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REVEALR-Based Genotyping of SARS-CoV-2 Variants of Concern in Clinical Samples

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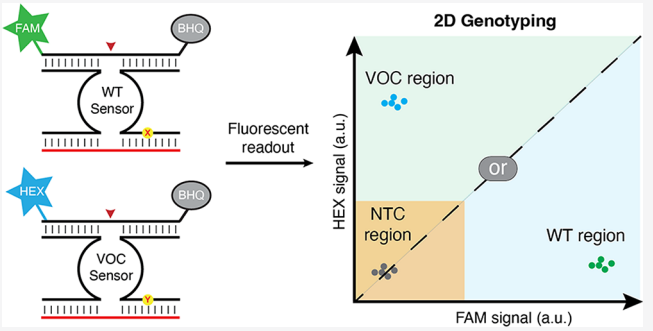
ABSTRACT: The SARS-CoV-2 virus has evolved into new strains that increase viral transmissibility and reduce vaccine protection. The rapid circulation of these more harmful strains across the globe has created a pressing need for alternative public health screening tools. REVEALR (RNA-encoded viral nucleic acid analytic reporter), a rapid and highly sensitive DNAzyme-based detection system, functions with perfect accuracy against patient-derived clinical samples. Here, we design REVEALR into a novel genotyping assay that detects single-base mismatches corresponding to each of the major SARS-CoV-2 strains found in the United States. Of 34 sequence-verified patient samples collected in early, mid, and late 2021 at the UCI Medical Center in Orange, California, REVEALR identified the correct variant [Wuhan-Hu-1, alpha (B.1.1.7), gamma (P.1), epsilon (B.1.427/9), delta (B.1.617.2), and omicron (B.1.1.529)] with 100% accuracy. The assay, which is programmable and amenable to multiplexing, offers an important new approach to personalized diagnostics.



INTRODUCTION

The coronavirus induced disease 19 (COVID-19) pandemic, caused by spread of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) across the globe, is responsible for nearly 6 million deaths worldwide and far-reaching socioeconomic disruptions.[1] Although the mutation rate of SARS-CoV-2 is relatively low due to the presence of an exonuclease enzyme that reduces the replication error rate by ~15−20-fold,[2] evolution of the virus has led to the emergence of novel viral lineages, including variants of concern (VOC) that threaten pandemic recovery by increasing viral transmissibility and reducing vaccine protection.[3] Following rapid fixation of the D614G substitution in early 2020,[4] new strains emerged that include the B.1.1.7 (alpha), B.1.351 (beta), P.1 (gamma), B.1.427/9 (epsilon), B.1.617.2 (delta), and B.1.1.529 (omicron) variants.[5] These new variants harbor mutations in the spike (S) glycoprotein that stabilize the receptor binding domain (RBD) in the up conformation, which supports increased binding of angiotensin-converting enzyme 2 (ACE2) on the host cell.[6−8] An increased risk of infection or reinfection,[9] coupled with reduced protection afforded by vaccines or neutralizing antibodies,[10] has created a pressing need for additional diagnostic tools that can facilitate COVID-19 detection in conjunction with strain identification.[11]

The COVID-19 pandemic has exposed critical gaps in point-of-care (POC) diagnostics that are needed to facilitate safe environments for social and economic activities.[12] Currently, whole genome sequencing is widely applied as a broad public health screening tool for monitoring epidemiological changes in the population, and more importantly, the early detection and prevalence of emerging SARS-CoV-2 variants (Figure 1a).[13] However, the diagnosis of individual patients for a specific VOC is best performed by sequencing the S-gene of viral samples collected from nasopharyngeal swabs, which is a slow and costly process that does not scale to the population. Other approaches, such as S-gene target failure,[14] TaqMan,[15] and LAMP,[16] suffer from limitations in their reliability, sensitivity, and specificity, making them suboptimal techniques for VOC genotyping.[17] Even CRISPR-based systems, though effective, have a narrow range of target sites due to constraints imposed by the PAM region.[18] This method also requires an extra base pair mismatch in the CRISPR RNA sequence that can lead to genotyping errors.[19] As such, new POC diagnostics are needed to rapidly detect nucleic acid sequences with high sensitivity and single-base specificity.[11]

Here, we report the design and clinical validation of a two-step REVEALR-based (RNA-encoded viral nucleic acid analytic reporter) nucleic acid detection system for SARS-CoV-2 genotyping. The REVEALR strategy is based on a

