Garrett Perchetti ORCID iD: 0000-0003-3786-0509

Patrick Mathias ORCID iD: 0000-0003-4266-561X

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Specific allelic discrimination of N501Y and other SARS-CoV-2 mutations by ddPCR detects B.1.1.7 lineage in Washington State

Garrett A. Perchetti[1,a], Haiying Zhu[1,a], Margaret G. Mills[1], Lasata Shrestha[1], Cassia Wagner[2,3], Shah Mohamed Bakhash[1], Michelle J. Lin[1], Hong Xie[1], Meei-Li Huang[1], Patrick Mathias[1,4], Trevor Bedford[2,3], Keith R. Jerome[1,3], Alexander L. Greninger[1,3,*,#], Pavitra Roychoudhury[1,3,*,#]

[1]Department of Laboratory Medicine and Pathology, Virology Division, University of Washington School of Medicine, Seattle, WA, United States

[2]Department of Genome Sciences, University of Washington, Seattle, WA, United States

[3]Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA, United States

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[4]Department of Biomedical Informatics and Medical Education, University of Washington School of Medicine, Seattle, WA, United States

[a]These authors contributed equally

*Co-senior authors

[#]Corresponding authors

Pavitra Roychoudhury, proychou@uw.edu

1616 Eastlake Ave E, Suite 320, Seattle, WA 98102

Box 358080

Phone: 206 667 7801

Alexander L. Greninger, agrening@uw.edu

1616 Eastlake Ave E, Suite 320, Seattle, WA 98102

Phone: 415 439 3448

Fax: 206 616 4340

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ABSTRACT

Real-time epidemiological tracking of variants of concern (VOCs) can help limit the spread of more contagious forms of SARS-CoV-2, such as those containing the N501Y mutation. Typically, genetic sequencing is required to be able to track VOCs in real-time. However, sequencing can take time and may not be accessible in all laboratories. Genotyping by RT-ddPCR offers an alternative to rapidly detect VOCs through discrimination of specific alleles such as N501Y which is associated with increased transmissibility and virulence. Here we describe the first cases of the B.1.1.7 lineage of SARS-CoV-2 detected in Washington State by using a combination of RT-PCR, RT-ddPCR, and next-generation sequencing. We initially screened 1,035 samples positive for SARS-CoV-2 by our CDC-based laboratory developed assay using ThermoFisher's multiplex RT-PCR COVID-19 assay over four weeks from late December 2020 to early January 2021. S gene target failures (SGTF) were subsequently assayed by RT-ddPCR to confirm four mutations within the S gene associated with the B.1.1.7 lineage: a deletion at amino acid (AA) 69-70 (ACATGT), deletion at AA 145, (TTA), N501Y mutation (TAT), and S982A mutation (GCA). All four targets were detected in two specimens; follow-up sequencing revealed a total of 9 mutations in the S gene and phylogenetic clustering within the B.1.1.7 lineage. Next, we continued screening samples for SGTF detecting 23 additional B.1.1.7 variants by RT-ddPCR and confirmed by sequencing. As VOCs become increasingly prevalent, molecular diagnostic tools like RT-ddPCR can be utilized to quickly, accurately, and sensitively distinguish more contagious lineages of SARS-CoV-2.

BACKGROUND

The first known case of the SARS-CoV-2 B.1.1.7 variant in the United States was reported in Colorado on December 29th 2020 and the next day, it was confirmed in California [1,2]. University of Washington (UW) Virology had begun surveillance a few days prior using PCR to screen SARS-CoV-2 positive samples for the absence, or "dropout" of the S gene, which encodes the spike (S) protein on the surface of the viral particle. The B.1.1.7 variant is characterized by 17 mutations, eight of which occur within the S gene domain [3,4]. This region of the SARS-CoV-2 genome is of interest due to the B.1.1.7 lineage being associated with increased transmissibility, but also because the approved vaccines in the United States target the S protein [4–10].

Over the course of four weeks, we screened more than a thousand SARS-CoV-2 positive samples for the S gene target failure (SGTF). We selected random clinical specimens that were positive using a CDC-based laboratory developed test (LDT) for SARS-CoV-2, and typically had a cycle threshold (C_T) under 35 [11–15]. These SARS-CoV-2 positive samples were then amplified with the TaqPath COVID-19 assay (ThermoFisher Scientific, Waltham, MA, USA), a multiplex RT-PCR targeting the S gene, as well as regions within the N gene and ORF1ab [16]. Candidates for the B.1.1.7 variant have a positive detection for the N gene and ORF1ab, with a negative for the S gene. SGTFs are indicative of the B.1.1.7 variant but not exclusive identifiers of B.1.1.7,, so genetic sequencing is typically used to confirm variants of concern (VOCs) [17]. However, sequencing can be a time-consuming and resource-intensive process that not all laboratories have

integrated into clinical workflows. Analysis of publicly available sequencing data by the Broad Institute revealed that it takes a median 85 days to get from sample to publicly available sequence in the United States [18,19].

