Quantitative SARS-CoV-2 Alpha Variant B.1.1.7 Tracking in Wastewater by Allele-Specific RT-qPCR

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Letter

Quantitative SARS-CoV-2 Alpha Variant B.1.1.7 Tracking in Wastewater by Allele-Specific RT-qPCR

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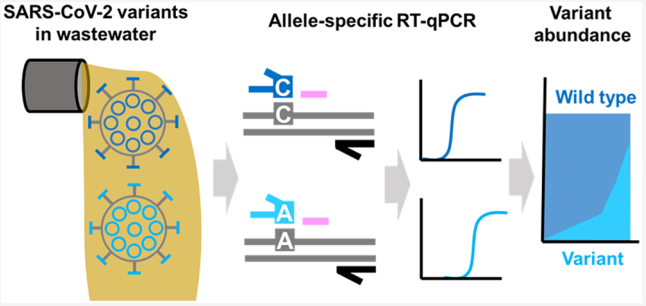
ABSTRACT: The critical need for surveillance of SARS-CoV-2 variants of concern has prompted the development of methods that can track variants in wastewater. Here, we develop and present an open-source method based on allele-specific RT-qPCR (AS RT-qPCR) that detects and quantifies the B.1.1.7 variant, targeting spike protein mutations at three independent genomic loci that are highly predictive of B.1.1.7 (HV69/70del, Y144del, and A570D). Our assays can reliably detect and quantify low levels of B.1.1.7 with low cross-reactivity, and at variant proportions down to 1% in a background of mixed SARS-CoV-2. Applying our method to wastewater samples from the United States, we track the occurrence of B.1.1.7 over time in 19 communities. AS RT-qPCR results align with clinical trends, and summation of B.1.1.7 and wild-type sequences quantified by our assays matches SARS-CoV-2 levels indicated by the U.S. CDC N1 and N2 assays. This work paves the way for AS RT-qPCR as a method for rapid inexpensive surveillance of SARS-CoV-2 variants in wastewater.



INTRODUCTION

Wastewater-based surveillance (WBS) has been shown to provide real-time, unbiased disease surveillance at the community level.[1−5] WBS programs have been established in the United States[6] and recommended for surveillance of SARS-CoV-2 and its variants in the European Union.[7,8] Variants of concern (VOCs), including B.1.1.7, contain spike protein mutations that have been reported to increase transmission[9,10] and confer increased resistance to neutralization by antibodies derived from convalescent patients' plasma and vaccine sera.[11] The potential public health challenges posed by B.1.1.7 warrant extensive real-time surveillance at the community level to inform efforts in controlling the pandemic.[8,12−15]

In light of new emerging VOCs, efforts have been made to utilize WBS in tracking SARS-CoV-2 variants that are circulating in the community.[7,16,17] However, wastewater presents unique methodological challenges, with low SARS-CoV-2 viral titers and mixtures of viral variants.[18,19] The latter requires assays to be variant-specific yet accurately quantitative for meaningful interpretation, and this poses a major hurdle for adaptation of methods developed for variant detection in clinical samples[20−22] for use in wastewater. Next-generation sequencing (NGS) is commonly applied to wastewater for variant monitoring.[17,23,24] However, many report inconsistent and/or low sequencing coverage, likely due to the low input concentrations of SARS-CoV-2 in wastewater.[16,23,25] Therefore, NGS-based approaches for WBS require considerable additional optimization before they can guide public health responses.[26] Several RT-qPCR-based assays for detection of VOCs in wastewater have been developed.[27−29] Challenges in assay development have included validating specificity and sensitivity at viral levels observed in the wastewater environment and demonstrating quantitation on par with widely used assays for wastewater SARS-CoV-2 RNA, such as the U.S. CDC N1 and N2 assays. A number of biomedical companies have also released products for the detection of SARS-CoV-2 VOCs in wastewater, involving RT-qPCR and/or RT-ddPCR; however, their primer and probe details are proprietary.[30−32] As such, there is a critical need for an open-source method for simple and accurate detection and quantitation of SARS-CoV-2 variants for WBS.

Here we develop a method that can detect specific SARS-CoV-2 mutations in wastewater samples and can be implemented using commercially available RT-qPCR proto-

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cols. Our approach is based on allele-specific (AS) PCR, a method developed more than 30 years ago[33,34] and commonly used for molecular genotyping in laboratory diagnostics.[35] AS PCR involves two parallel reactions, each including a wild-type (WT) or mutation-specific forward primer and a common reverse primer (or vice versa). The WT or mutation-specific primer is designed with its 3′ end to be complementary to the mutation, as a mismatch at a 3′ end is refractory to primer extension by *Thermus aquaticus* (Taq) polymerase. In addition, a synthetic mismatch is introduced near the 3′ terminal end of both primers to further destabilize the hybridization of nontarget sequences.[36] This combination enables single-nucleotide discrimination and allows for several orders of magnitude in specificity. Coupled with reverse transcription (RT) and quantitative PCR (qPCR), the method is termed AS RT-qPCR.

Our AS RT-qPCR panel for SARS-CoV-2 wastewater-based variant tracking consists of three pairs of primer−probe sets that target B.1.1.7-specific spike protein mutations HV69/70del, Y144del, and A570D.[10] These three pairs of primer−probe sets enable the discrimination of the SARS-CoV-2 variant B.1.1.7 sequence from the WT sequence. We performed analytical assessments of the primer sets to confirm that they have amplification efficiencies of >85%, possess similar sensitivities to the most commonly used and CDC-recommended N1 and N2 assays,[37] and reliably enable specific detection and accurate quantitation in a background of RNA of the opposite genotype (B.1.1.7 in WT and vice versa). We also applied this panel of assays for quantitative detection of B.1.1.7 in SARS-CoV-2 positive wastewater samples, where summation of B.1.1.7 and wild-type sequences quantified by our assays matches SARS-CoV-2 levels determined by the U.S. CDC N1 and N2 assays. The assay presented here uses the same workflow as existing SARS-CoV-2 RT-qPCR diagnostic assays, thus potentially allowing for its immediate implementation on a global scale. This work demonstrates the utility of AS RT-qPCR for the quantitative detection of variants in wastewater and could be readily applied to track the spread of B.1.1.7 and adapted to track other VOCs through WBS.