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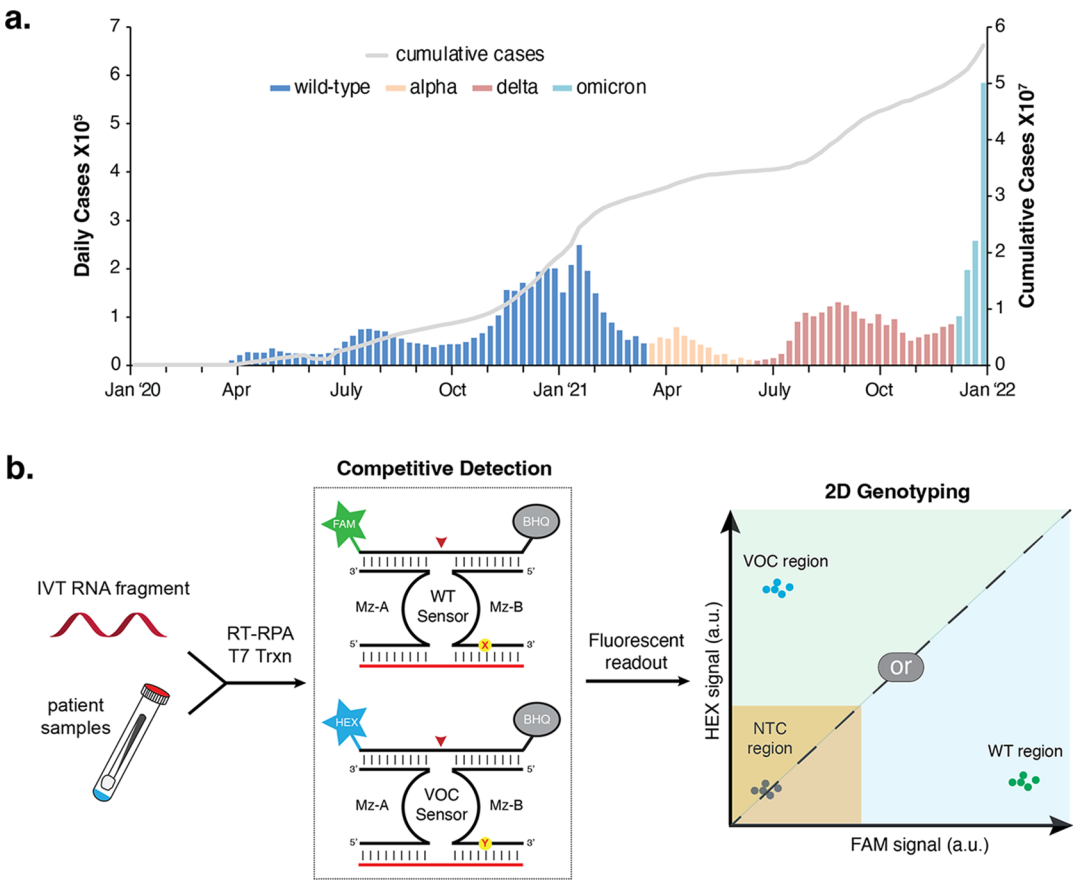

Figure 1. REVEALR-based detection of SARS-CoV-2 variants of concern. (a) Progression of COVID-19 cases in the United States from January 2020 to January 2022. Colors signify the dominant VOC observed at the collection date. Data points are based on a 7 day average. (b) Schematic view of competitive REVEALR genotyping. The SARS-CoV-2 region of interest is isothermally amplified by RT-RPA and T7 RNA polymerase to produce multiple copies of the RNA analyte. Competitive XNAzyme assembly on the viral RNA produces a fluorescent signal specific to the sample genotype via site-specific cleavage of a quenched fluorescent reporter. 2-D analysis shows WT or VOC detection based on the measured fluorescence observed for the FAM and HEX signal, respectively. Abbreviations: WT (wild-type); VOC (variant of concern); X, Y (SNP position); and au (arbitrary units). Red arrow indicates substrate cleavage site. Colors: RNA (red); DNA (black); green (WT, Fam signal); and blue (VOC, Hex signal).

multicomponent DNA enzyme (DNAzyme) that assembles in vitro to produce an output signal in response to the presence of a specific nucleic acid sequence.[20,21] Signal amplification, via cleavage of a quenched fluorescent reporter, occurs as long as the DNAzyme is bound to the target sequence,[22] which allows for highly sensitive COVID-19 detection in patient-derived clinical samples.[23] To facilitate VOC genotyping, we converted REVEALR into a competitive binding assay that detects single-base mismatches associated with each of the major SARS-CoV-2 variants. We then validated the assay against sequence-verified patient samples collected in early, mid, and late 2021 at the UCI Medical Center in Orange, California. REVEALR identified the correct VOC associated with each patient sample with 100% accuracy. The assay, which is programmable and amenable to multiplexing, has the potential to serve as a rapid, low cost, and scalable public health screening tool for symptomatic and asymptomatic detection of specific SARS-CoV-2 variants.