Here we describe the first detection of B.1.1.7 lineages in Washington State by a novel reverse-transcription droplet digital-PCR (RT-ddPCR) assay that specifically detects four mutations associated with the B.1.1.7 variant, particularly N501Y. This key mutation in the S gene is shared by the U.K., South African (B.1.351), and Brazilian (P.1) lineages [20]. Preliminary data has indicated B.1.1.7 to be more transmissible, while B.1.351 and P.1 are considered to be less well neutralized by antibodies induced by vaccines [3,20–22]. This RT-ddPCR assay can distinguish SARS-CoV-2 positive samples that carry this important N501Y mutation, flagging clinically relevant potential VOCs for genetic sequencing.

METHODS

Specimen Selection Criteria

From December 25th 2020 to January 20th 2021, 1,035 specimens positive for SARS-CoV-2 by our CDC-based LDT were screened for SGTFs (Fig. 1) [23–26]. Approximately 50% of these samples came from King County, followed by Pierce County with around 15%, Benton and Franklin Counties at 10%, and the remainder from other counties in Washington State. We selected samples with C_Ts<35 when available to reduce the effect of assay stochasticity, with consideration of initial data showing that the B.1.1.7 variant has been associated with higher viral loads (i.e. lower C_Ts) [27]. After detecting

the first positive B.1.1.7 cases, we assayed 23 additional B.1.1.7 variant samples previously confirmed by sequencing. Extracted nucleic acids were stored at 4^{o}C or -20^{o}C prior to amplification. This work was approved under a waiver of consent by the University of Washington institutional review board (STUDY00000408).

RT-PCR

RT-PCR was performed using TaqPath COVID-19 Combo Kit (ThermoFisher) with 10 µL of extracted nucleic acid used as template per 25 µL reaction. This multiplex real-time RT-PCR assay targets the S gene, N gene, and ORF1ab of SARS-CoV-2. Reactions utilized a kit-provided positive control (1x10^{4} copies/µL) diluted with TaqPath COVID-19 Control Dilution Buffer, and dH_2O as a negative template control. Amplifications were run on Applied Biosystems 7500 Real-Time PCR Systems (ThermoFisher) according to manufacturer's thermocycling parameters.

RT-ddPCR

Four sets of primers and probes were designed based on U.K. variant sequence hCoV-19/England/MILK-9E2FE0/2020 (EPI_ISL_581117, collection date 2020-09-21). To check for variation at primer and probe sites, 216 B.1.1.7 sequences were downloaded from the global initiative on sharing all influenza data (GISAID) on December 23rd, 2020, and aligned against the Wuhan-Hu1 reference sequence (NC_045512.2) using MAFFT v7.450 within Geneious Prime (https://www.geneious.com) (Supplementary Table 1). In addition, a G-block of 490bp was designed that includes four target

amplicons. All primer and probes were synthesized by ThermoFisher and the G-block was synthesized by IDT (Coralville, IA, USA). Primers were included at 900 nM and probes were used at 250 nM concentrations. All four targets are within the S gene domain: a deletion at amino acid (AA) 69-70 (ACATGT), deletion at AA 145, (TTA), N501Y mutation (TAT), and S982A mutation (GCA). The specific primers and probe sequences and characteristics are outlined in Table 1; the G-block sequence with underlined primer-binding sites and italicized probe-binding sites:

AACTCAGGACTTGTTCTTACCTTTCTTTTCCAATGTTACTTGGTTCC*ATGC*
*TATCTCTGGGACCAA*ATGGTACTAAGAGGTTTGATAACCCTGTCCTACCAT
TTAATGATGACGCTACTAATGTTGTTATTAAAGTCTGTGAATTTCAATTTT
GTAATG*ATCCATTTTTGGGTGTTTACCACA*AAAACAACAAAAGTTGGATG
GAAAGTGAGTTCAGAGTTTATTCTAGTGCGAATAATTGTACACACCTTGT
AATGGTGTTGAAGGTTTTAATTGTTACTTTCCTTTACAATCAT*ATGGTTTC*
*CAACCCACTTAT*GGTGTTGGTTACCAACCATACAGAGTAGTAGTACTTTC
TTTTGAACTTCTACATGCACCAGCAACCCAAACAACTTAGCTCCAATTTTG
GTGCAATTTCAAGTGTTTTAAATGATATCC*TTGCACGTCTTGACAAAGT*TG
AGGCTGAAGTGCAAATTGATAGGTTGATCACAGGC.