MATERIALS AND METHODS

Design of Probes and Allele-Specific Primers. Comparative genomics were performed on sequences of the B.1.1.7 variant and WT SARS-CoV-2, to identify loci that can distinguish B.1.1.7 from other variants (Table S1 and Supplemental Methods). Primers and probes were designed using the PrimerQuest Tool of Integrated DNA Technologies (IDT), with the mutation placed at the 3′ end of the forward or reverse primer. At least one primer and probe set was designed for each allele-specific (AS) primer direction (Figure S1). All primers were designed to have a T_m in the range of 59−65 °C, and all probes to have a T_m in the range of 64−72 °C. The probes were designed to anneal to the same strand as the AS primer, and as close to the 3′ end of the AS primers as possible, while avoiding guanines at the 5′ end of the probe. AS primers targeting one- or two-nucleotide mutations include an artificial mismatch near the 3′ terminal nucleotide to increase the degree of discrimination between WT and mutant sequences. All primers and probes (Table S2) were purchased from IDT.

RNA Standards. Twist Synthetic SARS-CoV-2 RNA Controls, control 2 or 4 (Wuhan-Hu-1 or USA/TX1/2020) and control 14 (England/205041766/2020), were used as RNA standards, representing WT and B.1.1.7, respectively. RNA standards were prepared as single-use aliquots. Controls 2, 4, and 14 were quantified by digital droplet PCR (ddPCR) (n = 3) (Figure S2) to be $(4.11 \pm 0.14) \times 10^5$, $(4.27 \pm 0.2) \times 10^5$, and $(1.14 \pm 0.04) \times 10^6$ (mean ± standard deviation) copies/μL, respectively.

Analysis of AS RT-qPCR Primer and Probe Sets. AS RT-qPCR was performed using the Taqman Virus 1-Step master mix (ThermoFisher catalog no. 4444434) with technical duplicates, in a final volume of 10 μL. A single reverse or forward primer and probe was used with each set of allele-specific forward or reverse primers (Table S3). The final concentrations of the primers were 500 nM, with that of the probe at 200 nM, with 1 μL of template. US CDC 2019-nCoV_N1 and 2019-nCoV_N2 (IDT) (sequences in Table S4) were used following recommendations, at final concentrations of 500 nM for primers and 125 nM for probes. No template controls were included for each assay, and none of them were amplified. The reactions were set up using electronic pipettes (Eppendorf) and performed on a Bio-Rad CFX384 real-time PCR instrument under the following conditions: 5 min at 50 °C and 20 s at 95 °C, followed by 45 cycles of 3 s at 95 °C and 30 s at 60 °C.

Accuracy of Detection of B.1.1.7 in the Background of WT RNA. Wastewater RNA was prepared from 8 h composites of SARS-CoV-2 negative wastewater obtained from the sanitary network of an office building in Singapore. Samples were pasteurized at 60 °C for 1 h and filtered through a 0.2 μm membrane (Millipore Sigma); 50 mL of clarified samples was concentrated with 10 kDa Amicon Ultra Centrifugal Filter units to ∼200 μL. RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, catalog no. 52906). One-step RT-qPCR was performed as described above, using 1 μL of wastewater RNA in a 10 μL reaction volume. Wastewater RNA contained PMMoV at an average of Ct 32−34, determined using primer−probe sequences listed in Table S5. The concentration of WT RNA used as a background for determining the accuracy of detection of varying B.1.1.7 copy numbers was 3.8×10^2 copies per reaction.

Analysis of Wastewater Samples Using an AS RT-qPCR Panel. The 24 h composite samples of raw sewage were obtained from the wastewater treatment plants and selected residential buildings across the United States as part of a regular wastewater surveillance service provided by Biobot Analytics, Inc. Samples were pasteurized at 60 °C for 1 h and vacuum filtered through a 0.2 μm membrane (Millipore Sigma). Fifteen milliliters of clarified samples was concentrated with 10 kDa Amicon Ultra Centrifugal Filter units (Sigma, catalog no. UFC9010) to ∼200 μL. Samples were subjected to RNA extraction (Qiagen RNeasy kit, catalog no. 74182). One-step RT-qPCR was performed as described above, using 1 μL of RNA in a 10 μL reaction volume. Ct values were converted to RNA copies based on standard curves established with Twist Synthetic SARS-CoV-2 RNA Controls. Standard curves shown in Figures S3 and S4 were applied across Ct values derived from samples, with technical variations across runs monitored and normalized with the use of interplate calibrators.

Data Analysis. Data were analyzed using Microsoft Excel and Graphpad prism. Graphs were created using Graphpad Prism. Results from wastewater samples were analyzed and

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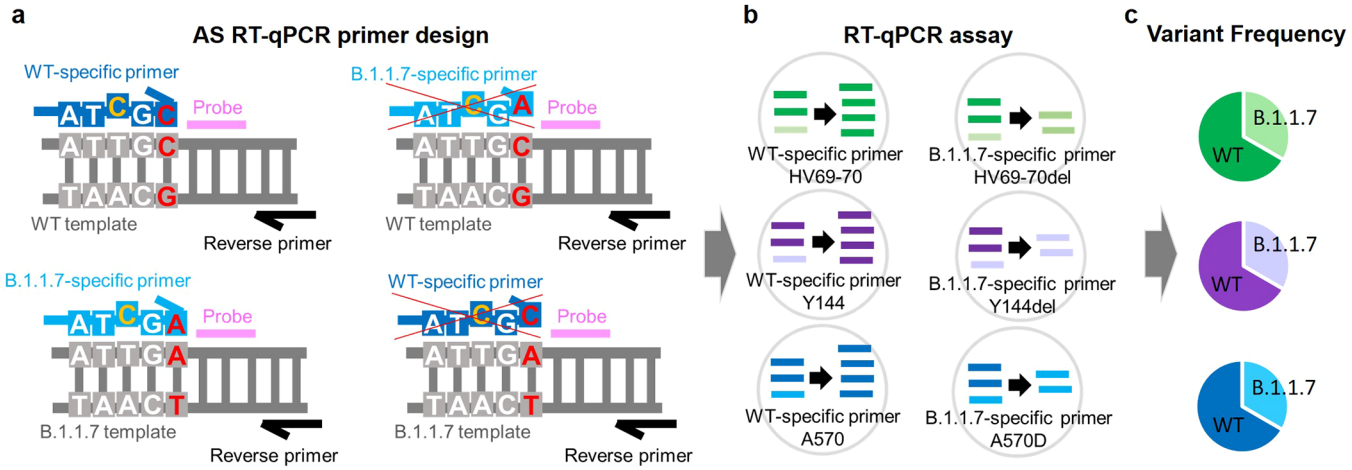