RESULTS

Transforming REVEALR into a Genotyping Assay. REVEALR is based on a split DNAzyme design strategy in which two halves of a catalytic core (Figure 1b) self-assemble in the presence of a viral RNA analyte to produce a functionally active catalyst that is capable of cleaving a quenched-fluorescent RNA oligonucleotide strand hybridized to the substrate binding arms of the DNA scaffold.[22] Signal amplification occurs via the multiple turnover activity of the enzyme, as long as the DNAzyme is bound to the viral RNA analyte, and ceases when the complex dissociates into its individual pieces.[22] Previous studies have shown that REVEALR functions with an analytical limit of detection (LoD) of ~20 aM (~10 copies/μL), which is equivalent to the CRISPR-based methods of SHERLOCK[19] and DETECTR[24] and below the average viral load of COVID-19 patients.[25]

Transforming REVEALR into a genotyping assay requires balancing differences in the energetics of hybridization between a perfectly matched viral RNA analyte and a viral analyte carrying a single-nucleotide mutation (*i.e.*, SNP). Since binding to a perfectly matched RNA strand is energetically more favorable than a mismatched strand, properly engineered sensors can be designed to detect a single mutation (transition or transversion) in a nucleic acid sequence.[26] To further enhance the sensitivity of detection, we designed a two-color competitive binding assay that challenges a wild-type and VOC-specific DNAzyme to recognize a genetic mutation within a small region of the viral RNA genome (Figure 1b). RNA substrates used in the competitive binding assay are

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equipped with fluorescent dyes (i.e., fluorescein (FAM) and hexachlorofluorescein (HEX)) that have non-overlapping spectral features and nucleic acid sequences that are complementary to their cognate DNAzyme (*e.g.*, wild-type and VOC). Data produced from the competitive assay is visualized in a two-dimensional (2-D) plot (Figure 1b) with the wild-type analyte producing a strong signal in the lower right quadrant and the VOC analyte generating an equivalently strong signal in the upper left quadrant depending on the identity of the viral RNA analyte present in the sample. By performing the assay against a panel of DNAzymes, each tailored to recognize a specific VOC, we reasoned that it should be possible to unambiguously identify the specific SARS-CoV-2 variant infecting a given patient-derived clinical sample.

Realizing that chemically modified nucleotides can increase the selectivity of SNP discrimination, we explored the use of locked nucleic acids (LNA) as a chemical tool for improving the activity of our DNAzymes.[27] LNA is a conformationally restricted nucleic acid analog that forces the ribose sugar to adopt a 3′-*endo* conformation by containing a methylene bridge between the *C*2′ and *C*4′ atoms.[28] Thermodynamic studies reveal that LNA increases the melting temperature of DNA oligonucleotides by 4−8 °C per residue when base paired with complementary strands of RNA.[29] Critically, LNA residues are known to enhance the SNP discrimination power of oligonucleotide probes by stabilizing the matched complex to a greater extent than the mismatched complex.[30] In our analysis, we positioned the SNP recognition site in the center of the right substrate binding arm, which was thought to be an optimal position based on prior work in the field.[31] We also evaluated the inclusion of LNA residues in both substrate binding arms, as LNA residues at these positions are known to increase the catalytic efficiency of the parent 10-23 DNAzyme.[32] We discovered that DNAzymes carrying LNA residues at both the SNP position and 5′ and 3′ and terminal positions of the substrate binding arms (Figure 2) function with higher sensitivity than an unmodified version of the DNAzyme or a DNAzyme carrying a single LNA residue at the SNP position. Titration assays reveal that all three sensors exhibit a linear downward correlation between fluorescence and analytic concentration (Figure S1) with the DNAzyme carrying LNA residues at the SNP position and substrate binding arms yielding the highest change in fluorescence between the matched and mismatched substrates across a concentration range of 5 to 100 nM analyte (Figure 2). This design configuration was therefore used as the molecular basis of our REVEALR genotyping technology.

