1 **Supplemental Methods:**

2

3 *Assay Design*

4 We used synthetic whole-genome RNA fragments as a wild-type control (Twist Bioscience, San Francisco, CA,
5 USA) diluted to 10^4 copies/µL in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA). Due to manufacturing restrictions
6 for SARS-CoV-2-specific dsDNA gene fragments, we pooled six individual ssDNA mutant amplicons (Elim
7 Biopharmaceuticals, Hayward, CA, USA) in equimolar ratios diluted to 10^4 copies/µL each in Tris-EDTA buffer (10
8 mM Tris, 1 mM EDTA) (Table S2). The two primer sets and three mutant probes used in this assay amplify two of
9 these six amplicons, while the other four were added for other candidate assays. Primers and dual-labeled BHQ-
10 quenched hydrolysis probes were ordered from the Stanford Protein and Nucleic Acid Facility (Table S1),
11 rehydrated to 100µM in Tris-EDTA buffer, and mixed to create bulk primer/probe mix.

12

13 Primer/probe mix (1 µL) was combined with a one-step RT-qPCR system (12.5 µL master mix + 0.5 µL *Taq*
14 polymerase, SuperScript™ III Platinum™ One-Step qRT-PCR Kit, Invitrogen, Carlsbad), nuclease-free water (6.0
15 µL), and template (5.0 µL) in a 25µL reaction (Table S3). All experiments were conducted on a BioRad CFX96
16 real-time PCR instrument in 96-well plates (BioRad, Hercules, CA, USA). One mutant control (pooled ssDNA
17 mutant amplicons) and one wild-type control (Twist whole-genome synthetic RNA) were included in each RT-
18 qPCR experiment. Cycling conditions were: 52°C for 15:00, 94°C for 2:00, and then 45 cycles of 94°C for 00:15,
19 57.0°C for 00:40, and 68°C for 00:20. Annealing temperature was optimized with a temperature gradient.

20

21 Fluorescence was collected in all channels (1, N501Y-FAM; 2, L452R-HEX; 3, N501-Cy3.5 (ROX); 4, E484K-Cy5;
22 5, no probe). Fixed fluorescence thresholds of 500 relative fluorescence units ([RFU], N501Y-FAM), 1000 RFU
23 (L452R-HEX), 50 RFU (N501-Cy3.5), and 200 RFU (E484K-Cy5) were used to determine the threshold cycle (C_t).
24 Assay interpretation is described in detail in Table S4.

25

26 *Analytical Performance*

27 To determine the lower limit of detection (LLOD), the pool of six mutant ssDNA oligonucleotides described above
28 was diluted to 100 copies/µL template, 10 copies/µL, 5 copies/µL, and 1 copies/µL in Tris-EDTA buffer in

29 replicates of 20. Any amplification crossing the fluorescence threshold (Table S4) was regarded as detection. The
30 95% LLOD was determined by fitting these data to a probit regression curve. The respective 95% LLODs for the
31 L452R, E484K, and N501Y targets were 1.5 (95% CI 1.0-3.1), 16.0 (11.1-40.3), and 23.6 (14.0-29.4) copies/μL
32 template (Table S5). Assay linearity was assessed from 0.0 to 6.0 log10 copies/μL template (Table S6). We
33 observed no non-specific N501 wild-type (Cy3.5) amplification even at high (10^6 copies/μL) mutant ssDNA copy
34 number; similarly, we observed no non-specific L452R, E484K, or N501Y non-specific amplification at high (10^6
35 copies/μL) wild-type TWIST synthetic RNA copy number (Table S6). Precision was not assessed for this
36 qualitative assay.
37
38 *Next-Generation Sequencing Validation*
39 A subset of wild-type and mutant specimens genotyped by RT-qPCR were assessed by WGS in a validation
40 dataset. Specimens selected for sequencing met one of several selection criteria: 1. Randomly-selected
41 specimens with original SARS-CoV-2 RT-qPCR diagnostic Ct value ≤30. 2. Specimens from patients with specific
42 clinical histories triaged for sequencing by the virology medical director. These included patients with suspected
43 COVID-19 re-infection, post-vaccination COVID-19 infection, or COVID-19 infection after travel from regions with
44 prevalent variants of interest/concern.3. Specimens with sufficient residual material and viral load (Ct ≤30) with
45 screen-detected L452R, E484K, or N501Y mutations by variant genotyping RT-qPCR.
46
47 We adapted an existing WGS pipeline for poliovirus genotyping to conduct SARS-CoV-2 whole-genome
48 amplicon-based sequencing.(1–3) For target enrichment, we designed 28 primersets to generate 28 overlapping
49 amplicons of approximately 1,200 nucleotides each using PrimalSeq.(2) Even- and odd-numbered primersets
50 were pooled separately, and then the whole genome was amplified in two reactions via long-range PCR using 10
51 μL New England BioLabs (NEB) Luna 2X buffer, 1 μL NEB enzyme mix, 2 μL nuclease-free water, 2 μL primer
52 pool (50nM each primer), and 5 μL extracted nucleic acid. Long-range PCR conditions were: 52°C for 30:00,
53 94°C for 2:00, and then 40 cycles of 94°C for 00:15, 55.0°C for 00:30, and 68°C for 02:00 prior to final 68°C
54 extension for 10:00.
55

