



Article

Supplementary Materials: Novel Nested-Seq Approach for SARS-CoV-2 Real-time Epidemiology and In-depth Mutational Profiling in Wastewater

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Table S1. Primers and qPCR probes for the *in house* nested PCR/real-time PCR assays

| Assay | Oligos | Sequence (5' – 3') | Product length |
|--|-----------------------|---------------------------------|----------------|
| N assay | | | |
| External set (1 st reaction) | Forward primer | TGGACTTCCCTATGGTGCTAACA | 310 bp |
| | Reverse primer | CAGCAAAGCAAGAGCAGCATC | |
| Internal set (2 nd reaction) | Forward primer | GCTGCAATCGTGCTACAACTTC | 125 bp |
| | Reverse primer | TTTCTTGAAGTGTGCGACTACG | |
| | qPCR probe (FAM-BHQ1) | AACATTGCCAAAAGGCTTCTACGCAGAA | |
| Helicase assay | | | |
| External set (1 st reaction) | Forward primer | CAGATGAGTTTTCTAGCAATGTTGC | 355 bp |
| | Reverse primer | TATATCTGCTGTCGTCTCAGGCA | |
| Internal set (2 nd reaction) | Forward primer | AGCTCTCTACTACCCTTCTGCTCG | 83 bp |
| | Reverse primer | TTAATGCCTTCTCACATAGTGCATC | |
| | qPCR probe (FAM-BHQ1) | TATACAGCTTGCTCTCATGCCGCTGTTGA | |
| NSP3 assay | | | |
| External set (1 st reaction) | Forward primer | AGGGCTGGTGAAGCTGCTAAC | 328 bp |
| | Reverse primer | CAGGTGGTGCTGACATCATAACA | |
| Internal set (2 nd reaction) | Forward primer | TGAGTTACTTGTTC AACATGCCA | 91 bp |
| | Reverse primer | AGGGTTGTCTGCTGTTGTCCAC | |
| | qPCR probe (FAM-BHQ1) | TTCTTGCAAAAGAGTCTTGAACGTGGTGTGT | |
| ORF3a assay | | | |
| External set (1 st reaction) | Forward primer | TGAACATGACTACCAGATTGGTGG | 192 bp |
| | Reverse primer | ACATGTTCTTCAGGCTCATCAACA | |
| Internal set (2 nd reaction) | Forward primer | GGGAATCTGGAGTAAAAGACTGTGT | 103 bp |
| | Reverse primer | TGTTCAACACCAGTGTCTGTACTCA | |
| | qPCR probe (FAM-MGB) | TCACTTCAGACTATTACCAGCTG | |

Table S2. Primers for the targeted DNA-seq analysis

| Assay | Oligos | Sequence (5′ – 3′) | Product length |
|--|----------------|----------------------------|----------------|
| D614G (23403A>G) assay - S gene | | | |
| External set (1 st reaction) | Forward primer | CCACAGACACTTGAGATTCTTGACA | 268 bp |
| | Reverse primer | CACCAATGGGTATGTCACACTCA | |
| Internal set (2 nd reaction) | Forward primer | TGTTCTTTTGGTGGTGTGTCAGTGTT | 151 bp |
| | Reverse primer | AACCTGTAGAATAAACACGCCAAGT | |
| Q57H (25563G>T) assay - ORF3a gene | | | |
| External set (1 st reaction) | Forward primer | CAAGGTGAAATCAAGGATGCTACTC | 245 bp |
| | Reverse primer | GCAACGAGCAAAAGGTGTGAGT | |
| Internal set (2 nd reaction) | Forward primer | CACTCCCTTTTCGGATGGCTTA | 122 bp |
| | Reverse primer | AACAAAGTGAACACCCTTGGAGA | |
| P323L (14408C>T) assay – ORF1ab/RdRP gene | | | |
| External set (1 st reaction) | Forward primer | TGTTAACTGTTTGGATGACAGATGC | 158 bp |
| | Reverse primer | GCTCTCTGAAGTGGTATCCAGTTGA | |
| Internal set (2 nd reaction) | Forward primer | TGCATTCTGCATTGTGCAAACCT | 117 bp |
| | Reverse primer | AGTTGAAACTACAAATGGAACACCA | |
| R203K (28881G>A) & G204R (28883G>C) assay – N gene | | | |
| External set (1 st reaction) | Forward primer | CAACATTGCCAAAAGGCTTCTAC | 221 bp |
| | Reverse primer | GCCTTTACCAGACATTTTGCTCTC | |
| Internal set (2 nd reaction) | Forward primer | CAGTCAAGCCTCTTCTCGTTCC | 160 bp |
| | Reverse primer | GCTCTCAAGCTGGTTCAATCTGT | |