Two multiplex RT-ddPCR reactions per sample were performed in parallel (as outlined in Table 1) using One-step RT-ddPCR Advanced Kit for Probes (Bio-Rad Laboratories, Hercules, CA, USA) with the Automated Droplet Generator (Bio-Rad) and C1000 Touch thermocycler (Bio-Rad). Template RNA for each clinical sample was diluted to an approximate N1 C_T of 30 before amplification, and 10 µl was used as template in each RT-ddPCR reaction. Thermocycling conditions were as follows: 50^{o}C for 60 min, 40

cycles at 95°C for 30 sec and 60°C for 1 min, then 98°C for 10 min. Droplet detection was performed using the QX200 Droplet Reader (Bio-Rad) and QuantaSoft Pro 1.0.596 version software. For SARS-CoV-2 B.1.1.7 detection, all four targets are amplified beyond the thresholds. For non-B.1.1.7 samples, 0-1 target(s) are amplified beyond the thresholds. Both the synthetic G-block and a known B.1.1.7 clinical sample are used as positive controls, and both a SARS-CoV-2 clinical positive control and dH_2O are used as negative controls in the B.1.1.7 assay.

Sequencing

For next-generation sequencing, 11 µL of extracted RNA was used for single-stranded complementary DNA synthesized using SuperScript IV First-Strand Synthesis System according to the manufacturer's protocol (ThermoFisher). Libraries were prepared using the Swift Normalase Amplicon Panel (SNAP) for SARS-CoV-2 (Swift Biosciences, Ann Arbor, MI, USA) as previously described [28]. For standards, a wild type clinical nasopharyngeal specimen positive for SARS-CoV-2 was used as a positive control and water was used as no-template negative control. The resulting libraries were quantified fluorometrically on Qubit 3.0 using the Quant-iT dsDNA high sensitivity kit (Life Technologies, Carlsbad, CA, USA). The libraries passing quality control, nucleic acid concentrations > [1.3 ng/µL], were normalized manually and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using MiSeq Reagent Kit v2 **(**2x150 reads).

Raw reads were analyzed using a custom bioinformatic pipeline (TAYLOR, https://github.com/greninger-lab/covid_swift_pipeline) [28]. Briefly,

raw reads were trimmed to remove adapters and low-quality regions and mapped to the Wuhan-Hu-1 reference sequence (NC_045512.2) using BBMap v38.86 (https:/jgi.doe.gov/data-and-tools/bbtools/). Aligned reads were then soft-clipped of PCR primers using the PrimerClip package from Swift Biosciences (https://github.com/swiftbiosciences/primerclip) and a consensus sequence was called using bcftools v1.9 [29]. Consensus sequences were aligned using MAFFT v7.450 within Geneious Prime (https://www.geneious.com) [30]. Mutations in the spike protein were manually reviewed in addition to automated variant calling within the pipeline. Clade assignment was performed using Nextclade v0.12.0 (https://github.com/nextstrain/nextclade). Sequences were deposited to Genbank (MW704463.1 and MW704464.1) and GISAID (EPI_ISL_861730 and EPI_ISL_861731); raw reads were deposited to the NIH's Sequence Read Archive (Bioproject PRJNA610428).

Phylogenetic tree construction utilized the Nextstrain pipeline to align sequences, reconstruct maximum-likelihood and time-resolved phylogenetic trees and to infer nucleotide and amino acid substitutions across the phylogeny [31]. The specific workflow for this analysis is available at: https://github.com/blab/ncov-wa-build. The tree includes the initial B.1.1.7 sequences described here, 1,586 SARS-CoV-2 samples available on GISAID collected from November 2020 through February 12th, 2021, and an additional 1,824 global contextual sequences from GISAID sampled based on genetic similarity to the Washington sequences (Supplementary Table 2). The tree, continually updated with additional Washington sequences collected between

November 2020 and February 2021, can be viewed at: https://nextstrain.org/groups/blab/ncov/wa/nov20-feb21.

