and probes were as follows: SARS-CoV-2 S gene (forward and reverse primer = 500 nM, probes = 200 nM for each probe (single and dual); B.1.1.7: forward primer = 600 nM, reverse primer = 800 nM, probes = 200 nM for each probe (single and dual); RNase P (forward and reverse primer = 250 nM, probe = 80 nM.

Analytical sensitivity (limit of detection)

To assess the analytical sensitivity of both our common SARS-CoV-2 S gene (S gene) and B.1.1.7 primer/probe sets, we used RNA isolated from a patient sample infected with the B.1.1.7 variant of SARS-CoV-2 as confirmed by sequencing. This RNA was diluted to 200 copies/µl and then serial dilutions were prepared by diluting the stock with a synthetic matrix "SARS-CoV-2 Negative" (Cat. No. COV000, Exact Diagnostics, TX, USA) containing genomic DNA at a concentration of 75,000 copies/ml, resulting in samples with concentrations of 8 copies/µl (= 40 copies/reaction), 2 copies/µl (= 10 copies/reaction), 0.8 copies/µl (= 4 copies/reaction), 0.4 copies/µl (= 2 copies/reaction) and 0.2 copies/µl (= 1 copy/reaction) that were used in the analytical sensitivity test. The assay was performed in 8 replicates for each prepared dilution.

Clinical performance evaluation

We evaluated the clinical utility of our SARS-CoV-2 S gene and B.1.1.7 primer/probe sets using a selected set of 65 SARS-CoV-2 positive clinical samples, which were confirmed by a reference method used for routine testing by regional public health authorities of the Slovak Republic. Further sequencing revealed 37 of these samples belonging to the B.1.1.7 lineage. The SARS-CoV-2 sequences were determined by sequencing of tiled ~ 2-kbp long amplicons on a MinION device (Oxford Nanopore Technologies, Oxford, UK) as described by Resende et al. (2020) [18]. All sequences have been deposited to the GISAID (https://www.gisaid.org/) database (**Table 6**).

Results

Analysis of sequences

Our analysis of 1,136 spike gene sequences (spanning 1 - 21 December 2020) revealed 228 sequences (20%) that contained both the ΔH69/ΔV70 and ΔY144 deletions (for country of origin, see **Table 1**). The shorter deletion (ΔY144) always co-occurred with the longer deletion (ΔH69/ΔV70), whereas the (ΔH69/ΔV70) deletion occurs independently in 17 sequences (1.5%). Pearson's correlation coefficient of the deletions is 0.953.

Analysis of the prevalence of both ΔH69/ΔV70 and ΔY144 deletions in lineages other than B.1.1.7 revealed a total of 29,872 sequences that possess both deletions, while 368,474 sequences do not have them. Based on the metadata file, we identified SARS-CoV-2 lineages across all called sequences with both deletions. Only five sequences (0.0167%) out of 29,872 records are not labelled as B.1.1.7. In other words, 99.98% of sequences containing both deletions belong to lineage B.1.1.7, highlighting the notion that these two deletions are highly specific for the B.1.1.7 variant and make ideal targets for primer/probe design.

Optimization of a S gene primer/probe set targeting both common SARS-CoV-2 and ΔH69/ΔV70 deletion variants

We began by designing a general S gene primer/probe assay (SARS-CoV-2 S gene) that could be used for screening purposes and would detect the most common strains of SARS-CoV-2 as well as variants containing the ΔH69/ΔV70, including lineage B.1.1.7. We designed a series of primers flanking the ΔH69/ΔV70 deletion and tested their performance during RT-qPCR using the wild type and ΔH69/ΔV70 templates (for primer/probes sequences, see **Table 2**). Although all primers/probe combinations amplified both wild type (**Figure 1A**) and ΔH69/ΔV70 (**Figure 1B**) templates, we selected the F1-P3-R1 set for further analysis since it resulted in the lowest overall Ct values and sufficient fluorescence intensity.

Dual probes enhance fluorescence intensity and sensitivity

Given that prior reports suggest that including a second TaqMan hydrolysis probe can be beneficial for specificity and sensitivity [19,20], we designed an additional hydrolysis probe (P4), identically labelled with the same reporter and quencher dyes, that would hybridize in tandem (i.e., on the same strand) with the first hydrolysis probe (P3). We compared single versus dual hydrolysis probe assays using three 10-fold dilutions of template RNA and

observed that dual probes resulted in greater fluorescent intensity at all dilutions (**Figure 1C**) as well as reduced Ct values at the first two dilutions (**Figure 1D**). Moreover, the efficiency of the RT-qPCR reaction was similar between single and dual probe assays. Therefore, we decided to incorporate dual probes into our SARS-CoV-2 S gene primer/probe set (F1-P3-P4-R1; **Table 2**)

Optimization of a S gene primer/probe set capable of differentiating between lineage B.1.1.7 and other SARS-CoV-2 variants

Lineage B.1.1.7 contains two deletions in the spike gene, including a six base pair deletion (bp: 21765-21770; ΔH69/ΔV70) and three base pair deletion (bp: 21991-21993; ΔY144). Since the co-occurance of these deletions is highly specific to B.1.1.7 (99.98%), we selected these deletions as candidate regions to target for a primer/probe set to differentiate B.1.1.7 from all other SARS-CoV-2 variants (see **Table 3** for primer and probe sequences). Our first approach consisted of designing several probes targeting the first deletion and determining specificity by conducting RT-qPCR using the forward and reverse primers from our SARS-CoV-2 S gene set (F1 and R1) to amplify both wild type and ΔH69/ΔV70 templates; however, none of the probes were capable of discriminating between wild type and ΔH69/ΔV70 templates, even though all probes were less efficient at detecting wild type template (**Figure 2A**). We then attempted to differentiate wild type template from ΔH69/ΔV70 template by designing several forward primers targeting the span of the six base pair deletion. We tested these forward primers using the same probe and reverse primer from our SARS-CoV-2 S gene set (P3 and R1) and found two forward primers (F2 and F3) that specifically amplified only ΔH69/ΔV70 template (**Figure 2B**). We selected F3 instead of F2 for further assay development based on its sensitivity to ΔH69/ΔV70 templates, fluorescence intensity, and profile of the amplification curve.

Since other SARS-CoV-2 variants share the ΔH69/ΔV70 deletion (e.g., B.1.1.298, B.1.160, B.1.177, B.1.258, B.1.375), we next sought to design a series of reverse primers to target the second, three base pair deletion (bp: 21991-21993; ΔY144). This approach would facilitate differentiating B.1.1.7 variants that contain both the ΔH69/ΔV70 and ΔY144 deletions from SARS-CoV-2 variants that contain only the ΔH69/ΔV70 deletion. To increase the specificity of the assay, we also utilized allele-specific PCR approaches. We introduced selective mismatch bases in the 3'-end of the primer to destabilize base complementary to sequences that lack the ΔY144 deletion, while retaining complementarity in sequences containing the ΔY144 deletion such as B.1.1.7 variants. We designed a series of reverse primers spanning the three base pair deletion (bp: 21991-21993; ΔY144) with and without

LNA-modified oligos to modify stability and tested the ability of these primer/probe sets to amplify three templates: wild type, ΔH69/ΔV70, and B.1.1.7. While our 3'-LNA-modified primers (R1-R6) were unable to differentiate between B.1.1.7 and ΔH69/ΔV70, several unmodified primers displayed reduced sensitivity towards ΔH69/ΔV70 with a Ct value difference greater than nine when compared with Ct values of B.1.1.7, demonstrating their capability to differentiate the two variants (**Figure 2C**). These primers also showed minimal reductions in sensitivity when compared to Ct values of B.1.1.7 template amplified using SARS-CoV-2 S gene set (**Figure 2D**). This data strongly suggests that the change in Ct value (ΔCt), that is to say, the relative difference in Ct values between the B.1.1.7 and SARS-CoV-2 S gene assays, could be used to confirm the presence of B.1.1.7 when the ΔCt value is minimal (e.g., ΔCt ≤ 5). Alternatively, a larger change in Ct values (e.g., ΔCt ≥ 8) could indicate the presence of a variant that contains the ΔH69/ΔV70 deletion, but not the ΔY144 deletion.