Non-competitive and Competitive REVEALR. In designing the REVEALR system, we were initially concerned about the potential for cross-reactivity between our DNAzymes. This drawback, which exists for all hybridization-based strategies, could make it difficult to accurately identify VOCs in clinical samples. To evaluate this problem, we compared the cross-reactivity of DNAzymes that were designed to discriminate the wild-type (Wuhan-Hu-1) and alpha (B.1.1.7) strains of SARS-CoV-2 by distinguishing a C → A transversion in the viral genome responsible for the A570D mutation in the S1 glycoprotein (Figure S2, Table S1). We measured the fluorescent signal generated by the wild-type and alpha DNAzyme sensors targeting a perfectly matched DNA analyte or mismatched DNA analyte. SNP detection assays were performed under non-competitive (individual DNAzymes) and

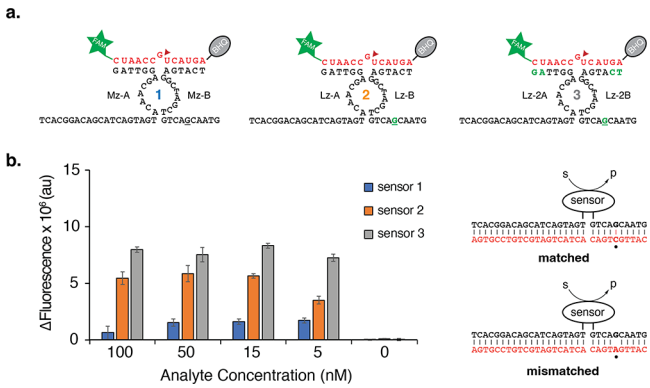

Figure 2. Sensor optimization. (a) Overview of multicomponent nucleic acid sensor. Two catalytic cores (Mz-A and Mz-B) self-assemble in the presence of a viral RNA analyte (not shown) to produce a functionally active catalyst with a separate active site that is capable of site-specific cleavage of a quenched fluorescent RNA reporter. Sensor 1 is DNA, sensor 2 contains a single LNA residue at the SNP position, and sensor 3 contains LNA residues at the SNP position and the 5′- and 3′-terminal positions of the substrate binding arms. Red arrow indicates the cleavage site and green nucleotides denote LNA residues. (b) Sensor optimization. Change in fluorescence observed for sensors 1−3 against decreasing concentration of a matched and mismatched DNA analyte corresponding to the A570D mutation observed in the S1 protein of the SARS-CoV-2 genome. Error bars represent the standard error of the mean (SEM) with $n = 3$. Abbreviations: au, arbitrary units; S, quenched 5′-fluorescent substrate; P, 5′-fluorescent product. Black dot denotes the SNP position.

competitive (both wild-type and VOC DNAzymes) binding conditions to compare the resolving power of nucleotide discrimination between the two assay formats (Figure S3). Fluorescence values collected across a range (5−500 nM) of analyte concentrations (Figure 3a,b) indicate that non-competitive binding conditions allow for accurate SNP detection at low analyte concentrations (5−15 nM range). However, the resolving power of this system is diminished when the analyte concentration reaches a higher level that is more reminiscent of viral RNA samples obtained after isothermal amplification by RT-RPA or LAMP (Figure 3a,b).[33] This problem is less severe in the competitive binding assay, which exhibits lower background fluorescence due to competition between DNAzymes for the same viral analyte. As an example, the wild-type sensor distinguishes the wild-type analyte (at 100 nM) from the alpha variant by a factor of 10:1 under competitive conditions but only 2:1 under non-competitive conditions. This result suggests that it should be possible to genotype patient samples across a broader range of analyte concentrations, as illustrated in the 2-D plot shown in Figure 3c.

Multicomponent DNAzyme Sensors for SARS-CoV-2 Variants of Concern. We evaluated 18 single-nucleotide mutations across all regions of the SARS-CoV-2 genome (Table S2) to establish a panel of multicomponent DNAzymes that could identify each of the major VOCs observed in the population over the last 24 months. To ensure high sensitivity for each VOC, we focused our analysis on genomic mutations that were prevalent in >90% of each genotypic lineage.[34] The screen identified 11 DNAzymes that functioned with high discriminating power against the wild-type strain in genotyping assays using in vitro transcribed (IVT) RNA analytes. The five most promising sensors (Figure 4a) are capable of distinguish-