Table S3. Primers for the mutational analysis of pike (S) gene of SARS-CoV-2

| Assay | Oligos | Sequence (5' – 3') | Alignment on NC_045512.2 | Product length |
|---|----------------|-------------------------------|--------------------------|----------------|
| Assay 1 | | | | |
| External set (1 st reaction) | Forward primer | GCTGTTATGTCTTTAAAAGAAGGTCAA | 21430-21456 | 481 bp |
| | Reverse primer | GGACTGGGTCTTCGAATCTAAAGTA | 21886-21910 | |
| Internal set (2 nd reaction) | Forward primer | CAGAGTTGTTATTTCTAGTGATGTTCTTG | 21516-21544 | 362 bp |
| | Reverse primer | AATCCAGCCTCTTATTATGTTAGACTTC | 21850-21877 | |
| Assay 2 | | | | |
| External set (1 st reaction) | Forward primer | GGACCAATGGTACTAAGAGGTTTGA | 21777-21801 | 425 bp |
| | Reverse primer | CACTAAATTAATAGGCGTGTGCTTAGA | 22175-22201 | |
| Internal set (2 nd reaction) | Forward primer | CTGTCCTACCATTTAATGATGGTGTT | 21807-21832 | 352 bp |
| | Reverse primer | CCATCAATATTCTTAAACACAAATTCC | 22132-22158 | |
| Assay 3 | | | | |
| External set (1 st reaction) | Forward primer | GCGAATAATTGCACTTTTGAATATG | 22049-22073 | 428 bp |
| | Reverse primer | GATTTC AACGTACACTTTGTTTCTGAG | 22450-22476 | |
| Internal set (2 nd reaction) | Forward primer | TCTTATGGACCTTGAAGGAAAACAG | 22087-22111 | 351 bp |
| | Reverse primer | GCACAGTCTACAGCATCTGTAATGGT | 22412-22437 | |
| Assay 4 | | | | |
| External set (1 st reaction) | Forward primer | TCAGGTTGGACAGCTGGTGC | 22328-22347 | 427 bp |
| | Reverse primer | CTGCATAGACATTAGTAAAGCAGAGATC | 22727-22754 | |
| Internal set (2 nd reaction) | Forward primer | GGGTTATCTTCAACCTAGGACTTTTC | 22363-22388 | 351 bp |
| | Reverse primer | GGAGACACTCCATAACACTTAAAAGTG | 22687-22713 | |
| Assay 5 | | | | |
| External set (1 st reaction) | Forward primer | AGATTTGCATCTGTTTATGCTTGG | 22598-22621 | 434 bp |
| | Reverse primer | AAGTAACAATTAACCTTCAACACCA | 23005-23031 | |
| Internal set (2 nd reaction) | Forward primer | AACTGTGTTGCTGATTATTCTGTCCT | 22640-22665 | 347 bp |
| | Reverse primer | GCCTGATAGATTTCAGTTGAAATATCTC | 22959-22986 | |
| Assay 6 | | | | |
| External set (1 st reaction) | Forward primer | CAATCTTGATTCTAAGGTTGGTGGT | 22879-22903 | 397 bp |
| | Reverse primer | TGTCAAGAATCTCAAGTGTCTGTGG | 23297-23321 | |

| | | | | |
|--|----------------|------------------------------|-------------|--------|
| Internal set (2 nd reaction) | Forward primer | TTGTTTAGGAAGTCTAATCTCAAACCTT | 22925-22952 | 355 bp |
| | Reverse primer | TAGTGTGAGCAATGTCTCTGCCA | 23257-23279 | |