We also attempted another approach by substituting a mismatched base in the 3'-end of the reverse primer to form a non-canonical base pair that would further destabilize 3'-end complementarity to a greater degree in templates that lack the ΔY144 deletion, a technique which can be useful for detecting single nucleotide polymorphisms [21–23]. These primers (R28-R37) showed the highest specificity with ΔH69/ΔV70 template amplifying over ten cycles later than B.1.1.7 template (**Figure 2C**). Although some of these reverse primers (R33-35) showed reduced sensitivity when compared to B.1.1.7 template amplified with our common SARS-CoV-2 S gene, we found that judicious placement of an LNA-modified thymine (LNA-T) towards the 3'-end mismatch retained high sensitivity (e.g., R36). This benefit had a limit, however, as placing the LNA-T too close to the mismatch was detrimental to sensitivity (R37; **Figure 2D**). Taken together, several of these primers are capable of discriminating between B.1.1.7 and other variants containing the ΔH69/ΔV70 deletion, provided that a second reaction is ran in parallel using the SARS-CoV-2 S gene set that can be used as a benchmark to assess the relative sensitivity. If the B.1.1.7 primer set amplifies the sample within five Ct cycles of the SARS-CoV-2 S gene primer set, then the sample is B.1.1.7 positive. Alternatively, if the B.1.1.7 primer set amplifies the sample in 8 or more Ct cycles relative to the SARS-CoV-2 S gene primer set, than the sample likely belongs to a variant that contains the ΔH69/ΔV70 deletion, but not the ΔY144 deletion, and hence is B.1.1.7 negative.

Comparison of B.1.1.7 S gene primer/probe sets

Following the clinical validation, we compared three different versions of B.1.1.7 primer/probe sets that varied according to the reverse primer (V1, V2, and V3 use reverse primers R14, R23, and R36, respectively) using a selected set of 46 samples, some of which

where sequenced to confirm lineage status. Given our interpretation criterion (**Table 4**), we determined that the V3 primer/probe set (F3-P3-P4-R36) performed the best since it correctly identified all B.1.1.7 and ΔH69/ΔV70 deletion samples, with the exception of one ΔH69/ΔV70 deletion sample that was interpreted as inconclusive (**Figure 3**). While V1 and V2 primer/probe sets correctly identified all B.1.1.7 samples, they performed poorly at calling ΔH69/ΔV70 deletion samples. V1 correctly identified only 11 samples, whereas V2 performed better by correctly idenfying 30 samples. Sequencing results on all B.1.1.7 samples and a subset (n=12) of ΔH69/ΔV70 deletion samples confirmed their lineage designation. Among the ΔH69/ΔV70 deletion samples that were sequenced, the V1, V2, and V3 primer/probes sets detected 25%, 67%, and 92%, respectively, of the samples identified as lineage B.1.258, thus confirming V3 as the most suitable primer/probe combination to identify other lineages containing only the ΔH69/ΔV70 deletion.

Analytical sensitivity and clinical evaluation of lineage B.1.1.7 S gene primer/probe set

With our final primer/probe sets for SARS-CoV-2 S gene and B.1.1.7, we multiplexed each assay with the US CDC human RNase P primer/probe set (for sequence, see [24]) as an internal control to assess RNA extraction and assay performance. We then assessed the analytical sensitivity using serial dilutions of RNA extracted from a B.1.1.7 positive sample. Both assays displayed high sensitivity (**Figure 4A**) with our SARS-CoV-2 S gene and B.1.1.7 assays reliably detecting down to only 2 copies/reaction (0.4 copies/µl) and 10 copies/reaction (2 copies/µl), respectively, placing them among the most sensitive SARS-CoV-2 RT-qPCR assays available.

We evaluated the clinical performance of our SARS-CoV-2 S gene and B.1.1.7 assays on 65 clinical samples that underwent sequencing to identify lineage status using interpretation criterion outlined in **Table 4**. Our SARS-CoV-2 S gene assay detected all 65 clinical samples regardless of lineage (**Table 5**) confirming its utility as a general screening assay for the most common SARS-CoV-2 variants. Out of 37 clinical samples classified as lineage B.1.1.7 by sequencing, our B.1.1.7 assay positively identified 36 samples, while only one sample was deemed inconclusive. The ΔCt of this sample was slightly greater than five cycles (e.g., Sample 40, ΔCt = 5.7) relative to the Ct value for the SARS-CoV-2 S gene assay, which exceeded our cut-off for a positive identification (**Figure 4B**).

Notably, our assay was also capable of identifying samples carrying the ΔH69/ΔV70 deletion such as those belonging to the B.1.258 lineage, provided that the sample contains sufficient viral load as other ΔH69/ΔV70 variants yield ΔCt values greater than 8 Ct cycles. For the 16 samples that carry only the ΔH69/ΔV70 deletion and belong to lineage B.1.258,

our B.1.1.7 assay correctly identified 13 out of 16 samples. Two samples had relatively high Ct values in the SARS-CoV-2 S gene assay (Sample 33, Ct = 30.1 and Sample 65, Ct = 28.9) and therefore were not detected by the B.1.1.7 assay, making confirmation of the ΔH69/ΔV70 status impossible with our cut-off criterion. One sample had a ΔCt outside the criterion for ΔH69/ΔV70 deletion confirmation and was deemed inconclusive. Detection of human RNase P showed high homogeneity in all analyzed samples, confirming the suitability of this assay as an internal control for collection and RNA extraction from a clinical sample (data not shown). Overall, the clinical evaluation confirmed the diagnostic utility of both our SARS-CoV-2 S gene and B.1.1.7 assays, which showed 100% (65/65) and 97.3% (36/37) diagnostic sensitivity, respectively. For an overview of the clinical evaluation data, lineage of each sample, and GISAID information, see **Table 6**. We have provided a decision tree (**Figure 4C**) that users may follow to implement this research use only test to directly detect the presence of the B.1.1.7 variant.

Discussion

The recent emergence of a novel SARS-CoV-2 variant called lineage B.1.1.7 has sparked global consternation as it has now been confirmed in over 70 countries and threatens to exacerbate an already dire pandemic. To mitigate the spread of the B.1.1.7 variant, it is imperative that countries have diagnostic tools that can quickly and accurately detect and track the prevalence of the variant in order to implement the appropriate epidemiological measures. Here we report a novel RT-qPCR test to differentiate the B.1.1.7 variant from other SARS-CoV-2 lineages. The test consists of running two S gene target assays, one specific for B.1.1.7 and the other for all SARS-CoV-2 strains, and performing a simple comparison of relative Ct values that allows the user to differentiate the B.1.1.7 variant from other variants that have the ΔH69/ΔV70 deletion. We validated this test on clinical samples that were sequenced to determine the exact SARS-CoV-2 lineage and the results demonstrated a high level of sensitivity in distinguishing the B.1.1.7. variant. This RT-qPCR test provides a positive identification of the B.1.1.7 variant, providing countries with a powerful tool to detect and track lineage B.1.1.7, especially countries that have considerable prevalence of variants carrying only the ΔH69/ΔV70 deletion [25–28], which are mistakenly identified as B.1.1.7 variants by currently used SGTF assays.