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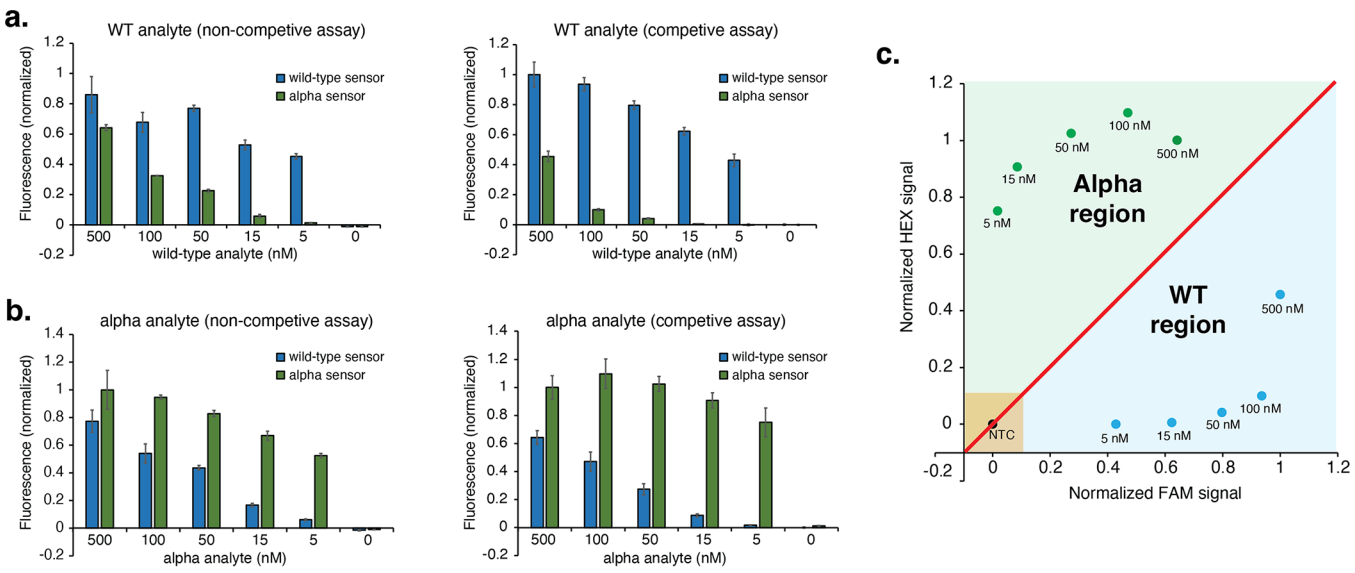

Figure 3. Nucleic acid detection assay. (a) Fluorescence signal generated by the wild-type and alpha sensors initialized by a segment of the wild-type analyte under non-competitive (left) and competitive (right) reaction conditions. (b) Fluorescence signal generated by the wild-type and alpha sensors initialized by a segment of the alpha variant under non-competitive (left) and competitive (right) reaction conditions. Error bars represent the standard error of the mean (SEM) with $n = 3$. (c) 2-D analysis showing wild-type and alpha variant detection under competitive reaction conditions. Each data point is the average of 3 replicates. NTC, no template control.