Figure 1. Schematic of our panel of AS RT-qPCR assays. (a) Each assay requires a common primer (black) and probe (pink), paired with a WT or mutation-specific primer (light and dark blue) in the reverse direction to the common primer. This allele-specific primer is designed to target the mutation of interest (red) at the 3′ end of the mutation-specific primer and contains a synthetic mismatch (orange) near the 3′ end to enhance assay specificity. The presented sequences and primer design represent the assay for locus A570D. (b) Two individual RT-qPCRs are needed to assay for variant frequencies at each locus. (c) Variant frequencies can be calculated after converting threshold cycles to genome copies using their respective standard curves.

visualized using Python version 3.9, with the code available on github at https://github.com/biobotanalytics/as-qpcr-lee.

Data Availability. The MIQE table is provided with this paper. Source data will be made available upon request.

RESULTS AND DISCUSSION

We developed an AS RT-qPCR panel for tracking SARS-CoV-2 variant B.1.1.7 in wastewater. This panel consists of three pairs of primer−probe sets that target individual SARS-CoV-2 mutations on B.1.1.7 (Figure 1 and Table S1). These three pairs of primers were derived from screening an initial set of 16 primer sets developed against eight target loci on B.1.1.7 (Figure S1 and Table S2) and selected on the basis of sensitivity and specificity assays (Figures S5−S10).

We demonstrate the specificity of the three AS RT-qPCR assays for their respective WT and mutant genome targets in SARS-CoV-2 RNA (HV69−70del, Y144del, and A570D) by screening them against full length synthetic RNA constructs of the B.1.1.7 and WT genotypes (Figure 2). Their amplification efficiencies were between 85% and 106% for the correct RNA (i.e., B.1.1.7 assay for B.1.1.7 RNA and WT assay for WT RNA). We determined the levels of cross-reactivity for RNA of the opposite genotype by testing each combination of WT and mutant primers. Cross-reactivity did not occur for WT HV69−70, and for WT Y144 primers, cross-reactivity was observed at only 10^5 copies of B.1.1.7 RNA (Figure 2a,c). WT A570 primers were less specific but still discriminated against the B.1.1.7 variant sequence (Figure 2e). Cross-reactivity for all three B.1.1.7-specific primers was minimal and observed for only $\geq 10^4$ copies of WT RNA per microliter (Figure 2b,d,f).

Using cycle threshold (Ct) values for the same input RNA as a proxy for the assay's sensitivity, we compared the Ct values of all three pairs of primer−probe sets to those of the U.S. CDC N1 and N2 primer−probe sets.[37] The three AS RT-qPCR primer−probe sets have comparable sensitivities to N1 and N2 assays for the same amount of input RNA across a 100-fold difference in target RNA concentration (Figure S11). The limits of detection for these RT-qPCR assays were not calculated due to the large number of primer sets involved. However, the Ct value of the intercepts of the three AS RT-qPCR primer−probe sets (Figure S4) was comparable to that of U.S. CDC N1 and N2 primer−probe sets (Figure S3), confirming similar assay sensitivities.

Wastewater samples likely contain a mixture of SARS-CoV-2 variant genotypes with variable abundance. To determine the quantitative accuracy of our panel with B.1.1.7 and WT RNA present in a mixture at highly skewed ratios, we examined the cycle thresholds (Ct) of primer−probe sets against their respective full length RNA in the presence or absence of $\leq 10^4$ copies of RNA of the opposite genotype (Figure S12). This constitutes a variant frequency of 0.1%, 1%, or 10%. WT assays were largely unaffected by the B.1.1.7 background, except for WT A570, which yielded higher concentrations (lower Ct values) in the B.1.1.7 background with 10 copies of WT input due to cross-reactivity with B.1.1.7, which results in its slight overestimation. The B.1.1.7 assays were unaffected by large quantities of WT (background) RNA. Overall, these results suggest that the assays were reliably quantitative in a background of RNA of the opposite genotype. When repeated in a wastewater RNA background at SARS-CoV-2 levels similar to those found in wastewater in this study and others,[5,38,39] our B.1.1.7-specific primers show a negligible rate of false positives and negatives, of one in 30 replicates (Figure S13), demonstrating the accuracy of detection of B.1.1.7.

We next validated the performance of our assay on wastewater samples. We tested samples collected from 16 urban and rural wastewater treatment plants and three buildings across 11 U.S. states. Samples were collected during three distinct time periods: fall 2020 (October 20 to November 5, 2020), when no B.1.1.7 was reported in clinical samples in the United States; January 2021 (January 20−29, 2021), when B.1.1.7 was first emerging in the United States; and between February 24 and March 8, 2021, when B.1.1.7 was circulating in most of the United States. All of the samples included in this experiment tested positive for SARS-CoV-2 with RT-qPCR using the U.S. CDC N1 and N2 assays. The WT sequence was detected in 55 of 58 samples (for at least two target loci), indicating that our WT AS RT-qPCR assays have sensitivity comparable to that of the routinely used N1/N2 RT-qPCR SARS-CoV-2 protocol (Figure 3a).