Assay 7

| | | | | |
|--|----------------|----------------------------|-------------|--------|
| External set (1 st reaction) | Forward primer | AATTTCAACTTCAATGGTTTAACAGG | 23180-23205 | 429 bp |
| | Reverse primer | CCGAGGAGAATTAGTCTGAGTCTGA | 23584-23608 | |
| Internal set (2 nd reaction) | Forward primer | ACTGAGTCTAACAAAAAGTTTCTGCC | 23219-23244 | 347 bp |
| | Reverse primer | GCACCAATGGGTATGTCACACTC | 23543-23565 | |

Assay 8

| | | | | |
|--|----------------|----------------------------|-------------|--------|
| External set (1 st reaction) | Forward primer | TGGCGTGTATTATTCTACAGGTTCTA | 23459-23483 | 438 bp |
| | Reverse primer | GGTGTTTTTGTCTTGTTCAACAGC | 23873-23896 | |
| Internal set (2 nd reaction) | Forward primer | AATAGGGGCTGAACATGTCAACA | 23512-23534 | 345 bp |
| | Reverse primer | CGGTTTAATTGTGTACAAAACTGC | 23832-23856 | |

Assay 9

| | | | | |
|--|----------------|-----------------------------|-------------|--------|
| External set (1 st reaction) | Forward primer | CCAGTGTCTATGACCAAGACATCAGTA | 23744-23770 | 445 bp |
| | Reverse primer | CAGAAGTGTATTGAGCAATCATTTC | 24163-24188 | |
| Internal set (2 nd reaction) | Forward primer | ACTGAATGCAGCAATCTTTTGTTG | 23801-23824 | 356 bp |
| | Reverse primer | AGCAAAGGTGGCAAAACAGTAAG | 24130-24156 | |

Assay 10

| | | | | |
|--|----------------|--------------------------|-------------|--------|
| External set (1 st reaction) | Forward primer | CTGGCTTCATCAAACAATATGGTG | 24054-24077 | 427 bp |
| | Reverse primer | ATTGCACCAAAATTGGAGCTAAG | 24458-24480 | |
| Internal set (2 nd reaction) | Forward primer | TTGCTGCTAGAGACCTCATTGTG | 24093-24116 | 343 bp |
| | Reverse primer | GCTTGTGCATTTTGTTGACC | 24415-24435 | |

Assay 11

| | | | | |
|--|----------------|---------------------------|-------------|--------|
| External set (1 st reaction) | Forward primer | GATTGCCAACCAATTTAATAGTGCT | 24328-24352 | 433 bp |
| | Reverse primer | AGTCACATGCAAGAAGACTACACCA | 24735-24760 | |
| Internal set (2 nd reaction) | Forward primer | AAGACTCACTTTCTTCCACAGCAAG | 24366-24390 | 350 bp |
| | Reverse primer | GGACATAAGATGATAGCCCTTTCC | 24692-24715 | |

Assay 12

| | | | | |
|--|----------------|----------------------------|-------------|--------|
| External set (1 st reaction) | Forward primer | TTAATTAGAGCTGCAGAAATCAGAGC | 24596-24621 | 416 bp |
| | Reverse primer | TCCTTGAATGAGTCTAATTCAGGTTG | 24986-25011 | |

| | | | | |
|--|----------------|----------------------------|-------------|--------|
| Internal set (2 nd reaction) | Forward primer | TGTCAGAGTGTGTACTTGGACAATCA | 24648-24673 | 338 bp |
| | Reverse primer | CAAAGGATCATAAACTGTGTTGTTGA | 24960-24985 | |