RESULTS

RT-PCR

For initial SGTF screening, means, medians, and the ranges of C_Ts detected for the S gene, N gene, and ORF1ab targets are characterized in Supplementary Table 3. Originally, seven samples were candidates for SGTF, out of 1,035 samples initially screened for the B.1.1.7 variant. Of these, 5/7 had C_Ts >33.5 for all targets with a mean and median C_T of 35.7 and 35.6 for N gene, and C_Ts 37.8 and 37.5 for ORF1ab, respectively. Two samples however, had strong fluorescent signal for the N gene and ORF1ab targets, but no detected S gene signal (Fig. 2). Candidate 1 (55538) had C_Ts of 23.4 and 23.7 for N gene and ORF1ab targets respectively, without S gene amplification. Candidate 2 (55545) had C_Ts of 26.7 and 26.4 for N gene and ORF1ab. Consequently, we decided to RT-ddPCR and sequence these specimens.

RT-ddPCR

For the first two B.1.1.7 cases detected, both specimens showed clear fluorescent amplification above analytical thresholds of all four targets for both sets of RT-ddPCR reactions (Fig. 3). Quantification of virus copies/mL is characterized in Table 2 for all 25 B.1.1.7 samples detected by RT-ddPCR. With back-calculations considering RNA dilutions and extraction compressions, U.K. Variant #1 (55538) was quantified to have 764,000-

860,000 virus copies/mL and U.K. Variant #2 (55545) was quantified to have 60,400-72,400 virus copies/mL depending on target amplicon.

Next, for specificity, we continued assaying samples with SGTFs as well as 62 additional wild type (WT) SARS-CoV-2 specimens. 100% of samples considered WT SARS-CoV-2 by sequencing genotyped as WT by RT-ddPCR. A mix of 12 clinically relevant respiratory viruses such as adenovirus, bocavirus, non-SARS-CoV-2-coronavirus, human metapneumovirus, influenza A, influenza B, respiratory syncytial virus, parainfluenza 1-4, and rhinovirus, was also included to evaluate cross pathogen specificity [32–34]. No cross-reaction amplification was detected for other respiratory viruses, or for 16 SARS-CoV-2 negative clinical samples. Moreover, during prospective genotyping efforts, two samples without SGTF by the TaqPath COVID-19 assay (ThermoFisher) were identified as VOCs by RT-ddPCR; one typed as B.1.1.7 and the other typed as either B.1.351 or P.1. These samples were later confirmed by sequencing to be B.1.1.7 and P.1 respectively.

While the primer sites for all four targets are present in WT SARS-CoV-2 viral RNA as well as B.1.1.7 RNA, the probes for each target are designed to bind specifically to the regions mutated in B.1.1.7. Depending on the difference in melting temperature between the WT and variant sequences for a given target, the probe may show some binding to the WT sequence as well, but at a lower efficiency than to the variant sequence. In RT-ddPCR, because individual template strands are amplified in separate droplets, inefficient probe binding can be identified as lower-amplitude fluorescence

from each droplet. Thus, even an A to T single nucleotide polymorphism (SNP) such as that present in the N501Y mutation (S1B) is easily distinguishable by RT-ddPCR by screening for droplets with S1B probe amplitude above a fluorescent threshold of 5,700.

Sequencing

We obtained quality consensus genomes for both initial B.1.1.7 samples, with more than 20,000X average coverage across the SARS-CoV-2 genome (Supplementary Table 4). Both samples were classified as 20I/501Y.V1 using Nextclade (v.0.12.0) and had 100% pairwise nucleotide identity in a whole genome alignment). For each sample, a total of 9 mutations were found in the spike protein: H69-70-, Y144-, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H. In a phylogenetic tree, these samples represented a unique cluster within clade 20I/501Y.V1 (Figure 4). They are 6 mutations diverged from the genetically closest sample available in GISAID, England/ALDP-BB47ED/2020, sampled in the United Kingdom in November 2020. Other B.1.1.7 samples collected in Washington did not cluster with them, suggesting that these samples represent a unique introduction into the state.