56 Libraries were prepared using NEBNext library preparation reagents for Illumina sequencing instruments (New
57 England BioLabs, Ipswich, MA). Products from the two long-range PCR reactions (40 µL) were pooled and
58 purified with 36 µL (0.9X ratio) AMPure XP beads (Beckman Coulter, Brea, CA) and 80% ethanol, then
59 resuspended into 32 µL AVE elution buffer. Purified cDNA was quantified using the Qubit dsDNA broad range
60 assay. Libraries were then fragmented at 37°C for 30:00 using 2 µL 10X fragmentation buffer, 1 µL 200mM
61 $MgCl_2$, 2 µL fragmentase, and 15 µL purified cDNA from long-range PCR.
62
63 Fragmented libraries (20 µL) were then purified with 36 µL (1.8X ratio) AMPure XP beads and 80% ethanol, then
64 resuspended into 32 µL AVE elution buffer. Purified fragmented cDNA (30 µL) was subject to end-repair/dA tailing
65 using 1.5 µL NEB end-repair enzyme mix and 3.5 µL end-repair reaction buffer (20°C for 30:00, 65°C for 30:00).
66 We ligated single-index NEBNext adapters according to manufacturer recommendations in a 45 µL reaction
67 containing 35 µL end-repaired product. This product was again purified with 0.9X AMPure XP beads and 80%
68 ethanol, then resuspended in 28 µL buffer.
69
70 We indexed libraries using NEB single index primers in a 50 µL reaction containing 25 µL NEBNext high-fidelity
71 2X master mix, 1 µL universal primer, 1 µL index primer, and 23 µL adapter-ligated cDNA. Thermocycler settings
72 for indexing were: 94°C for 00:30, then 12 cycles of 94°C for 00:10, 65.0°C for 00:30, and 72°C for 00:30.
73 Indexed libraries were then purified with 18 µL (0.9X ratio) AMPure XP beads and 80% ethanol, then
74 resuspended into 32 µL AVE elution buffer.
75
76 Indexed library fragment size and concentration were measured with a BioAnalyzer 2100, then diluted to 15pM.
77 Each sequencing run contained one no template control, one wild-type TWIST synthetic whole-genome RNA
78 control, and up to 46 clinical specimens. Libraries were sequenced on an Illumina MiSeq using single-end 150-
79 cycle sequencing using the MiSeq reagent kit V3.
80
81 Genomes were assembled via a custom assembly and bioinformatics pipeline using NCBI NC_045512.2 as
82 reference. Whole-genome sequences with at least 90% genome coverage to a depth of at least 10 reads were