Although there are hundreds of approved RT-qPCR tests for the detection of SARS-CoV-2, none of them are capable of directly differentiating the B.1.1.7 variant from common

variants of SARS-CoV-2. Paradoxically, failed RT-qPCR tests have been instrumental in identifying putative B.1.1.7 positive samples and tracking its prevalence [25,29]. These RT-qPCR tests contain multigene assays, with at least one assay targeting the spike gene, and during routine testing a “drop-out” in the spike gene assay may occur, while other gene targets yield positive signals. This SGTF can indicate the presence of the B.1.1.7 variant and flag samples for confirmation by sequencing. It is important to note, however, SGTPs are produced by other variants that contain the ΔH69/ΔV70 deletion, including the B.1.1, B1.258, and the mink cluster V (B.1.1.298) lineages [8]. This highlights the importance of follow up sequencing of SGTF samples to determine lineage status. Indeed, an analysis of SGTFs and corresponding sequencing data by Public Health England revealed that SGTF assays were poor proxies for the presence of B.1.1.7 in early October with only 3% of SGTFs assays positively identifying a B.1.1.7 variant.

The SGTF assays only became useful proxies when the variant spread and became more dominant in late November when the assays then detected over 90% of the variant [30]. Volz and colleagues [6] reached a similar conclusion, suggesting that the success of SGTF assays depends on the location, time, and frequency of other variants that contain the ΔH69/ΔV70 deletion. This is particularly problematic, since the SGTF assays are the least accurate at the time when the B.1.1.7 variant is at low prevalence, precisely the time when an accurate test is needed most in order to establish effective mitigation strategies. Our test outlined here makes significant strides in this effort by accurately differentiating the B.1.1.7 variant with a test that does not rely on a SGTF, thus providing a rapid, accurate test that eliminates the need to conduct expensive and laborious sequencing to confirm lineage status.

Besides the SGTF tests, two recent preprint papers from the same group describe a deep learning approach that was used to design two primer/probe sets targeting a synonymous mutation C16176T in the ORF1a gene or a nonsynonymous mutation in the spike gene (S982A) that were found to be specific to the B.1.1.7 variant [31,32]. However, the primer design raises questions because both mutations in the ORF1a and S genes appear in the middle of the respective forward primers, making it unlikely that either primer set would specifically amplify only the B.1.1.7 variant, since discrimination of SNPs generally requires the mutated base to be located near the 3’-terminal to inhibit extension by the polymerase. Also, the primer sets were only validated *in silico* so it is critical to assess their performance and specificity on clinical samples that have been verified by sequencing. Our approach targeted both the ΔH69/ΔV70 and ΔY144 deletions in the spike gene with the 3’-end of both forward and reverse primers spanning the region of the deletions. We also leveraged allele-specific PCR approaches where we introduced a mismatch base that resulted in enhanced differentiation of the B.1.1.7 variant from other ΔH69/ΔV70 variants, effectively allowing one

to discriminate between B.1.1.7, other ΔH69/ΔV70 variants, and the common SARS-CoV-2 in one test. As a testament to the specificity of this assay, our analysis of all GISAID sequences containing both the ΔH69/ΔV70 and ΔY144 deletions revealed that a staggering 99.98% of all these sequences belong to lineage B.1.1.7, ensuring that users can have high confidence that a positive B.1.1.7 assay result is a true positive.

The Grubaugh lab developed another test using primers that flanked both the ΔH69/ΔV70 and ΔY144 deletions and two different probes to mimic the S gene "drop-out" assay and permit a B.1.1.7 "detection" assay [33]. While the S gene "drop-out" assay successfully detected B.1.1.7 and other ΔH69/ΔV70 variants, the "detection" assay failed to distinguish between B.1.1.7, ΔH69/ΔV70 variants, and common SARS-CoV-2 variants. In essence, they created an open-source version of the SGTF RT-qPCR test that could be used for screening purposes. In an updated protocol [34], they also multiplex their S gene drop-out assay with another drop out primer/probe set that targets the Δ3675-3677 SGF deletion in the ORF1a gene observed in B.1.1.7 and both the B.1.351 and P.1 variants recently detected in South Africa and Brazil. By combining these primer/probe sets with the CDC N1 primer/probe set, the user can putatively differentiate B.1.1.7 from B.1.351 and P.1 as well as variants containing only the ΔH69/ΔV70 deletion, providing a unique assay to screen for these emerging variants. While the B.1.351 and P.1 assay remains to be validated on clinical samples, the test still relies on target gene failures and therefore retains the limitations for SGTF assays and necessity to confirm lineage status by sequencing.

To differentiatie B.1.1.7, we took an alternative approach by targeting the ΔY144 deletion using allele-specific PCR methods combined with judicious placement of LNA oligonucleotides. This allowed us to stabilize the 5'-end and mismatched base, and to shorten the 3'-terminal of the reverse primer. Together, these modifications provided us with a primer/probe set that retained specificity for B.1.1.7 variants and reduced specificity to other variants containing the ΔH69/ΔV70 deletion. Our test, instead of relying on target failures to identify putative variants, provides a positive signal in the presence of B.1.1.7 and ΔH69/ΔV70 deletion variants that can easily be differentiated by comparing their relative Ct values to a common SARS-CoV-2 S gene primer/probe set that serves as a benchmark.

We have provided interested users with the primer and probe sequences to implement this B.1.1.7 assay in their own laboratories with the hope this can rapidly scale the ability of countries to identify the B.1.1.7 variant and implement epidemiological measures to mitigate its spread. This test is also available as a research use only kit called rTest COVID-19 B.1.1.7 qPCR kt (https://www.multiplexdx.com/products/rtest-covid-19-b-1-1-7-qpcr-kit, MultiplexDX, Inc., Bratislava, Slovakia) that contains lyophilized SARS-CoV-2 S gene and B.1.1.7 primer/probe sets both multiplexed with human RNase P (200 reactions per assay) and a

lyophilized positive control containing B.1.1.7 RNA spiked with human RNA that can be used to validate all primer/probe sets. The kit utilizes the room temperature-stable SOLIScript® 1-step CoV Kit (SolisBiodyne) and laboratory tests show that all kit components are stable for at least one month at room temperature, eliminating cold chain shipping/storage and freeing valuable freezer storage space (e.g., for storage of mRNA-based vaccines). This test can provide labs with a powerful tool to directly confirm the presence of the B.1.1.7 variant in a sample previously determined SARS-CoV-2 positive by an approved screening test, thus avoiding the use of target gene failure assays that can be plagued with low specificity and obviating the need to conduct burdensome and costly genomic sequencing. This is particularly important for countries that are experiencing extensive circulation of variants harboring only the ΔH69/ΔV70 deletion as current RT-qPCR assays that rely on SGTFs erroneously classify these samples as presumptive B.1.1.7 variants.

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Table 1. Origin and genomic characterization of GISAID sequences used for alignment and primer/probe design.