Assay 13

| | | | | |
|--|----------------|----------------------------|-------------|--------|
| External set (1 st reaction) | Forward primer | CACAAAGGAATTTTATGAACCACA | 24876-24900 | 447 bp |
| | Reverse primer | AGCAGGATCCACAAGAACAACAG | 25300-25322 | |
| Internal set (2 nd reaction) | Forward primer | ATTTGTGTCTGGTAACTGTGATGTTG | 24922-24947 | 360 bp |
| | Reverse primer | CAACTGGTCATACAGCAAAGCATAA | 25257-25281 | |

Assay 14

| | | | | |
|--|----------------|--------------------------|-------------|--------|
| External set (1 st reaction) | Forward primer | TTGGCTAGGTTTTATAGCTGGCTT | 25210-25233 | 380 bp |
| | Reverse primer | TTGAGGGTTATGATTTTGGAAGC | 25567-25589 | |
| Internal set (2 nd reaction) | Forward primer | GCTTGATTGCCATAGTAATGGTGA | 25230-25253 | 308 bp |
| | Reverse primer | CAACAATAAGCCATCCGAAAGG | 25515-25537 | |

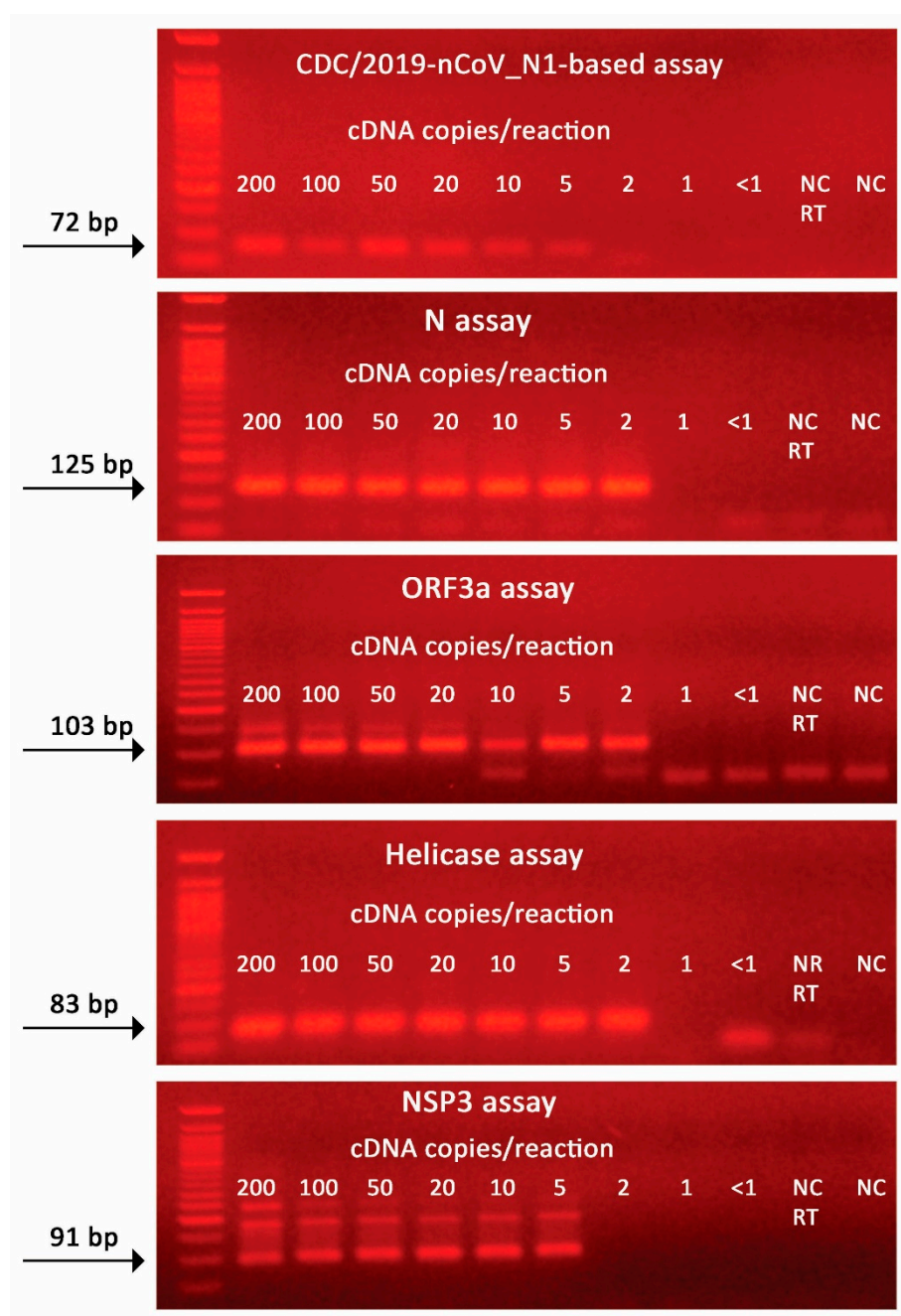


Figure S1. Agarose-gel electrophoresis of the PCR products of the in house CDC/2019-nCoV_N1-based assay and our nested PCR assays, A. N assay, B. ORF3a assay, C. Helicase assay and D. NSP3 assay, against serial dilutions of SARS-CoV-2 complete genome RNA control. M: 50 bp DNA ladder, PC: positive control, NC: PCR negative control, NCRT: reverse transcription negative control.