DISCUSSION

The particular variants circulating in an area affect the utility of public health measures intended to control the SARS-CoV-2 pandemic. For example, the B.1.1.7 variant has been shown to be more transmissible and associated with more severe disease than the WT lineage [4,7–10]. One of the

mutations thought to be responsible for this increased transmissibility, N501Y, is shared by other VOCs such as B.1.351 and P.1, which include additional mutations in S gene thought to reduce the neutralization capacity of antibodies generated against WT virus[22,35,36]. Unfortunately, this means recently developed vaccines may have diminished vaccine efficacy. Pfizer reported that via plaque reduction neutralization testing, neutralization of the B.1.351 variant was reduced by two-thirds compared to USA-WA1/2020 [37]; in the same day, Moderna reported their vaccine elicited sixfold less antibodies against the B.1.351 lineage [38]. These mutations have tremendous real-world impact as South Africa recently halted using the Oxford-AstraZeneca vaccine due randomized control trial data indicating substantially decreased efficacy against the B.1.351 variant [39–41]. Therefore, detection of the N501Y mutation flags samples that may harbor mutations associated with immune evasion as well as increased transmissibility [42].

Our experience with the SGTF screening underscores both the enduring utility and the limitations of multiplex RT-PCR assays. RT-PCR remains a powerful tool in screening large volumes of viral samples for variant detection. Other clinical laboratories from San Francisco to Lyon have also recently implemented modified RT-PCR-based screening methods to hone in on samples with potential to be VOCs [43–45]. However, RT-PCR can distinguish between alleles of varying length more reliably than it can distinguish between SNPs, so most RT-PCR assays rely on insertions or deletions that are incidental but not necessarily functional in VOCs.

Furthermore, the TaqPath primers/probe that fortuitously detect the 6 nucleotide deletion in B.1.1.7, when multiplexed with other primer/probe sets, can occasionally miss even B.1.1.7 variants as we observed in our screen of putative WT samples from TaqPath when we identified a B.1.1.7 sample that the TaqPath assay missed. The large number of genomic mutations associated with B.1.1.7, including SNPs (A570D, D614G, P681H, T716I, S982A, and D1118H) highlight the need to rapidly distinguish these subtle mutations in emerging VOCs[46]. RT-ddPCR is more sensitive than RT-PCR at resolving SNPs, and is better suited for allelic discrimination to differentiate the B.1.1.7 lineage or other variants of concern [47–49]. At the time of manuscript writing, no assay was commercially available to definitively distinguish B.1.1.7 from other lineages without sequencing. Since then, multiple commercial and publicly available assays have become available. The new TaqMan SARS-CoV-2 Mutation Panel (ThermoFisher) is one example, however is not multiplexed and must be run in separate parallel assays. Other assays targeted N501Y as well, or used melt-curve analysis, but the ABI 7500 PCR machines we used for SGTF screening are not well-suited for melt-curve analysis, and N501Y alone is not sufficient to definitely characterize a SARS-CoV-2 lineage without sequence analysis [50–52].

According to GISAID, the United States has only sequenced 3 out of every 1,000 positive SARS-CoV-2 samples [53]. Helix, a population genomics company, initially reported that of the positive COVID-19 tests screened, less than 1% had SGTF, however, of those SGTFs, more than one-third were confirmed B.1.1.7 lineages [54]. RT-ddPCR has potential to prioritize specimens for sequencing, allowing more efficient allocation of strain surveillance

resources. Although our surveillance was limited, the variant positivity at time of initial detection was approximately 1 in 500 (2 out of 1,035) randomly selected SARS-CoV-2 samples. However, reports indicate that due to B.1.1.7's increased transmissibility, it is already rising in frequency and is now the dominant strain in the United States as of April 2021 [6,9,42,54].

As genetic surveillance becomes increasingly relevant in efforts to track and understand new SARS-CoV-2 variants of concern in real-time, RT-ddPCR continues to cement its place in the clinical laboratory armamentarium. However, RT-ddPCR technology is still not ubiquitous in clinical laboratory settings. Increased adoption and investment in this technology can allow labs to rapidly estimate prevalence of existing variants and perform sample screening to better allocate limited sequencing resources.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