Country of origin	Sequences	Omitted	No deletions	ΔH69/ΔV70 only	ΔY144 only	ΔH69/ΔV70 and ΔY144
Australia	21	0	20 (95.2 %)	0	0	1 (4.8 %)
Denmark	107	0	99 (92.5 %)	8 (7.5 %)	0	0
UK	965	5	725 (75.5 %)	8 (0.8 %)	0	227 (23.7 %)
New Zealand	13	0	13	0	0	0
Sweden	2	0	2	0	0	0
Thailand	3	0	3	0	0	0
USA	25	0	25	0	0	0
Total	1136	5	887	16	0	228

Table 2. Oligonucleotide primers and probes for common SARS-CoV-2 S gene primer/probe set.

Oligonucleotide	Sequence	Tm (ºC)	Secondary structure potential (kcal/mol)
SARS-CoV-2 S gene - **F1**	TCT**t**T**t**CCAATGTTACTTGGTTC	54.3	-1.52
SARS-CoV-2 S gene - **F2**	TCT**t**T**t**CCAATGTTACTTGGT**t**C	55.9	-1.52
SARS-CoV-2 S gene - **F3**	T**t**ACCT**t**TCT**t**TTCCAATGTTAC C	54.5	1.51
SARS-CoV-2 S gene - **F4**	CT**t**ACCT**t**TCT**t**TTCCAATGT**t**AC	56.4	1.51
SARS-CoV-2 S gene - **P1**	AGAGGTTTGATAACCCTGTCCTACCA	59.1	-1.97
SARS-CoV-2 S gene - **P2**	AGAGGTTTGATAACCCTGTCC**t**ACCA	60.3	-1.97
SARS-CoV-2 S gene/B.1.1.7 - **P3**	AGAGGTTTGATAACCCTG**t**CC**t**ACCA	61.9	-1.97
SARS-CoV-2 S gene/B.1.1.7- **P4**	T**t**TGCTTCCACTGAGAAGTC**t**AACAT	59.0	-1.48
SARS-CoV-2 S gene - **R1**	AGTAGGGACTGGGTCTTCGAATCT	58.9	-0.94
SARS-CoV-2 S gene - **R2**	GTAGGGACTGGGTCTTCGAATCTA	57.3	-0.94

Primers/probes highlighted in green comprise the final common SARS-CoV-2 S gene primer/probe set
Nucleotides in lowercase and bold denote LNA-modified bases
F, forward primer; P, probe; R, reverse primer

Table 3. Oligonucleotide primers and probes for lineage B.1.1.7 S gene primer/probe set.

Oligonucleotide	Sequence	Tm (ºC)	Secondary structure potential (kcal/mol)
Forward primers (F1-F4) targeting the 1st deletion (ΔH69/ΔV70)			
B.1.1.7 - **F1**	GTTACTTGGTTCCATGCTATCTCTG	55.3	1.11
B.1.1.7 - **F2**	GT**t**ACTTGGTTCCATGCTATCTCT	56.3	1.11
B.1.1.7 - **F3**	GT**t**ACT**t**GGTTCCATGCTATCTC	56.8	1.11
B.1.1.7 - **F4**	GTTCCATGCTATCTCTGGGACC	57.1	-0.62
Detection probes (P1-P4) targeting the 1st deletion (ΔH69/ΔV70)			
B.1.1.7 - **P1**	ATGCTATCTCTGGGACCAATGGTACT	59.1	-0.96
B.1.1.7 - **P2**	ATGCTATCTCTGGGACCAATGG**t**ACT	60.9	-0.96
B.1.1.7 - **P3**	TGCTATCTCTGGGACCAATGGTACT	59.1	-0.96
B.1.1.7 - **P4**	TGCTATCTCTGGGACCAATGG**t**ACT	61.0	-0.96
Detection probes (P3-P4) targeting all SARS-CoV-2 variants			
SARS-CoV-2 S gene/B.1.1.7 - **P3**	AGAGGTTTGATAACCCTG**t**CC**t**ACCA	61.9	-1.97
SARS-CoV-2 S gene/B.1.1.7- **P4**	T**t**TGCTTCCACTGAGAAGTC**t**AACAT	59.0	-1.48
Reverse primers (R1-R37) targeting the 2nd deletion (ΔY144)			
B.1.1.7 - **R1**	T**t**TGTTGTTTTTGTGGTAA**a**CACC	55.2	-1.51
B.1.1.7 - **R2**	T**t**GTTGTT**t**TTGTGGTAA**a**CACC	56.9	-1.51
B.1.1.7 - **R3**	TGTTGTT**t**TTGTGGTAA**a**CACCC	57.1	-1.51
B.1.1.7 - **R4**	GTTGTT**t**TTGTGGTAA**a**CACCC	55.6	-1.51
B.1.1.7 - **R5**	T**t**GT**t**GTT**t**TTGTGGTAA**a**CAC	56.6	-1.51
B.1.1.7 - **R6**	TG**t**TG**t**TTT**t**GTGGTAA**a**CAC	56.1	-1.51
B.1.1.7 - **R7**	CAAC**t**TTTGTTGTTTTTGTGGTAAACAC	55.6	-3.34
B.1.1.7 - **R8**	CAAC**t**T**t**TGTTGTTTTTGTGGTAAACAC	56.7	-3.34
B.1.1.7 - **R9**	CA**a**C**t**TTTGTTGTTTTTGTGGTAAACAC	56.6	-3.34
B.1.1.7 - **R10**	CAAC**t**T**t**TGTTGTTTTTGTGGTAAACA	56.0	-2.38
B.1.1.7 - **R11**	CA**a**C**t**T**t**TGTTGTTTTTGTGGTAAACA	57.0	-2.38
B.1.1.7 - **R12**	CA**a**C**t**T**t**TGTTGTTTTTGTGGTAAAC	55.8	-2.14
B.1.1.7 - **R13**	CAAC**t**T**t**TGTTGTTTTTGTGGTAAAC	54.7	-2.14
B.1.1.7 - **R14**	CAAC**t**TT**t**GTTGTTTTTGTGGTAAAC	55.0	-2.14
B.1.1.7 - **R15**	CAACT**t**T**t**GTTGTTTTTGTGGTAAAC	55.3	-2.14
B.1.1.7 - **R16**	CAACTTTT**t**GTTGTTTTTGTGGTAAAC	54.1	-2.14
B.1.1.7 - **R17**	CAACT**t**TTGTTGTTTTTGTGGTAAAC	53.8	-2.14
B.1.1.7 - **R18**	CAAC**t**TTTGTTGTTTTTGTGGTAAAC	53.6	-2.14
B.1.1.7 - **R19**	CAACTTTT**t**GTTG**t**TTTTGTGGTAAAC	55.4	-2.14