83 accepted for interpretation. Mutation calling required a depth of at least 12 reads with a minimum variant
84 frequency of 20%. Lineage name was assigned using PANGOLIN.(3)
85
86 *Clinical Specimen NAAT Platforms*
87 Prior to genotyping RT-qPCR, initial respiratory SARS-CoV-2 NAAT was conducted on a variety of platforms
88 (Table 1).(4–6) These included: 1) a previously-described laboratory-developed reverse transcription quantitative
89 polymerase chain reaction (RT-qPCR) targeting the envelope gene (*E* gene) on the Rotor-Gene Q (Qiagen,
90 Germantown, MD)(4–6); 2) a laboratory-developed RT-qPCR assay utilizing a PerkinElmer kit targeting the
91 ORF1ab and nucleocapsid gene ([*N* gene] PerkinElmer, San Jose, CA); 3) Panther Fusion SARS-CoV-2 (Hologic,
92 Marlborough, MA), a high-throughput RT-qPCR method targeting open reading frame 1ab (ORF1ab); 4) Aptima
93 SARS-CoV2 (Panther System, Hologic), a transcription mediated amplification method targeting ORF1ab; 5)
94 GeneXpert Xpress SARS-CoV-2 (Cepheid, Sunnyvale, CA), a rapid RT-qPCR method targeting both *E* and *N*
95 genes; 6) cobas Liat SARS-CoV-2 & Influenza A/B (Roche, Indianapolis, IN), a point-of-care RT-PCR method
96 targeting ORF1ab and *N* gene; 7) e-Plex SARS-CoV-2 (Genmark, Carlsbad, CA), a rapid RT-PCR method
97 targeting the *N* gene. All specimens testing positive for SARS-CoV-2 by NAAT with RT-qPCR $C_t \leq 30$ or
98 transcription-mediated amplification relative light units (RLU) ≥ 1,100 during this period were subject to multiplex
99 allele-specific genotyping RT-qPCR. The small subset (288/4,049, 7.1%) of included specimens tested by rapid
100 NAATs (Cepheid GeneXpert, Roche Liat, Genmark Eplex) did not have C_t values available and were included
101 irrespective of viral load. All assays were conducted according to manufacturer and emergency authorization
102 instructions.(7, 8)
103
104 *Nucleic Acid Extraction*
105 Because residual eluate is not available for specimens originally tested on the Hologic or rapid NAAT (Cepheid
106 GeneXpert, Roche Liat, Genmark Eplex) platforms, we re-extracted these genotyped samples from the original
107 respiratory swab specimens on the same platform, provided there was sufficient material. Total nucleic acids were
108 extracted from 300 µL viral transport media, universal transport media, or phosphate-buffered saline and eluted
109 into 60 µL elution buffer (PerkinElmer Janus G3 Reformatter, Chemagic 360 nucleic acid extractor, and Chemagic
110 Viral DNA/RNA 300 Kit).

111 All other specimens were genotyped from the residual diagnostic eluate.

112

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137 **Supplemental Table 1.** Primer and Probe Oligonucleotide Sequences and Characteristics

138

Oligonucleotide	Sequence (5' → 3')	5' Mod	3' Mod	Tm[a] Match (°C)	Tm[a] Mismatch (°C)	Sequence Conservation			
						NCBI Pre-12/2020 (n=31,027)	GISAID B.1.427/B.1.429 (n=622)	GISAID B.1.1.7 (n=7,864)	GISAID B.1.351 (n=341)
L452R_FWD	CTCTCTCAAAAGGTT TGAGATTAGACT	-	-	62.7	-	99.6% (n=30,903)	100.0% (n=622)	99.9% (n=7,856)	99.7% (n=340)
L452R_REV	CTTGATTCTAAGGTT GGTGGTAA	-	-	60.5	-	99.0% (n=30,695)	99.5% (n=619)	99.8% (n=7,850)	98.8% (n=337)
L452R_MT_HEX	CCTAAACAATCTATA CCGGTAATT	HEX	BHQ	58.7	51.6	<0.1% (n=19)	100.0% (n=622)	0.0% (n=0)	0.0% (n=0)
E484K_FWD	CTGAAATCTATCAGG CCGGTA	-	-	61.2	-	99.4% (n=30,823)	99.7% (n=620)	99.7% (n=7,843)	99.7% (n=340)
E484K_REV	GAAAGTACTACTACT CTGTATGG	-	-	57.4	-	98.6% (n=30,584)	99.7% (n=620)	99.8% (n=7,850)	98.5% (n=336)
E484K_MT_CY5	CTTGTAATGGTGTTA AAGGTTT	CY5	BHQ	57.6	53.7	<0.1% (n=13)	0.0% (n=0)	<0.1% (n=1)	99.7% (n=340)
N501Y_MT_FAM[b]	TTTCCAACCCACTTA TGGT	FAM	BHQ	59.0	54.8	0.1% (n=38)	0.0% (n=0)	100.0% (n=7,861)	99.7% (n=340)
N501_WT_CY3.5[b]	TTTCCAACCCACTAA TGGT	CY3.5	BHQ	59.0	56.8	99.2% (n=30,773)	100.0% (n=622)	0.0% (n=0)	0.0% (n=0)

Mod, modification; Tm, melt temperature; FWD, forward; REV, reverse; WT, wild-type; MT, mutant. A hyphen (-) indicates this cell is not applicable for this row (e.g., no 5' modification for the L452R_FWD primer).