Table 1. Design information for B.1.1.7 identification assay

	Target	Primer/Probe Name	Sequence	Primer/ProbeLength (nt)	T_M (^oC)	Amplicon Length (nt)	RT-ddPCR reaction	Analysis Threshold	
								Wild Type (Amplitude)	B.1.1.7 (Amplitude)
S1A	Deletion 69-70 (ACATGT)	SForward 1A	tgttcttacctttctttttccaatgttactt	30	60				
		SReverse 1A	aatggtaggacagggttatcaaacct	26	60.2	91	Set 1	3,200	6,000
		SProbe1A	FAM_atgcta**tc**tctgggaccaa_MGB	19	69				
S1B	N501Y (A->T)	SForward 1B	atggtgttgaaggtttaattgttacttt	29	58.8				
		SReverse 1B	gtgcatgtagaagttcaaaagaaagtacta	30	58	82	Set 1	3,500	5,700
		SProbe1B	VIC_atggtttccaaccacct**t**at_MGB	20	72				
S2A	Deletion 145 (TTA)	SForward 2A	tgttgttattaaagtctgtgaatttcaatttt	32	59.9				
		SReverse 2A	tcgcactagaataaactctgaactcact	28	59.1	120	Set 2	2,100	7,000
		SProbe2A	FAM_atccatttttggtgt**tt**taccaca_MGB	24	70				
S2B	S982A (T->G)	SForward 2B	aattttggtgcaatttcaagtgttt	25	58.9	113	Set 2	--	5,000
		SReverse 2B	acctatcaatttgcacttcagcct	24	59.2				

SProbe2B	VIC_actttgtcaaga cgtg**c**aa_MGB	19	68

Primer and probe sets used in B.1.1.7 genotyping RT-ddPCR assay for SARS-CoV-2 variant detection. In the “Sequence” column, the bolded and underlined nucleotides reflect the region of deletion or mutation. For instance, for the probe used in S1A to detect the 69-70 deletion, the sequence ACATGT is present between the bolded and underlined thymine and cytoside nucleotides in wild-type virus. In the probe used for S1B, a thymine (uracil) nucleotide is included in place of adenosine at position 18 in the probe sequence. Notably, in S2B, although the mutation is a T to G transversion, the probe sequence is a reverse complement and so is reflected by a cytosine nucleotide at position 17 in the S2B probe sequence. Primers were included at 900 nM and probes were used at 250 nM concentrations. The analysis threshold units are in terms of fluorescent amplitude.

Abbreviations: T_M, primer melting temperature; FAM, 6-carboxyfluorescein; MGB, Minor Groove Binder; VIC, 2’-chloro-7’phenyl-1,4-dichloro-6-carboxy-fluorescein

Table 2. RT-ddPCR genotyping of four key SARS-CoV-2 B.1.1.7 mutations

Sample	69-70del (S1A)	501Y (S1B)	145del (S2A)	982A (S2B)	Lineage
1	8.60E+05	7.64E+05	8.08E+05	8.40E+05	B.1.1.7
2	7.24E+04	6.04E+04	6.68E+04	6.87E+04	B.1.1.7

3	4.61E+07	4.17E+07	4.36E+07	4.64E+07	B.1.1.7
4	4.04E+05	3.81E+05	3.71E+05	4.10E+05	B.1.1.7
5	2.92E+04	2.88E+04	2.37E+04	2.73E+04	B.1.1.7
6	1.95E+04	1.85E+04	1.92E+04	1.85E+04	B.1.1.7
7	2.23E+05	1.87E+05	2.06E+05	1.72E+05	B.1.1.7
8	3.53E+02	3.09E+02	6.24E+02	7.07E+02	B.1.1.7
9	2.47E+07	2.72E+07	3.15E+07	3.66E+07	B.1.1.7
10	3.01E+05	2.87E+05	2.80E+05	3.13E+05	B.1.1.7
11	4.21E+05	4.13E+05	3.97E+05	4.14E+05	B.1.1.7
12	4.10E+04	3.88E+04	4.14E+04	4.03E+04	B.1.1.7
13	2.05E+06	1.82E+06	1.90E+06	1.92E+06	B.1.1.7
14	1.05E+06	9.39E+05	9.95E+05	1.02E+06	B.1.1.7
15	7.45E+05	6.32E+05	7.26E+05	7.49E+05	B.1.1.7
16	4.14E+06	3.73E+06	3.98E+06	4.06E+06	B.1.1.7
17	7.05E+06	5.44E+06	5.85E+06	6.01E+06	B.1.1.7
18	2.64E+06	2.33E+06	2.59E+06	2.64E+06	B.1.1.7
19	7.04E+06	7.04E+06	1.25E+09	1.25E+09	B.1.1.7
20	1.12E+04	9.88E+03	1.05E+04	1.04E+04	B.1.1.7

21	4.39E+04	3.98E+04	3.99E+04	4.52E+04	B.1.1.7
22	6.25E+08	6.25E+08	1.25E+09	1.25E+09	B.1.1.7
23	1.24E+03	9.65E+02	1.56E+03	1.01E+03	B.1.1.7
24	5.50E+06	3.91E+06	6.78E+06	4.85E+06	B.1.1.7
25	2.46E+07	2.34E+07	2.08E+07	2.00E+07	B.1.1.7
Pos[1]	1.65E+08	1.83E+08	1.63E+08	2.08E+08	B.1.1.7
Pos[2]	4.46E+08	3.71E+08	4.09E+08	4.08E+08	B.1.1.7
Neg[1]	0	0	0	0	WT
Neg[2]	0	0	0	0	N/a