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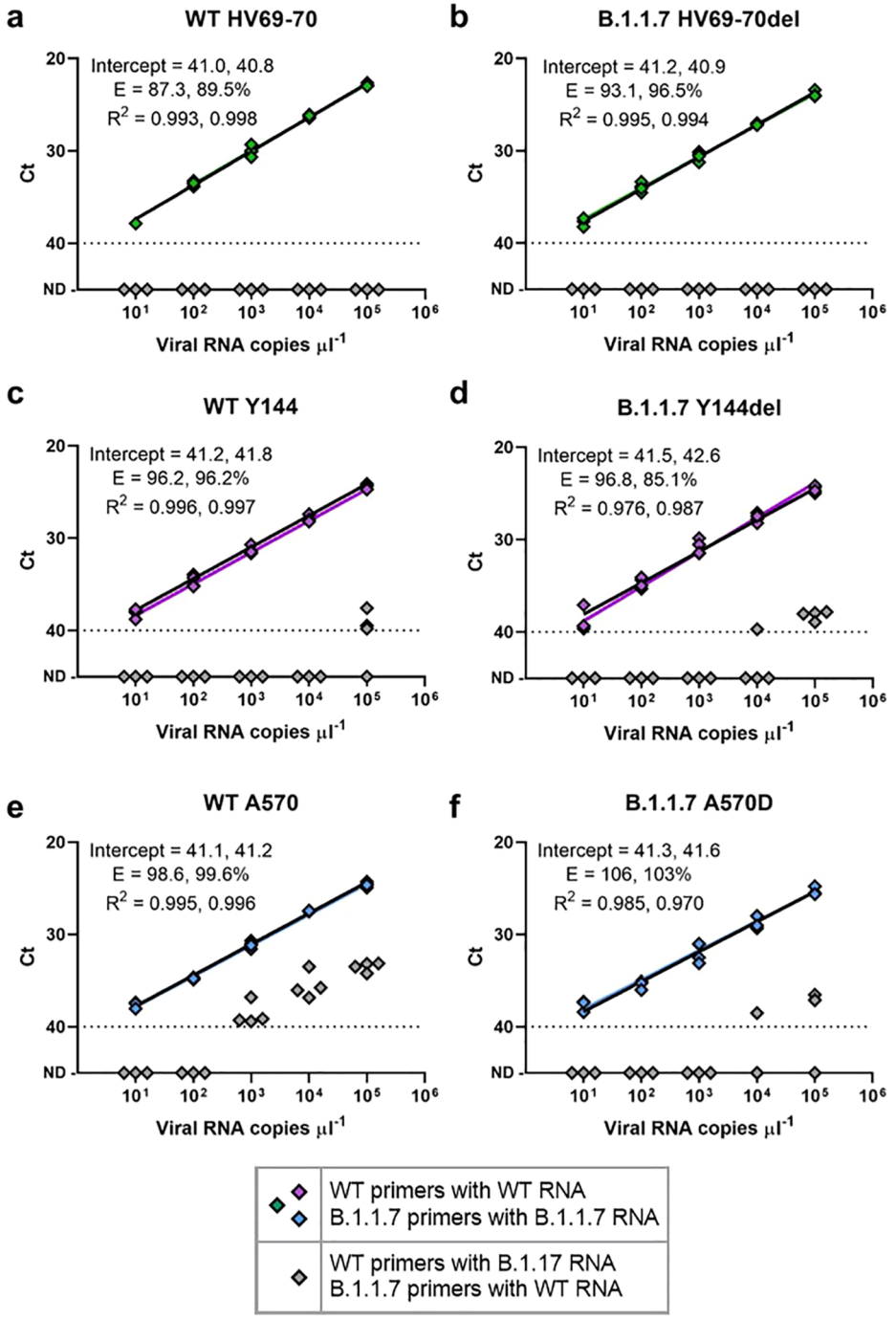

Figure 2. Specificity and cross-reactivity of AS RT-qPCR primers against WT and B.1.1.7 RNA in nuclease-free water. PCR efficiency and *y*-intercept cycle threshold (Ct) values were calculated for each of six primer−probe sets against 10-fold dilutions of synthetic full length SARS-CoV-2 RNA. The presented data reflect two sets of independent measurements taken on different days. Green, purple, and blue diamonds represent tests against the matching genotype (WT-specific primers to WT RNA and B.1.1.7-specific primers to B.1.1.7 RNA), and gray diamonds denote tests against RNA of the opposite genotype.

B.1.1.7 targets were not detected in the October 2020 samples, reflecting the absence of clinically confirmed B.1.1.7 in the United States at that time. In January 2021, four samples were positive for at least two target B.1.1.7 loci. Three of these B.1.1.7 positive samples were from Florida, which agrees with the clinical trend that Florida was the U.S. state with the highest fraction of B.1.1.7 cases in January 2021 (Figure 3b). For comparison, 19.1% of GISAID sequences in Florida in January 2021 were of the B.1.1.7 lineage, compared to 2.8% nationwide.

The fraction of the B.1.1.7 variant increased >3-fold at all tested locations between January and February/March 2021 (Figure 3c). The average fraction of B.1.1.7 for all non-building locations increased from 2.6% to 19.2%. These results are consistent with the average fraction of B.1.1.7 cases in the United States according to GISAID for these time periods (4.2% and 13.3%, respectively). Interestingly, one building-level sample was found to contain exclusively B.1.1.7 in February/March 2021. This is consistent with our expectation, as for an individual building we could expect only one strain to