[a] Calculated using IDT OligoAnalyzer (https://www.idtdna.com/pages/tools/oligoanalyzer) using qPCR conditions (DNA, 0.2μM [oligonucleotide], 50mM [Na^+], 3mM [Mg^{2+}],0.8mM [dNTPs]. Mismatch Tm is for wild-type → mutant or mutant → wild-type nucleotide annealing.

[b] Anneals to E484K_FWD/REV amplicon downstream of E484K_MT_CY5.

139

140 **Supplemental Table 2.** Mutant Amplicon Controls

141

Oligonucleotide[a]	NC_045512 Polymorphism	Amplicon Length	Sequence (5' → 3')
ssDNA_L452R_MT	22917 T>G	79	CTCTCTCAAAAGGTTTGAGATTAGACTTCCTAAACAATCTATACCGGTAATTATAATTACCACCAACCT TAGAATCAAG
ssDNA_del69-70_MT	del21765-70	113	ACATTCAACTCAGGACTTGTTCTTACCTTTCTTTTCCAATGTTACTTGGTTCCATGCTATCTCTGGGAC CAATGGTACTAAGAGGTTTGATAACCCTGTCCTACCATTTAATG
ssDNA_K417N_MT	22813G>T	101	CATTTGTAATTAGAGGTGATGAAGTCAGACAAATCGCTCCAGGGCAAACTGGAAATATTGCTGATTATA ATTATAAATTACCAGATGATTTTACAGGCTGC
ssDNA_E484K_MT	23012G>A	133	CTGAAATCTATCAGGCCGGTAGCACACCTTGTAATGGTGTTAAAGGTTTTAATTGTTACTTTCCTTTAC AATCATATGGTTTCCAACCCACTTATGGTGTTGGTTACCAACCATACAGAGTAGTAGTACTTTC
ssDNA_N501Y_MT	23063A>T	134	GTTTTAATTGTTACTTTCCTTTACAATCATATGGTTTCCAACCCACTTATGGTGTTGGTTACCAACCAT ACAGAGTAGTAGTACTTTCTTTTGAACTTCTACATGCACCAGCAACTGTTTGTGGACCTAAAAAG
ssDNA_P681H_MT	23604C>A	99	CAGGTATATGCGCTAGTTATCAGACTCAGACTAATTCTCATCGGCGGGCACGTAGTGTAGCTAGTCAAT CCATCATTGCCTACACTATGTCACTTGGTG

MT, mutant.
[a] Individual ssDNA amplicons were pooled in equimolar ratios diluted to 10^4 copies/µL each in Tris-EDTA buffer to create a mutant control.

142

143 **Supplemental Table 3.** Multiplex RT-PCR Reagents and Concentrations

144

Reagent	Stock Concentration	Volume (µL)	PCR Reaction Concentration
L452R_FWD	9000 nM	1.0[a]	360 nM
L452R_REV	9000 nM	-	360 nM
E484K_FWD	9000 nM	-	360 nM
E484K_REV	9000 nM	-	360 nM
L452R_MT_HEX	2000 nM	-	80 nM
E484K_MT_CY5	2000 nM	-	80 nM
N501Y_MT_FAM	2000 nM	-	80 nM
N501_WT_CY3.5	2000 nM	-	80 nM
SuperScript™ III Platinum™ One-Step qRT-PCR Kit 2X Mix[b]	2X	12.5	1X
SuperScript™ III Platinum™ One-Step qRT-PCR Kit *Taq* Mix[b]	-	0.5	-
Nuclease-free Water	-	6.0	-
Template[c]	-	5.0	-
Total	**-**	**25.0**	**-**

MT, mutant; WT, wild-type; nM, nanomolar. A hyphen (-) indicates this cell is not applicable for this row (e.g., PCR template does not have a known stock concentration or PCR reaction concentration).
[a] 1.0µL primer/probe mix at stock concentrations listed above.
[b] Catalog Numbers 11732-020 and 11732-088
[c] Wild-type TWIST whole-genome synthetic RNA control, pooled mutant amplicon control (Supplemental Table 2), or extracted nucleic acids from clinical upper respiratory swabs.