B.1.1.7 - **R20**	CAACT**t**TTGTTG**t**TTTTGTGGTAAAC	55.1	-2.14
B.1.1.7 - **R21**	CAACT**t**T**t**GTTGTTTTTGTGGTAAA	54.4	-2.14
B.1.1.7 - **R22**	CAACTTT**t**GTTG**t**TTTTGTGGTAAA	54.5	-2.14
B.1.1.7 - **R23**	CAAC**t**TT**t**GTTG**t**TTTTGTGGTAAAC	56.3	-2.14
B.1.1.7 - **R24**	CAACT**t**T**t**GTTG**t**TTTTGTGGTAAAC	56.6	-2.14
B.1.1.7 - **R25**	CAAC**t**TT**t**GTTGTTTTTGTGGTAAACA	56.2	-2.38
B.1.1.7 - **R26**	CAACT**t**T**t**GTTGTTTTTGTGGTAAACA	56.5	-2.38
B.1.1.7 - **R27**	CAACTTT**t**GTTG**t**TTTTGTGGTAAACA	56.6	-2.38
B.1.1.7 - **R28**	CAAC**t**TT**t**GTTGTTTTTGTGGTAACC	56.5	-2.26
B.1.1.7 - **R29**	CAAC**t**TT**t**GTTGTTTTTGTGGTAAGC	56.7	-2.26
B.1.1.7 - **R30**	CAACT**t**T**t**GTTGTTTTTGTGGTAACC	56.9	-2.26
B.1.1.7 - **R31**	CAACT**t**T**t**GTTGTTTTTGTGGTAAGC	57.1	-2.26
B.1.1.7 - **R32**	CAACT**t**T**t**GTTGTTTTTGTGGTACAC	56.8	-2.26
B.1.1.7 - **R33**	CAAC**t**TT**t**GTTG**t**TTTTGTGGTAAGC	58.0	-2.26
B.1.1.7 - **R34**	CAAC**t**TT**t**GTTGTT**t**TTGTGGTAAGC	58.0	-2.26
B.1.1.7 - **R35**	CAAC**t**TT**t**GTTGTTTT**t**GTGGTAAGC	58.2	-2.26
B.1.1.7 - **R36**	CAAC**t**TT**t**GTTGTTTTTG**t**GGTAAGC	58.2	-2.26
B.1.1.7 - **R37**	CAAC**t**TT**t**GTTGTTTTTGTGG**t**AAGC	58.4	-2.26

Primers/probes highlighted in green comprise the final lineage B.1.1.7 S gene primer/probe set
Nucleotides in lowercase and bold denote LNA-modified bases
Nucleotides in red font indicate mismatch bases used for SNP detection
F, forward primer; P, probe; R, reverse primer

Table 4. Interpretation of SARS-CoV-2 test results and corresponding actions

SARS-CoV-2 S gene	ΔCt between SARS-CoV-2 S gene and B.1.1.7	Human RNase P	Result interpretation	Report
+	Max 5 Ct	+/ND	SARS-CoV-2 B.1.1.7 detected	SARS-CoV-2 B.1.1.7 positive
+	Min 8 Ct	+/ND	SARS-CoV-2 ΔH69/ΔV70 deletion detected	SARS-CoV-2 B.1.1.7 negative
+	Min 20 Ct	+/ND	Other lineage of SARS-CoV-2 detected	SARS-CoV-2 B.1.1.7 negative
+	ND	+/ND	Consensus or other lineage of SARS-CoV-2 detected	SARS-CoV-2 B.1.1.7 negative
ND	+	+/ND	Inconclusive result	Inconclusive
ND	ND	ND	Invalid result	Invalid

ND, not detected

Table 5. Clinical performance of SARS-CoV-2 S gene and B.1.1.7 primer/probes sets.

		rTEST COVID-19 B.1.1.7 qPCR				
Analyzed samples	**Sequencing**	**SARS-CoV-2 S gene**	**B.1.1.7 (ΔH69/ΔV70 + ΔY144)**	**ΔH69/ΔV70 only**	**Other lineage of SARS-CoV-2**	**Inconclusive**
B.1.1.7 (ΔH69/ΔV70 + ΔY144)	37	37	36	0	0	1
ΔH69/ΔV70 only	16	16	0	13	2	1
Other lineages of SARS-CoV-2	12	12	0	0	12	0

Table 6. Overview of clinical sample RT-qPCR results, lineage, and GISAID information

	rTEST COVID-19 qPCR B.1.1.7 kit			Sequencing		
Sample ID	**B.1.1.7 PCR [Ct]**	**S gene PCR [Ct]**	**ΔCt**	**Sequencing outcome (GISAID lineage)**	**Name in GISAID**	**Accesion ID in GISAID**
1	23.8	23.6	0.2	**B.1.1.7**	UKBA-706	EPI_ISL_875525
2	18.0	18.1	-0.1	**B.1.1.7**	UKBA-707	EPI_ISL_875526
3	33.9	34.5	-0.5	**B.1.1.7**	UKBA-801	EPI_ISL_831667
4	36.4	36.0	0.5	**B.1.1.7**	UKBA-802	EPI_ISL_831668
5	32.3	31.5	0.8	**B.1.1.7**	UKBA-708	EPI_ISL_875527
6	37.5	35.2	2.3	**B.1.1.7**	UKBA-803	EPI_ISL_831672
7	23.7	25.0	-1.3	**B.1.1.7**	UKBA-714	EPI_ISL_875521
8	25.3	24.8	0.5	**B.1.1.7**	UKBA-713	EPI_ISL_875520
9	No CT	30.6	-	**B.1.160**	UKBA-701	EPI_ISL_875530
10	28.8	28.5	0.3	**B.1.1.7**	UKBA-703	EPI_ISL_875522
11	32.0	26.9	5.0	**B.1.1.7**	UKBA-705	EPI_ISL_875524
12	25.2	22.9	2.3	**B.1.1.7**	UKBA-704	EPI_ISL_875523
13	32.2	24.5	7.7	**B.1.258**	UKBA-702	EPI_ISL_875528
14	No CT	28.8	-	**B.1.1.243**	UKBA-715	EPI_ISL_875516
15	No CT	29.6	-	**B.1.177**	UKBA-716	EPI_ISL_875533
16	No CT	24.4	-	**B.1.177**	UKBA-717	EPI_ISL_875534
17	34.3	29.6	4.7	**B.1.1.7**	UKBA-718	EPI_ISL_875517
18	30.8	30.2	0.6	**B.1.1.7**	UKBA-719	EPI_ISL_875518
19	25.2	22.6	2.6	**B.1.1.7**	UKBA-720	EPI_ISL_875519
20	23.2	13.2	10.1	**B.1.258**	UKBA-722	EPI_ISL_875529
21	No CT	12.7	-	**B.1.1.170**	UKBA-723	EPI_ISL_875532
22	No CT	25.8	-	**B.1.1.170**	UKBA-724	EPI_ISL_875538