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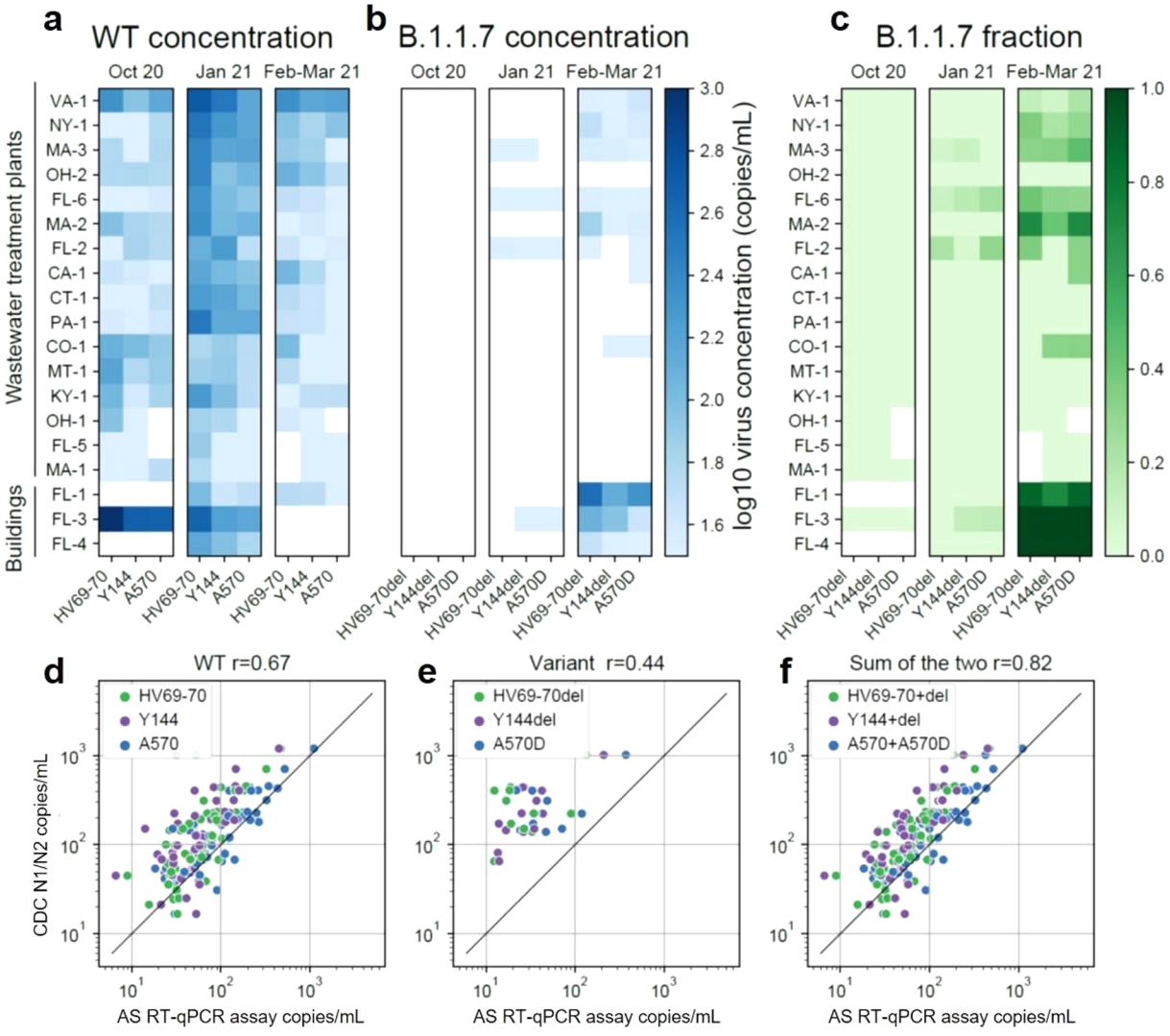

Figure 3. Application of the AS RT-qPCR panel to 16 wastewater treatment plant samples and three building wastewaters collected from across the United States. Detection and quantitation of WT and B.1.1.7 RNA in wastewater samples collected in the United States from October 2020 to March 2021. Locations are ordered from maximum (top) to minimum (bottom) N1/N2 concentration for the most recent (February/March 2021) samples. Plot (blue) showing the wastewater concentrations for (a) WT and (b) B.1.1.7 loci. (c) Plot (green) showing the relative fraction of B.1.1.7 to the total SARS-CoV-2 viral titer derived from the individual pairs of AS RT-qPCR primers. Data shown in white denote a readout below the limit of detection or data not available. (d–f) Comparison of SARS-CoV-2 RNA concentrations from CDC N1 and N2 assays and concentrations measured by the AS RT-qPCR assay. Each point represents one measurement. Each sample has three measurements, one per target locus. (d) Comparison of N1/N2 with the respective WT loci. (e) Comparison with the respective B.1.1.7 loci. (f) Comparison of N1/N2 concentrations with the sum of WT and B.1.1.7 (sum of the total copies, divided by the input volume).

be circulating in a small number of people. In all, our assays have demonstrated the potential of AS RT-qPCR to produce interpretable and sensible results when applied to quantitation and detection of B.1.1.7 and WT SARS-CoV-2 in wastewater samples.

Finally, we compared the concentrations of B.1.1.7 and WT SARS-CoV-2 measured in wastewater by our assay with the SARS-CoV-2 concentrations measured using U.S. CDC N1 and N2 assays. For each site, the combined concentration of WT and mutant sequences at the AS RT-qPCR loci correlated well with concentrations predicted by the N1 and N2 assays (R = 0.82) and fell along a 1:1 line (Figure 3d–f and Figure S14). This result verifies that our quantification of WT and B.1.1.7 virus in wastewater is consistent with those of the U.S. CDC N1 and N2 assays.

Wastewater surveillance is instrumental for tracking the spread and abundance of SARS-CoV-2 and its variants at the population level.[6,7] Next-generation sequencing that involves either sequencing of the entire SARS-CoV-2 genome or targeted sequencing of specific loci has been reported;[16,17,25] however, these methods lack the sensitivity necessary to detect low variant abundances in dilute and mixed wastewater samples,[40] while being time-consuming and expensive. A qPCR-based approach,[27−29] on the contrary, is better suited for targeted detection at low viral concentrations, is widely available at relatively low cost in laboratories conducting clinical diagnostics, and provides quantitative data for the determination of variant abundances. However, qPCR-based methods, as with our AS RT-qPCR, have limitations. Our method detects B.1.1.7 by targeting three mutations specific to B.1.1.7. However, wastewater represents a pooled sample in

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which detections of individual mutations are unlinked. In addition, our approach cannot discover new variants. However, there can be synergy between clinical genomic surveillance and WBS, where new VOCs identified locally via clinical surveillance can be followed up with rapid development of AS RT-qPCR assays to target VOC-specific mutations, and finally adopted broadly into WBS campaigns to quickly start monitoring their spread.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.1c00375.