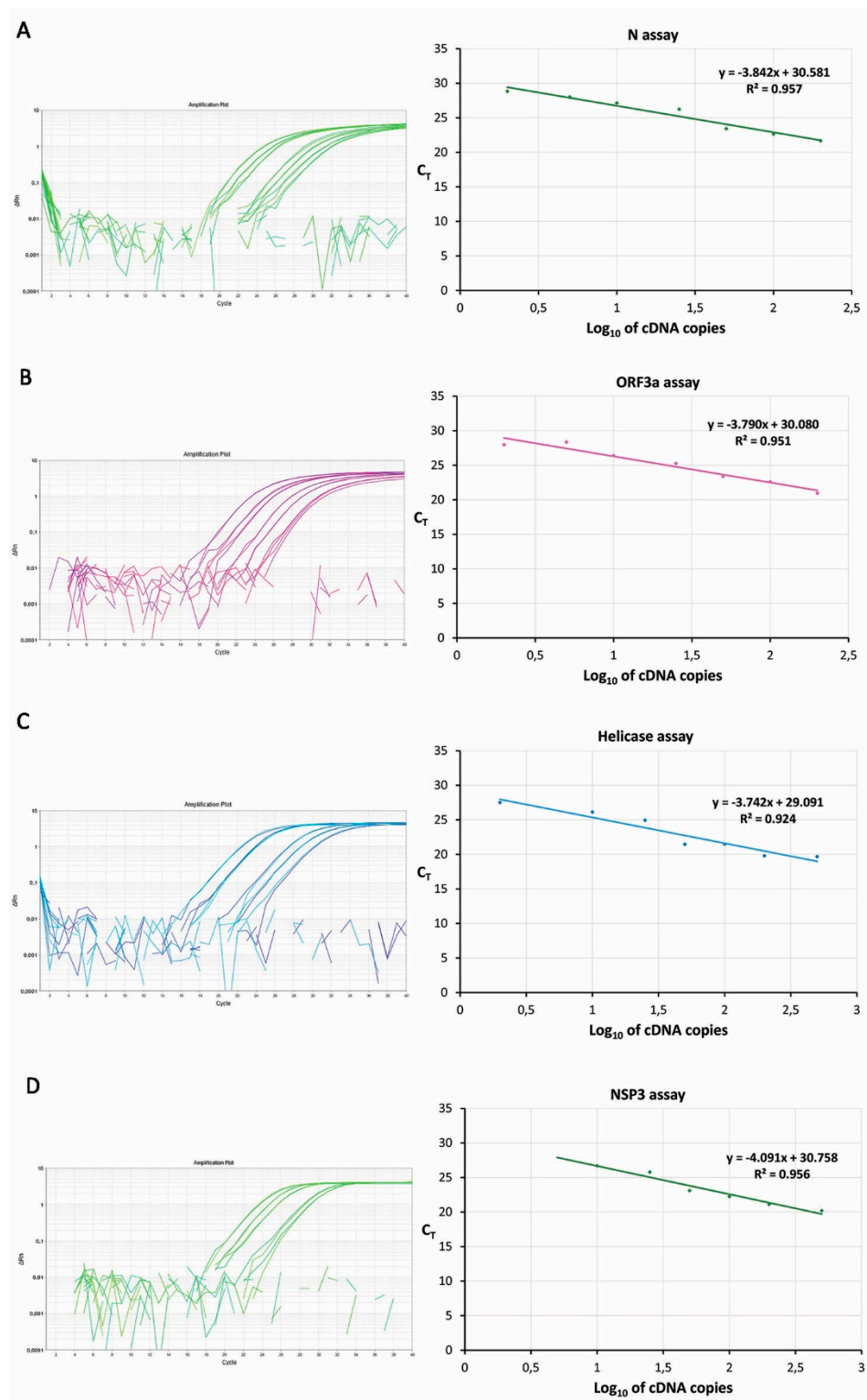


Figure S2. Amplification plots (left) and standard curves (right) of our developed nested real-time PCR assays: A. N assay, B. ORF3a assay, C. Helicase assay, D. NSP3 