23	21.1	18.8	2.3	**B.1.1.7**	UKBA-804	EPI_ISL_831669
24	29.5	26.9	2.6	**B.1.1.7**	UKBA-805	EPI_ISL_831670
25	22.8	20.8	1.9	**B.1.1.7**	UKBA-806	EPI_ISL_831673
26	26.2	24.2	2.0	**B.1.1.7**	UKBA-807	EPI_ISL_831671
27	21.3	18.6	2.6	**B.1.1.7**	UKBA-808	EPI_ISL_831674
28	26.7	15.2	11.5	**B.1.258**	UKBA-809	EPI_ISL_831676
29	30.0	27.8	2.3	**B.1.1.7**	UKBA-814	EPI_ISL_831675
30	23.5	21.8	1.6	**B.1.1.7**	UKBA-815	EPI_ISL_831663
31	22.9	18.7	4.1	**B.1.1.7**	UKBA-816	EPI_ISL_831664
32	28.5	24.2	4.3	**B.1.1.7**	UKBA-817	EPI_ISL_831665
33	No CT	30.1	-	**B.1.258**	UKBA-818	EPI_ISL_831666
34	25.4	24.0	1.4	**B.1.1.7**	UKBA-501	EPI_ISL_779651
35	29.4	29.1	0.3	**B.1.1.7**	UKBA-502	EPI_ISL_779652
36	30.7	28.9	1.8	**B.1.1.7**	UKBA-503	EPI_ISL_779653
37	31.5	30.0	1.5	**B.1.1.7**	UKBA-504	EPI_ISL_779654
38	34.3	29.9	4.4	**B.1.1.7**	UKBA-505	EPI_ISL_779655
39	31.5	30.8	0.7	**B.1.1.7**	UKBA-506	EPI_ISL_779656
40	37.9	32.2	5.7	**B.1.1.7**	UKBA-507	EPI_ISL_779657
41	30.2	29.1	1.1	**B.1.1.7**	UKBA-508	EPI_ISL_779658
42	37.6	34.4	3.2	**B.1.1.7**	UKBA-509	EPI_ISL_779659
43	39.7	35.2	4.5	**B.1.1.7**	UKBA-512	EPI_ISL_779660
44	25.8	14.7	11.1	**B.1.258**	UKBA-1001	EPI_ISL_903980
45	No CT	23.1	-	**B.1.1.170**	UKBA-1002	EPI_ISL_903981
46	24.4	15.2	9.2	**B.1.258**	UKBA-1004	EPI_ISL_903983
47	35.8	27.1	8.7	**B.1.258**	UKBA-1005	EPI_ISL_903984
48	29.1	18.3	10.8	**B.1.258**	UKBA-1006	EPI_ISL_903985
49	15.6	16.8	-1.2	**B.1.1.7**	UKBA-1007	EPI_ISL_903986
50	25.4	16.1	9.3	**B.1.258**	UKBA-1008	EPI_ISL_903987
51	No CT	16.5	-	**B.1.160**	UKBA-1009	EPI_ISL_903988
52	19.9	17.4	2.5	**B.1.1.7**	UKBA-1010	EPI_ISL_903989
53	27.6	25.5	2.1	**B.1.1.7**	UKBA-1011	EPI_ISL_903990
54	37.1	26.0	11.1	**B.1.258**	UKBA-1012	EPI_ISL_903991
55	No CT	15.1	-	**B.1.177**	UKBA-1013	EPI_ISL_903992
56	28.0	19.6	8.4	**B.1.258**	UKBA-1014	EPI_ISL_903993
57	No CT	23.4	-	**B.1.1.277**	UKBA-1015	EPI_ISL_903994
58	35.5	26.9	8.6	**B.1.258**	UKBA-1016	EPI_ISL_903995
59	No CT	16.3	-	**B.1.160**	UKBA-1017	EPI_ISL_903996
60	33.2	22.9	10.3	**B.1.258**	UKBA-1018	EPI_ISL_903997
61	26.5	17.5	9.0	**B.1.258**	UKBA-1020	EPI_ISL_903999
62	19.8	20.7	-0.9	**B.1.1.7**	UKBA-1021	EPI_ISL_904000
63	No CT	18.9	-	**B.1.221**	UKBA-1022	EPI_ISL_904001
64	35.2	25.3	9.9	**B.1.258**	UKBA-1023	EPI_ISL_904002
65	No CT	28.9	-	**B.1.258**	UKBA-1024	EPI_ISL_904003

Figure 1.

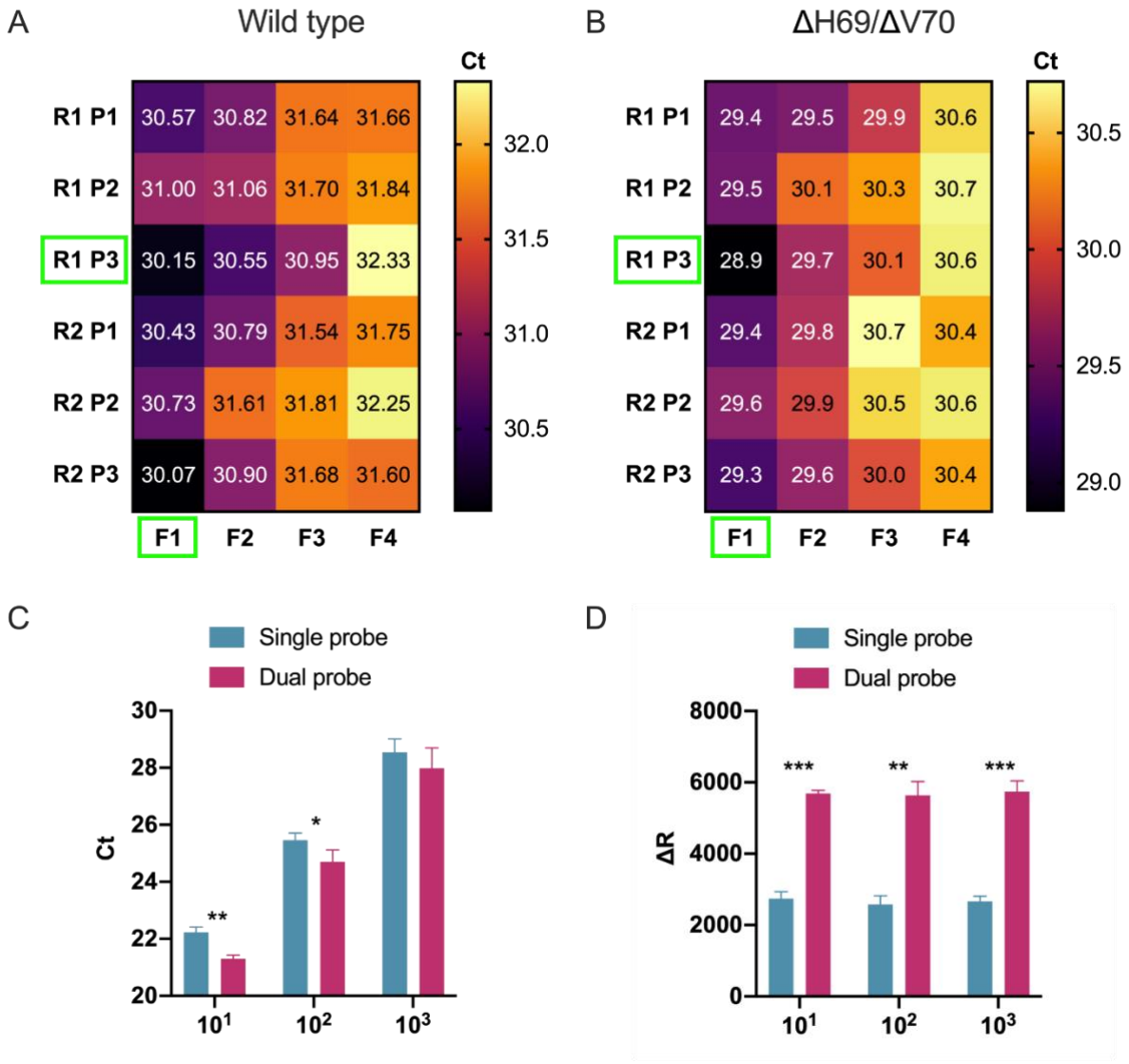

Figure 1. Development and optimization of a general SARS-CoV-2 S gene primer/probe set for all SARS-CoV-2 variants.
A, B) Heatmaps illustrate oligonucleotide primer and probe combinations designed to target conserved sequences within the spike gene, including all SARS-CoV-2 variants that were contained in our bioinformatics analysis. Combinations of forward (F1-F4) and reverse primers (R1-R2) and hydrolysis probes (P1-P2) were tested using two separate SARS-CoV-2 variants, a common SARS-CoV-2 variant (Wild type, panel **A**) and a variant containing the ΔH69/ΔV70 deletion (**B**). Green rectangle boxes indicate best performing primer/probe combinations. **C, D)** Bargraphs compare RT-qPCR performance of a single probe versus an additional identically labelled dual probe using three 10-fold (10^1, 10^2, 10^3) dilutions of SARS-CoV-2 template ran in triplicates. Evaluation of the performance was done by comparing raw Ct values (**C**) and fluorescence intensity values (**D**). Statistical analysis was performed using paired t-test (***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$).