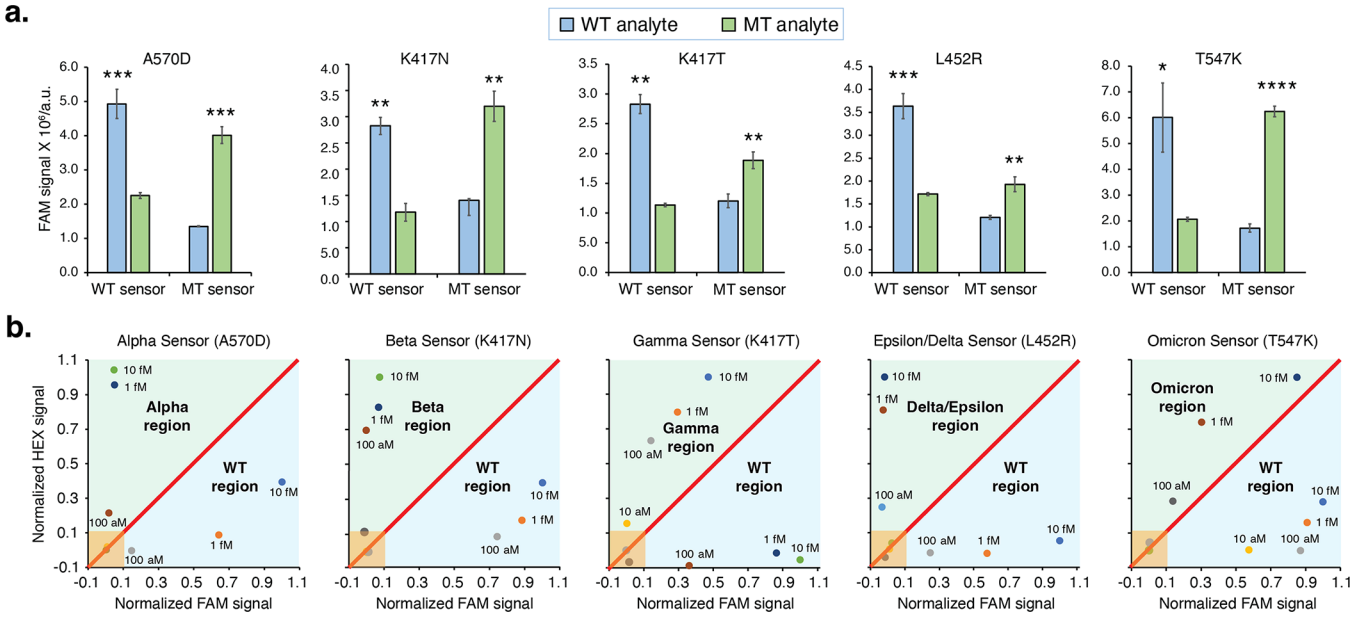

Figure 4. Sensitivity of REVEALR genotyping under competitive conditions. (a) Fluorescent signal generation for 15 nM of the A570D, K417N/K417T, L452R, and T547K DNA analyte after incubation for 30 min at 37 °C. Measurements are mean ± standard error (S.E.M), $n = 3$. Two tailed Student's t test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (b) Data are presented in 2-D plots showing wild-type and VOC detection across a range of concentrations. Assays were performed by separately delivering either the wild-type or VOC analyte to the reaction mixture. Each data point is the average of 3 replicates.

ing the A570D mutation observed in the alpha (B.1.1.7) variant, the K417N mutation observed in the beta (B.1.351) variant, the K417T mutation observed in the gamma (P.1) variant, the L452R mutation observed in the epsilon (B.1.427/9) and delta (B.1.617.2) variants, and the T547K mutation observed in the omicron (B.1.1.529) variant. Another six DNAzymes (Figure S4) showed strong discrimination against mutations observed in the alpha, beta, gamma, delta, and omicron strains, indicating that these sensors offer an additional layer of sensitivity for future diagnostic assays. The remaining 7 DNAzymes were unable to differentiate the wild-type and mutant analytes (Figure S5) at this time but could potentially be improved through the use of additional chemical modifications or further optimization of the SNP recognition site.

In the context of a REVEALR-based detection assay, where IVT RNA is pre-amplified and detected in a two-step assay, the five most promising sensors were found to function with an analytic LoD of 10−100 aM (Figure 4). In each case, wild-type and VOC-specific DNAzymes were separately challenged with

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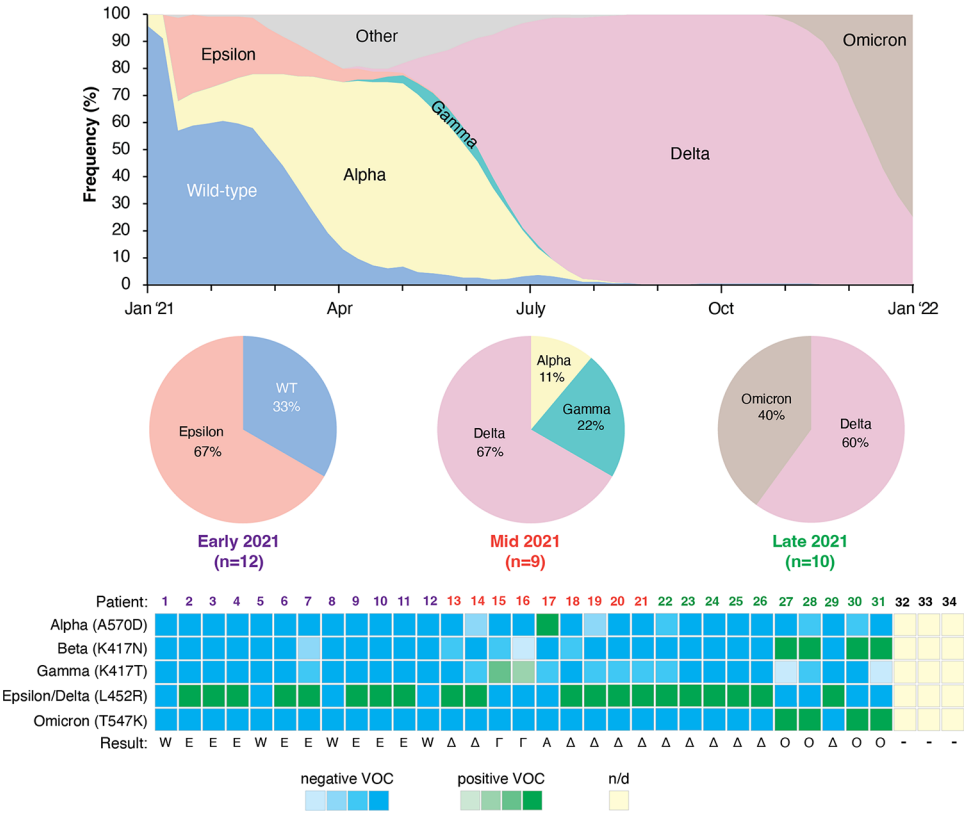