145

146 **Supplemental Table 4.** RT-qPCR Assay Interpretation and Reporting

147

Template	Instrument Result[a]				Triage	Final Report			
	N501 (Cy3.5)	**N501Y (FAM)**	**E484K (Cy5)**	**L452R (HEX)**	**Action**	**L452R Mutation**	**E484K Mutation**	**N501Y Mutation**	**Interpretative Comment[b]**
Wild-type TWIST Control	C_t<38	ndet	ndet	ndet	Negative QC Passed	-	-	-	-
Mutant ssDNA Control	ndet	C_t<38	C_t<38	C_t<38	Positive QC Passed	-	-	-	-
Patient Samples: Upper Respiratory Swab	C_t ≤40	ndet	ndet	ndet	Report	Not Detected	Not Detected	Not Detected	1 (below)
	C_t ≤40	ndet	C_t ≤40	ndet	Report	Not Detected	Detected	Not Detected	2 (below)
	C_t ≤40	ndet	ndet	C_t ≤40	Report	Detected	Not Detected	Not Detected	3 (below)
	ndet	C_t ≤40	C_t ≤40	ndet	Report	Not Detected	Detected	Detected	4 (below)
	ndet	C_t ≤40	ndet	ndet	Report	Not Detected	Not Detected	Detected	5 (below)
	ndet	ndet	ndet	ndet	Repeat PCR[c]	-	-	-	-
	C_t ≤40	C_t ≤40	C_t ≤40	C_t ≤40	Director Review[d]	-	-	-	-
	C_t ≤40	C_t ≤40	C_t ≤40	ndet	Director Review	-	-	-	-
	C_t ≤40	C_t ≤40	ndet	C_t ≤40	Director Review	-	-	-	-
	C_t ≤40	C_t ≤40	ndet	ndet	Director Review	-	-	-	-
	C_t ≤40	ndet	C_t ≤40	C_t ≤40	Director Review[e]	-	-	-	-
	ndet	C_t ≤40	C_t ≤40	C_t ≤40	Director Review	-	-	-	-
	ndet	C_t ≤40	ndet	C_t ≤40	Director Review	-	-	-	-
	ndet	ndet	C_t ≤40	C_t ≤40	Director Review[f]	-	-	-	-
	ndet	ndet	C_t ≤40	ndet	Director Review	-	-	-	-
	ndet	ndet	ndet	C_t ≤40	Director Review	-	-	-	-

C_t, cycle threshold; ndet, not detected; RFU, relative fluorescence units, QC, quality control. A hyphen (-) indicates this cell is not applicable for this row (e.g., wild-type TWIST control and mutant ssDNA pool control results are for quality control and are not reported to the medical record).
[a] Channel thresholds: 500 RFU (FAM), 1000 RFU (HEX), 50 RFU (Cy3.5), 200 RFU (Cy5).

[b] Interpretative comments reported to medical record:

1. "These results do not rule out the presence of mutations other than L452R, E484K, and N501Y, or the possibility that L452R, N501Y, and/or E484K mutations are present below the assay lower limit of detection. These results should not be used as the sole basis for patient management decisions."

2. “The presence of the E484K mutation without the N501Y mutation has been reported most commonly in SARS-CoV-2 lineages P.2, B.1.525, and B.1.526. It can also be seen sporadically, and in other lineages, and therefore cannot be used to definitively assign a variant/lineage/clade identity. Detection of E484K does not preclude the possibility of a mixed infection containing both mutant and wildtype SARS-CoV-2. These results do not rule out the presence of mutations other than L452R, E484K, and N501Y, or the possibility that N501Y and/or L452R are present at levels below the assay limit of detection. These results should not be used as the sole basis for patient management decisions.”

3. “The presence of the L452R mutation has been reported most commonly in SARS-CoV-2 lineages B.1.429 and B.1.427. It may be seen sporadically, and in other lineages, and therefore cannot be used to definitively assign a variant/lineage/clade identity. Detection of L452R does not preclude the possibility of a mixed infection containing both mutant and wildtype SARS-CoV-2. These results do not rule out the presence of mutations other than L452R, E484K, and N501Y, or the possibility that N501Y and/or E484K are present at levels below the assay limit of detection. These results should not be used as the sole basis for patient management decisions.”

4. “The presence of the E484K mutation and the N501Y mutation has been reported most commonly in SARS-CoV-2 lineages B.1.351 and P.1. They might also be seen sporadically and therefore cannot be used to definitively assign a variant/lineage/clade identity. Detection of these mutations does not preclude the possibility of a mixed infection containing both mutant and wildtype SARS-CoV-2. These results do not rule out the presence of mutations other than L452R, E484K, and N501Y, or the possibility that L452R is present at levels below the assay limit of detection. These results should not be used as the sole basis for patient management decisions.”