Figure 2.

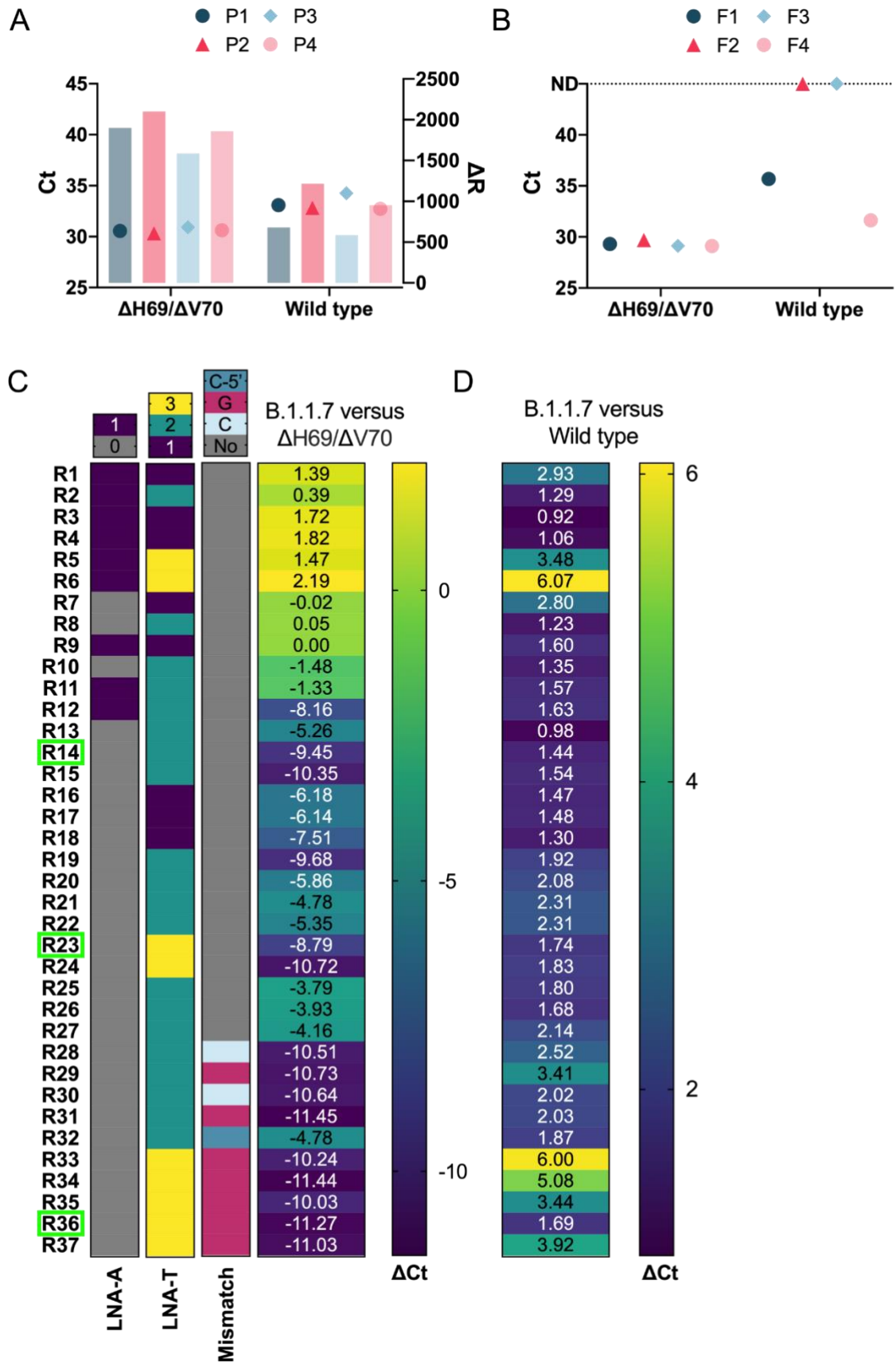
A
P1
P3
P2
P4
Ct
ΔR
45
40
35
30
25
2500
2000
1500
1000
500
0
ΔH69/ΔV70
Wild type
B
F1
F3
F2
F4
ND
Ct
40
35
30
25
ΔH69/ΔV70
Wild type
C
1
0
3
2
1
C-5'
G
C
No
B.1.1.7 versus
ΔH69/ΔV70
R1 1.39
R2 0.39
R3 1.72
R4 1.82
R5 1.47
R6 2.19
R7 -0.02
R8 0.05
R9 0.00
R10 -1.48
R11 -1.33
R12 -8.16
R13 -5.26
R14 -9.45
R15 -10.35
R16 -6.18
R17 -6.14
R18 -7.51
R19 -9.68
R20 -5.86
R21 -4.78
R22 -5.35
R23 -8.79
R24 -10.72
R25 -3.79
R26 -3.93
R27 -4.16
R28 -10.51
R29 -10.73
R30 -10.64
R31 -11.45
R32 -4.78
R33 -10.24
R34 -11.44
R35 -10.03
R36 -11.27
R37 -11.03
0
-5
-10
ΔCt
LNA-A
LNA-T
Mismatch
D
B.1.1.7 versus
Wild type
2.93
1.29
0.92
1.06
3.48
6.07
2.80
1.23
1.60
1.35
1.57
1.63
0.98
1.44
1.54
1.47
1.48
1.30
1.92
2.08
2.31
2.31
1.74
1.83
1.80
1.68
2.14
2.52
3.41
2.02
2.03
1.87
6.00
5.08
3.44
1.69
3.92
6
4
2
ΔCt

Figure 2. Development and optimization of a spike gene primer/probe set specific for the B.1.1.7 SARS-CoV-2 variant.

A) Comparison of probes (P1-4) targeting the ΔH69/ΔV70 deletion in B.1.1.7 using the best forward and reverse primers from the SARS-CoV-2 S gene primer/probe set (from **Figure 1**/**Table 2**). The performance between ΔH69/ΔV70 deletion template and wild type template is depicted by comparing Ct values (colored symbols) and fluorescence intensity (ΔR, colored bars). **B)** Assessment of forward primers (F1-4) targeting the ΔH69/ΔV70 deletion in B.1.1.7 using the best reverse primer and probe (from **Figure 1**/**Table 2**). Symbols compare Ct values of the ΔH69/ΔV70 variant and wild type templates. Dotted line indicates samples that were not detected (ND) within 45 cycles. **C)** Overview of reverse primer designs targeting the ΔY144 deletion of the B.1.1.7 variant and their effects on specificity by comparing the relative ΔCt when amplifying either B.1.1.7 or ΔH69/ΔV70 variants as template. Darker colors in the heatmap represent a greater ΔCt and consequently better specificity. Green rectangle boxes indicate reverse primers selected for further optimization. LNA-A depicts primers containing an LNA modified adenine base located at either the 3'- or 5'-end of the reverse primer. LNA-T displays the number (1-3) of LNA-modified thymine bases for each reverse primer. Mismatch base represents design modifications to introduce either a guanine (G) or cytosine (C) mismatch base in either the penultimate base (G or C) or the 3rd from last base (C-5') relative to the 3'-end of the reverse primer. **D)** Heatmap shows ΔCt value comparison of B.1.1.7 primer/probe set to SARS-CoV-2 S gene primer/probe set using the B.1.1.7 variant as template. Darker colors indicate smaller ΔCt and consequently better specificity.

Figure 3.