Checklist MIQE (PDF)

Frequencies of targeted mutations in SARS-CoV-2 B.1.1.7 (Table S1), AS RT-qPCR primer design (Figure S1), AS RT-qPCR primer sequences (Table S2), quantitation of Twist Synthetic SARS-CoV-2 RNA Controls by ddPCR (Figure S2), AS RT-qPCR panel for B.1.1.7 and WT (Table S3), U.S. CDC N gene assay primers and probe sequences (Table S4), primers and probe sequences used to quantify PMMOV levels (Table S5), standard curves for N1 and N2 primer−probe sets (Figure S3), standard curves for B.1.1.7 and WT in analysis of U.S. wastewater samples (Figure S4), AS RT-qPCR primer screening, initial screen of AS RT-qPCR primer sets against WT RNA (Figure S5), difference in cycle threshold between WT primers and B.1.1.7 primers against WT RNA (Figure S6), screen of primer sets against synthetic DNA containing WT or B.1.1.7 sequences (Figure S7), difference in cycle threshold between specific and cross amplification for WT and B.1.1.7 primers (Figure S8), Ct values for the primer sets against DNA containing WT or B.1.1.7 target sequences (Figure S9), effect of an engineered mismatch on amplification of nontarget sequences (Figure S10), Ct values for the AS RT-qPCR primers in comparison to those of U.S. CDC N1 and N2 assays (Figure S11), detection and quantification of SARS-CoV-2 RNA in the presence of 10^4 copies of RNA of the opposite genotype (Figure S12), occurrence of false positive or nondetections of B.1.1.7 (Figure S13), comparison between N1 and N2 concentrations measured by the AS RT-qPCR assay (Figure S14), Supplemental Methods, and sequences of DNA standards containing WT or B.1.1.7 sequences (Table S6) (PDF)

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Author Contributions

E.J.A. and J.T. conceptualized the project. W.L.L. and K.A.M. designed the experiments. W.L.L., M.I., K.A.M., C.D., M.L., F.W., A.X., and K.M. analyzed the data. E.J.A., J.T., N.G., and M.M. supervised the project. W.L.L., K.A.M., X.G., F.A., F.C., H.C., S.M., R.F.-O., M.M.P., S.T.W., K.L.J.B., and C.Y.J.L. performed experiments. All authors contributed to the writing of the manuscript. All authors read and approved the manuscript.

Notes

The authors declare the following competing financial interest(s): M.M. and N.G. are co-founders of Biobot Analytics, Inc. E.J.A. is advisor to Biobot Analytics, Inc. K.A.M., M.I., C.D., S.M., R.F.-O., M.M.P., and S.T.W. are employees at Biobot Analytics, Inc., and all of these authors hold shares in the company.

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■ REFERENCES

(1) Hart, O. E.; Halden, R. U. Computational Analysis of SARS-CoV-2/COVID-19 Surveillance by Wastewater-Based Epidemiology Locally and Globally: Feasibility, Economy, Opportunities and Challenges. *Sci. Total Environ.* **2020**, *730*, 138875.

(2) Polo, D.; Quintela-Baluja, M.; Corbishley, A.; Jones, D. L.; Singer, A. C.; Graham, D. W.; Romalde, J. L. Making Waves: Wastewater-Based Epidemiology for COVID-19 − Approaches and Challenges for Surveillance and Prediction. *Water Res.* **2020**, *186*, 116404.

(3) Thompson, J. R.; Nancharaiah, Y. V.; Gu, X.; Lee, W. L.; Rajal, V. B.; Haines, M. B.; Girones, R.; Ng, L. C.; Alm, E. J.; Wuertz, S. Making Waves: Wastewater Surveillance of SARS-CoV-2 for Population-Based Health Management. *Water Res.* **2020**, *184*, 116181.

(4) O'Reilly, K. M.; Allen, D. J.; Fine, P.; Asghar, H. The Challenges of Informative Wastewater Sampling for SARS-CoV-2 Must Be Met: Lessons from Polio Eradication. *Lancet Microbe* **2020**, *1* (5), e189−e190.

(5) Wu, F.; Xiao, A.; Zhang, J.; Moniz, K.; Endo, N.; Armas, F.; Bonneau, R.; Brown, M. A.; Bushman, M.; Chai, P. R.; Duvallet, C.; Erickson, T. B.; Foppe, K.; Ghaeli, N.; Gu, X.; Hanage, W. P.; Huang, K. H.; Lee, W. L.; Matus, M.; McElroy, K. A.; Nagler, J.; et al. SARS-CoV-2 Titers in Wastewater Foreshadow Dynamics and Clinical Presentation of New COVID-19 Cases. *medRxiv* **2021**, DOI: 10.1101/2020.06.15.20117747.

(6) National Wastewater Surveillance System (NWSS). https://www.cdc.gov/coronavirus/2019-ncov/cases-updates/wastewater-surveillance.html (last accessed 2021-06-29).

(7) Commission recommendation on a common approach to establish a systematic surveillance of SARS-CoV-2 and its variants in wastewaters in the EU. https://ec.europa.eu/environment/pdf/water/recommendation_covid19_monitoring_wastewaters.pdf (last accessed 2021-06-29).

(8) Priesemann, V.; Balling, R.; Brinkmann, M. M.; Ciesek, S.; Czypionka, T.; Eckerle, I.; Giordano, G.; Hanson, C.; Hel, Z.; Hotulainen, P.; Klimek, P.; Nassehi, A.; Peichl, A.; Perc, M.; Petelos, E.; Prainsack, B.; Szczurek, E. An Action Plan for Pan-European Defence against New SARS-CoV-2 Variants. *Lancet* **2021**, *397* (10273), 469−470.

(9) Davies, N. G.; Abbott, S.; Barnard, R. C.; Jarvis, C. I.; Kucharski, A. J.; Munday, J. D.; Pearson, C. A. B.; Russell, T. W.; Tully, D. C.; Washburne, A. D.; Wenseleers, T.; Gimma, A.; Waites, W.; Wong, K. L. M.; van Zandvoort, K.; Silverman, J. D.; Diaz-Ordaz, K.; Keogh, R.; Eggo, R. M.; Funk, S.; et al. Estimated Transmissibility and Impact of SARS-CoV-2 Lineage B.1.1.7 in England. *Science (Washington, DC, U. S.)* **2021**, *372*, eabg3055.