5. “The presence of N501Y mutation without the E484K mutation has been reported in a variety of SARS-CoV-2 lineages, most notably B.1.1.7. Detection of these mutations does not preclude the possibility of a mixed infection containing both mutant and wildtype SARS-CoV-2. These results do not rule out the presence of mutations other than L452R, E484K, and N501Y, or the possibility that E484K and/or L452 are present at levels below the assay limit of detection. These results should not be used as the sole basis for patient management decisions.”

[c] Possibly due to extraction failure, setup failure, or viral load below limit of detection. Consider repeating PCR with 11µL eluate in same reaction volume (no water). If same result after repeat, suggest reporting as “unable to genotype”.
[d] Conflicting results of N501 and N501Y detected. Possibilities include mixed infection, contamination, and/or nonspecific amplification, among others.
[e] Mutations uncommonly observed together. Possibilities include new/rare variant, mixed infection, contamination, or nonspecific amplification, among others.
[f] Failure of N501 locus to amplify. Possibilities include low viral load leading to other probes out-competing N501/N501Y, alternate mutation in N501 site, and nonspecific amplification, among others.

148

149 **Supplemental Table 5.** Analytical Performance: Multiplex Genotyping RT-qPCR Lower Limit of Detection

150

Template and Concentration	Number of Detected Replicates			
Mutant ssDNA Control	**N501 Wild-Type (Cy3.5)**	**N501Y Mutant (FAM)**	**E484K Mutant (Cy5)**	**L452R Mutant (HEX)**
1.0 copies / µL template	0/20	8/20	7/20	17/20
5.0 copies / µL template	0/20	9/20	11/20	20/20
10.0 copies / µL template	0/20	14/20	16/20	20/20
100.0 copies / µL template	0/20	20/20	20/20	20/20
95% LLOD (95% CI) - copies / µL template	(appropriate ndet[a])	23.6 (14.0-29.4)	16.0 (11.1-40.3)	1.5 (1.0-3.1)
95% LLOD (95% CI) - copies / µL specimen[b]	(appropriate ndet[a])	117.8 (70.0-147.2)	79.9 (55.7-201.4)	7.5 (5.0-15.6)

LLOD, lower limit of detection; CI, confidence interval; ndet, not detected.
[a] Appropriate absence of Cy3.5 amplification (N501 wild-type) in all replicates.
[b] Extrapolated from nucleic acid extraction protocol: 300 µL respiratory swab specimen extracted into 60 µL elution buffer.

151

152 **Supplemental Table 6.** Analytical Performance: Multiplex Genotyping RT-qPCR Linearity in Replicates of Three Across Seven Orders of Magnitude

153 for the L452R (HEX), E484K (Cy5), and N501Y (FAM) Allele-Specific Hydrolysis Probes.

154

Control Template	Concentration (copies/ µL template)	Replicate C_t Values				Coefficient of Variation (%)		
		N501 Wild-Type (Cy3.5)[a]	**N501Y Mutant (FAM)**	**E484K Mutant (Cy5)**	**L452R Mutant (HEX)**	**N501Y Mutant (FAM)[b]**	**E484K Mutant (Cy5)[b]**	**L452R Mutant (HEX)[b]**
Mutant ssDNA	10^0	ndet x 3	42.61, 43.10, 43.70	42.95, 42.62, 42.56	43.35, 43.34, 43.34	98.99%	10.58%	32.26%
	10^1	ndet x 3	39.43, 40.40, 41.50	39.62, 40.24, 41.03	39.67, 40.02, 40.32	31.92%	9.58%	23.06%
	10^2	ndet x 3	37.31, 36.48, 36.06	37.18, 36.83, 36.50	36.25, 36.43, 36.25	9.00%	1.47%	5.21%
	10^3	ndet x 3	34.29, 34.16, 34.42	34.01, 33.88, 33.93	33.33, 33.20, 33.16	1.38%	0.87%	0.70%
	10^4	ndet x 3	30.09, 30.02, 30.12	30.25, 30.00, 30.27	29.54, 29.53, 29.57	0.38%	0.15%	1.14%
	10^5	ndet x 3	26.94, 26.72, 26.66	26.78, 26.70, 26.71	26.04, 26.05, 26.02	0.87%	0.09%	0.26%
	10^6	ndet x 3	23.14, 23.03, 22.98	23.25, 23.22, 23.17	22.65, 22.61, 22.64	0.40%	0.10%	0.20%
	R^2	-	0.9925	0.9955	0.9995	-	-	-
Wild-Type TWIST	10^4	22.64	ndet	ndet	ndet	-	-	-
	10^6	27.33	ndet	ndet	ndet	-	-	-

C_t, cycle threshold. A hyphen (-) indicates this cell is not applicable for this row (e.g., no applicable coefficient of variation for the L452R, E484K, and N501Y mutations for the TWIST control, as these mutations are neither present nor detected in the wild-type template).