Figure 5. Surveillance testing of SARS-CoV-2 in the United States and Orange County, California. Surveillance of SARS-CoV-2 from GISAID shows the chronological frequency of variants of concern observed in the United States between January 2021 and January 2022 (top plot) using a subsampled dataset with time points documented at 7 day increments. Pie charts depict the distribution of sequence-verified SARS-CoV-2 variants observed in patients treated at the UCI Medical Center in Orange, California in early, mid, and late 2021. All 31 clinical samples were positively genotyped by competitive REVEALR. Although patient samples 27, 28, 30, and 31 yielded positive results for both the beta and omicron variants, due to a common K417N mutation shared between both strains, these samples were labeled omicron. This decision was based on a positive test for the T547K mutation, and the fact that the beta variant was not observed in the United States and its presence elsewhere in the world preceded the sample collection date. Each data point is the average of 3 replicates. Clinical samples 32−34 are negative controls obtained from healthy patients. Colors: blue, positive for wild-type; green, positive for VOC; yellow, clinical samples that are negative for COVID-19. Abbreviations: W, wild-type; A, alpha; E, epsilon; Γ, gamma; Δ, delta; and O, omicron; and n/d or -, not detected.

either the wild-type or mutant analyte in a competitive binding format to assess the detection limit for a simulated viral target. When plotted in the 2-D format (Figure 4b), wild-type and VOC analytes show strong signal separation along the diagonal axis, illustrating the resolving power of the assay to clearly distinguish wild-type and VOC analytes.

Clinical Validation of REVEALR Genotyping for SARS-CoV-2 Surveillance. Surveillance testing in the United States, both nationally and locally, reveals the spread of SARS-CoV-2 variants of concern across the country. Beginning in January 2021, the country witnessed the chronological rise of five major VOCs, including the alpha (B.1.1.7), gamma (P.1), epsilon (B.1.427/9), delta (B.1.617.2), and omicron (B.1.1.529) strains, along with several other minor variants (Figure 5). The rapid circulation of these more harmful strains in the public created a need for rapid and accurate genotyping assays that could be deployed as an alternative to traditional sequencing methods, which are slow, costly, and difficult to scale to the population. As a possible solution to this problem, we evaluated our two-step REVEALR genotyping assay on heat-inactivated patient samples collected at the UCI Medical Center in Orange, California. RNA extracted from nasopharyngeal swaps obtained from 34 patients treated in early, mid, and late 2021 were individually assayed in the competitive binding format for the wild-type, alpha, beta, gamma, epsilon/delta, and omicron variants. The patient samples were each analyzed for activity against the set of five VOC sensors, with each VOC sensor competing against the wild-type sensor. In total, 170 diagnostic assays were performed against the set of 34 clinical samples, which reflect 31 COVID positive patients and 3 COVID negative patients (Table S3). All of the samples were sequence verified, either at the UCI Medical Center or in our lab on the main campus. REVEALR identified each VOC with perfect accuracy, including patients infected with the wild-type strain, and yielded a clear negative result for each of the three COVID negative swabs (Figure 5). Importantly, samples collected in early, mid, and late 2021 reflect the abundance of SARS-CoV-2 strains observed locally and nationally at that time, indicating that REVEALR offers a powerful new tool for population surveillance and patient diagnosis. This latter observation could have an important impact on options for therapeutic intervention, as emerging strains, such as delta and omicron, have different virulence levels that can affect patient treatment.

DISCUSSION

The COVID-19 pandemic, caused by the spread of SARS-CoV-2 across the globe, represents the greatest threat to U.S.

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health and prosperity since the Great Depression. Controlling the spread of the disease will require the broad deployment of highly sensitive, low cost, and simple to use point-of-care diagnostics that are readily available for routine healthcare monitoring. Critical to this effort is the need to rapidly identify and triage patients infected with variants of concern that increase viral transmissibility and reduce vaccine protection. Current whole genome sequencing efforts designed to monitor epidemiological changes in the population are insufficient for this purpose, as this approach is used to provide a global picture of disease spread in local communities and across the nation. By contrast, the diagnosis of individual patients for a specific VOC is currently performed by Sanger sequencing, which is a slow and costly process that does not scale to the population.