(10) Rambaut, A.; Loman, N.; Pybus, O.; Barclay, W.; Jeff, B.; Carabelli, A.; Connor, T.; Peacock, T.; Robertson, D. L.; Volz, E. Preliminary genomic characterisation of an emergent SARS-CoV-2 lineage in the UK defined by a novel set of spike mutations. https://virological.org/t/preliminary-genomic-characterisation-of-an-emergent-sars-cov-2-lineage-in-the-uk-defined-by-a-novel-set-of-spike-mutations/563 (accessed 2021-06-10).

(11) Wang, P.; Nair, M. S.; Liu, L.; Iketani, S.; Luo, Y.; Guo, Y.; Wang, M.; Yu, J.; Zhang, B.; Kwong, P. D.; Graham, B. S.; Mascola, J. R.; Chang, J. Y.; Yin, M. T.; Sobieszczyk, M.; Kyratsous, C. A.; Shapiro, L.; Sheng, Z.; Huang, Y.; Ho, D. D. Antibody Resistance of SARS-CoV-2 Variants B.1.351 and B.1.1.7. *Nature* **2021**, *593*, 130.

(12) CDC. Genomic Surveillance for SARS-CoV-2 Variants. https://www.cdc.gov/coronavirus/2019-ncov/cases-updates/variant-surveillance.html (last accessed 2021-06-29).