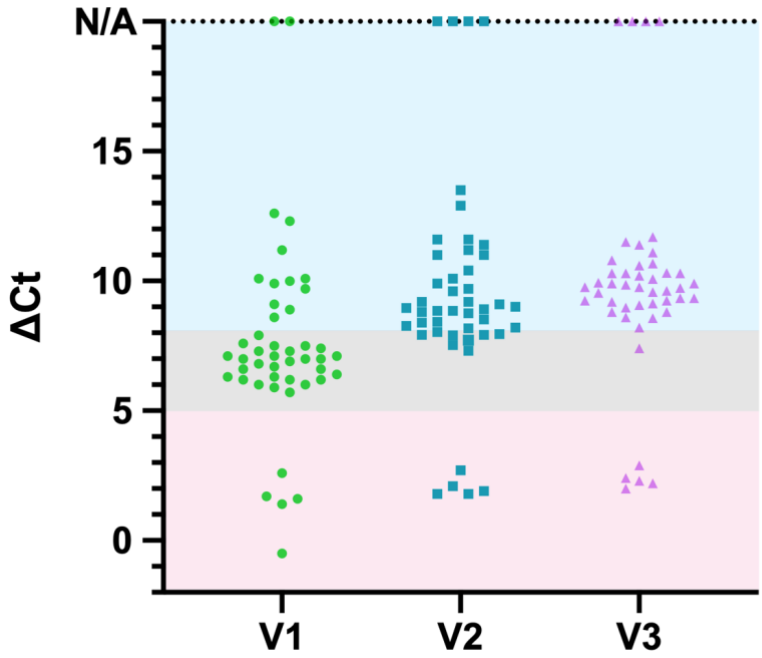

Figure 3. Overview of B.1.1.7 assay performance on clinical samples.
Three different versions (V1, V2, V3) of primer/probe sets for detection of the B.1.1.7 variant were directly compared on a selected panel of 46 SARS-CoV-2 positive clinical samples, some of which were confirmed B.1.1.7 and B.1.258 variants by sequencing. ΔCt values correspond to SARS-CoV-2 S gene assay Ct – B.1.1.7 assay Ct. Colored boxes within the plot define boundaries for corresponding variant interpretation, red (ΔCt ±5) for B.1.1.7, blue (ΔCt 8-20) for ΔH69/ΔV70, grey (ΔCt 5-8) for inconclusive samples. N/A represents samples which were detected only in SARS-CoV-2 S gene assay and therefore are interpreted as consensus SARS-CoV-2.

Figure 4.

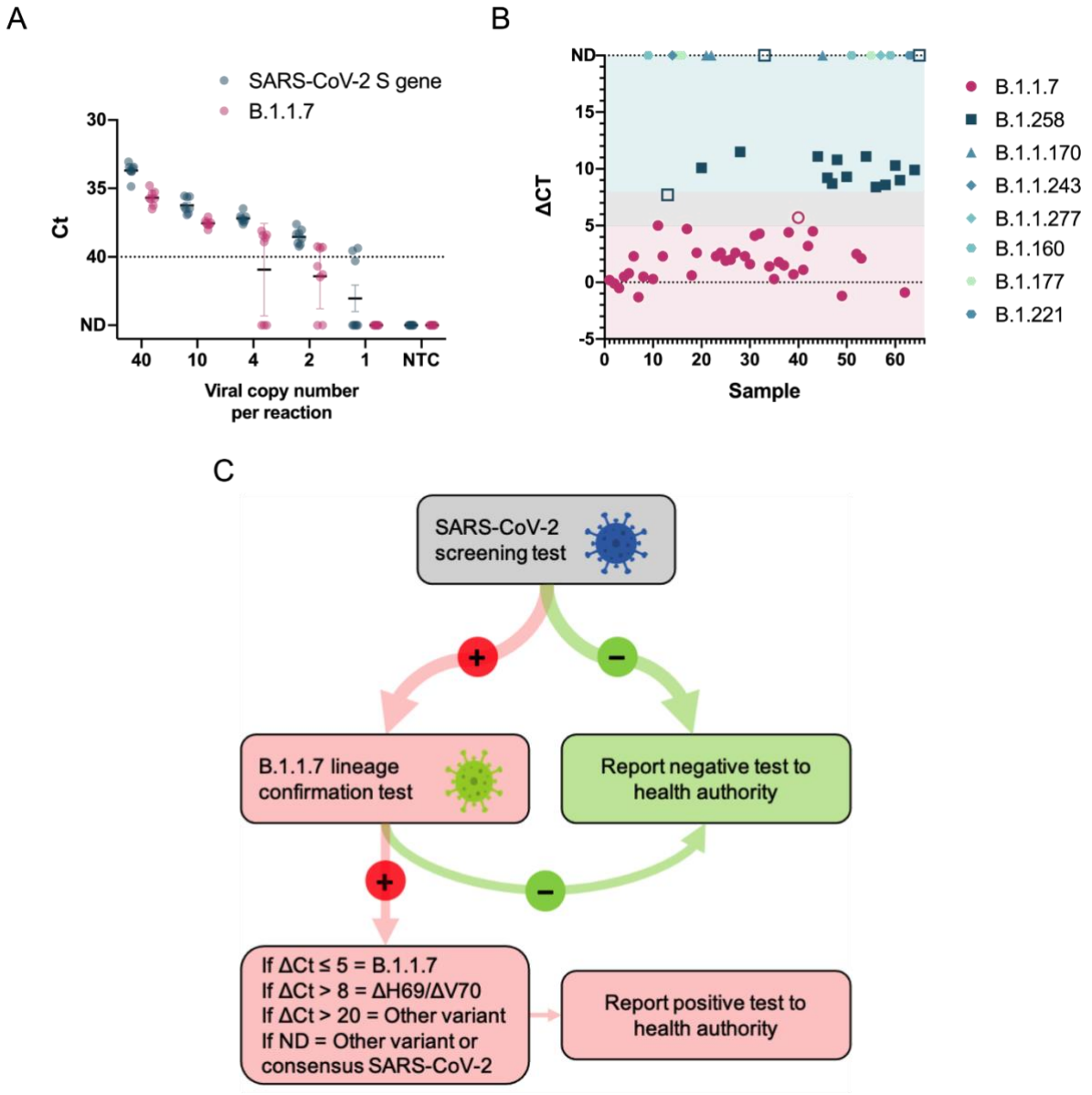

Figure 4. Analytical sensitivity and clinical validation of SARS-CoV-2 S gene and B.1.1.7 assays.
A) The limit of detection was determined for both SARS-CoV-2 S gene and B.1.1.7 assays by serial dilutions of isolated viral B.1.1.7 RNA. Data depict the mean and SD of eight replicates per each dilution. The dotted line at Ct 40 serves as a threshold after which amplification is considered invalid. **B)** Overview of ΔCt values (= SARS-CoV-2 S gene assay Ct. – B.1.1.7 assay Ct) for each sample in the clinical validation. Symbols represent the various SARS-CoV-2 lineages that were identified by sequencing. Closed symbols represent samples correctly identified by either the SARS-CoV-2 S gene or B.1.1.7 assays, whereas open symbols denote samples that did not meet the criterion established for variant identification. The shaded background shows ΔCt ranges that correspond with the criterion to report a sample as B.1.1.7 positive (pink), ΔH69/ΔV70 deletion positive (teal), and inconclusive (gray). ND, not detected. **C)** Decision tree demonstrating the proper workflow, interpretation criterion, and actions to implement the SARS-CoV-2 S gene and B.1.1.7 assays a testing regime to identify B.1.1.7 positive samples.