Here, we describe the design, optimization, and clinical validation of a two-step genotyping system that is capable of precisely identifying the specific VOCs infecting COVID-19 patients. Our technology platform, called REVEALR, is based on a multicomponent DNAzyme strategy in which wild-type and VOC-specific DNAzymes compete to recognize single-nucleotide mutations in the viral RNA of patient-derived nasopharyngeal swaps. Assays performed in the competition format exhibit lower background fluorescence and higher discriminating power across a broader range of analyte concentrations than assays performed in a conventional single-reagent format. We demonstrated the viability of REVEALR as a rapid and highly sensitive genotyping assay for detecting SARS-CoV-2 variants of concern by evaluating 34 samples collected from patients treated at the UCI Medical Center in Orange, CA. REVEALR identified the correct viral strain in all 31 of the COVID positive samples and yielded a clear negative result for each of the 3 COVID negative samples. In addition to strong positive and negative predictive capabilities, results obtained from the clinical validation study were consistent with local and national COVID-19 surveillance efforts.

We wish to note that the epsilon and delta variants analyzed in the current study were distinguished based on their sample collection dates (Figure 5, Table S3), as both strains share a common L452R mutation for which a sensor was available. In the future, it may be possible to distinguish these strains using the T19R and E156G backup sensors (Figure S4), which are specific to the delta strain. Likewise, it should also be possible to distinguish new omicron subvariants using a hierarchical system in which an initial positive result for omicron is followed by a second genotyping assay to elucidate the identity of the specific subvariant in a patient sample (Figure S6).

Although REVEALR is conceptually similar to known CRISPR-based approaches in terms of analyte detection and signal generation, the platform has several unique advantages that warrant consideration. These include (1) broader targetability due to the absence of sequence constraints imposed by the PAM motif, (2) lower background fluorescence as a result of the competitive binding assay, (3) lower risk of viral or bacterial contamination by avoiding the need for protein expression and purification, and (4) increased simplicity and lower cost because the sensor is based entirely on the self-assembly of synthetic oligonucleotides and does not require recombinant protein or RNase inhibitors as reagents common to the CRISPR system. Together, these features make REVEALR an attractive system for SARS-CoV-2 genotyping.

Looking ahead to the future, it is clear that REVEALR could benefit from further improvements that lead to higher sensitivity, high specificity, greater throughput, and increased user friendliness. Such advances could, for example, include the transition from a fluorescent-based detection system to a simpler lateral flow device as well as the creation of an amplification-free multiplex detection platform for routine healthcare monitoring. We have previously demonstrated the ability for REVEALR to function in a lateral flow system for COVID detection,[23] suggesting that similar systems could be used as a diagnostic for patient genotyping. We could also optimize the chemistry and position of the SNP discrimination site in the sensor to allow for greater sensitivity of mismatch detection. Other less technical advances for improving signal detection may include changes to the virus inactivation protocol, which currently uses elevated temperatures that could damage the viral RNA analyte. More efficient lysis methods, such as glass bead-based ultrasonic power as applied to the Cepheid GeneXpert platform, could improve assay performance.[35] Finally, the incorporation of redundancy into the assay through the use of multiple sensors per VOC would increase the confidence of SARS-CoV-2 genotyping. This last step could be done by utilizing backup sensors identified in this study, discovering new sensors, or by optimizing existing sensors for improved activity.

CONCLUSIONS

In summary, we have shown that REVEALR is a versatile and efficient method for genotyping SARS-CoV-2 strains in COVID-positive patients. This strategy offers a valuable new approach for improving the sensitivity and specificity of single-nucleotide detection assays in far reaching applications that include pathogen detection, antibiotic resistance, genetic diseases, and cancer. Future developments could enable routine testing in hospitals, clinical diagnostic laboratories, and possibly even local activities involving businesses and schools.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c03420.

Table S1: List of oligonucleotides used in this study; Table S2: Single-nucleotide mutations evaluated in the SARS-CoV-2 genome; Table S3: Summary of patient-derived clinical samples; Figure S1: DNAzyme sensor design; Figure S2: Genotype of SARS-CoV-2 variants; Figure S3: Schematic overview of multicomponent enzyme screening; Figure S4: Back-up sensors identified for SNP discrimination; Figure S5: Multicomponent sensors that did not qualify for SNP detection; Figure S6: Possible REVEALR-based genotyping strategy for omicron subvariants (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors have filed a patent application on REVEALR genotyping.

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