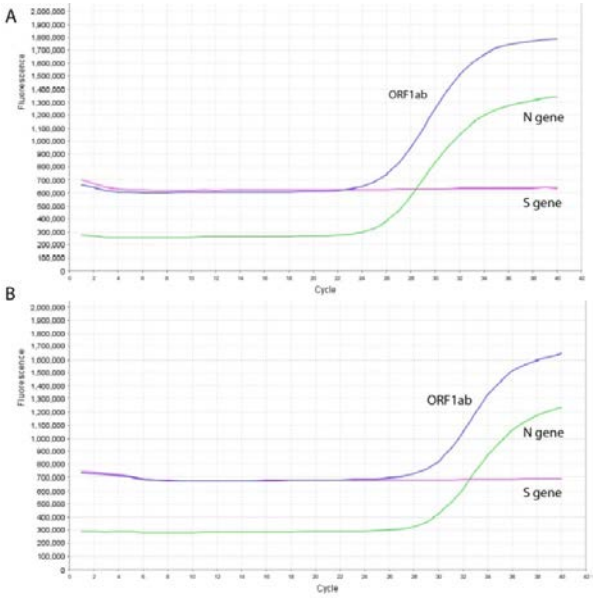
Copies/mL of RNA for each target amplified in multiplex RT-ddPCR genotyping reaction. Pos control = [1]Previously-identified and sequenced B.1.1.7 clinical sample; [2]G-block. Neg control = [1]Clinical (non-B.1.1.7) SARS-CoV-2 positive; [2]water. Samples with >5 x10^5 copies/mL have viral titers that exceed the upper limit of absolute quantification by RT-ddPCR.

FIGURES

Figure 1. RT-PCR, RT-ddPCR, and sequencing surveillance effort for initial detection of B.1.1.7 lineage

Samples sent to UW Virology for SARS-CoV-2 molecular detection were initially screened for S gene transcript failure (SGTF) by multiplex RT-PCR as a proxy for variant detection. SARS-CoV-2 positive samples with SGTF were subsequently genotyped for specific allelic discrimination by RT-ddPCR. Both SGTF samples that genotyped as B.1.1.7 lineage by RT-ddPCR were also sequenced and clustered with N501Y.V1 clades in the United States.

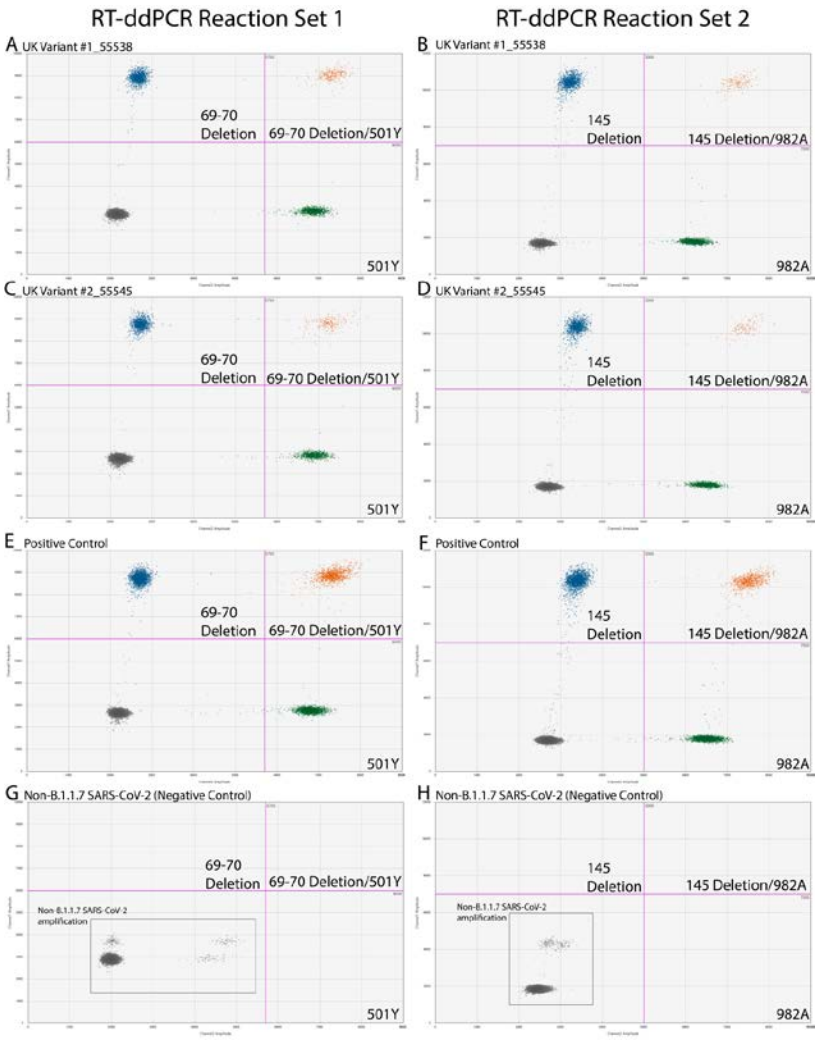
Figure 2. Multiplexed qRT-PCR fluorescence of S gene dropout in SARS-CoV-2 positive samples