(13) Walensky, R. P.; Walke, H. T.; Fauci, A. S. SARS-CoV-2 Variants of Concern in the United States—Challenges and Opportunities. *JAMA* **2021**, *325* (11), 1037−1038.
(14) The Lancet Microbe. Spot the Variant-the Pursuit of Greater Vigilance. *Lancet Microbe* **2021**, *2* (3), e88.
(15) Mascola, J. R.; Graham, B. S.; Fauci, A. S. SARS-CoV-2 Viral Variants—Tackling a Moving Target. *JAMA* **2021**, *325*, 1261.
(16) Jahn, K.; Dreifuss, D.; Topolsky, I.; Kull, A.; Ganesanandamoorthy, P.; Fernandez-Cassi, X.; Bänziger, C.; Stachler, E.; Fuhrmann, L.; Jablonski, K. P.; Chen, C.; Aquino, C.; Stadler, T.; Ort, C.; Kohn, T.; Julian, T. R.; Beerenwinkel, N. Detection of SARS-CoV-2 Variants in Switzerland by Genomic Analysis of Wastewater Samples. *medRxiv* **2021**, DOI: 10.1101/2021.01.08.21249379.
(17) Crits-Christoph, A.; Kantor, R. S.; Olm, M. R.; Whitney, O. N.; Al-Shayeb, B.; Lou, Y. C.; Flamholz, A.; Kennedy, L. C.; Greenwald, H.; Hinkle, A.; Hetzel, J.; Spitzer, S.; Koble, J.; Tan, A.; Hyde, F.; Schroth, G.; Kuersten, S.; Banfield, J. F.; Nelson, K. L. Genome Sequencing of Sewage Detects Regionally Prevalent SARS-CoV-2 Variants. *medRxiv* **2020**, DOI: 10.1101/2020.09.13.20193805.
(18) Foladori, P.; Cutrupi, F.; Segata, N.; Manara, S.; Pinto, F.; Malpei, F.; Bruni, L.; La Rosa, G. SARS-CoV-2 from Faeces to Wastewater Treatment: What Do We Know? A Review. *Sci. Total Environ.* **2020**, *743*, 140444.
(19) Kitajima, M.; Ahmed, W.; Bibby, K.; Carducci, A.; Gerba, C. P.; Hamilton, K. A.; Haramoto, E.; Rose, J. B. SARS-CoV-2 in Wastewater: State of the Knowledge and Research Needs. *Sci. Total Environ.* **2020**, *739*, 139076.
(20) Clark, A. E.; Wang, Z.; Cantarel, B.; Kanchwala, M.; Xing, C.; Chen, L.; Irwin, P.; Xu, Y.; Oliver, D.; Lee, F.; Gagan, J. R.; Filkins, L.; Muthukumar, A.; Park, J. Y.; Sarode, R.; SoRelle, J. A. Multiplex Fragment Analysis Identifies SARS-CoV-2 Variants. *medRxiv* **2021**, DOI: 10.1101/2021.04.15.21253747.
(21) Wang, H.; Miller, J. A.; Verghese, M.; Sibai, M.; Solis, D.; Mfuh, K. O.; Jiang, B.; Iwai, N.; Mar, M.; Huang, C.; Yamamoto, F.; Sahoo, M. K.; Zehnder, J.; Pinsky, B. A. Multiplex SARS-CoV-2 Genotyping PCR for Population-Level Variant Screening and Epidemiologic Surveillance. *medRxiv* **2021**, DOI: 10.1101/2021.04.20.21255480.
(22) Vogels, C. B. F.; Breban, M. I.; Alpert, T.; Petrone, M. E.; Watkins, A. E.; Ott, I. M.; de Jesus, J. G.; Claro, I. M.; Magalhães Ferreira, G.; Crispim, M. A. E.; Singh, L.; Tegally, H.; Anyaneji, U. J.; Hodcroft, E. B.; Mason, C. E.; Khullar, G.; Metti, J.; Dudley, J. T.; MacKay, M. J.; Nash, M. PCR Assay to Enhance Global Surveillance for SARS-CoV-2 Variants of Concern. *medRxiv* **2021**, DOI: 10.1101/2021.01.28.21250486.
(23) Prado, T.; Fumian, T. M.; Mannarino, C. F.; Resende, P. C.; Motta, F. C.; Eppinghaus, A. L. F.; Chagas do Vale, V. H.; Braz, R. M. S.; de Andrade, J. da S. R.; Maranhão, A. G.; Miagostovich, M. P. Wastewater-Based Epidemiology as a Useful Tool to Track SARS-CoV-2 and Support Public Health Policies at Municipal Level in Brazil. *Water Res.* **2021**, *191*, 116810.
(24) Izquierdo-Lara, R.; Elsinga, G.; Heijnen, L.; Munnink, B. B. O.; Schapendonk, C. M.E.; Nieuwenhuijse, D.; Kon, M.; Lu, L.; Aarestrup, F. M.; Lycett, S.; Medema, G.; Koopmans, M. P.G.; de Graaf, M. Monitoring SARS-CoV-2 Circulation and Diversity through Community Wastewater Sequencing, the Netherlands and Belgium. *Emerging Infect. Dis.* **2021**, *27* (5), 1405−1415.
(25) Fontenele, R. S.; Kraberger, S.; Hadfield, J.; Driver, E. M.; Bowes, D.; Holland, L. A.; Faleye, T. O. C.; Adhikari, S.; Kumar, R.; Inchausti, R.; Holmes, W. K.; Deitrick, S.; Brown, P.; Duty, D.; Smith, T.; Bhatnagar, A.; Yeager, R. A.; Holm, R. H.; von Reitzenstein, N. H.; Wheeler, E. High-Throughput Sequencing of SARS-CoV-2 in Wastewater Provides Insights into Circulating Variants. *medRxiv* **2021**, DOI: 10.1101/2021.01.22.21250320.
(26) Gwinn, M.; MacCannell, D.; Armstrong, G. L. Next-Generation Sequencing of Infectious Pathogens. *JAMA* **2019**, *321* (9), 893−894.
(27) Graber, T. E.; Mercier, É.; D'Aoust, P. M.; Hoang, H.-D.; Tian, X.; Tasneem, S.; Bhatnagar, K.; Delatolla, R. An Allele-Specific Primer Extension Assay to Quantify the Proportion of B.1.1.7-Specific SARS-CoV-2 RNA in Wastewater. *medRxiv* **2021**, DOI: 10.1101/2021.02.22.21252041.
(28) Wurtzer, S.; Waldman, P.; Levert, M.; Mouchel, J. M.; Gorgé, O.; Boni, M.; Maday, Y.; Marechal, V.; Moulin, L. Monitoring the Propagation of SARS CoV2 Variants by Tracking Identified Mutation in Wastewater Using Specific RT-QPCR. *medRxiv* **2021**, DOI: 10.1101/2021.03.10.21253291.
(29) Yaniv, K.; Ozer, E.; Plotkin, N.; Bhandarkar, N. S.; Kushmaro, A. RT-QPCR Assay for Detection of British (B.1.1.7) and South Africa (B.1.351) Variants of SARS-CoV-2. *medRxiv* **2021**, DOI: 10.1101/2021.02.25.21252454.
(30) GTMolecular. All-in-one Multiplex PCR Test Kits. https://www.gtmolecular.com/pcrkits (last accessed 2021-06-29).
(31) Bio-Rad. ddPCR and RT-PCR Assays for SARS-CoV-2 Variant Surveillance. https://www.bio-rad.com/featured/en/sars-cov-2-variants-pcr-assays.html (last accessed 2021-06-29).
(32) Seegene. Allplex SARS-CoV-2 Variants Assay. https://www.seegene.com/advantages/complete_solution_for_the_covid_19_response (last accessed 2021-06-29).
(33) Petruska, J.; Goodman, M. F.; Boosalis, M. S.; Sowers, L. C.; Cheong, C.; Tinoco, I. J. Comparison between DNA Melting Thermodynamics and DNA Polymerase Fidelity. *Proc. Natl. Acad. Sci. U. S. A.* **1988**, *85* (17), 6252−6256.
(34) Wu, D. Y.; Ugozzoli, L.; Pal, B. K.; Wallace, R. B. Allele-Specific Enzymatic Amplification of Beta-Globin Genomic DNA for Diagnosis of Sickle Cell Anemia. *Proc. Natl. Acad. Sci. U. S. A.* **1989**, *86* (8), 2757−2760.
(35) Coleman, W. B.; Tsongalis, G. J. *Molecular Diagnostics: For the Clinical Laboratorian*; Humana Press: Totowa, NJ, 2007.
(36) Newton, C. R.; Graham, A.; Heptinstall, L. E.; Powell, S. J.; Summers, C.; Kalsheker, N.; Smith, J. C.; Markham, A. F. Analysis of Any Point Mutation in DNA. The Amplification Refractory Mutation System (ARMS). *Nucleic Acids Res.* **1989**, *17* (7), 2503−2516.
(37) CDC. CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel. https://www.fda.gov/media/134922/download (last accessed 2021-06-29).
(38) Wu, F.; Xiao, A.; Zhang, J.; Moniz, K.; Endo, N.; Armas, F.; Bushman, M.; Chai, P. R.; Duvallet, C.; Erickson, T. B.; Foppe, K.; Ghaeli, N.; Gu, X.; Hanage, W. P.; Huang, K. H.; Lee, W. L.; Matus, M.; McElroy, K. A.; Rhode, S. F.; Wuertz, S. Wastewater Surveillance of SARS-CoV-2 across 40 U.S. States. *medRxiv* **2021**, DOI: 10.1101/2021.03.10.21253235.
(39) Wu, F.; Zhang, J.; Xiao, A.; Gu, X.; Lee, W. L.; Armas, F.; Kauffman, K.; Hanage, W.; Matus, M.; Ghaeli, N.; Endo, N.; Duvallet, C.; Poyet, M.; Moniz, K.; Washburne, A. D.; Erickson, T. B.; Chai, P. R.; Thompson, J.; Alm, E. J. SARS-CoV-2 Titers in Wastewater Are Higher than Expected from Clinically Confirmed Cases. *mSystems* **2020**, *5* (4), e00614−20.
(40) Fuqua, J. L.; Rouchka, E. C.; Waigel, S.; Sokoloski, K.; Chung, D.; Zacharias, W.; Zhang, M.; Chariker, J.; Talley, D.; Santisteban, I.; Varsani, A.; Moyer, S.; Holm, R. H.; Yeager, R. A.; Smith, T.; Bhatnagar, A. A Rapid Assessment of Wastewater for Genomic Surveillance of SARS-CoV-2 Variants at Sewershed Scale in Louisville, KY. *medRxiv* **2021**, DOI: 10.1101/2021.03.18.21253604.

https://doi.org/10.1021/acs.estlett.1c00375
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