[a] Appropriate absence of Cy3.5 amplification (N501 wild-type) in all replicates.

[b] Based on standard curves: N501Y-FAM: copies/µL template = $10^{(12.888-0.295 \cdot C_t)}$; E484K-Cy5: copies/µL template = $10^{(13.095-0.302 \cdot C_t)}$; L452R-HEX: copies/µL template = $10^{(12.542-0.289 \cdot C_t)}$

155

156 **Supplemental Table 7.** Whole Genome Next-Generation Sequencing Quality Measures for Subset of 229 Sequenced Specimens Genotyped by RT-

157 qPCR

158

Sequencing Quality Measure	**Median (Interquartile Range)**
Number of specimens	229
Diagnostic specimen NAAT C_t value	17.9 (15.6 - 20.9)
Number of aligned reads	664,369 (512,314 - 816,797)
Whole-genome coverage at 10X depth	99.3% (99.0 - 99.4%)
Whole-genome mean coverage	834.7 (689.5 - 911.4)
Spike protein coverage at 10X depth	>99.9% (99.9 - >99.9%)
Spike protein mean coverage	829.2 (615.1 - 954.5)

NAAT, nucleic acid amplification test.

159

160 **Supplemental Table 8.** GISAID Accession Identification Numbers for 229 Sequenced Specimens Genotyped by RT-qPCR

RT-qPCR Genotype Group	GISAID Accession ID
L452, E484, N501	>hCoV-19/USA/CA-Stanford-01_S03/2020|EPI_ISL_1379719|2020-12-10
	>hCoV-19/USA/CA-Stanford-01_S04/2020|EPI_ISL_1424067|2020-12-10
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	>hCoV-19/USA/CA-Stanford-01_S16/2020|EPI_ISL_1379722|2020-12-15
	>hCoV-19/USA/CA-Stanford-01_S20/2020|EPI_ISL_1379723|2020-12-17
	>hCoV-19/USA/CA-Stanford-01_S21/2020|EPI_ISL_1379724|2020-12-19
	>hCoV-19/USA/CA-Stanford-01_S25/2020|EPI_ISL_1424068|2020-12-06
	>hCoV-19/USA/CA-Stanford-01_S26/2020|EPI_ISL_1424069|2020-12-06
	>hCoV-19/USA/CA-Stanford-01_S27/2020|EPI_ISL_2009071|2020-12-09
	>hCoV-19/USA/CA-Stanford-01_S38/2020|EPI_ISL_1424070|2020-12-07
	>hCoV-19/USA/CA-Stanford-01_S39/2020|EPI_ISL_1424071|2020-12-07
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	>hCoV-19/USA/CA-Stanford-02_S15/2021|EPI_ISL_1379737|2021-01-12
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	>hCoV-19/USA/CA-Stanford-02_S25/2021|EPI_ISL_1379742|2021-01-12
	>hCoV-19/USA/CA-Stanford-02_S27/2021|EPI_ISL_1379743|2021-01-12
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	>hCoV-19/USA/CA-Stanford-02_S30/2021|EPI_ISL_1379746|2021-01-12
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	>hCoV-19/USA/CA-Stanford-07_S37/2021|EPI_ISL_1379863|2021-01-30
	>hCoV-19/USA/CA-Stanford-07_S41/2021|EPI_ISL_1379865|2021-01-30
	>hCoV-19/USA/CA-Stanford-08_S35/2021|EPI_ISL_1424105|2021-01-31
	>hCoV-19/USA/CA-Stanford-08_S44/2021|EPI_ISL_1379882|2021-01-28
	>hCoV-19/USA/CA-Stanford-13_S41/2021|EPI_ISL_2027147|2021-01-19
	>hCoV-19/USA/CA-Stanford-18_S44/2021|EPI_ISL_2027225|2021-01-21
	>hCoV-19/USA/CA-Stanford-18_S45/2021|EPI_ISL_2027228|2021-01-21
	>hCoV-19/USA/CA-Stanford-18_S46/2021|EPI_ISL_2027140|2021-01-24
L452, E484K, N501	>hCoV-19/USA/CA-Stanford-02_S40/2021|EPI_ISL_1364498|2021-01-13
	>hCoV-19/USA/CA-Stanford-04_S01/2021|EPI_ISL_1364506|2021-01-23
	>hCoV-19/USA/CA-Stanford-06_S07/2021|EPI_ISL_1364507|2021-01-29
	>hCoV-19/USA/CA-Stanford-07_S13/2020|EPI_ISL_1364508|2020-12-31
	>hCoV-19/USA/CA-Stanford-07_S17/2021|EPI_ISL_1364509|2021-01-19
	>hCoV-19/USA/CA-Stanford-07_S18/2021|EPI_ISL_1364510|2021-01-19
	>hCoV-19/USA/CA-Stanford-07_S21/2021|EPI_ISL_1364513|2021-02-01
	>hCoV-19/USA/CA-Stanford-07_S22/2021|EPI_ISL_1364514|2021-02-01
	>hCoV-19/USA/CA-Stanford-07_S23/2021|EPI_ISL_1364515|2021-01-20
	>hCoV-19/USA/CA-Stanford-09_S01/2021|EPI_ISL_1364516|2021-01-27
	>hCoV-19/USA/CA-Stanford-09_S07/2021|EPI_ISL_1364520|2021-01-22
	>hCoV-19/USA/CA-Stanford-09_S10/2021|EPI_ISL_1364523|2021-02-11
	>hCoV-19/USA/CA-Stanford-11_S06/2021|EPI_ISL_2027150|2021-02-09
	>hCoV-19/USA/CA-Stanford-12_S41/2021|EPI_ISL_2027167|2021-02-28
L452, E484, N501Y	>hCoV-19/USA/CA-Stanford-03_S44/2021|EPI_ISL_1364502|2021-01-15
	>hCoV-19/USA/CA-Stanford-03_S46/2021|EPI_ISL_1364504|2021-01-15
	>hCoV-19/USA/CA-Stanford-03_S47/2021|EPI_ISL_1364505|2021-01-18
	>hCoV-19/USA/CA-Stanford-04_S22/2021|EPI_ISL_2009049|2021-01-19
	>hCoV-19/USA/CA-Stanford-07_S20/2021|EPI_ISL_1364512|2021-01-19
	>hCoV-19/USA/CA-Stanford-09_S02/2021|EPI_ISL_1364517|2021-02-05
	>hCoV-19/USA/CA-Stanford-09_S03/2021|EPI_ISL_1364518|2021-01-26
	>hCoV-19/USA/CA-Stanford-09_S08/2021|EPI_ISL_1364521|2021-02-09
	>hCoV-19/USA/CA-Stanford-09_S09/2021|EPI_ISL_1364522|2021-02-11
	>hCoV-19/USA/CA-Stanford-10_S09/2021|EPI_ISL_1424116|2021-02-24
	>hCoV-19/USA/CA-Stanford-10_S10/2021|EPI_ISL_1424117|2021-02-24