assay, against serial dilutions of SARS-CoV-2 complete genome RNA control.

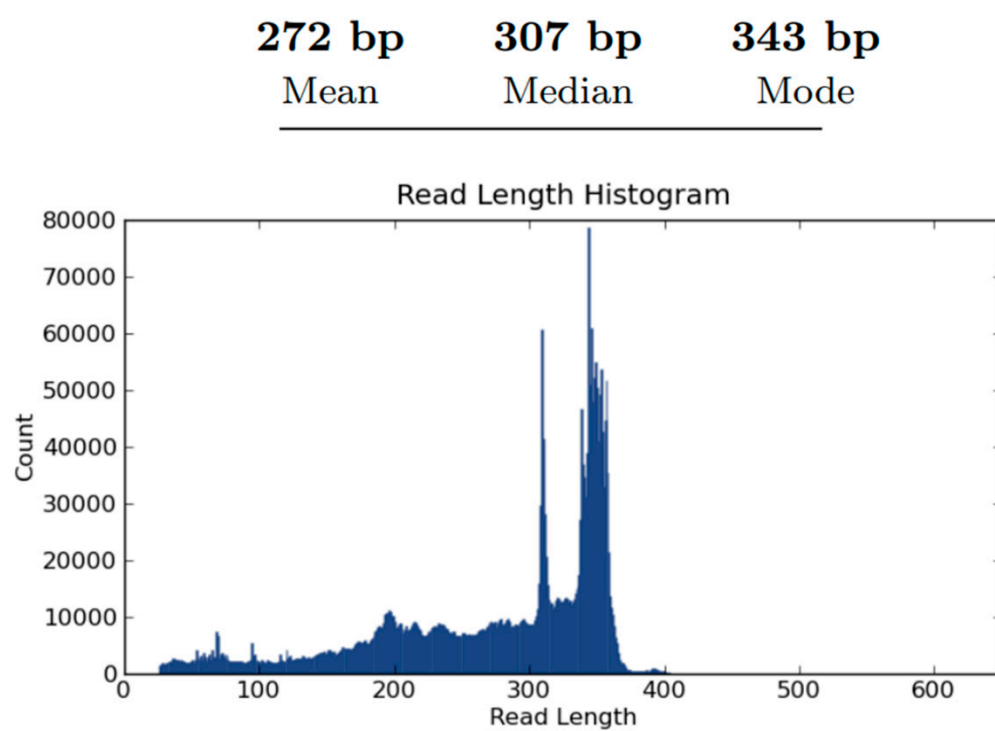


Figure S3. Histogram of sequencing reads length of S gene targeted DNA-seq in March 2021 samples.