Multicomponent amplification plots are shown for (A) U.K. Variant #1 (55538) and (B) U.K. Variant #2 (55545). PCR cycle is plotted on the X axis, with

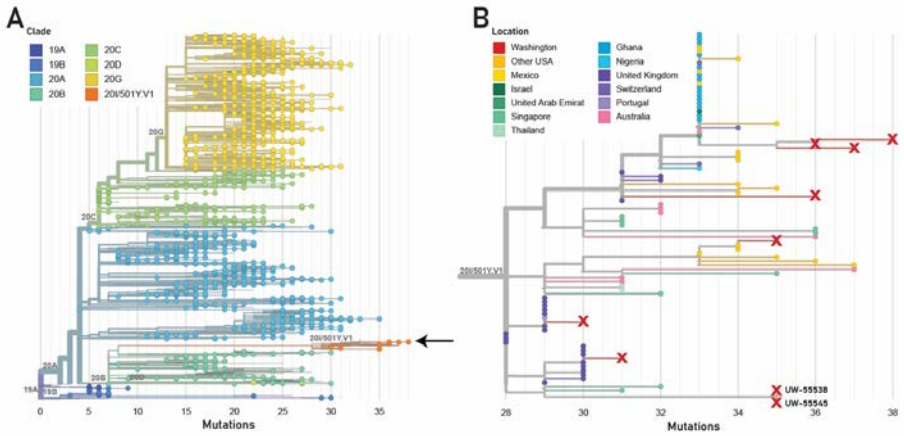
quantity of fluorescence detected in real-time on the Y axis. Two out of three fluorophores are detected by qRT-PCR for both samples on the 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) and analyzed on 7500 software v.2.3 (Life Technologies). VIC and FAM are reporters for N gene and ORF1ab, respectively and are seen here with robust amplification. ABY, the probe for the S gene, is present in the reaction mix, but is not detected by fluorescence, indicating a potential B.1.1.7 SARS-CoV-2 variant.

Figure 3. RT-ddPCR amplification results for SARS-CoV-2 B.1.1.7 lineage



(A) U.K. Variant Sample #1 (55538) and (C) U.K. Variant Sample #2 (55545) have amplification for AA69-70del and N501Y mutation. (E) Positive control demonstrates amplification for AA69-70del and N501Y mutation and (G) B.1.1.7 Negative control (Non-B.1.1.7 SARS-CoV-2 and water) shows no amplification for AA69-70del and N501Y mutation for RT-ddPCR amplicon set 1. (B) U.K. Variant Sample #1 (55538) and (D) U.K. Variant Sample #2 (55545) have amplification for AA145 deletion and S982A mutation. (F) Positive control demonstrates amplification for AA145 deletion and S982A mutation and (H) B.1.1.7 Negative control (Non-B.1.1.7 SARS-CoV-2 and water) shows no amplification for AA145 deletion and S982A mutation for RT-ddPCR amplicon set 2.

Figure 4. Phylogenetic tree focused on Washington State SARS-CoV-2 samples collected from November 2020 through February 2021



In (A) the phylogenetic tree is filtered to only show Washington samples; 501Y.V1 (B.1.1.7) samples are shown in orange (arrow). (B) 501Y.V2 clade showing Washington samples (red X) in context of global SARS-CoV-2,

selected by genetic proximity to the Washington samples. UW-55538 and UW-55545 separately cluster at the bottom of the clade.

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