	>hCoV-19/USA/CA-Stanford-10_S11/2021|EPI_ISL_1424118|2021-02-19 >hCoV-19/USA/CA-Stanford-10_S12/2021|EPI_ISL_1424119|2021-02-21 >hCoV-19/USA/CA-Stanford-10_S13/2021|EPI_ISL_1424120|2021-02-22 >hCoV-19/USA/CA-Stanford-10_S14/2021|EPI_ISL_1424121|2021-02-18 >hCoV-19/USA/CA-Stanford-10_S15/2021|EPI_ISL_1424122|2021-02-19 >hCoV-19/USA/CA-Stanford-10_S16/2021|EPI_ISL_1424123|2021-02-19 >hCoV-19/USA/CA-Stanford-10_S19/2021|EPI_ISL_1424124|2021-02-12 >hCoV-19/USA/CA-Stanford-10_S21/2021|EPI_ISL_1424125|2021-02-26 >hCoV-19/USA/CA-Stanford-12_S37/2021|EPI_ISL_2027133|2021-02-28 >hCoV-19/USA/CA-Stanford-12_S38/2021|EPI_ISL_2027138|2021-03-03 >hCoV-19/USA/CA-Stanford-12_S46/2021|EPI_ISL_2027136|2021-03-07
L452, E484K, N501Y	>hCoV-19/USA/CA-05_S19_JAN/2021|EPI_ISL_1335872|2021-01-06 >hCoV-19/USA/CA-07_S15_JAN/2021|EPI_ISL_1335871|2021-01-19 >hCoV-19/USA/CA-Stanford-07_S10/2021|EPI_ISL_1424066|2021-01-27

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