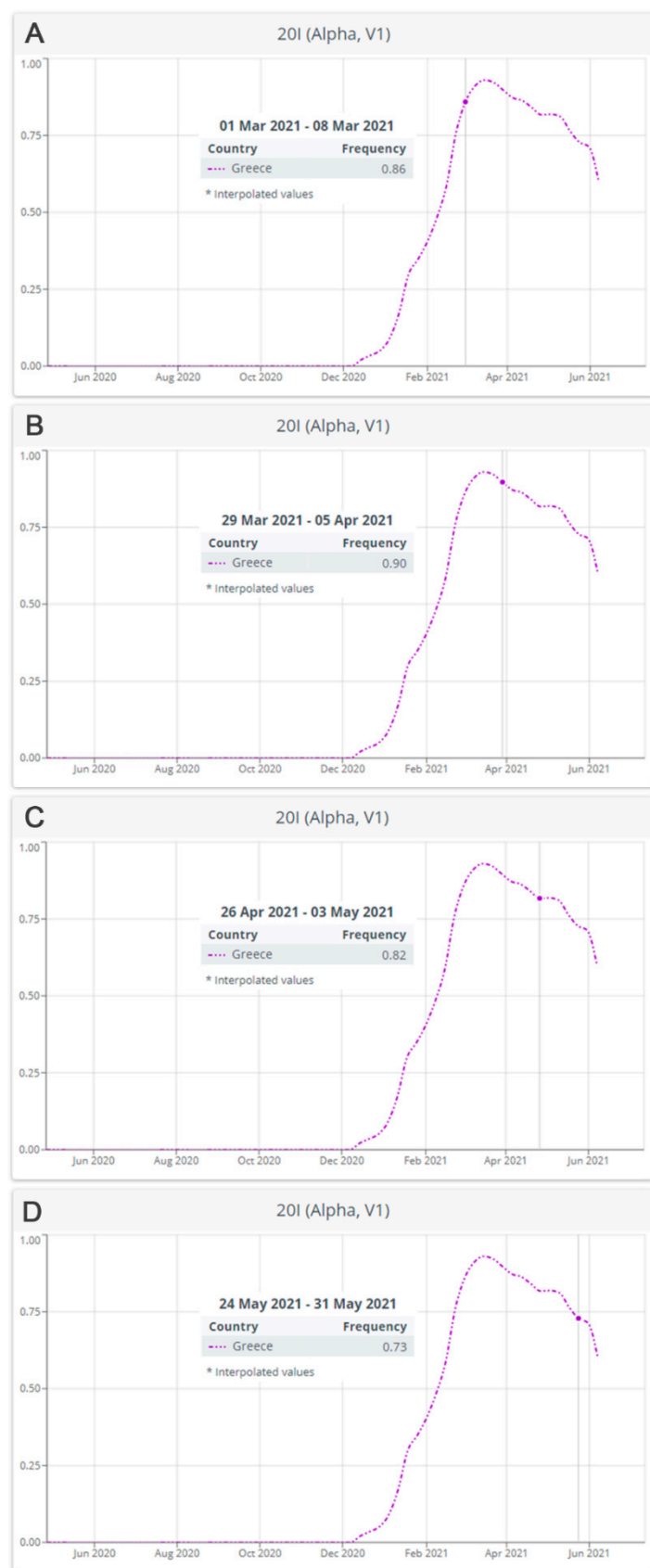


Figure S4. Frequencies of B.1.1.7/alpha (20I/501Y.V1) variant of concern in Greece clinical samples during early March (A), end of March (B), end of April (C) and end of May (D) 2021. Plots and analysis are provided by CoVariant (<https://covariants.org/per-variant>) using the data deposited in GISAID (<https://www.gisaid.org/>).

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